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Yeast interaction in New Zealand Sauvignon Blanc

by

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Abstract

Interaction between fermenting yeast has a major impact on the aroma of wine. This thesis investigates the interaction of different fermenting yeast in New Zealand Sauvignon Blanc and their impact on the key aromas in the resulting wine. Volatile thiols 3-mercaptohexanol (3MH) and 3-mercaptohexyl acetate (3MHA), reminiscent of tropical aromas like passionfruit and grapefruit have been identified as key aroma compounds in New Zealand Sauvignon Blanc. Yeast interactions were monitored via change in population dynamics, fermentation kinetics, aroma concentration and change in protein and metabolite profiles of un-inoculated or controlled ferments.

In-depth analyses of population dynamics of the yeast involved in the 2008 Kumeu River Sauvignon Blanc led to the identification of 11 non-commercial species, of which S. bayanus was found to be the dominant fermenting yeast. A high level of thiol 4-mercapto-4-methylpentan-2-one (4MMP) was identified in this wine, this is a characteristic of Sauvignon Blanc made using S. bayanus isolates. A temperature limited growth model was able to predict the early dominance of the cryotolerant S. bayanus isolates.

In order to identify potential yeast interactions which produced unique aroma characters in New Zealand Sauvignon Blanc, a full factorial experiment was designed. This experiment consisted of a range of temperature and nitrogen conditions at which New Zealand Sauvignon Blanc fermentation was carried out by S. bayanus (SBJ1d) isolate and its co-ferments with T. delbrueckii, P. anomala and P. kluyveri (PKKR1) or S. cerevisiae (VL3) and its co-ferment with P. kluyveri (PKKR1).

The selected Kumeu River S. bayanus isolate SBJ1d had a high fermentation rate compared to S. cerevisiae (VL3) and was not perturbed by the presence of non-Saccharomyces yeasts. Aroma analyses from ferments showed that S. bayanus (SBJ1d) was capable of producing significantly more varietal thiols 3MH and 4MMP along with 2-phenyl ethyl ethanol (PEE) and beta-phenyl ethyl acetate (BPEA) compared to S. cerevisiae (VL3) potentially influencing floral aroma in New Zealand Sauvignon Blanc. On comparison of S. bayanus (SBJ1d) ferments with co-ferments, it was shown that presence of non-Saccharomyces yeast further enhanced the varietal aroma of the resulting New Zealand Sauvignon Blanc. Concentrations of isobutanol, isoamyl alcohol, isoamyl acetate and 4MMP were found to be greater (two-fold higher) in co-ferments of SBJ1d compared to S. bayanus (SBJ1d)-only ferments at low temperature. Thus, potential interactions between S. bayanus (SBJ1d) and selected isolates of P. kluyveri (PKKR1), T. delbrueckii and P. anomala have been identified which are capable of influencing aroma in New Zealand Sauvignon Blanc.
Systems biology approach was used in understanding the biochemical pathways involved in the interaction between *S. cerevisiae* (VL3) and *P. kluyveri* (PKKR1) in co-ferment of Sauvignon Blanc. Mono and co-ferments of New Zealand Sauvignon Blanc by these yeasts were analysed using 8-plex iTRAQ proteomic and extracellular metabolomic techniques. Analyses of extracellular metabolomics yielded in the identification of Leucine, Isoleucine and Glutamic acid as potential biomarkers and 34 different metabolic pathways as being important in distinguishing between *S. cerevisiae* (VL3)–only ferment and co-ferment. However, proteomic analyses using iTRAQ methodology was unable to identify key proteins or protein pathways important in the interaction between these two yeasts. Both the ‘omics approaches had their limitations when analysing a large-scale data-set. However, controlled co-fermentation coupled with targeted metabolomic analyses is a viable option to delineate yeast species interaction during fermentation.
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<tr>
<td>#, n</td>
<td>number</td>
</tr>
<tr>
<td>%</td>
<td>per cent</td>
</tr>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>(NH₄)₂SO₂</td>
<td>ammonium sulphate</td>
</tr>
<tr>
<td>3MH</td>
<td>3-mercaptohexan-1-ol</td>
</tr>
<tr>
<td>3MHA</td>
<td>3-mercaptohexyl acetate</td>
</tr>
<tr>
<td>4M2M2MB</td>
<td>4-methoxy-2-methyl-2-mercaptobutane</td>
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<tr>
<td>4MMP</td>
<td>4-mercapto-4-methylpentan-2-one</td>
</tr>
<tr>
<td>AS</td>
<td>Activity Score</td>
</tr>
<tr>
<td>AWRI</td>
<td>Australian Wine Research Institute</td>
</tr>
<tr>
<td>BHA</td>
<td>butylated hydroxyanisole</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BPEA</td>
<td>Beta phenylethyl acetate</td>
</tr>
<tr>
<td>CBS</td>
<td>Centraalbureau voor Schimmelcultures (Utrecht, The Netherlands)</td>
</tr>
<tr>
<td>cfu</td>
<td>colony forming unit</td>
</tr>
<tr>
<td>CID</td>
<td>Collision Induced Dissociation</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>DAP</td>
<td>diammonium phosphate</td>
</tr>
<tr>
<td>DMDC</td>
<td>dimethyl dicarbonate</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxy ribo nucleic acid</td>
</tr>
<tr>
<td>dNTP(s)</td>
<td>deoxy nucleotide triphosphate(s)</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>E</td>
<td>exponential notation</td>
</tr>
<tr>
<td>e</td>
<td>Euler’s constant (~2.718)</td>
</tr>
<tr>
<td>eV</td>
<td>electron Volt</td>
</tr>
<tr>
<td>FRST</td>
<td>Foundation for Research, Science and Technology</td>
</tr>
<tr>
<td>g</td>
<td>acceleration due to Earth’s gravity at sea level</td>
</tr>
<tr>
<td>GCMS</td>
<td>gas chromatography coupled mass spectrometry</td>
</tr>
<tr>
<td>²H</td>
<td>Deuterium</td>
</tr>
<tr>
<td>h, min, s</td>
<td>hour, minute, second</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrochloric acid</td>
</tr>
<tr>
<td>H₂S</td>
<td>hydrogen sulphide</td>
</tr>
<tr>
<td>IAA</td>
<td>isoamyl alcohol</td>
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<tr>
<td>IAAc</td>
<td>isoamyl acetate</td>
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<tr>
<td>IB</td>
<td>isobutanol</td>
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<tr>
<td>iTRAQ</td>
<td>isobaric Tags for Relative and Absolute Quantification</td>
</tr>
<tr>
<td>ITS</td>
<td>internal transcribed spacer</td>
</tr>
<tr>
<td>Kb</td>
<td>kilo base</td>
</tr>
<tr>
<td>Kg, g, µg, ng</td>
<td>kilogram, gram, microgram, nanogram</td>
</tr>
<tr>
<td>L, mL, µL</td>
<td>litre, millilitre, microliter</td>
</tr>
<tr>
<td>LCMS</td>
<td>liquid chromatography coupled mass spectrometry</td>
</tr>
<tr>
<td>Ln</td>
<td>natural log (inverse of e)</td>
</tr>
<tr>
<td>Log₂</td>
<td>log in base 2</td>
</tr>
<tr>
<td>Log₁₀</td>
<td>log in base 10</td>
</tr>
<tr>
<td>M, mM, µM</td>
<td>molar, milimolar, micromolar</td>
</tr>
</tbody>
</table>
M, μM  | meter, micrometer
---|---
m | Malthusian Fitness
MCF | methyl chloroformate
MgCl₂ | magnesium chloride
MSI | Ministry of Science and Innovation
mVP | model co-ferment
m/z | mass to charge ratio
N₂ | nitrogen gas
OD | optical density
P | probability value
PAPi | Pathway Activity Profiling
PCA | principal component analysis
PCR | polymerase chain reaction
PEE | Phenyl ethyl ethanol
pHMB | sodium-4-(hydroxymercuri)benzoate
PKKR1 | P. kluyveri 1 a.k.a Viniflora® FrootZen™
R | R statistical programming language
r | growth rate
rDNA | ribosomal DNA
RFLP | Restriction Fragment Length Polymorphism
rpm | revolutions per minute
SBJ1d | S. bayanus isolate J1d from 2008 Kumeu River Sauvignon Blanc
SB+PKKR1 | co-ferment of SBJ1d and PKKR1 with inoculum ratio of 1:9
SB+PA | co-ferment of SBJ1d and P. anomala with inoculum ratio of 1:9
SB+TD | co-ferment of SBJ1d and T. delbrueckii with inoculum ratio of 1:9
SEM | standard error of the mean
SGM | synthetic grape medium (Sauvignon Blanc)
TA | titratable acidity
(v/v) | volume of solute/volume of solution
V, mV | Volt, millivolt
VL3 | S. cerevisiae VL3
VL3+PKKR1 | co-ferment of VL3 and PKKR1 with initial inoculum ratio of 1:9
Vmax | maximum rate of fermentation
w | Darwinian Fitness
YAN | yeast available nitrogen
YPD | yeast extract, peptone, dextrose rich medium
General Introduction
1.1 General introduction

This chapter provides a broad overview of concepts spanning microbial ecology, fermentation and wine aroma. It also provides background information on the various techniques used to test various hypotheses during my doctoral research. This chapter summarizes the literature on both the theoretical and technical background necessary to outline the general objectives of this thesis. In-depth introduction to specific topics are provided in the appropriate results chapters.

1.2 Microbes in nature

Microbes contribute roughly half of all the biomass on earth (Prosser et al., 2007). Microbes have been discovered thriving in diverse geographical locations from arid deserts to the depths of oceans and under extreme environmental conditions such as sub-zero temperatures of Polar Regions to thermal vents (Prosser et al., 2007). The capacity of microbes to exist in diverse conditions can be attributed to their ability to adapt and utilize various compounds for nutrition and evolve unique metabolic pathways that enable their survival. Due to their diverse capacities microbes play an important role in biogeochemical cycling of key elements like carbon, nitrogen and sulphur essential for maintaining life on earth (Jessup et al., 2004; Prosser et al., 2007).

The diverse nature of microbes has seen their application in food production, waste water treatment, clean-up pollution and harness bio-energy among other uses (Jessup et al., 2004). Indeed, useful microbes and biotechnology have contributed to the advancement of our civilization. Although microbes are an indubitable part of our lives, their existence was not uncovered until Antoine van Leeuwenhoek discovered micro-organisms in pond scum in 1674 (Leeuwenhoek, 1703). The word microbe is derived from the Greek mikrós meaning “small” which is used to describe any organism that is either single celled, in cell clusters or acellular (no cell) like viruses (Jessup et al., 2004).

1.2.1 Microbial communities

With the advent of modern microbiology and more recently molecular biology techniques, research is slowly unveiling the nature and species composition of microbes inhabiting various niches (Jessup et al., 2004; Klitgord and Segrè, 2011). Many microbes are capable of occupying and thriving in a variety of niches and in combinations give rise to a diversity of microbial communities (Prosser et al., 2007).

Some of the key factors driving microbial communities, irrespective of the niche, are nutrient availability and environmental factors such as availability of sunlight, water, pH, level of oxygen,
temperature and barometric pressure. Microbes which are able to adapt to different nutrient levels and varying environmental conditions are able to thrive in a variety of niches (Jessup et al., 2004; Kent et al., 2007; Prosser et al., 2007). The presence of more than one species of microbe in a niche often results in some form of interaction (direct or indirect) between the different species. Microbial interactions have a major impact on the biotic and abiotic factors responsible for the functioning of that niche (Kent et al., 2007).

1.2.2 Species co-existence and interactions within microbial communities

The study of community ecology is built upon the conceptual foundation of species co-existence (Hubbell, 2001). Theoretically species are able to co-exist because each (species) has a demographic advantage when rare which allows for the propagation and survival of a species; conversely some manner of demographic disadvantage prevents a species from excluding other species from a particular niche (Siepielski and McPeek, 2010). The flux in microbial communities is greatly dependent on the nature of interactions between species (Jessup et al., 2004; Klitgord and Segrè, 2011). Species interactions in microbial communities can be direct including predator-prey, parasitic (including infectious diseases), co-operative interactions such as symbiotic and commensalism through to sexual interactions or indirectly by altering the abiotic environment and or competing for resources (Jessup et al., 2004; Klitgord and Segrè, 2011). All these interactions have been well documented in microbial communities. At their core these interactions constitute the movement and exchange of metabolites between organisms (Klitgord and Segrè, 2011).

Metabolites can be defined as small molecules which are intermediates and/or products of an organism’s metabolism (Klitgord and Segrè, 2011; Nielsen and Oliver, 2005). Metabolites are involved in both primary functions such as growth development and reproduction, as well as secondary functions such as communication, defence and maintaining the survivability of an organism. Interactions between metabolites of the different microbes in a niche have a major impact on their life (Klitgord and Segrè, 2011).

For example: communication among same or different species of microbe termed quorum sensing is an important tool used to moderate one another’s activities through the exchange of metabolic intermediates (Jessup et al., 2004). These dynamic interactions have been shown to be a product of natural selection within Pseudomonas populations and therefore increase fitness (survivability) of microbes in some manner (Rainey and Travisano, 1998). Other model systems in ecology have been reviewed by Jessup et al. (2004) and include the interaction between T4 bacteriophage and E. coli (K-12) used to understand predator-prey dynamics along with other multi-trophic interactions (Bohannan and Lenski, 2000). Also, the rocky intertidal community comprising algae Endocladia muricata, acorn barnacles Balanus glandula; mussels Mytilus californianus; and sea star Pisaster ochraceus;
which have been used to understand ecological processes such as competition, predation and disturbance which are important factors shaping community structures (Paine and Levin, 1981).

Despite recent technological innovations; critical empirical tests to understand the fundamental rules governing community structure and the evolution of these interactions are lacking (Siepielski and McPeek, 2010). We can comprehend these interactions by firstly quantifying them and then testing whether they are adaptive using experimental ecology and evolution (Siepielski and McPeek, 2010). Such work would enhance our understanding of the dynamics that regulate microbial communities (Jessup et al., 2004; Rainey and Travisano, 1998). However, research in this area is almost completely lacking (Jessup et al., 2004; Prosser et al., 2007). The purpose and inspiration for this doctoral research was to elucidate and quantify the metabolic interactions between species in a naturally occurring microbial community.

Fundamental to this research is the availability of an appropriate experimental system. Such a system must consist of organisms that are molecularly tractable and easy to propagate; these organisms must be known to interact, and above all this system must be easy to replicate experimentally yet be realistic (Replansky et al., 2008; Siepielski and McPeek, 2010). One such system comprises of various species of yeast that exist naturally during the un-inoculated fermentation of grape juice to wine (Replansky et al., 2008). The elegance of this system derives from a few aspects. First, this is a system that is not as simplistic as an artificial two-species laboratory construct, yet not as complicated as the microbial community that one may find in, say, a woodland. This system is an ideal ‘half-way house’ which is controlled yet constitutes natural species. Second, the microbes we chose to focus on in this system – Hemiascomyte yeasts – are experimentally tractable from a microbiological and molecular genetic perspective: indeed the dominant wine yeast, Saccharomyces cerevisiae, has long been a model eukaryote and is arguably the best molecularly characterised organism on the planet (Replansky et al., 2008).

1.3 General history and process of winemaking

The process of converting grape juice to wine is one of the oldest biotechnological tools utilized by mankind (Chambers and Pretorius, 2010; Pretorius, 2000; Querol and Fleet, 2006). There is historical evidence to suggest that deliberate fermentation of grape juice was being carried out in ancient China and present-day Iran as early as 7000BC along with other civilizations such as the Greeks, romans and Egyptians (Querol and Fleet, 2006). The Romans were responsible for spreading the knowledge of viticulture and winemaking to into central and northern Europe around 100AD, these European nations are considered as the old world wine producing countries (Chambers and Pretorius, 2010).
Countries in North and South America, Australia and New Zealand were introduced to winemaking by European pioneers in the 16th, 17th and 18th centuries and are looked upon as the new world wine producers (Robinson, 2006).

Historically, during winemaking, the microbial flora from the surrounding vineyard, winery and other equipment used were allowed to carry out the fermentation process (Fleet, 2003; Pretorius, 2000). Many winemakers from old world countries (European) still prefer this technique of un-inoculated or natural fermentation due to its propensity to deliver complex and unique flavour profiles in the resulting wine (Robinson, 2006).

An increasing number of studies show that winemaking is a complex process involving a number of ecological and biochemical interactions between various species of yeasts, bacteria fungi and associated viruses (Chambers and Pretorius, 2010; Fleet, 2003). Un-inoculated or un-inoculated ferments are repositories of microbial communities with a diversity of microbes including large population of yeasts (Querol and Fleet, 2006; Replansky et al., 2008). Grape must (juice) represents a complete growth media for microbes, but certain characteristics like low pH and high sugar content make it more favourable for yeasts which are the principal fermenting agents responsible for the alcoholic fermentation of grape sugars into ethanol (Querol and Fleet, 2006). The diversity and number of yeasts can depend on numerous factors such as the geographical location, climate, grape variety, physical damage to the berries, viticulture practices and method of winemaking (Pretorius, 2000).

1.3.1 Winemaking

Winemaking is a complex physical and biological process which starts during the ripening of the grape berries and continue on after the bottling procedure (Chambers and Pretorius, 2010; Jackson, 2000). An outline of the general winemaking process is given in figure 1.1.

In general, the physical process of winemaking involves the crushing and pressing of grape berries to obtain grape juice which is then introduced to yeasts (inoculated or un-inoculated) to commence fermentation following the fermentation process the resulting wine is separated from the settled yeast cells (lees). The wine is then temperature stabilized and refined of any undesirable solids and proteins. After this point the wine is filtered and blended before being bottled for consumption (Fleet, 2003; Jackson, 2000).

Biologically, winemaking process involves the modification of grape juice and subsequently wine by yeasts and bacteria (Querol and Fleet, 2006). Winemaking process involves the delicate balance of harnessing the beneficial properties of fermenting yeasts and bacteria but preventing spoilage and...
undesirable aromas. The grape variety and the fermentation process are the two main factors responsible for delivering the final flavour and aroma in a wine (Querol and Fleet, 2006).

![General outline of the physical and biological processes involved in winemaking.](image)

**Figure 1.1:** General outline of the physical and biological processes involved in winemaking. The options in the star-shapes are usually dependent on type of wine and winemaking techniques. Adapted from (Jackson, 2000).

### 1.3.2 Fermentation

Fermentation involves the conversion of glucose, fructose and other nutrients present in grape juice by yeasts into ethanol and carbon dioxide (Jackson, 2000). Along with the production of ethanol and carbon dioxide, yeasts also release various secondary metabolites which are responsible for some of the aroma and flavour of the resulting wine (Fleet, 2008; Pretorius, 2000).

Un-inoculated ferments are unpredictable and are usually started by a diverse variety of low-alcohol tolerant apiculate yeasts such as *Hanseniaspora* (*Kloeckera*) which account to about 50-75% of the
yeast population, less prevalent are species of Candida, Brettanomyces, Kluyveromyces, Pichia and the pink yeast Rhodotorula (Fleet, 2003). Initially, Saccharomyces species are present at low numbers of 10-100 cfu/mL. This might suggest that grape must is not the primary source of this elliptical yeast which comes to dominate and finish the ferment (Martini et al., 1996). Various conclusions have been reached by different researchers as to the source of Saccharomyces in un-inoculated fermentations. However one thing is certain that the fermenting grape must undergoes a rapid change brought on by establishment of anaerobic conditions, depleting nutrients and increasing levels of ethanol all of which increase the selectivity of the media (Gao and Fleet, 1988; Heard and Fleet, 1985). Indeed, Saccharomyces cerevisiae is considered the top wine yeast as it is known to engineer its niche during fermentation to deter other yeast species and make available valuable nutrition (Goddard, 2008). The non-Saccharomyces yeasts proliferate to final populations of $10^6$-$10^7$ cfu/ml and decline after 3-4 days (mid-fermentation) when the ethanol production by Saccharomyces exceeds 5-7%. The dominating Saccharomyces is then able to utilize the sugars and complete ferments (Gao and Fleet, 1988; Heard and Fleet, 1985). Interestingly some species of Candida and Pichia are known to survive till the end of the fermentation process (Querol and Fleet, 2006). In the event of Saccharomyces being unable to dominate and complete the ferment, it often results in stuck or sluggish fermentation, this is because most non-Saccharomyces yeasts are unable to respire anaerobically and consequently are poor fermenters (Querol and Fleet, 2006). This could also result in the production of off-odours and other unacceptable characteristics in the resulting wine (Fleet, 2003). Due to these risk factors most winemakers in NZ prefer to inoculate the grape must with commercial strains of wine yeast (personal communication Mr Michael Brajkovich MW).

1.3.3 New World wines and modern commercial winemaking

With the advent of microbiology, and the elucidation of the fermentation process by Louis Pasteur in 1863, the importance of yeast during alcoholic fermentation and their impact on the flavour and aroma was made evident to winemakers (Fleet and Heard, 1993; Querol and Fleet, 2006). In 1890, Müller-Thurgau grape variety was responsible for a major step towards modern commercial winemaking with the concept of inoculating ferments with a suitable pure yeast strain (Chambers and Pretorius, 2010; Robinson, 2006). Emil Christian Hansen from Carlsberg brewery was the first to isolate and propagate cultures derived from a single yeast cell. These advances in microbiology and winemaking led to the development of active dry wine yeasts in the 20th century which are widely used in modern commercial winemaking (Chambers and Pretorius, 2010; Pretorius, 2000).

Commercial wine yeast preparations usually consist of either a single strain of Saccharomyces cerevisiae or a tailored mixture of yeast belonging to the Saccharomyces sensu stricto (Ciani et al., 2010; Pretorius, 2000). These yeasts are usually subjected to selective breeding to express and
enhance various oenological traits; some of which are outlined in table 1.1. Most New World winemakers prefer to utilize a commercial wine yeast strain to inoculate grape must to produce wine of consistent quality. However, a potential downside to commercial inoculated fermentation in the lack of complex flavour profile in the resulting wine due to the absence of contribution from other yeast species (Ciani et al., 2010).

<table>
<thead>
<tr>
<th>Increased:</th>
<th>Decreased:</th>
<th>Technological properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fermentation efficiency</td>
<td>Temperature optimum</td>
<td>Resistance to desiccation</td>
</tr>
<tr>
<td>Ethanol tolerance</td>
<td>Nitrogen requirement</td>
<td>Zymocidal characteristics</td>
</tr>
<tr>
<td>Osmo-tolerance</td>
<td><em>Formation of off-odours and harmful chemicals such as:</em></td>
<td>Enhanced autolysis</td>
</tr>
<tr>
<td>Sulphur tolerance</td>
<td>Sulphites, sulphides, DMS,</td>
<td>Moderate biomass production</td>
</tr>
<tr>
<td>Esterase activity</td>
<td>Volatile acidity</td>
<td>Compact sedimentation</td>
</tr>
<tr>
<td><em>Production of:</em></td>
<td>Higher alcohols</td>
<td>Limited foam formation</td>
</tr>
<tr>
<td>Glycosylated flavour compounds</td>
<td>Biogenic amines</td>
<td></td>
</tr>
<tr>
<td>Glycerol</td>
<td></td>
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</tr>
</tbody>
</table>

Table 1.1: Some of the desirable characteristics of commercial wine yeast. This table was compiled using information from (Fleet, 2008; Jackson, 2000; Pretorius, 2000; Swiegers et al., 2005).

1.3.4 Emerging concept of Multi-starter yeast cultures

A trade-off exists between the consistency and control attributed to inoculated fermentation and the potential flavour and complexity benefits of un-inoculated ferments. As a result there have been studies in the recent past exploring the idea of multi-species starter cultures and co-fermentation process(Ciani et al., 2006). Numerous studies have shown that by inoculating grape must with multiple yeast species can improve the complexity and the depth of flavour of a resulting wine when compared to wine produced from a single species starter culture (Ciani et al., 2006; Howell et al., 2006). In recent years increasingly the contribution of non-*Saccharomyces* yeast to wine aroma and quality is being studied (Ciani and Comitini, 2011). The concept of co-inoculating or co-fermenting grape must with non-*Saccharomyces* yeast along with a conventional yeast species is gaining popularity (Ciani and Comitini, 2011; Ciani et al., 2010). Studies focused on co-fermentation
involving different species of *Saccharomyces* and/or non-*Saccharomyces* have shown that indeed these multiple yeast combinations are capable of enhancing specific wine features and also improving the varietal character (Ciani *et al.*, 2010; Howell *et al.*, 2006). Various starter cultures in wine and their properties are discussed in depth in chapter 2.1. A foundation to these concepts has been the thorough understanding of the underlying forces that drive the yeast population dynamics during a natural/ un-inoculated fermentation process.

### 1.4 Factors determining the yeast population dynamics during un-inoculated fermentation

The molecular and physiological state of yeasts during anaerobic respiration responsible for alcoholic fermentation can be broadly explained as a response to a stressful environment (Attfield, 1997; Fleet, 2008). Fermenting yeasts are exposed to a variety of chemical and physical stresses ranging from mild to severe. Some of these stressors are abiotic while others can be deemed as biotic; biotic stresses are those that are affecting one organism due to the biological activity of another in the same niche (Fleet, 2003; Ivorra *et al.*, 1999).

#### 1.4.1 Influence of stress factors on yeast

In a un-inoculated fermentation, the natural yeast population is initially dominated by species from the surrounding environment. These yeasts are immediately faced with a highly osmotic environment of the grape juice at the beginning of fermentation; most species that are incapable of adapting to this environment are quickly replaced by yeasts resistant to the high sugar concentrations (Querol and Fleet, 2006). The start of anaerobic respiration results in the decreased availability of nitrogen and oxygen, this coupled with increasing temperature and ethanol levels is responsible for the decline of most of the non-*Saccharomyces* yeast species (Pérez-Nevado *et al.*, 2006). Different stress factors are responsible for the decline of different non-*Saccharomyces* yeast species during early fermentation (Hohmann, 1997).

Research shows that some non-*Saccharomyces* yeast (*T. delbrueckii* and *K. thermotolerans*) are less tolerant to an oxygen limiting environment compared to *Saccharomyces cerevisiae* which can be attributed to their early death in a mixed ferment, suggesting that non-*Saccharomyces* species might require an aerobic environment to function better (Pérez-Nevado *et al.*, 2006). On the other hand, it was suggested that wine strains of *H. uvarum*, *H. guillermondi* and *C. stellata* were poor fermenters due to a deficient uptake in sugars rather than low ethanol tolerance (Albergaria *et al.*, 2003a; Albergaria *et al.*, 2003b). Conversely, it was reported that many strains of *H. guillermondi* and *C.
**stellata** were able to withstand high ethanol concentrations (Pina *et al.*, 2004b). It has also been shown that when the ferment temperature is lower than 15-20°C it decreases the ethanol sensitivity of non-*Saccharomyces* yeasts, thus they are able to have a significant impact on the complexity of the wine flavour (Charoenchai *et al.*, 1998).

Additionally, if non-*Saccharomyces* species consume a lot of the available amino acids and vitamins during initial fermentation they can prevent the growth of *Saccharomyces*, hence causing sluggish ferments (Bisson, 1999; Mortimer, 2000). In conclusion, there are many factors responsible for the success of *Saccharomyces* and the initial death of the non-*Saccharomyces* species, some of which are known and if controlled appropriately contribute to the complexity of the wine (Fleet, 2003). But, it can be said that *Saccharomyces* species has adapted to fermentation and ensures maximum availability of nutrients for itself by engineering its environment via anaerobic respiration resulting in increased ethanol concentration and temperature making it toxic for other microbes (Goddard, 2008). This phenomenon is not unusual because when the niches of individual microbes overlap its likely to result in an interaction between them. These interactions can either be direct or indirect. The impact of these interactions can be evaluated on the basis of the population sizes of the interacting species during fermentation (Querol and Fleet, 2006). This is discussed in depth in chapter 3.1.

### 1.4.2 Growth kinetics of yeast during fermentation

Typically both *Saccharomyces* species and non-*Saccharomyces* species exhibit batch growth kinetics during fermentation. They all have a lag phase, an exponential growth phase, stationary phase and death phase (Fleet and Heard, 1993). However, they differ in their exponential growth phase resulting in different cell populations in the stationary phase, i.e. the non-*Saccharomyces* have restricted exponential phase compared to *Saccharomyces* species and consequently have lower cell populations. These differing cell populations can be attributed to the various kinds of nutrient limitations posed by grape must during fermentation (Houtman *et al.*, 1980; Nissen *et al.*, 2003). It is a known fact that *Saccharomyces* increases their biomass in the presence of a nitrogen source, along with nitrogen availability nutrients such as oxygen, salts and other trace elements influence cell populations (Bely *et al.*, 2003 ; Fleet and Heard, 1993). The biomass generated by *Saccharomyces* species is greater compared to other species; it reflects that *Saccharomyces* are able to propagate at lower nutrient availability compared to their counterparts (Fleet, 2003). Most of the current literature ascribes the inability of non-*Saccharomyces* species to survive in grape must and hence their early death to increased temperature coupled with low ethanol tolerance. Few studies have been aimed at understating the mechanisms involved in this phenomenon. In this thesis, research was conducted to understand the impact of different environmental parameters such as temperature and nitrogen...
availability on the fermentation kinetics of *S. cerevisiae*-only ferments and co-ferments of *S. cerevisiae* with non-*Saccharomyces* yeast. This topic is discussed in depth in chapter 3.1.

### 1.4.3 Secondary metabolites and yeast survival

During fermentation, secondary yeast metabolites like medium chain fatty acids (e.g.: acetic, hexanoic, octanoic and decanoic acids) are produced which can induce cell death in certain yeast species, some strains of yeast are able to produce specific extracellular glycoproteins and are termed killer positive yeast strains these strains are capable of killing sensitive strains of yeast (Musmanno, 1999). For example some species from the genera of *Hanseniaspora, Pichia, Candida* and *Saccharomyces* have been identified which exhibit this killer toxin activity (Querol and Fleet, 2006). Killer toxins produced by certain yeast species during fermentation maybe responsible for inhibiting the growth of non-*Saccharomyces* yeast which are more sensitive to these compounds in comparison to *Saccharomyces* species (Querol and Fleet, 2006). In prokaryotes there is a growth regulatory mechanism called quorum sensing responsible for the onset of stationary growth phase, and in higher eukaryotes cell density is regulated by a cell-cell contact mechanism (Nissen and Arneborg, 2003). Neither mechanism has been conclusively observed in *Saccharomyces* the model winemaking organism. However, research carried out by Nissen *et al.* (2003) suggested that there was some cell-cell contact mediated mechanism responsible for the early death of *T. delbrueckii* and *K. thermotolerans* in a mixed ferment with *S. cerevisiae*. Another study by Pérez-Nevado *et al.* (2006) using killer toxin sensitive strains of *S. cerevisiae* and killer neutral strains of *H. uvarum* and *H. guillermondi* showed that cell-free extracts of a *S. cerevisiae* ferment was able to cause cell death in non-*Saccharomyces* species for ferments. Results from Pérez-Nevado *et al.* (2006) indicated that *S. cerevisiae* produced some toxic compounds responsible for the early deaths of these species. The toxic compounds and the underlying mechanisms are yet to be determined but are significant enough to challenge the importance of the role of classic selective pressures in the succession of yeast population.

Thus it can be said that, there are many other not so straightforward mechanisms involved in the continual change in population dynamics of an indigenous ferments and that there is some sort of indirect interaction between the *Saccharomyces* and non-*Saccharomyces* yeasts during fermentation and that *Saccharomyces* species are capable of antagonizing its counterparts by producing a whole suite of secondary metabolites and are better able to utilize the available nutrients (Nissen and Arneborg, 2003; Pérez-Nevado *et al.*, 2006).
1.4.4 Microbial species interactions and their impact on winemaking

On grapes, yeasts usually co-exist with a many different fungi and bacteria, some of which can contribute to the fermentation process. For example; many red wines require a secondary bacterial or a malolactic fermentation to develop a final complexity in their qualities (Fleet, 2003). Researchers have studied the importance of these reactions and concluded that yeasts used for the first ferment has an impact on the subsequent secondary bacterial growth (Fleet, 2003). Effects such as micronutrient availability and excretion of inhibitory chemicals by yeast to inhibit the bacterial growth have been observed. Conversely yeasts play an indirect beneficial role during secondary fermentation: autolysis of yeast cells release amino acids and vitamins back into the media thus encouraging bacterial growth, their cell walls might also act as a bio-adsorbent and remove toxic substances from the wine and the released proteolytic enzymes might break down components in grape juice and make it available for consumption by bacteria (Fleet, 2003). These mechanisms are indirect and are thus not species interaction per se. One study suggested a possible quorum sensing mechanism as a factor in the ability of the bacteria P. aeruginosa to kill C. albicans, this analogy could be extended to other bacteria-yeast interaction during wine fermentation (Hogan and Kolter, 2002).

A study conducted by Lüthi (1957) showed that it was not possible to obtain a characteristic property in a wine when it was inoculated with a certain strain of bacteria which were isolated from the same type of wine with that particular character, it was suggested that some sort of synergism between the fermenting organisms was responsible and not just the mere addition of certain bacteria for the development of the desired characters in that wine. Similarly there are reports that Botrytis, cinerea, Aspergillus spp. And Penicillium spp. produces metabolites and toxins, for example: ochratoxin A, which might inhibit yeast growth (Fleet, 2003). A mannoprotein produced by B. cinerea might be an anti-yeast factor; β-glucans produced by this fungus might inhibit yeast activity (Ribéreau-Gayon, 1985). On the other hand fungal growth on grapes favour growth conditions for acetic acid bacteria which might inhibit yeast growth during fermentation (Fleet 2003). There have be studies which report that yeast species such as Pichia, Candida, Metschnikowia etc. show strong anti-fungal activity; they are capable of producing 1, 3 β-glucanases which can destroy fungal cell walls (Fleet, 2003).

These studies are preliminary and few, there is much that needs to be explored in terms of interactions between yeasts and other microbes and consequently their impact on the winemaking process (Ciani et al., 2010). To my knowledge there has been no known global analysis of multi-species interaction during fermentation process utilizing advanced molecular biology techniques such as proteomics, transcriptomics or metabolomics. As part of this research; interaction between S. cerevisiae and non-Saccharomyces yeast during fermentation was analysed using global proteomic
and metabolomics techniques to understand their impact on the final wine quality. This is discussed in detail in chapter 5.

1.4.5 Saccharomyces yeast species interaction during fermentation

*Saccharomyces cerevisiae* is the dominant wine yeast species, and various strains of this species have been commercialised for winemaking purposes due their ability to impart specific oenological characteristics (Fleet, 2008). It showed that using multiple *S. cerevisiae* starters resulted in a remarkably different wine in comparison to the blended wine made from individually fermented wines using *S. cerevisiae* from the original multi-starter inoculum (Howell *et al.*, 2006). Howell *et al.* (2006) were able to show that it was due to the *Saccharomyces* yeast interacting with one another that resulted in the unique character of the wine not reproduced by post ferment blending. This finding is interesting as there have been a few studies which have focussed on the population dynamics of the different strains of *S. cerevisiae* in un-inoculated ferments (Gayevskiy and Goddard, 2012; Pramateftaki *et al.*, 2000; Torija *et al.*, 2001). Other *Saccharomyces* species such as *S. bayanus* and *S. uvarum* have also been studied for their role in un-inoculated fermentation of wine which has shown them to have distinct organoleptic properties when compared to *S. cerevisiae* (Dubourdieu *et al.*, 2006; Eglinton *et al.*, 2000; Favale *et al.*, 2007; Muratore *et al.*, 2007; Tosi *et al.*, 2009). These diverse strains of *Saccharomyces* yeast could potentially contribute to the complexity of the resulting wine and hence present an untapped resource for further study. An in-depth study of a un-inoculated New Zealand Sauvignon Blanc was conducted partially to unearth/discover the fermentation potential of as yet untapped strains of *Saccharomyces* yeast. Along with commercial usage, these studies could provide valuable insight into the mechanisms of niche construction and species interaction at a metabolic level it is discussed in chapter 2.

1.4.6 Yeast interactions and their impact on wine quality

Yeast-yeast interaction can be broadly classified as those between different species of *Saccharomyces* with non-*Saccharomyces* yeast, between different non-*Saccharomyces* yeast and those between different species/strains of *Saccharomyces* yeast during fermentation (Ciani *et al.*, 2010). These interactions range from being antagonistic, neutral and beneficial to one another and play an important role in the survival of yeast during fermentation (Ciani *et al.*, 2010; Fleet, 2003). From a commercial point of view, these interactions can be potentially harnessed to drive wine flavour and quality. Indeed, there is an increasing demand to commercialize multi-starter yeast mixtures that are capable of imparting complex character in wine (Ciani *et al.*, 2010). This demand has seen the commercialization of multi-starter yeast mixtures comprising different strains of *Saccharomyces* yeast
or a blend of both *Saccharomyces* and non-*Saccharomyces* yeast (Ciani and Comitini, 2011). These concepts are discussed in detail in chapter 3 and 4.

Howell *et al.* (2006) demonstrated the importance of *Saccharomyces* yeast interaction and their impact on chardonnay, while Ciani *et al.* (2010) have outlined the range of interactions between non-*Saccharomyces* and *Saccharomyces* yeast impacting on final wine quality. Another study carried out by Anfang *et al.* (2009) on the ability of an isolate of *P. kluyveri* to influence varietal aroma of Marlborough (New Zealand) Sauvignon Blanc when co-fermented with *S. cerevisiae*. Most of the studies to date have focussed on the impact of non-*Saccharomyces* yeast on *S. cerevisiae*; however, there have been studies which have shown that other *Saccharomyces* yeast are capable of dominating and driving fermentation (Eglinton *et al*., 2000; Naumov *et al*., 2000). There has been little research conducted evaluating the interaction between non-*Saccharomyces* yeast and those that belong to *Saccharomyces sensu-stricto* (apart from *S. cerevisiae*). These concepts are discussed in chapter 3, 4 and 5.

Many studies have focussed primarily on specific oenological characters when analysing the interaction between different yeasts (Ciani *et al*., 2010). However, a study was conducted on 34 non-*Saccharomyces* yeast isolated from winemaking environments and their range of responses to several winemaking conditions like response to SO₂ levels, β-glucosidase activity, protease activity, ester-hydrolase activity and killer toxin character were monitored (Ciani and Comitini, 2011). Also selected non-*Saccharomyces* yeast from this study were co-fermented with commercial *S. cerevisiae* EC1118 with three different inoculation concentrations, wherein researchers found unique responses from the non-*Saccharomyces* yeast to EC1118 and also to altered inoculation ratios resulting in variable growth kinetics and differences in both soluble and volatile metabolite production (Ciani and Comitini, 2011). This study sets a good example to show that there is untapped biodiversity and oenological phenomena in the natural yeast associated with wine and that these mixed fermentations are highly dependent on their environment. Therefore it is also important to undertake research into behaviour of co-fermentations under a range of environmental parameters to unearth the full oenological potential of these interactions.

One of the aims of my thesis was to unearth yeast-species interactions apart from the obvious antagonistic behaviour of *Saccharomyces* during un-inoculated fermentation (Goddard, 2008). This aim is supported by numerous studies carried out to unravel the dynamics of yeast during the vinification of a variety of wines from all over the world (Lopandic *et al*., 2008; Naumov *et al*., 2002; Pramateftaki *et al*., 2000; Torija *et al*., 2001). There have been studies exploring the change in population dynamics of yeast during the un-inoculated fermentation of a variety of wine (Lopandic *et al*., 2008). Although, *Saccharomyces* species came to dominate the ferment in most of these studies, the changes to the non-*Saccharomyces* yeast species dynamics were varied (Lopandic *et al*., 2008;
Naumov et al., 2002; Pramateftaki et al., 2000; Torija et al., 2001). The variation in the population dynamics of non-\textit{Saccharomyces} yeast in these studies can be attributed to differences in winemaking conditions, type of wine and sampling of yeast species. Therefore these studies have emphasised the uniqueness of different un-inoculated fermentations. As a result of these studies, yeast population dynamics of a un-inoculated ferment of New Zealand Sauvignon Blanc was examined, this is discussed in detail in chapter 2. The yeast population uncovered was then studied under different physiological conditions both individually and in co-ferments to un-earth potentially novel yeast-yeast interactions, these concepts are discussed in chapters 2, 3 and 4.

1.5 History and prominence of New Zealand Sauvignon Blanc

1.5.1 History of winemaking in New Zealand

Samuel Marsden planted the first grape vines in 1819 in Keri Keri in New Zealand (Scott, 1964). It took more than 150 years before New Zealand’s wine growers and wine makers started producing internationally acknowledged wines. In 1974, Matua Valley Wines produced the first trial wine from Sauvignon Blanc at Waimauku, near Auckland, in 1979; Montana released its first Sauvignon Blanc from Marlborough and commercially established that variety (Cooper and McDermott, 1996). In the 1980s Montana’s piercingly herbaceous, a more subtle and riper Cloudy Bay and Hunter’s wines were responsible for alerting the world to the unique quality of New Zealand Sauvignon Blanc (Cooper and McDermott, 1996). Sauvignon Blanc wines were single-handedly responsible for introducing New Zealand wine to the world. In particular, Sauvignon Blanc from the Marlborough region located at the top of New Zealand’s South Island is known for its own distinctive style. Marlborough Sauvignon Blanc is intensely fragrant, more obviously fruity than the Loire prototype, with just a hint of both grass and sweetness and, occasionally, gooseberries and asparagus. That blend of tropical fruit flavour (melons, pineapple and passion fruit) coupled with gooseberry and capsicum based herbaceous character is usually regarded as New Zealand’s national Sauvignon Blanc wine style (Robinson, 2006).

1.5.2 Marlborough Sauvignon Blanc

The distinctiveness of Marlborough Sauvignon Blanc can also be attributed to the different winemaking techniques utilized by New Zealand winemakers when compared to Sauvignon Blanc produced in the old world (Sancerre and Loire) (Lund et al., 2009; Nicolau et al., 2006). In France, the use of oak barrels in quite common with Sauvignon Blanc whereas in New Zealand, winemakers choose to employ simpler techniques focusing on enhancing varietal fruity characters rather than aiming for complexity and longevity (Lund et al., 2009; Nicolau et al., 2006). Fermentation is
carried out in stainless steel tanks usually at low temperatures (12-18°C). These winemaking techniques together with viticultural practices, soil and climate are thought to be responsible for the unique New Zealand style of Marlborough Sauvignon Blanc.

Sauvignon Blanc is the most planted grape variety in New Zealand, and the Marlborough region has the largest area under vine and is the biggest producer of this wine variety. According to the 2011 winegrowers annual report, 69.1% of the grapes harvested were Sauvignon Blanc indicating the ever-growing dependence of New Zealand wine export market on this wine variety. In 2011 the wine industry was the ninth biggest export industry in NZ with an export value of 1.1 billion dollars making it an important economic product (2011). However, in the last 30 years, the diversity of white wines and winemaking techniques have steadily declined to accommodate an increasingly globalized wine market catering to standardized consumer tastes (Ciani et al., 2010). This has lead towards a general trend in wine producers imitating a few universally appreciated wine models such as Marlborough Sauvignon Blanc. As a result there is now direct competition for Marlborough Sauvignon Blanc from cooler wine producing countries such as Chile, South Africa and certain parts of USA. Therefore there is an ever-increasing need to further drive and develop the New Zealand style of Sauvignon Blanc.

Since 2004, a research initiative on New Zealand Sauvignon Blanc has been funded by The New Zealand Winegrowers, the Foundation for Research, Science and Technology (FRST) and later by Ministry of Science and Innovation (MSI) involving the University of Auckland, Lincoln University, the Plant and Food Research Institute of New Zealand Ltd, and the Marlborough Wine Research Centre. Along with the support of New Zealand wine industry the goal of this research programme is to understand the different aspects of the viticulture and winemaking that result in the distinctive style of Marlborough Sauvignon Blanc. Different disciplines such as Microbiology, Chemistry, Molecular Biology and Sensory Science have been brought together in the programme to provide the industry with new insights and knowledge. My doctoral research was funded and aided by this programme and focuses primarily on the influence of yeast interactions on Marlborough Sauvignon Blanc.

1.6 Characteristics of Sauvignon Blanc

Sauvignon Blanc is a green-skinned grape variety, and has its origins from the Bordeaux region of France. This variety is planted widely around the world; it can be made into a varietal wine or blended to produce dessert wine (Darriet et al., 1995). Depending on the climate Sauvignon Blanc wine flavour can range from very grassy to sweetly tropical (Darriet et al., 1995). The must from Sauvignon Blanc is “simple flavoured” and almost odourless, however the wine produced from these musts have the above mentioned characteristic aromas relatively specific to this variety (Nicolau et
Peynaud and Spencer, 1984). Darriet et al. (1995) and Tominaga et al. (1998b) showed that volatile sulphur compounds called thiols present in wine were responsible for the characteristic aromas present in Sauvignon Blanc wines. Aroma of New Zealand Sauvignon Blanc is influenced by thiols called 3-mercaptohexan-1-ol (3MH) and 3 mercaptohexyl acetate (3MHA), which are responsible for the characteristic notes of passionfruit and grapefruit (Lund et al., 2009; Nicolau et al., 2006).

1.6.1 Importance of volatile thiols in Sauvignon Blanc

Volatile thiols are present in extremely low concentrations in wine in the range of a few hundred nano grams per litre, however they have extremely low perception threshold in humans which make their contribution significant to the aroma and flavour of Sauvignon Blanc wine (Tominaga et al., 1998a). Initial experiments from Darriet et al. (1995) who produced the first aroma gram for Sauvignon Blanc wine showed that there were two aroma zones which evoked the characteristic aromas of Sauvignon Blanc which became odourless with the addition of copper to the wine. In addition, one can perceive Sauvignon like aroma after consuming the nearly odourless must through retro-olfaction (Peynaud and Spencer, 1984; Tominaga et al., 1998c). This information coupled with experiments conducted by Tominaga et al. (1995 and 1998c) who used cell free extracts of Eubacterium limnosum for the in vitro production of thiols 4MMP, 4MMPOH and 3MH from Sauvignon must, suggested the involvement of a S-cysteine β-lyase like activity for the liberation of thiols from S-cysteine-conjugates which are odourless and probably present in grape must. These precursor molecules were then formally identified by Tominaga et al. (1998b). Important varietal thiols and their odour characteristics are discussed in chapter 4.

It has been suggested that thiol precursors are odourless S-cysteine-conjugates present in grape must, and they are converted into volatile thiols by yeasts during the fermentation process (Swiegers et al., 2007; Tominaga et al., 2000). Although the genes and pathways involved are yet to be elucidated, it can be said that yeasts have an important role in producing characteristic qualities in Sauvignon Blanc wine. Apart from thiols, yeast are also responsible for the production of a whole suite of volatile sulphur compounds ranging from hydrogen sulphide, methanethiol, dimethylsulfide, methylthioesters like S-methylthioacetate, S-methyl thiopropanoate etc. which contribute to odours ranging from rotten eggs to cooked cabbages to cheesy aromas (Swiegers et al., 2007; Swiegers et al., 2009). These compounds can be less or more attractive or contribute to off odours depending on their absolute and relative concentrations (Swiegers et al., 2006b). For example, excessive production of the volatile thiol 4MMP during fermentation can give an intense cat’s pee aroma to the Sauvignon Blanc wine,
whereas 4MMP in lower concentrations can be a pleasant aroma of blackcurrants (Nicolau et al., 2006). Modulating these quality controlling compounds in wine in accordance to consumer requirements poses a challenge to the winemakers. The strain of yeast used for fermentation, ferment conditions and nutritional status of the wine has an impact on the concentrations of these compounds, little is known about different parameters regulating production of volatile sulphur compounds (Swiegers et al., 2006a).

1.6.2 Unravelling the mechanism of thiol production

In recent years, the French and Australian wine research in Sauvignon Blanc has concentrated on the mechanism of thiol production by yeast. This research is mostly based on Tominaga’s hypothesis that a S-cysteine β-lyase like activity is required to liberate thiols from precursors (Tominaga et al., 2000). Howell et al. (2005) conducted research wherein genes encoding putative yeast S-cysteine β-lyase were deleted in the lab strain of S. cerevisiae and tested for their ability to release 4MMP from Cys-4MMP. Four different genes were identified as being important; this suggested that a complex pathway involving many genes might be responsible in thiol production by yeast (Howell et al., 2005). However, Swiegers et al. (2007b) over-expressed a heterologous β-lyase in the wine strain of S. cerevisiae VL3: they showed that the engineered wine yeast was then capable of releasing 10 times more 4MMP from the precursor Cys-4MMP when compared to control ferment. Therefore it is possible to engineer wine yeast to produce more thiols during fermentation. But there is still a low acceptance of engineered yeast for the large-scale production of commercial wine. However, in recent years many alternate genes and pathways have been suggested for their involvement in thiol production, these studies are reviewed in chapter 4.

1.6.3 A holistic approach to understanding the mechanism of thiol production during fermentation

There are many studies which support the holistic approach to understanding the mechanism of thiol liberation by yeast during ferment. Firstly, there is evidence that different commercial strains have varying ability to liberate thiols during fermentation; for example, S. cerevisiae strains VL3 and EG8 release more thiols compared to VL1 and 522d, S. bayanus strains are able to release more 4MMP compared to VL3 and EG8 and S. cerevisiae/S. bayanus hybrids are shown to elevate thiols levels in a ferment (Dubourdieu et al., 2006; Howell et al., 2004; Murat et al., 2001a). Secondly, experiments conducted by Howell et al. (2006) showed that co-ferments of various commercial wine yeast showed a unique and different thiol profile when compared to the profile of wine made with the same yeast which were then blended This suggested that the final aroma of the thiols perceived from a wine is a lot more complex than the mere presence or absence of these aroma compounds (Howell et al., 2006).
Also, that co-fermenting yeasts interact with one another in an unpredictable manner adding to the final complexity and aroma of the wine. Therefore winemakers need to consider the yeast species interactions as an important parameter in enhancing the aroma and complexity of wine (Ciani and Comitini, 2011).

1.6.4 Importance of non-\textit{Saccharomyces} yeast in aroma production in Marlborough Sauvignon Blanc

Most of the research conducted on elucidating mechanisms for thiol production has concentrated on wine strains belonging to the \textit{Saccharomyces sensu stricto}. But, Anfang \textit{et al.} (2009) have been able to show that non-\textit{Saccharomyces} yeast species are capable of producing high levels of thiols on their own and are also able to elevate the thiol levels in Sauvignon Blanc wine when co-fermented with certain commercial wine yeast strains of \textit{S. cerevisiae}. They have been able to show that a natural isolate of \textit{Pichia kluyveri} when co-fermented with wine yeast (\textit{S. cerevisiae}) VL3 in the inoculating ratio of 9:1 will predictably elevate 3MH levels by up to 7 times in co-ferments compared to control ferments (Anfang \textit{et al.}, 2009). This is not only evidence for some sort of synergistic interaction between these two yeasts but also a predictable tool for winemakers wishing to enhance thiols in their wine. This approach has given us an insight into the complex mechanism of thiol production in that it has revealed a reproducible way of yeast species interaction in the fermenting media; the in-depth analysis of which could provide key answers that determine the production of thiols by yeast during fermentation (Anfang \textit{et al.}, 2009).

1.6.5 Techniques involved in analysis of fermentation and of aroma production in wine

Increasingly there is evidence that non-\textit{Saccharomyces} yeasts are involved in aroma production during fermentation. A study showed that a mixed ferment of \textit{S. cerevisiae} with \textit{H. guilliermondi} and with \textit{P. anomala} yielded higher concentrations of aromatic acetate esters compared to single ferments of \textit{S. cerevisiae}, indicating that there is an underlying interaction between these yeast species which results in increased acetate ester formation (Rojas \textit{et al.}, 2003). Studying the above mentioned species interactions using advanced techniques in transcriptomics, proteomics and metabolomics could provide insights into the mechanisms involved in aroma production by yeast. Most research in aroma production by yeast in wine has concentrated on the microbiology, molecular biology and organic chemistry techniques (Querol and Fleet, 2006). There have been small-scale studies analysing different wine yeast for their abilities to produce various odorant chemicals (Ribéreau-Gayon \textit{et al.}, 2006a). There have also been microarray analysis and gene expression studies carried out for specific compounds under specific conditions (Borneman \textit{et al.}, 2007). But there have been few systemic studies to understand complex reactions and pathways involved during fermentation, furthermore
most of these studies concern the impact of winemaking strains of *S. cerevisiae* and none take into account the impact of non-*Saccharomyces* yeast (Borneman *et al.*, 2007). Few scientists have tried to analyse the impact of the fermenting conditions on *S. cerevisiae*; for example researchers used a transcriptomic and proteomic approach to understand the adaptation of *S. cerevisiae* to fermenting conditions (Zuzuarregui *et al.*, 2006). They focused on comparing the difference in mRNA and proteomic profiles of two commercial wine yeast strains ICV 16 and ICV 27 at a time point when the strains entered a stationary growth phase; they identified many differences in gene expression and protein profiles which could explain specific characteristics of that particular wine strain (Zuzuarregui *et al.*, 2006). They discovered an over-accumulation of proteins involved in sulphur assimilation pathways in protein profiles of ICV 27 in comparison to ICV 16 which could explain the organoleptic properties of the resulting wine (Zuzuarregui *et al.*, 2006). This study also revealed some of the key proteins involved in pathways important during fermentation such as glucose metabolism, methionine biosynthesis and stress-related mechanisms etc. interestingly they were able to correlate some of the transcriptomic changes to changes in protein expression profiles (Zuzuarregui *et al.*, 2006). This study encourages further use of proteomics and transcriptomics on yeast during fermentation.

Another study relating to the identification of various beer brewing strains of *S. cerevisiae* the researchers were able to successfully identify and characterize 9 different strains of *Saccharomyces* yeasts based on their metabolisms during fermentation; furthermore they were able to differentiate species using their metabolites when their genomes could not be differentiated (Pope *et al.*, 2007). These initial studies pave the way for a systems biology approach to understanding the fermentation process and the underlying mechanisms by which yeast manipulate their environment and the various stress related mechanisms that help them survive in harsh conditions (Borneman *et al.*, 2007). Understanding these processes will help in engineering better winemaking strains or in tailoring winemaking conditions in a specific way to obtain a certain character. Nevertheless these approaches will definitely enhance the tools available to winemakers and help them make wine to suit the consumer demands (Borneman *et al.*, 2007). Indeed, one of my research aims was to analyse the global yeast proteome along with the extracellular metabolites in wine resulting from a co-ferment of Marlborough Sauvignon Blanc by *P. kluyveri* and *S. cerevisiae*. Holistic approach of this study could be used as a potential pipeline for the in-depth study of microbial species interactions in wine as outlined in figure 1.2.
Figure 1.2: A systems biology pipeline to identify and characterise yeast interactions in wine fermentation. The pipeline shaded grey is the protocol used in this research (although transcriptomics work not carried out by me) to obtain in-depth information regarding a specific yeast interaction. Overall, this pipeline can be used to mine, identify and obtain in-depth information regarding yeast community interactions in fermentation. Adapted from (Zoetendal et al., 2008).
1.7 Research aims

Microbial species interaction is the driving force of bio-geo-chemical cycling on this planet and knowledge of the fundamental rules that govern these interactions is of vital importance (Jessup et al., 2004). In this research, yeast species interaction during alcoholic fermentation of New Zealand Sauvignon Blanc grape must (juice) was explored and studied. Apart from gaining an understanding of specific yeast interactions, the primary aim of this research was to explore the applicability of yeast species interactions in driving a unique style of New Zealand Sauvignon Blanc. These aims were supported by crucial findings on the yeast interactions capable of enhancing the concentration of varietal aroma of New Zealand Sauvignon Blanc (Anfang et al., 2009). Several studies have been published regarding the influence of multiple yeast species on final wine quality; however, little is understood about these interactions and the range of environmental conditions under which these operate (Ciani and Comitini, 2011; Fleet, 2003). Therefore in order to understand the above mentioned concepts, the aims of this research were as follows:

1. Examine the population dynamics of yeast in a un-inoculated ferment (commercial quality) of Marlborough Sauvignon Blanc (Chapter 2)
2. Test the influence of a range of oenological conditions on the growth and fermentation capabilities of different yeast species (Chapter 3)
3. Test for yeast interactions and their impact on wine aroma in a range of oenological conditions (Chapter 4)
4. Minutely examine the interaction between S. cerevisiae VL3 and P. kluyveri PKKR1 during the fermentation of Marlborough Sauvignon Blanc using systems biology tools (Chapter 5)
Isolation and characterization of yeasts from a uninoculated Marlborough Sauvignon Blanc ferment
2.1 Introduction

Despite increasingly favourable opinions of non-Saccharomyces yeast and natural strains of Saccharomyces yeast, very little research has been conducted to elucidate the un-inoculated yeast population dynamics in the new world wines, even less is known about their involvement in the production of favourable aromas (Cocolin et al., 2001; Jolly et al., 2003, 2006; Mercado et al., 2007). New Zealand has a unique biodiversity of yeasts associated with winemaking process; however, only one of these yeasts is used in the commercial sector (Goddard et al., 2010). In this study I wanted to elucidate the natural yeast community present in a un-inoculated ferment of New Zealand Marlborough Sauvignon Blanc and their relevance in producing varietal characters to the wine.

2.2 Importance of yeast in modern winemaking

The composition and properties of many grape varieties have been extensively examined (Peynaud and Spencer, 1984; Ribéreau-Gayon et al., 2006a). The quality of a wine is highly dependent on the components in the grape juice and the microorganisms (yeast and bacteria) involved in fermentation (Chambers and Pretorius, 2010; Fleet, 2003; Querol and Fleet, 2006; Romano et al., 2003). Winemaking is an ancient art that has undergone many changes throughout history and has now adapted to a modern industrialised process. Commercial winemaking involves inoculating the pre-treated juice with any of the widely available active dry cultures of Saccharomyces cerevisiae to produce wine of consistent quality (Chambers and Pretorius, 2010; Lambrechts and Pretorius, 2000; Pretorius, 2000). Fermentation is usually carried out in temperature controlled steel tanks (for white wine), filtration, fining and finally bottling is now performed automatically under controlled industry standards (Ribéreau-Gayon et al., 2006b; Swiegers et al., 2006a). Despite the many advantages of commercial winemaking, it is known to diminish the natural yeast population present in the fermentation process, and this could lead to a lack of variety in sensory properties of the wine (Ciani et al., 2010). It is argued that although commercial yeast are developed to withstand a range of conditions and to impart specific oenological characters to wine, their use can somehow diminish the distinctiveness and regionality of a wine (Ciani et al., 2010). In fact, some of the old world wines are indeed coveted for their regional specific characters and still maintain traditional winemaking techniques (Ribéreau-Gayon et al., 2006a). However, the role of native yeasts in the production of region specific wines is proving to be increasing valuable but it is yet to be completely understood (Pretorius, 2000; Romano et al., 2003). A few studies have examined the impact of un-inoculated fermentation on varietal aroma (Clemente-Jimenez et al., 2004; Francesca et al., 2010).
2.3 Marlborough Sauvignon Blanc

Although, New Zealand is a relative new-comer to the world’s wine market, it is well known for its unique wines (Robinson, 2006). Especially, Sauvignon Blanc from the Marlborough region is well regarded for its distinctive character. The distinctiveness of Marlborough Sauvignon Blanc has been attributed to its high concentrations of varietal aromas (Lund et al., 2009; Nicolau et al., 2006). In winemaking, varietal aromas are compounds which define or have a high impact on the final aroma profile of a particular wine (Fleet, 2003; Ribéreau-Gayon et al., 2006a; Romano et al., 2003). In New Zealand Sauvignon Blanc, volatile sulphur containing compounds called polyfunctional thiols are one of the major impact chemicals which define its final aroma (others include esters, C₆ alcohols and methoxypyrazines). The thiols 3-mercaptotetrahexan-1-ol (3MH), 3-mercaptohexyl acetate (3MHA), and 4-mercapto-4-methylpentan-2-one (4MMP) to a lower extent play an important role in attributing distinctiveness to Marlborough Sauvignon Blanc (Nicolau et al., 2006). Their aroma properties and chemistry is discussed more in detail in chapter 4.

Aromatic thiols are produced during fermentation and their final concentration can vary depending on the oenological parameters used, one being the yeast species used to ferment the wine (Swiegers and Pretorius, 2007). About 90% of New Zealand Sauvignon Blanc is produced by employing commercial winemaking techniques which include inoculating with a single overseas strain of *S. cerevisiae* (personal communication, M. Brajkovich MW). However, Anfang et al. (2009) were able to show that varietal aroma of Marlborough Sauvignon Blanc could be elevated by the usage of a strain of *Pichia kluyveri* isolated from a New Zealand vineyard and co-fermented with certain commercial strains of *S. cerevisiae*. Therefore, it is feasible to propose that natural isolates of NZ yeast, if allowed to ferment, might possess desirable attributes and be able to introduce a sense of regionality to New Zealand Sauvignon Blanc.

There are a few commercial wineries which produce New Zealand Sauvignon Blanc made using naturally occurring yeast. For example distinctive and award winning Marlborough Sauvignon Blanc has been made at Kumeu River, located in West Auckland, using un-inoculated fermentation. Therefore an in-depth analysis of this type of ferment and wine would provide an opportunity to understand the effect of local yeasts on Marlborough Sauvignon Blanc.
2.4 Natural or Un-inoculated fermentation of wine

The natural fermentation of grape juice is a complex biochemical process during which a succession of local yeast flora utilize the various nutrients present and transform the grape sugar into ethanol and carbon dioxide and a range of secondary metabolites (Fleet, 2008; Lambrechts and Pretorius, 2000). These wine yeasts play an important role in influencing the composition, aroma, flavour and therefore the quality of the final wine (Fleet, 2008). A variety of microorganisms are naturally present in grape juice, one recent NZ study identified 18 species of fungi present on ripe Chardonnay and Syrah fruit (Gayevskiy and Goddard, 2012). In this thesis, only the yeast community involved in un-inoculated fermentation of Sauvignon Blanc grape juice to wine was examined. The fermenting yeast community is classically divided into the yeast belonging to genus *Saccharomyces*, and others being designated as non-*Saccharomyces* yeast (Fleet and Heard, 1993).

Typically in a successful natural fermentation, non-*Saccharomyces* yeasts dominate early on and are then replaced by *Saccharomyces* yeast species which carry out the majority of alcoholic fermentation (Fleet and Heard, 1993; Goddard, 2008; Martini, 1993). In the early stages of fermentation, the most common non-*Saccharomyces* genera typically encountered are: *Candida*, *Hanseniaspora*, *Kloeckera*, *Pichia*, *Rhodotorula*, *Torulaspora* and occasionally *Issatchenkia* and *Metschnikowia* (Clemente-Jimenez et al., 2004; Fleet, 2003; Herraiz et al., 1990; Romano et al., 2003). Studies have shown that these yeasts are capable of reaching considerable population sizes up to $10^7$ to $10^8$ cells/mL (Fleet, 2003; Sipiczki et al., 2001; Torija et al., 2001). Some non-*Saccharomyces* yeast have been shown to survive into the later stages of fermentation where ethanol concentrations are higher (around 7%), while some species of *Candida* have been shown to be surviving even at the latter stages of fermentation (Ciani and Picciotti, 1995; Fleet, 2003).

*Saccharomyces* species are the predominant wine yeasts, they are also known as Crabtree positive yeast in context of fermentation (Goddard, 2008). The Crabtree effect refers to the phenomenon of anaerobic assimilation of sugar by yeast in a high sugar solution such as grape juice, even in the presence of oxygen. *Saccharomyces cerevisiae* in particular is favoured by winemakers as it has evolved a way of engineering the fermentation environment to actively eliminating other competing yeast by toxifying the environment with the production of ethanol, CO$_2$ and heat (Goddard, 2008). Even though majority of sugar assimilation is carried out by *S. cerevisiae* it’s rarely found in early stages of fermentation, additionally, there is also a large genetic diversity with many strains fermenting the grape must (Goddard et al., 2010; Torija et al., 2001). In a commercial ferment, the inoculated *S. cerevisiae* is present in large numbers compared to any other naturally present yeast, but in a un-inoculated ferment it has to co-exist with a suite of other yeast species. Therefore it can be hypothesised that the metabolism of *S. cerevisiae* during natural fermentation is in contrast to its...
metabolism in a commercial ferment (a non-competitive sterile medium), and this could be an important distinction in the development of complex aroma in wine (Ciani et al., 2010; Fleet, 2008). Understanding the diversity of Saccharomyces yeast in a un-inoculated ferment of Marlborough Sauvignon Blanc (MSB) could prove to be an important factor in making distinctive NZ wines.

2.5 Importance of non-Saccharomyces yeast in fermentation

In the past decade there has been a growing demand for developing and tailoring yeast capable of fermenting different varieties and styles of wine (Ciani et al., 2010; Fleet, 2008). An increasingly popular approach to improve chemical and sensory properties of wine is by using multi-starter cultures made of a mixture of non-Saccharomyces and Saccharomyces wine yeast strains (Bisson and Kunkee, 1993; Boulton et al., 1998; Swiegers et al., 2005). In the last couple of decades there have been investigations looking at persistence of non-Saccharomyces yeast in both inoculated and un-inoculated fermentations and their impact on the oenological properties on wine (Moreira et al., 2005; Romano et al., 2003; Swiegers et al., 2005; Viana et al., 2008; Zironi et al., 1993). Various non-Saccharomyces yeast species have shown β-D-xylosidase and β-glucosidase activities, these enzymes are involved in the production of favourable wine aromas (Ferreira et al., 2001; Manzanares et al., 1999; Strauss et al., 2001). Aside from the presence of favourable enzymes, it was showed that mixed starter cultures of Hanseniaspora guilliermondi and H. uvarum with S. cerevisiae are capable of enhancing desirable esters without increasing levels of undesirable sulphur compounds (Moreira et al., 2008). In a NZ study, it was showed that a natural isolate of Pichia kluyveri when co-fermented with an industrial yeast strain is capable of increasing varietal wine aroma (Anfang et al., 2009).

Most non-Saccharomyces yeast are incapable of fermentation, but an increasing number of studies have shown that these yeasts are capable of having a positive impact on final wine aroma (Ciani and Maccarelli, 1998; Fleet and Heard, 1993; Gil et al., 1996; Herraz et al., 1990; Jolly et al., 2006; Rojas et al., 2001; Viana et al., 2008; Zironi et al., 1993). Many non-Saccharomyces yeast have been found to be good co-fermenting partners to Saccharomyces cerevisiae strains (both commercial and natural). Ciani et al. (2010) review the use of non-Saccharomyces yeast to enhance wine aroma.

There is now evidence that natural yeast populations are capable of having a beneficial impact on the final aroma of wine (Erten, 2002; Fleet, 2008). However, the natural population of yeast on grapes is different under different conditions dictated by various environmental factors such as geographical location, climate, seasonal changes related to humidity, temperature etc. (Gayevskiy and Goddard,
2012; Heard and Fleet, 1985; Lambrechts and Pretorius, 2000). Therefore it is important to identify and characterise the native yeast population separately for each region.

It was shown that non-\textit{Saccharomyces} yeasts were greatly affected by vinification temperature while changes to pH and sugar content seemed not to alter their growth (Charoenchai \textit{et al}., 1998). Charoenchai \textit{et al}. (1998) investigated 22 species belonging to the genera of \textit{Candida}, \textit{Pichia}, \textit{Kloeckera}, \textit{Torulaspora}, and \textit{Saccharomyces}. The vinification factors pH and sugar content did not result in significant growth advantages for any of the yeasts in this study. Differing temperatures can significantly impact the growth dynamics of the yeasts and probably affect the ecology of fermentations (Charoenchai \textit{et al}. , 1998; Goddard, 2008). Lower temperatures allowed faster growth of non-\textit{Saccharomyces} species in comparison to \textit{S. cerevisiae} (Charoenchai \textit{et al}. , 1998). Other oenological conditions such as availability of assimilable nitrogen also play an important role in the population dynamics of a un-inoculated ferment (Bell and Henschke, 2005). In order to understand the exact nature of the contribution of natural yeasts to wine; along with their identity, their diversity, growth kinetics, biochemical properties and sensitivity to oenological parameters need to be elucidated (Lambrechts and Pretorius, 2000). Therefore, in this chapter, the identity, changes in population dynamics of both \textit{Saccharomyces} and non-\textit{Saccharomyces} yeast along with production of varietal aromas were all examined simultaneously from a un-inoculated fermentation of Marlborough Sauvignon Blanc.

Accurate identification and characterization of yeast is a time-consuming process and requires the usage of modern molecular biology techniques along with techniques to characterise morphology and physiology (Chambers and Pretorius, 2010; Ribéreau-Gayon, 1985). Some of the current molecular biology techniques used for identification of yeast is discussed below.

2.6 Evaluation of the molecular techniques for identifying and understanding the diversity of the natural yeast population

In the past few decades, molecular techniques have been increasingly applied to identifying yeast species and strains. White \textit{et al}. (1990) developed a protocol of amplifying the ribosomal Internal Transcribed Spacer (ITS) region. Profiling of ITS along with the coding 5.8 rDNA regions have been useful in differentiating between yeast species (Esteve-Zarzoso \textit{et al}., 1999; Kurtzman, 1992; White \textit{et al}., 1990). The ribosomal regions evolve in a concerted manner which has rendered them to have low intraspecific differences but have high interspecific variability; thus a technique based on their amplification and subsequent restriction digestion with certain endonucleases enabled Esteve-Zarzoso \textit{et al}. to identify and differentiate between 132 yeast species encompassing telomorphic and
anamorphic *Ascomycetes* and *Basidiomycetes* (Esteve-Zarzoso et al., 1999). These methods are highly reliable, however if the yeast sequences are different by more than 1% to that held in a database, a second method of detection is required; sequencing the 26 S rDNA is a powerful diagnostic tool for identifying the different yeast (Kurtzman and Robnett, 1995). In this thesis all the yeast isolates were identified both by ITS-RFLP technique and partial 26 S rDNA sequencing and were compared to the sequences deposited in the CBS (Centraalbureau voor Schimmelcultures Utrecht, The Netherlands) database.

The ITS-RFLP techniques and partial sequencing 26 S rDNA are powerful tools for identifying different yeasts but are limited in their use for phylogenetic and diversity examinations (Kurtzman and Fell, 1998). A study concluded that examination of the diversity of *Saccharomyces* yeast species requires different techniques (Redžepović et al., 2002). Another technique routinely used to fingerprint yeast is to amplify the highly variable microsatellites (simple sequence repeats) dispersed throughout the yeast genome (Field and Wills, 1998; Legras et al., 2005; Richards et al., 2009). Based on several studies on *S. cerevisiae*, microsatellite method was found to be reproducible in differentiating between strains, thus providing a powerful tool to examine diversity along with the others discussed above (Schuller et al., 2004). Based on previous microsatellite work, an in house protocol was developed to differentiate *S. cerevisiae* and *S. bayanus* (un-published) capable of accurately differentiating between a billion strains (Richards et al., 2009).

### 2.7 Aims

Little work has been conducted on the relationship between yeast species and resulting thiol levels in a Marlborough Sauvignon Blanc (Anfang et al., 2009). Thus understanding the diversity of fermenting *Saccharomyces* yeast in an un-inoculated ferment is the key first step in unearthing yeast species capable of producing novel ranges of characteristic aromas. These yeasts can potentially help tailor New Zealand Sauvignon Blanc wine. In this chapter I examined a commercial un-inoculated fermentation of MSB, and the resulting wine. The flow chart (Figure 2.1) presented summarises the sampling, isolation, identification and monitoring protocol for yeast and also the analysis of varietal aroma from a un-inoculated MSB.
The general aims of the work communicated in this chapter are:

1) To identify the yeasts involved in a commercial un-inoculated Marlborough Sauvignon Blanc ferment.

2) Monitor the change in yeast population during a commercial un-inoculated Marlborough Sauvignon Blanc fermentation.

3) Track the evolution of important thiols during the fermentation of a commercial un-inoculated Marlborough Sauvignon Blanc ferment.
2.8 Methods and Materials

2.8.1 Sample handling, collection and storage

All juice and wine samples were collected from a un-inoculated commercial ferment of Marlborough Sauvignon Blanc from Tank 47 at Kumeu River Winery in West Auckland between 7th and 28th April 2008. The head winemaker of Kumeu River, Mr Michael Brajkovich (MW), provided the information regarding juice parameters: pH 3.2, Brix° 24.7, TA 7.7 g/L and YAN 254 g/L. The juice/wine was monitored for changes in density (sugar concentration), temperature and yeast population throughout this time period. Initially the temperature of the ferment was not controlled and when it reached around 20°C, the fermentation tank was stabilised to that temperature. Overall twelve samples were collected starting from 7th till the 28th April mostly on alternate days except during peak fermentation rate when they were taken every day (between 17th to 20th April). At each sample time-point, the tap outlet on the tank was thoroughly rinsed with hot water before being cleaned with 70% ethanol solution, after which the tap was left open for about 20 seconds before collecting approximately 300mL of sample in 6-50 mL capacity sterile Falcon tubes, it was then transported back to the lab on ice (~4°C). The 6 falcon tubes were mixed vigorously before 300 µL of the sample was taken out from each and placed in a 15 mL capacity sterile tube to perform tests. Next, the 6 sample tubes were centrifuged for 10 min at 6000g at room temperature, the clear supernatant was transferred into sterile 50 mL containers while some of the pellet (~6mL volume) was stored in 15% glycerol and the remaining stored in separate sterile containers. Both the pellets and supernatant was stored at -80°C for further analysis.

2.8.2 Determination of yeast cell counts and viability via dye reduction and Haemocytometer counting chamber

A dye reduction technique was used to differentiate viable and dead yeast cells from the fermentation samples collected in section 1.2.1. The dye, Methylene Blue changes in colour from blue to colourless when it is used as a Hydrogen acceptor instead of Oxygen, this occurs at the surface of viable cells: hence the viable cells render methylene blue colourless while the dead cells retain a blue-black appearance (Jackson, 2000). Methylene blue solution was prepared by adding 0.2g of Methylene blue into a 100mL of 0.2% solution of tri-sodium citrate solution in water, it was then stirred thoroughly before being filtered using a Whatmans’s No 1 filter paper. Equal parts of the dye and sample were mixed in a 1.5 mL eppendorf tube for the early parts of the fermentation for enumeration while a 1:100 dilution of the sample was carried out before being mixed in equal parts with the dye for enumeration of yeast from later time-points as explained below. The mixture was incubated at room temperature for approximately 10 min before enumeration of cells.
Enumeration of cells was carried out by placing approximately 10µL of sample in an Improved Neubauer Haemocytometer’s counting chamber under a glass coverslip. It was then placed under a light microscope and magnified 20 times. Cells in 4 random squares within the 4 sets of 16 large squares were counted from both the top and bottom counting chambers (Figure 2.2). Cells touching the top and left-hand side of the squares were counted while those on the bottom and right were not. Both the viable and colourless yeast cells and dead and blue coloured cells were counted. Enumeration was carried out in duplicate for each sample. The number of yeast cells present in the original sample was calculated using the following formula

\[
\text{Cfu/mL} = \frac{\text{number of cells counted}}{(\text{proportion of chamber counted})(\text{volume of chamber})} \left( \frac{\text{volume of sample dilution}}{\text{volume of original mixture in sample}} \right)
\]

Equation 2.1: Equation to calculate the cell population of yeast using a Neubauer Haemocytometer.

This was applied to both the colourless and blue yeast cells, thus the total number of cells and the number alive and dead were calculated for each sample.

![Figure 2.2: Counting chambers of a Neubauer Haemocytometer.](image)

2.8.3 Isolation and cfu estimation of yeast by culture technique

The cell counts obtained via haemocytometer were used as an estimate to determine the dilution factor required for plating. Appropriate serial decimal dilution was carried out; a thousand fold dilution range of the sample was spread plated separately to obtain a range of yeast present at different dilution and to enumerate the colonies. A nutrient rich medium of YPD was used to plate the samples, YPD agar is made up of 10g/L yeast extract, 20g/L casein-peptone and 20g/L of D-glucose and 15g/L of agar before being sterilised by autoclaving at 121 psi for 20 min and poured into petri dishes. The sample plates were then allowed to incubate at room temperature for 2-3 days to enable various yeasts
to form colonies. The colonies on each plate were counted and the average number of colonies from the triplicates for different dilutions was then multiplied with the respective dilution factor to obtain the cfu/mL. Simultaneously, 96 random colonies were picked using sterile toothpicks from the sample plates across different dilution series and sub-cultured on fresh YPD square plates formatted into eight rows and twelve columns as shown in figure 2.3 to carry out further analysis.

![Figure 2.3: Layout of yeast colonies on square YPD plates.](image)

### 2.8.4 Long term storage of colony isolates

Twelve samples were taken at various stages of fermentation, 96 colonies were isolated from each of these time-points as described in section 2.8.3. The sub-cultured isolates were then incubated for a further 2-3 days to allow for the development of colonies with a diameter of 1-3mm. These colonies were then suspended in 100-200µL (depending on the size of the colony) of 15% (v/v) glycerol solution in water sterilised by filtering through a membrane filter with pore size of 0.25µM. The glycerol stocks were then stored at -80°C in sterile plates with 96 wells in the same pattern the colonies were sub-cultured. All future work of identification and profiling was carried out by regrowing these colonies on YPD agar at room temperature from these glycerol stocks.

### 2.8.5 Yeast species identification

#### 2.8.5.1 ITS PCR and RFLP analysis

Firstly, yeast DNA was obtained by adding the desired yeast colony into 100-300 µL of 5% chelex solution mixed vigorously to distribute the colony into the chelex solution and incubated at 100°C for 10 min following which the mixture was mixed again and centrifuged at 13,000 rpm for 2 min. 

(Bradbury, 2004). A volume of supernatant containing DNA was then added to 4 times the volume of
ice cold 100% absolute ethanol to precipitate the DNA. This solution was left for 5 min followed by mixing and centrifugation at 13,000rpm for 2 min. The supernatant was then discarded and any ethanol was allowed to evaporate at room temperature in a Laminar flow. The resulting DNA pellet was then re-suspended in 50 µL of sterile water and stored at -20°C for future use.

ITS PCR was carried out using the primers ITS1 and ITS4 (table 2.1) as described by White et al. (1990). The reaction mixture consisted of 5µL of PCR buffer (10x stock solution without MgCl₂), 1.5 µL MgCl₂ (50mM), 5 µL dNTP (2 mM), 1µL ITS1 (10 pmol/µL), 1µL ITS4 (10 pmol/µL), 0.2 µL of Taq polymerase (5 U/ µL) about 2 µL of the DNA of the sample and sterile water to make the total volume up to 50 µL. The thermal cycling conditions followed were as described by White et al. (1990) with 54°C annealing temperature and 35 cycles. The band size of the ITS PCR amplified samples were then determined by electrophoresis through a 1% agarose gel at 100mV for 40 min followed by staining with ethidium bromide and imaging and photographing the gel under UV light using GEL DOC system using QUANTITY ONE software. A 1Kb+ ladder supplied by Invitrogen was run along with samples to enable ITS PCR product size estimation. To further confirm the identity of the yeast species of a sample the PCR product was then digested with restriction endonucleases Hae III and HinfI in separate digests. One unit of enzyme was used for 1 µL of amplified DNA along with the addition of appropriate buffer supplied with each enzyme kit to aid digestion. Digestion was carried out at 37°C for at least 4 h, and the digestion pattern was then determined by running them on 1% agarose gel as described above. This lead to yeast samples being grouped into cohorts of distinctive ITS and RFLP patterns, representative samples from each cohort was further processed before final identification.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITS 1</td>
<td>5’ TCC GTA GGT GAA CCT GCG G 3’</td>
</tr>
<tr>
<td>ITS 4</td>
<td>5’ TCC TCC GCT TAT TGA TAT GC 3’</td>
</tr>
<tr>
<td>NL 1</td>
<td>5’ GCA TAT CAA TAA GCG GAG GAA AAG 3’</td>
</tr>
<tr>
<td>NL 4</td>
<td>5’ GGT CCG TGT TTC AAG ACG G 3’</td>
</tr>
</tbody>
</table>

Table 2.1: ITS and 26S primers for yeast species identification.

2.8.5.2 26 S rDNA sequencing of samples for yeast species identification

ITS PCR followed by RFLP using HaeIII and HinfI allowed the isolates to be grouped into cohorts. DNA from the 26s ribosomal unit was then amplified for 3-4 isolates from each cohort followed by sequencing using the protocol detailed below to identify the isolates at a species level. The purified DNA from chosen isolates was amplified at its 26S rDNA region using the primers NL1 and NL4 (primer sequence listed in table 2.1) at following reaction conditions: 94°C for 2 min, followed by 35
cycles of 94°C for 1 min, 52°C for 2 min and 72°C for 2 min, and finished with 72°C for 10 min. The success of PCR amplification was assessed by gel electrophoresis. The PCR products were then purified using instructions and materials provided in the Quiagen™ PCR purification kit, the concentration of the purified product was determined using 1µL of the sample on the Nanodrop™ instrument. The concentration of amplified DNA was then adjusted to 10ng/µL following which 1µL was used for both forward (NL1) and reverse (NL4) sequencing.

Following sequence determination by the ABI 3700, the software GENEIOUS™ was used for the alignment of forward and reverse sequences of a sample followed by manual removal of ambiguous data (at both 5’ and 3’ ends) and generate a consensus sequence. Following editing, the consensus sequence of a sample was searched against all the yeast sequences available the CBS database by using its BLAST algorithm. Running the BLAST algorithm built in the CBS database resulted in a list of sequences homologous to the DNA submitted, 98%> homology was used as a cut-off to identify the query DNA. The query DNA was then identified as belonging to a specific species based on its homology to the respective type-strain in the CBS database (where available). Type-strains are a reliable source of species-level identification on the CBS database.

2.8.6 Microsatellite profiling of Saccharomyces bayanus and Saccharomyces cerevisiae

2.8.6.1 Strain differentiation within S cerevisiae

A series of di and tri-nucleotide microsatellites found on different chromosomes of S. cerevisiae have been used by various researchers to reliably differentiate between different strains (Field and Wills, 1998; Richards et al., 2009). This method is cheaper and acts as a short-hand to whole genome comparisons as shown by Richards et al. (2009), this is a powerful tool to discriminate between different S. cerevisiae strains given the number and frequency of alleles at each locus. In this study, any S. cerevisiae isolated was further differentiated using the protocol developed by Richards et al. (2009) which amplifies repeat regions from 10 un-related microsatellite on various chromosomes in S. cerevisiae along with a MAT-α locus which distinguishes isolates as being haploid or diploid. A master primer mix (10 x concentration) was prepared using the appropriate concentrations of fluorescently labelled primers for amplifying the different microsatellites (see table 2.2 for details). A multiplex PCR reaction using 1 µL of the primer mix was set up using the manufacturer’s protocol for QUIAGEN multiplex PCR kit. The thermal cycling for amplification required 95°C for 15 min followed by 35 cycles of 94°C for 30 sec, 54°C for 90 sec and 72°C for 60 sec followed by 60°C for 30 min. The amplified products were separated using ABI 3730 on a DS 30 matrix, the size of the individual bands were analysed using the ABI software GENEMAPPER and manually adjusted to the nearest base value as recommended by Richards et al. (2009). This technique has previously been used by Goddard et al. (2010) to differentiate different strains of NZ S. cerevisiae.
Visualising the strains based on the microsatellite data was achieved firstly by generating a genetic distance matrix; this was created by comparing and ranking the strains at all their loci using the co-dominant genotype algorithm on GenAIEx 6.3 macros for Microsoft Excel 2007 (Peakall and Smouse, 2006). The distance matrix generated was then visualised using Splitstree 4 (version 4.11.3) software (Huson and Bryant, 2006). The relatedness of isolates was displayed as an un-rooted network.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Gene</th>
<th>Chr</th>
<th>Repeat</th>
<th>Primer sequence (5’-3’)</th>
<th>Size range</th>
<th>Conc in µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>YOR267C</td>
<td>HRK1</td>
<td>XV</td>
<td>CAA</td>
<td>GTGTCTCGATGTGGATGATTG-HEX GTGTCTTGAAACATGGTAGAGTGACG</td>
<td>373–512</td>
<td>0.2</td>
</tr>
<tr>
<td>VML091C</td>
<td>RPM2</td>
<td>XIII</td>
<td>AAT</td>
<td>GTGTCTAAAGCCCTTTCAAGCATGAC-NED GTGTCTTGAAACATGGTAGAGTGACG</td>
<td>212–362</td>
<td>0.5</td>
</tr>
<tr>
<td>VOL109 W</td>
<td>ZEO1</td>
<td>XV</td>
<td>TAA,TAG</td>
<td>GTGTCTAGGAGAAAAATGCTGTTATTCGACC-HEX TTTTCTCCGGGACGTAAATA</td>
<td>237–362</td>
<td>0.75</td>
</tr>
<tr>
<td>YFR028C</td>
<td>CDC14</td>
<td>VI</td>
<td>GT</td>
<td>GTGTCTTGACACAGAATAGGCACTCA-HEX GCAAGCGACTAGAACAACACAC</td>
<td>109–180</td>
<td>0.1</td>
</tr>
<tr>
<td>YPL099C</td>
<td>–</td>
<td>XVI</td>
<td>CTT</td>
<td>GTGTCTGGTTTGGATTTCATGGA-FAM GTGTCTTCAATTTCTCTTCACCA</td>
<td>387–497</td>
<td>0.2</td>
</tr>
<tr>
<td>YDR160 W</td>
<td>SSY1</td>
<td>IV</td>
<td>AAT</td>
<td>GTGTCTTGGAGCCGAAATGGACAG-NED CACCTGAAGATGCTTTTAG</td>
<td>347–484</td>
<td>1</td>
</tr>
<tr>
<td>YLR049 W</td>
<td>–</td>
<td>XII</td>
<td>TA</td>
<td>GCAACATAATGATTTGAGGT-GGTGTACTAGAACAACACACAC</td>
<td>260–332</td>
<td>0.75</td>
</tr>
<tr>
<td>YBR240C</td>
<td>THI2</td>
<td>II</td>
<td>TA</td>
<td>TTTCACCAAATCTCGCA-FAM CATTGTCTGTGCGG</td>
<td>330–368</td>
<td>1</td>
</tr>
<tr>
<td>YGL014 W</td>
<td>PUF4</td>
<td>VII</td>
<td>TAA</td>
<td>GTGTCTCAGGTCGTTCAACGTTGAAATG-NED GCTGTGGCTGGTAGATTGT</td>
<td>115–168</td>
<td>0.25</td>
</tr>
<tr>
<td>YGL139 W</td>
<td>–</td>
<td>VII</td>
<td>CAA</td>
<td>GTGTCTCTTTTTATTCAAACAGGGCCAT-FAM AAATCTCATGGCTGGTAGGGGTAT</td>
<td>95–135</td>
<td>0.1</td>
</tr>
<tr>
<td>–</td>
<td>MATA</td>
<td>III</td>
<td>CAA</td>
<td>GTGTCTTTTTTTATTACCAATGGCCAT-FAM CAAATGATGAAATAGGATGTCGG-HEX</td>
<td>468, 492</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 2.2: Microsatellite primers used to differentiate *S. cerevisiae* strains obtained from Richards et al. (2009).

**2.8.6.2 Strain differentiation within *S. bayanus***

The protocol used for *S. bayanus* strain differentiation is similar to one used for *S. cerevisiae* as described in the previous section (section 2.8.6.1), except the primer sets used are developed from *S. bayanus* genome and there are no primers for MAT locus. The primers used and their labelling is shown in the table below (table 2.3).
<table>
<thead>
<tr>
<th>Name</th>
<th>Repeat</th>
<th>Position</th>
<th>Primer sequence (5'-3')</th>
<th>Size</th>
<th>Chromosome</th>
</tr>
</thead>
<tbody>
<tr>
<td>NB10H</td>
<td>TG</td>
<td>contig 123, 21766-21814</td>
<td>TCCACAACGATATCAAGACA GTGCTCTCGATGTATATATTTATGTAGTGATG TGT-HEX</td>
<td>104</td>
<td>Chromosome XII</td>
</tr>
<tr>
<td>NB10R</td>
<td></td>
<td></td>
<td>CAACATTCTTTTGTCACATA GTGCTCAAACAAGAAACTGTGGTAGTTGTCGT-HEX</td>
<td>118</td>
<td>Possible XV</td>
</tr>
<tr>
<td>NB9F</td>
<td>AT</td>
<td>contig 57, 5217-5245</td>
<td>CACTAAGAATGATGGGGAAG GTGCTCGTGGACACAAAGATAACAAFAM</td>
<td>144</td>
<td>possible IV</td>
</tr>
<tr>
<td>NB9R</td>
<td></td>
<td></td>
<td>CACTAAGAATGATGGGGAAG GTGCTCGTGGACACAAAGATAACAAFAM</td>
<td>182</td>
<td>possible VII</td>
</tr>
<tr>
<td>NB5H</td>
<td>GCT</td>
<td>contig 215, 2294-2339</td>
<td>GTGCTTTAATTTCAGAAACA</td>
<td>200</td>
<td>Chromosome XII</td>
</tr>
<tr>
<td>NB5R</td>
<td></td>
<td></td>
<td>GTGCTCAAACAAGAAACTGTGGTAGTTGTCGT-HEX</td>
<td>242</td>
<td>Chromosome V</td>
</tr>
<tr>
<td>NB6F</td>
<td>AAT</td>
<td>contig 5, 35419-35477</td>
<td>GTGCTCGTGGACACAAACGATAACAAFAM</td>
<td>298</td>
<td>chromosome VIII</td>
</tr>
<tr>
<td>NB6R</td>
<td></td>
<td></td>
<td>GTGCTCGTGGACACAAACGATAACAAFAM</td>
<td>343</td>
<td>chromosome XII</td>
</tr>
<tr>
<td>NB1H</td>
<td>ATG</td>
<td>contig 123, 28427-28468</td>
<td>ATCCTTTAATGCTGCTAGTGTG</td>
<td>429</td>
<td>chromosome XVI</td>
</tr>
<tr>
<td>NB1R</td>
<td></td>
<td></td>
<td>ATCCTTTAATGCTGCTAGTGTG</td>
<td>429</td>
<td>chromosome XVI</td>
</tr>
<tr>
<td>NB2F</td>
<td>CAA</td>
<td>contig 87, 29890-29937</td>
<td>ATCCTTTAATGCTGCTAGTGTG</td>
<td>429</td>
<td>chromosome XVI</td>
</tr>
<tr>
<td>NB2R</td>
<td></td>
<td></td>
<td>ATCCTTTAATGCTGCTAGTGTG</td>
<td>429</td>
<td>chromosome XVI</td>
</tr>
<tr>
<td>NB3F</td>
<td>AAC</td>
<td>contig 185, 7389-7551</td>
<td>ATCCTTTAATGCTGCTAGTGTG</td>
<td>429</td>
<td>chromosome XVI</td>
</tr>
<tr>
<td>NB3R</td>
<td></td>
<td></td>
<td>ATCCTTTAATGCTGCTAGTGTG</td>
<td>429</td>
<td>chromosome XVI</td>
</tr>
<tr>
<td>NB4H</td>
<td>TGT</td>
<td>contig 123, 20539-20594</td>
<td>ATCCTTTAATGCTGCTAGTGTG</td>
<td>429</td>
<td>chromosome XVI</td>
</tr>
<tr>
<td>NB4R</td>
<td></td>
<td></td>
<td>ATCCTTTAATGCTGCTAGTGTG</td>
<td>429</td>
<td>chromosome XVI</td>
</tr>
<tr>
<td>NB8F</td>
<td>TGT</td>
<td>contig 19, 22831-22995</td>
<td>ATCCTTTAATGCTGCTAGTGTG</td>
<td>429</td>
<td>chromosome XVI</td>
</tr>
<tr>
<td>NB8R</td>
<td></td>
<td></td>
<td>ATCCTTTAATGCTGCTAGTGTG</td>
<td>429</td>
<td>chromosome XVI</td>
</tr>
</tbody>
</table>

Table 2.3: Microsatellite primer sets for *S. bayanus* strain differentiation.

2.8.7 Varietal Aroma analysis

2.8.7.1 Extraction of thiols from wine

The three volatile thiols; 3-mercaptohexan-1-ol (3MH), 3-mercaptohexyl acetate (3MHA), and 4-mercapto-4-methylpentan-2-one (4MMP) play an important role and are major contributors of flavour and aroma in Marlborough Sauvignon Blanc. The analysis of these thiols is based on the method described by Tominaga *et al.* (1998b) with modifications by Dr Laura Nicolau and colleagues in the Wine Science Department of University of Auckland. Table 2.4 lists all the reagents required and their supplier.
<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>3MH(3 mercaptopohexanol-1-ol)</td>
<td>98%</td>
<td>Interchim, France</td>
</tr>
<tr>
<td>4MMP (4 mercapto-4methylpentan-2-one)</td>
<td>1% in PEG</td>
<td>Interchim, France</td>
</tr>
<tr>
<td>3MHA (3 mercaptohexyl acetate)</td>
<td>98%</td>
<td>Oxford Chemicals Ltd, UK</td>
</tr>
<tr>
<td>4M2M2MB (4methoxy-2-methyl-2-mercaptopbutane)</td>
<td>98%</td>
<td>Acros Organics, USA</td>
</tr>
<tr>
<td>BHA (Butylated hydroxyanisole)</td>
<td>≥98%</td>
<td>Sigma-Aldrich, Germany</td>
</tr>
<tr>
<td>Dichloromethane: SupraSolv for GC</td>
<td>≥99.8%</td>
<td>Merck, Germany</td>
</tr>
<tr>
<td>DOWEX Resin 1x2, chlorine form, 50-100mesh</td>
<td>n.a.</td>
<td>Sigma-Aldrich, Germany</td>
</tr>
<tr>
<td>Ethanol</td>
<td>≥99.5%</td>
<td>Ajax Finechem, Australia</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>99.7%</td>
<td>Sigma-Aldrich, Germany</td>
</tr>
<tr>
<td>Hydrochloric acid</td>
<td>37%</td>
<td>Scharlau, Spain</td>
</tr>
<tr>
<td>L-Cysteine hydrochloridehydrate</td>
<td>99%</td>
<td>Sigma-Aldrich, Germany</td>
</tr>
<tr>
<td>pHMB(p-hydroxymercurybenzoate)</td>
<td>≥95% (Hg)</td>
<td>Sigma-Aldrich, Germany</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>≥99%</td>
<td>Scharlau, Spain</td>
</tr>
<tr>
<td>Sodium hydroxide</td>
<td>≥99%</td>
<td>Scharlau, Spain</td>
</tr>
<tr>
<td>Sodium sulphate anhydrous</td>
<td>≥99%</td>
<td>Scharlau, Spain</td>
</tr>
<tr>
<td>TRIS ultrapure</td>
<td>≥99%</td>
<td>AppliChem, Germany</td>
</tr>
</tbody>
</table>

Table 2.4: Reagents required for the analysis of important thiols in Sauvignon Blanc.

Thiol extraction and analysis required 50 mL of room temperature wine, the following reagents were added sequentially to the wine, firstly, 0.5 mL of 2 mM BHA ethanol solution and 5 mL of 1 mM pHMB in 0.1 M TRIS were added along with 24 µL of 2.5 nM of internal standard 4M2M2MB and 50 µL solution containing 22 µM of [1-2H2] 3MH and 2.8 µM of [1-2H2] 3MHA (duterated forms of 3MH and 3MHA) and stirred for 10 min at 500 rpm. The use of duterated forms of 3MH and 3MHA as internal standards reduced the analysis to be done in duplicates instead of triplicates, internal standard 4M2M2MB was used to extrapolate the concentration of 4MMP (Hebditch et al., 2007). After stirring for 10 min, the pH of the wine was adjusted to 7 using sodium hydroxide to allow for the formation of pHMB-thiol complex, the sample was now ready for extraction.

The pH adjusted wine sample was loaded onto an activated DOWEX resin bed (basic anion exchange) and passed through with a flow-rate of 1 drop per 7 s; this allowed the pHMB-thiol complexes to bind to the resin. The resin column was then washed with 50 mL of sodium acetate buffer (0.1M Sodium acetate, pH6, 0.02M BHA) at a flow rate of 1 drop per 5 s. Following washing, the thiols were liberated from their pHMB complex by eluting with 50 mL of L-cysteine buffer (0.1 M sodium acetate, 0.02 mM BHA, 400 mg cysteine-HCl, pH 6) at a flow rate of 1 drop every 7 s.

Liquid-liquid extraction was performed on the thiol elute solution, 0.5 mL of ethyl acetate was added to the elute followed by double extraction by addition of 4 mL then 2 mL of dichloromethane. After
stirring the elute-dichloromethane mixture for 5 min at 1000 rpm, the lower organic phase was recovered by using a glass separating funnel. The organic phase obtained by the double extraction was combined and removed of all residual water and dried by adding ~ 1-2 mg of anhydrous sodium sulphate. The dried organic phase (~ 4 mL) was then transferred to a new glass test tube and concentrated to a volume of 15 µL under continuous Nitrogen gas flow. The sample was then transferred to a GC vial for analysis on GCMS.

2.8.7.2 Analysis of thiols using GCMS

Thiol analysis was carried out by injection of 1-3 µL of the concentrate prepared from the above protocol (section 2.8.7.1) into an Agilent Chromatograph 6890N coupled to an Agilent 5973 mass-selective detector. The column used for analysis was either a BP20 from SGE (50 M x 220 µM x 0.25 µM) or a HP-Innowax (60 M x 252 µM x 0.25 µM). The injector was operated at 240°C with Helium being used as a carrier gas at 112 kPa and a flow rate of 30 mL/min. The sample was injected in a split mode with a split flow of 5mL/min (split ratio 5:1). The flow was fixed at 1.0 mL/min for 52 min, after the elution of the compounds of interest it was increased to 1.5 mL/min and then finally to 2.4 mL/min for 12 min. The column temperature was programmed to include, 40°C for 10 min and to increase to 115°C at a rate of 3°C/min and held for 22 min, followed by an increase to 150°C at 40°C/min and held for 3 min, further raised to 173°C at 3°C/min, finally raised to 250°C at 70°C/min and held for 17 min. After that the temperature was brought back to 50°C at 40°C/min and held for 3 min resulting in a total run time of 64.3 min. The interface temperature of the detector was at 230°C, the ion source was in EI mode at 70eV and held at 230°C and the quadrupole was set at 150°C with EM voltage being 2047 V.

2.8.7.3 Quantification of thiols

Thiols were analysed in the selected ion monitoring (SIM) mode. The ions used for quantification of thiols are given in the table below (table 2.5). The area of the quantifier ion peak was compared to the area of quantifier ion peak for its duterated counterpart and the concentration of a particular thiol was then extrapolated from a calibration curve for that thiol. Peak detection and integration was carried out using MSD Chemstation D.01.02.16 (Agilent). Calibration curves for quantification of thiols was obtained by adding a range of known amounts of 3MHA (0-2000 ng/L), 3MH (0-20000 ng/L) and 4 MMP (0-600 ng/L) into 50 mL of Corbans White Label Sauvignon Blanc in 5-9 doses of increasing concentrations. These adjusted wine samples were then analysed for their thiol concentrations as described above (section 2.8.7.1 and 2.8.7.2). Representative example of the standard curves obtained for the different thiols are provided in Appendix 1. The linear equations and correlation coefficients ($R^2$) values obtained for each thiol is also provided in appendix 1.
<table>
<thead>
<tr>
<th>Thiols</th>
<th>Qualifier ions [m/z]</th>
<th>Quantifier ion[m/z]</th>
</tr>
</thead>
<tbody>
<tr>
<td>4M2M2MB</td>
<td>134,75</td>
<td>134</td>
</tr>
<tr>
<td>4MMP</td>
<td>132,75,99</td>
<td>132</td>
</tr>
<tr>
<td>[1-²H₂]3MHA</td>
<td>118,103</td>
<td>118</td>
</tr>
<tr>
<td>3MHA</td>
<td>116,101</td>
<td>116</td>
</tr>
<tr>
<td>[1-²H₂]3MH</td>
<td>136,102</td>
<td>136</td>
</tr>
<tr>
<td>3MH</td>
<td>134,100</td>
<td>134</td>
</tr>
</tbody>
</table>

Table 2.5: List of Quantifier and Qualifier ions for the varietal thiols in Sauvignon Blanc.

2.8.8 Growth of *Saccharomyces* isolates under controlled conditions

To understand the ability of the yeast isolates to grow under defined conditions, the growth media and temperature were strictly controlled. The growth rate of yeasts were then measured by monitoring the changes in turbidity of the media using spectrophotometer, the log- growth phase was then calculated followed by statistical analyses. Growth conditions and post-hoc data analyses procedure is detailed below.

2.8.8.1 Preparation of growth media

Yeast isolates underwent growth in aerobic conditions in Marlborough Sauvignon Blanc. Marlborough Sauvignon Blanc juice from vintage 2008 was kindly provided by Pernod-Ricard NZ and stored at -20°C. The juice had a pH of 3.28 TA(titratable acidity) of 6, Brix° 17.8 was thawed slowly to room temperature from -20°C in a warm water bath, followed by chemical sterilization using DMDC (dimethyl dicarbonate). One litre of thawed juice was sterilized with the addition of 200 µL of DMDC and incubated at 25°C with 100 rpm shaking for 12 hours before inoculation with desired yeast.

2.8.8.2 Micro-fermentation set up, monitoring and growth evaluation

Pure yeast colonies were obtained by streak plating isolates on YPD agar (recipe requires adding 10 g of agar to YPD media prior to autoclaving) from glycerol stock (section 2.8.4). Single colonies were then pre-cultured in YPD media at 28 °C; 150 rpm for 48 hours to obtain a high cfu count (obtained using the protocol in section 2.8.3). The pre-cultured isolates were then separated from the YPD media by centrifugation at 13000 rpm for 2 min and discarding the supernatant and replacing with
same volume of sterile water. The cells were then re-suspended in fresh media and ready to be used in the experiment.

Micro-fermentation was carried out in 400µL capacity 96 well transparent plates with clear covers. The growth of each isolate to be tested was done in quadruplicate. Each well contained 200 µL of SB juice and inoculated with the respective isolate at a concentration of 2.5 million cfu/mL. Sterile media was used in each plate as negative control to monitor for contamination. The plates were incubated at 12.8°C and 15.8°C and shaken at 100 rpm.

Growth of the yeast isolates was monitored by recording the change in turbidity of the media. Optical Density measurements of the growing isolates was recorded by using a plate reader Spectra Max-Pro 340 (Molecular Devices) set at a wavelength of 595 nm. The data generated was then transferred to Microsoft Excel 2007 for further analysis. OD readings were recorded at close time intervals.

Changes to the OD measurements were used to calculate the exponential growth rate of yeast isolates. The formula used to calculate the rate of growth or k is provided below

\[ N_t = N_0 \ e^{kt} \]

\[ k = \frac{\ln(N_t-N_0)}{t} \]

\( t \) = time
\( N_0 \) = Cells at time 0
\( N_t \) = Cells at time t
\( k \) = growth rate
\( e \) = natural log

Equation 2.2: Equation for calculating exponential growth of yeast
2.9 Results and Discussion

2.9.1. Growth of yeast during un-inoculated ferment of 2008 Kumeu River Sauvignon Blanc

Growth of the microbes in the ferment was monitored by viable staining and enumeration of colony forming units as outlined in section (section 2.8.2 and 2.8.3). From sampling days 1-7 the methylene blue test, which employs direct observation, indicated a larger cell density in comparison to the cell counts obtained from culturing on YPD plates. The difference between the two methods was the greatest at on days 1-3 and days 5-7 (figure 2.4). A student’s t-test showed that these differences were not significant (P-value 0.32), it could be due to small sample sizes (methylene blue n=2, plate count n=3). However, this phenomena where the cell numbers observed in a sample compared to the number of colonies they produce on standard media has been noted in several other environmental studies carried out on other microbes, it was called the ‘the great plate count anomaly’ by Staley and Konopka in 1985, since then various research groups have tried to overcome this discrepancy (Nichols, 2007; Staley and Konopka, 1985). From sample point E onwards the two methods showed similar cell counts: this coincided with the rapid assimilation of sugar and temperature stabilization (refer figure 2.5 for temperature and sugar utilization measurements). The end-point samples showed a few discrepancies between cell counts obtained from the two methods, in this case, the t-test between the two methods reveals the differences were significant (P-values shown on figure 2.4); one reason could be due to the nature of the methylene blue test which is a reductive dye that is capable of crossing the cell membrane of dead yeast cells. At the end of ferment, the yeast cells after having undergone both temperature and osmotic stresses along with increased ethanol and depleted nutrients appear dead when examined using methylene blue test but could possibly grow on YPD agar.

In this study, the focus was on yeasts that were in sufficiently high numbers and could be grown under standard growth conditions and important in determining fermentation characteristics of the wine. The sample points with un-identified yeast were before the rigorous assimilation of sugar, I chose not to identify the un-culturable yeast which could potentially provide interesting insights about the yeast community present in grape juice. Culture independent molecular techniques like ARISA, Pyro-sequencing, PCR DGGE need to be employed to identify these yeasts (Hamady and Knight, 2009). The cultured yeasts were used to determine the yeast community during fermentation.
Figure 2.4: Growth estimation of yeast during the un-inoculated fermentation of Kumeu River Marlborough Sauvignon Blanc 2008 using two different techniques. Haemocytometer counts using methylene blue (blue line) (n=2; ± SEM), plate counts (red line) (n=3; ±SEM), * indicates P-value ≤0.05, ** indicates P-value ≤ 0.01 and *** indicates P-value ≤ 0.001.
2.9.2 Molecular identification of yeast in the un-inoculated ferment of 2008 Kumeu River Sauvignon Blanc

As detailed in the methods section each sample point was examined in depth to identify the yeast community during fermentation (section 2.8.5). The yeasts were grouped into 22 different cohorts based on visually analysing their ITS-RFLP profiles. But upon Sequencing of 26 S rDNA from the different cohorts, it resulted in the identification of 11 different yeast species, as shown in table 2.6. Similarity of sequences within each cohort (n=3 or 4) was analysed using the Geneious™ software, it ranged between 99.1-100%; this indicated that the samples/colonies chosen to represent a cohort belonged to a single species. However, more than one cohort of samples belonged to the same species upon comparison with the CBS database. This resulted in the identification of only 11 different species from 22 cohorts. All the sequences within each cohort were compared to the corresponding yeast species sequences in the CBS database, and all showed a homology between 99.48-100%. The difference between the samples within a cohort and the difference between the samples and the corresponding type strain in CBS database were less than 1 %, and this is within the threshold recommended for species level differentiation of yeasts allowing for a positive classification (Kurtzman and Fell, 1998). However, the lack of a 100% similarity to a CBS type strain and to one another within a cohort even with low sample numbers shows diversity and suggests a diverse population exhibiting minor differences in the 26 S rDNA sequence within this ferment (Kurtzman and Robnett, 1998). The cohorts can be classified as strains of Pichia anomala, Candida galbrata, Saccharomyces cerevisiae, Torulaspora delbrueckii, Pichia kluyveri, Saccharomyces bayanus, Issatchenkia orientalis, Candida oleophila, Metschnikowia pulcherrima and Candida railenensis after comparing with the CBS database.

It is interesting to note that while both S. cerevisiae and S. bayanus showed variation within their cohorts, the chosen isolates had a 100% homology to the partial sequence of 26 S rDNA in the CBS database. Therefore as suggested by Shuller et al. (2004) an alternate method was used to obtain the species diversity using Microsatellite profiling.
<table>
<thead>
<tr>
<th>PCR size</th>
<th>HaeIII profile</th>
<th>HinfI profile</th>
<th>Species</th>
<th>Homology within cohort (%)</th>
<th>CBS no</th>
<th>Homology to CBS database (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>650</td>
<td>600,50</td>
<td>310,310</td>
<td><em>Pichia anomala</em></td>
<td>100</td>
<td>CBS 248</td>
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<td>800</td>
<td>650</td>
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<td><em>Candida galbrata</em></td>
<td>99.9</td>
<td>CBS 4692</td>
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<tr>
<td>880</td>
<td>320,230,180,150</td>
<td>360,150</td>
<td><em>Saccharomyces cerevisiae</em></td>
<td>99.1</td>
<td>CBS 5794</td>
<td>100</td>
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<tr>
<td>800</td>
<td>800</td>
<td>400,380</td>
<td><em>Torulaspora delbrueckii</em></td>
<td>99.9</td>
<td>CBS 6749</td>
<td>100</td>
</tr>
<tr>
<td>450</td>
<td>370,80</td>
<td>250,200</td>
<td><em>Pichia kluyveri</em></td>
<td>100</td>
<td>CBS 188</td>
<td>99.8</td>
</tr>
<tr>
<td>880</td>
<td>500,220,140</td>
<td>360,150</td>
<td><em>Saccharomyces bayanus</em></td>
<td>99.8</td>
<td>CBS 1604</td>
<td>100</td>
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<tr>
<td>500</td>
<td>400,100</td>
<td>300,100,100</td>
<td><em>Issatchenkia orientalis</em></td>
<td>99.9</td>
<td>CBS 5147</td>
<td>100</td>
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<td>600</td>
<td>380,120,90</td>
<td>300,290</td>
<td><em>Candida oleophila</em></td>
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<td>CBS 2223</td>
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<td>280,100</td>
<td>200,190</td>
<td><em>Metschnikowia pulcherrima</em></td>
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<td>CBS 9704</td>
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<td>450,150</td>
<td>350</td>
<td><em>Candida railenensis</em></td>
<td>99.1</td>
<td>CBS 8165</td>
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<tr>
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<td>200</td>
<td>Unidentified <em>Metschnikowia</em></td>
<td>n.a</td>
<td>AM161116</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 2.6: List of species identified in a un-inoculated ferment of 2008 Kumeu River Sauvignon Blanc based on molecular methods and comparison with strains deposited in CBS database.
2.9.3 Yeast community dynamics during un-inoculated ferment

2.9.3.1 Non-Saccharomyces yeast population

The ITS-RFLP patterns were elucidated for 96 yeast isolates at each sample point. This enabled the estimation and tracking of changes in frequency of each species through the ferment (figure 2.5). Non-Saccharomyces yeast species from genus Candida, Pichia, Metschnikowia, Torulaspora and Issatchenkia dominated the early part of the ferment in the grape must (days 1-3) when the temperature was below 12.5°C with a high sugar concentration. These yeasts were unable to compete with Crabtree positive yeasts and were quickly replaced; nonetheless they are known to contribute to the desirable qualities of the wine (Fleet, 2003; Querol and Fleet, 2006). To my knowledge this is the first study to estimate the yeast community dynamics in a new world Sauvignon Blanc. However, comparing the community profiles of white wines for non-Saccharomyces yeast revealed similarities to those studied at different geographical locations (Ciani and Picciotti, 1995; Jolly et al., 2006; Pretorius, 2000).

Metschnikowia, Candida and Pichia species were identified in commercial Dolce wine from California (blend of Semillon and Sauvignon Blanc) (Cocolin et al., 2001). The notable absence from the Kumeu River yeast community were species from the genus Hanseniaspora and Rhodotorula which were identified by other studies, although this could be because only juice and ferment were sampled in my study and didn’t include grape berries where these species have previously been isolated (Fleet, 2003; Martini et al., 1996). In this study, Torulaspora delbrueckii was able to tolerate and persist till the end of the fermentation process, this is also similar to reports from other studies (Ciani et al., 2006; Torija et al., 2001). Different studies have shown that C.stellata, T. delbrueckii, P. kluyveri and P. anomala are capable of improving wine quality when co-fermented either in a multi-starter, or sequential additions with S. cerevisiae by enhancing glycerol content, varietal aroma and reducing un-desirable characteristics like volatile acidity and off-odours (Ciani et al., 2010). It is possible these yeasts had a favourable impact on the resulting wine.
2.9.3.2 *Saccharomyces* yeast population

ITS-RFLP, 26S rDNA and Microsatellite sequence analysis were used to identify and estimate the population of *Saccharomyces* yeast during fermentation. Temperature of the ferment was recorded to be between 13-14 °C around Days 5 and 7, this coincided with the establishment of Crabtree positive yeast in this case *Saccharomyces bayanus* as the dominant yeast species. Subsequent increase in the number of *S. bayanus* resulted in the rapid assimilation of sugar into ethanol. Thus it can be concluded that the un-inoculated ferment of 2008 Kumeu River Sauvignon Blanc was primarily carried out by *S. bayanus*. This finding is contrary to common findings from other researchers were *S. cerevisiae* is the main fermenting yeast (Pretorius, 2000).

Although, there have been a few studies which have showed *S. bayanus* as the main fermenting species in the un-inoculated fermentation; like in white wines Folle Blanche and Hondarrabi zuri from the Basque appellation in Spain, here the researchers noted a complete absence of *S. cerevisiae* in these ferments(Rementeria *et al.*, 2003). There have also reported the presence of *S. bayanus* species to be dominating ferments in Burgundy, Alsace, Champagne and Val de Loire region, *S. bayanus* isolates were also found in Tokay wines in Slovakia, in Muscat (Crimea, Ukraine), Recioto and Amarone (Italy) wines (Demuyter *et al.*, 2004; Massoutier *et al.*, 1998; Naumov *et al.*, 2000; Naumov *et al.*, 2002; Torriani *et al.*, 1999; Tosi *et al.*, 2009). Lopandic *et al.* (2008) examined un-inoculated
fermentations at different climates in Austria and found S. bayanus was persistent in high numbers at the beginning at 4:0 and middle of ferment at 4:9 compared with S. cerevisiae and was also identified at the end of the ferment at 1:9 in Zweigelt (red wine) from St. Georgen region. S. bayanus var. uvarum was also isolated from natural fermentations of botrytized grape must (Antunovics et al., 2005; Naumov et al., 2000; Naumov et al., 2002; Sipiczki et al., 2001). Notably all of the above studies analysed fewer samples compared to 96 samples at each time point and could not provide an in-depth estimation of abundance of this species.

In this study, Saccharomyces cerevisiae was identified as being present at the start of the ferment at a ratio of 1:10 with S. bayanus isolates but subsequent tracking showed that it was unable to dominate and ferment the grape must with ratios of 1:46 on day 7, and 1:95 on day 11 as it was outcompeted by S. bayanus isolates. But S. cerevisiae was able to tolerate the fermenting niche and was noted as being present at the end of the ferment at a ratio of 1:95 with S. bayanus (refer figure 2.5). The ratios of the numbers of S. cerevisiae compared to S. bayanus isolates obtained from our study suggests at some innate advantage for S. bayanus isolates in the un-inoculated ferment of 2008 Kumeu River Sauvignon Blanc.

It has been reported in NZ Chardonnay, that un-inoculated fermentation contains many strains of S. cerevisiae during rapid sugar assimilation (Goddard et al., 2010). However, there is no information regarding the strain variation in S. bayanus dominating ferments. Also, the mechanism of interaction between the two Saccharomyces species is poorly studied, except to look for a possible reduction in acetaldehyde levels (Cheraiti et al., 2005). Recently the growth rates of these two species were compared along with the production of discriminative aromatic acetates (Masneuf-Pomarède et al., 2010). Virtually nothing is known about the dominance of one species of Saccharomyces over another during fermentation of grape must. However, differences in growth at specific temperatures between the different yeast within the Saccharomyces genus in a defined media has been studied by many (Salvadó et al., 2011; Sampaio and Gonçalves, 2008; Sweeney et al., 2004).

Salvadó et al. (2011) have tested for the minimum, maximum and optimum temperatures of the different species within the Saccharomyces genus. Serra et al. (2005) found that a strain of S. bayanus var uvarum reacted in a similar manner to that of S. cerevisiae VL3c in decreasing its productivity with decrease in temperature however, at temperatures lower than 21°C, S. bayanus var uvarum was more stable attesting to its cryotolerant property. Therefore in order to test the above mentioned characteristics, the next important step was to understand the strain diversity within S. bayanus in Marlborough Sauvignon Blanc.
2.9.4 Strain diversity of *Saccharomyces* species involved in un-inoculated fermentation

2.9.4.1 Microsatellite analysis to identify *Saccharomyces* species

Whilst 26srDNA sequencing based techniques are valuable tools in identification and differentiating between species, they rarely provide information regarding diversity within species (Schuller *et al.*, 2004). For the purposes of this study, we utilised the protocol developed in house to identify the strain diversity within isolates of *S. cerevisiae* and *S. bayanus* identified during fermentation using 10 *S. cerevisiae* loci and 8 *S. bayanus* loci (Richards *et al.*, 2009). The ITS PCR RFLP technique identified 907 isolates as *S. bayanus*, since it was time and resource consuming to obtain all the microsatellite profiles, 25 isolates was randomly selected for further genotypic analysis.

Figure 2.6 is the UV visualised Gel of the PCR amplified microsatellite profiles of the 25 random isolates of *S. bayanus* amplified using both *S. cerevisiae* and *S. bayanus* primer sets. All of the isolates except 4 showed PCR products only when using *S. bayanus* primer sets (the blank lanes in *S. bayanus* were later successfully re-amplified using *S. bayanus* primers). This showed that the majority (n=21) of the isolates were *S. bayanus* and they were only amplified by *S. bayanus* microsatellite primers. However, the four isolates showed amplification products for both *S. cerevisiae* and *S. bayanus* primers suggesting that they are hybrids between the two species. Previously from ITS-RFLP technique they were classified as *S. bayanus* due to their restriction digestion pattern matching that of *S. bayanus* using the Hae III endonuclease (table 2.6). Due to a vast number of isolates showing ITS-RFLP profile similar to *S. bayanus* (n=907), it is difficult to predict the number and diversity of these hybrids and would require an in-depth examination of the yeast community from Marlborough Sauvignon Blanc. In the past, to my knowledge, researchers have successfully made *S. bayanus* and *S. cerevisiae* hybrids in laboratories but these are the first naturally isolated hybrids between the two *Saccharomyces* species from wine (Masneuf *et al.*, 1996; Masneuf *et al.*, 2002). The PCR products were then used to obtain the microsatellite profile of the isolates using the ABI sequencer for analysis of yeast strain diversity refer to appendix 3 for the microsatellite profiles.
Figure 2.6: UV visualisation of microsatellite profiles of *Saccharomyces* species using both *S. cerevisiae* and *S. bayanus* primers. These PCR products were used to obtain exact sequences of the different isolates.

2.9.4.2 Testing if the *S. bayanus* isolates were from a natural population

Even though Kumeu River Winery does not utilize commercial strains of yeast to carry out fermentation, they are known to source grape juice from vineyards/wineries using commercial yeasts. Also majority of NZ wineries use commercial yeast to ferment wine including a few *S. bayanus*/*S. uvarum* yeasts such as Lalvin’s EC1118, DV10, QA 23 and S6U (Lalvin yeast, 2012). There have been reports from Europe where commercial yeasts are known to spread-out from wineries and establish in vineyards (Valero *et al.*, 2005). Therefore it could be possible that these *S. bayanus* isolates could have a potential commercial origin. It was important to establish the nature and origin of the *S. bayanus* isolates from this study.

*S. bayanus* isolates were compared against certain commercial yeast strains (VL3,L3,X5,VIN7,VIN13,IOC,QA23,CY3079,EC1118,DV10,Siha7,SVG,S6U,Rhone4600, Fermivin (DSM) and Alchemy II) which could have been present in the source vineyard to rule out any similarities. Figure 2.7 shows the Hae III endonuclease digest profiles of these commercial yeast strains and four randomly selected isolates from Kumeu River Sauvignon Blanc (L7a, L7d, L7e and L7f). Initial visual analysis showed that apart from two commercial yeast strains; S6U and Alchemy, the profiles of amplified KR isolates were different when compared to commercial yeast. Next, all the commercial yeasts including the two ambiguous yeast strains (S6U and Alchemy) and the 4 KR isolates were sequenced using Microsatellite primers for both *S. cerevisiae* and *S. bayanus* (microsatellite profile data not shown for commercial isolates, refer appendix 2 for KR isolates).
Figure 2.7: Restriction digestion pattern of commercial yeast strains and 4 Kumeu River S. bayanus isolates using Hae III endonuclease.

Upon Microsatellite analysis, most of the commercial yeast showed a profile only for S. cerevisiae including Alchemy II which had previously shown similar ITS-RFLP pattern to that of the KR isolates, however, the 4 KR isolates had only S. bayanus profiles. Also, Alchemy II is classified as S. cerevisiae by manufacturer (Anchor wine yeast 2012). The other ambiguous commercial yeast was Lalvin S6U a natural hybrid between S. cerevisiae and S. uvarum, it showed a profile for both sets of microsatellite primers, (refer to appendix 2 for S. bayanus microsatellite profile of S6U) (Ciolfi, 1992). So the next step was to analyse the relatedness of the microsatellite profile of S6U (using S. bayanus primers) to more S. bayanus isolates from Kumeu River (n=20, including 4 KR isolates) winery using GenAIEx 6.3 macros for Microsoft Excel 2007 developed by Peakall and Smouse to compare genetic distances (Peakall and Smouse, 2006). In this program, the differences in genotype are converted into genetic distance matrix between the different strains, this matrix was then visualised using Splitstree software and is shown in Figure 2.8. (Refer section 2.8.6.1 for method).

From figure 2.8, we can see that there is a very distant relationship between S6U and other S. bayanus isolates suggesting that Kumeu River isolates are more closely related to one another when compared to S6U. Also, none of S. bayanus isolates share even 50% of their microsatellite alleles with S6U (appendix 2). Also, observing the un-rooted network only isolates F12b, E2a and J1a and J5d shared similar profiles. The network shows that there is large diversity within a small sample of Kumeu River S. bayanus isolates, this is similar to the findings on S. cerevisiae strains isolated by Goddard et al. (2010) from a un-inoculated ferment of NZ Chardonnay must. Therefore from this study, it can be concluded that none of the tested commercial isolates were closely related to the randomly selected S. bayanus isolates from Kumeu River Sauvignon Blanc. Thus, the S. bayanus isolates are most likely from a natural population.
Figure 2.8: Un-rooted distance network of 20 isolates of *S. bayanus* from Marlborough Sauvignon Blanc based on pair-wise microsatellite profile comparison. Also shown is the genetic distance between the isolates compared to a commercial *S. bayanus* hybrid (S6U in red).
2.9.5 Relevance of temperature as a vinification factor influencing *S. bayanus*

population in the un-inoculated ferment

There have been reports of presence of *S. bayanus* species in the colder wine growing regions of France, Ukraine, Northern Spain, Italy and Slovakia, there have also been suggestions that *S. bayanus* is cryotolerant and is therefore capable of surviving in these regions (Naumov *et al.*, 2000). Apart from an old study testing for their (*S. bayanus*) winemaking capabilities at different temperatures, no attempt has been made to understand the reason for the presence of and subsequent dominance of this species in certain wines (Usseglio-Tomasset *et al.*, 1980, information obtained from Naumov *et al.* 2000).

The importance of physical, chemical and biotic factors influencing both autochthonous and allochthonous yeasts populations have been emphasised by various authors (Pretorius, 2000; Walker, 1998). In this study, Sauvignon Blanc grapes were harvested, berries crushed, juice extracted and chilled in Marlborough before being transported to Auckland to ferment utilizing minimal winemaking techniques. It is important to note the difference in climatic conditions between Auckland and Marlborough region. The average Auckland temperature is around 15.8°C while it is 12.8°C in Marlborough (MetService, 2012). Harvesting in Marlborough could have played a crucial role in establishment of native micro flora in the juice. There is a high possibility that the cryotolerant *S. bayanus* isolates originated from the cooler Marlborough as previous sampling of yeast from relatively warmer Kumeu River winery environment (from chardonnay and pinot noir ferments, soil and winery equipment) did not lead to the isolation of *S. bayanus* (Gayevskiy and Goddard, 2012; Goddard *et al.*, 2010).

Supporting the theory of establishment of cryotolerant *S. bayanus* under Marlborough conditions can be seen in figure 2.4, were higher numbers of *S. bayanus* isolates were observed compared to *S. cerevisiae* isolates (10:1) in the initial juice sample with a low temperature of 12.4°C. Until recent molecular biology developments to identify species, these two *Saccharomyces* species were differentiated by their ability to grow at different temperatures, with *S. bayanus* being more cryotolerant (Giudici *et al.*, 1998; Kishimoto and Goto, 1995). In a recent study, the ability of *S. bayanus* to grow and ferment at low temperature was validated by monitoring fermentation of *S. bayanus var uvarum* and *S. cerevisiae* (Masneuf-Pomarède *et al.*, 2010). However, in this study, comparisons were made on *S. bayanus* and *S. cerevisiae* isolates found in NZ climactic conditions.
2.9.5.1 Testing the hypothesis that *S. bayanus* is capable of faster growth than *S. cerevisiae* under defined conditions

Previous work in the Goddard laboratory have found only *S. cerevisiae* strains driving un-inoculated ferments of Chardonnay and Pinot noir in the Auckland region (Gayevskiy and Goddard, 2012; Goddard *et al.*, 2010). While both *S. cerevisiae* and *S. bayanus* were present in the Kumeu River MSB juice, it was determined that *S. bayanus* became dominant and drove the alcoholic fermentation (figure 2.4). In principle this dynamic between the two species could be due to either random or deterministic factors; one would have to sample multiple un-inoculated ferments from both Auckland and Marlborough regions to determine if any pattern exists for the distribution of these two species among these two regions.

Given the previous work showing that *S. bayanus* is more cryotolerant than *S. cerevisiae*, and the average temperature in the Marlborough (12.8°C) region is lower than in Auckland (15.8°C), it can be hypothesised that differential adaption to temperature explains this observation of not only the presence but dominance of *S. bayanus* in 2008 Kumeu River fermentation (Naumov *et al.*, 2000; Salvadó *et al.*, 2011; Serra *et al.*, 2005).

One prediction arising from the above hypothesis is that *S. bayanus* will be competitively (growth) superior at lower temperatures compared to *S. cerevisiae*. To test this hypothesis 5 random isolates (in quadruplets) of *S. bayanus* and 5 isolates of *S. cerevisiae* (in quadruplets) previously isolated from the 2008 Kumeu River ferment were grown in Marlborough Sauvignon Blanc juice at the average temperature of 12.8°C observed in Marlborough and also at average temperature of Auckland at 15.8°C. Growth rates of the two different species were monitored via the optical density method.

2.9.5.2 Testing for difference in growth rates of *S. bayanus* and *S. cerevisiae* isolates

A graph of weight loss over time was used to determine the maximal growth of the different yeast isolates at the different temperatures. (Figure 2.10 and 2.11) For *S. bayanus* isolates (j1a, l7e, e1a, f1h, g10a), the maximal growth in this experiment was calculated using the OD readings measured between 67 and 119 hours at 12.8°C and between 27.5 hours and 64.5 hours at 15.8°C. Similarly for *S. cerevisiae* isolates (k1a, a6a, a6c, e7d, and h2c) the maximal growth was calculated between 23.5 and 84.5 hours at 12.8°C, and between 40.5 and 83 hours at 15.8°C. It can be seen that for *S. cerevisiae*, at 12.8°C, the log phase was calculated between 23.5 and 84.5 hours, as there was very little growth (change in OD readings) observed under this temperature (figure 2.9). On the other hand, *S. bayanus* isolates were capable of considerable growth at both temperatures. The OD readings were monitored regularly for only 140 hours after inoculation.
Figure 2.9: Growth curve of \textit{S. bayanus} and \textit{S. cerevisiae} isolates at 12.8\(^{\circ}\)C in Marlborough Sauvignon Blanc juice. The OD readings were natural log transformed, error bars are ±SEM (n=4).

From figure 2.9, it can be seen that different isolates of both \textit{S. bayanus} and \textit{S. cerevisiae} exhibit different growth rates, suggesting that these isolates are from a natural population. Indeed, the isolate e1a (\textit{S. bayanus}) has higher OD readings compared to other \textit{S. bayanus} isolates. While f1h (\textit{S. bayanus}) is incapable of growing (little change in OD over 140 hours). Therefore, more analysis was required regarding the growth and ferment rates of the different natural isolates of \textit{S. bayanus} (this is discussed in detail in chapter 3). Also, isolates l7e and f1h (both \textit{S. bayanus}) are incapable of growth similar to other \textit{S. bayanus} isolates.

Growth was slow in general for \textit{S. cerevisiae} isolates at 12.8\(^{\circ}\)C, except for isolate k1a (identified by microsatellite profile as \textit{S. cerevisiae}; see appendix 2), which showed change in OD readings similar to \textit{S. bayanus} samples. These differences in growth profiles of the two species could be an indicator that these isolates came from a natural population consisting of yeast with varying abilities (Goddard \textit{et al.}, 2010).
Figure 2.10: Growth curve of *S. bayanus* and *S. cerevisiae* isolates at 15.8°C in Marlborough Sauvignon Blanc juice. The OD readings were natural log transformed, error bars are ±SEM (n=4)

Interestingly, the *S. cerevisiae* isolates were capable of similar growth to *S. bayanus* isolates at 15.8°C (figure 2.10). A Student’s t-test performed on the log transformed growth rates (measured from log phase growth of the two species using equation 2.1) of the two species at the two different temperatures revealed that *S. bayanus* had marginally higher growth rate compared to *S. cerevisiae* at the Marlborough temperature of 12.8°C (P-value = 0.042) but had no advantage at the Auckland temperature (15.8°C; P-value 0.23). This is in line with the idea that *S. bayanus* is present, and dominant in the Marlborough region as it has a selective advantage over *S. cerevisiae* at the lower Marlborough temperature conditions.

In figure 2.11, further examination showed that *S. bayanus* had a growth rate of 0.011 r/h (±0.002 r/h) and *S. cerevisiae* which was growing at 0.005 r/h(±0.001r/h) at 12.8°C, and had growth rates of 0.039 r/h (±0.001r/h) and 0.033 r/h (±0.001 r/h) respectively at 15.8°C. At both the temperature conditions *S. bayanus* had a faster growth rate compared *S. cerevisiae* by 0.006 r/h. The growth kinetics for *S. bayanus* and *S. cerevisiae* were then used to predict the observed population changes between these two species in Kumeu River fermentation.
**2.9.5.3 Prediction of change in population of S. bayanus and S. cerevisiae isolates in Kumeu River ferment**

Despite the differences in the growth rates of the different isolates, at the lower temperature of 12.8°C, *S. bayanus* had more than twice the growth rate of *S. cerevisiae*. This suggests that during early fermentation period of Kumeu River Sauvignon Blanc (days 1-5), when the temperature ranged between 12.4-13.5°C, *S. bayanus* being cryotolerant, rapidly doubled in cell numbers in comparison to *S. cerevisiae* thus establishing itself as the dominant yeast species. By day 9 when the temperature was around 16°C, *S. bayanus* probably would have lost its growth advantage over *S. cerevisiae*. One way of testing this hypothetical phenomenon would be predicting the change in the population of *S. bayanus* in Kumeu River ferments between days 1-5 based on the growth rates observed in 2.9.5.2.

![Average growth rate of isolates of *S. bayanus* (blue) and *S. cerevisiae* (red) at temperatures of 12.8°C and 15.8°C in Marlborough Sauvignon Blanc juice. The error bars indicate ±SEM (n=20) and P-values shown are from a pair-wise Student’s t-test.](image)

**Figure 2.11:** Average growth rate of isolates of *S. bayanus* (blue) and *S. cerevisiae* (red) at temperatures of 12.8°C and 15.8°C in Marlborough Sauvignon Blanc juice. The error bars indicate ±SEM (n=20) and P-values shown are from a pair-wise Student’s t-test.

The first interesting observation from the empirical growth rate data, is that the average difference in growth rate between *S. cerevisiae* and *S. bayanus* is the same (*r* = 0.006 h⁻¹) at both 12.8 °C and 15.8 °C. The prediction of change in population of *Saccharomyces* isolates is based on the proportion of *S. bayanus* and *S. cerevisiae* isolates observed at each time-point (where n=96). The calculations were made using the difference in growth rates calculated at 12.8°C and 15.8 °C, which was the same in this instance. Malthusian fitness (*m*) or the difference in rate of exponential increase of *S. bayanus* and *S. cerevisiae*, was calculated and converted to percentage Darwinian fitness (*w*) since *w* = 100(exp(*m*)-1) (Goddard, 2008). Based on the average growth rates calculated from figure 2.9 at 12.8°C, *w* was calculated to be 0.6 per cent h⁻¹ fitness advantage for *S. bayanus* isolates. By using the
equation provided by Hartl and Clark (1997), change in the ratio of *S. cerevisiae: S. bayanus* can be predicted at different days, the equation 2.2 is as follows;

\[
t = \frac{1}{m} \ln \left( \frac{p q_0}{q t p_0} \right)
\]

where: \( t \) = time (h),

\( m \) = Malthusian fitness,

\( p \) is proportion of *S. cerevisiae*

\( q \) is proportion of *S. bayanus*

\( 0 \) = initial time-point, \( t \) = time point

Equation 2.3: Equation to calculate the change in ratio of *S. cerevisiae: S. bayanus* using Malthusian fitness (\( m \)) (Hartl and Clark, 1997).

Theoretically, this simple model which only accounts for temperature comes reasonably close to predicting the time it took to achieve the observed ratio of these two species, given their starting proportions (refer 2.9.3.2 for ratios). Given the difference in growth as a function of temperature only, the model predicts 4.6 days to reach the ratio of 1:46 which was actually observed in 7 days in the 2008 Kumeu River Sauvignon Blanc ferment. However, this equation cannot be used to predict the ratios at later stages due to considerable change in fermenting temperature of Kumeu River ferment (figure 2.5). Also, unsurprisingly it is clear this model does not encapsulate all the complexity that explains the dynamics in this ferment; for example; the 5 random isolates of *S. bayanus* and *S. cerevisiae* used to determine growth rates may or may not have been the dominant strains in Kumeu River ferment. Also the juice composition was different between the growth and fermentation studies and the fact that other yeasts which were present in commercial ferment were unaccounted.

Therefore from using the above mentioned equation on limited data, we can only presume that the initial growth advantage for *S bayanus* was an important factor which allowed *S. bayanus* isolates to dominate and carry out rapid assimilation of sugar in the un-inoculated ferment of 2008 Kumeu River Sauvignon Blanc. More work needs to be conducted to elucidate the niche occupied by *S. bayanus* in un-inoculated ferments and the degree to which different environmental factors effect whether *S. bayanus* or *S. cerevisiae* dominate the fermentation process.
2.9.6 Impact of \textit{S. bayanus} on the varietal aroma of 2008 Kumeu River Sauvignon Blanc

Along with yeast species identification and molecular characterization during un-inoculated fermentation, samples were also analysed for varietal aroma. Thiols; 3MH, 3MHA and 4MMP are important aromatic sulphur compounds released from their non-aromatic pre-cursors by yeast during the fermentation of Sauvignon Blanc from Marlborough region (Lund \textit{et al.}, 2009; Tominaga \textit{et al.}, 1998b).

Figure 2.12 shows the evolution of thiols during different stages of fermentation. Many studies have monitored the level of various aroma compounds during various stages of fermentation using GCMS techniques (Mallouchos \textit{et al.}, 2002; Vas \textit{et al.}, 1999; Vianna and Ebeler, 2001). However, aroma studies in Sauvignon Blanc wine have mostly looked at the impact of different strains of commercial \textit{S. cerevisiae} on volatile thiol production (Howell \textit{et al.}, 2006; Howell \textit{et al.}, 2004; Swiegers \textit{et al.}, 2009). One study has looked at impact of co-fermentation of \textit{S. cerevisiae} with non-\textit{Saccharomyces} on thiol production in Sauvignon Blanc (Anfang \textit{et al.}, 2009). A couple of studies have analysed the level of thiols in Sauvignon Blanc made using \textit{S. bayanus} and its hybrids (Dubourdieu \textit{et al.}, 2006; Masneuf-Pomarède \textit{et al.}, 2006). Most of these studies have been carried out in a synthetic wine media or under controlled laboratory conditions or by inoculation of commercial yeast, so far to my knowledge this is the first study to have monitored the release of thiols during a commercial un-inoculated ferment of Sauvignon Blanc.

As shown in figure 2.12; 3MH was the first thiol to be released on day 7 of fermentation followed by both 3MHA and 4MMP from day 9 onwards. The release of aroma coincided with the rapid rate of assimilation of grape sugar by \textit{S. bayanus}, with fluctuations in thiol concentration during fermentation (figure 2.12). At the end of fermentation 3MH was approximately 3330 ng/L (±79 ng/L), 3MHA was 211 ng/L (± 10.5 ng/L) and 4MMP was 135 ng/L (±5 ng/L). It is interesting to note that the 4MMP concentration in the un-inoculated ferment was 168.5 times greater than its odour perception threshold of 0.05 ng/L as shown in table 2.7(compiled from data obtained from F.Benkwitz) (Lund \textit{et al.}, 2009). The concentration of 3MH and 3MHA were however within the range described by F. Benkwitz (Benkwitz, 2009). The aroma profile of commercial yeast fermented Marlborough Sauvignon Blanc is usually influenced by methoxypyrazines and varietal thiols 3MH and 3MHA, rarely does it show a high level of the thiol 4MMP (Benkwitz, 2009; Lund \textit{et al.}, 2009).

One possible explanation for the lack of thiol 4MMP in commercial wines is due to the fact that almost all of these ferments were carried out by wine yeast \textit{S. cerevisiae}; most of the commercial \textit{S. cerevisiae} lack the gene \textit{IRC 7} which has been shown to be important in releasing the thiol 4MMP during fermentation, although the mechanism of 4MMP production is yet to be elucidated (Roncoroni \textit{et al.}, 2011). Aromatic thiol production during fermentation is discussed more in detail in chapter 4.
<table>
<thead>
<tr>
<th>Thiol</th>
<th>Aroma</th>
<th>Perception Threshold ng/L</th>
<th>Final Concentration in KRSB 2008 ng/L</th>
<th>Typical range over the perception threshold for MSB</th>
<th>Range over perception threshold for KRSB 2008</th>
</tr>
</thead>
<tbody>
<tr>
<td>4MMP</td>
<td>Boxwood, blueberry, passion fruit, grapefruit</td>
<td>0.8</td>
<td>135</td>
<td>7-20</td>
<td>168.5</td>
</tr>
<tr>
<td>3MH</td>
<td>Passion fruit, grapefruit</td>
<td>60</td>
<td>3172</td>
<td>30-100</td>
<td>53</td>
</tr>
<tr>
<td>3MHA</td>
<td>Gooseberry, guava</td>
<td>4</td>
<td>221</td>
<td>10-100</td>
<td>55</td>
</tr>
</tbody>
</table>

Table 2.7: Perception threshold and typical range of important thiols in Marlborough Sauvignon Blanc.

There have been reports that *S. bayanus* isolated from a un-inoculated ferment in Sancerre region was capable of releasing more 4MMP compared to *S. cerevisiae* when fermenting the same Sauvignon Blanc must (Dubourdieu et al., 2006). Coincidentally, *S. bayanus* isolates studied by Roncorini et al. (2011) were known to carry a full copy of the IRC7 gene important in 4MMP production during fermentation. This information suggests that a high level of 4MMP in Sauvignon Blanc could be due to *S. bayanus* dominating the fermentation process. However, more work was required to definitely establish the relationship between *S. bayanus*, fermenting conditions and 4MMP levels. This work was carried out in chapter 4.

Figure 2.12: Levels of varietal aroma of 3MH, 3MHA and 4MMP at different time points during un-inoculated fermentation of 2008 Kumeu River Sauvignon Blanc. Primary Y axis is used to depict 3MHA (  ) and 4MMP (  ) levels. Secondary Y axis is used to depict 3MH (  ) due to vast difference in their respective concentrations.
2.10 Conclusion

In recent years, there have been several reports of the influence on aroma and wine character by several isolates of both *Saccharomyces* and non-*Saccharomyces* yeast from un-inoculated ferments (Ciani *et al.*, 2010). This was the first study to comprehensively analyse the community of a un-inoculated commercial ferment of a Marlborough Sauvignon Blanc. The major culturable non-*Saccharomyces* yeast present in 2008 Kumeu River Sauvignon Blanc at the start were similar to those found in other white wines (Ciani *et al.*, 2006; Ciani *et al.*, 2010; Fleet, 2003; Torija *et al.*, 2001). Fortuitously these non-*Saccharomyces* yeast have been shown to have positive influence on wine along with *S. cerevisiae* (Ciani *et al.*, 2010).

*S. bayanus* was the dominating yeast species during fermentation, this agrees with other reports of un-inoculated ferments carried out by *S. bayanus* in cold climate wine regions of Europe (Lopandic *et al.*, 2008; Naumov *et al.*, 2000; Rementeria *et al.*, 2003). It is possible that *S. bayanus* being cryotolerant is capable of growing faster in cold temperature and establishing itself as the dominant species in the early part of fermentation. Although this domination by *S. bayanus* was not seen in the red wine ferment in St. Georgen region (cold climate) of Austria, it could be because red wine has a higher pH and conducted at a higher temperature (typically between 20-30°C) and thought to harbour a different yeast community compared to white wines (Fleet, 2003; Lopandic *et al.*, 2008). This hypothesis is to be tested to further understand the high prevalence of *S. bayanus* isolates in un-inoculated ferments of certain wines.

There was a difference in growth ability of the *S. bayanus* and *S. cerevisiae* isolates from 2008 Kumeu River Sauvignon Blanc at lower temperature (12.8°C). Thus, by calculating Malthusian fitness (m) of these *Saccharomyces* yeast a limited growth model was able to predict the initial ratio *S. cerevisiae*: *S. bayanus* in 2008 Kumeu River Sauvignon Blanc. Thus it can be suggested that the initial temperature of 2008 Kumeu River Sauvignon Blanc ferment could have conferred a growth advantage to the *S. bayanus* isolates allowing them to dominate the fermentation. Although other factors such as the killer activity of *S. bayanus* and *S. cerevisiae* isolates were not tested and could prove to be important in dictating the population dynamics during fermentation. Finally, the origin and the source of these yeasts were not confirmed during this study.

Tracking the evolution of thiols during fermentation showed that they are liberated at the height of alcoholic fermentation, it could be possible that high levels of 4MMP seen in 2008 Kumeu River Sauvignon Blanc is due to *S. bayanus* carrying out the majority of ferment as this thiol is rarely observed in commercially fermented Marlborough Sauvignon Blanc (Lund *et al.*, 2009). Thus monitoring an un-inoculated commercial Marlborough Sauvignon Blanc has found yeast isolates potentially capable of influencing varietal aroma of Sauvignon Blanc.
Impact of oenological conditions on growth and fermentation kinetics of yeast
3.1 Introduction

Oenological conditions influencing fermentation of grape must play an important role in the population dynamics and kinetics of fermenting yeasts and also on the quality of the wine (Chambers and Pretorius, 2010; Fleet, 2003). These oenological conditions could be due to quality of grape must or winemaking style which in turn influence yeast activities (Fleet and Heard, 1993; Houtman et al., 1980). For example; *S. cerevisiae* activities initiates a host of biotic and abiotic factors during fermentation, and these various factors modulate the environment to various degrees (Goddard, 2008). The modulation of the ferment environment by one species can in-turn affect other yeast species, these effects may range from mild to severe (Fleet, 2003). The molecular and physiological response of fermenting yeast to these oenological (fermentation) conditions can be defined as ‘stress response’ (Ivorra et al., 1999). Tolerance (response) to stress plays a crucial role in the establishment, nutrient consumption and survival of specific yeast species during fermentation which consequently affects the quality of wine (Attfield, 1997; Ciani et al., 2010; Fleet, 2003; Goddard, 2008). It has been shown that there is direct correlation between survival of a yeast and resistance to various stresses imposed on it during fermentation (Ivorra et al., 1999). In this chapter, the responses (tolerance) of fermenting yeast to different oenological conditions (stress) and also to the presence of other yeast were examined further.

3.2 Oenological conditions

Chemical stress factors during fermentation includes availability of assimilatory sugars, yeast available nitrogen, oxygen levels, vitamins, minerals, availability of certain unsaturated fatty acids and ergosterol (Bisson, 1999; Hohmann, 1997). Equally important are physical stressors like changing temperatures, changes in grape juice pH and osmotic pressure (Charoenchai et al., 1998; Hohmann, 1997). Inhibitory compounds due to yeast activity also induce stress, these include; (but not limited to) ethanol, acetic acid, certain fatty acids, carbon dioxide, sulphites, mycotoxins, bacterial toxins, phenolic phytoalexins and agrichemical residues (Bauer and Pretorius, 2000; Fleet, 2003). The continual adaptation of yeast during fermentation to the various stresses not only ensures its survival but also impacts on the resulting wine through the constant modification of grape juice. There are numerous studies focussing on specific genes, proteins or metabolites which are affected by a diauxic shift in yeast growth during fermentation, in this study, however, only the main oenological conditions which have an impact on *Saccharomyces* and non-*Saccharomyces* yeast during fermentation are discussed (Zaman et al., 2008).
3.2.1 Yeast response to abiotic oenological conditions during fermentation

It is important to note that winemaking industry has developed sophisticated methods to control various oenological conditions impacting fermentation to suit the type of wine being produced (Bauer and Pretorius, 2000). Thus in most cases, controlled industrial fermentation does not allow for exploration of the complex nature and impact of various oenological conditions mentioned above. Therefore the oenological conditions driving a un-inoculated or natural fermentation process were reviewed (only white wine). Also the described (below) oenological conditions would have played an important roles in the 2008 Kumeu River fermentation (un-inoculated) of Marlborough Sauvignon Blanc, some of the yeasts examined in this chapter were isolated from this ferment.

3.2.1.1 Osmotic stress response

In un-inoculated fermentation, the initial yeast population is dominated by yeasts found naturally in vineyards and on grapes and winery equipment (Querol and Fleet, 2006). These yeasts are faced with a highly osmotic environment of grape juice with high nutrient and hexose concentrations (Querol and Fleet, 2006). Most yeasts are adapted to varying degrees to the highly osmotic (high sugar) environment of grape juice (Bauer and Pretorius, 2000; Scanes et al., 1998). They respond to hyperosmotic shock by modifying cell wall and cytoskeleton structures, and also by using an osmolyte to re-establish the osmotic balance of the cell (Bauer and Pretorius, 2000; Blomberg et al., 1992; Hohmann, 1997). Various yeasts and fungi utilize different osmolytes like potassium ions, proline, sugars and alcohols, these compounds are either accumulated actively from the environment or biosynthesized (Hohmann, 1997). Saccharomyces cerevisiae synthesises glycerol to maintain its osmotic balance in a fermenting environment (Blomberg et al., 1992; Hohmann, 1997).

3.2.1.2 Ethanol induced stress response

It was shown that yeast strains with high ethanol and temperature tolerance were the ones dominating the latter stages of fermentation (Torija et al., 2003). Most non-Saccharomyces species die after initial stages of fermentation as they are unable to tolerate high temperatures, ethanol concentrations and lack the ability to ferment grape juice (Pina et al., 2004b; Querol and Fleet, 2006; Torija et al., 2003). In fact Erten (2002) showed that low temperatures of below 15°C favoured the growth of K. apiculata and that temperatures greater than 20°C supported the growth of S. cerevisiae. Additionally, another study showed that lower temperatures also improved ethanol tolerance in the non-Saccharomyces they examined (Charoenchai et al., 1998). Indeed Gao and Fleet (1988) found that Kloeckera apiculata and Candida stellata were able to survive ethanol concentrations of up to 15% below 16°C. However, anaerobic fermentation is an exogenic reaction and can heat up the juice by about 30°C under ideal conditions (Williams, 1982). Goddard (2008) suggested that Saccharomyces cerevisiae increases its
fitness in the fermenting niche by producing ethanol and increasing temperature through the anaerobic assimilation of sugar: this increase in ethanol and temperature is very effective in deterring the growth of non-\textit{Saccharomyces} yeast species.

3.2.1.3 Yeast response to pH and nutrient imbalance and availability of oxygen during fermentation

In addition to ethanol production and increasing temperature another by product of fermentation is the release of carbon dioxide; mostly by \textit{Saccharomyces} yeast which purges oxygen from wine (Bauer and Pretorius, 2000; Hohmann, 1997; Holm Hansen \textit{et al}., 2001). Oxygen availability is key to the survival of some non-\textit{Saccharomyces} yeast like \textit{Kluyveromyces thermotolerans} and \textit{Torulaspora delbrueckii} are unable to survive in an anaerobic environment (Holm Hansen \textit{et al}., 2001).

Non-\textit{Saccharomyces} species are better adapted to variations in pH, and Charoenchai \textit{et al}.

Apart from adapting to potentially severe environments, yeasts at the latter stages of fermentation also face nutrient depletion and imbalance. Nutrient depletion faced by the dominant yeast could be due to grape juice related factors or due to the consumption of vital nutrients by other microbes (Bauer and Pretorius, 2000). On the other hand nutrient imbalance occurs when there is a shortage of yeast available nitrogen (YAN) compared to the available hexose sugars, this is mainly due to the inefficient usage of nitrogen compounds by \textit{S. cerevisiae} (Bauer and Pretorius, 2000). YAN consists of free amino acids (but not limited to) like arginine, alanine, glutamine, glutamate, serine and threonine, ammonium ions and γ-aminobutyrate (Bell and Henschke, 2005). \textit{S. cerevisiae} uses different sources of nitrogen throughout fermentation and its preference is based on the ease of uptake of these nitrogen sources (Magasanik and Kaiser, 2002). Nitrogen Catabolite repression is induced in \textit{S. cerevisiae} during fermentation which controls the uptake of these nitrogen sources by varying gene expression (Bell and Henschke, 2005; Deed \textit{et al}., 2011; Vilanova \textit{et al}., 2007).

Nutritional stress can result in retarded growth and stuck fermentation (Bisson, 1999). Depletion of nutrients is prevented partially by producing certain proteolytic and pectolytic enzymes capable of hydrolysing proteins and pectins in grape juice (Fleet, 2003). The autolysis of large amount of biomass in the mid to late stages releases vitamins and amino acids allowing for continued growth of yeast throughout fermentation (Fleet, 2003). Nutritional limitation of non-\textit{Saccharomyces} yeast during fermentation is not well understood.

Yeast biomass also acts to adsorb and remove toxic substances like metal ions and grape phenols from the fermentation process (Fleet, 2003). In the final stages of fermentation \textit{S. cerevisiae} shows reduced
fermentation efficiency due to the increased ethanol toxicity and lack of oxygen which compromises cell wall integrity and uptake of vital nutrients (Fleet, 2003). However there are several winemaking techniques evolved to alleviate various stressors faced by yeast during fermentation; such as addition of DAP, temperature control and use of yeast bred for specific ferment conditions, but these techniques are usually not utilized during the un-inoculated fermentation at Kumeu River winery (Jackson, 2000).

To my knowledge, very little is known about the response of non-Saccharomyces yeast to depleted YAN or their interactions with Saccharomyces yeast during fermentation. One of the aims of my research was to study the impact of oxygen depletion and nutrient availability on the species dynamics between Saccharomyces and non-Saccharomyces yeast. A key focus of Goddard laboratory has been the interaction between Pichia kluyveri and S. cerevisiae (VL3) in Marlborough Sauvignon Blanc under specified inoculum ratios and environmental conditions (Anfang et al., 2009). This interaction was selected as there is detailed information on the individual dynamics of these two yeast species under controlled oenological conditions which enabled further experimentation (Anfang, 2010). The experimental design is discussed in methods section 3.4.1.

3.2.2 Yeast response to biotic factors (other microbes) during fermentation

There are numerous biotic factors responsible for the growth and survival of yeast during un-inoculated fermentation, where, biotic factors can be defined as activities or metabolites induced exclusively due to the presence of other organisms and do not comprise the general mechanisms and compounds induced during fermentation (Ciani et al., 2010; Fleet, 2003; Villas-Bôas and Bruheim, 2007). Examining various un-inoculated ferments have shown that there is a plethora of microbes present initially, ranging from metazoans, filamentous fungi and a variety of yeast and bacterial species (Fleet, 2003; Torija et al., 2001). Here the dynamics of yeast-filamentous fungi, yeast-bacteria and yeast-yeast interactions and their positive and negative impact on alcoholic fermentation are broadly discussed from a fermentation point of view (grape must). The oenological properties resulting from these interactions are discussed in depth in chapter 4.

3.2.2.1 Yeast-fungi interaction

The presence of filamentous fungi belonging to genera Botrytis, Alternaria, Plasmopara, Aspergillus, Penicillium, Rhizopus, Odium and Cladosporum on grape surface can lead to spoilage (Blomberg et al., 1992; Fleet, 2003). Grape spoilage in turn affects the ecosystem and thus the natural yeast population (Fleet, 2003). Apart from interfering with the ecosystem some fungi also produce mycotoxins like ochratoxin A which can retard the growth of various yeast species (Walker et al., 1995). Fungal growth can result in high acetic acid bacteria numbers which in turn produce acetic acid and prevent yeast growth (Drysdale and Fleet, 1988; Ribéreau-Gayon, 1985). However, certain non-
**Saccharomyces** species belonging to genera *Pichia, Candida, Metschnikowia* among others have anti-fungal activities (Fleet, 2003; Zironi *et al.*, 1993). A few of these yeasts produce 1, 3 β glucanases which are capable of destroying fungal cell walls. Certain killer toxins produced by yeast can also inhibit fungal growth (Walker *et al.*, 1995). The 1, 3 β glucans in yeast cell walls are also capable of absorbing mycotoxins. In contrast, there have been early works which show a stimulatory effect of certain fungi on alcoholic fermentation (Fleet, 2003).

### 3.2.2.2 Yeast- bacteria interaction

Yeast-bacterial interaction on grape and in grape juice and wine has been well examined (Fleet, 2003; Osborne and Edwards, 2005). These interactions can be both beneficial and harmful to wine production. Grape juices from healthy and mature grapes have low concentrations of bacterial population (< 10⁴ cfu/mL). The two important groups present are lactic acid and acetic acid bacteria, and persistence of these bacteria is dependent on their pH and ethanol tolerance (Drysdale and Fleet, 1988; Fleet, 2003; Osborne and Edwards, 2005). Lactic acid bacteria from genera *Lactobacillus* and *Pediooccus* of which *Oenooccus oeni* is commercially important due to their usage in malolactic fermentation (Osborne and Edwards, 2005). Commonly isolated acetic acid bacteria include *Acetobacter aceti, Acetobacter pasteurianus, Gluconobacter oxydans* (Osborne and Edwards, 2005).

### 3.2.2.3 Yeast-yeast interaction

Since the industrialization of winemaking, many strains of *S. cerevisiae* have been examined and cultivated to suit particular ferment conditions (Fleet, 2003). There has been considerable research into the mechanisms of ethanol production, and yeast metabolism. It is now well understood that yeast metabolism is key to imparting flavour and aroma to wine (Chambers and Pretorius, 2010; Swiegers *et al.*, 2005). In the last couple of decades, there has also been an increasing interest in the non-*Saccharomyces* yeast present in wine. It is now widely accepted that not all non-*Saccharomyces* yeast are associated with wine spoilage and stuck fermentation but some are capable of contributing positively to wine flavour (Ciani and Comitini, 2011). But, it has only been in the last 10 years that there have been investigations into the interactions between different yeast species during fermentation. There is now evidence suggesting that not all yeast species co-exist passively during un-inoculated fermentation and that certain strains interact with one another and as a result have an un-predictable impact on wine quality (Ciani *et al.*, 2010; Clemente-Jimenez *et al.*, 2004; Romano *et al.*, 2003) Research in these interactions is partly due to the growing need to match the fermentation style to the grape variety and produce unique flavour characters in wines (Fleet, 2008). In recent years it has become common for winemakers to utilize multi-starter cultures to inoculate grape juice and also introduce various yeast species at different stages of fermentation termed sequential fermentation to improve wine quality (Fleet, 2008). This trend can be noticed as a proprietary blend of *S. cerevisiae/K. thermotolerans/T. delbrueckii* has been commercialized as Vinflora® and the
commercial availability of non-Saccharomyces yeast for co-inoculation with commercial S. cerevisiae like Pichia kluyveri marketed as Viniflora Frootzen® (both by Christian Hansen) to improve wine quality. In the following paragraphs, both Saccharomyces-Saccharomyces species interactions and Saccharomyces-non-Saccharomyces interactions that occur during winemaking will be discussed.

3.2.2.3.1 Interaction between Saccharomyces and non-Saccharomyces yeast

As described previously in this chapter, S. cerevisiae releases several compounds during fermentation like ethanol, short-medium chain fatty acids, acetaldehyde and acetic acid that prevent the growth of some non-Saccharomyces yeast and a few sensitive S. cerevisiae (Bauer and Pretorius, 2000). Another inhibitory mechanism discovered in certain species of genera Saccharomyces, Hanseniaspora, Pichia, Candida, Kluyveromyces, Zygosaccharomyces, Metschnikowia and Cryptococcus is the killer toxin activity (van Vuuren and Jacobs, 1992). Indeed, certain strains (killer positive strains) have been discovered to produce toxic glycoproteins capable of killing sensitive strains of yeast (killer sensitive strains) during winemaking (van Vuuren and Jacobs, 1992).

However, not all inhibitory compounds have been discovered; it was showed that a killer toxin sensitive S. cerevisiae was directly responsible for the death of two non-Saccharomyces yeast Hanseniaspora guilliermondii and H. uvarum during winemaking, the mechanism of which remains unknown (Pérez-Nevado et al., 2006). Interestingly it has also been shown that cell-cell interactions are also responsible for direct inhibition, wherein, the presence of high concentrations of S. cerevisiae cells (certain strains only) was sufficient to inhibit/kill selected strains of T. delbrueckii and K. thermotolerans (Nissen and Arneborg, 2003; Nissen et al., 2003).

Not all Saccharomyces – non- Saccharomyces interactions are antagonistic. Ciani et al. (2010) have reviewed some these interactions and their beneficial organoleptic properties these will be discussed in detail in chapter 4. To my knowledge, there is scare literature regarding the fermentation kinetics of these mixed species multi-starter inoculations.

3.2.2.3.1.1 Fermentation kinetics of Saccharomyces-non-Saccharomyces co-ferments

One of my research aims was to understand the yeast dynamics of un-inoculated fermentation of Kumeu River Sauvignon Blanc (2008). Thus, experiments were carried out in Sauvignon Blanc juice with the dominant S. bayanus yeast co-fermenting with three non-Saccharomyces yeast; P. kluyveri (PKKKR1) studied by Nicole Anfang and P. anomala and T. delbrueckii that were identified in 2008 Kumeu River Sauvignon Blanc at a range of controlled temperature and YAN conditions. These non-Saccharomyces yeast were used to co-ferment with S. bayanus to emulate the kinetics of a un-inoculated ferment. A range of oenological conditions were used to determine the dynamics of
ferment kinetics (discussed in section 3.3). These ferments were carried out as part of a full-factorial experiment to understand the fermentation potential of NZ isolates of *S. bayanus* and non-*Saccharomyces* yeast under different oenological conditions and their impact on quality of resulting wine. Below is an overview of research conducted by other groups and the impact of *Saccharomyces* and non-*Saccharomyces* yeast interaction on wine.

Single inoculations of non-*Saccharomyces* yeast were compared with *Saccharomyces* yeast on diluted Trebbiano Toscano grape juice and found that although *S. cerevisiae* had an increased growth compared to most of the other yeasts, non-*Saccharomyces* yeast were able to grow and increase in biomass and ferment the sugars to a certain extent (Ciani and Picciotti, 1995). *K. apiculata* was able to survive longer in the presence of *S. cerevisiae* compared to on its own in a synthetic grape juice media (Mendoza *et al.*, 2007). However, in co-fermentation both the yeasts showed lower growth rates. This phenomenon was also observed for pure and mixed cultures of *S. cerevisiae* and *H. uvarum* (Mendoza *et al.*, 2007). This could be due to the altered glucose and fructose consumption rates by both *Saccharomyces* and non-*Saccharomyces* yeast during single and mixed fermentations (Mendoza *et al.*, 2007). Mendoza *et al.* (2007) also noted a reduction in the alcohol content of mixed fermentations. Anfang *et al.* (2009) also showed that both *S. cerevisiae* and *P. kluyveri* had lower cell numbers in co-ferments compared to monoculture in Sauvignon Blanc but clearly increased in the level of aromatic thiols liberated. This trend in lowered growth rates and reduced alcohol content could prove to be important when considering industrial scale fermentations.

Other co-ferments studied have been between *S. cerevisiae* and *I. orientalis* (1:1 co-fermentation ratio), *S. cerevisiae* and *P. anomala* (1:1 ratio), *S. cerevisiae* with *C. stellata* (1:10) (Kim *et al.*, 2008; Soden *et al.*, 2000). Most of these interactions were studied in synthetic media simulating grape juice and carried out at temperatures ranging from 18-25⁰C. But in 2002, Erten showed that at higher temperatures (20⁰C) *S. cerevisiae* dominated the fermentation completely in co-ferment, whereas, when co-fermentation was carried out at 10-15⁰C *K. apiculata* was able to dominate the fermentation for a short period of time. Therefore more work needs to be done to understand the growth dynamics of mixed fermentations at different temperatures.

There is an interest in commercializing yeasts/yeast mixtures that are capable of imparting complex character in wine. But, winemaking is a dynamic process carried out at a range of temperatures pH and other varietal specific treatments (Charoenchai *et al.*, 1998). An important caveat of the studies on mixed fermentation is that it is carried out under specific oenological conditions and that these mixed species interactions are not well understood at a range of different winemaking conditions and on the various grape varieties (Houtman *et al.*, 1980). One recent study conducted on 34 non-*Saccharomyces* yeast isolated from winemaking environments and observed a range of responses from these yeasts to several winemaking conditions like response to SO2 levels, β-glucosidase activity, protease activity,
ester-hydrolase activity and killer toxin character (Ciani and Comitini, 2011). Also upon co-fermenting selected non-\textit{Saccharomyces} yeast with commercial \textit{S. cerevisiae} EC 1118 with three different inoculation concentrations, they found unique responses from the non-\textit{Saccharomyces} yeast to EC 1118 and also to altered inoculation ratios resulting in variable growth kinetics and differences in both soluble and volatile metabolite production (Ciani and Comitini, 2011). This study sets a good example to show that there is untapped biodiversity and oenological phenomena in the natural yeast associated with wine and that these mixed fermentations are highly dependent on their environment. Therefore it is also important to undertake research into co-fermentations under a range of environmental parameters to unearth the full oenological potential of specific yeast interactions.

\textbf{3.2.2.3.2 \textit{Saccharomyces-Saccharomyces} interaction during fermentation}

Although \textit{Saccharomyces cerevisiae} is the dominant wine yeast species, there are various strains of this species that have been commercialized for winemaking due to their specific characteristics. It is well understood that usage of different strains as inoculum to grape juice yields wine with distinct oenological characters (Eglinton \textit{et al.}, 2000; Muratore \textit{et al.}, 2007). Howell \textit{et al.} (2006) showed that fermenting chardonnay juice using multiple \textit{S. cerevisiae} starters resulted in a remarkable different wine in comparison to a blended wine made of wines fermented with individual starter cultures of \textit{S. cerevisiae} used in the multi-starter inoculum. Thus they showed that it was due to the \textit{Saccharomyces} yeast interacting with one another that resulted in the unique character of the wine not reproduced by post ferment blending (Howell \textit{et al.}, 2006). This study also noted that there was differences in the completion of fermentation of the mixed starter wines in comparison with the individual inoculum wines, however there is no indication of statistically significant difference. They also noted that both multi-starter and respective monoculture ferments consisted of the same number of viable cells during fermentation (Howell \textit{et al.}, 2006).

Their finding is interesting as there have been a few studies which have monitored the population dynamics of \textit{S. cerevisiae} in un-inoculated ferments and have reported the presence of more than a 100 distinctive strains (Goddard \textit{et al.}, 2010; Pramateftaki \textit{et al.}, 2000; Torija \textit{et al.}, 2001). Therefore the presence of the vast diversity of \textit{S. cerevisiae} strains in un-inoculated ferments could potentially add to the complexity of wines otherwise lacking when commercially fermented.

Kumeu River fermentation of Sauvignon Blanc (2008) was carried out by \textit{S. bayanus}. Various strains of \textit{S. bayanus} were involved in this process, the impact of different strains of \textit{S. cerevisiae} on fermentation is known whereas virtually nothing is known regarding the impact of various strains of \textit{S. bayanus} on wine quality or their ability to ferment. One of my research aims was to identify the fermentation kinetics of different strains of \textit{S. bayanus} isolated. This study was necessary to pursue further understanding of aroma potential of \textit{S. bayanus} capable of fermenting Sauvignon Blanc.
3.2.2.3.2.1 Comparison between *S. bayanus* and *S. cerevisiae*

As discussed in chapter 2, very little is understood regarding the strain diversity of *S. bayanus* during un-inoculated fermentation or their contribution to wine character, only the fact that they were known to dominate and complete fermentations of certain wines (Masneuf-Pomarède *et al.*, 2006; Naumov *et al.*, 2000). Also there is no literature regarding the interaction between different strains of *S. bayanus* under controlled oenological conditions. However, there have been a few studies that have compared the fermentation kinetics and wine aroma between *S. cerevisiae* and *S. bayanus* var. *uvarum* (Eglinton *et al.*, 2000; Favale *et al.*, 2007; Tosi *et al.*, 2009). The consensus from these studies is that there are differences in the ethanol production and temperature tolerance between these two yeast species. It was noted that *S. cerevisiae* isolates were able to tolerate higher ethanol concentrations and completed fermentation in 29 days whereas it took 37 days for the *S. bayanus* var. *uvarum* strains to finish fermenting Amarone wine (Tosi *et al.*, 2009). They also produced distinct organoleptic characteristics in wines starting from the same grape juice (Tosi *et al.*, 2009).

Co-fermentation with *S. bayanus* var *uvarum* and *S. cerevisiae* isolates have been studied (Cheraiti *et al.*, 2005). Cheraiti *et al.* (2005) showed that there seemed to be communication between the two species through the production of acetaldehyde. It was shown that these two species had different metabolisms, sequential fermentation with *S. bayanus* followed by *S. cerevisiae* and vice versa resulted in wines with different profiles, and these profiles were also different compared to the respective mono-ferments (Favale *et al.*, 2007). However, there is no information regarding co-fermentation behaviour of *S. bayanus* with non-*Saccharomyces* yeast.

There is now knowledge that *S. bayanus* is one of the key wine fermenting yeast species capable of producing unique wine characters, but *S. bayanus* is yet to be studied as comprehensively as *S. cerevisiae* (Masneuf-Pomarède *et al.*, 2010). The complete genome of *S. bayanus* will prove useful in further studies to understand its oenological traits (Kellis *et al.*, 2003). There is a vast biodiversity of *S. bayanus* as yet un-tapped for commercial use. Co-fermentation of *S. bayanus* with non-*Saccharomyces* yeast is yet to be examined; this could prove to be of similar commercial success as co-fermentation using *S. cerevisiae*-non-*Saccharomyces* yeast. Along with commercial usage, these studies could provide valuable insight into the mechanisms of niche construction and species interaction at a metabolic level.
3.3 Full-factorial experimental set-up to analyse interaction between

Saccharomyces and non-Saccharomyces yeast under controlled oenological conditions

The onus of my PhD was to identify and understand yeast interaction in New Zealand Sauvignon Blanc ferment and their impact on varietal aroma of wine. I achieved this partly by designing and performing a full-factorial experiment based on results obtained in chapter 2 and chapter 3 (section 3.5.1 and 3.5.2) (Box et al., 2005). Here, the impact of three important oenological conditions on the outcome of fermentation of New Zealand Sauvignon Blanc was investigated. With respect to the experimental design, a two-step procedure was applied, i.e., ferments were performed at between a range (high and low) of the oenological conditions under study, followed by extensive data collection (all ferments were carried out in triplicates with measurements for fermentation kinetics, and aroma compounds) (Box et al., 2005; Mertens et al., 2012).

This experiment consisted of three discrete factors (non-confounding) with different levels whose experimental units (sample values) could be used to test the effect of each factor or even the interaction between the different factors (Box et al., 2005). Full factorial designs are commonly used to monitor microbial response and optimise industrial processes in food microbiology (Mertens et al., 2012). There were two aims for this experiment; one was to compare mono-ferments (from fermentation kinetics through to aroma analyses of wine) of a selected S. bayanus isolate from 2008 Kumeu River Sauvignon Blanc ferment and VL3 and also their respective co-ferments with PKKR1. Another aim was to compare the co-ferments of selected S. bayanus isolate with different isolates of non-Saccharomyces yeast identified from 2008 Kumeu River Sauvignon Blanc.

Assimilable nitrogen is often the limiting factor during alcoholic fermentation of grape must by yeast (Bell and Henschke, 2005). However, increasing the YAN of a grape must increase the yeasts’ biomass and their fermentation rate (Bely et al., 2003). The absolute minimum concentration of YAN required for successful completion of fermentation is hard to determine, however, many studies have reported a range of YAN concentration to guarantee completion of fermentation (Bely M et al., 1990; Martínez-Moreno et al., 2012; Mendes-Ferreira A et al., 2004). The reported YAN concentrations range from as low as 120 mg/L to 267 mg/L, there is at least a two-fold difference between these values and they are supposedly impacted by the type of juice, yeast strain utilised and the quality of nitrogen source (ratio between amino acids and ammonia) (Bely M et al., 1990; Martínez-Moreno et al., 2012; Mendes-Ferreira A et al., 2004). In this study, the source juice had a relatively high YAN of 390 mg/L (compared to the different studies), so a three-fold difference juice.
with YAN of 1170 mg/L (via DAP addition to the original juice) was used to determine the overall fermentation potential of the yeast strains. Literature suggests that yeast cannot utilise YAN of more than 400-500 mg/L, however, in this study the aim was to understand the outcome (both positive and negative) of high and low level of YAN on selected yeast (Martínez-Moreno et al., 2012). As these yeast have not be studied previously (except VL3 and PKKR1), this experimental set-up provided an opportunity to understand the impact of high level of YAN on fermentation kinetics and aroma potential by these species. The outcome would perhaps further our understanding of stuck/sluggish fermentations, however, this level of YAN (1170 mg/L) is not used in commercial winemaking.

Differences in temperature of fermentation are known to impact on the survival and nutrient utilization of both Saccharomyces and non-Saccharomyces yeast (Charoenchai et al., 1998; Goddard, 2008; Molina et al., 2007). Therefore to understand the fermentation potential of the S. bayanus isolate SBJ1d two different temperature conditions were utilised. The temperature of 12.8 °C and 20 °C were selected primarily to understand fermentation kinetics at both low and high temperature conditions, 12.8 °C was selected as it’s the average Marlborough temperature (MetService) and the possible origin of SBJ1d, and 20 °C was selected as it was final fermenting temperature of 2008 Kumeu River Sauvignon Blanc.

The full-factorial experiment consisted of three factors; temperature, YAN and yeast species. The yeast species factor consisted of six levels including mono-ferments of S. bayanus isolate SBJ1d and S. cerevisiae (VL3) and co-ferments of S. bayanus isolate with non-Saccharomyces yeast T. delbrueckii, P. anomala and P. kluyveri (PKKR1) and co-ferment of VL3 with PKKR1 (the experimental set-up and layout discussed in methods section 3.4.3). All the co-ferments had an initial inoculum ratio of 1:9 for Saccharomyces and non-Saccharomyces yeast. The temperature factor consisted of two levels at 12.8°C and 20°C. The YAN factor consisted of two levels of YAN in Sauvignon Blanc juice used in fermentation with Y1 juice being 390 mg/L and Y2 juice being 1170 mg/L. The responses measured for all these ferments were fermentation kinetics (this chapter), concentration of varietal aroma and other volatile aromas (chapter 4). This experiment was carried out as a step in understanding the potential of New Zealand isolates of S. bayanus in commercial Sauvignon Blanc fermentation.

This chapter discusses primarily the impact of oenological conditions on fermentation kinetics of various mono and co-ferments. The results discussed here are from testing null hypotheses from independent experiments as well as a part of the full-factorial experiment.
3.3.1 Aims

The objectives of this chapter are outlined below.

1) Test the null hypothesis that a change in environment (temperature and oxygen availability) has no impact on the population dynamics between *S. cerevisiae* VL3 and *P. kuyveri* PKKR1 under controlled conditions.

2) Test the null hypothesis that there is no difference in the fermentation kinetics of different *S. bayanus* isolates from the same natural population in the same environment (temperature) under controlled conditions.

3) Test the null hypothesis that there is no difference in fermentation kinetics between *S. bayanus* and *S. cerevisiae* when fermented at a range of oenological environments (temperature, Yeast Available Nitrogen, presence of a non-Saccharomyces yeast) under controlled conditions.

4) Test the null hypothesis that there is no difference in the fermentation kinetics of *S. bayanus* with 3 different non-Saccharomyces yeast under controlled conditions.
3.4 Materials and methods

This chapter addresses four aims and the materials and methods utilised to test the pertinent hypotheses are listed under separate sub-headers.

3.4.1 Experimental set up to examine the impact of oxygen and nutrient availability on species interaction between *Pichia kluyveri* PKKRI and *Saccharomyces cerevisiae* VL3.

3.4.1.1 Pre-culture of yeast to be used in the experiment

PKKR1 and VL3 were pre-cultured in media and conditions described in chapter 1 section (2.8.3).

3.4.1.2 Preparation of Synthetic grape juice media (SGM)

This experiment was performed in a synthetic media (SGM) imitating grape juice as described by Henschke and Jiranek (1993), but with modified amino acid and Di ammonium phosphate (DAP) concentration to resemble the Sauvignon Blanc juice from the Marlborough region in New Zealand between the years 2004-2006 (data not shown). The list of all the compounds used and their concentration in SGM is shown in table 3.1. Stock solutions of sugar and major salts, trace minerals, vitamins and nitrogen and amino acids were prepared and combined in a sterile manner to the desired volume with the final concentration as shown in table 3.1. All the solutions were autoclaved as described in chapter 2 section 2.8.3 and stored in a cool dark place at room temperature, except the Vitamins solution and iron(II) sulphate solutions which were filter sterilized using Minsart® 0.20 µm filters supplied through Sartorius™ and stored at 4°C.
Table 3.1: Synthetic Grape juice Medium (SGM) resembling Marlborough Sauvignon Blanc, with a total YAN of 300mg/L (Harsch, 2009).
3.4.1.3 Experimental set up to monitor population dynamics between PKKR1 and VL3 under defined conditions

*P. kluyveri* PKKR1 and *S. cerevisiae* VL3 were grown under aerobic and anaerobic conditions, respectively. Total initial inoculum into each of the flasks was as prescribed by Anfang et al. (2009) to achieve elevated thiol levels in resulting Sauvignon Blanc wines. The inoculum consisted of 2.5 million cells/mL of which 90% (2.25 million cells/mL) constituted PKKR1 and remaining 10% (0.05 million cells/mL) was VL3. Apart from the cultures being aerobic or anaerobic they were either shaking or static to control the availability of nutrients. Samples were taken on alternate days for 20 days to monitor the change in population dynamics as prescribed by Anfang (2010).

### 3.4.1.3.1 Aerobic and Anaerobic shaking condition

Aerobic shaking condition included three 250 mL conical flasks with rubber sealed side-ports. Each flask contained 200 mL of synthetic grape media inoculated with 2.5 million cells/mL of 9:1 mixture of PKKR1 and VL3, the mouth of the flask was covered with a cotton gauze pad to prevent contamination. The flasks were incubated at 14.5°C and shaken at 100 rpm.

Anaerobic shaking condition was similar to aerobic shaking set-up using three rubber sealed side-port conical flasks, only exception involved covering the mouth of the flasks with an air-lock filled with water to render the flasks anaerobic.

Once every two days, 100 µL the ferment was collected from the triplicates (both aerobic and anaerobic) using sterile disposable 0.25 mm gauge needle through the rubber sealed side-port. Following serial dilution, the sample was plated on YPD agar plate (see section 2.8) to enumerate the cells. It is possible to morphologically differentiate between PKKR1 which form powdery wrinkly colonies compared to the smooth rounded shiny colonies formed by VL3; this enabled determining the change in the population of these two species during fermentation. A morphological difference in colony formation is shown in figure 3.1.

**Figure 3.1:** Morphology of yeast on YPD agar plates. (A) *P. kluyveri* PKKR1 and (B) *S. cerevisiae* VL3.
3.4.1.3.2 Aerobic and Anaerobic static treatment

The aerobic static treatment involved destructive sampling to minimise disturbance of samples. Thirty, 15mL capacity sterile plastic tubes were set up with 10 mL of the synthetic grape medium in each and inoculated with 2.5 million cells/mL at a ratio of 9:1 for PKKR1 and VL3. The flasks were kept aerobic by covering them with a cotton gauze pad. They were incubated at 14.5°C in a static incubator.

The anaerobic static treatment was similarly set up with the exception of sealing the plastic tubes to make them air tight with a single tiny hole in the lid to allow for the escape of carbon dioxide during fermentation, this hole was closed using a breathable tape to prevent contamination.

Destructive sampling was carried out (both aerobic and anaerobic) where three 15 mL tubes for each sample type were taken on alternate days and their contents mixed gently by pipetting before serial dilution followed by plating on YPD agar to monitor change in population dynamics.

All the conditions had controls set-up and monitored for contamination at the end of the experiment by plating 100µL undiluted media on YPD agar.

3.4.2 Microvinification set up to monitor impact of temperature on rate of fermentation of a natural population of *Saccharomyces bayanus* isolated from Marlborough Sauvignon Blanc

Twenty *S. bayanus* isolates identified previously (see appendix 2) using microsatellite analysis were allowed to ferment separately in Marlborough Sauvignon Blanc grape must at two different temperatures of 12.8°C and 20°C in triplicates, their rate of fermentation was calculated using the weight loss data recorded (Bely et al., 1990). Data analysis was carried out using R 2.12 and Microsoft Excel 2007 (Team, 2011).

3.4.2.1 Juice sterilization and experiment set-up

Similar to the procedure outlined in chapter 2 section 2.8.8, Marlborough Sauvignon Blanc juice from 2008 vintage provided by Pernod Ricard (pH of 3.28, TA (titratable acidity) of 6, Brix° 17.8) was thawed slowly to room temperature from -20°C in a warm water bath, followed by chemical sterilization using DMDC (dimethyl dicarbonate).

The *S. bayanus* isolates were pre-cultured as described previously in chapter 2 section 2.8.8. A list of isolates and their microsatellite profiles are listed in appendix 2. Individual isolates were then inoculated into 10 mL of sterilised Marlborough Sauvignon Blanc with an initial inoculum of 2.5 million cells/mL. Fermentation was carried out in 15 mL capacity sterile plastic tubes with sealed lids.
with tiny hole to allow for release of carbon dioxide. Each *S. bayanus* isolate was fermented in triplicate at both the temperatures of 12.8°C and 20°C while shaking at 100 rpm.

The weight loss of each of the ferments was monitored daily and was used to calculate the average maximum rate of fermentation (Vmax). This method assumed that the rate of weight loss was directly proportional to the rate of carbon dioxide release as described by Bely *et al.* (1990). Thus, fermentation rate (V) was calculated for the triplicates and the average maximum rate of fermentation (Vmax) was obtained using the equation 3.1:

\[
V = \frac{dWL}{dt}
\]

where; \(WL\) = weight loss in grams,

\(V\) = fermentation rate

\[dWL = (WL_j - WL_k)\]

\[dt = (t_j - t_k)\]

\(j, k = \) time periods j, k

Equation 3.1: Equation to calculate the fermentation rate of fermentation based on weight loss

3.4.3 Experimental set up to monitor impact of Yeast Available Nitrogen (YAN) level and temperature on *Saccharomyces* yeast and their interaction with non-*Saccharomyces* yeast

Fermentation of Marlborough Sauvignon Blanc juice from Stoneleigh (pH of 3.33, TA (titratable acidity) of 6, Brix° 21.4) was carried out in 1 L bottles using selected yeast (table 3.2). Fermentation was conducted under controlled conditions in triplicates and two different temperature and YAN levels (refer section 3.3). Weight loss data was monitored on a daily basis to calculate average maximum rate of fermentation (Vmax) as described in the previous section (section 3.4.2.1). Maximum rate of fermentation (Vmax) was the parameter used to determine impact of environment (YAN and Temperature) on yeast during anaerobic fermentation. All the calculations were carried out in Microsoft office Excel 2007 and R 2.12 software (Team, 2011).
3.4.3.1 Fermentation set up

3.4.3.1.1 Yeast pre-culture

The yeast used and their proportions in initial inoculum is listed in the table below (table 3.2). The selected yeasts were pre-cultured in YPD medium at 28°C for 48 hours with 150 rpm shaking. Following pre-culture, the media containing cells were centrifuged gently at 2000 rpm for 5 min to pelletize cells, the pellets were washed to remove unwanted media and cells were re-suspended in water and used immediately to inoculate sterile grape juice with a starting inoculum of 2.5 million cells/mL. Initial inoculation of co-fermentations contained 10% of desired *Saccharomyces* yeast and 90% of non-*Saccharomyces* yeast.

<table>
<thead>
<tr>
<th>Species</th>
<th>Code</th>
<th>Library annotation</th>
<th>Proportion of initial inoculum</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Saccharomyces cerevisiae</em> VL3</td>
<td>VL3</td>
<td>VL3</td>
<td>100% or 10%</td>
</tr>
<tr>
<td><em>Saccharomyces bayanus</em> SBJ1d</td>
<td>SB</td>
<td>J1d</td>
<td>100% or 10%</td>
</tr>
<tr>
<td><em>Pichia kluyveri</em> PKKRI</td>
<td>PKKRI</td>
<td>PKKRI</td>
<td>90%</td>
</tr>
<tr>
<td><em>Pichia anomala</em></td>
<td>PA</td>
<td>A1c</td>
<td>90%</td>
</tr>
<tr>
<td><em>Torulaspora delbrueckii</em></td>
<td>TD</td>
<td>D1a</td>
<td>90%</td>
</tr>
</tbody>
</table>

Table 3.2: Yeast species and their proportion in initial inoculum used in ferments in the full-factorial experiment.

3.4.3.1.2 Juice treatment and fermentation set up

The juice used in this experiment (pH 3.4, Brix° 22.1, TA (titratable acidity) 7.4 g/L) was kindly provided by Stoneleigh (2010) and transported from Marlborough under chilled (below 4°C) conditions. Yeast available nitrogen (YAN) of the juice comprises of free ammonium ions, all the primary amino acids (except proline which is not available for yeast consumption during fermentation) and side chains of L-arginine present (this requires hydrolysis by a yeast arginase during fermentation) (*Jiranek et al.*, 1995). YAN of the juice was calculated to be 390 mg/L by using a commercially available kit from Megazyme©.

A full-factorial experimental design was used to understand the fermentation kinetics and aroma potential (section 3.3) of selected *Saccharomyces* –only and *Saccharomyces* and non-*Saccharomyces* co-ferments (*Box et al.*, 2005). The experimental set-up consisted of 72, 1L bottles and 4 control bottles, 38 of the bottles were to have sterile juice with the original YAN and the other half were to contain sterile juice with YAN that was 1170 mg/L, three times the original value of 390 mg/L. A total of 60 Litres of juice was used. The original juice was shaken vigorously to mix the contents and separated into two 30L sterile carboys. One of the carboys was treated with Di ammonium phosphate.
(DAP), which is a chemical widely used in winemaking to increase the YAN of a grape juice to the desired level (Bell and Henschke, 2005). Addition of 1 g/L of DAP ((NH₄)₂HPO₄) into grape juice increases the YAN by 258 mg/L. Therefore the 30L of juice was treated with 45.33 g of DAP to achieve a total YAN of 1170 mg/L. Both the carboys were then treated with DMDC and chemically sterilized.

Fermentation was set up where a 1L bottle received ~650 mL of sterilized juice (original (Y1) or DAP treated (Y2)) and yeast (mono or co-ferments) and was then incubated at either 12.8°C or 20°C without shaking. The full-factorial experimental set-up consisted of triplicate 1L bottles of S. bayanus (SB) only inoculum, S. cerevisiae (VL3) only inoculum, co-inoculum of S. bayanus (SB) plus Pichia kluyveri (PKKR1), S. bayanus plus Pichia anomala (SB), S. bayanus (SB) plus T. delbrueckii (TD) and S. cerevisiae (VL3) plus Pichia kluyveri (PKKR1) at 12.8°C and also at 20°C. These ferments were also containing either the original juice or the DAP treated juice, for a flow chart representation refer figure 3.2.

![Flow chart representation of the full-factorial fermentation set up including various yeast under different temperature and YAN conditions. S. bayanus (SB) only inoculum, S. cerevisiae (VL3) only inoculum, co-inoculum of S. bayanus (10%) plus Pichia kluyveri (90%) (PKKR1+SB), S. bayanus (10%) plus Pichia anomala (90%) (PA+SB), S. bayanus (10%) plus Torulaspora delbrueckii (90%) (TD+ SB) and S cerevisiae (10%) plus Pichia kluyveri (90%) (PKKR1 +VL3) at temperature of 12.8°C and 20°C and also at YAN levels of 390 mg/L and 1170 mg/L](image-url)
3.4.3.1.3 Bottling of the ferments and storage

The ferments took between 16-32 days, weight loss was monitored daily and when there was less than 0.01g of weight loss in 48 hours the bottle was removed from the incubator and stored in cool room (4°C) and bottled within 24-48 hours. Each bottle was topped up with sterile glass marbles and food grade Nitrogen gas to reduce headspace to prevent oxidation and sealed using screw caps and stored in cool room(4°C) for aroma analysis (refer chapter 4).
3.5 Results and Discussion

Winemaking process creates a continuously evolving niche especially for fermenting yeasts where oenological conditions such as temperature, oxygen, nutrient availability, ethanol level and presence of competing microbes all have a major impact on their growth and survival, consequently on wine quality. Yeasts have evolved key mechanisms to grow and thrive in the fermenting wine niche; indeed some of these mechanisms can potentially be used to develop wine of distinct quality. The purpose of this chapter was to examine both growth (aerobic) and fermentation kinetics of selected yeast under controlled oenological conditions. This was done by testing the null hypotheses laid out in the introduction of this chapter.

3.5.1 Test the null hypothesis that a change in oxygen availability has no impact on the population dynamics between *S. cerevisiae* VL3 and *P. kluyveri* PKKR1 under controlled conditions

While the initial inoculum ratio and temperature of fermentation can be controlled even in large-scale fermentation, industrial winemaking is carried out in fermenting vessels ranging from steel tanks to oak barrels utilizing various techniques; potentially this could have a large impact on yeast interaction (Houtman *et al.*, 1980). The difference in shape and size and porosity (e.g. oak barrels) of fermenting vessel can impact on the oxygen available to yeast during fermentation (Houtman *et al.*, 1980; Jackson, 2000). It has been shown that non-*Saccharomyces* yeast survive longer in presence of oxygen, this could potentially alter the interaction between PKKR1 and VL3 and impact on the final aroma of Sauvignon Blanc (Anfang *et al.*, 2009; Ciani *et al.*, 2010; Erten, 2002). The study conducted by Anfang *et al.* (2009) did not take into account other oenological conditions at which the co-ferment could be used to enhance aroma thus providing winemakers with a spectrum for interaction between PKKR1 and VL3.

In this study; according to Anfang *et al.* (2009), the population dynamics of PKKR1 and VL3 with the prescribed inoculum ratio (1:9) and temperature of 14.5 °C were examined while simulating different oxygen availabilities. Here, co-fermentation was carried out in synthetic grape media under aerobic, anaerobic and shaking (100 rpm) and static treatments. Where, aerobic ferments potentially allowed for the availability of oxygen throughout the fermentation process as did the shaking of flasks which allowed for the uniform distribution of oxygen throughout the fermentation.
As shown in figure 3.3, it can be seen that there is a marked difference in the population dynamics (enumerated using method described in 3.4.1.3) between VL3 and PKKR1 under different fermenting conditions. In both aerobic and anaerobic static fermentations, VL3 dominated over PKKR1. In the aerobic static treatment, the frequency of VL3:PKKR1 changed from 1:9 to 4:6 on day 2 of sampling, followed by a >90% domination for the remainder of the duration of fermentation. However, VL3 was able increase in frequency by day 2 in the anaerobic static fermentation and change from initial 1:9 of VL3:PKKR1 to 7:3 respectively and clearly dominated the fermentation process.

In case of aerobic shaking fermentation trial, the frequency of PKKR1 was high throughout the sampling time period with VL3 seen in very low frequencies (< 0.1 % cfu/mL). This abundance of PKKR1 under shaking treatments is directly contrasting with the observation made under static treatments in an aerobic environment. Under anaerobic shaking treatment however, there was a gradual decrease in the frequency of PKKR1 and an increase in number of VL3, sample taken on day 10 of fermentation showed that there was approximately equal numbers of VL3 and PKKR1.
Interestingly, day 12 saw an increase in frequency of PKKR1 from 0.5 to 0.8 and a corresponding decrease in numbers of VL3 (0.5 to 0.2), the next few sample points showed mixed trend in the frequencies of the two different species. This fluctuation in population dynamics was different when compared to dynamics observed by Anfang et al. (2009).

Figure 3.4: Change in cell numbers of *S. cerevisiae* VL3 (blue) and *P. kluyveri* PKKR1 (red) under different conditions. (a) aerobic and static (b) anaerobic and static (c) aerobic and shaking (d) anaerobic and shaking. Y-axes represents the number of cells present in total cfu/mL, X-axes represents the days the population was sampled. All values plotted are the mean ± SEM (n=3).

A close look at the cell numbers in all the four conditions (figure 3.4) showed that there were differences in growth of the two species under different conditions (oxygen availability). In a study, it was showed that both VL3 and PKKR1 reached maximum cell numbers by day 3 and gradually decreased afterwards (Anfang, 2010). However, in synthetic grape juice ferments monitored, the two species took varying amounts of time to reach maximal cell numbers (figure 3.4). Under aerobic static treatment, both VL3 and PKKR1 had maximum cell numbers on day 10 with the population size being 2.3x10^7 and 2.2x10^6 cfu/mL respectively with total cell numbers in the end at 1.7x10^7 cfu/mL in the anaerobic static treatment, VL3 reached 1.5x10^7 cfu/mL and PKKR1 reached 1.23x10^6 cfu/mL on
day 8, there was a late surge in VL3 numbers with its population reaching $2.13 \times 10^7$ cfu/mL on day 16 and had a total end point cell count of $9.5 \times 10^6$ cfu/mL. Under anaerobic shaking treatment VL3 reached $1.12 \times 10^7$ cfu/mL on day 10 whereas PKKR1 reached $1.98 \times 10^7$ cfu/mL on day 8 with a total end point cell/ml of $1.7 \times 10^7$ cfu/mL. In the aerobic shaking treatment, VL3 reached $2.27 \times 10^6$ cfu/mL the lowest number observed for all the four conditions on day 10 whereas PKKR1 reached $1.23 \times 10^8$ cfu/mL on day 10 which is almost 2 fold greater than VL3. Under aerobic shaking treatment, the total number at end-point was noted to be around $9.52 \times 10^7$ cfu/mL; the highest amongst all of the conditions studied. It should be noted that the high cell numbers observed at the end time point in the four conditions is different from those observed by Anfang (2010). However, unlike measurements taken by Anfang (2010) at the end of fermentation, the end time point was at 20 days for all the 4 conditions and completion of fermentation (less than 2 % residual sugar) was not considered, therefore the yeasts in these experiments could still be growing.

From the results discussed above, it can be stated that there is a difference in population dynamics between PKKR1 and VL3 despite maintaining the inoculum ratio and temperature. However, to test the null hypothesis that availability of oxygen has no impact on the population dynamics between PKKR1 and VL3 during fermentation, the following equation (3.2) was used:

$$O_0 S_0 V P d_t = O_1 S_0 V P d_t = O_0 S_1 V P d_t = O_1 S_1 V P d_t$$  

$O_0$ = Anaerobic

$O_1$ = Aerobic

$S_0$ = Static

$S_1$ = Shaking

$d_t$ = change in time (2-20 days)

$VP$ = VL3:PKKR1

**Equation 3.2: Equation to test null hypothesis that there is no difference between the co-ferments of VL3 and PKKR1 at different oxygen availabilities**

To test the null hypotheses (equation 3.2), a 3 factor ANOVA was performed (the factors being; O, S and D). The factor O had a P-value of ($<2.2e^{-16}$), S had a P-value of ($<2.2e^{-16}$) and D had a P-value of ($5.641e^{14}$), the P-value for interaction between the 3 factors was also significant (2.773e$^{07}$). These P-values were highly significant thus providing strong evidence against the null hypothesis.
The availability of oxygen has an impact on the population dynamics observed. However, in this experiment, the impact of the composition of the synthetic Sauvignon Blanc media on population dynamics should not be discounted even if it cannot be quantified (Houtman et al., 1980).

Shaking the flasks can increase the oxygen availability in a ferment and can aid the growth of PKK1 (figure 3.4). It has been shown that non-Saccharomyces yeasts are reliant on oxygen for their survival during fermentation (Erten, 2002; Holm Hansen et al., 2001). Also it can be reasoned that the initial consumption of oxygen by the yeast combined with lack of shaking could create an anaerobic environment in the aerobic static treatment. This anaerobic environment can facilitate the growth of Crabtree positive VL3; it can also explain the initial dominance of PKK1 for 2 days under aerobic static treatment. One reason for the fluctuation observed could be due to a semi-aerobic environment inside the flasks which would support the growth of PKK1, another reason for the observed difference could be due to the usage of synthetic grape media.

Anfang et al. (2009) conducted the fermentation in Sauvignon Blanc juice; perhaps the observed fluctuation is due to the presence or absence of certain vital component in synthetic grape juice (SGM) when compared to natural grape juice. This study shows that although synthetic grape juice media is prepared to closely resemble natural grape juice, media composition should still be carefully considered before using in experiments to mimic natural fermentation process.

It can therefore be said that it took both VL3 and PKK1 longer to grow in synthetic grape juice compared to natural grape juice. The aerobic and anaerobic shaking treatments favoured the maximum growth of PKK1, whereas the static condition (both aerobic and anaerobic) favoured the growth of VL3, this agrees with results from other studies (Erten, 2002; Mendoza et al., 2007). Additionally, this experiment has shown that changes to media i.e. synthetic or natural grape juice also has an impact on the size of population which could potentially contribute to change in population dynamics. Thus, extra care needs to be taken while extrapolating experiments carried out under controlled laboratory conditions to harness any potential commercial value.
3.5.2 Comparison of fermentation kinetics of *S. bayanus* isolates from the same natural population

The population biology of *Saccharomyces* yeast in New Zealand is being examined and is one of the areas of focus of Dr Matthew Goddard’s Laboratory. Monitoring the 2008 Kumeu River fermentation population dynamics showed that *S. bayanus* was the dominating yeast species; indeed many natural isolates of *S. bayanus* from this fermentation was isolated and identified. This was the first instance where a uninoculated commercial ferment of Sauvignon Blanc was identified as being fermented by *S. bayanus* in New Zealand (chapter 2). Although there have be reports of *S. bayanus* isolates capable of fermenting wine from Europe and Australia, little is known of their population biology and fermentation potential (Eglinton *et al.*, 2000; Masneuf-Pomarède *et al.*, 2010; Naumov *et al.*, 2002).

Many factors such as temperature and ethanol content are responsible for the survival and establishment of fermenting yeast, these factors could also be responsible for the population structure of yeast species during fermentation (Goddard, 2008). In this study, to determine the dominant fermenting *S. bayanus* strain from a natural population, two factors were considered; it has to be able to achieve a reasonably high rate of fermentation compared to other strains and also it has to establish itself quicker than other strains in order to dominate the fermentation (Bely M *et al.*, 1990; Bisson, 1999; Fleet and Heard, 1993).

907 yeast isolates were identified (and stored in a library) as *S. bayanus* from the 2008 Kumeu River ferment of Sauvignon Blanc as discussed in the previous chapter (Chapter 2). From these, 20, *S. bayanus* isolates were selected randomly and were analysed for their microsatellite profiles in order to estimate the potential diversity of *S. bayanus* isolates from a natural population (refer chapter 2 figure 2.8). Of the 20 isolates examined, 16 showed unique microsatellite profiles, suggesting a vast diversity within the same species. These isolates were randomly selected from various time points throughout the fermentation of 2008 Kumeu River Sauvignon Blanc and are the same isolates described previously (See Appendix 2 for microsatellite profiles). Two parameters were examined for all the 20 isolates; the maximum specific rate of fermentation (*V*max) of an isolate and the time it took to achieve this rate at two temperature conditions of 12.8°C and 20°C in order to understand their dynamics at both low and high fermenting conditions (Salvadó *et al.*, 2011). The methods and materials section (3.4.2) details the experimental set up and method used to calculate the *V*max values for the isolates. These values were used to test the null hypothesis that there is no difference in the fermentation dynamics between these isolates at a particular temperature.
Figure 3.5: Scatter plot of average maximum fermentation rates (Vmax) of 20 *S. bayanus* isolates at two different temperatures; 20°C (blue), 12.8 °C (green). The average Vmax value (n=3) of each isolate at both temperature is shown on the Y axis. The X-axis shows the time (hours) it took for each of the isolate to reach Vmax at both the temperatures.

Figure 3.5 shows that there is a difference amongst the isolates at both the temperatures for Vmax values and also the time it took to reach those Vmax values. At 20°C the average Vmax of the isolates is 0.013 r/h compared to 0.005 r/h observed at 12.8 °C. A t-test between the Vmax values of the isolates at 20°C and 12.8°C was significant (P-value = 4.26e^{-18}). This demonstrates that these isolates behaved differently at different temperature with respect to their ability to ferment. This suggests that temperature of fermentation can have a major impact on the population structure of *S. bayanus*. This result is similar to observation of different growth and fermentation ability of *S. cerevisiae* from a natural population (Anfang, 2010; Goddard, 2008; Legras JL *et al.*, 2007)

Furthermore, at 20 °C the isolates can be divided into two groups. One group had a Vmax ranging from 0.015 r/h to 0.012 r/h (n=15) which was achieved 50 hours after inoculation into sterile grape juice. The second group of isolates had a Vmax of around 0.011 r/h (n=5) which was achieved at 72 hours after inoculation. A t-test between the Vmax of these two groups fermenting at 20 °C was
significant (P-value = 6.69e-08), this suggests that there is a difference in Vmax within these isolates, which indicates differences in their ability to ferment.

Similarly, at 12.8 °C the isolates can be categorised into three groups based on the time at which they achieved Vmax at 72, 97 and 121.5 hours, but there was little variation in the overall Vmax values between these three groups for the three comparisons (72 v 97, 97 v 121.5 and 72 v 121.5), with Vmax values ranging between 0.004 r/h to 0.006 r/h (for single factor ANOVA for the three groups P-value =0.29). Thus it was not possible to differentiate between their ability to ferment at 12.8°C.

An overall comparison of the time taken by the 20 isolates to reach Vmax at 20°C and 12.8 °C is shown in table 3.3. Cross-tabulation of isolates based on the time taken to reach Vmax at two temperature conditions allowed for identification of difference in behaviour of S. bayanus isolates at each condition.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Time to achieve Vmax (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>50</td>
</tr>
<tr>
<td>12.8°C</td>
<td>0</td>
</tr>
<tr>
<td>20°C</td>
<td>15</td>
</tr>
<tr>
<td>Total</td>
<td>15</td>
</tr>
</tbody>
</table>

*Table 3.3: Distribution of 20 S. bayanus isolates based on the time taken to achieve Vmax at 12.8°C and 20 °C during fermentation Sauvignon Blanc juice.*

In table 3.3, the 20 S. bayanus isolates are distributed differently at two different temperatures in their ability to reach Vmax. The isolates can be divided into three groups at 12.8 °C and 2 groups at 20 °C. Because only 20 isolates from a huge pool of natural population of S. bayanus were examined, Fisher’s exact test was used to test for any difference in distribution of these isolates based on the time taken to achieve Vmax at 12.8 °C and 20 °C. Upon performing the test, a P-value of 2.133 e-09 was obtained; this shows that there is a significant difference in the time taken by the 20 isolates to achieve Vmax between 12.8°C and 20 °C.

In summary it has been shown that there is a significant difference in the Vmax of S. bayanus isolates fermenting at 12.8 and 20°C. The Vmax values for S. bayanus isolates could be divided into 2 groups within 20°C where there was a significant difference between their Vmax values. Although the Vmax values at 12.8°C weren’t significantly different, the time it took to achieve Vmax was more spread out in comparison with their behaviour at 20°C.
There is a statistically significant difference in the distribution of isolates at 12.8°C and 20 °C based on the time taken to reach Vmax. Also evident is that not all isolates are capable of achieving high growth rates. Thus the null hypothesis that there is no difference in the growth of different S. bayanus isolates can be rejected. These results are similar to those observed in a natural population of NZ S. cerevisiae by Goddard et al. (2010) and are the first step in understanding the behaviour of different isolates in natural population.

These differences in fermentation potential of S. bayanus isolates from the same natural population is interesting to note because, usually commercialization of yeast for winemaking involves a single yeast isolate which is examined thoroughly for its various physiological characteristics and its ability to ferment, thus ignoring the vast fermentation potential of a natural population (Henschke, 1997; Romano et al., 2008). Few yeast isolates are able to adapt and ferment rapidly at a range of temperature conditions; therefore care needs to be taken when selecting yeast for specific vinification processes (Chambers and Pretorius, 2010; Henschke, 1997; Pretorius, 2000). The ability of yeast to adapt rapidly to the grape juice environment is crucial for its survival and subsequent domination (Goddard, 2008). More S. bayanus isolates from 2008 Kumeu River Sauvignon Blanc need to be examined in order to unmask the fermentation potential in their natural population.

The above work was helpful in determining the selection of a natural S. bayanus isolate (SBJ1d) which was able to perform well at both 12.8°C (Vmax 0.0056 r/h taking 72 hours) and 20°C (Vmax of 0. 152 r/h taking 50 hours). The isolate SBJ1d had the highest Vmax at 20°C and even though it did not have the highest Vmax at 12.8°C it could reach reasonable Vmax quicker (Appendix 3 for growth curves of the isolates). Therefore it was used to conduct further vinification experiments to compare its fermentation potential with that of a commercial S. cerevisiae isolate and understand mixed species interaction involving S. bayanus.
3.5.3 Fermentation kinetics of a natural *S. bayanus* isolate SBJ1d in comparison with *S. cerevisiae* VL3 under controlled oenological conditions

The previous section (3.5.2) has shown that there is diverse potential among the natural isolates of *S. bayanus* isolated from 2008 Kumeu River Sauvignon Blanc fermentation. Among them a single isolate SBJ1d was chosen due to its robust specific growth rate taking into account its Vmax and time it took to reach the Vmax for further vinification experiments. The ultimate goal was to identify a New Zealand natural isolate with a potential to be used in commercial fermentation of Sauvignon Blanc. Therefore, this isolate (SBJ1d) was compared to commercial isolate VL3 (*S. cerevisiae*) against a range of oenological conditions. These comparisons were part of a full factorial experiment set-up and only VL3 was used in these comparisons due to limit on availability of Sauvignon Blanc from single source. The whole experiment utilised Marlborough Sauvignon Blanc from Stoneleigh (2010) to prevent any juice related bias. (Section 3.4.3.1 for set-up and method)

In this study, comparison was made between *S. bayanus* isolate from Kumeu River Sauvignon Blanc (SB J1d) and *S. cerevisiae* VL3 in their ability to ferment at two temperatures (12.8°C and 20°C) and different YAN values (390 mg/L(Y1) and 1170 mg/L(Y2)) under controlled laboratory conditions. The oenological conditions of YAN and temperature were manipulated for the reasons discussed in section 3.3.

The experiments discussed below were conducted to test the null hypothesis that there is no difference between *S. bayanus* (SBJ1d) and *S. cerevisiae* (VL3) when fermenting under similar oenological conditions. The experimental set-up and calculation of Vmax are detailed in methods section (3.4). Both *S. bayanus* and *S. cerevisiae* ferments were carried out in triplicates at the two different temperature and YAN conditions. Note that the Vmax here is a representation of maximum weight loss of ferment within a minimum amount of time (Bely et al., 1990).

Weight loss of the fermentation was monitored daily for the first ten days (except day 7) and every alternate day for 32 days. Both *S. bayanus* and *S. cerevisiae* were able to finish fermentation at 20 °C (lose 20% of original weight), however, *S. bayanus* completed fermentation by day 16 with Y2 juice compared to 18 days for Y1 juice and *S. cerevisiae* finished fermentation by day 18 for Y2 juice and at day 20 had completed the fermentation. At 12.8 °C, *S. bayanus* was able to finish fermentation by day 22 for both Y1 and Y2 juice. On the other hand, *S. cerevisiae* was able to achieve its weight loss by day 26 for Y2 juice and day 28 for Y1 juice.

It is important to note that there was more variation in weight loss within the replicates at 12.8°C compared to 20 °C (refer figure 3.6 a). The main purpose of recording weight loss was to determine Vmax. At 20 °C, both the species achieved maximum average weight loss between days 2-3, whereas, at 12.8 °C Vmax was achieved between days 4-5 with *S. cerevisiae* achieving Vmax slower
(day 5) compared to *S. bayanus* (day 4). Appendix 4 shows the weight loss of the VL3 and SBJ1d ferments over time. A graph of average weight loss of ferments for the two species over time (rate of fermentation) for the different conditions is shown below; this was used to calculate Vmax (figure 3.6).

![Graph showing rate of fermentation](image)

**Figure 3.6:** Rate of fermentation (g/Day) of *S. bayanus* (SBJ1d) and *S. cerevisiae* (VL3) ferments under different oenological conditions: (a) values of SBJ1d and VL3 at 12.8°C in both Y1 and Y2 juice, (b) values of SBJ1d and VL3 at 20°C in both Y1 and Y2 juice. Labels; *S. bayanus* Y ( ), *S. bayanus* Y2 ( ), *S. cerevisiae* Y1 ( ), *S. cerevisiae* Y2 ( ) the values are average rate of weight loss per day ±SEM (n=3).
Figure 3.7: Average \( V_{\text{max}} \) values of \( S. \) bayanus (SBJ1d) and \( S. \) cerevisiae VL3 under the oenological conditions: 12.8\(^\circ\)C+Y1 ( ), 12.8\(^\circ\)C +Y2 ( ), 20\(^\circ\)C+ Y1 ( ) and 20\(^\circ\)C+Y2 ( ), \( V_{\text{max}} \) is the mean with ±SEM (n=3). The comparison (Tukey’s HSD) between the two species indicated by * P-value < 0.05, ** P-value <0.01, *** P-value <0.001.

From figure 3.7 the maximum average rate of weight loss or \( V_{\text{max}} \) was obtained for the two species under different conditions. An ANOVA with two factors, temperature (12.8 °C and 20 °C) and YAN (Y1 and Y2) was performed on these values, it showed that both the factors contributed significantly to the differences between fermentation kinetics of \( S. \) bayanus (SBJ1d) and \( S. \) cerevisiae VL3, both factors with a P-value of < 0.0001. Therefore a Tukey’s HSD (Honestly Significant Difference) test was performed to investigate the factor combinations causing the differences (table 3.4). As seen in figure 3.7, SBJ1d has greater average \( V_{\text{max}} \) values compared to VL3 irrespective of the oenological conditions. Nonetheless, there are some significant differences between the overall fermentation rates.

<table>
<thead>
<tr>
<th>Yeast</th>
<th>Average ( V_{\text{max}} ) (r) (n=3)</th>
<th>P-value (Y1 vsY2)</th>
<th>( 20 , ^\circ)C Y1</th>
<th>( 20 , ^\circ)C Y2</th>
<th>P-value (Y1 vsY2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SB</td>
<td>7.33</td>
<td>9.70</td>
<td>0.062</td>
<td>18.63</td>
<td>21.06</td>
</tr>
<tr>
<td>VL3</td>
<td>4.73</td>
<td>6.23</td>
<td>0.0007</td>
<td>15.60</td>
<td>16.73</td>
</tr>
<tr>
<td>P-value (SB vs. VL3)</td>
<td>0.042</td>
<td>0.003</td>
<td>0.004</td>
<td>0.0003</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.4: Average \( V_{\text{max}} \) (r) values of \( S. \) bayanus and \( S. \) cerevisiae (VL3) (in bold) and corresponding P-values for the different oenological conditions.
Firstly, when comparing the average Vmax rates of *S. bayanus*-only ferments under different oenological conditions, it can be seen that there is a significant difference in Vmax between 12.8 °C and 20 °C (including both Y1 and Y2 values) with P-value of < 0.001. Also, there is a significant difference between Vmax of *S. bayanus* at Y1 and Y2 at 12.8 °C with a P-value 0.037 and 20 °C with a P-value of 0.012 (refer figure 3.7 and table 3.4). This result is similar to the increased fermentation rates of yeast observed in juice with higher YAN compared to juice with lower YAN by Bely *et al.* (2003).

Similarly looking at *S. cerevisiae*-only ferments, there is a significant difference in the Vmax between 12.8 and 20 °C with P-value < 0.0001. Addition of YAN had a greater impact on fermentation rates at 12.8 °C (P-value 0.0011) compared to 20 °C with a P-value of 0.04. It is important to note that addition of DAP to increase the YAN levels of the juice significantly increased Vmax of both species irrespective of the temperature. This result is in accordance with other studies linking increased biomass during exponential growth in early phase of alcoholic fermentation with nitrogen availability (Bely *et al.*, 2003; Hernandez-Orte *et al.*, 2006; Varela *et al.*, 2004). Although it should be noted that the YAN values in this study are considerably higher when compared to other studies (Vilanova *et al.*, 2007).

In comparing average Vmax values between *S. bayanus* (SBJ1d) and *S. cerevisiae* VL3 it can be seen that at 20 °C, SBJ1d has a higher rate compared to VL3 irrespective of nitrogen levels (Y1 P-value: 0.004, Y2 P-value: 0.0003). Similarly, the Vmax values at 12.8 °C are also significantly different between SBJ1d and VL3 at both nitrogen levels (Y1 P-value = 0.04, Y2 P-value 0.003). The greater Vmax of SBJ1d (Kumeu River isolate) compared to VL3 could help explain the dominance of *S. bayanus* in 2008 Kumeu River Sauvignon Blanc where the fermenting temperature ranged between 12 °C and 20 °C, despite the identification of a few *S. cerevisiae* isolates (refer chapter 2 figure 2.5).

These values and statistical significance are contrasting with the results shown by Tosi *et al.* (2009), where the rate of ethanol production and maximum rate of ethanol production of the natural isolate of *S. cerevisiae* (CA1, max value = 16.60 g/day) from Amarone juice during fermentation at 21-23 degrees was not different compared to natural isolate of *S. bayanus var uvarum* (UF2, max value =16.2 g/day). The experiment described above and the experiment conducted by Tosi *et al.* (2009) can be compared because, rate of ethanol production is directly proportional to weight loss due to release of CO₂ from the fermentation (Bely *et al.*, 1990). It is also important to consider that the differences observed between the two studies could be strain specific for both the *Saccharomyces* yeast species tested. Importantly it could also be due to a variation in the composition of Amarone and Sauvignon Blanc grape juice used or the adaptation of the yeast species to these juices. An important contributing factor between SBJ1d and VL3 is perhaps that *S. bayanus* is a cryotolerant species capable of fermenting efficiently at lower temperatures (Masneuf-Pomarède *et al.*, 2006).
Therefore from above discussion, the null hypothesis stating that there is no difference between SBJ1d and VL3 when fermenting under similar oenological conditions can be disproved irrespective of the oenological condition utilised. However, more work would elucidate the nature of the difference between the two species. Conducting a similar experiment with more isolates of *S. bayanus* and *S. cerevisiae*, also a comparison between commercial isolates of *S. bayanus* and *S. cerevisiae* will give an insight into the generality of these results. These studies can help understand fermentation potential of *S. bayanus* yeast species and help discern the usage of appropriate yeast for specific winemaking techniques (Pretorius, 2000).
3.5.4 Comparison between the co-fermentation kinetics of natural *S. bayanus* isolate SBJ1d and *P. kluyveri* PKKR1 with *S. cerevisiae* VL3 and *P. kluyveri* PKKR1 under controlled oenological conditions

It has been reported that the strain of *P. kluyveri* PKKR1 when co-fermented with *S. cerevisiae* VL3 under specific conditions can alter final aroma profile of Marlborough Sauvignon Blanc (Anfang et al., 2009). However, in this chapter (section 3.5.1) it was shown that the oenological condition can potentially alter the dynamics between these two yeast species which can prevent any beneficial interaction. Similarly, in the previous section (3.5.4) it was shown that *S. bayanus* (SBJ1d) co-ferments were impacted by oenological conditions.

There is literature showing the altered growth of *S. bayanus* var *uvarum* and *S. cerevisiae* when co-fermented with each other or sequentially fermented (Favale et al., 2007; Tosi et al., 2009). To my knowledge there has been no study comparing the co-fermenting behaviour of these two species of *Saccharomyces* yeast with the same non-*Saccharomyces* yeast under controlled oenological conditions. Therefore the aim of this study was to compare the interactions of two different *Saccharomyces* yeast (SBJ1d and VL3) with single non-*Saccharomyces* yeast (PKKR1) to understand the *Saccharomyces* species specific dynamics with the same non-*Saccharomyces* yeast. Fermentation rates of both the co-ferments at 12.8°C (Y1 and Y2) and 20°C Y1 and Y2 are shown in figure 3.8.
Figure 3.8: Rate of fermentation (g/Day) of *S. bayanus* SBJ1d and *P. kluyveri* PKKR1 compared to *S. cerevisiae* VL3 and *P. kluyveri* PKKR1 co-ferments under different oenological conditions: (a) values of SB+PKKR1 and VL3+PKKR1 at 12.8°C in both Y1 and Y2 juice, (b) values of SB+PKKR1 and VL3+PKKR1 20°C in both Y1 and Y2 juice. Labels; SB+PKKR1 Y1 (---), SB+PKKR1 Y2 (---), VL3+PKKR1 Y1 (---), VL3+PKKR1 Y2 (---). The values are based on average rate of weight loss per day ±SEM (n=3).

The null hypothesis tested here stated that there is no difference in the fermentation kinetics between co-ferments of *S. bayanus* (SBJ1d) and PKKR1 and *S. cerevisiae* (VL3) and PKKR1. The oenological conditions are described in methods section (3.4.3.1) and are similar to those explained in the previous section. The time taken to complete fermentation was different for the different co-ferments at different oenological conditions (refer figure 3.8) At 12.8°C and Y1; SB+PKKR1 took 28 days.
compared to 32 for VL3+PKKR1 and at Y2 condition they took the same time to undergo a 20% weight loss. At 20°C and Y1 and Y2 conditions, it took 16 days for SB+PKKR1 while it took 20 days for VL3+PKKR1.

An ANOVA with two factors, temperature (12.8 °C and 20 °C) and YAN (Y1 and Y2) was performed on these values, it showed that both the factors contributed significantly to the differences between fermentation kinetics of SB+PKKR1 and VL3+PKKR1; P-values (temperature = < 2.2e\textsuperscript{-16}, YAN = 0.003). Therefore a Tukey’s HSD (Honestly Significant Difference) test was performed to investigate the factor combinations causing the differences (table 3.5). The observed P-values are highly statistically significant (P-value < 0.01 for all comparisons) thus suggesting a difference in fermentation kinetics of the different *Saccharomyces* yeast co-ferments (figure 3.9).

<table>
<thead>
<tr>
<th>Yeast</th>
<th>Average Vmax (r) (n=3)</th>
<th>P-value (Y1 vs Y2)</th>
<th>P-value (SB+PKKR1 vs VL3+PKKR1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12.8°C Y1</td>
<td>12.8°C Y2</td>
<td>20 °C Y1</td>
</tr>
<tr>
<td>SB+PKKR1</td>
<td>7.60</td>
<td>8.90</td>
<td>0.033</td>
</tr>
<tr>
<td>VL3+PKKR1</td>
<td>4.76</td>
<td>5.30</td>
<td>0.317</td>
</tr>
<tr>
<td>P-value (SB+PKKR1 vs. VL3+PKKR1)</td>
<td>0.006</td>
<td>0.003</td>
<td>0.003</td>
</tr>
</tbody>
</table>

Table 3.5: Average Vmax (r) values of SB+PKKR1 and VL3+PKKR1 (in bold) and corresponding P-values for the different oenological conditions.
Figure 3.9: Average Vmax values of co-fermentation of *S. bayanus* SBJ1d and *S. cerevisiae* VL3 with *P. kluyveri* PKKR1 under similar oenological conditions: 12.8°C + Y1 ( ), 12.8°C + Y2 ( ■), 20°C + Y1 ( ) and 20°C + Y2 ( ■), Vmax is the mean with ±SEM (n= 3). The indicated P-values are direct comparison (Tukey’s HSD) between co-ferments of *S. bayanus* (SBJ1d) and PKKR1 and *S. cerevisiae* (VL3) and PKKR1; ** P-value < 0.01.

It is interesting to note that there are similar trends to Vmax values for the different co-ferments under similar oenological conditions (refer table 3.4 and table 3.5). There is a slight increase in Vmax values at Y2 condition at 12.8 °C compared to Y1 condition and a slight decrease in Vmax values at Y2 condition at 20 °C compared to the corresponding Y1 condition. These results are perhaps due to the observed difference between *S. bayanus* (SBJ1d) and *S. cerevisiae* (VL3) under similar conditions (Section 3.5.2), and PKKR1 contributing little to these differences. However, the differences in Vmax values between SB+PKKR1 and VL3+PKKR1 are lower under co-fermentation condition when compared to mono-ferment condition at 20°C (section 3.5.2). The lowered co-fermentation rates in this study is similar to the trend noted in other studies (Anfang et al., 2009; Kim et al., 2008; Mendoza et al., 2007) These results suggest that it’s important to consider co-fermenting yeasts as there are differences in response from the two different *Saccharomyces* yeast to the presence of *P. kluyveri* PKKR1 which can be observed through a difference in their corresponding Vmax values.
3.5.5 Impact of co-fermentation with non-Saccharomyces yeast on S. bayanus fermentation rates under controlled oenological conditions

There have been studies showing the altered fermentation dynamics of both non-Saccharomyces yeast and S. cerevisiae when co-fermenting in grape juice (Anfang et al., 2009; Erten, 2002; Mendoza et al., 2007). To my knowledge there has been no information regarding the co-fermenting behaviour of S. bayanus isolates with non-Saccharomyces yeast.

The aim of this study was to test the null hypothesis that there is no difference in the fermentation kinetics of mono-ferment of S. bayanus with co-ferments of S. bayanus with selected non-Saccharomyces yeast. Here, non-Saccharomyces yeast species isolates of Torulaspora delbrueckii and Pichia anomala from 2008 Kumeu River Sauvignon Blanc were used for co-fermentation with S bayanus (SBJ1d). Pichia kluyveri (PKKR1) was also co-fermented with SBJ1d (as discussed in 3.5.4) under controlled oenological conditions. These were similar to the oenological conditions described in previous section, they were: fermenting temperature of either 12.8°C or 20 ºC in combination with a YAN level of 390 mg/L (Y1) or 1170 mg/L(Y2) of Sauvignon Blanc juice as described in methods section (2.3.3). The ferments were labelled SBPKKR1 (SBJ1d + PKKR1), SBTD (SBJ1d + Torulaspora delbrueckii), SBPA (SBJ1d + Pichia anomala) and SB (SBJ1d only).

Weight loss at 12.8 ºC and 20 ºC at both the nitrogen levels (Y1 and Y2) were monitored for all the ferments till they were considered dry (loss of 20 % of the initial weight). As mentioned earlier, the objective was to obtain the maximum rate of fermentation for the different ferments between smallest intervals of time under varying oenological conditions. Appendix 4 shows the weight loss of the mono-ferment of SBJ1d and co-ferments over time.

From figure 3.10 the average weight loss (g/day) of the different ferments can be identified. At 12.8 ºC it can be seen that S. bayanus-only ferments reached Vmax 4 days after inoculation, whereas, the co-ferments were slower in reaching Vmax ranging from days 5-6 after inoculation. However, at 20 ºC fermentation, all the co-ferments and S. bayanus-only ferments reached Vmax after 2 days of inoculation. Perhaps the difference in time taken to reach Vmax at 12.8 ºC between the co-ferments and S. bayanus-only ferments is due to longer survival of non-Saccharomyces yeast at 12.8 ºC causing S. bayanus in the co-ferment to take longer to start fermentation.

Erten (2002) noted that non-Saccharomyces yeast persist for longer at lower temperatures and also noted that under co-fermentation with S. cerevisiae, the non-Saccharomyces yeast was able to dominate the ferment for a short period of time. Another study noted that at non-Saccharomyces yeast compete with Saccharomyces yeast by limiting nutrients such as essential vitamins and assimilable nitrogen, however, they conducted their experiments at 25 ºC which could impact the yeasts in this study differently (Medina et al., 2012). Anfang (2010) noted that there was reduction in the rate of
weight loss for co-ferment between PKKR1 and *S. cerevisiae* VL3 compared to fermentation by VL3 on its own at identical oenological conditions. Therefore it could be due similar interaction between *S. bayanus* and selected non-*Saccharomyces* yeast that the *S. bayanus* co-ferments achieved V-max at a later time compared to *S. bayanus*-only ferments.

It is interesting to note that Anfang (2010) recorded a slight increase in the cell numbers of *S. cerevisiae* VL3 around days 8-10 in single and co-fermentation with PKKR1 at 14 ºC after reaching the maximum cell number around 2 days after inoculation, perhaps this increase impacted on the rate of weight loss of single and co-ferment and could possibly explain the slight increase in rate of weight loss observed at 12.8 ºC for *S. bayanus* and co-ferments with *S. bayanus* with selected non-*Saccharomyces* yeast around 10 days after inoculation (appendix 4 and figure 3.10). This increase in rate of weight loss is not noticeable in fermentation at 20 ºC.
Figure 3.10: Rate of fermentation of Marlborough Sauvignon Blanc by co-ferments of select non-Saccharomyces yeast with S. bayanus (SBJ1d) and S. bayanus SBJ1d-only ferments under controlled oenological conditions; 12.8 °C and Y1 (a) and Y2 (b) and 20 °C and Y1 (c) and Y2 (d). Values are ±SEM (n=3) are shown on Y-axis (g/day) vs. days on X-axis for co-ferments SB+PKKR1 ( ), SB+TD ( ), SB+PA ( ) in comparison with SB-only ( ) ferments under similar conditions.
For the purpose of answering the null hypotheses posed, only certain comparisons were made. In the future this study can potentially provide data for other similar studies.

Figure 3.11: Average Vmax values of co-fermentation between SB+PKKR1, SB+TD, SB+PA and SB-only ferments under different oenological conditions: 12.8°C+Y1 ( ), 12.8°C +Y2 ( ), 20°C+ Y1 ( ) and 20°C+Y2 ( ), Vmax is the mean with ±SEM (n=3). The indicated P-values are an individual comparison (Tukey’s HSD) between S. bayanus-only and respective co-ferments. Any significant difference in Vmax of co-ferments are indicated by; * P-value < 0.05, ** P-value < 0.01, *** P-value <0.001, f P-value < 0.001.

<table>
<thead>
<tr>
<th>Yeast</th>
<th>Average Vmax (r) (n=3)</th>
<th></th>
<th></th>
<th></th>
<th></th>
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<tr>
<td></td>
<td>12.8°C Y1</td>
<td>12.8°C Y2</td>
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<td>20 °C Y2</td>
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<td>SB</td>
<td>7.33</td>
<td>9.70</td>
<td>0.062</td>
<td>18.63</td>
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</tr>
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<td>PKKR+SB</td>
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<td>P-value (SB vs. PKKR+SB)</td>
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<td>0.137</td>
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<tr>
<td>TD+SB</td>
<td>7.50</td>
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<td>0.002</td>
<td>17.94</td>
<td>17.40</td>
</tr>
<tr>
<td>P-value (SB vs. TD+SB)</td>
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<td>0.008</td>
<td></td>
</tr>
<tr>
<td>PA+SB</td>
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<td>8.26</td>
<td>0.023</td>
<td>17.18</td>
<td>15.20</td>
</tr>
<tr>
<td>P-value (SB vs. PA+SB)</td>
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<td>0.023</td>
<td>0.134</td>
<td>0.00003</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.6: Average Vmax (r) values of SB+PKKR1, SB+TD, SB+PA and SB-only ferments (in bold) and corresponding P-values under different oenological conditions.
To test the null hypotheses that there is no difference between SB-only ferment and selected co-ferments, an ANOVA with three factors, temperature (12.8 °C and 20 °C), YAN (Y1 and Y2) and yeast species (SB, PKKR1+SB, TD+SB, PA+SB) was performed. It showed that all the factors contributed significantly (P-value of yeast species = 2.134e-11, temperature = < 2.2e-16 and YAN = 0.002) to the differences between fermentation kinetics of S. bayanus (SB1d)-only ferment and selected co-ferments (PKKR1+SB, TD+SB, PA+SB). Therefore a Tukey’s HSD (Honestly Significant Difference) test was performed to investigate the factor combinations causing these differences (table 3.6).

The co-ferments with non-Saccharomyces yeast were directly compared with S. bayanus-only ferments under similar oenological conditions. It can be seen in figure 3.11, only a few comparisons were significant. Notably, there was no difference between the co-ferments and S. bayanus-only ferments at both the temperatures of 12.8 °C and 20 °C under Y1 conditions (refer table 3.6 for average Vmax values). Therefore nitrogen levels played a significant role in differentiating between co-ferments and S. bayanus-only ferments. It is interesting to note that while addition of nitrogen (Y2) increased the average Vmax values of S. bayanus-only ferments irrespective of temperature (3.5.3), it had a different impact on co-ferments. At 12.8 °C there seem to be a significant increase in Vmax of co-ferments at Y2 condition compared to Y1 condition (P-value <0.05 for all the three co-ferments) this was similar to the trend observed in S. bayanus-only ferments. Importantly, the Vmax values of co-ferments with T. delbrueckii and P. anomala(Pina et al., 2004a) were significantly lower compared to S. bayanus-only ferments at 12.8 °C and Y2 condition, although it was not significant for co-ferment with PKKR1. At 20 °C under the Y2 condition, all the three co-ferments had a significantly lower Vmax value compared to S. bayanus-only ferment, here it can been seen that there is a decrease in the Vmax value of the co-ferments whereas an increase in Vmax value of S. bayanus-only ferment compared to Y1 condition.

Perhaps the combined factor of a threefold increase in yeast available nitrogen (Y2) and a higher temperature (20°C) provided an unexpected resistance from selected non-Saccharomyces yeast to niche engineering and subsequent dominance of S. bayanus yeast in the co-ferments. Medina et al. (2012) found that despite the relatively high temperature, non-Saccharomyces yeasts were able to compete with S. cerevisiae for nutrition thereby reducing the overall fermentation rate. Also, this finding seems to contrast partially with the study by Erten (2002) which showed that non-Saccharomyces yeast survive longer at lower temperature, however in this study both temperature and YAN levels played important roles and perhaps a combination (YAN*temperature interaction had P-value 0.015) of both the oenological conditions had a different impact.

To my knowledge, there is no literature on the impact of DAP on the survival of non-Saccharomyces yeast during co-fermentation with Saccharomyces yeast. However, in a study the impact of varying
YAN and temperature and inoculum sizes on wine fermentation addition of YAN seems to significantly increase cell growth of yeast only at higher temperature (Carrau et al., 2010). This study can be partially related to my study where the combined effect of higher YAN and temperature increased the Vmax values of Saccharomyces-only ferments. However, work needs to be done to elucidate the nitrogen usage of non-Saccharomyces yeast and their impact on the Saccharomyces yeast species during co-fermentation. These results will be valuable when selecting co-fermenting partners and winemaking techniques to distinguish NZ Sauvignon Blanc.

From the above discussion the null hypothesis stating that there is no difference between the fermentation kinetics of co-ferments with non-Saccharomyces yeast and S. bayanus-only ferment can be negated due to the differences observed by addition of DAP to juice (Y2 conditions) The DAP addition in this study was excessively high and does not represent YAN levels observed naturally, therefore care has to be taken before translating these results for practical purposes. The three fold difference in YAN between Y1 and Y2 was used to gauge the range of potential for fermentation of the yeasts used. In the future, by observing the changes to these yeasts at a protein expression level, a more meaningful interpretation of these results can be achieved. However, from this study it can be shown that oenological conditions can have a major impact on the fermentation kinetics of both mono and co-ferments of the selected yeast.
3.6 Conclusion

Important conclusions that can be drawn from the above set of experiments are that there is variability within the selected natural isolates of *S. bayanus*. Altering oenological conditions could potentially impact on the interaction between *S. cerevisiae* VL3 and *P. kluyveri* PKKR1. There is a significant difference in the fermentation kinetics of *S. bayanus* and *S. cerevisiae* irrespective of oenological conditions. Also there is difference between closely related *Saccharomyces* yeast VL3 and SBJ1d in their interaction with non-*Saccharomyces* yeast PKKR1 during fermentation. Altering oenological conditions has a significant impact on co-fermentation kinetics of *S. bayanus* with *T. delbrueckii* and *P. anomala*. Another important observation is that the natural isolate of *S. bayanus* SBJ1d is capable of performing on par with commercial *S. cerevisiae* VL3 yeast. Only fermentation kinetics was monitored to determine these differences, more work needs to be done to elucidate the exact nature of these differences and the cellular mechanisms involved.

The differences in wine aroma resulting from these ferment dynamics are examined in chapter 4. These results provide an important indicator for winemakers choosing to co-ferment both commercial and natural *Saccharomyces* yeast with non-(*Saccharomyces*) yeast. Finally, these experiments also help emphasise the importance of oenological conditions such as assimilable nitrogen and temperature on fermentation, which should not be ignored when considering scale-up or industrialization of selected yeast. The overall evaluation of the impact of different oenological conditions on New Zealand Sauvignon Blanc is discussed in the general conclusions chapter (section 6.2).
Influence of oenological conditions on the concentration of aroma in New Zealand Sauvignon Blanc
4.1 Aroma Characteristics of New Zealand Sauvignon Blanc

The aroma and flavour of a wine is dependent on hundreds of volatile chemical compounds arising from various processes of winemaking. Key sensory components of a wine variety can be driven by viticulture practices through to the bottling of the finished wine (Ciani et al., 2010; Fleet, 2003; Styger et al., 2011). The typical aroma of a Sauvignon Blanc wine is described as containing vegetative, grassy, herbaceous, gooseberry, asparagus and green capsicum descriptors (Swiegers et al., 2009; Tominaga et al., 2000). However, the aroma of Marlborough Sauvignon Blanc from New Zealand differs considerably from those originating from other geographical locations; Marlborough Sauvignon Blanc shares common ‘green’ aroma descriptors in addition to a distinctive intense ‘tropical’ signature driven by gooseberry, guava, passionfruit and grapefruit aromas (Lund et al., 2009).

Consumer preference and key impact aroma compounds of Marlborough Sauvignon Blanc have been studied. Chemical analysis of aroma compounds of Marlborough Sauvignon Blanc found the volatile compounds 3MH and 3MHA and 3-isobutyl-2-methoxypyrazine (IBMP) to be present in high concentrations compared to Sauvignon Blanc wines from other regions, and these compounds were proposed to play an important role in the consumer perceived distinctiveness of the Marlborough Sauvignon Blanc wine (Lund et al., 2009; Nicolau et al., 2006). Other studies have shown that 4-mercapto-4-methylpentan-2-one (4MMP) is also important to Sauvignon Blanc wine aroma (Des Gachons et al., 2005; Dubourdieu et al., 2006; Swiegers et al., 2009; Tominaga et al., 2000).

4.1.2 Important aromas in Marlborough Sauvignon Blanc

In order to study the impact of oenological conditions on Sauvignon Blanc wine aroma it is necessary to examine the nature and mechanism of production of key varietal aromas. There have been numerous studies exploring aroma compounds in Sauvignon Blanc and these are discussed below.

4.1.2.1 Methoxypyrazines

The ‘green’ character of a Sauvignon Blanc wine is mostly driven by methoxypyrazines. Methoxypyrazines are nitrogen containing compounds synthesised by the plant and found in grape berries (Allen et al., 1994). Important methoxypyrazines in Sauvignon Blanc are 3-isobutyl-2-methoxypyrazine (IBMP) and 3-isopropyl-2-methoxypyrazine (IPMP) imparting green capsicum and earthy aromas respectively (Allen et al., 1994; Swiegers et al., 2006a). Levels of Methoxypyrazines in Sauvignon Blanc are driven by grape quality and post-harvest processing of grapes to produce juice and is not impacted by the fermentation process (Lund et al., 2009). Due to these characteristics of Methoxypyrazines, they were not included in the analysis of final aroma which only took into account fermentation impacted aroma compounds.
4.1.2.2 Volatile thiols

However, the ‘tropical’ characteristics of Sauvignon Blanc are driven by a range of fermentation-derived volatile sulphur containing compounds called polyfunctional thiols (also referred to as thiols) (Dubourdieu et al., 2006; Swiegers et al., 2009). The thiols 4-mercapto-4-methylpentan-2-one (4MMP), 3-mercaptohexan-1-ol (3MH) and 3-mercapto-hexylacetate (3MHA) are responsible for passionfruit, grapefruit, gooseberry and guava like aromas. Volatile thiols are potent chemicals with human odour perception thresholds with 0.8 ng L\(^{-1}\) for 4MMP, 60 ng L\(^{-1}\) for 3MH and 4 ng L\(^{-1}\) for 3MHA (Dubourdieu et al., 2006; Swiegers et al., 2009). Thiols 3MH and 3MHA are present in very high concentrations in NZ Marlborough Sauvignon Blanc compared to rest of the world (table 4.1).

In recent times these thiols have been renamed as 4-methyl-4-sulfanylpentan-2-one, 3-sulfanylhexan-1-ol and 3-sulfanylhexyl acetate, respectively (Roncoroni et al., 2011). It has also been found that both R- and S-enantiomers of 3MH and 3MHA are responsible for aroma in dry white wines and also in botrytized (dessert) wines (Tominaga et al., 2006). The ratios of the enantiomers vary according to the type of wine (50:50 and 30: 70 of R and S enantiomers found in dry white wine and sweet wine respectively). The different enantiomers have slightly different aroma profiles and also have different perception thresholds in humans (Tominaga et al., 2006). The distribution of the different stereoisomers of 3MH and 3MHA are as yet unknown in Marlborough Sauvignon Blanc. The development of thiols levels in Sauvignon Blanc during fermentation is yet to be fully understood, however, its known that the fermenting yeast species play an important role (Roncoroni et al., 2011).

<table>
<thead>
<tr>
<th>Thiol Description</th>
<th>OTH (ng/L)</th>
<th>Marlborough n=28 (2004-'05)</th>
<th>Bordeaux n=4</th>
<th>Sancerre n=4</th>
<th>South Africa n=4 (2004)</th>
<th>Australia n=9 (2004-'05)</th>
<th>USA n=8 (2004-'05)</th>
</tr>
</thead>
</table>
Table 4.1: Aromatic thiols of importance in Marlborough Sauvignon Blanc. OTH: odour threshold value. The values for each region shown are times over OTH. The OTH values for 3MH, 3MHA and 4MMP were calculated based on the values obtained from Benkwitz (2010); values for Bordeaux and Sancerre were adapted from Dubourdieu et al. (2006). Calculation of OTH is described in methods section according to Louw et al. (2010).

### 4.2 Enzymes and mechanisms in yeast relevant to thiol production

Unlike methoxypyrazines, volatile thiols are mostly absent in Sauvignon Blanc grape juice (Tominaga et al., 1998b). Initially, addition of copper to Sauvignon Blanc wines resulted in the removal of tropical aroma of the wine. This lead to the conclusion that the compounds responsible for this aroma had an active SH group which bound to copper and were hypothesized to be volatile thiols. These volatile thiols are formed during fermentation of grape juice by yeasts (Darriet et al., 1995; Tominaga et al., 1998a; Tominaga et al., 1998b; Tominaga et al., 1995). Therefore it was proposed that fermenting yeasts are responsible for the formation of volatile thiols in wine. However, yeasts are incapable of de novo synthesis of 4MMP and 3MH (Darriet et al., 1995). Identification of non-aromatic, non-volatile cysteine bound conjugates; S-4-(4-methylpentan-2-one)-l-cysteine (Cys-4MMP) and S-3-(hexan-1-ol)-l-cysteine (Cys-3MH) in Sauvignon Blanc grape juice and subsequent discovery of release of 4MMP from Cys-4MMP by using a cell-free bacterial carbon-sulphur lyase lead to an early hypothesis. This hypothesis proposed a carbon-sulphur lyase enzyme mechanism of yeast during fermentation for the release of volatile thiols from its respective non-aromatic cysteine conjugates found in grape juice (Darriet et al., 1995; Tominaga et al., 1998a; Tominaga et al., 1998b; Tominaga et al., 1995).
4.2.1 Role of ATF1 gene

Further research from AWRI showed that deletion of any of the four putative Carbon-sulphur Lyase enzyme coding genes (BNA3, GLO1, METCand CYS3) from the lab strain of S. cerevisiae lead to a diminished ability in the production of 4MMP from Cys-4MMP (Howell et al., 2004). This implied that release of thiols from yeast could be dependent on a complex multi enzyme pathway. However, it was shown that the thiol 3MHA is absent in grape juice and was produced from 3MH through alcohol acetyl transferase mechanism of fermenting yeast (Swiegers et al., 2006b). This enzyme is encoded by the ATF1 gene, interestingly another gene IAH1 coding for an esterase was found to significantly reduce 3MHA formation suggesting a reverse mechanism for the conversion of 3MHA into 3MH (Swiegers et al., 2006b). In another study, chemically synthesized Cys-3MH in model ferments decreased in concentration with a corresponding increase in 3MHA concentration in the final wine, albeit, only a small proportion of the synthetic Cys-3MH was converted to 3MHA (Dubourdieu et al., 2006). Activity of ATF1 gene in the conversion of 3MH to 3MHA during fermentation showed a link between ester production and thiol metabolism in fermenting yeast. In another study involving Cabernet Sauvignon and Merlot wines the total amount of 3MH produced in the model wine positively correlated with the amount of Cys-3MH precursor (Murat et al., 2001b). However, only 3.2% of the precursor was converted into volatile thiol irrespective of the concentration. Final yields of all three thiols are considerably lower compared to the relative concentration of the precursors, and the calculated yields range from <1% to about 5% (Dubourdieu et al., 2006; Murat et al., 2001b). More recently, through the use of duterated cysteine precursors of thiols, it was shown that their conversion to thiols was very low and accounted for only a small portion of the total thiol level in wine (Subileau et al., 2008a). This shows a limited capability of yeast strains to liberate thiols from their non-aromatic Cys-conjugates.

4.2.2 Glutathione conjugates

Similarly, glutathione conjugates of both 4MMP and 3MH have been identified in juice (Capone et al., 2010; Fedrizzi et al., 2009; Roland et al., 2010a). Glutathionylated 4MMP is converted to 4MMP through a similar mechanism in yeast. Both Glutathionylated and cysteinylated 4MMP precursors showed similar efficiency in converting to 4MMP. It has been estimated that the former contributes up to 20% of the total 4MMP based on its relative abundance in juice (Roland et al., 2010b). Glutathionylated 3MH is also converted to 3MH by yeast, but it has a lower conversion efficiency compared to Cys-3MH (Grant-Preece et al., 2010; Kobayashi et al., 2010). A recent theory is that the conversion of aromatic precursors to thiols by yeast involves production of the cysteinylated form as an intermediate (Grant-Preece et al., 2010). It is currently estimated that there could be anywhere up to a 100 fold difference in the ratio of glutathione to cysteine precursors in juice for 3MH (Capone et al., 2010; Roland et al., 2010c).
It has recently been shown that the gene IRC7 if present in a full copy greatly increases the levels of 4MMP produced by yeast (Roncoroni et al., 2011). This gene has beta-lyase enzymatic activity and shows a preference for cys-4MMP compared to cys-3MH, although insertion of this gene into Zymafour F15 increased its potential to release all the three thiols (Roncoroni et al., 2011). Despite recent discoveries of important genes involved in thiol metabolism, our current knowledge regarding the relative contribution of both the Glutathionylated and cysteinylated precursors to the final concentration of varietal thiols in wine is incomplete, as is our knowledge of the pathways involved in both Saccharomyces and non-Saccharomyces yeast (Grant-Preece et al., 2010). Figure 4.1 is the diagrammatic representation of summary of the research done on the possible yeast mechanisms and pre-cursor molecules.

4.2.3 Alternate pathway

Schneider et al. (2006) proposed an alternative pathway for the formation of 4MMP and 3MH during fermentation; they proposed mesityl oxide and E-2-hexanal as precursors to 4MMP and 3MH respectively. These compounds require the addition of sulphur either directly through the addition of hydrogen sulphide or through conjugation with cysteine and are later cleaved by yeast enzymes to liberate thiols (Schneider et al., 2006). However, mesityl oxide is not found in grape must and remains a theoretical precursor to 4MMP. Two studies using the using duterated E-2-hexenal as precursor in juice showed that its contribution to the final concentration to 3MH in wine was anywhere between 1 to 10 % (Schneider et al., 2006; Subileau et al., 2008a). Recently it was shown that addition of either E-2-hexenal or glutathione to grape must was capable of increasing the level of varietal thiols in the final wine (Roland et al., 2010c). This could represent a distinct pathway or maybe an earlier entry point in the pathway outlined above.
Figure 4.1: Diagrammatic representation of the role of thiol pre-cursors and yeast metabolism to produce volatile thiols. Information was adapted from literature discussed in section 4.2.

4.3 Importance of other aroma compounds

At least 600-800 aromatic compounds have been identified in wine (Guth, 1997; Swiegers et al., 2009). Among them ethyl esters have been reported to have a strong influence on the aroma profiles of various wine varieties (Guth, 1997; Ribéreau-Gayon et al., 2006a). The most abundant ester is perhaps ethyl acetate, which is formed from ethanol and acetic acid found in wine. The formation of esters during fermentation can be described as a dynamic process, the average ester production and the relative proportions of each ester are highly dependent on the yeast strain and the influence of other parameters, such as temperature, oxygen and nitrogen, other nutrients present and must solids (Boulton et al., 1998; Styger et al., 2011; Sumby et al., 2010; Vilanova et al., 2007). In assessment of final aroma of Marlborough Sauvignon Blanc, rarely are aromas other than varietal thiols and methoxypyrazines considered, in this chapter the other class of aroma compounds mentioned below were considered to examine the broad range of impact on aroma due to ferment manipulations.

4.3.1 Esters

Chemically, esters are formed by reaction between alcoholic and carboxylic acid functional groups with the removal of a water molecule. Esters are mainly formed by enzymatic yeast activity during fermentation and also during wine maturation (Guth, 1997). Many enzymes such as esterases, lipases
and acyltransferases are involved in the synthesis of esters. There is potentially a large variety of esters due to the presence of a wide range of organic acids and alcohols (Guth, 1997; Jackson, 2000; Swiegers et al., 2005).

4.3.2 Higher alcohols

Compounds such as higher alcohols (1-propanol, isobutanol, isoamyl alcohol and 2-phenylethanol) are generated from α-ketoacids derived from sugars and by other anabolic reactions involving branched chain amino acids through the Ehrlich pathway (Guth, 1997; Swiegers et al., 2005). They can impart a pungent smell or contribute to complexity of wine if they are present in concentrations less than 0.30 g/L (Eden et al., 2001; Rapp and Mandery, 1986; Sumby et al., 2010). Acetate esters are produced by yeast from the reaction of acetyl-coA with a higher alcohol. The formation of these compounds is favoured in slow, low temperature, anaerobic fermentations and is generally in excess of their equilibrium constants (Styger et al., 2011). This results in the hydrolization back to their component alcohols and acetic acid (Jackson, 2000). These esters lend intense unusual odours such as banana, pear-drops, and apple to wines.

4.3.3 Fatty acids

During alcohol fermentation, yeasts produce small amounts of caproic (Hexanoic), caprylic (Octanoic) and capric (Decanoic) acids. These fatty acids in high concentrations can impart cheesy, fatty, rancid, goat like aromas, but when found in concentrations below their odour threshold can influence the complexity and depth of flavour in a wine (Styger et al., 2011; Sumby et al., 2010; Swiegers et al., 2005). These acids may also become toxic to Saccharomyces cerevisiae and inhibit the fermentation process (Dubourdieu et al., 2006). Fatty acid ethyl esters are produced by yeast activity during fermentation via ethanolysis of the acylCoA that is formed during fatty acid synthesis or degradation and reach a maximum concentration during fermentation (Styger et al., 2011). These esters impart pleasant aromas of wax, sweet, fruity, honey, and floral to red wines.

Table 4.2 lists some of the main aromatic alcohols, esters, ester acetates and fatty acids and their characteristic aroma and their odour perception thresholds which were analysed in this study, also shown are the levels of some of the compounds found in Marlborough Sauvignon Blanc (Benkwitz, 2009; Louw et al., 2010). The concentration of these aromas in Marlborough Sauvignon Blanc was obtained from Dr F. Benkwitz as part of his doctoral research (Benkwitz, 2009).
### Table 4.2: A list of aromatic compounds analysed in this study apart from volatile thiols. Descriptions and OTH values adapted from Louw et al. (2010). Times over OTH were adapted from F. Benkwitz (2010). In this table “Na” denotes that the data is not available.

<table>
<thead>
<tr>
<th>Name</th>
<th>Abbreviation</th>
<th>Description</th>
<th>OTH (mg/L)</th>
<th>Times over OTH Marlborough n=28</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoamyl alcohol</td>
<td>IAA</td>
<td>Alcoholic, harsh</td>
<td>40</td>
<td>7 to 8</td>
</tr>
<tr>
<td>Isobutanol</td>
<td>IB</td>
<td>Fusel, alcohol</td>
<td>30</td>
<td>below OTH</td>
</tr>
<tr>
<td>Hexanol</td>
<td>H</td>
<td>Grassy</td>
<td>8</td>
<td>1 to 3</td>
</tr>
<tr>
<td>2-phenyl ethanol</td>
<td>PEE</td>
<td>Roses</td>
<td>14</td>
<td>2 to 6</td>
</tr>
<tr>
<td>Isoamyl acetate</td>
<td>IAAc</td>
<td>Bananas</td>
<td>0.03</td>
<td>17 to 53</td>
</tr>
<tr>
<td>Ethyl hexanoate</td>
<td>EH</td>
<td>Green apple</td>
<td>0.014</td>
<td>1 to 30</td>
</tr>
<tr>
<td>Hexyl acetate</td>
<td>HAC</td>
<td>Apple, cherry, floral</td>
<td>1.5</td>
<td>below OTH</td>
</tr>
<tr>
<td>Ethyl lactate</td>
<td>EL</td>
<td>Lactic, buttery, fruity</td>
<td>154.6</td>
<td>Na</td>
</tr>
<tr>
<td>Ethyl octanoate</td>
<td>EO</td>
<td>Sweat, soapy, fruity</td>
<td>0.005</td>
<td>1 to 3</td>
</tr>
<tr>
<td>Ethyl decanoate</td>
<td>ED</td>
<td>Grape, soapy, fruity</td>
<td>0.2</td>
<td>below OTH</td>
</tr>
<tr>
<td>2-phenyl ethyl acetate</td>
<td>BPEA</td>
<td>Rose, honey, tobacco</td>
<td>0.25</td>
<td>below OTH</td>
</tr>
<tr>
<td>Isobutyl acetate</td>
<td>IBA</td>
<td>Fruity, apple, banana</td>
<td>1.6</td>
<td>Na</td>
</tr>
<tr>
<td>Hexanoic acid</td>
<td>HEA</td>
<td>Sweat, cheesy</td>
<td>0.42</td>
<td>Na</td>
</tr>
<tr>
<td>Octanoic acid</td>
<td>OA</td>
<td>Rancid, harsh, sweaty</td>
<td>0.5</td>
<td>Na</td>
</tr>
<tr>
<td>Decanoic acid</td>
<td>DA</td>
<td>Rancid, fatty</td>
<td>1</td>
<td>Na</td>
</tr>
</tbody>
</table>

4.4 Impact of oenological conditions on final aroma of wine

4.4.1 Impact of temperature on aroma levels

Several researchers have monitored the influence of temperature on the secondary metabolites produced by yeast strains (both Saccharomyces and non-Saccharomyces) during fermentation and its impact on final wine quality (Antonelli et al., 1999; Aragon et al., 1998; Dubourdieu et al., 2006; Erten, 2002; Howell et al., 2004; Masneuf-Pomarède et al., 2006; Torija et al., 2003). It was shown that the level of 4MMP released from its cysteine conjugate during fermentation of a synthetic medium was higher at a higher fermentation temperature of 28 °C when compared with fermentation at 18 °C. This was true for the yeast strains CWY7 and CWY8. However, CWY1 was capable of releasing similar levels of 4MMP at both the temperatures. This indicated that the effect of temperature for the release of 4MMP was dependent on the strain of yeast utilized (Howell et al., 2004). Similar results were reported by the French study conducted on commercial wine yeasts and a hybrid between S. cerevisiae and S. bayanus where they reported a positive impact of temperature for the release of both 3MH and 4MMP from Sauvignon Blanc grape must. However, they didn’t find any direct impact of temperature on the levels of 3MHA regardless of the yeast strain used in the
study (Masneuf-Pomarède et al., 2006). The Australian study utilised synthetic grape medium and used 10,000 times the concentration of cys-4MMP to monitor corresponding release of 4MMP, the French study reported only low concentrations of the three thiols, therefore more work needs to be conducted to understand the release of thiols in Marlborough Sauvignon Blanc (Howell et al., 2004; Masneuf-Pomarède et al., 2006). Interestingly these studies seem to negate popular hypothesis that low fermentation temperatures enhance the aromatic characteristics of wines, possibly because of greater synthesis and a greater retention of volatile compounds (Molina et al., 2007; Styger et al., 2011).

It is widely accepted that lower temperature ferments are capable of improving aroma profiles of wine (Masneuf-Pomarède et al., 2006). Although as previously stated thiol liberation in Sauvignon Blanc wine increased with higher temperature fermentation (Masneuf-Pomarède et al., 2006). Curiously, apart from thiols, other aroma compounds have not been a major focus when studying aroma of Marlborough Sauvignon Blanc. These other molecules are important fermentative chemicals capable of altering the final wine quality. Not much work has been conducted on the importance of temperature on esters production during Sauvignon Blanc fermentation. In a recent study, levels of esters and other aromatic compounds were analysed for young wines from South Africa from 2005 to 2007; the study found ester compounds to be important in distinguishing between Sauvignon Blanc and Chardonnay (Louw et al., 2010). Therefore, it could be possible that these compounds can render distinguishing properties to Sauvignon Blanc from Marlborough region and perhaps help differentiate Marlborough Sauvignon Blanc wine made using different wine yeasts. One of the aims of this chapter was to understand the impact of temperature manipulation on the final aroma of Saccharomyces- non-Saccharomyces co-ferments of Marlborough Sauvignon Blanc.

### 4.4.2 Impact of YAN on final aroma

Growth of yeast during fermentation of grape juice is highly dependent on the concentration of available nitrogen (Martínez-Moreno et al., 2012). Nitrogen supplementation by addition of diammonium phosphate (DAP) is widely practiced throughout the winemaking industry (Jackson, 2000). Only recently has the impact of DAP on organoleptic properties been examined (Torrea et al., 2011; Vilanova et al., 2007). Bely et al. (2003) found that increasing YAN of grape must resulted in increased biomass and fermentation rates of \( S. \) \( cerevisiae \); it also increased the volatile acidity of the wine. However, the impact of adding DAP on the varietal aroma of wine has mixed views among winemakers and researchers. Addition of DAP induces Nitrogen Catabolite Repression (NCR) a universal control mechanism in yeast during fermentation, this has an overall impact on yeast metabolism (Deed et al., 2011). Thibon et al. (2008) and Subileau et al. (2008b) found evidence that suggested that expression or repression of GAP1 gene related to NCR is responsible for the release of
thiol from its cysteine conjugate into the synthetic wine media by yeast. It was shown that not all NCR related genes were repressed by DAP in wine yeast during fermentation and that addition of DAP had little impact on the thiol levels of the resulting wines (Subileau et al., 2008b; Thibon et al., 2008). It was suggested that alternative pathways in yeast were capable of overriding the NCR genes (Deed et al., 2011). Interestingly the 4MMP related gene IRC7 was not impacted by NCR (Deed et al., 2011). However, NCR and DAP additions are important factors in fermentation and clearly more work needs to be conducted on the relevance of NCR on aromatic potential of Sauvignon Blanc wines.

Unlike the impact of NCR on thiols, the impact of nitrogen additions and NCR on the formation of esters has been frequently examined by various groups (Carrau et al., 2008; Hernandez-Orte et al., 2006; Vilanova et al., 2007). The compounds most impacted by nitrogen content in grape must include acetate and ethyl esters, higher alcohols, medium chain fatty acids (MCFA) and branched-chain acids. These compounds can impart fruity and floral aromas, solvent, fusel odours, soapy, cheesy, sweaty or rancid odours (Bell and Henschke, 2005; Francis and Newton, 2005; Torrea et al., 2011). Thus Yeast Available Nitrogen (YAN) has a huge impact on final aroma of wine. However, impact of YAN on Saccharomyces-non-Saccharomyces co-ferments is yet to be underpinned. It was one of the aims of this chapter to understand the impact of YAN concentration on co-ferments.

4.4.3 Impact of yeast species on aroma levels

It has been shown that the strain of wine yeast Saccharomyces cerevisiae used, plays an important role in releasing 4MMP into final wine (Dubourdieu et al., 2006; Howell et al., 2006; Howell et al., 2005; Murat et al., 2001a; Swiegers et al., 2006b). However, usage of different strains seems to have no impact on the level of 3MH released (Murat et al., 2001a). It has also been shown that the co-inoculation of Saccharomyces cerevisiae var VL3 used in combination with Pichia kluyveri (PKKR1) at a specific starting ratio into Marlborough Sauvignon Blanc must is capable of increasing the levels of the thiol 3MHA in final wine compared to inoculation with VL3 alone (Anfang et al., 2009). Another study showed that Saccharomyces bayanus species was capable of producing more 4MMP compared to wine yeast VL3 in Sauvignon Blanc (Dubourdieu et al., 2006; Masneuf-Pomarède et al., 2010). Thus, by using different yeasts or specific combinations of Saccharomyces and non-Saccharomyces yeast it is possible to alter the levels of specific thiols in Sauvignon Blanc wines. Therefore it is possible that using novel Saccharomyces yeast isolates either as single inoculum or co-inoculated with non-Saccharomyces yeast can be used to bring about characteristic release of thiols in Sauvignon Blanc wines.
The physiological role of both aromatic thiols and esters are not completely understood. Current literature suggests that yeasts synthesize esters to balance toxicity caused by long and medium chained fatty acids and to maintain acetyl-CoA/CoA ratio (Mason and Dufour, 2000; Sumby et al., 2010). Several research groups have stressed the importance of yeast strain used in winemaking on the ester profile of the finished product (Fleet, 2003; Miller et al., 2007; Rojas et al., 2001; Romano et al., 2003; Soles et al., 1982; Swiegers et al., 2005). Although rarely examined, other microbes and non-Saccharomyces yeast present during initial fermentation of grape must can add to the aroma complexity of the finished wine (Ciani and Comitini, 2011; Jolly et al., 2006). A few non-Saccharomyces yeast were capable of producing thiols on their own in sterilized grape juice (Anfang, 2010). Screening of non-Saccharomyces yeast by various groups resulted in identification of various strains of Hanseniaspora such as H. osmophila, H. gullierimondi, H. uvarum and K. apiculata as capable of increasing aromatic compounds such as acetaldehyde, phenyl-ethyl acetate, and other acetonin stereoisomers and contribute favourably to final wine quality (Ciani et al., 2010; Moreira et al., 2008; Moreira et al., 2005; Romano et al., 2003; Viana et al., 2008). Interestingly, cryotolerant yeast such as S. bayanus and S. uvarum are known to produce less acetic acid in ferments (Eglinton et al., 2000). Clearly, more work needs to be done to understand the role of yeast species on final aroma of Marlborough Sauvignon Blanc.

4.5 Background to experiments

The final aroma is one amongst the many factors used to distinguish the calibre of wine (Ribéreau-Gayon et al., 2006a). Marlborough Sauvignon Blanc is renowned for its distinctiveness based on its geographical location and also its unique varietal aroma attributes. One of the reasons Marlborough Sauvignon Blanc is popular is due to its high levels of aromatic thiols when compared to its counterparts from across the world (Lund et al., 2009). However, there is little knowledge regarding the production/manipulation of the concentration of these aroma compounds in the final wine. Factors ranging from different viticulture practices through to the fining and bottling of wine could play an important role in production/manipulation of aromatic thiols in Marlborough Sauvignon Blanc.

This chapter focused on the production of thiols and other potentially important aroma compounds during fermentation and examined the impact of oenological conditions on their final concentrations in the resulting wine. In a previous study, it was shown that non-Saccharomyces yeast were capable of producing thiols on their own in Sauvignon Blanc, albeit unable to finish the fermentation process. (Anfang, 2010). Though, some non-Saccharomyces yeast such as Pichia kluyveri (PKKR1) were able to increase the production of 3MHA when co-fermented with an industrial Saccharomyces strain
(VL3) (Anfang et al., 2009). However, this phenomenon was only observed under a particular temperature and co-ferment inoculation ratio conditions and not much is understood regarding production of esters by co-ferments in Sauvignon Blanc (Anfang et al., 2009).

In industrial scale winemaking, factors such as temperature and YAN and the strain of yeast inoculated play an important role and can vary between winemakers and vintages, therefore it is important to test key aroma levels and their relationship to the various oenological conditions.

The main aim of this PhD was to elucidate different yeast interactions in Sauvignon Blanc ferment and their impact on varietal aroma. Therefore a full-factorial experiment (where there is no confounding between the different factors) was designed to understand the interactions between selected Saccharomyces and non-Saccharomyces yeast at a range of temperature and YAN (Discussed in chapter 3 section 3.3) (King, 2006). These interactions were monitored via any observable changes in the fermentation kinetics (Chapter 3) and final aroma (this chapter) in resulting wine.

This chapter discusses part of the results (thiol and other volatile aroma) obtained from the full-factorial experiment set up to understand yeast interaction in New Zealand Sauvignon Blanc and their impact on the aroma. The objective was to compare mono-culture ferments of S. bayanus SBJ1d and S. cerevisiae VL3 along with their co-ferment with P. kluyveri PKKR1. Also examined were the three co-ferments of SBJ1d with three non-Saccharomyces isolates identified in Kumeu River Sauvignon Blanc (2008). In this study, mono-ferments using yeast VL3 (referred to as VL3or vl3) and S. bayanus strain (SBJ1d, hereon referred to as SB or sb) isolated in chapter 2, co-ferments of PKKR1 and VL3 (PKKR1+VL3 or vl3pkkr), PKKR1 and S. bayanus (PKKR1 + SB or sbpkkr), strain of P. anomala (A1c) isolated as described in chapter 2 and S. bayanus (PA+SB or sbpa) and strain of T. delbrueckii (D1a) and S. bayanus (TD+SB or sbtd) with an initial inoculum ratio of 9:1(2.5 million cells/mL) were conducted in triplicates under temperatures 12.8 and 20°C with an initial YAN of 390 mg/L (Y1) or 1170 mg/L (Y2). A layout of this full-factorial experimental design is shown below in figure 4.2 and also in figure 3.2 (chapter 3).
Figure 4.2: Flow chart representation of full-factorial fermentation set up with three factors. Where 6 different yeast combinations were fermented at two different temperature and YAN conditions:

Temperature conditions were 12.8°C and 20°C, YAN conditions were 390 mg/L (Y1) (no DAP addition) and 1170 mg/L (Y2). Yeast combinations used were as follows: S. bayanus-only (SB), PKKR1 and S. bayanus (PKKR1+SB), P. anomala and S. bayanus (PA+SB), T. delbrueckii and S. bayanus (TD+ SB), S. cerevisiae-only (VL3) and PKKR1 and S. cerevisiae (PKKR1+VL3). All co-ferments had an inoculum ratio of 9:1, non-Saccharomyces to Saccharomyces yeast and were in triplicates for all conditions.

The focus of this chapter was to characterise the aroma profile from Marlborough Sauvignon Blanc under the different fermenting parameters such as the strain of yeast used, temperature of fermentation and yeast nitrogen availability. This was done by testing the null hypothesis that manipulation of oenological conditions (YAN and Temperature and Yeast) examined had no impact on the final aroma concentration in wine. This study was conducted to understand the relevance of the above mentioned oenological conditions in differentiating Marlborough Sauvignon Blanc.
4.5.1 Aims

1) Compare and contrast the overall aroma profiles of Marlborough Sauvignon Blanc made under different oenological parameters to test the null hypothesis that the manipulation of biological and environmental factors has no measurable effect on the compounds that contribute to MSB aroma and flavour.

2) Examine any significant interactions between the different oenological conditions investigated; i.e. fermenting temperature, YAN and inoculating yeast species.
4.6 Methods and Materials

4.6.1 Fermentation set-up and aroma analysis
See chapter 3 section 3.4.3.1

4.6.2 Thiol analysis
See chapter 2 section 2.8.7

4.6.3 Analysis of other aromatic compounds
The extraction of other non-thiol volatile compounds such as C₆ aldehydes and alcohols, higher alcohols, fatty acids, esters, and ethyl phenols was performed in duplicate according to the method described previously (Sabon et al., 2002). 100 mL of sample wine was spiked with 50 µL of DL-3-octanol (1830 mg/L) as internal standard, followed by stirring at 500 rpm for 20 min for equilibration. Aromatic compounds were extracted three times from the same sample with 4, 2 and 2 mL of diethyl-ether: hexane (1:1 v/v) by stirring for 5 min at 1000 rpm. Each time the organic phase was separated from the aqueous phase using a glass separating funnel into a Teflon® centrifuge tube. Finally the two phases were separated by centrifugation for 5 min at 8000 rpm. The aqueous phase at the bottom was removed using a Pasteur pipette and the organic phase was dried over anhydrous sodium sulphate and filtered through glass wool. Finally the filtered organic phase was concentrated under nitrogen gas to approximately 0.5 mL; this was stored in a GC vial and injected into GCMS.

4.6.3.1 GCMS parameters
All compounds were analysed with GCMS using an Agilent 19091N-136 HP-Innowax polyethylene glycol capillary column (0.25mm x 60m x 0.25µm). The injector temperature was kept at 230°C and 2 µL of the concentrated sample was injected in splitless mode. A pulsed splitless injection with a pulse and purge time at 1.00 min, purge flow of 12.0 mL/min, and total flow of 16.6 mL/min was delivered with helium as a gas carrier onto the Innowax column. The oven parameters were an initial temperature of 60°C and maximum temperature of 255°C; the oven ramps can be found in the table below (4.3). The GCMS was operated in selective ion monitoring (SIM) mode with a total run time of 88 minutes.
<table>
<thead>
<tr>
<th>Ramp</th>
<th>Rate (°C/min)</th>
<th>Next Temp (°C)</th>
<th>Hold (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>200</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>70</td>
<td>250</td>
<td>40</td>
</tr>
<tr>
<td>3</td>
<td>40</td>
<td>60</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 4.3: The GCMS oven ramps used and their parameters to identify aromatic compounds.

### 4.6.3.2 Calibration curves for volatile compounds

A calibration curve was created for each volatile compound studied by spiking a standard commercial Sauvignon Blanc (Corbans White Label, 2009) with increasing concentrations of each compound using standard solutions of known concentrations. The extraction protocol was performed as previously explained (4.2.3) and the GCMS parameters remained the same. The calibration equations attained after subtracting the values obtained from un-spiked standard wine replicates and were used for quantification of each compound’s concentration in the research wine samples. The concentration ranges, peak ions, and calibration equations can be seen in table 4.4.

<table>
<thead>
<tr>
<th>Name</th>
<th>CAS no</th>
<th>Supplier</th>
<th>Peak Ion</th>
<th>Concentration ranges in µg/L except * with mg/L</th>
<th>Calibration curve equations</th>
<th>Correlation coefficients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isobutanol</td>
<td>555-82-1</td>
<td>Acros</td>
<td>43</td>
<td>14.8 to 295.4*</td>
<td>(y = 151.87x + 8.9835)</td>
<td>(R^2 = 0.9965)</td>
</tr>
<tr>
<td>Phenyl ethyl ethanol</td>
<td>60-12-8</td>
<td>Acros</td>
<td>91</td>
<td>26.3 to 657.7*</td>
<td>(y = 18.042x - 51.328)</td>
<td>(R^2 = 0.9364)</td>
</tr>
<tr>
<td>Isoamyl alcohol</td>
<td>505-10-2</td>
<td>Acros</td>
<td>55</td>
<td>25.4 to 635.8*</td>
<td>(y = 60.94x - 29.588)</td>
<td>(R^2 = 0.988)</td>
</tr>
<tr>
<td>Hexanol</td>
<td>111-27-3</td>
<td>Acros</td>
<td>56</td>
<td>281.7 to 5634.4</td>
<td>(y = 3479.9x - 231.86)</td>
<td>(R^2 = 0.9731)</td>
</tr>
<tr>
<td>2-phenyl ethyl acetate</td>
<td>101-97-3</td>
<td>Acros</td>
<td>104</td>
<td>9.1 to 182.2</td>
<td>(y = 8.2177x + 3.1827)</td>
<td>(R^2 = 0.9014)</td>
</tr>
<tr>
<td>Hexyl acetate</td>
<td>142-92-7</td>
<td>Acros</td>
<td>56</td>
<td>44.9 to 898.9</td>
<td>(y = 1483.6x - 4.4662)</td>
<td>(R^2 = 0.9944)</td>
</tr>
<tr>
<td>Isoamyl acetate</td>
<td>110-19-0</td>
<td>Fluka</td>
<td>70</td>
<td>113.7 to 2273.6</td>
<td>(y = 23.738x - 192.98)</td>
<td>(R^2 = 0.9523)</td>
</tr>
<tr>
<td>Isobutyl acetate</td>
<td>123-92-2</td>
<td>Univar</td>
<td>43</td>
<td>16.9 to 338.3</td>
<td>(y = 3105.2x + 1.8345)</td>
<td>(R^2 = 0.9968)</td>
</tr>
<tr>
<td>Ethyl lactate</td>
<td>687-47-8</td>
<td>Acros</td>
<td>45</td>
<td>16.2 to 323.5</td>
<td>(y = 25957x - 12.478)</td>
<td>(R^2 = 0.9898)</td>
</tr>
<tr>
<td>Ethyl hexanoate</td>
<td>123-66-0</td>
<td>Fluka</td>
<td>88</td>
<td>24.6 to 492</td>
<td>(y = 1188.6x - 38.05)</td>
<td>(R^2 = 0.9111)</td>
</tr>
<tr>
<td>Ethyl octanoate</td>
<td>106-32-1</td>
<td>Acros</td>
<td>88</td>
<td>48.7 to 974.2</td>
<td>(y = 567.11x + 3.0488)</td>
<td>(R^2 = 0.986)</td>
</tr>
<tr>
<td>Ethyl decanoate</td>
<td>110-38-3</td>
<td>Aldrich</td>
<td>88</td>
<td>11 to 219.8</td>
<td>(y = 639.66x - 3.9667)</td>
<td>(R^2 = 0.9927)</td>
</tr>
<tr>
<td>Hexanoic acid</td>
<td>142-62-1</td>
<td>Acros</td>
<td>60</td>
<td>764.9 to 15297.8</td>
<td>(y = 3928.3x - 655.48)</td>
<td>(R^2 = 0.9457)</td>
</tr>
<tr>
<td>Octanoic acid</td>
<td>124-07-2</td>
<td>Acros</td>
<td>60</td>
<td>223.7 to 4474.8</td>
<td>(y = 3050.5x + 3.4378)</td>
<td>(R^2 = 0.9694)</td>
</tr>
<tr>
<td>Decanoic acid</td>
<td>334-48-5</td>
<td>Acros</td>
<td>60</td>
<td>178.2 to 3564</td>
<td>(y = 1491.3x - 44.371)</td>
<td>(R^2 = 0.9845)</td>
</tr>
</tbody>
</table>

Table 4.4: List of aromatic compounds analysed using GCMS with their respective calibration curves.
The raw data was obtained for the research wines from GCMS to that explained in section 2.8.7. The raw data was then tabulated and processed using Microsoft Excel 2007. The values of each of the aroma compounds were interpolated from its corresponding calibration curve using the equations obtained (as shown in table 4.4). The average of the three biological replicates, and their two technical replicates were calculated for each sample and each aroma compound along with their respective standard deviations.

Factorial analysis of variance (3 ways ANOVA) tests were performed using the R Statistical Computing Program (v 2.13.1) on the total volatile aroma data (thiols and esters) to determine the differences between the different environmental conditions of temperature and Yeast available nitrogen (YAN) concentrations (Team, 2011). These tests also estimated the impact of possible Temperature*YAN*Yeast species interactions. A 2D Q-PCA (Principal component Analysis) was also performed using R 2.13.1 to obtain the information regarding variance in the data, complimentary analysis of scores and loading plots were carried out to analyse the relationship between samples and variables. Furthermore, two-tailed Students’ T-test with unequal variance were also performed on R 2.13.1 taking into account False Discovery Rates (fdr) while obtaining P-values to explore the patterns observed from 2D R-PCA. The relevant R codes are available in appendix 6.

For each volatile compound in all of the research samples, Odour activity value (OAV) was calculated as reported in literature (Guth, 1997). Odour activity values were calculated by dividing the average concentrations of a compound by its odour threshold (OTH). Compounds with OAV ≥ 1 were considered as having an impact on the aroma profile of that wine (Guth, 1997).
4.7 Results and Discussion

4.7.1 Source of variation in final aroma of Marlborough Sauvignon Blanc made under controlled oenological conditions

4.7.1.1 Raw data and Standardisation

The varietal aroma and other volatile aroma analysed from the full-factorial experiment is analysed in this chapter. The final concentration of 18 aroma compounds (thiols and other aroma compounds) were obtained for each sample as described in the methods section above. This resulted in multi-factorial complex dataset. Figure 4.3 below is a box and whisker’s plot which shows the range for each aroma compound under different oenological condition (Temperature (12.8°C and 20°C) and YAN Y1 (390mg/L) and Y2 (1170, g/L)) tested. For a detailed description of experimental set-up see section 4.5 and figure 4.2. For example; 12.8y1 hexyl acetate, the box plot depicts the final concentration of hexyl acetate for all the six yeast combinations and their replicates (total n = 18) under the oenological conditions; 12.8°C and YAN y1. All the aroma values are to the log 10 scale as there are vast differences in their concentration in the final wine. Before any meaning or comparisons could be gleaned from these values; they had to be standardised to remove any bias (King, 2006).

From figure 4.3, differences can be observed within an aroma under different oenological conditions for aromas like isoamyl alcohol and isoamyl acetate, and within specific conditions like 12.8y2 PEE and 20y2 PEE. Also there were 100-1000 fold differences in concentration in the overall dataset. Standardization of the entire data set removes the above mentioned bias (King, 2006). Here statistical standardisation (geometrical mean standardisation) was achieved by subtracting the mean value of an aroma compound (obtained from 18 samples including six yeast combinations) from the sample value followed by dividing it by the standard deviation for that aroma compound (obtained from 18 samples including the six yeast combinations; vl3, vl3pkkr, sb, sbpkkr, sbtd and sbpa as described in section 4.5 and in figure 4.2) (King, 2006). This method was used on all the aroma compounds under all the oenological conditions in order to centre the mean value of concentration of each sample around zero. This ensures a less biased comparison both within and between the oenological conditions. The standardised values are shown in figure 4.4 as box and whiskers plots.
Figure 4.3: Box plot of log10 values of aroma compounds under different temperature and YAN conditions from the full-factorial experiment. Each box-plot shows the mean value and range of a particular compound under a particular condition (temperature and YAN) for the six different fermenting yeast combinations (n=18; vl3, vl3pkkr, sb, sbpkkr, sbtd and sbpa). There are vast log-scale differences both between and within compounds.
Figure 4.4: Box-plot of standardised concentrations of aroma compounds examined under different temperature and YAN conditions from the full-factorial experiment. Each box-plot shows the mean value and range of a particular compound under a particular condition (temperature and YAN) for the six different fermenting yeast combinations (n=18; vl3, vl3pkkr, sb, sbpkkr, sbtd and sbpa) Standardisation centred the means of the box-plots on zero.
4.7.1.2 Three-way ANOVA

To understand the impact of different oenological conditions from a full-factorial design and any significant interaction among them on the final concentration of the key aromas measured in this study; a three-way ANOVA (with factors being; temperature, YAN and species) was performed for each aroma compound (King, 2006). Table 4.5 reports the P-values revealed by the ANOVA and indicate the effect of each of the oenological conditions on specific aroma compounds. Also shown in the table are any potential combinations of oenological conditions which have a significant impact on the final levels of specific aroma compounds. This test allowed the overall examination of relationships of oenological conditions and their level of impact on key aroma compounds and any interactions between them.
Table 4.5: P-values revealed by a 3-way ANOVA (temperature, YAN and species as factors) on aroma compound concentration obtained from full-factorial experiment using Marlborough Sauvignon Blanc. A significant P-value indicates an effect by the respective factor.

<table>
<thead>
<tr>
<th>Name of compound</th>
<th>Temperature</th>
<th>YAN</th>
<th>Species</th>
<th>Temperature:YAN</th>
<th>Temperature:Species</th>
<th>YAN:Species</th>
<th>Temperature:YAN:Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexyl acetate</td>
<td>1.61E-02</td>
<td>9.65E-09</td>
<td>3.11E-13</td>
<td>1.63E-11</td>
<td>2.20E-16</td>
<td>3.68E-07</td>
<td>5.42E-08</td>
</tr>
<tr>
<td>2-phenyl-ethyl acetate</td>
<td>1.36E-12</td>
<td>2.20E-16</td>
<td>2.20E-16</td>
<td>2.55E-11</td>
<td>3.94E-04</td>
<td>2.20E-16</td>
<td>6.91E-06</td>
</tr>
<tr>
<td>Decanoic acid</td>
<td>2.20E-16</td>
<td>3.34E-08</td>
<td>2.20E-16</td>
<td>2.04E-01</td>
<td>2.42E-05</td>
<td>4.00E-04</td>
<td>4.65E-05</td>
</tr>
<tr>
<td>Ethyl decanoate</td>
<td>2.20E-16</td>
<td>2.20E-16</td>
<td>2.20E-16</td>
<td>2.20E-16</td>
<td>2.20E-16</td>
<td>2.20E-16</td>
<td>4.31E-16</td>
</tr>
<tr>
<td>Ethyl hexanoate</td>
<td>4.74E-10</td>
<td>2.20E-16</td>
<td>1.50E-06</td>
<td>2.37E-07</td>
<td>2.20E-16</td>
<td>5.82E-12</td>
<td>3.21E-04</td>
</tr>
<tr>
<td>Ethyl lactate</td>
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<td>2.02E-06</td>
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<td>1.08E-09</td>
<td>4.94E-02</td>
<td></td>
</tr>
<tr>
<td>Ethyl octanoate</td>
<td>1.39E-13</td>
<td>2.20E-16</td>
<td>2.20E-16</td>
<td>1.38E-10</td>
<td>2.20E-16</td>
<td>1.64E-15</td>
<td>6.24E-03</td>
</tr>
<tr>
<td>Hexanoic acid</td>
<td>1.34E-07</td>
<td>2.20E-16</td>
<td>2.20E-16</td>
<td>2.20E-16</td>
<td>2.20E-16</td>
<td>1.03E-01</td>
<td></td>
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<td>Hexanol</td>
<td>2.20E-16</td>
<td>9.97E-15</td>
<td>2.20E-16</td>
<td>1.89E-01</td>
<td>8.79E-01</td>
<td>8.45E-06</td>
<td>7.93E-04</td>
</tr>
<tr>
<td>Isoamyl alcohol</td>
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<td>2.20E-16</td>
<td>4.68E-06</td>
<td>2.20E-16</td>
<td>3.70E-05</td>
<td>1.79E-02</td>
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<td>2.20E-16</td>
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<td>2.20E-16</td>
<td>2.20E-16</td>
<td>2.20E-16</td>
<td></td>
</tr>
<tr>
<td>Isobutanol</td>
<td>2.20E-16</td>
<td>1.20E-15</td>
<td>2.20E-16</td>
<td>2.20E-16</td>
<td>2.20E-16</td>
<td>1.80E-12</td>
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<td>Isobutyl acetate</td>
<td>2.20E-16</td>
<td>2.20E-16</td>
<td>2.20E-16</td>
<td>2.20E-16</td>
<td>2.20E-16</td>
<td>2.20E-16</td>
<td></td>
</tr>
<tr>
<td>Octanoic acid</td>
<td>2.20E-16</td>
<td>3.15E-03</td>
<td>2.20E-16</td>
<td>1.80E-04</td>
<td>1.21E-14</td>
<td>9.31E-01</td>
<td>3.65E-02</td>
</tr>
<tr>
<td>Phenyl-ethyl-ethanol</td>
<td>2.20E-16</td>
<td>2.20E-16</td>
<td>2.20E-16</td>
<td>6.31E-03</td>
<td>9.21E-11</td>
<td>8.48E-16</td>
<td>1.35E-03</td>
</tr>
<tr>
<td>3MH</td>
<td>7.41E-01</td>
<td>2.80E-02</td>
<td>2.20E-16</td>
<td>7.12E-04</td>
<td>2.20E-16</td>
<td>1.14E-07</td>
<td>7.03E-05</td>
</tr>
<tr>
<td>3MHA</td>
<td>2.20E-16</td>
<td>4.25E-02</td>
<td>2.20E-16</td>
<td>2.71E-02</td>
<td>7.50E-12</td>
<td>1.55E-05</td>
<td>4.03E-05</td>
</tr>
<tr>
<td>4MMP</td>
<td>6.70E-04</td>
<td>4.14E-09</td>
<td>2.20E-16</td>
<td>9.75E-12</td>
<td>6.81E-11</td>
<td>1.80E-11</td>
<td>4.07E-13</td>
</tr>
</tbody>
</table>
From table 4.5, most oenological conditions (ANOVA factors) were significantly impacting on each of the key aroma compound measured, as were the different combination of these parameters. Some notable exceptions where the parameters were not significant were temperature on level of 3MH, lack of impact of temperature and nitrogen interaction on decanoic acid or hexanol. Also there is a lack of interaction between temperature and species on hexanol, along with a lack of interaction between three parameters on hexanoic acid.

All that could be concluded from the three-way ANOVA was that all the three oenological conditions had a significant impact on the final concentration of these aroma in Marlborough Sauvignon Blanc and also that the interaction between these parameters had a significant impact on most of these compounds. Therefore more in-depth analyses were required to obtain information regarding specific relationships between oenological conditions (factors) and aroma compounds.

**4.7.1.3 Analysis of differences in aroma concentration between oenological conditions using Tukey’s HSD**

Table 4.6 shows the differences in the concentrations of aroma compounds when comparing specific oenological conditions. Here the difference in the concentration of aroma compounds between 20°C and 12.8°C, between Y2 and Y1 and also between the different yeast species are shown. A green colour indicates an increase in concentration while red indicates a decrease. All values are at log 10 scale and a bold-type font indicates a significant P-value for a comparison as obtained by performing Tukey’s HSD.

From the table (Table 4.6) it can be observed that both temperature and YAN conditions tested had a significant impact on all the aroma compounds measured. However, the trends observed for the differences in concentration of aroma compounds were unique. Similarly, there was a unique and complex relationship between concentration of aroma compounds and yeast used in fermentation. Isoamyl acetate seemed particularly sensitive exhibiting four-fold increase or decrease depending on the oenological conditions.

However, the caveat is that specific response of aroma compounds (their concentration) to individual oenological conditions cannot be garnered from this data at this level of analyses. Therefore the response of aroma compounds to different conditions required a different type of analyses of the raw data. This is discussed in the section below.
Table 4.6: Differences in final concentration of the aroma compounds obtained by comparing within the three oenological conditions examined; i.e. temperature (20°C and 12.8°C), YAN (Y1 and Y2) and fermenting yeasts (sb, sbpkkr, sbtd, sbpa, vl3 and vl3pkkr) using Tukey’s HSD. The differences in concentration are shown as log 10 of the original values, those values marked in bold had P-values < 0.05, while those not in bold were not statistically significant. Green gradient indicates an increasing trend, while red gradient indicates decreasing trend in aroma concentration.
4.7.2 Principal Component Analysis (PCA)

The dataset for comparing the impact of various oenological conditions on key aroma compounds in Marlborough Sauvignon Blanc was composed of 36 samples (considering biological and technical replicates) and 18 variables (key aromas analysed) in four conditions (12.8ºC: Y1, 12.8ºC: Y2, 20ºC: Y1 and 20ºC: Y2). Although there was high dimensionality of the data, there were not enough degrees of freedom (not enough samples) to carry out informative multivariate statistics (Holland, 2008; King, 2006). In order to obtain an overview of this complex dataset, it was not sufficient to plot two variables against one another, however, by assuming that the direction of the data was based on the largest variance in the dataset it was possible to reduce its dimensionality and thus obtain meaningful relationship between samples and aroma compounds under different temperature and YAN conditions (Holland, 2008).

By using PCA, the n-dimensional data space (n=18) was rotated in such a way that the directions of the largest variance (in the dataset) become the co-ordinate axes, and the resulting new axes (Principal components) are orthogonal to one another, are not correlated and sorted according to decreasing variances (Holland, 2008). Interpretation of PCA is subject to normality of the dataset as outliers and skewness in data have a major impact, so upon examination of the raw data (Figure 4.3), geometrical mean standardization was used to normalize the entire dataset (Figure 4.4) (Holland, 2008; King, 2006).

The normalised data matrix was then subjected to R-PCA (applicable to correlation matrix) where the loadings-plot and the scores-plot with PC1 and PC2 axes were drawn for conditions 12.8ºC: Y1, 12.8ºC: Y2, 20ºC: Y1 and 20ºC: Y2 respectively to analyse any possible relationships (figures 4.5, 4.6, 4.7 and 4.8) (Holland, 2008). In PCA of this data-matrix, the score-plot depicted the summary of relationships between the samples (under the given condition e.g.: 12.8ºC: Y1) and it was complimentary and superimposable to the reciprocal loading-plot which was the summary of the relationship of the variables (aroma compounds) (Holland, 2008).

Also, the samples and the aroma compounds that could be grouped together on score and loading plots respectively showed similar behaviour under the given conditions. Thus by observing the score and loading plots individually it was possible to understand the samples and aromas that behaved most similar (or dissimilar) to one another under a given condition. Also, by comparing the score and loading-plots for a given condition it was possible to understand the complimentary relationships of aroma compounds to the different samples (Holland, 2008; Vilanova et al., 2007).
Figure 4.5: PCA for 12.8°C:Y1: (a) score-plot, (b) loading-plot. PC1 accounted for 52.6% variation and PC2 for 24.8%, circles on the plots depicted regions of similar behaviour in aromas (b) and also in samples (a). The circles 1, 2, 3 and 4 in blue on the loading plot refer to aroma compounds with similar behaviour under 12.8°C:Y1, they are complimentary to circles 1, 2, 3 and 4 on the score plot where red circles indicate a positive relationship of similar samples to respective aroma compounds in the blue circle whereas a black circle indicates a negative relationship between the plots in those regions. The full forms of the abbreviated compounds can be found in table 4.2 in section 4.3.3.
4.7.2.1 Score and Loading-plot for oenological condition of 12.8°C:Y1

Reduction in dimensionality and focussing on a few Principal Components (PC) instead of many variables is the goal of PCA (Holland, 2008). There are many criteria and theories as to how many PCs need to be investigated in order to gain insights into the relationships between samples and variables; in this study, PC1 and PC2 for all the comparisons were examined as the other PCs provided little or no extra information (data not shown for other PCs) (Holland, 2008). The variance explained by a PC (PC1 and PC2) was calculated as a percentage of total variance observed using the respective eigenvalues (Holland, 2008). The relevant R codes for obtaining PCA data is provided in appendix 6 (Team, 2011).

Under the 12.8°C:Y1 condition, PC1 accounts for 52.6% while PC2 for 24.8% of the overall variance observed. The loadings plot (figure 4.5 b) can be divided into four groups of aroma compounds with similar behaviour under the given oenological condition. The score plot (figure 4.5a) could be divided into three similar groups of yeast species used in fermentation; with sb, sbpkrk, sbpa and sbtd and vl3 and vl3pkkr forming distinct groups. The information on score and loadings plots are complimentary and superimposable and was used to draw information regarding the behaviour of aroma compounds in certain samples under the given oenological condition (Holland, 2008; Vilanova et al., 2007). As shown in figure 4.5, the red circles 1, 2 and 4 marked regions within which the samples showed a positive correlation to aroma compounds encircled within blue circles 1, 2 and 4 respectively on the loadings plot. However, the black circle 3 on score plot had a negative correlation to the aroma compounds encircled within the blue circle 3 on the loadings plot.

Briefly, the aroma compounds isoamyl acetate, isoamyl alcohol, isobutanol, and isobutyl acetate were positively correlated to samples of sbpa. The aroma compounds 4MMP, 3MH and ethyl octanoate were positively correlated most to sbpkrk samples. While hexanol, hexyl acetate and hexanoic acid were positively correlated to vl3 and vl3pkkr samples, whereas compounds such as PEE, BPEA and 3MHA were negatively correlated to these samples.

The aroma correlation to mono-ferments of S. bayanus and S. cerevisiae (VL3) were considerably different from these initial observations. Also notable was the difference between single and co-ferments of S. bayanus in contrast with the lack of similar observation between single and co-ferment of S. cerevisiae (VL3) in relation to their correlation to aroma compounds. Further analysis was required to define and obtain statistical relevance from these comparisons.
Figure 4.6: PCA for 12.8°C:Y2: (a) score-plot, (b) loading-plot. PC1 accounted for 48.5% variation and PC2 for 29.8%, circles on the plots depicted regions of similar behaviour in aromas (b) and also in samples (a). The circles 1 and 2 in blue on the loading plot refer to aroma compounds with similar behaviour under 12.8°C:Y2, they are complimentary to circles 1 and 2 on the score plot where red circles indicate a positive relationship of similar samples to the respective aroma compounds in the blue circle whereas a black circle indicates a negative relationship between the plots in those regions. The full forms of the abbreviated compounds can be found in table 4.2 in section 4.3.3.
4.7.2.2 Score and Loading-plot for oenological condition of 12.8ºC:Y2

At the oenological condition 12.8ºC:Y2, PC1 explained 48.5% and PC2 29.8% of the total variance observed. The loadings plot (Figure 4.6b) can be divided into two regions of similarly behaving aroma compounds. The score plot (Figure 4.6a) could be divided into 3 regions of similarly behaving samples; these groups consisting of sb, S. bayanus co-ferments and vl3 and vl3pkkr were distinguishable similar to that observed in figure (4.5 a). On the score-plot, the samples within red circles 1 and 2 were positively correlated to aroma compounds within the blue circle 1 and 2 respectively on the loading plot. However, the samples within the black circle 2 were negatively correlated with aroma compounds within the blue circle 2. Aromas such as BPEA, PEE, 4MMP, 3MH, ethyl decanoate and decanoic acid were positively correlated to samples such as sbpa and sbtd, whereas these aroma compounds were negatively correlated to vl3 and vl3pkkr. On the other hand vl3 and vl3pkkr were positively correlated to compounds such as hexyl acetate, hexanoic acid and ethyl hexanoate.

In the score plot, the samples were group similarly to the pattern observed from the oenological condition 12.8ºC:Y1. Also similarities in the relationships between samples and aroma compounds at the different nitrogen conditions under the same fermenting temperature was observed; for example, thiols such as 4MMP and 3MH and other compounds such as PEE and BPEA were positively correlated to co-ferments of S. bayanus at 12.8ºC irrespective of nitrogen condition. Also compounds such as hexanoic acid and hexyl acetate were positively correlated to VL3 and VL3pkkr samples at 12.8ºC irrespective of nitrogen. Therefore it could be hypothesised that different fermenting yeast combinations were capable of producing unique aroma profiles in final wine made from same juice under the same controlled conditions. Similar observations have been made by other researchers in other wines (Ciani et al., 2010; Howell et al., 2006). Thus by selecting the yeast/yeast combinations, it is possible for winemakers to alter the final aroma of Sauvignon Blanc from the same vintage. Although further statistical analysis of the data was required to associate specific aroma to specific fermenting yeast combinations.
4.7.2.3 Score and Loading-plot for oenological condition of 20°C:Y1

The score and loading plot for 20°C: Y1 was remarkably different to those observed in figure 4.5 and 4.6. Here, PC1 accounted for 75.5% and PC2 8.4% of the total variation in the data. In the loadings plot (figure 4.7b), all the aroma compounds studied with the exception of hexanol and 3MH were grouped together when PC1 was plotted against PC2, further PCs showed little variance (data not shown) thus PC1 vs. PC2 plots were retained. Similarly, in the score plot (Figure 4.7a); the samples were divided into two groups of S. bayanus and its co-ferments and vl3 and vl3pkkk. Thus it could be hypothesised that by increasing the fermenting temperature to 20°C the distinction between mono and co-ferments of both S. bayanus and S. cerevisiae (VL3) were lost.

However, the distinction between fermentation carried out using S. bayanus and co-ferments and vl3 and vl3pkkk were retained. Importantly, by comparing the relationships between the score and loadings plot it can be seen that the aroma profiles are more distinct between the mono and co-ferments of the two different Saccharomyces yeast species. Altering the fermenting temperature had a major impact on the final aroma of wine in this study, the distinction between mono and co-ferments were reduced at 20°C compared to 12.8°C. Thus it can be hypothesised that certain temperature conditions can induce specific aroma compounds in fermentation using defined yeast/yeast combinations.

Alternately, an aromatic phenomenon observed at a particular temperature when using a certain yeast/yeast combination may not be observed at another temperature. This finding is important to winemakers trying to tailor wine using yeast.
Figure 4.7: PCA for 20°C:Y1: (a) score-plot, (b) loading-plot. PC1 accounted for 75.5% variation and PC2 for 8.4%, circles on the plots depicted regions of similar behaviour in aromas (b) and also in samples (a). The blue circle on the loading plot refer to aroma compounds with similar behaviour under 20°C:Y1, they are complimentary to circles on the score plot where red circle indicate a positive relationship of similar samples to the respective aroma compounds in the blue circle whereas a black circle indicates a negative relationship between the plots in those regions. The full forms of the abbreviated compounds can be found in table 4.2 in section 4.3.3.
Figure 4.8: PCA for 20°C:Y2: (a) score-plot, (b) loading-plot. PC1 accounted for 73.6% variation and PC2 for 8.1%, circles on the plots depicted regions of similar behaviour in aromas (b) and also in samples (a). The blue circles 1 and 2 on the loading plot refer to aroma compounds with similar behaviour under 20°C:Y2, they are complimentary to circles on the score plot where red circles indicate a positive relationship of similar samples to the respective aroma compounds in the blue circles whereas a black circles indicates a negative relationship between the plots in those regions. The full forms of the abbreviated compounds can be found in table 4.2 in section 4.3.3.
4.7.2.4 Score and Loading-plot for oenological condition of 20°C:Y2

In contrast to observations made using figure 4.5, figure 4.6 shows a remarkable difference especially in the score plot, where four clear distinctions could be made. In the score plot (figure 4.8a), the samples sb, *S. bayanus* co-ferments, vl3 and vl3pkkr can be differentiated. It was here that vl3 and vl3pkkr could be differentiated, compared to the other three oenological conditions examined. In this oenological condition, PC1 accounted for 73.6% while PC2 was 8.1% of the total variance in the data. In the loadings plot, BPEA, PEE, 4MMP, octanoic acid, decanoic acid and hexyl acetate consisted of one group while 3MHA,3MH,ethyl hexanoate and ethyl octonoate made up the other (group 2).

Group one of similar aroma compounds were positively correlated to *S. bayanus* co-ferments while being negatively correlated to vl3, also group two on loadings plot was positively correlated to sb samples while being negatively correlated to vl3pkkr samples. Thus from this condition it could be hypothesised that excessive nitrogen was responsible for differentiating the different yeast groups more so at 20°C than at 12.8°C.

4.7.2.5 Summary of findings from PCA with further data analyses strategy

Thus, PCA was useful in understanding the relationships between aroma compounds with samples and also used to distinguish the differences between yeast/yeast combinations used at the oenological conditions examined. In summary, from observing the score-plots for 12.8°C (both Y1 and Y2) it is clear that *S. bayanus*-only ferments differed from its co-ferment counterparts as well as between *S. cerevisiae*-only and *S. cerevisiae* and *P. kluyveri* co-ferment. However, at 20°C and Y1, the difference between *S. bayanus*-only and its co-ferment counterparts was diminished in comparison to the trend observed at 12.8°C. Upon addition of DAP (i.e. Y2 juice) at 20°C the mono-ferments of *S. bayanus* and *S. cerevisiae* had different aroma profile compared to one another and their co-ferment. However, further statistical analyses were required to examine the trends observed in the data-set.

Some of the important trends observed and analysed further were;

(a) the differences in aroma profile between *S. bayanus* and VL3 mono-ferments
(b) the differences between the co-ferments sbpkkr and vl3pkkr which shared the same non-*Saccharomyces* yeast
(c) The differences in aroma between *S. bayanus*-only and *S. bayanus*-co-ferments at the four oenological conditions.

These trends were then examined using a student’s t-test assuming a two-tailed data distribution with unequal variances. The P-values obtained were corrected to account for false discovery rates (fdr).
4.7.3 Comparison between Marlborough Sauvignon Blanc made using *S. bayanus* (SBJ1d) and *S. cerevisiae* VL3

In this study, three aromatic thiols and fifteen other aroma compounds were examined from the Sauvignon Blanc produced by *S. bayanus* (SBJ1d) and *S. cerevisiae* VL3 under different conditions. From the tables (table 4.7 and table 4.8) below it can be seen that ten of the fifteen esters appear to impact the aroma profile of the resulting wines based on their respective Odour Activity Values (OAV). The statistical analyses and calculation of OAV is described in methods section (4.6.4). Compounds that were significant to the aroma profiles of these wines were: higher alcohols such as Isoamyl alcohol, Isobutyl alcohol and 2-Phenyl ethyl ethanol, acetate ester of isoamyl alcohol (isoamyl acetate), fatty acids such as hexanoic, octanoic and decanoic acids and their respective ethyl esters (ethyl hexanoate, ethyl octanoate, ethyl decanoate). All of the three thiols 4MMP, 3MH and 3MHA were also important in characterising the aroma profile of the wines. The average concentration of aroma compounds found in these ferment is shown in table 4.7 and 4.8.

Aroma grams were also constructed for wine from each category based on the yeast species, temperature and YAN used to visualize differences between the overall aromas of these wines (figure 4.9).

Comparisons between the two ferment were made by testing the null hypothesis that there is no difference in aroma profile between Marlborough Sauvignon Blanc made using *S. bayanus* SBJ1d and *S. cerevisiae* VL3 under the oenological conditions described in the full-factorial experiment.
<table>
<thead>
<tr>
<th>Aroma compound</th>
<th>Descriptor</th>
<th>OTH</th>
<th>12.8 Y1</th>
<th>12.8 Y2</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td>sb</td>
<td>vl3</td>
<td>sb</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mg/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Average (n=6)</td>
<td>S.E.M</td>
<td>OAV</td>
<td>Average (n=6)</td>
</tr>
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<td>Isobutanol</td>
<td>Fusel, alcohol</td>
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<td>51.67</td>
<td>1.33</td>
</tr>
<tr>
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Table 4.7: Volatile composition of Marlborough Sauvignon Blanc made using *S. bayanus* and *S. cerevisiae* VL3 (VL3) at temperature of 12.8°C and nitrogen conditions Y1 (390 mg/L) and Y2 (1170 mg/L). OTH: Odour threshold; S.E.M standard error of mean, OAV: Odour Activity Value; P-value: Student’s t-test P-value (fdr corrected) comparing between sb and vl3 within Y1and Y2. P-values marked in Green indicate at least 2-fold increase in concentration of aroma in sb ferments, while those marked in Red indicate at least 2-fold increase in concentration of aroma in vl3.
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</table>

Table 4.8: Volatile composition of Marlborough Sauvignon Blanc made using S. bayanus and S. cerevisiae VL3 (VL3) at temperature of 20°C and nitrogen conditions Y1 (390 mg/L) and Y2 (1170 mg/L). OTH: Odour threshold; S.E.M: Standard error of mean; OAV: Odour Activity Value; P-value: Student’s t-test P-value (fdr corrected) comparing between sb and v13 within Y1 and Y2. P-values marked in Green indicate at least 2-fold increase in concentration of aroma in sb ferments, while those marked in Red indicate at least 2-fold increase in concentration of aroma in v13.
Figure 4.9: Illustration of differences in aroma profile of Marlborough Sauvignon Blanc made using *S. bayanus* (-) and *S. cerevisiae* VL3 (-) at 12.8°C Y1 (a) and Y2 (b) and 20°C Y1 (c) and Y2 (d) nitrogen conditions. The aroma gram represents all aromas studied with Y-axis indicating the intensity of the aroma based on Log10 conversion of its OAV, where an increase by one unit indicates an actual 10-fold increase in aroma concentration.
It can be observed that *S. bayanus* inoculated wines contained significantly higher levels (greater than two fold) of PEE, BPEA and 4MMP compared to those with VL3 (refer table 4.7 and 4.8 for concentration and Pairwise t-test values). Addition of YAN and increasing the fermentation temperature from 12.8°C to 20°C had mixed impact on their concentration. On the other hand, isobutyl alcohol was present in significantly higher quantities in wines made using VL3 compared to *S. bayanus* but only when fermentation was carried out at 12.8°C. Addition of YAN and increasing temperature significantly decreased the impact of this compound on the wine profiles made using VL3. These reports are similar to other studies where increasing nitrogen in the grape must (pinot noir and chardonnay) was attributed to lower levels of higher alcohols (Maigre, 2002; Torrea *et al*., 2011).

Another study compared the effects of two different yeast strains and various fermentation temperatures revealing elevated fusel alcohol levels for both strains at lower fermentation temperatures (Girard *et al*., 2001). Other studies in Canada and France noted a similar trend (Girard *et al*., 1997; Massoutier *et al*., 1998). These results contradict earlier statements of increased fusel alcohol levels with higher fermentation temperatures (Dubourdieu *et al*., 2006; Jackson, 2000).

Isoamyl acetate had a very significant impact on VL3 wines fermented at 12.8°C compared to *S. bayanus* wines at the same temperature but this impact diminished by a 100 fold when fermentation was carried out at 20°C. On the other hand the levels of isoamyl acetate while lower in *S. bayanus* were somewhat increased with higher fermentation temperatures. Addition of YAN had a minor negative impact.

Octanoic acid was present at really high levels in wines made using both *S. bayanus* and VL3 compared to other fatty acids. Wines made using VL3 had increased levels of this fatty acid compared to wine made using *S. bayanus* at 12.8°C, although this impact was diminished in VL3 wines fermented at higher temperature.

*S. bayanus* wines showed a high impact from ethyl octanoate compared to VL3 wines. The addition of nitrogen seemed to decrease this impact. However, increasing temperature significantly increased the impact of this compound in *S. bayanus* wines while decreased in impact in VL3 wines.

All of the three thiols were significantly higher in *S. bayanus* wines compared to wines made using VL3 irrespective of temperature or YAN. Interestingly, increasing temperature and YAN increased all the three thiols in wines made by *S. bayanus* wines while higher temperature resulted in complete loss in impact by 4MMP in VL3 wines although addition of YAN seemed to increase levels of 3MH. This seems to contradict the findings by Subileau *et al.* (2008) where they showed addition of DAP to Sauvignon Blanc juice reduced the levels of 3MH in the resulting wines. Therefore it is crucial to account for yeast strain and source of grape must to understand the global changes to thiol metabolism during fermentation.
Compounds such as Isobutanol, 2-phenyl ethyl alcohol, isoamyl acetate, octanoic acid, decanoic acid and ethyl hexanoate were present in higher concentration compared to South African Sauvignon Blans studied (Louw et al., 2010). However, compounds such as ethyl acetate, ethyl butyrate acetic acid and butyric acid had significant impact on South African Sauvignon Blanc were not assessed in this study.

A factorial (2 way) ANOVA (table 4.9) on the Sauvignon Blanc data showed that both temperature and YAN had significant impacts on the aroma profile of wine made using S. bayanus, with the exception of octanoic acid. However, temperature*YAN interaction had a significant impact on levels of Hexanoic acid, ethyl octanoate and ethyl decanoate.

Interestingly, wines made using VL3 were either impacted by YAN or temperature conditions. Notably there was a lack of significant impact of YAN on the levels of thiol, whereas temperature was a significant parameter in the levels of thiols produced by VL3 during fermentation with 3MH and 3MHA increasing with increasing temperature and a complete loss of 4MMP with increased fermentation temperature. This phenomenon could be due to two separate mechanisms being present to produce 4MMP and 3MH in S. cerevisiae or changes to pre-cursor preferences at different temperatures.
Table 4.9: Two-way ANOVA P-values indicating whether there are significant Temperature or YAN effects and a Temperature*YAN interaction among Marlborough Sauvignon Blanc made using S. bayanus (sb-only) and S. cerevisiae (vl3-only). All significant values are indicated in bold font. P-values denoted as 0 are very highly significant.

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<th>YAN</th>
<th>Temp * YAN</th>
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<th>Temperature</th>
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<td>Roses</td>
<td>0</td>
<td>0.001</td>
<td>0.918</td>
<td>0</td>
<td>0.75</td>
<td>0.074</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>PEE</td>
<td>Rose, honey, tobacco</td>
<td>0</td>
<td>0.061</td>
<td>0.368</td>
<td>0</td>
<td>0.989</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Thiols</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4MMP</td>
<td>Boxwood, cat's pee</td>
<td>0.001</td>
<td>0</td>
<td>0.893</td>
<td>0.647</td>
<td>0</td>
<td>0.647</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>3MHA</td>
<td>Passionfruit, grapefruit</td>
<td>0</td>
<td>0</td>
<td>0.092</td>
<td>0.399</td>
<td>0.023</td>
<td>0.001</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>3MH</td>
<td>Gooseberry, guava</td>
<td>0.013</td>
<td>0</td>
<td>0.672</td>
<td>0.327</td>
<td>0</td>
<td>0.026</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

In summary, it can be seen that S. bayanus (SBJ1d) only ferments were capable of delivering more complex fruity aromas in Marlborough Sauvignon Blanc compared to vl3-only ferments. Although S. bayanus ferments produced lower quantities of aromatic esters, differences in temperature of fermentation didn’t impact these levels. Whereas in vl3-only ferments, greater quantities of aromatic ester compounds were found at lower temperature, the advantage of these compounds were lost at higher temperature fermentations. Overall, S. bayanus only ferments produced more thiols especially 4MMP and phenyl ethyl ethanol (PEE) and beta phenyl ethyl acetate (BPEA) at both the temperatures levels compared to vl3-only ferments. Thus from the above data, the null hypothesis that there is no difference between the aroma profile of S. bayanus only and vl3-only ferments under controlled conditions can be disproved.
4.7.4 Comparison between Marlborough Sauvignon Blanc fermented using co-ferments

*S. bayanus* (SBJ1d) and *P. Kluyveri* (PKKR1) and *S. cerevisiae* VL3 and *P. kluyveri* (PKKR1)

Multiple studies have been carried out to monitor changes to the organoleptic properties of wine by using non-*Saccharomyces* yeast in conjunction with *S. cerevisiae* (Ciani *et al.*, 2010). Recently it was shown that co-inoculating Marlborough Sauvignon Blanc with *S. cerevisiae* VL3 along with an isolate of *P. kluyveri* (PKKR1) produced wines with higher 3MHA when compared to VL3 only wines (Anfang *et al.*, 2009). However, that study was carried out at a specific temperature and YAN. In this study the impact of both high (20\(^{\circ}\)C) and low (12.8\(^{\circ}\)C) temperature fermentation and two different initial YAN conditions Y1 and Y2 on aroma profiles of the wine were assessed.

In addition to VL3 and PKKR1 co-fermentation, co-fermentation between *S. bayanus* (SBJ1d) and PKKR1 was also carried out under similar oenological conditions. The aim of this study was to not only assess the impact of temperature and nitrogen on an established interaction between VL3 and PKKR1, but also to observe for changes in other aroma compounds. The interaction between *S. bayanus* and PKKR1 can help understand the similarities and differences between the two different *Saccharomyces* yeasts in co-ferments.

All the aroma compounds assessed for the co-ferments are shown in the tables below (4.10 and 4.11). The aroma grams (Figures 4.10) display the similarities and differences in aroma profiles of the two co-ferments.

Comparisons between the two co-ferments were made by testing the null hypothesis that there is no difference in aroma profile between Marlborough Sauvignon Blanc made using *S. bayanus* SBJ1d and PKKR1 and *S. cerevisiae* VL3 and PKKR1 under the oenological conditions described in the full-factorial experiment.
<table>
<thead>
<tr>
<th>Aroma compound Descriptor</th>
<th>OTH</th>
<th>12.8 y1</th>
<th>12.8 y2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>m/L</td>
<td>sbpkkr Average (n=6)</td>
<td>sbpkkr Average (n=6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SEM OAV</td>
<td>SEM OAV</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>isobutanol</td>
<td>30.00</td>
<td>103.75 3.46 3.46 54.18</td>
<td>111.70 2.80 3.72 3.82E-06</td>
</tr>
<tr>
<td>isobutyl acetate</td>
<td>1.60</td>
<td>0.36 0.22 0.23 0.14</td>
<td>0.44 0.28 0.41 0.26</td>
</tr>
<tr>
<td>iso-amyl alcohol</td>
<td>40.00</td>
<td>212.86 7.92 5.32 116.10</td>
<td>210.46 8.74 5.26 122.76</td>
</tr>
<tr>
<td>isoamyl acetate</td>
<td>0.03</td>
<td>127.83 3.26 426.16 66.44</td>
<td>140.35 3.85 4678.41 140.79</td>
</tr>
<tr>
<td>PEE</td>
<td>14.00</td>
<td>164.18 8.04 11.73 7.19</td>
<td>118.09 1.91 8.43 0.00</td>
</tr>
<tr>
<td>BPEA</td>
<td>0.25</td>
<td>0.07 0.00 0.29 0.03</td>
<td>0.06 0.00 0.23 0.00</td>
</tr>
<tr>
<td>hexanol</td>
<td>8.00</td>
<td>1.66 0.04 0.21 2.06</td>
<td>1.64 0.02 0.20 1.66</td>
</tr>
<tr>
<td>heptyl acetate</td>
<td>1.50</td>
<td>0.81 0.01 0.54 0.93</td>
<td>0.81 0.02 0.54 1.18</td>
</tr>
<tr>
<td>decanoic acid</td>
<td>1.00</td>
<td>2.34 0.08 2.34 2.17</td>
<td>2.68 0.09 2.68 1.82</td>
</tr>
<tr>
<td>ethyl decanoate</td>
<td>0.20</td>
<td>1.17 0.07 5.84 0.84</td>
<td>0.94 0.02 4.70 0.72</td>
</tr>
<tr>
<td>hexanoic acid</td>
<td>0.42</td>
<td>4.55 0.09 10.83 6.25</td>
<td>4.06 0.04 9.66 7.24</td>
</tr>
<tr>
<td>ethyl hexanoate</td>
<td>0.01</td>
<td>1.74 0.04 124.47 1.87</td>
<td>1.24 0.02 88.89 2.06</td>
</tr>
<tr>
<td>octanoic acid</td>
<td>0.50</td>
<td>1.96 0.04 3.91 1.91</td>
<td>2.12 0.06 4.24 1.83</td>
</tr>
<tr>
<td>ethyl octanoate</td>
<td>0.01</td>
<td>2.68 0.06 536.47 2.01</td>
<td>2.15 0.04 430.96 1.94</td>
</tr>
<tr>
<td>ethyl lactate</td>
<td>154.60</td>
<td>6.88 0.13 0.04 5.25 0.16 0.03 3.04E-05</td>
<td></td>
</tr>
<tr>
<td>Thiol</td>
<td>ng/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4mmf</td>
<td>0.80</td>
<td>220.32 20.71 275.40 36.48</td>
<td>103.16 6.78 128.94 29.67</td>
</tr>
<tr>
<td>3nm</td>
<td>60.00</td>
<td>3919.21 219.90 65.32 2195.75</td>
<td>3089.14 80.98 51.49 1295.41</td>
</tr>
<tr>
<td>3mha</td>
<td>4.00</td>
<td>188.02 4.63 47.01 170.60</td>
<td>198.61 1.96 49.65 174.55</td>
</tr>
</tbody>
</table>

Table 4.10: Volatile composition of Marlborough Sauvignon Blanc made using *S. bayanus* + *P. kluyveri* (sbpkkr) and VL3 + *P. kluyveri* (vl3pkkr) at temperature of 12.8°C and nitrogen conditions Y1 (390 mg/L) and Y2 (1170 mg/L). OTH: Odour threshold; SEM: Standard error of mean; OAV: Odour Activity Value; P-value: Student’s t test P-value (fdr corrected) comparing between sbpkkr and vl3pkkr within Y1 and Y2. P-values marked in Green indicate at least 2-fold increase in concentration of aroma in sbpkkr ferments, while those marked in Red indicate at least 2-fold increase in concentration of aroma in vl3pkkr.
<table>
<thead>
<tr>
<th>Aroma compound Descriptor</th>
<th>OTH</th>
<th>20 y1</th>
<th>20 y2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>sbpkr Average (n=6)</td>
<td>vL3pkr Average (n=6)</td>
</tr>
<tr>
<td></td>
<td>mg/L</td>
<td>SEM</td>
<td>OAV</td>
</tr>
<tr>
<td>isobutanol</td>
<td>Fusel, alcohol</td>
<td>30.00</td>
<td>87.51</td>
</tr>
<tr>
<td>isobutyl acetate</td>
<td>Fruit, apple banana</td>
<td>1.60</td>
<td>0.11</td>
</tr>
<tr>
<td>iso-amyl alcohol</td>
<td>Alcoholic, harsh</td>
<td>40.00</td>
<td>75.06</td>
</tr>
<tr>
<td>isoamyl acetate</td>
<td>Bananas</td>
<td>0.03</td>
<td>9.51</td>
</tr>
<tr>
<td>PEE boxwood</td>
<td>Roses</td>
<td>14.00</td>
<td>160.03</td>
</tr>
<tr>
<td>BPEA Rose, honey, tobacco</td>
<td>0.25</td>
<td>0.10</td>
<td>0.01</td>
</tr>
<tr>
<td>hexanol</td>
<td>Grassy</td>
<td>8.00</td>
<td>1.57</td>
</tr>
<tr>
<td>hexyl acetate</td>
<td>Apple, cherry, floral</td>
<td>1.50</td>
<td>0.97</td>
</tr>
<tr>
<td>decanoic acid</td>
<td>Rancid, fatty</td>
<td>1.00</td>
<td>3.25</td>
</tr>
<tr>
<td>ethyl decanoate</td>
<td>Grape, soapy, fruity</td>
<td>0.20</td>
<td>3.20</td>
</tr>
<tr>
<td>hexanoic acid</td>
<td>Sweat, cheesy</td>
<td>0.42</td>
<td>6.06</td>
</tr>
<tr>
<td>ethyl hexanoate</td>
<td>Green apple</td>
<td>0.01</td>
<td>2.00</td>
</tr>
<tr>
<td>octanoic acid</td>
<td>Rancid, harsh, sweaty</td>
<td>0.50</td>
<td>1.65</td>
</tr>
<tr>
<td>ethyl octanoate</td>
<td>Sweat, soapy, fruity</td>
<td>0.01</td>
<td>3.11</td>
</tr>
<tr>
<td>ethyl lactate</td>
<td>Lactic, buttery, fruity</td>
<td>154.60</td>
<td>6.80</td>
</tr>
<tr>
<td>Thiols</td>
<td>ng/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4mmp</td>
<td>Boxwood, cat's pee</td>
<td>0.80</td>
<td>134.47</td>
</tr>
<tr>
<td>3mh</td>
<td>Gooseberry, guava</td>
<td>60.00</td>
<td>2701.07</td>
</tr>
<tr>
<td>3mha</td>
<td>Passionfruit, grapefruit</td>
<td>4.00</td>
<td>220.84</td>
</tr>
</tbody>
</table>

Table 4.11: Volatile composition of Marlborough Sauvignon Blanc made using S. bayanus + P. kluyveri (sbpkr) and VL3 + P. kluyveri (vL3pkr) at temperature of 20°C and nitrogen conditions Y1 (390 mg/L) and Y2 (1170 mg/L). OTH: Odour threshold; SEM: Standard error of mean; OAV: Odour Activity Value; P-value: Student’s t-test P-value (fdr corrected) comparing between sbpkr and vL3pkr within Y1 and Y2. P-values marked in Green indicate at least 2-fold increase in concentration of aroma in sbpkr ferments, while those marked in Red indicate at least 2-fold increase in concentration of aroma in vL3pkr.
Figure 4.10: Illustration of differences in aroma profile of Marlborough Sauvignon Blanc made using sbpkkr (--) and vl3pkkk (—) at 12.8°C Y1 (a) and Y2 (b) and 20°C Y1 (c) and Y2 (d) nitrogen conditions. The aroma gram represents all aromas studied with Y-axis indicating the intensity of the aroma based on Log10 conversion of its OAV, where an increase by one unit indicates an actual 10-fold increase in aroma concentration.
Among the higher alcohols analysed, 2-phenyl-ethanol is present only in *S. bayanus* and PKKR co-ferment at OAV > 1. The impact of this compound decreases with increased temperature of the fermentation (20°C), and addition of YAN seems to have a negative impact on the level of Phenyl-ethanol (PEE). Isobutanol and Isoamyl alcohol are significantly higher in both sbpkkr and vl3pkkr at 12.8°C compared to their OAV at 20°C (refer to table 4.10 and 4.11 for all the aroma compounds with at least 2-fold difference between the two co-ferments). These data again contradict popular opinions which suggest increasing temperature to increase levels of higher alcohols (Gil *et al.*, 1996; Lambrechts and Pretorius, 2000).

Addition of nitrogen has mixed impact on values of these compounds in vl3pkkr and sbpkkr ferments (refer tables 4.10 and 4.11 for aroma concentration and Pairwise t-test values). Addition of YAN at lower temperatures seemed to increase concentration of higher alcohols in vl3pkkr while decrease their concentration in sbpkkr. The increase in YAN at 20°C seemed to have negative impact on both co-ferments. This is similar to reports on chardonnay (*S. cerevisiae* only ferments) from AWRI which was fermented at 18°C (Torrea *et al.*, 2011). However, it shows that temperature of fermentation is an important factor to consider when studying the impact of YAN on aroma.

The sbpkkr co-ferments had more impact from all of the varietal thiols compared to vl3pkkr co-ferments on the overall aroma profile (figure 4.10). The highest levels of 4MMP and 3MH were found in wines made at 12.8°C and Y1 conditions (table 4.10). The addition of nitrogen seemed to have a negative impact at lower temperatures but 4MMP-values were elevated at Y2 conditions compared to Y1 for wines made at 20°C (table 4.11). In addition there was a 3.62 fold increase in 4MMP-values in sbpkkr ferments compared to sb-only ferments (P-value < 2.12 e^{-16}) indicating a potential interaction between the two species.

On the other hand, vl3pkkr co-ferments produced lower amounts of thiols 3MH and 3MHA when fermentation was carried out at 12.8°C compared to 20°C. However, vl3pkkr co-ferments were incapable of producing 4MMP at a higher temperature. These results agree with the study carried out on French Sauvignon Blanc where they observed higher levels of thiols to be formed at higher fermentation temperature (Masneuf-Pomarède *et al.*, 2010).

There also seemed to be little or no difference between the amounts of 3MHA produced between the vl3- only and vl3pkkr co-ferments under the oenological conditions (three-way ANOVA with factors; temperature P-value = 0.042, YAN P-value = 0.62 and yeast species P-value = 0.42) tested. It could be because the elevation of 3MHA levels in co-ferments is noticeable only at specific temperatures (14.5°C) (Anfang *et al.*, 2009). Thus it was shown that oenological conditions play an important role in expression of 3MHA in vl3pkkr co-ferment.
Observing the single and co-ferment two-way ANOVA data (table 4.12), it can be seen that oenological conditions have different impact on levels of 4MMP compared to level of thiols 3MH and 3MHA (irrespective of the yeast species). *S. bayanus* ferments (single or co-ferments) seem to have a significant (P-values < 0.05) ability to produce 4MMP and 3MH compared to vl3 ferments, and seem less impacted by temperature and nitrogen conditions. This observation can be related to findings on the IRC 7 gene, where nitrogen had little impact compared to the yeast strain (Roncoroni *et al.*, 2011).

### Table 4.12: Two-way ANOVA P-values indicating whether there are significant Temperature or YAN effects and a Temperature*YAN interaction among Marlborough Sauvignon Blanc made using sbpkkr and vl3pkkr. All significant values are indicated in bold font. P-values denoted as 0 are very highly significant.

<table>
<thead>
<tr>
<th>Aroma compound</th>
<th>Descriptor</th>
<th>sbpkkr</th>
<th>vl3pkkr</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Esters</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>isobutyl acetate</td>
<td>Fruit, apple banana</td>
<td>0.017</td>
<td>0.294</td>
</tr>
<tr>
<td>hexanoic acid</td>
<td>Sweat, cheesy</td>
<td>0.001</td>
<td>0.002</td>
</tr>
<tr>
<td>octanoic acid</td>
<td>Rancid, harsh, sweaty</td>
<td>0.551</td>
<td>0.001</td>
</tr>
<tr>
<td>decanoic acid</td>
<td>Rancid, fatty</td>
<td>0.882</td>
<td>0.039</td>
</tr>
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<td>ethyl hexanoate</td>
<td>Green apple</td>
<td>0.342</td>
<td>0.716</td>
</tr>
<tr>
<td>ethyl octanoate</td>
<td>Scent, soapy, fruity</td>
<td>0.001</td>
<td>0.002</td>
</tr>
<tr>
<td>ethyl decanoate</td>
<td>Grape, soapy, fruity</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>isobutanol</td>
<td>Fused, alcohol</td>
<td>0.107</td>
<td>0.058</td>
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<tr>
<td>hexyl acetate</td>
<td>Apple, cherry, floral</td>
<td>0.133</td>
<td>0.015</td>
</tr>
<tr>
<td>hexanol</td>
<td>Grassy</td>
<td>0.01</td>
<td>0.092</td>
</tr>
<tr>
<td>isoamyl acetate</td>
<td>Bananas</td>
<td>0.101</td>
<td>0.054</td>
</tr>
<tr>
<td>iso-amyl alcohol</td>
<td>Alcoholic, harsh</td>
<td>0.808</td>
<td>0.321</td>
</tr>
<tr>
<td>ethyl lactate</td>
<td>Lactic, buttery, fruity</td>
<td>0.02</td>
<td>0.444</td>
</tr>
<tr>
<td>PEE</td>
<td>Roses</td>
<td>0</td>
<td>0.606</td>
</tr>
<tr>
<td>BPEA</td>
<td>Rose, honey, tobacco</td>
<td>0.002</td>
<td>0.014</td>
</tr>
</tbody>
</table>

| Thiols         |            |        |         |
| 4MMP           | Boxwood, cat's pee | 0    | 0.294  |
| 3MHA           | Passionfruit, grapefruit | 0.95  | 0.003  |
| 3MH            | Gooseberry, guava | 0    | 0.049  |

In summary, sbpkkr wines have significantly more aroma levels compared to vl3pkkr ferments, especially, levels of higher alcohols, which are the next most important set of aroma compounds after thiols (especially 4MMP) for these co-fermented wines. There was a 100-fold increase in isoamyl acetate and 3.62 fold increase in 4MMP-values in sbpkkr compared to sb-only ferment suggesting a synergistic interaction between *S. bayanus* SBJ1d and *P. kluyveri* PKKR1. Also, *S. bayanus* and VL3 have different relationship with PKKR1 resulting in different aroma compounds being impacted by these unique interactions. Furthermore, there was no significant increase in 3MHA levels in vl3pkkr co-ferment compared to vl3-only ferments irrespective of the oenological conditions.
4.7.5 Comparisons between Marlborough Sauvignon Blanc fermented using *S. bayanus* (SBJ1d) and PKKR1 with co-ferments of *S. bayanus* (SBJ1d) and *P. anomala* (sbpa) and *S. bayanus* and *T. delbrueckii* (sbtd)

Aroma profiles from the previous section (4.7.3.2) indicated that the non-*Saccharomyces* yeast PKKR1 interacted differently with *S. bayanus* and *S. cerevisiae*, resulting in wines with unique character. Recent studies using *T. delbrueckii* in mixed and sequential cultures with *S. cerevisiae* has shown that together, these yeasts are capable of reducing volatile acidity (Ciani et al., 2010). One of the earliest studies recommended the usage of *T. delbrueckii* to reduce acetic acid content in wines (Bely et al., 2008; Castelli, 1955). The complex nature of mixed and multi-starter cultures of *S. cerevisiae* and *T. delbrueckii* were studied by Herraiz et al. (1990), where the analysis of volatile composition showed marked difference in aroma profiles of *S. cerevisiae* only and mixed fermentations. These and other studies have led to a positive industry contribution through the development of Vinflora®, an active dried yeast with a proprietary blend of *S. cerevisiae*, *T. delbrueckii* and *K. thermotolerans* (Ciani et al., 2006; Zironi et al., 1993).

Based on the above mentioned studies it would be interesting to understand the interactions of *S. bayanus* (SBJ1d) with other non-*Saccharomyces* yeast isolated from 2008 Kumeu River Marlborough Sauvignon Blanc. Thus co-ferments using the two non-*Saccharomyces* yeast from 2008 Kumeu River Sauvignon Blanc and *S. bayanus* (SBJ1d) could provide information regarding the interaction of yeast during un-inoculated ferment. These co-ferments could be a viable option for producing unique Marlborough Sauvignon Blanc.

In this section, the various aroma profiles of the different co-ferments using non-*Saccharomyces* yeast with *S. bayanus* (SBJ1d) are compared to the single ferment with *S. bayanus* (SBJ1d) by testing the null hypothesis that there is no difference in the aroma profiles between the mono and co-ferments of *S. bayanus* (SBJ1d) with selected non-*Saccharomyces* yeast under the oenological conditions defined in the full-factorial experiment. Student’s t-tests were carried out between biological replicates of *S. bayanus* only inoculated ferment and different co-ferments under various oenological conditions (Table 4.13, 4.14, 4.15 and 4.16)
<table>
<thead>
<tr>
<th>Aroma compound</th>
<th>Descriptor</th>
<th>OTH</th>
<th>12.8 Y1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/L</td>
<td>sb</td>
<td>SEM</td>
</tr>
<tr>
<td>isobutanol</td>
<td>Fusel, alcohol</td>
<td>30.00</td>
<td>5.67</td>
</tr>
<tr>
<td>isobutyl acetate</td>
<td>Fruit, apple banana</td>
<td>1.60</td>
<td>0.05</td>
</tr>
<tr>
<td>iso-amyl alcohol</td>
<td>Alcoholic, harsh</td>
<td>40.00</td>
<td>7.32</td>
</tr>
<tr>
<td>isoamyl acetate</td>
<td>Bananas</td>
<td>0.03</td>
<td>0.96</td>
</tr>
<tr>
<td>PEE</td>
<td>Roses</td>
<td>14.00</td>
<td>118.27</td>
</tr>
<tr>
<td>BPEA</td>
<td>Rose, honey, tobacco</td>
<td>0.25</td>
<td>0.07</td>
</tr>
<tr>
<td>hexanol</td>
<td>Grassy</td>
<td>8.00</td>
<td>1.68</td>
</tr>
<tr>
<td>heptyl acetate</td>
<td>Apple, cherry, floral</td>
<td>1.50</td>
<td>0.72</td>
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<td>Grape, soapy, fruity</td>
<td>0.20</td>
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Table 4.13: Volatile composition of Marlborough Sauvignon Blanc made using *S. bayanus*-only (sb), *S. bayanus + P. kluyveri* (sbpkk), *S. bayanus + P. anomala* (sbpa) and *S. bayanus + T. delbrueckii* (sbtd) at temperature of 12.8°C and nitrogen condition Y1 (390 mg/L). OTH: Odour threshold; SEM: Standard error of mean; OAV: Odour Activity Value; P-value: Student’s t-test P-value (fdr corrected) comparing between *S. bayanus* only with sbpkk, sbpa and sbtd. P-values marked in Green indicate at least 2-fold increase in concentration of aroma in sb ferments, while those marked in Red indicate at least 2-fold increase in concentration of aroma in sbpkk, sbpa or sbtd respectively.
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<td>P-value</td>
<td>Average (n=6)</td>
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Table 4.14: Volatile composition of Marlborough Sauvignon Blanc made using *S. bayanus*-only (sb), *S. bayanus*+ *P. kluyveri* (sbpkkr), *S. bayanus* + *P. anomala* (sbpa) and *S. bayanus* + *T. delbrueckii* (sbdtd) at temperature of 12.8°C and nitrogen condition Y2(1170 mg/L). OTH: Odour threshold; SEM: Standard error of mean; OAV: Odour Activity Value; P-value: Student’s t-test P-value (fdr corrected) comparing between *S. bayanus*-only with sbpkkr, sbpa and sbdtd. P-values marked in Green indicate at least 2-fold increase in concentration of aroma in sb ferments, while those marked in Red indicate at least 2-fold increase in concentration of aroma in sbpkkr, sbpa or sbdtd respectively.
Table 4.15: Volatile composition of Marlborough Sauvignon Blanc made using *S. bayanus*-only (sb), *S. bayanus + P. kluyveri* (sbpkkr), *S. bayanus + P. anomala* (sbpa) and *S. bayanus + T. delbrueckii* (sbtnd) at temperature of 20°C and nitrogen condition Y1(390 mg/L). OTH: Odour threshold; SEM: Standard error of mean; OAV: Odour Activity Value; P-value: Student’s t-test P-value (fdr corrected) comparing between *S. bayanus* only (sb) with sbpkkr, sbpa and sbtd. P-values marked in Green indicate at least 2-fold increase in concentration of aroma in sb ferment, while those marked in Red indicate at least 2-fold increase in concentration of aroma in sbpkkr, sbpa or sbtd respectively.
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<th>Aroma compound</th>
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<th>OAV</th>
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<th>SEM</th>
<th>OAV</th>
<th>T test</th>
<th>Sbtd Average (n=6)</th>
<th>SEM</th>
<th>OAV</th>
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<td>0.06</td>
<td>4.31E-01</td>
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<td>0.00</td>
<td>0.07</td>
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<td>Lactic, buttery, fruity</td>
<td>154.60</td>
<td>5.91</td>
<td>0.21</td>
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<td>3.24E-02</td>
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<tr>
<td>4mmp</td>
<td>Boxwood, cat's pee</td>
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<td>131.95</td>
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<td>7.41</td>
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<td>3794.46</td>
<td>279.48</td>
<td>63.24</td>
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<td>3mha</td>
<td>Passionfruit, grapefruit</td>
<td>4.00</td>
<td>254.35</td>
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<td>63.59</td>
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<td>2.73E-05</td>
<td>227.88</td>
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Table 4.16: Volatile composition of Marlborough Sauvignon Blanc made using S. bayanus-only (sb), S. bayanus+ P. kluyveri (sbpkk), S. bayanus + P. anomala (sbpa) and S. bayanus+ T. delbrueckii (sbd) at temperature of 20°C and nitrogen condition Y2 (1170 mg/L). OTH: Odour threshold; SEM: Standard error of mean; OAV: Odour Activity Value; P-value: Student’s t-test P-value (fdr corrected) comparing between S. bayanus-only with sbpkk, sbpa and sbtd. P-values marked in Green indicate at least 2-fold increase in concentration of aroma in sb ferments, while those marked in Red indicate at least 2-fold increase in concentration of aroma in either sbpkk, sbpa or sbtd respectively (in this instance none of the compounds had a 2-fold change between different ferments).
Figure 4.11: Illustration of differences in aroma profile of Marlborough Sauvignon Blanc when comparing \textit{S. bayanus}–only (sb) in bold (–) and sbpkkr (-----), sbpa (----) and sbtd (-----) in dotted lines at (a) 12.8°C: Y1, (b) 12.8°C: Y2, (c) 20°C: Y1 and (d) 20°C: Y2 conditions. The aroma gram represents all aromas studied with Y-axis indicating the intensity of the aroma based on Log10 conversion of its OAV, where an increase of 1 unit corresponds to a 10-fold increase in aroma concentration.
Observing the aroma profiles of co-fermented wines at 12.8°C and Y1 conditions, it can be seen that both sbpkkr and sbtd ferments showed a greater than 2-fold increase in higher alcohols (isobutanol, and isoamyl alcohol) along with isoamyl acetate and the thiol 4MMP compared to S. bayanus-only ferments (figure 4.11 and table 4.13). The concentrations of higher alcohols isobutanol and isoamyl alcohol and their acetates were too low to be detected in sbpa co-ferments at 12.8°C and Y1 condition. However, 4MMP levels in sbpa were greater than sb-only ferments. Addition of extra nitrogen to juice (12.8°C and Y2 condition) had the same impact on all the three co-ferments; it reduced the levels of thiols thus making their OAV similar to that seen in S. bayanus-only ferments. However, the higher alcohols and isoamyl acetate were still significantly higher (greater than 2-fold) in co-ferments compared to S. bayanus only ferments (table 4.14). In addition it can be seen that co-fermented wine with T. delbrueckii had more impact from ethyl decanoate compared to the S. bayanus only ferments adding to the fruitiness of the wine.

Increasing the temperature of the fermentation (20°C instead of 12.8 °C, at Y1) led to a loss of difference in the aroma profiles of single and co-ferments (table 4.15). Addition of YAN (Y2 instead of Y1 at 20 °C) at higher temperature led to an overall decrease in aroma content and was similar to the impact it had on S. bayanus only ferment (table 4.16). The levels of 3MH were lower in co-ferments with PKKR and P. anomala compared to S. bayanus-only ferments. On the other hand, co-ferments with T. delbrueckii showed a decrease in impact from ethyl esters of fatty acids compared to S. bayanus-only ferments.
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<th>Aroma compound</th>
<th>descriptor</th>
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<th>YAN</th>
<th>Temperature</th>
<th>Temp * YAN</th>
<th>Sbpkkr</th>
<th>YAN</th>
<th>Temperature</th>
<th>Temp * YAN</th>
<th>sbpa</th>
<th>YAN</th>
<th>Temperature</th>
<th>Temp * YAN</th>
<th>sbsd</th>
<th>Temperature</th>
<th>Temp * YAN</th>
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<td>Fruit, apple banana</td>
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<td>0.267</td>
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<tr>
<td>hexanoic acid</td>
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<td>0.008</td>
<td>0.001</td>
<td>0</td>
<td>0.001</td>
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<tr>
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<td>0.002</td>
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<td>0</td>
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<td>BPEA</td>
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Table 4.17: Two-way ANOVA P-values indicating whether there are significant Temperature or YAN effects and a Temperature*YAN interaction among Marlborough Sauvignon Blanc made using S. bayanus-only (sb), S. bayanus + P. kluyveri (sbpkkr), S. bayanus + P. anomala (sbpa) and S. bayanus + T. delbrueckii (sbsd). All significant values are indicated in bold font. P-values denoted as 0 are very highly significant.
Non-Saccharomyces yeast PKKR1 and T. delbrueckii yeast used in co-ferments behaved almost identically. However, P. anomala isolate selected for this study was unable to produce higher alcohols isobutanol and isoamyl alcohol and their acetates at 12.8°CY1 apart from that, though there were no major differences in their aroma profiles, some of the aromas were slightly significantly different when comparing the different co-fermentations (table 4.17). A two-fold difference in isobutanol, isoamyl alcohol, isoamyl acetate and 4MMP was observed between co-ferments sbpkkr and sbtd at 12.8°CY1 condition. Addition of DAP (Y2 juice) and increasing temperature (20°C) diminished the difference between co-ferments and S. bayanus-only ferments.

Thus in summary, the null hypothesis that there is no difference between S. bayanus-only ferment and the three different S. bayanus co-ferments is disproved under the given test conditions. These aroma profiles agree with other studies showing that lower temperature is able to sustain non-Saccharomyces yeast for longer periods during fermentation and increase aroma potential of the resulting wines (Erten, 2002; Mendoza et al., 2007; Viana et al., 2008). However, impact of YAN on non-Saccharomyces yeast is not well understood (Vilanova et al., 2007).

One factor that was not considered during these studies (hence excluded from experimental design) was the possibility of impact on aroma profiles of co-ferments based on different non-Saccharomyces to Saccharomyces yeast initial inoculum ratios. In a study, the ratio of non-Saccharomyces to Saccharomyces yeast used to inoculate grape must had a major influence on the aroma profile of the resulting wine (Ciani and Comitini, 2011). However, this study was carried out on synthetic media at high temperature of 25°C and is yet to be tested in wine (Ciani and Comitini, 2011). Interestingly, oenological conditions of YAN and temperature seemed to impact different co-ferments uniquely, suggesting at the underlying mechanisms as being unique and complex (table 4.17).
4.7.6 Impact of oenological conditions on acetylation ratio

Volatile esters are largely responsible for the fruity character of a wine (Swiegers et al., 2005). They are produced during fermentation due to the activity of yeast. The concentration of these aromas is dependent on a host of factors including but not limited to yeast strain, temperature, pH, grape variety etc. (Styger et al., 2011) Availability of certain amino acids (Leucine, Isoleucine, Valine etc.) during fermentation is an important criterion for the production of aromatic esters (Rojas et al., 2003; Styger et al., 2011). Synthesis of esters by various strains of S. cerevisiae and non-Saccharomyces yeast has been studied by many researchers (Rojas et al., 2001; Swiegers et al., 2009). Swiegers et al. (2009) noted that different S. cerevisiae had differing capacity to convert 3MH to 3MHA. Notably, formation of acetate esters is catalysed by alcohol acetyltransferases (Atf1p and Atf2p); especially isoamyl alcohol acetyltransferase is involved in the conversion of isoamyl alcohol (IAA) to isoamyl acetate (IAAc) and alcohol acetyl transferase ATF1 is involved in converting 3MH into 3MHA (Rojas et al., 2003; Styger et al., 2011; Swiegers et al., 2009). Also, formation of ethyl esters (PEE) two acyl-CoA: ethanol O-acyltransferase enzymes (Eeb1p and Eht1p) (Styger et al., 2011).

In this study, the ratio of conversion (capacity) of IAA to IAAc, 3MH to 3MHA and PEE to BPEA was different for different yeast under different oenological conditions (table 4.18). Several interesting observations were made. Notably, there is a 100-fold increase in the level of isoamyl acetate in all of ferments (except sbpa) when compared to S. bayanus-only (sb) ferments, however, high concentrations of isoamyl acetate is not necessarily desired in wines. This result contradicts report by Rojas et al. (2001) which identified P. anomala to be good producer of Isoamyl acetate and BPEA. This was due to lack of data for isoamyl alcohol and isoamyl acetate for sbpa at 12.8°C Y1. However, P. anomala seems to be a good producer of Isoamyl acetate at 12.8°C Y2. Also observed is a significant decrease in the acetylation of isoamyl alcohol into isoamyl acetate in all the ferments (except sb) with the increase in ferment temperature irrespective of YAN (Y1 or Y2). The difference in acetylation ratio of IAAc: IAA between S. bayanus (SBJ1d) and its co-ferments suggests at a possible interaction between the Saccharomyces and non-Saccharomyces yeast in these ferments. Also, S. cerevisiae-only ferments have a higher ratio of conversion compared to S. bayanus-only ferments; thus suggesting differences in the genetic ability of these two yeasts. Although this difference could be strain specific.

Phenyl ethyl ethanol (PEE) and BPEA were significant in discriminating between ferments of S. bayanus and S. cerevisiae. In general, S. bayanus-only ferments and its co-ferments were able to produce higher concentrations of these esters compared to VL3 and VL3+PKKR1 ferments. However, interestingly, the conversion of PEE to BPEA in VL3-only ferment was 5-10 fold higher compared to
*S. bayanus*-only ferments under different oenological conditions. Thus, suggesting a complex mechanism of aroma production during fermentation.

The difference in conversion of 3MH to 3MHA in the various ferments was complex. Firstly, *S. bayanus*-only ferment had a significantly higher rate of conversion at lower temperature (12.8°C Y1 and Y2) compared to its co-ferments with non-*Saccharomyces* yeast (although not a 2 fold change). However, increase in temperature (20°C Y1 andY2) reversed these ratios wherein, co-ferments were capable of a higher rate of conversion of 3MH to 3MHA. More importantly, *S. cerevisiae*-only ferments had a greater conversion of 3MH to 3MHA compared to *S. bayanus*-only ferments irrespective of oenological condition of the ferments. These differences in conversion capacity highlight the strain differences. Interestingly, there was no significant difference between the conversion rates of 3MH to 3MHA in VL3-only and VL3-PKKR1 co-ferments under most oenological conditions (except at 12.8°C Y2). Therefore, the synergistic interaction observed by Anfang *et al.* (2009) is not universal and it can be concluded that this interaction is dependent on oenological conditions of fermentation.
<table>
<thead>
<tr>
<th>Ratio</th>
<th>Ferment</th>
<th>sb (%) (n=6)</th>
<th>SEM (%)</th>
<th>sbpkkr (%) (n=6)</th>
<th>SEM (%)</th>
<th>P-value</th>
<th>sbpa (%) (n=6)</th>
<th>SEM (%)</th>
<th>P-value</th>
<th>v13 (%) (n=6)</th>
<th>SEM (%)</th>
<th>P-value</th>
<th>vl3pkkr (%) (n=6)</th>
<th>SEM (%)</th>
<th>P-value</th>
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<td>NA</td>
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<td>0.25</td>
</tr>
<tr>
<td>3MHA</td>
<td>12.8°C Y1</td>
<td>7.03</td>
<td>0.23</td>
<td>4.86</td>
<td>0.25</td>
<td>8.0E-05</td>
<td>5.18</td>
<td>0.12</td>
<td>0.0001</td>
<td>4.42</td>
<td>0.09</td>
<td>2.6E-05</td>
<td>8.01</td>
<td>0.20</td>
<td>0.008</td>
</tr>
<tr>
<td></td>
<td>12.8°C Y2</td>
<td>6.13</td>
<td>0.17</td>
<td>6.45</td>
<td>0.14</td>
<td>0.18</td>
<td>5.55</td>
<td>0.15</td>
<td>0.02</td>
<td>5.07</td>
<td>0.08</td>
<td>0.0009</td>
<td>8.56</td>
<td>0.20</td>
<td>3.6E-06</td>
</tr>
<tr>
<td></td>
<td>20°C Y1</td>
<td>6.10</td>
<td>0.38</td>
<td>8.20</td>
<td>0.21</td>
<td>0.001</td>
<td>0.07</td>
<td>0.00</td>
<td>0.01</td>
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<td>8.54</td>
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<td>0.86</td>
<td>6.87</td>
<td>0.18</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Table 4.18: Acetylation ratios for the various mono and co-ferments from the full-factorial experiment. Acetylation ratios of conversion of IAA to IAAc, PEE to BPEA and 3MH to 3MHA were calculated for *S. bayanus*-only (sb), *S. bayanus* *P. kluyveri* (PKKR1) (sbpkkr), *S. bayanus* + *P. anomala* (sbpa), *S. bayanus* + *T. delbrueckii* (sbd), *S. cerevisiae* (VL3) –only (vl3) and *S. cerevisiae* (VL3) + *P. kluyveri* (PKKR1) (vl3pkkr) ferments at different oenological conditions. P-value\(^1\) shows P-values for *S. bayanus*-only ferment when compared to various other ferments. P-value\(^2\) shows the values from comparison between VL3-only and VL3-PKKR1 co-ferment, whereas P-value\(^3\) shows the comparison between sbpkkr and vl3pkkr at different conditions. All P-values were corrected for fdr. NA indicates lack of data.
4.8 Conclusion

Observing the aroma profile of ferments from the full-factorial experiment it was determined that there was a significant interaction between all the three factors for all of the aromas (table 4.5). Thus, a two-dimensional PCA was utilised to further discern the trends observed at 12.8°C Y1, 12.8°C Y2, 20°C Y1 and 20°C Y2 conditions. Furthermore, three trends observed in PCA were further analysed, they were; overall comparison between \textit{S. bayanus}-only and \textit{S. cerevisiae}-only ferments, overall comparison between \textit{S. bayanus} co-ferments with PKKR1 and VL3-PKKR1 co-ferment and overall comparison between the different \textit{S. bayanus} co-ferments with non-\textit{Saccharomyces} yeast.

From the above comparisons it was found that \textit{S. bayanus} (SBJ1d) ferments were capable of producing unique aroma profiles in Marlborough Sauvignon Blanc compared to commercial \textit{S. cerevisiae} VL3 ferments. The unique aroma profiles of \textit{S. bayanus} (SBJ1d) can be attributed to higher levels of 4MMP and higher alcohols, especially phenyl ethanol and its acetate. These results seem to comply with previous studies comparing aroma of French Sauvignon Blanc (Dubourdieu et al., 2006; Masneuf-Pomarède et al., 2006). However, levels of 2 phenyl ethyl acetate were lower in Marlborough Sauvignon Blanc made using \textit{S. bayanus} SBJ1d compared to French Sauvignon Blanc (Dubourdieu et al., 2006; Masneuf-Pomarède et al., 2006). It implies that there are many subtle factors contributing to the release of aroma from juices such as their origin from different geographical locations.

It is important to consider that this study was done on juice obtained from a single vintage and that resultant aroma profiles could have been influenced significantly by the character of the source juice. Also aroma profiles of \textit{S. cerevisiae} and \textit{S. bayanus} ferments are similar to those observed in Australian studies, although esters seem to have more impact when compared to thiols on the final aroma of Sauvignon Blanc from Australia (King et al., 2008).

According to this study, aroma compounds analysed had an increased impact with increasing temperature of fermentation, but impact of added nitrogen on aroma concentration was more complex and was dependant on the temperature of fermentation.

\textit{S. bayanus} co-ferments with PKKR1 were quantified as having more aroma compared to vl3pkkr co-ferment. Notably, presence of PKKR1 increased the levels of isoamyl acetate by increasing the acetylation ratio in the resulting wines compared to single ferments, an increase in thiols 3MH and 4MMP were also observed.

The non-\textit{Saccharomyces} yeast all had similar impact (except sbpa at 12.8°CY1) on aroma profile of the \textit{S. bayanus} co-ferment wines. A two-fold increase in higher alcohols and 4MMP in co-ferments
(sbpkkr, sbtd and sbpa) compared to *S. bayanus*-only ferments are a potential indicator of interaction between *S. bayanus* (SBJ1d) and the selected non-*Saccharomyces* yeast. Co-ferments at lower temperatures with *S. bayanus* and the selected non-*Saccharomyces* yeast isolates from 2008 Kumeu River Sauvignon Blanc at an initial inoculum ratio of 1:9 (*S. bayanus*: non-yeast) is a viable option for producing unique aroma profiles in Marlborough Sauvignon Blanc.

Thus in the future, co-ferments are best conducted at lower temperatures to have a positive impact on the aroma profile of Marlborough Sauvignon Blanc. More importantly, co-ferments with *S. bayanus* at lower temperatures can potentially increase the impact of other aroma compounds while maintaining high levels of thiols, as observed in high temperature *S. bayanus* only ferments. The overall evaluation of the impact of different oenological conditions on New Zealand Sauvignon Blanc is discussed in the general conclusions chapter (section 6.2).
Analysis of interaction between *S. cerevisiae* VL3 and *P. kluyveri* PKKR1 during fermentation using Systems Biology
5.1 Interaction between yeast species

As seen in the previous chapters, numerous studies have been carried out to understand the population biology of *S. cerevisiae* (Legras JL et al., 2007; Liti G et al., 2009; Schacherer J et al., 2009). Its growth and fermentation kinetics, its aroma producing capabilities, its interaction with both *Saccharomyces* and non-*Saccharomyces* yeast during fermentation of grape must and the ability to engineer its niche (Ciani et al., 2010; Delfini et al., 2001; Fleet, 2003; Gil et al., 1996; Goddard, 2008; Jolly et al., 2006; Salvadó et al., 2011). All these interactions between environmental and other microbial factors elicit the expression of genes and proteins in *S. cerevisiae* which control the levels of internal and external metabolites. Indeed the way biological communities are structured and function are largely dependent on the interaction between the species contained within (Jessup et al., 2004; Siepielski and McPeek, 2010).

One way species interactions in a niche can be examined and perhaps be quantified is by monitoring the movement and exchange of metabolites between organisms (Goddard, 2008; Mapelli et al., 2008). Such metabolic interactions may encompass a range of inter-species interactions ranging from predator-prey, parasitic, through to co-operative interactions such as commensalism, symbiotic relationships and to the emergence of multi-cellularity (Biofilms) and sexual interactions (Hubbell, 2001; Mapelli et al., 2008; Paine and Levin, 1981). These species interactions are a product of natural selection and therefore increase the fitness of the interacting organisms in some manner (Rainey and Travisano, 1998; Siepielski and McPeek, 2010). One example of metabolic interaction seen among different microbes is quorum sensing where the microbes are capable of moderating one another’s activities through the exchange of metabolic intermediates (Jessup et al., 2004).

5.1.1 Abiotic and biotic interactions of *S. cerevisiae*

*S. cerevisiae* has been shown to be interacting specifically and respond to both abiotic and biotic factors. *S. cerevisiae* has also been shown to produce Tyrosol (aromatic alcohol) in response to a high cell density and low nitrogen concentration to regulate transition from unicellular to filamentous growth (Chen and Fink, 2006). Few studies have been conducted examining the interaction of *S. cerevisiae* with other yeasts in wine; Cheraiti et al. (2005) identified the redox interactions between *S. cerevisiae* and *S. uvarum* by monitoring the exchange of the metabolite Acetaldehyde between the two species. Howell et al. (2006) showed that chardonnay made using mixed population of *S. cerevisiae* produced a different volatile metabolite profile compared to post ferment blending of chardonnay from the individual *S. cerevisiae*, importantly they also showed that the volatile profile contribution of yeast species could not be predicted based on its cell population. Similarly Anfang et al. (2009) showed that concentration of varietal aroma produced from a mixed ferment between *S. cerevisiae* VL3 and *P. kluveri* PKK1 was not just an additive contribution by the two species.
Nissen et al. (2003) tried to identify a possible quorum sensing mechanism responsible for the arrest in growth of non-\textit{Saccharomyces} yeast in the presence of \textit{S. cerevisiae}; instead they concluded that the growth arrest was due to as yet unknown cell-cell mediated interaction between the two species. Contrarily, Perez-Nevado et al. (2006) showed that it was not cell-cell mediated contact but some unknown inhibitory mechanism that arrested the growth of \textit{H. guilliermondii} and \textit{H. uvarum} in the presence of \textit{S. cerevisiae}. Indeed, these studies suggest that \textit{S. cerevisiae} interacts with its environment and with other yeast.

### 5.1.2 Model experimental system to investigate species interaction

Very little is understood regarding the fundamental principles driving metabolic interactions. The key first step is the availability of the right experimental system. Such a system must consist of organisms that are molecularly tractable and easy to propagate; these organisms must be known to interact, and above all this system must be easy to replicate experimentally yet be realistic (Replansky et al., 2008; Siepielski and McPeek, 2010). The interaction between yeasts during wine fermentation fits the criteria for such an investigation. Firstly, controlled fermentation of grape juice (a complex media) by a specific set of species is not as far removed from nature, nor is it as simplistic as an artificial laboratory experiment. Furthermore, co-fermentation with two yeast species is not as complicated and uncontrolled as the microbial community contributing to commercial un-inoculated fermentations, thus allowing for control and monitoring of species interaction (Querol and Fleet 2006).

The co-fermentation of Marlborough Sauvignon Blanc by \textit{S. cerevisiae} VL3 and \textit{P. kluyveri} PKK1 under controlled conditions as shown by Anfang et al. (2009) with initial inoculum ratio of 1:9 respectively at 15\(^\circ\)C, resulted in wine with increased varietal aroma characteristics due to as yet unknown synergistic interaction between the two species. This co-fermentation of Sauvignon Blanc by PKK1 and VL3 is an ideal ‘half-way house’, it can be controlled yet constitutes a natural environment (grape juice). Secondly, the \textit{Hemiascomycete} yeasts in this co-ferment are experimentally tractable from a microbiological and molecular genetic perspective: indeed the dominant wine yeast, \textit{Saccharomyces cerevisiae}, has long been a model eukaryote and is arguably the best molecularly characterised organism on the planet (Llorente et al., 2000). However, little else is known about this co-fermentation process. Therefore along with a Master’s degree project (Yael 2009 data not discussed in this thesis) analysing the transcriptome, I undertook the analysis of proteome and extracellular metabolites from a replica of co-fermentation to further understand the underlying mechanisms of interaction between \textit{S. cerevisiae} VL3 and \textit{P. kluyveri} PKK1.

Taking a holistic approach to understanding the interaction between PKK1 and VL3 is not only commercially beneficial but also provide a working model to test the principles of species interactions. Alcoholic fermentation of grape juice is a dynamic process and the yeasts involved are constantly adapting to the changes in their environment (Fleet, 2003). Predominantly, grape nutrients
are broken down and converted into alcohol and other secondary metabolites produced and vital nutrients taken up by yeast during fermentation through the action of numerous proteins and enzymes (Querol and Fleet, 2006). The co-fermentation of PKKR1 and VL3 not only results in changes to the final thiol concentration but also the fermentation kinetics of the individual yeasts (Anfang et al., 2009). Therefore it can be hypothesized that the main fermenting yeast VL3 is interacting with PKKR1 during fermentation and this interaction is resulting in a difference in the final wine quality compared to a VL3-only mono-ferment.

5.1.3 PKKR1 and its interaction with VL3

*Pichia kluysteri* PKKR1 is an isolate identified in 2006 by Casey Jun while investigating the yeast ecology and diversity at Kumeu River winery and the adjoining vineyard (Casey Jun 2006). Kumeu River winery in Auckland was selected for this study because it is one of the few wineries in New Zealand which relies completely on natural fermentation (no inoculation with commercial yeast) for the production of its commercial wines. Yeasts were isolated and identified from various sources such as soil, grape vines, and grape juices. PKKR1 was isolated from a settling tank in the winery using standard microbiology followed by ITS (Internal Transcribed Spacer) PCR of isolated DNA and stored at -80°C for future use (Anfang, 2010; Anfang et al., 2009).

PKKR1 along with other natural isolates were tested for their ability to produce varietal aromas of Marlborough Sauvignon Blanc such as 3MH and 3MHA (Anfang, 2010). The levels of thiols released by the natural isolates were compared against mono-ferment of *S. cerevisiae* used as control. Most of the non-*Saccharomyces* yeast although producing relatively high levels of thiols were incapable of completely fermenting the grape must. Therefore co-fermentation of these non-*Saccharomyces* yeast with various commercial *S. cerevisiae* was carried out. Various initial inoculum ratios between the natural non-*Saccharomyces* yeast isolates and commercial *S. cerevisiae* were trialled, of them; the co-fermentation of PKKR1 with VL3 yielded the most marked result. The specific combination of 9:1 of PKKR1 and VL3 respectively in the initial inoculum was responsible for elevating the level of the thiol 3MHA by at least two-fold when fermenting at 14.5°C when compared to mono-ferment by VL3 on its own (Anfang et al., 2009).

A potential to increase varietal aroma in Marlborough Sauvignon Blanc is considered hugely beneficial by commercial winemakers. In addition, tasting notes of co-ferments compared to mono-ferments by experienced winemakers described the additional of *P. kluysteri* PKKR1 as adding tropical notes, complexity and palate weight to the wines. After three years of commercial trials and product development in collaboration with CHR Hansen, this strain is now commercially available under the trade name Viniflora® FrootZen™.
However, the interaction between PKKR1 and VL3 as described by Anfang et al. (2009) is dependent on the environmental parameters (results chapter 3 and chapter 4). The previous chapter (chapter 3.5.1) in this thesis showed that population dynamics of co-fermentation between PKKR1 and VL3 differed significantly with different nutrient/oxygen availability conditions. Based on the results discussed in chapter (4.7.3.2), it can be concluded that temperature (12.8°C and 20°C), YAN and the presence or absence PKKR1 had no impact on levels of 3MHA. Therefore it can be assumed that the synergistic interaction between PKKR1 and VL3 is observed only at a specific temperature (14.5°C), and the population dynamics between these two species is affected by availability of oxygen and possibly other oenological conditions (Houtman et al., 1980).

One way of understanding the mechanism involved in this interaction is by testing the null hypothesis that there is no difference in proteome or extracellular metabolome between VL3+ PKKR1 co-ferment and VL3-only and PKKR1-only ferments. Direct comparison between mono and co-ferments were assumed to demonstrate any differences between these ferments, thereby identifying the contribution of PKKR1 in VL3-PKKR1 co-ferment. The null hypothesis was tested by analysing both the ever-changing metabolites (small molecules) in the must/wine and the levels and types of proteins present inside the yeast cells in a co-ferment scenario and comparing it to the levels of metabolites and proteins in cells from a mono-ferment under similarly controlled conditions. By monitoring yeast’s internal environment (proteins) and its external environment (metabolites in grape must/wine) it is possible to track its response to environmental stimuli including any interaction with another organism (Figure 5.1).

Figure 5.1: The systems biology potential of a yeast cell. Yeast’s interaction with both abiotic and biotic environmental factors can be examined by monitoring and identifying key genes, mRNAs, proteins or metabolites (both external and internal metabolites).
5.2 Systems biology approach to examining the interaction between VL3 and PKKR1 during fermentation

The complete genome of the industrial wine yeast strain VL3 has very recently been sequenced and published (Borneman et al., 2011). The initial version of the sequence and related protein annotations are publically available on NCBI (National Centre for Biotechnology Information Accession numbers CM001126.1 to CM001141.1). The availability of complete genome data was crucial for the holistic approach to understanding the interaction between VL3 and PKKR1. In this study, the interaction between VL3 and PKKR1 was considered only from VL3’s perspective as there is very little sequence/protein information regarding PKKR1 (there were none in the genbank database for this isolate and only 118 sequences for *P. kluyveri*). Therefore the modified null hypothesis tested was that there is no difference in either the protein profile or extracellular metabolite profile of VL3 in single and VL3-PKKR1 co-ferments. To test this hypothesis, the appropriate systems biology tools were required. The following paragraphs will discuss the different methods available and the methods that most powerfully test the above hypotheses.

5.2.1 Selection of a large-scale proteomic technique to study the interaction between VL3 and PKKR1

The word “proteome” was coined by Wilkins et al. (1996) to define all the proteins present in given sample of cell, tissue, organelle or a pathological state. The term “proteomics” refers to all the techniques that can be used for the large-scale identification proteins and can also include techniques used to analyse protein structure and discerning protein function (Wilkins, 1996). Proteomics encompasses a vast array of techniques such as antibody based protein chips, high throughput crystallography screening, yeast two-hybrid screens and a number of Gel and Mass Spectrometry based techniques (Becker and Bern, 2011; Wang et al., 2008).

Recent years have seen numerous mass spectrometry based techniques capable of identifying a vast array of proteins from a single sample. Most of these techniques also include some sort of quantitative comparison of proteins in the samples. Figure 5.2 is a summary of the most commonly used techniques in large-scale comparative proteomics from Wang et al. (2008).
Mass spectrometry functions by measuring the mass of a highly purified substance by identifying its mass to charge ratio (m/z). Accurate measurement of mass thus enables in the identification of the substance (Glish and Vachet, 2003; Wang et al., 2008). Mass spectrometry on its own has limited capacity in identifying peptides and proteins but on the other hand tandem mass spectrometry or MS/MS has practical use in large scale proteomics (Glish and Vachet, 2003). Tandem MS is not only capable of analysing the m/z ratio of fragments but also the amino acid composition from a peptide thus allowing for accurate identification of the peptide based on its mass (figure 5.3) (Glish and Vachet, 2003). Complexity arises when multiple proteins or mixtures of peptides have to be analysed through MS/MS as the peptide mixture needs to be pure for accurate identification (Glish and Vachet, 2003). Upstream manipulation of the protein sample so as to provide pure mixtures of peptides for MS/MS thus becomes imperative. Upstream manipulation of protein samples can be performed in many ways (Becker and Bern, 2011; Wang et al., 2008).

Figure 5.2: Representation of the different Mass Spectrometry based techniques used in large-scale comparative proteomics. (A) Outline of work flow in 2-DE, (B) outline of work flow in ICAT and iTRAQ and (C) outline of work flow in SILAC. This figure is modified from Wang et al. (2008).
5.2.2 Upstream manipulation of protein samples

As outlined in figure 5.2, the upstream separation and purification of complex protein samples can be achieved by 2D SDS–PAGE where protein samples are separated and selected gel spots are further analysed using MS/MS (Becker and Bern, 2011; Wang et al., 2008). This method while providing high resolution has limitations in the types of protein that can be reproducibly separated. On the other hand techniques such as affinity tag purification and functional protein arrays are highly advantageous only when focussing on specific protein complexes or protein types (Wang et al., 2008).

Large-scale non targeted or global analyses of proteins from sample to treatment require techniques that include either metabolic labelling (SILAC) or chemical labelling of samples (ICAT or iTRAQ) (Becker and Bern, 2011; Wang et al., 2008). For the purposes of this study SILAC was disregarded as it requires cell cultures to be labelled with heavy or light isotopes of elements like carbon, nitrogen, hydrogen, oxygen. Media containing numerous labelled isotopes are cost prohibitive when required in large scale. Due to suitability of method (can compare between many samples) and availability of resources the iTRAQ method was selected. A brief description of the method and analyses is discussed below (Becker and Bern, 2011; Wang et al., 2008).
5.2.2.1 iTRAQ methodology

iTRAQ (isobaric tags for relative and absolute quantification) is the result of a recent innovation in proteomics which allow a direct quantitative comparison of proteomes from multiple samples, and this has been commercialised by Applied Biosystems (Ross et al., 2004). Typically, using the iTRAQ technique, the primary amine groups from the trypsin digested proteins from different samples (cell populations) are labelled using four to eight (in this study an eight-plex system was used) amine specific isobaric reagents, and the samples are pooled following labelling (Ross et al., 2004). The pooled samples are then fractionated using a single dimensional liquid chromatography (usually reverse phase LC) or multi-dimensional LC based on its complexity. Following LC separation the labelled proteins are ionised usually by electrospray ionization (Wang et al., 2008). A potential is applied to the elute from the LC when passing through the ioniser needle resulting in a fine spray of droplets containing the sample which is heated prior to entry into the mass spectrometer allowing for desolvation and ionization (Becker and Bern, 2011; Wang et al., 2008). Ionization of the samples allows for further MS/MS analyses leading to protein/peptide identification. Because of the isobaric nature of the label, same peptide from each sample appears as a single peak in the MS spectrum which greatly reduces complexity of data (figure 5.4) (Ross et al., 2004). Upon collision induced dissociation (CID) during MS/MS the iTRAQ tagged peptides fragment to release reporter ions which can then be used to assess the relative abundance of peptides and in turn the protein from which it was derived (Glish and Vachet, 2003). Since multiple samples are analysed at the same time, the direct comparison of protein levels between samples can be performed following bioinformatics analyses of the overall data (Becker and Bern, 2011; Karp et al., 2010).

For all the MS/MS based techniques including iTRAQ; measurements are made at the peptide level which then has to be identified (as belonging to a particular species) combined, computed and summarised to obtain protein levels from which biological inferences can be obtained (Becker and Bern, 2011; Karp et al., 2010). This process of identifying and quantifying peptide/protein profiles is aided considerably by the availability of complete genome and annotated proteome of the selected organism by enabling accurate data analysis (Becker and Bern, 2011; Karp et al., 2010). Due to the complex nature of data obtained from MS/MS experiments, some sort of automation is required in analysis. Many free and proprietary software are available to analyse iTRAQ data (Karp et al., 2010). Also, these data-sets require the usage of appropriate statistical analyses to prevent distortion or exaggeration of biological meaning (Becker and Bern, 2011; Karp et al., 2010).
5.2.3 Analyses of the yeast’s extracellular environment through metabolic footprinting

5.2.3.1 What is Metabolomics?

In systems biology, metabolomics is a tool used to identify and quantify all metabolites with molecular weight lower than 1.5 kDa that are produced and transformed by the cells (Villas-Boas et al., 2007). Metabolomics was initially developed as a functional genomics tool in late 1990’s to verify the end products of manipulation of cellular regulatory processes (Oliver et al., 1998). However, during the past years, metabolomics has quickly evolved into its own field, and it is now applied to various areas of global analyses including disease diagnosis, discovery of new metabolic pathways, bioremediation among others (Madsen et al., 2010; Mapelli et al., 2008; Villas-Bôas et al., 2005; Villas-Bôas and Bruheim, 2007).
Metabolome analysis is generally categorised into three types: (1) metabolic fingerprinting/footprinting, which aims to capture the intracellular/extracellular metabolite profiles sometimes without enquiring as to the identity of metabolites, (2) targeted analysis, which aims to detect, identify and quantify specific metabolites in the samples, and (3) metabolite profiling, which aims to detect and identify as many metabolites as possible in a quantitative or at least in a semi-quantitative manner (figure 5.5) (Mapelli et al., 2008; Nielsen and Oliver, 2005; Smart et al., 2010; Vivanco et al., 2011). In this study, an approach with metabolite footprinting involving identification of metabolites in samples was used to track the changes in the extracellular environment in various ferments. This data was used to test the null hypothesis that there is no difference between the extracellular metabolite profiles between single and co-ferments.

**Figure 5.5:** Diagrammatic representation of the different approaches and applications of metabolomics in winemaking. Figure adapted from Vivanco et al. (2011).

### 5.2.3.2 Strategies to identify biomarkers using metabolomics

The main feature of metabolomics is its ability to identify and relatively quantify the metabolic phenotype of cells under different conditions; this technique was complimentary to iTRAQ proteome analyses (Mapelli et al., 2008; Smart et al., 2010). As metabolites are not encoded by genes, metabolomics data can be directly linked to metabolic pathways in the cell and hence the enzymes involved, and coupled with proteomics; metabolomics can potentially be used to verify the physiology of the cell (Mapelli et al., 2008; Smart et al., 2010; Villas-Boas et al., 2007). Metabolomics can be a
powerful technique to identify metabolic biomarkers that are different between unperturbed and perturbed systems (in this study; mono and co-ferment) (Villas-Boas et al., 2007). Such analyses of biomarkers can be very useful in generating hypotheses about the regulatory mechanisms underlying the observed phenotype (figure 5.5) (Vivanco et al., 2011).

Therefore, in this study, proteomics and metabolomics were used to test the null hypotheses proposed above; where data from each of the ‘omics were used separately to examine the interaction between VL3 and PKKR1 during co-fermentation. Although transcriptomics analyses were performed on the same ferments; those results will not be discussed in this thesis (Yahel 2009).

Presently, it is not possible to analyse the complete set of metabolites in a cell or organism due to their diversity and complexity of chemical properties (Nielsen and Oliver, 2005). Various analytical approaches have been developed in the field of metabolomics to capture as much information as possible (Villas-Boas et al., 2007). In recent years, methods involving chromatographic separation of metabolites from a sample (cell population) coupled to either mass spectrometry (MS) or nuclear magnetic resonance (NMR) detector have been recognised as offering the most effective analytical platforms (Villas-Boas et al., 2007). A GCMS platform combines the separation efficiency of GC and sensitivity of MS and is capable of resolving complex biological matrices and allow for the analyses of a large number of metabolites from a wide range of chemical properties (Villas-Boas et al., 2007). In this study, gas chromatography (GC) coupled to MS was employed for obtaining extracellular metabolites from mono and co-ferments of P. kluyveri PKKR1 and S. cerevisiae VL3.

5.2.3.3 Sample preparation in Metabolomics

One of the caveats for GCMS analysis is that it requires analytes to be volatile and thermally stable in order for them to be injected into the GC system (Villas-Boas et al., 2007). Many of the metabolites do not meet this requirement; therefore a chemical derivitization is commonly employed to convert the metabolites into their volatile derivatives. There are two main types of derivitization techniques commonly used in metabolomics namely; silylation and alkylation. While both techniques are used robustly, they have their strengths in derivitizing different classes of metabolites (Villas-Bôas et al., 2003). An alkylation technique developed by Villas-Bôas and colleagues provides an effective solution in the analyses of metabolites of the central carbon and nitrogen metabolism (Villas-Boas et al., 2007). Metabolites in central carbon and nitrogen metabolism have either amino or carboxyl groups or both and MCF derivitization is capable of converting these functional groups into their volatile counterparts namely; carbamates and esters respectively (Villas-Boas et al., 2007). Yeasts rely heavily on central carbon and nitrogen metabolic pathways during alcoholic fermentation; it would be useful to track changes in the mono and co-ferments of VL3 and PKKR1 using MCF.
derivitization to test the null hypothesis (Bell and Henschke, 2005). Therefore MCF derivitization technique was employed in this study.

Metabolomics studies involving yeast (predominantly S. cerevisiae) have been on the increase in the recent years (Beltran et al., 2006; Daran-Lapujade et al., 2004; Kresnowati et al., 2006; Rossouw and Bauer, 2009; Villas-Boas et al., 2007). These studies have provided unique perspective into the molecular mechanisms involved in yeast’s response to environmental stimuli such as fermentation temperature and availability of certain substrates. Kresnowati et al. (2006) showed that there was an increase in trehalose-6p which played an important role in homeostasis following relief from glucose limitation. They also showed the down regulation of three hexokinase genes involved in the response to prevent glucose-accelerated death in yeast. Most of these studies have coupled transcriptomic and metabolomic analyses to understand the mechanisms involved in yeast under different conditions. Another study relating to the identification of various beer brewing strains of S. cerevisiae, the researchers were able to successfully identify and characterize 9 different strains of Saccharomyces yeasts based on their metabolisms during fermentation; furthermore they were able to differentiate species using their metabolites when their genomes could not be differentiated (Pope et al., 2007). However, using global metabolomics techniques, variation in key aroma production pathways are yet to be studied. Also, these techniques have not been used to study interaction between two yeast species.

5.3 Systems biology studies on oenological yeast

Although, S. cerevisiae is a well-studied eukaryote, its application in winemaking has not been well explored through the use of proteomic and metabolic analyses tools. A few studies have been conducted in the recent years, they involved 2D gel electrophoresis coupled to LC-MS/MS and are usually used as a tool to confirm global transcriptomic analyses (Rossignol et al., 2009). Systems biology approach has been used to analyse, the stress response to osmotic pressure, analyses of glycoproteins of grape and yeast in wine and yeast biomass propagation (Gómez-Pastor et al., 2010; Jiménez-Martí et al., 2011; Palmisano et al., 2010; Salvadó et al., 2008). A few studies have concentrated on the effect of fermentation and media on both lab yeast strains and industrial yeast strains (Rossignol et al., 2009; Rossouw et al., 2010). Among them a study conducted by Rossouw et al. (2010) to demonstrate the differences between lab yeast and industrial yeast in their ability to utilize sugar during fermentation involved the use of iTRAQ technique. This is the only study to my knowledge that has utilised iTRAQ analyses to study fermenting yeast. On the other hand, no study has been conducted as yet to understand the global impact of one yeast species on another during fermentation. There is no study to my knowledge comparing non-targeted metabolite profiles between
different ferments. The testing of the null hypothesis proposed is the first study aiming to understand the impact of non-Saccharomyces yeast on an industrial Saccharomyces cerevisiae.

5.4 Aims and objectives

There are two main aims for this chapter, they are as follows:

1) Test the null hypothesis that there is no influence of PKKR1 on VL3 during co-fermentation by using the protein profiles obtained from iTRAQ analyses of the co-ferment and individual mono-ferments
   (a) The null hypothesis was tested by comparing protein profiles between VL3–only and VL3 and PKKR1 co-ferment.
   (b) The null hypothesis was also tested by comparing between the protein profiles of VL3 – PKKR1 co-ferment and a model co-ferment derived from profiles of VL3-only plus PKKR1-only ferments. This comparison tested for the possibility that the co-ferment was just an additive profile of the two species, thus allowing the confirmation of the null hypothesis.

2) Test the null hypothesis that there is no influence of PKKR1 on VL3 during co-fermentation by using the extracellular metabolite profile obtained from footprinting analyses of the co-ferment and individual mono-ferments
   (a) The null hypothesis was tested by comparing the extracellular metabolite profiles between VL3–only and VL3 and PKKR1 co-ferment.
   (b) The null hypothesis was also tested by comparing between the extracellular metabolite profiles of VL3–PKKR1 co-ferment and a model co-ferment derived from profiles of VL3-only plus PKKR1-only ferments. This comparison tested for the possibility that the co-ferment was just an additive profile of the two species, thus allowing the confirmation of the null hypothesis.
5.5 Materials and methods

5.5.1 Fermentation set-up and monitoring

5.5.1.1 Yeast strains

*Saccharomyces cerevisiae* strain VL3 and *Pichia kluyveri* strain PKKR1 were used in this fermentation set up (from Goddard yeast library). Pure colonies of these yeasts were grown at 28°C in sterile YPD media for 48 hours with 100rpm shaking (refer section 2.8.3). The cells were pelleted by centrifugation at 4000rpm for 10 minutes. The yeast pellet was re-suspended in sterile MiliQ water and cell count enumerated by using a Haemocytometer (refer section 2.8.2). The required amount was then transferred into appropriate flasks containing sterile grape juice. The intended total inoculum of each flask was 2.5 million cells/mL.

5.5.1.2 Juice sterilization

Sauvignon Blanc grape juice was sourced from Marlborough and kindly provided by Pernod-Ricard NZ (pH of 3.28 TA (titratable acidity) of 6, Brix° 17.8). The juice was stored at -20°C until use. This juice was slowly thawed at 4°C for 2 days and transferred into sterile 20L carboy. The thawed juice was chemically sterilized by adding 0.5mL/L DMDC (see section 2.8.8.1) and a further 2 days were allowed at 4°C to allow grape solids to settle. Clear juice was then siphoned off for immediate use. Sterility of the juice was confirmed by plating 200µL of un-diluted juice on YPD Agar and culturing for 24 hours at 28°C.

5.5.1.3 Fermentation set-up and conditions

All fermentation was carried out in sterile 250 mL capacity Erlenmeyer flasks with a single side-port. A volume of 200 mL of grape juice was used for fermentation in these flasks. Following addition of juice and inoculation with appropriate yeasts the neck of each flask was closed with a sterile air-lock filled with sterile water. The side port of the flask was stoppered with a sterile rubber stopper. Both the air-lock and rubber stopper were coated by sterile glycerol to ensure perfect closure.

In total, 28 flasks were setup for this experiment. Nine flasks were set up with an initial inoculum of *S. cerevisiae* VL3 only, nine flasks inoculated with *Pichia kluyveri* PKKR1 only and a further nine flasks with an initial inoculum consisting of a 9:1 ratio of PKKR1 to VL3. The total inoculum of each flask was 2.5 million cells/mL. A control flask was also setup similarly consisting only of the sterile grape juice and was monitored throughout the experiment.

The inoculated grape juice in the flasks was incubated at 14.5°C with a continuous shaking of 100rpm in an INFORS HT Multitron incubator to allow for controlled fermentation.
5.5.1.4 Monitoring and sampling during fermentation

5.5.1.4.1 Weight loss measurements

The amount of weight loss of each of these flasks were measured and recorded daily throughout the experiment. The amount of weight lost by a flask during fermentation is directly proportional to the rate of release of carbon dioxide during fermentation (Bely et al., 1990). Rate of release of carbon dioxide during fermentation was used to calculate the rate of fermentation (for ferment kinetics see Appendix 5).

5.5.1.4.2 Population dynamics

Change in population of the yeast was monitored during fermentation; samples were taken once every two days from all the flasks and plated on YPD agar (see section 2.8.3). Enumeration of the number of colonies on the YPD agar plates allowed for the calculation of the number of colony forming units per mL of grape juice. The population of VL3 and PKKR1 in the co-ferments were also enumerated based on their differences in colony morphology (refer figure 3.1).

5.5.2 Destructive sampling for protein and metabolite analysis

During fermentation, samples were collected for protein and metabolite analyses on days 2, 9 and 16. These days were selected based on the previous data collected from monitoring population dynamics of VL3 and PKKR1 co-fermentation (Anfang et al., 2009). On day 9 the ratio of the population of VL3 and PKKR1 was expected to be around 1:1 and on day 2 the population was expected to be a high proportion of (at least 80%) PKKR1 and on day 16 the fermentation was expected to be mostly dominated by VL3 (at least 80%).

On days 2, 9 and 16, destructive sampling was carried out on each treatment in triplicate. Each of the flasks was removed from the incubator and shaken gently to homogenise the culture. The air lock was dismantled quickly and the contents were poured into four separate 50mL capacity sterile centrifuge tubes. The yeast was pelleted out by centrifugation at 4000 rpm for 5-10 min. Following centrifugation, the supernatant was separated from the pellet and stored at -80°C in separate sterile 50 mL capacity containers. The collected pellet was re-suspended in sterile water and centrifuged a further 5 min at 4000 rpm, this allowed for the removal of most of the grape solids and other grape juice material from the yeast pellet. The cleaned yeast pellets were stored at -80°C for further analysis.

The cleaned yeast pellets were used for protein analysis, while the fermenting grape juice (yeast cells removed) was utilised for analysis of extracellular metabolites or metabolic footprint during fermentation.
5.5.3 Processing of yeast pellets for large-scale protein analysis

5.5.3.1 Protein extraction

All the chemicals used were purchased from Merck. An alkaline buffer was freshly prepared on the day of protein extraction and it consisted of 7M Urea, 7M Thiourea, and 5 mM Dithiothreitol (DTT), 0.1% Triton in 50mM Ammonium bicarbonate buffer with a pH of 8.0. A 1:1 ratio (v/v) of buffer and yeast pellet mixture was prepared in micro-centrifuge tubes and this mixture was sonicated using a Soniprep 150 probe sonicator. Each sample was sonicated for 30 s followed by cooling in an ice bath for 30 s, this was repeated 5 times. The sonicated samples were then centrifuged at 16,000 g to separate yeast solids from supernatant. The supernatant from the samples were carefully collected and transferred to a new micro centrifuge for downstream processing.

5.5.3.2 Protein quantitation, reduction, alkylation and trypsin digestion

The concentration of protein in the supernatant of the samples was analysed by using an EZQ kit (Invitrogen). The proprietary fluorescent dye in the kit was used to stain the protein sample of which 1µl was spotted on a prepared Whatman paper followed by quantitation of fluorescence via a plate reader. Following protein quantitation, aliquots of samples containing 50 µg of protein were then used for further preparation before iTRAQ labelling.

The aliquoted protein samples then underwent reduction and alkylation on the same day to prevent coagulation. Reduction of protein samples involved incubating the samples in 10mM DTT (dithiothreitol) at 56⁰C for 1h. Following reduction, the protein samples underwent alkylation. Alkylation involved incubating the samples in 30mM iodoacetamide at pH 8.0 for 1h in darkness. Following alkylation the samples were immediately quenched with a further DTT to prevent over-alkylating the samples.

The quenched protein samples then underwent trypsin digestion to allow for labelling of peptides with isobaric iTRAQ dyes. Trypsin digestion involved incubating the samples with 2 µg of trypsin (Promega, Madison, WI) at 37⁰C overnight.

The digested peptides were then de-salted using 10 mg Oasis SPE cartridges (Waters Corporation, MA). The samples were then dried down using a speed vacuum concentrator (Thermo Savant, Holbrook, NY). The samples were now ready to be labelled with iTRAQ reagents. I followed the protocol for preparing samples for iTRAQ analyses as directed by Mr Martin Middleditch based on work by Jüllig et al. (2007).
5.5.3.3 iTRAQ labelling of samples

An 8-plex iTRAQ reagent kit (Applied Biosystems, Foster City, CA) was used for labelling samples. The dried protein digests were reconstituted using 30 µL of the dissolution buffer from the iTRAQ reagent kit followed by labelling of the samples with different iTRAQ labels according to the manufacturer’s instructions. Four 8-plex kits were used to label all the samples as shown in the table below (table 5.1 discussed in results). Following labelling the samples of the different runs were pooled separately and acidified, and de-salted as described above to prevent introduction of salts into the LCMS. The samples were concentrated to a volume of 50 µL before being finally diluted to 250 µL in 0.1% formic acid. These samples were now ready for Chromatographic separation followed by Mass Spectrometry.

5.5.3.4 Chromatographic separation of iTRAQ labelled samples

A pooled 8-plex labelled sample was fractionated on-line a BioSCX II 0.3 by 35-mm column (Agilent Technologies, Santa Clara, CA). The pooled sample was fractionated by utilizing five salt steps of 50, 100, 150, 200 and 500 mM KCl. A HPLC (High Pressure Liquid Chromatography) gradient of 6 µL/min was formed between Buffer A(0.1% formic acid in distilled water) and Buffer B (0.1% formic acid in acetonitrile) it is as follows: 10% buffer B for the first 3 min, increasing to 35% buffer B by 80 min, increasing to 95% buffer B by 84 min, held at 95% until 91 min, back to 10% buffer B at 91.5 min, and held there until 100 min. The peptides were captured on a PepMap cartridge (0.3 by 5 mm) (LC Packings, Dionex Corporation, Sunnyvale, CA) before being separated by a Zorbax 300SB-C18 column (0.3 by 100 mm) (Agilent Technologies, Santa Clara, CA).

5.5.3.5 Mass Spectrometry conditions for analysing iTRAQ labelled samples

For each iTRAQ run the effluent from the HPLC was directed into an IonSpray source of a QStar XL hybrid quadrupole time-of-flight mass spectrometer (Applied Biosystems). The scanning range was set to 300 to 1600 m/z. The top three most abundant peptides with multiple charges were selected for a further MS/MS analysis with a scanning range of 55 to 1600 m/z. Both the HPLC and Qstar were under the control of the Analyst QS software package throughout the run (Applied Biosystems).

5.5.3.6 Data Analysis of the iTRAQ runs

All the data files from the four separate iTRAQ analyses using 2-D LC MS/MS experiment were searched as a single set using the software ProteinPilot 2.0.1 (Applied Biosystems) against the annotated proteins from the newly sequenced VL3 genome. The search criteria while using ProteinPilot 2.0.1 to generate data summaries were; enzyme trypsin, Cys alkylation, iodoacetamide, search effort: rapid. The protein sequences for VL3 were published by Borneman et al. in 2011 and made available through NCBI (National Center for Biotechnology Information). The proteome for
VL3 consists of 4201 entries and was downloaded in June 2011 into the ProteinPilot 2.0.1 software. Performing identical searches using the same database with all sequences reversed was used to estimate false-discovery rates for protein identifications (Elias and Gygi 2007). In this case, any proteins with an ‘unused’ score of < 2 were excluded based on the reverse search results. This rate suggests that most of the peptides recognised were well matched to the peptide sequences from the VL3 database. The proteomic data set is available as supplemental material.

5.5.3.7 Bias correction, data normalization and statistical testing

Bias correction in the proteomic data set was performed to remove peptides which could be false positives and introduce inaccurate information. Bias correction was carried out in Microsoft Excel 2010 according to method adapted from Jüllig et al. (2007). Firstly, all peptides with a used score of 0 were excluded. Also any spectra matched to a peptide with a COOH-terminal proline (P) known to interact with label 116 m/z were excluded. This was followed by filtering out all peptides containing the amino acids PQ next to each other anywhere in the sequence, as these peptides interacted with iTRAQ label 115. These label interactions are based on the tendency of peptides to fragment before Proline (Jüllig et al., 2007). Applied Biosystems and other studies strongly recommend a bias correction step when using iTRAQ labels for shot-gun proteomics (Jüllig et al., 2007; Karp et al., 2010). Following bias correction any poorly quantitated spectra or shared spectra were removed by selecting only spectra denoted “auto”. The resulting Excel spread sheets were merged and saved as a comma delimited (CSV) file for downstream data analyses.

Data normalization is a pre-requisite for MS based protein quantification methods like iTRAQ as the abundance measurements are made at peptide levels which are to be combined to identify and quantify proteins for specific organisms (Karp et al., 2010). R 2.12.1 and R 2.13.1 software were used for data normalization process with the package PreprocessCore installed through the Bioconductor website (www.bioconductor.org) (Bolstad et al., 2003; Team, 2011). Data normalization and statistical testing will be discussed in the results section and relevant R codes available in appendix 6.

5.5.4 Extracellular metabolite profiling

5.5.4.1 Sample preparation and derivitization

The extracellular soluble metabolites found in ferments were profiled as described below. In section 5.5.2 the supernatant from samples taken at different time points during fermentation was stored at -80°C. Before MCF derivitization, 50 µL of each of the samples was lyophilized using a freeze dryer (Labconco 12L, Labconco Corporation, Kansas City, MI, USA). The protocol required metabolite
derivitization followed by GCMS analysis, processing of raw data to identify the metabolites and finally visualization of metabolite profiles.

Methyl chloroformate (MCF) method of chemical derivitization was developed by Villas-Boas et al. (2003). Chemical derivitization is capable of converting the non-volatile polar metabolites to less-polar, volatile and thermally stable derivatives suitable for GCMS analysis. Each of the samples (n=3 at each time point) at day 2, 9 and 16 of fermentation for VL3-only, PKKR1-only and VL3-PKKR1 co-ferment were analysed in triplicate.

For derivitization, 50 µL of the lyophilized sample was dissolved in 180 µL of 1M sodium hydroxide solution in a salinized test tube, to this solution 20 µL of an internal standard (10 mM of 2, 3, 3, 3-d4 DL-Alanine dissolved in 1 M sodium hydroxide) was added. This solution was then mixed with 67 µL of methanol and 34 µL of pyridine. The derivitization process was initiated by addition of 20 µL of MCF, the mixture was then mixed vigorously for 30 s, a further 20 µL of MCF was added and mixed again for 30 s; following MCF addition, 400 µL of chloroform was added to the mixture and vigorously mixed for 10 s. The addition of MCF and extraction into chloroform were steps that had to be performed sequentially without a lapse in time. 400 µL of 50 mM sodium bicarbonate solution was added to the mixture and mixed for 10 s. The upper aqueous layer was discarded and the lower organic phase was dried of any residual water by the addition of 5-10 mg of anhydrous sodium sulphate powder. The dried organic phase was then transferred using a glass pipette into a salinized GC vial for GCMS analysis.

5.5.4.2 GCMS analysis

Analysis of the derivitised samples on GCMS was performed according to the protocol described by Villas-Boas et al. (2007). A GC7890 gas chromatograph coupled to a MSD 5975 quadrupole mass spectrometer (Agilent technologies, Inc., Santa Clara, CA, USA) operating at 70eV in the EI mode was used, this was equipped with a ZB-1701 capillary column (30 M x 250 µM, 0.15µM) (Phenomenex Inc., Torrance, CA, USA). Helium was used as the carrier gas at a flow-rate of 1 mL/min. The temperature settings were: 290 °C for the injector, 250 °C for the interface and 200°C for the quadrupole. Sample volume of 1µL was injected under pulsed splitless mode with 1.8 bars until 1 min followed by 20 ml/min split flow after 1.01 min. The oven temperature was programed to 45 °C for 2 min, then ramped up-to 180 °C at 9°C/min and held for 5 min and then ramped to 220 °C at 40 °C/min and held for a further 5 min, followed by ramping to 240 °C at 40 °C/min and held for 11.5 min and finally ramped to 280°C at 40 °C/min with a final hold time of 2 min. The GCMS was controlled by ChemStation (Applied Biosystems) throughout the run. Data analysis and metabolite profiling was performed on the raw files generated form these runs.
5.5.4.3 Data clean-up

The raw GCMS files for metabolite profiling were deconvoluted using the Automated Mass Spectral Deconvolution and Identification System (AMDIS) package (National Institute of Standards and Technology (NIST), Department of Commerce, Washington D.C, USA). AMDIS package is capable of extracting spectra for individual components in a GCMS data file and deconvoluting them to identify target compounds by matching the unique (deconvoluted) spectra against an in-house reference library of pure metabolite standards (Courtesy: Dr Silas Villas Boas). Here an AMDIS report file was generated containing identified metabolites in samples using an 80% “match factor” and default deconvolution settings. The combined data file was stored as a CSV file for downstream processing.

The data clean-up was performed using R 2.13.1 using a package called “Metab” available from www.bioconductor.org (Aggio et al., 2011). It involved automated correction of identified metabolites by comparing their ‘Observed Retention Time’ with their corresponding ‘Expected Retention Time’ based on the standards in the reference library using the command `clean.fix` and following instructions. This was followed by normalization of all the samples within the data set to the intensity of internal standard (D4 Alanine) within each sample using the command `norm.internal`. This was followed by applying the command `norm.medium` to subtract the log intensity of each metabolite identified in control juice from the log intensities of corresponding metabolites identified in the biological samples to enable metabolite footprint analyses. The resulting dataset was then subjected to the command `norm.biomass` to normalize for differences in metabolite levels due to different population sizes. Although `norm.biomass` is developed to normalize for the sample biomass, it can also be used to normalize samples by cell population density (cfu/mL). Both the metabolite intensities and population size were log transformed. All the commands and other additional package installation were performed as described by Aggio et al. (2011). Statistical testing of the normalised GC MS data and visualization was performed using R 2.13.1 and will be discussed in the results section (Team, 2011).

5.5.5 Pathway Activity Profiling or PAPi of extracellular metabolites

PAPi is an R package developed by Aggio et al. (2010) it can be found on www.bioconductor.org to be installed on R packages. PAPi used normalised external metabolite data was to detect the activities of various cellular pathways using Pathway Activity Profiling (PAPi). A data frame containing the normalised metabolites with their unique KEGG codes and their relative abundance in the sample was created. The KEGG compound code for metabolites can be found at KEGG website (http://www.genome.jp/dbget-bin/www_bfind?compound) (Kanehisa and Goto., 2000). Furthermore, PAPi is able to retrieve information from KEGG database (has API allowing access to external software) of all biochemical pathways (pre-selected for Fungi) for which each metabolite is known
to play a part in all identified pathways are score based on the abundance/relative abundance of the metabolite from all samples to which it is linked. Then the total number of metabolites associated with each pathway is recorded, following this, the pathways are ranked according to the number of metabolites with which they are associated. The percentage of detected metabolites from all samples is calculated for all the identified biochemical pathways finally a sum of scores is obtained of the pathways called the total pathway score which is then normalised by dividing by the proportion of metabolites detected from its respective pathway. The normalized score of each pathway represents the Activity Score (AS) or the level of its activity inside the cell, where the higher the AS the lower the pathway activity these functions can be carried out on all the samples for all experimental conditions.

In this study, for the analysis of extracellular metabolites the profiles of metabolites were previously normalised (subtracted) by the metabolites identified in the sterile grape juice from the control flask before analysis by PAPi as recommended by Aggio et al. (2011). PAPi also performs two sample t-test or ANOVA (test the global hypothesis of difference between samples) on AS, before presenting a list of all the pathways that were statistically significant (P-value <0.05). PAPi also is able to produce a graph of metabolic activity which was significantly different for the different treatments. All the R codes used in the PAPi package was obtained from Aggio et al. (2011).
5.6 Results and Discussion

In this section, data from mono and co-fermentation of Marlborough Sauvignon Blanc using *S. cerevisiae* VL3 and *P. kluyveri* PKKR1 was analysed to understand the mechanism of interaction between the two species. Firstly, the change in population and fermentation kinetics was analysed and compared to the population dynamics observed by Anfang *et al.* (2009). Next, the Proteomic data obtained from the iTRAQ study was analysed using appropriate methods for normalization and quantitation. Also, extracellular metabolite profiles of wine from all the samples were analysed as described by Aggio *et al.* (2010 and 2011). Next, iTRAQ data and the data obtained from extracellular metabolite footprinting were used independently to test the null hypothesis that there is no impact of PKKR1 on VL3 during co-fermentation as laid out in section 5.3.

5.6.1 Population dynamics and fermentation kinetics of the co-fermentation between PKKR1 and VL3

The aim here was to monitor the change in population of co-ferment of VL3 and PKKR1 and also the mono-ferments of VL3-only and PKKR1-only ferments. This information was later used to build the predictive model for testing various null hypotheses discussed below. The observed population dynamics in this experiment had similar trends to those reported by Anfang (2010). It was re-assuring to note the reproducibility of this population dynamic under controlled conditions as this co-ferment is used to improve varietal aroma in Marlborough Sauvignon Blanc. There were a few notable differences in the population size of mono-ferments of PKKR1 and VL3. These are explained below.

*P. kluyveri* PKKR1 on its own is not capable of fermentation according to Anfang *et al.* (2009), it was also observed in this experiment (Appendix5). However, the maximum population sizes of co-ferments \((1.321 \times 10^{08} \text{cfu/mL})\) and mono-ferments of VL3\((5.06 \times 10^{08} \text{cfu/mL})\) and PKKR1\((1.66 \times 10^{08} \text{cfu/mL})\) were greater than those observed by Anfang *et al.* (2009) (figure 5.6 a).

Here it was observed that the cell numbers of VL3 and PKKR1 in co-ferments are lower than their mono-ferment counterparts. A Student’s test comparing the population sizes of VL3-only ferment to that of the population of VL3 in VL3-PKKR1 co-ferments on day 2 was significant (0.039) and day 8 was not significant (0.12) and day 16 was significant (0.002). Similarly, comparing the population size of PKKR1-only ferments with the population size of PKKR1 in VL3-PKKR1 co-ferment yielded; no significant difference on day 2 (0.19), and on day 8 (0.42) but significant on day 16 (0.002). Population sizes were calculated from three biological replicates for each ferment type. Days 2, 8 and 16 were chosen as they were closest to sampling days of 2, 9 and 16 for proteomic and metabolite analyses.
Also, for VL3, the largest population size observed in co-ferment was lower than its counterpart in mono-ferment by approximately 2.7 times, while, for PKKRI the co-ferment population was lower than mono-ferment population by about 1.35 times. These trends are similar to those observed by Anfang but differed in the fold-changes in the populations (5.5 times for VL3 and 2.2 for PKKRI) (Anfang, 2010). In co-ferments, initially the population was dominated by PKKRI for 8 days at which time-point the population sizes of VL3 and PKKRI in co-ferment were roughly equal (50:50) followed by VL3 being dominant till the end of sampling period while PKKRI persists at much lower proportions (figure 5.6 b). The proportion of PKKRI was higher in the latter stages of co-ferment when compared to the study by Anfang et al. (2009); this could be due to the high cell numbers observed in this study. The overall differences in cell numbers could be due to differences in juice characteristics. However, this interaction was nicely reproducible in terms of how the populations dominate the co-ferment. This was important while trying to understand biomarkers that underpin the interaction between these two species.
Figure 5.6: Change in population dynamics of mono-ferments of *S. cerevisiae* VL3 and *P. kluyveri* PKKR1 along with their co-fermentation. (a) change in population size of VL3-only (solid blue), PKKR1-only (solid red) and that of VL3 (dotted blue) and PKKR1 (dotted red) within co-ferments over the sampling period, (b) population dynamics between VL3 (dotted blue) and PKKR1 (dotted red) in co-ferments. The sample size at each time point was n=3 for all measurements with error bars of ± SEM.
5.6.2 Experimental set-up and data gathering for large-scale systems biology analyses of the interaction between VL3 and PKKR1

Acquiring the transcriptome (Omri Yahel’s Masters Project), proteome and extracellular metabolites allowed for the identification of any potential biomarkers (mRNA proteins and metabolites) underpinning the interaction between VL3 and PKKR1 (Wang et al., 2008). Therefore it was important to design the experiment to capture the influence of these biomarkers during the co-fermentation process (Becker and Bern, 2011). As discussed in the previous section, the population dynamics between the two species was monitored, this information was used in this study along with population dynamics information in available in Anfang et al. (2009). Based on the population dynamics between VL3 and PKKR1 in co-ferments, three time points during fermentation of both co-ferments and mono-ferments were selected for further analyses using systems biology;

a) Day 2 of co-fermentation when the population size of PKKR1 is vastly greater than that of VL3

b) Day 9 when the population size of PKKR1 and VL3 are approximately 50:50

c) Day 16 when the population size of VL3 is greater than that of PKKR1

Thus, at each time point 3 samples of each of the VL3-only, PKKR-only and VL3-PKKR co-ferments were taken, to total nine per time point, for further analyses of mRNA, protein and extracellular metabolites.

In total, 27 samples were collected from the VL3-only (n=9), PKKR1-only (n=9) and co-ferments (n=9) at three different time points the wine samples were processed separately to analyse mRNA, protein and extracellular metabolites along with varietal aroma analyses (methods section 5.5.4). To obtain proteomic data, exactly 1 mL of yeast cells (v/v) from the different replicates were used for protein extraction. Also, exactly 50 µg of protein extract from each sample was used in the iTRAQ labelling and downstream processing. Extracellular metabolite data was obtained from wine from each sample.
5.6.3 Proteomic analyses of interaction between *S. cerevisiae* (VL3) and *P. kluyveri*

**PKKR1**

5.6.3.1 iTRAQ labelling strategy for proteomic analyses of the data set

As described in section (5.5.3.3), a maximum of 8 samples can be analysed in a single iTRAQ run. Thus it required 4 8-plex iTRAQ runs to completely analyse all the samples. A labelling strategy was used and it was driven by the number of 8-plex iTRAQ runs and comparisons between samples (VL3-only and VL3-PKKR). A Latin square design was used to ensure that each iTRAQ run was designed to incorporate the comparison between VL3-only (V) and VL3-PKKR1 (VP) fermentations from the three different sample time-points (Mertens *et al.*, 2012). This strategy was also used to avoid biological replicates of samples from being labelled using the same isobaric tags to prevent bias in data analyses as suggested by Karp *et al.* (2010). iTRAQ labels have a slight bias to specific peptides ending in amino acids proline (P) and Glutamine (Q) (Jüllig *et al.*, 2007; Karp *et al.*, 2010). The V and VP comparisons required 6 of the 8-plex labels; the remaining two labels were used to tag PKKR-only (P) samples. In total there were three 8-plex runs and a fourth run requiring only 3 labels for P samples, remaining 5 labels were used to re-run 1 previously failed sample and four other data poor samples to capture the complete proteomic data from fermentation samples. The layout of the samples in each run and their labels are shown in table 5.1.

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Table 5.1: The labelling system for ferment samples in iTRAQ (8-plex) runs. With samples identified as *S. cerevisiae* VL3-only (V), *P. kluyveri* PKKR1-only (P) and VL3-PKKR1 co-ferment (VP). Labels 1-3 refer to samples from day 2, 4-6 from day 9 and 7-9 from day 16. * indicates repeated samples.

5.6.3.2 Proteomic data generation and quality assessment

Each 8-plex iTRAQ run resulted in a single raw data file which incorporated data from the five salt steps used to fractionate the samples (see section 5.5.3.4 for the exact protocol). All the raw data files from the 2D LC-MS/MS runs were individually searched against the proteome of VL3 (fasta file...
format) using the ProteinPilot 2.0.1. although other strains of *S. cerevisiae* have been sequenced and have more proteomic information available in the public domain, VL3 genome was used in this study, the reasons for its use apart from being the organism used in this experiment is given below. The genome and proteome of VL3 was recently published by Borneman *et al.* (2011) from AWRI (Australian Wine Research Institute), in total, 4201 proteins were annotated, the genome of VL3 is different from that of the lab strain of *S. cerevisiae* S288C which is very well curated by SGD (*Saccharomyces* Genome Database, USA).

It was found that the industrial strain of *S. cerevisiae* VL3 displayed chromosomal copy number variation (CNV) and had an amplified Chr VIII. (Borneman *et al.*, 2011). Recent sequencing efforts of industrial yeasts such as EC 1118 have ignored heterozygous regions of its chromosomes during analyses, but Borneman *et al.* (2011) have shown that it is important to sequence the whole organism to understand the heterozygous variation in Insertions and Deletions (InDels) in SNPs (Single Nucleotide Polymorphisms) and also larger DNA fragments. It was suggested that these InDels (both heterozygous and homozygous) could contribute to differences in gene expression (Borneman *et al.*, 2011).

There were also differences in the location of a number of genes and importantly, around 20 novel genes were also identified. Therefore it was clear that using S288c proteome information in this study could yield unclear/incomplete data and since only the haploid genome of EC 1118 is currently available. VL3 proteome was appropriately used as the reference strain here. One disadvantage to using the current VL3 proteome was that it consisted of only 4201 proteins compared to S288c (21731, includes other S288c derived, isogenous strains, NCBI) and EC 1118 (6179, NCBI). An important fact of this study was that it also contained PKKR1, and there is little information available regarding this species’ genome and proteome (118 gene sequences and 6 protein sequences for *P. kluyveri* in NCBI). Therefore, the peptides from four iTRAQ runs were identified using only the VL3 proteome. Any peptide identified in the PKKR-only samples were considered for further data analyses to be homologous to VL3 as a 99% match factor was used in ProteinPilot 2.0.1. As the genome of PKKR1 is not sequenced, there is little information regarding the number of genes and proteins homologous to *S. cerevisiae* VL3 (Llorente *et al.*, 2000). Therefore a complete exclusion of all the peptides that were positively identified in PKKR1-only ferments from the entire data-set (VL3-only and VL3-PKKR1 protein profiles) could have severely limit the scope of this experiment. Furthermore, it was shown by Llorente *et al.* (2000) that there is a degree of gene redundancy in the genome of the *hemiascomyote* yeasts they studied and showed that around 130 proteins were homologous to *S. cerevisiae* proteins from *Pichia angusta* and *Pichia sorbitophila*, therefore it could be that similar homologous proteins exist in *P. kluyveri* PKKR1 when compared to VL3. This
reinforced the decision to consider any homologous peptides between PKK1 and VL3 for further data analyses.

A caveat of this study was that it was not possible to distinguish the individual contributions of VL3 and PKK1 in the protein profiles of VL3-PKK1 co-ferment samples. Another possibility that cannot be verified by this data-set is that there might be proteins/peptides only from PKK1 which are homologous to proteins from VL3 present in co-ferment which could potentially be altering the abundance of those proteins.

However, the aim of the study was to identify biomarkers (proteins) that were different between VL3-only ferment when compared to VL3-PKK1 co-ferments, irrespective of the source of the yeast in these conditions. The idea being that any identified biomarkers (irrespective of the yeast source) were responsible for any changes observed and will provide information regarding the mechanism of interaction between VL3 and PKK1.

Furthermore, when peptides were identified using ProteinPilot 2.0.1 using VL3 proteome, a simultaneous search with reversed sequence of the peptides was performed under similar condition to identify false discovery rates. This strategy is commonly used and was popularised by Gygi and others (Elias and Gygi, 2007). Also a bias correction was applied to remove any incorrect dissociation of cross linked peptides containing Proline (P) and Glutamine (Q) which could potentially contribute to incorrect assessment of the intensity of iTRAQ labels 115 and 116 (Jüllig et al., 2007). A conservative approach was utilised and any peptide spectra with a weak signal was not used in generating the raw identified peptide files (Jüllig et al., 2007).

5.6.3.3 Normalization of peptide data for statistical analyses

The raw identified peptide file, after bias correction, consisted of thousands of individual peptide peak area intensity information along with their associated identifications (if known), mass-to-charge ratios (m/z), charge states (z), and chromatographic retention times or characteristics (Becker and Bern, 2011). The total sum of peak area intensities of all the peptides belonging to a single protein was obtained for all the iTRAQ runs. The total intensities of different proteins had different numbers of individual peptide intensities contributing to their final score; this resulted in a range of total intensities for proteins. Also total intensities of the proteins (sum of individual peptide intensities for each protein) were different across the different iTRAQ runs. Sometimes the peptides representing a protein were completely absent. To reduce non-biological variance in the data normalization was performed before statistical analyses (Karp et al., 2010).
A common data normalization technique is to log\(_2\) transform the protein intensities across all the iTRAQ runs (Becker and Bern, 2011; Karp et al., 2010). However, in this study, log transformation was insufficient for normalizing the dataset (see figure 5.7).

![Figure 5.7: Box and Whisker plot of Log\(_2\) transformed iTRAQ data.](image)

Another commonly used technique is the geometric mean normalization, where the geometric mean of the total sum of the intensities of all the proteins identified within an iTRAQ label is calculated; this is followed by dividing all the individual protein intensities within that label with its geometric mean (Karp et al., 2010; King, 2006). When this normalization was performed for all the labels within all the different iTRAQ runs, it was found that the data sets were not completely normalized (boxplot not shown). This method can sometimes result in bias towards proteins with higher intensities which contribute more towards calculation of the mean compared to proteins with low intensities, i.e. this method is very sensitive to the outliers in the data set. Also, due to incomplete sequence information in my data set, this method would have introduced vast numbers of false positive discoveries. Therefore other robust techniques were investigated for normalization of the data set from this study.

### 5.6.3.4 Quantile-Quantile normalization of the proteomic data-set

Quantile normalization technique is commonly used in analyses of Proteome and Microarray datasets (Bolstad et al., 2003; Karp et al., 2010). It uses data from across all the different runs from an experiment for normalisation. In this scenario, quantile normalization was a non-parametric procedure which assumed that there was no difference in protein intensities of proteins across the different samples from different iTRAQ runs. Here, the 27 samples run in different iTRAQ runs were quantile normalised relative to each other. This method of normalization is based on the ideal that if two
samples had similar distributions, their quantile-quantile plot would show a straight diagonal line, if they were not similar they would not resemble a diagonally straight line. When this concept is extended to \( n \) data sets, if all the \( n \) data vectors are plotted on a quantile-quantile plot of \( n \) dimensions it would yield a straight line along the line given by the unit vector \((1/\sqrt{n}, \ldots, 1/\sqrt{n})\) (Bolstad et al., 2003).

Using the above method, the proteomic data set was made to have the same distribution by projecting the points of the \( n \) dimensional quantile plot of the data set onto a diagonal (Bolstad et al. 2003). This method is useful when there is a magnitude of difference in protein abundance data, which was seen in the proteomic data set with the samples from different ferments (e.g.: VL3 vs. VL3PKKR1 samples from day 2, 9 and 16).

Quantile normalization was performed using the ‘quantile.normalize’ command in the R package ‘PreprocessCore’ (www.bioconductor.org) (Bolstad et al., 2003). Figure 5.8 shows the normal quantile-quantile plot of the data set, the diagonal is tending towards being a straight line. This method is less affected by outliers (e.g.: difference in protein abundance from VL3PKKR day 2, day 9 and day 16) in sample compared to the geometric mean normalization and allowed for the rigorous testing of the null hypothesis.

Figure 5.8: Quantile-Quantile plot of Quantile normalised iTRAQ data set. The diagonally straight line is present to demonstrate the quantile normalization of the data set. Quantile normalisation was performed using “PreprocessCore” R package (Bolstad et al., 2003).
Log₂ transformation of the quantile normalised data set showed normal distribution (figure 5.9). Therefore quantile normalised proteomic data set was then used for all of the following statistical analyses to test the null hypothesis.

![Figure 5.9: Box and whisker plot of Log₂ transformed quantile normalised iTRAQ proteomic data set.](image)

5.6.3.5 Overall assessment of proteomic data

In this study, the iTRAQ analysis of samples from VL3-only, PKKR1-only and VL3-PKKR1 co-ferments from three different time-points identified 237 individual proteins, this constituted 5.6% of all the protein entries for VL3 in NCBI. The individual protein hits from the four different runs are tabulated below (table 5.2). There were proteins unique to a single iTRAQ run, and 161 proteins present in at least two iTRAQ runs and 67 were found in all the four runs.

This dataset consisted of information from fewer proteins compared to the only other iTRAQ study on wine yeast (Rossouw et al., 2010). 436 proteins were identified by Rossouw et al. (2010) using 8-plex iTRAQ when examining the differences in wine yeast fermentation kinetics between VIN13 (Anchor Yeast, South Africa) and BM45 (Lallemand Inc., Canada). Ten salt steps were utilised to fractionate protein samples before MS/MS and also S288c proteome information from SGD was used in their identification (Rossouw et al., 2010). In this study, many factors could have contributed to the low numbers of proteins being identified, such as; the use of VL3 proteome with 4201 protein entries (NCBI protein entries) to identify proteins, when compared to 19,262 entries for S. cerevisiae S288c.
and 6,171 entries for EC1118 and the fact that only five salt steps were utilised to fractionate the samples before analysis through MS/MS (Borneman et al., 2011).

The 2D gel electrophoresis coupled MS analyses of EC1118 during fermentation yielded in the identification of 59 proteins by Rossignol et al. (2009). In another study, 2D gel-electrophoresis study on a wild-type *S. cerevisiae*, only about 33 proteins were unambiguously identified (Trabalzini et al., 2003). Thus in comparison, iTRAQ methodology was capable of identifying more proteins from oenological yeasts.

<table>
<thead>
<tr>
<th>Run</th>
<th>peptides</th>
<th>Total proteins</th>
<th>Proteins unique to run</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1143</td>
<td>134</td>
<td>16</td>
</tr>
<tr>
<td>2</td>
<td>1725</td>
<td>163</td>
<td>27</td>
</tr>
<tr>
<td>3</td>
<td>1602</td>
<td>152</td>
<td>17</td>
</tr>
<tr>
<td>4</td>
<td>1353</td>
<td>127</td>
<td>18</td>
</tr>
</tbody>
</table>

Table 5.2: Number of Peptides and proteins identified in different iTRAQ runs from mono and co-ferments of *S. cerevisiae* VL3 and *P. kluyveri* PKKR1.

### 5.6.3.6 Testing the null hypothesis using proteomic data

Testing the null hypothesis required the direct comparison of protein profiles between VL3-only and VL3-PKKR1 co-ferments. This was done by performing a two-way ANOVA with interactions (ferment-type and time-point as the two factors), followed by a student’s t-test to compare individual proteins between these two fermentation conditions. Since multiple comparisons were being made to test the null hypothesis, the P-value obtained from student’s t-test was subjected to a False Discovery Rate (fdr) control (King, 2006). In this study q-value for the comparisons was directly estimated rather than setting a level to control for fdr.

Here a linear model ANOVA was used to relate the response of two different samples (VL3-only and VL3-PKKR1 co-ferment) to three different time-points (day 2, day 9 and day 16) during fermentation. A two-way ANOVA P-value was statistically insignificant (ferment-type = 0.9, time-point = 1, and the interaction between the two factors: ferment-type: time-point = 1) for proteomic profile comparison between VL3-only and VL3-PKKR1 ferments. Thus, the null hypothesis that there is no difference between VL3 and VL3-PKKR1 profiles is supported by this data set. Within the constraints
of this experiment and analysis, the addition of PKKR1 in co-ferment did not significantly alter the proteome of VL3.

5.6.3.7 Pair-wise comparison of individual proteins between VL3-only and VL3-PKKR1 co-ferments

Although, the null hypotheses held true for proteomic data-set as a whole, the pair-wise comparison of individual proteins was undertaken to identify any potentially significant change in biomarkers (proteins). A student’s t-test with adjusted P-values (fdr control) yielded 8 proteins with P-value < 0.1 (with 5 proteins with P-value < 0.05) that were significantly different between VL3-only and VL3-PKKR1 co-ferments. They are listed in the table below (Table 5.3). It can be noted that none of these proteins show at-least two fold difference between the two profiles which is usually an indicator of significant alteration in biological activity (Becker and Bern, 2011).

<table>
<thead>
<tr>
<th>Protein name</th>
<th>VL3-PKKR1/ VL3-only</th>
<th>P-value (fdr)</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aco1p</td>
<td>1.05</td>
<td>0.004</td>
<td>Aconitate hydratase I (Carbohydrate Metabolism)</td>
</tr>
<tr>
<td>Act1p</td>
<td>1.06</td>
<td>0.001</td>
<td>actin beta/gamma 1 (Phagosome)</td>
</tr>
<tr>
<td>Rps26bp</td>
<td>0.95</td>
<td>0.020</td>
<td>small subunit ribosomal protein (Translation)</td>
</tr>
<tr>
<td>Pdi1p</td>
<td>1.05</td>
<td>0.008</td>
<td>protein disulfide-isomerase A1 (Protein processing in endoplasmic reticulum)</td>
</tr>
<tr>
<td>Atp1p</td>
<td>1.10</td>
<td>0.004</td>
<td>F-type H+-transporting ATPase subunit alpha (Oxidative phosphorylation)</td>
</tr>
<tr>
<td>(P-value &lt;0.1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tma19p</td>
<td>0.95</td>
<td>0.071</td>
<td>Hypothetical protein</td>
</tr>
<tr>
<td>Atp2p</td>
<td>1.06</td>
<td>0.095</td>
<td>F-type H+-transporting ATPase subunit beta (Oxidative phosphorylation)</td>
</tr>
<tr>
<td>Eft2p</td>
<td>1.01</td>
<td>0.057</td>
<td>elongation factor 2</td>
</tr>
</tbody>
</table>

Table 5.3: A list of proteins significantly different between VL3-only and VL3-PKKR1 co-ferments. The functions of the listed proteins were obtained from KEGG database (Kanehisa and Goto., 2000). A ratio of 1 suggests no difference between the two ferments, greater than 1 indicates higher abundance of protein in VL3-PKKR1 while lower than 1 indicates higher abundance in VL3-only ferment.
5.6.3.8 ANOVA and pair-wise comparison of protein profiles at different time points within VL3-only and VL3-PKKR1 ferments

There were differences in population sizes and ferment kinetics within VL3-only and VL3-PKKR1 co-ferments during the sampling periods. Therefore, an ANOVA was performed to test for any differences in the overall protein profiles of VL3-only and VL3-PKKR1 co-ferments at individual time-points (at day 2, day 9 and day 16). This ANOVA, resulted in insignificant P-value for both VL3-only (P-value= 0.9) and VL3-PKKR1 (P-value=0.9). Suggesting that there was no significant change in the overall protein profile of either VL3-only or VL3-PKKR1 ferments through the different time points during fermentation.

This result is contrary to studies monitoring change in gene expression in S. cerevisiae during fermentation (Puig and Perez-Ortin, 2000). During fermentation of grape must to wine; S. cerevisiae gene expression is driven by Stress Response Elements (STRE) (Puig and Perez-Ortin, 2000). Also during the initial shift of S. cerevisiae metabolism from respiration to fermentation, many stress related genes such as HSP12 and HSP26 are up-regulated (Puig and Perez-Ortin, 2000).

However, the common conundrum of systems biology is the lack of correlation between data using different ‘omes (Newman et al., 2006). Also, Newman et al. (2006) suggested that proteins involved in responding to environmental stimuli such as those involved in stress response are subject to stochastic variation. This variation could be attributed to difference in response of individual yeast cell to stimuli which is the cornerstone of phenotypic variability. However, the contribution of this noise from a yeast population (from different replicates) increases the complexity of the protein data. In this study, stochastic noise in protein levels in the various ferments coupled with insufficient sequence data and a rigorous data normalisation could have contributed to the lack of difference between mono and co-ferments (Newman et al., 2006; Rossouw and and Bauer, 2009).

However, an ANOVA performed on individual proteins between time-points within a single ferment-type resulted in the identification of a few proteins significantly different between the different time points within VL3-only and VL3-PKKR1 ferments (fdr control included, with all protein hits above q-value ≥ 0.05 being excluded). Interestingly, there were 11 common proteins that were significantly different at the different time points for both VL3-only and VL3-PKKR1 ferments, while 6 proteins differed significantly between the three time points for VL3-only ferments and 7 proteins differed in VL3-PKKR1 co-ferment.

When comparing the actual fold-change differences between these (significantly different) proteins at different time points for both VL3-PKKR1 and VL3-only ferments, none were greater than two-fold (Becker and Bern, 2011). In fact the differences between the proteins abundance at different times were negligible. Also, there were negligible differences when comparing the protein abundance of the
11 common proteins between the ferments. Therefore I refrained from making physiological inferences. In the table below (Table 5.4), fold-change of the significant proteins from day 2 - day 9 and day 2 - day 16 are shown for both VL3-PKKR1 ferments and VL3-only ferments. Only Heat Shock Proteins (hsp12p, hsp26p) showed any considerable change from the initial time point. These proteins are heavily involved in buffering stress on the yeast cells and seem to be important in tolerance of ethanol stress in S. cerevisiae (Stanley et al., 2010; Zaman et al., 2008). These results could also be due to the lack of reproducibility among biological replicates within each time-point as none of these proteins were significantly different enough to cause a global change in protein profile (Newman et al., 2006).

<table>
<thead>
<tr>
<th>Name</th>
<th>Function</th>
<th>Day9/day2</th>
<th>Day16/day2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tdh1p</td>
<td>glyceraldehyde 3-phosphate dehydrogenase</td>
<td>1.099</td>
<td>1.165</td>
</tr>
<tr>
<td>YHR138C-like protein</td>
<td>hypothetical protein</td>
<td>1.127</td>
<td>1.217</td>
</tr>
<tr>
<td>Hsp12p</td>
<td>Heat Shock Protein</td>
<td>1.080</td>
<td>1.259</td>
</tr>
<tr>
<td>Eft2p</td>
<td>translation elongation factor 2</td>
<td>0.985</td>
<td>0.982</td>
</tr>
<tr>
<td>Cpr1p</td>
<td>Peptidyl prolyl isomerase</td>
<td>1.082</td>
<td>1.111</td>
</tr>
<tr>
<td>Pglk1p</td>
<td>phosphoglycerate kinase</td>
<td>1.017</td>
<td>1.017</td>
</tr>
<tr>
<td>Hsp26p</td>
<td>Heat Shock Protein</td>
<td>1.260</td>
<td>1.304</td>
</tr>
<tr>
<td>Tef1p</td>
<td>Translation elongation factor EF-1beta</td>
<td>0.971</td>
<td>0.972</td>
</tr>
<tr>
<td>Ygp1p</td>
<td>Asparaginase (amidohydrolase)</td>
<td>1.035</td>
<td>1.149</td>
</tr>
<tr>
<td>Asc1p</td>
<td>activating signal co-integrator 1 complex subunit 1</td>
<td>0.931</td>
<td>0.915</td>
</tr>
<tr>
<td>Met17p</td>
<td>O-acetyl homoserine (thiol)-lyase</td>
<td>1.080</td>
<td>1.112</td>
</tr>
<tr>
<td>Tdh3p</td>
<td>glyceraldehyde 3-phosphate dehydrogenase</td>
<td>0.968</td>
<td>m</td>
</tr>
<tr>
<td>Act1p</td>
<td>actin beta/gamma 1</td>
<td>0.983</td>
<td>m</td>
</tr>
<tr>
<td>Rps6bp</td>
<td>small subunit ribosomal protein S6e</td>
<td>0.942</td>
<td>m</td>
</tr>
<tr>
<td>Atp1p</td>
<td>F-type H⁺-transporting ATPase subunit alpha</td>
<td>0.974</td>
<td>m</td>
</tr>
<tr>
<td>Vma4p</td>
<td>V-type H⁺-transporting ATPase subunit E</td>
<td>1.075</td>
<td>m</td>
</tr>
<tr>
<td>Rps11bp</td>
<td>small subunit ribosomal protein S11e</td>
<td>0.944</td>
<td>m</td>
</tr>
<tr>
<td>Ilv5p</td>
<td>ketol-acid reductoisomerase</td>
<td>1.035</td>
<td>m</td>
</tr>
<tr>
<td>Pep4p</td>
<td>Saccharopepsin</td>
<td>m</td>
<td>1.065</td>
</tr>
<tr>
<td>Uga1p</td>
<td>4-aminobutyrate aminotransferase / (S)-3-amino-2-methylpropionate transaminase</td>
<td>m</td>
<td>1.169</td>
</tr>
<tr>
<td>Rpl1bp</td>
<td>large subunit ribosomal protein L10Ae</td>
<td>m</td>
<td>0.921</td>
</tr>
<tr>
<td>Cdc19p</td>
<td>pyruvate kinase</td>
<td>m</td>
<td>0.987</td>
</tr>
<tr>
<td>Gph1p</td>
<td>starch phosphorylase</td>
<td>m</td>
<td>1.187</td>
</tr>
<tr>
<td>Sam1p</td>
<td>S-adenosyl methionine synthetase</td>
<td>m</td>
<td>0.932</td>
</tr>
</tbody>
</table>

Table 5.4: A List of proteins that are different in VL3-only and VL3-PKKR1 ferments; day9/day2 and day16/day2 comparisons are shown. (m) Indicates missing values. Only protein abundances with significant q-value of ≤ 0.05 are included in this table. A ratio of 1 suggests no difference between the two ferments; greater than 1 indicates higher abundance of protein in VL3-PKKR1 while lower than 1 indicates higher abundance in VL3-only ferment.
5.6.3.9 Developing a model co-ferment

Although, ANOVA showed that there was no difference between the protein profiles between VL3-only and VL3-PKKR1 co-ferments, there was evidence that co-fermentation altered the growth of VL3 when compared to VL3-only ferment. Firstly, the population size of both VL3 and PKKR1 were lower in co-ferment compared to their mono-ferment counterparts as seen in figure 5.6. Secondly, the fermentation kinetics was different between VL3-only and VL3-PKKR1 ferments (appendix 5). Also, pair-wise comparison of individual protein abundances along with pair-wise comparison of change in protein abundance over different time points yielded in the identification of significant proteins. Finally, the level of varietal thiol 3MHA was significantly higher in VL3-PKKR1 when compared to VL3-only ferment on day 2 (P-value < 0.0001) and day 9 (P-value = 0.001) despite lower population size (discussed further in section 5.6.6). Therefore, a model ferment was constructed to test for the impact of PKKR1 in co-fermentation, it was based on the model constructed by Anfang et al. (2009) to test for an additive effect on 3MHA production(VL3 + PKKR1) in co-ferment compared to single ferment(VL3-only or PKKR1-only).

The null hypothesis in the study by Anfang et al. (2009) stated that the observed increase in levels of 3MHA in co-ferments was due to an additive effect of the levels of 3MHA produced individually by VL3 and PKKR1 in co-ferment. This hypothesis was proposed due to the fact that the population size of VL3 and PKKR1 in co-ferments were lower than their mono-ferment counterparts. Since there was no significant difference between the proteome of VL3-only and VL3-PKKR1 co-ferment, it was proposed that the protein profile of VL3-PKKR1 co-ferment could be explained by adding the profiles of VL3-only and PKKR1-only co-ferments at the same time-points during fermentation after considering for differences in their population sizes. In other words, as there was an apparent lack of interaction between VL3 and PKKR1 causing changes to VL3 proteome in co-ferment, the proteome of the co-ferment was the sum of VL3 and PKKR1 proteomes. The model for a single protein, thus the whole proteome at a single time-point can therefore be described below:

\[
(n \times x) + (q \times y) = z
\]

\(n = \text{Proportion of VL3 in co-ferment}\)

\(q = \text{Proportion of PKKR1 in co-ferment}\)

\(x = \text{Concentration of Protein A in VL3-only ferment}\)

\(y = \text{Concentration of Protein A in PKKR1-only ferment}\)

\(z = \text{Concentration of protein A in mVP}\)

\textit{Equation 5.1: Equation to calculate theoretical protein abundance in model co-ferment mVP.}
Thus, a model proteome (mVP) for co-ferment was constructed based on the equation for a single protein as shown above for the three different time-points. The proportion of VL3 and PKKR1 in co-ferments on day 2, day 9 and day 16 were obtained by monitoring the change in population dynamics (see figure 5.6 for population dynamics). The supplemental data for this model proteome is available on CD provided.

### 5.6.3.10 ANOVA between VL3-PKKR1 and mVP

A null hypothesis was proposed that there was no difference between the proteome of VL3-PKKR1 co-ferment and the model co-ferment mVP. This null hypothesis was proposed to explain a possible additive effect of the proteins from VL3 and PKKR1 in co-ferment, thus explaining the lack of difference between VL3-only and VL3-PKKR1 proteomes identified using only the published VL3 proteome (Borneman et al., 2011). A two-way ANOVA (ferment-type and time-point as the two factors) was performed to test the modified null hypothesis, the resulting P-value was statistically insignificant (ferment-type = 0.9, time-point = 0.9, the interaction between two factors: ferment-type: time point = 0.9). Thus, the null hypothesis held true, i.e. the co-ferment protein profile is the result of an additive protein profile of its respective yeasts when fermented individually.

Although, the null hypothesis could not be disproved, many caveats to this study should be taken into account; lack of coverage of PKKR1 proteins, assumption of the homology of proteins identified in PKKR1 to VL3 and the availability of only a partial proteome for VL3 (Borneman et al., 2011).

However, the observed differences in the level of 3MHA (thiol) produced by co-fermentation in this experiment and by Anfang et al. (2009) cannot be ignored. Also the differences in population size and ferment kinetics between mono and co-ferments cannot be discounted. The difference in 3MHA levels between VL3-only and VL3-PKKR1 co-ferment could be due to changes in one or few proteins not sufficient to produce a large-scale change in the proteome of VL3 necessary to disprove the proposed null hypothesis (Newman et al., 2006). The differences observed could also be due to changes in levels of 3MHA produced by PKKR1 in response to the presence of VL3 in co-ferments as PKKR1 on its own is capable of producing 3MHA (Anfang, 2010). According to this proteomic data set, the above mentioned caveats and any changes to the proteome of PKKR1 cannot be directly confirmed using shot-gun proteomics, due to lack of genome and proteome information. Moreover, extracellular metabolite profile for the same experiment was also obtained. This data set and the varietal thiol data for the ferments are discussed in the next section.
5.6.4 Analyses of extracellular metabolite data from mono and co-ferments of VL3 and PKKR1

5.6.4.1 Data normalisation

Data normalisation of the extracellular metabolite profiles from different samples was straightforward compared to that of the proteomic data-set for this experiment. When preparing the samples for extracellular metabolite analyses, a known concentration of an internal standard (D4 Alanine) was added, this allowed for rigorous normalisation of the entire data-set by the abundance of the internal standard in each sample. Also this method of derivitizing metabolites from a sample followed by prescribed data analyses was adapted from Smart et al. (2010) using the “Metab” R package (Aggio et al., 2011).

Following normalisation by internal standard, the samples were also normalised for their population size, this normalisation was carried out to prevent exaggeration of metabolite abundance in the various mono and co-ferments (Aggio et al., 2011). Population size normalisation was unique to extracellular metabolite profiling as proteomic data was obtained from a defined volume of cells. Here even though a defined volume of wine was used (50 µL), the population size contributing to the metabolite profile in that volume was different.

Finally, the extracellular metabolite profile was also normalised (subtracted by) the metabolite profile of sterile grape juice used as control. This is especially important as grape juice is a different media compared to wine being produced by the various ferments and importance was given to metabolites useful in identifying any differences between these ferments (Aggio et al., 2011).

5.6.4.2 Overall assessment of the normalised metabolite data

Overall, analyses of extracellular metabolites from all the samples resulted in the identification of 83 metabolites from the reference library, of which 53 were identified in all the replicates. To my knowledge, this data set is the only one where the extracellular metabolites from Sauvignon Blanc fermentation (mono and co-ferments of VL3 and PKKR1) that were analysed using MCF derivitization (Villas-Bôas et al., 2003). It is possible that some of the metabolites were unable to be analysed using MCF derivitization due to either their thermo-labile property or their inefficiency in being derivitised by methyl chloroformate (Villas-Bôas et al., 2003). The number of metabolites identified were also extremely limited by the number of metabolites present in the reference library used in their identification (n= 243, personal communication R.Aggio), although this limitation could
be circumvented by analysis of raw peak data. However, raw peak data analysis was not performed as it was beyond the scope of this thesis.

5.6.4.3 Testing the null hypothesis using extracellular metabolites

The null hypothesis proposed that there was no difference in extracellular metabolite profiles of VL3-only ferments compared to VL3-PKKR1 co-ferments across three different time-points during fermentation. A pair-wise student’s t-test was also performed with fdr control to identify any metabolites with at-least two-fold change between VL3 and VL3-PKKR1 ferments (Biomarkers).

Upon performing a two-way ANOVA on VL3-only and VL3-PKKR1 co-ferments, a statistically significant P-value was obtained for the ferment-type effect, showing that the metabolite profiles of the two different ferments were different irrespective of the three time-points (ferment=0.02*, time-point=0.9, interaction effect ferment-type: time-point=0.12). This meant that the null hypothesis could be negated as P-values showed that the VL3-only ferment had a significantly different profile of extra cellular metabolites compared to VL3-PKKR1 co-ferments.

5.6.4.4 Pair-wise t-test on extracellular metabolite profile of VL3-only and VL3-PKKR1 ferments

Pair-wise t-test on all the identified extracellular metabolites in VL3-only and VL3-PKKR1 ferments yielded 15 metabolites which were significantly different between the two ferment-treatments (table 5.5). Among the significantly different metabolites; three metabolites had a greater than two-fold change comparing VL3-PKKR1 over VL3-only ferment, they were; Leucine (2.88 fold), Glutamic acid (4.6 fold) and Isoleucine (-4.5 fold), they can be considered as potential biomarkers to indicate the differences between the two ferments (Becker and Bern, 2011). However, further research is required before they provide biological inference. Any negative value indicates a greater abundance of a metabolite in VL3-only ferment compared to its abundance in VL3-PKKR1 co-ferment.

Metabolites such as Threonine (-1.34 fold), 2- isopropyl malic acid (1.5 fold), 2-oxoglutaric acid (1.25 fold) and Glutaric acid (1.08 fold) played an important role in contributing to the differences in the resulting wine from the two ferments.
Table 5.5: A list of metabolites significantly different between VL3-only and VL3-PKKR1 co-ferments. Metabolites with * indicate fold-change difference significant enough to be classified as a potential biomarker (Becker and Bern, 2011). † indicates metabolites tending towards being potential biomarkers. A negative fold-change indicates that a particular metabolite was in greater abundance in VL3-only ferment compared to VL3-PKKR1 co-ferment.

<table>
<thead>
<tr>
<th>Metabolite name</th>
<th>VL3-PKKR1/VL3-only</th>
<th>P-value (fdr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leucine*</td>
<td>2.8873</td>
<td>0.0294</td>
</tr>
<tr>
<td>Threonine†</td>
<td>-1.3479</td>
<td>0.0003</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.5274</td>
<td>0.0056</td>
</tr>
<tr>
<td>Glyceral acid</td>
<td>0.2135</td>
<td>0.0255</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.0885</td>
<td>0.0007</td>
</tr>
<tr>
<td>Glutamine</td>
<td>0.5170</td>
<td>0.0011</td>
</tr>
<tr>
<td>Acotinic acid</td>
<td>0.5055</td>
<td>0.0133</td>
</tr>
<tr>
<td>Glyoxylic/glyoxalic acid</td>
<td>0.7708</td>
<td>0.0189</td>
</tr>
<tr>
<td>Glutamic acid*</td>
<td>4.6336</td>
<td>0.0140</td>
</tr>
<tr>
<td>Isoleucine*</td>
<td>-4.5373</td>
<td>0.0000</td>
</tr>
<tr>
<td>Serine</td>
<td>0.4659</td>
<td>0.0300</td>
</tr>
<tr>
<td>2-isopropylmalicacid†</td>
<td>1.5009</td>
<td>0.0000</td>
</tr>
<tr>
<td>2-oxoglutaricacid†</td>
<td>1.2523</td>
<td>0.0139</td>
</tr>
<tr>
<td>Glutaric acid†</td>
<td>1.0840</td>
<td>0.0125</td>
</tr>
</tbody>
</table>

5.6.4.5 Pair-wise Student’s t-test on extracellular metabolite profile between VL3-only and VL3-PKKR1 ferments at each time point

Pair-wise Student’s t-tests performed (time point as factor) on metabolite profile between VL3-only and VL3-PKKR1 co-ferments at the three different sample time-points to observe and track metabolite changes (with all metabolite hits with a P-value ≥ 0.05 being excluded). At day 2 only 4, at day 9 only 6 and on day 16 only 8 of the metabolites were found to be different between VL3-only and VL3-PKKR1 co-ferment. Furthermore, none of the metabolites were found to be significantly different between the two ferments at the three sample time points. However, a lack of reproducibility cannot be discounted as a possible explanation. The table below (table 5.6) compares the fold-
difference between these metabolites in VL3-PKKR1 over VL3-only ferment. Negative value indicates greater abundance in VL3-only ferments across the three time points.

<table>
<thead>
<tr>
<th>Metabolite name</th>
<th>day 2</th>
<th>day 9</th>
<th>day 16</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glyceric acid</td>
<td>-0.5149</td>
<td>m</td>
<td></td>
</tr>
<tr>
<td>Succinic acid</td>
<td>2.0886</td>
<td>m</td>
<td>0.7212</td>
</tr>
<tr>
<td>Acotinic acid</td>
<td>1.4286</td>
<td>m</td>
<td>3.3381</td>
</tr>
<tr>
<td>Alanine</td>
<td>1.5531</td>
<td>m</td>
<td>0.2982</td>
</tr>
<tr>
<td>Oxaloacetic acid</td>
<td>M</td>
<td>-1.6665</td>
<td>m</td>
</tr>
<tr>
<td>Itaconic acid</td>
<td>M</td>
<td>-0.0446</td>
<td>m</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>M</td>
<td>0.5623</td>
<td>0.4987</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>M</td>
<td>4.5700</td>
<td>m</td>
</tr>
<tr>
<td>2-isopropylmalic acid</td>
<td>M</td>
<td>1.4362</td>
<td>1.5712</td>
</tr>
<tr>
<td>Caproic acid</td>
<td>M</td>
<td>0.9159</td>
<td>1.5465</td>
</tr>
<tr>
<td>Caprinate</td>
<td>M</td>
<td>m</td>
<td>m</td>
</tr>
<tr>
<td>Caprylate</td>
<td>M</td>
<td>m</td>
<td>0.9514</td>
</tr>
<tr>
<td>Isocitric acid</td>
<td>M</td>
<td>m</td>
<td>-0.2741</td>
</tr>
</tbody>
</table>

Table 5.6: A list of extracellular metabolites that are significantly different between both VL3-only and VL3-PKKR1 ferments; day2, day 9 and day 16 comparisons are shown; (m) indicates missing values. Only protein abundances with a significant q-value of ≤ 0.05 were included. Negative metabolite values indicate that abundance was greater in VL3-only ferment.

5.6.4.6 ANOVA between extracellular metabolite profile of VL3-PKKR1 co-ferment and model ferment (mVP)

A two-way ANOVA was carried out between VL3-PKKR1 co-ferment and a model ferment derived from the additive model used by Anfang et al. (2009) as shown in the equation 5.1 in section 5.6.3.9 to account for any additive effects of extracellular metabolites from PKKR1 and VL3 in co-ferment. Any additive effects precludes from concluding an interaction between PKKR1 and VL3 in co-ferment.

Before conducting the ANOVA, the entire data-set consisting of VL3-only, PKKR1-only and VL3-PKKR1 co-ferment were normalised only by internal standard and from the metabolite profile of sterile juice, biomass normalisation was not performed. Following normalisation, the model co-
ferment metabolite profile was obtained by adding together the metabolite profile of PKKR1-only and VL3-only ferments at different time points accounting for their difference in population size.

A two-way ANOVA with factors of ferment-type and time-point was performed, it resulted in a significant P-value for the factor ferment-type (P-value =3.863e^{-12}) while the P-values for time point (P- value = 0.39) and the interaction effect (ferment-type: time point P-value = 0.37) were not statistically significant. Therefore in conclusion, there were significant differences between the ferment types with both direct comparison (VL3-only and VL3-PKKR1) and comparison between co-ferment and model ferment (mVP) was carried out. This suggests that there are differences in global extracellular metabolite profiles between VL3-only and VL3-PKKR1 co-ferment which cannot be explained in terms of an additive effect. Thus, the differences observed in extracellular metabolite profiles between VL3-only and VL3-PKKR1 can be attributed to an interaction between VL3 and PKKR1.
5.6.5 Conclusions from testing the null hypothesis using proteomic and extracellular metabolite data

The proteomic data set identified 237 proteins overall, 67 were found in all the 4 iTRAQ runs, null hypothesis testing that there is no difference between VL3-only and VL3-PKKR1 co-ferment using both against the actual data set and also a model data set were not disproved. However, pair-wise t-test on entire data set yielded 5 proteins that were significantly different between VL3-only and VL3-PKKR1 co-ferments, although none were different by more than two-fold which is commonly used as an indicator of a biomarker (Becker and Bern, 2011). Pair-wise t-test comparing between VL3-only and VL3-PKKR1 co-ferment at individual time points yielded proteins that were significant within a ferment-type and also 11 common proteins differing at different time points. Heat shock proteins Hsp12p and Hsp16p showed a noticeable change between time points, this is to be expected as they play an important role in cell’s stress response mechanism (Stanley et al., 2010; Zaman et al., 2008).

Extracellular metabolite data identified 83 metabolites overall, of which 53 were commonly present. Null hypothesis testing for a lack of difference between VL3-only and VL3-PKKR1 co-ferment showed that there was a significant difference between the two ferment-types for both the actual and model dataset comparisons. However, significant changes in overall metabolite profiles with time was not observed; this result although ties in with lack of difference observed in proteomic data with change in time, it does not agree with other studies examining the diauxic shift in *S. cerevisiae* (Rossouw and and Bauer, 2009; Stanley et al., 2010). Pair-wise t-test of individual metabolites yielded Leucine, Glutamine and Isoleucine as potential biomarkers with greater than two fold difference between the ferment-types. These metabolites are important in pathways involved in nitrogen metabolism and production of fusel alcohols (Rossouw and and Bauer, 2009; Styger et al., 2011). In the future, a targeted experiment with isotope labelled metabolites need to be conducted to reliably use these metabolites as biomarkers identifying the interaction between VL3 and PKKR1 in co-ferment (Vivanco et al., 2011).

Thus in conclusion, upon testing the null hypothesis that there is no difference in proteomic or extracellular metabolite profile between VL3-only and VL3-PKKR1 co-ferment, I obtained mixed results. The extracellular metabolite comparison between the two ferments is not in line with findings from the proteomic data. There are many reasons for this discrepancy; firstly, the data interpretation and normalisation of proteomic data could have had a major impact on the outcome of the null hypothesis testing. Secondly the biological nature of the two different ‘omes contributed significantly to the overall outcome of null hypothesis testing (Newman et al., 2006; Rossouw and and Bauer, 2009).
Data interpretation and normalisation techniques between the two ‘omic profiles varied vastly (Rossouw and and Bauer, 2009). Proteomic data was limited by the lack of genomic/proteomic information on PKK1, also the genome annotation of VL3 is still in progress and has lower hits compared to other S. cerevisiae which have been sequenced (Borneman et al., 2011). Lack of internal standard in the in proteomic data-set coupled with incomplete protein information forced a very conservative approach to the normalisation (Bolstad et al., 2003). Also only 5 salt steps were used to extract all the different peptide information from the 4 different iTRAQ runs, usually, 10 to 20 salt steps are utilised to obtain a greater depth (numbers) of proteomic data (Jüllig et al., 2007; Rossouw et al., 2010). Perhaps in the future, a targeted hypothesis coupled with molecular genetics technique such as gene-knockout could be used to delineate a specific interaction between PKK1 and VL3.

On a biological level, proteins are species specific and protein expression, purification and identification involves complex and often cost prohibitive processes (Wang et al., 2008). On the other hand metabolites are small organic compounds which are identical across species and are routinely used to verify end products of gene expression (Oliver et al., 1998). In this instance lack of genome and protein data of the two species VL3 and PKK1 was not a hindrance to analysing any potential interaction between them.

However, the metabolite profiles generated for this study were severely limited by the number of metabolites in the reference library; the results obtained do not by any means provide a complete picture of the interaction between VL3 and PKK1. Furthermore, numerous biological replicates and at least 6 technical replicates are usually recommended to accurately confirm the metabolite abundances, this rigorous testing is a physical limiting factor when testing multiple hypotheses (Villas-Boas et al., 2007). Also not all metabolites are sensitive to the protocol utilised in the above section due to issues in polarity and thermo sensitivity making them unsuitable for analyses (Villas-Boas et al., 2007). Perhaps a semi targeted metabolite analyses of volatile aroma compounds can provide a more direct result as with 3MHA level in co-ferments of VL3 and PKK1 (Anfang et al., 2009).
5.6.6 Comparison of 3MHA and 3MH levels between VL3-only, VL3-PKKR1 and model co-ferment (mVP)

Quantification of the thiol 3MHA by Anfang et al. (2009) followed by comparison between mono-ferments of VL3 and co-ferment of VL3-with PKKR1 led to their conclusion that, a synergistic interaction between the two species was probably responsible for the more than two-fold change observed in the thiol (3MHA) level. As discussed in the section above, they used an additive model to disprove the null hypothesis of no interaction between the two species during co-fermentation.

Here, 3MHA levels were quantified using similar techniques to those described by Anfang et al. (2009) for VL3-only and VL3-PKKR1, subsequently 3MHA values were also calculated for the additive model based on the population size of VL3-only and PKKR1-only samples (as described in section 5.6.3.9). Thiol levels were quantified only on days 2, 9, 16 which were also the sampling days to obtain proteomic and metabolomics data (refer section 2.8.7.3 for method for quantification of thiols). Fold difference was calculated for VL3-PKKR1 over VL3 similar to Anfang et al. (2009.

Additionally, fold difference was also calculated for VL3-PKKR1 over mVP to detect any discrepancy between the additive model and the actual co-ferment.

A Student’s t-test was used to identify any significant differences in the 3MHA profiles (figure 5.10). It can be seen that there is less than two-fold difference observed between co-ferment and model ferment (mVP) at all the three time-points. Interestingly, the difference in 3MHA between VL3-PKKR1 and mVP in significant on day 2 (P-value < 0.001) and day 9 (P-value < 0.001) and not significant at day 16 (P-value 0.14). Thus, it can be seen that additive model co-ferment generally differs from the actual VL3-PKKR1 co-ferment except on day 16 .However, these data are not in line with the findings from Anfang et al. (2009) as there was a lack of two fold change in the 3MHA concentration between VL3-PKKR1 and VL3-only ferments (final concentration).
Figure 5.10: Level of thiol 3MHA (ng/L) in VL3-PKKR1, VL3-only and model co-ferment (mVP) at three different time-points. Error bars are indicative of ±SEM (n=3). The top number indicates fold-change for VL3-PKKR1 over mVP; the bottom number indicates fold-change for VL3-PKKR1 over VL3-only ferments. ** indicates P-value < 0.001, *** indicates P-value < 0.0001

The differences observed in the 3MHA levels between this study and one conducted by Anfang et al. (2009) could be attributed to both yeast population size and the difference in juice composition. Firstly, at time-point 16 there was a magnitude of difference with PKKR1 = 1.26 \times 10^7 \text{cfu/mL} and VL3 = 5.12 \times 10^7 \text{cfu/mL} compared to < 1.0 \times 10^7 \text{cfu/mL} for both PKKR1 and VL3 observed by Anfang et al. (2009). There was a difference in the population dynamics of these species, although VL3 and PKKR1 followed similar pattern in population dynamics until day 9, PKKR1 seemed to persist in higher numbers in ferment carried out for this study (see section 5.6.1.1). Nearly 20% of the population on day 16 of the co-ferment was PKKR1 as opposed to only around 10% on day 16 observed by Anfang et al. (2009). The composition of juice used for the two studies were also markedly different with juice used in this study consisting of 17.8° Brix, YAN of 280 mg/L and TA of 6 compared to 22.2° Brix, YAN of 330 mg/L and TA of 9.9 found in Sauvignon Blanc juice used by Anfang et al. (2009). The increased cell numbers of yeast coupled with lower YAN, Brix and TA could have contributed to the altered population dynamics after day 9 which could have impacted on the final 3MHA level of the wine (Anfang et al. 2009, Anfang 2010).

It is possible that the 3MHA in co-ferment could have been enzymatically converted into 3MH (Swiegers et al., 2006b). However, it should be noted that in this study, (Figure 5.11) the level of 3MH in the co-ferment (P-value =< 0.0001) and the additive model co-ferment (P-value =< 0.0001) were significantly lower compared to VL3-only ferment. Overall there was a significant difference in the co-ferment in its population size, dynamics and levels of 3MHA and 3MH (figure 5.11) compared
to co-ferment conducted by Anfang et al. (2009). Therefore other subtle yet complex parameters such as juice composition should be taken into account when studying yeast interaction during fermentation (Houtman et al., 1980).

Although few trends could be observed such as similarity in initial population dynamics and initial differences in levels of 3MHA between co-ferment and model co-ferment (mVP). These differences could have significantly impacted on the proteomic and extracellular metabolite profiles examined.

Figure 5.11: Level of thiol 3MH (ng/L) in VL3-PKKR1, VL3-only and model co-ferment (mVP) at three different time-points. Error bars are indicative of ± SEM (n=3). The top number indicates fold-change for VL3-PKKR1 over mVP; the bottom number indicates fold-change for VL3-PKKR1 over VL3-only ferments. *** indicates P-value < 0.001
5.6.7 Biochemical pathway activity in VL3-only and VL3-PKKR1 ferments

From the above sections it can be seen that there was mixed results from testing the null hypothesis using proteomics and extracellular metabolite profiles of VL3-only and VL3-PKKR1 co-ferments. The lack of differences in protein profiles between the two ferments could have been due to the lack of significant change in VL3 proteome due to the presence of PKKR1, however, any changes to the proteome of PKKR1 could not be explained from this study. A lack of change in protein levels did not translate into a change in metabolite levels, as the null hypothesis was disproved using the extracellular metabolites between the two ferments. The differences in protein and metabolite profiles could be due to the possibility that the proteins present were utilised differently between the two conditions; e.g. most enzymes are capable of catalysing both the forward and backward catalysis of substrates and can be involved in different pathways; this could have been the cause of differences in metabolism without change in the levels of enzymes themselves (Becker and Bern, 2011; Newman et al., 2006). Another possibility was that the metabolite changes observed were mostly due to the activity of enzymes from PKKR1 during co-ferment.

An individual protein/enzyme can potentially play an important role in driving the reactions of multiple pathways, similarly, an individual metabolite can participate in numerous metabolic pathways, thus making the analysis of pathway activity inside a cell difficult to interpret (Aggio et al., 2010; Rossouw and and Bauer, 2009). The correlation of metabolite levels and pathway activity is still difficult to achieve, but many proprietary software packages have been developed by companies (SpectralWorks Ltd, Agilent Technology and Applied Biosystems) and institutions (e.g. Bioconductor) to understand the change in pathway activities (Aggio et al., 2010).

In addition, many web-based databases are publically available with information regarding, genes, metabolites, metabolic pathways, proteins, enzyme reactions, etc (Kopka et al., 2005). One such database is Kyoto Encylopaedia of Gene and Genomes (KEGG, http://www.genome.jp/kegg/); KEGG is one of the more popular web-based databases as it has application programming interface (API) which allows for computational tools to automatically access, extract and manipulate the information in the web database (Aggio et al., 2010; Kanehisa and Goto., 2000). One such tool is Pathway Activity Profiling or PAPI, and is an R package developed to generate and compare the activity of various metabolic pathways between experimental conditions (Aggio et al., 2010).

5.6.7.1 PAPI

PAPI relies on measurement of AS or Activity Score of a metabolic pathway, AS is calculated in PAPI based on the number of metabolites identified in a pathway and their abundances; the greater the number of identified metabolites and their abundances for a particular pathway the greater its Activity Score (Aggio et al., 2010). Activity Scores are generated based on two assumptions; one that a
metabolic pathway is more active if more metabolic intermediates are identified for that pathway, the other that greater the activity of metabolic pathway, the lower the abundance of intermediates in that pathway due to the high turnover of these metabolites or the high flux of the metabolic pathway (Aggio et al., 2010). The software PAPi was used to generate Activity Scores (AS) and subsequently significant pathway activity in VL3-only and VL3-PKKR1 co-ferments. The pathway activity profile was generated only for day 16 to gauge the difference in activity of S. cerevisiae VL3 in mono and co-ferment at a time point where it was the dominant fermenting yeast.

The extracellular metabolite data set was previously normalised to internal standard, biomass and influence of sterile grape juice before being used in the PAPi package. Figure 5.12 shows the metabolic pathways that were significantly different (P-value $\leq 0.05$) between VL3-only ferment and VL3-PKKR1 co-ferment on day 16. The metabolites that were significantly different between the two conditions were due to the differences in the activity of the pathways responsible for their biosynthesis/degradation (Aggio et al., 2010).

In Figure 5.12, it can be seen that PAPi identified 34 biochemical pathways as being significantly different between VL3-only and VL3-PKKR1 co-ferment. These observations were made based on the activity score calculated for each metabolic pathway followed by pair-wise comparison of the activity score (AS) from VL3-only and VL3-PKKR1 co-ferment. Activity scores are dependent on the number of metabolites identified for each pathway along with their abundance; accumulation of metabolites upstream of a pathway suggests low activity of that pathway in a particular ferment and is shown as negative fold change. Whereas, accumulation of metabolites downstream of a pathway suggests higher activity for that pathway in a particular ferment and is shown as positive fold-change. (Aggio et al., 2010).
Figure 5:12: Comparison of pathway activity (based on AS using extracellular metabolite concentration) between VL3-only (-) and VL3-PKKR1 (-) co-ferments using PAPi for extracellular profiles from day 16. Error bars indicate ±SEM (n=9) (P-value ≤0.05). Negative fold-change indicates low pathway activity with accumulation of upstream metabolic intermediates, whereas positive fold-change indicates accumulation of downstream metabolic intermediates in a particular pathway.
5.6.7.2 Role of extracellular metabolites in influencing fermentation and aroma of Sauvignon Blanc

From the figure (figure 5.12), it can be seen that there is a vast difference in the PAPi profile between VL3-only and VL3-PKKR1 co-ferment. VL3-PKKR1 co-ferments have at least 19 metabolic pathways which are significantly down regulated compared VL3-only co-ferments. Most of these pathways are important in central carbon and nitrogen metabolism of yeast during fermentation (Stanley et al., 2010; Styger et al., 2011). This could be due to lower fermentation rate observed in the co-ferment compared to VL3-only ferment (Appendix 5). Although other aroma compounds (other than thiols) were not analysed for this experiment, comparisons can be drawn from aroma information for co-ferments from chapter 4 and the significance of certain metabolites analysed in this chapter.

From chapter 4, (figure 4.6) higher alcohols and ester acetates were present in higher concentration in Saccharomyces yeast co-ferments with PKKR1 compared to respective mono-ferments. Higher alcohol concentration in wine is dependent on amino acid metabolism (Lysine, Alanine, Leucine etc.) and that of esters is dependent sugar and TCA metabolic pathways in yeast (Styger et al., 2011; Swiegers et al., 2005). From table 5.5 most of the metabolites (except isoleucine and threonine) were present in greater abundance in VL3-PKKR1 co-ferment compared to VL3-only ferment, thus producing a lowered Activity Score (AS) in PAPi analyses (figure 5.12). Most of these metabolites are involved in amino acid metabolism or TCA cycle; therefore a negative correlation between the activity of the amino acid metabolism and TCA cycle with concentration of aroma in co-ferments is proposed. This idea is supported by finding higher concentrations of isoamyl alcohol in co-ferments (chapter 4) coupled with identifying Leucine as a potential biomarker; uptake of Leucine and TCA cycle plays an important role in isoamyl alcohol production during fermentation (Swiegers et al., 2005).

In other words; lowered fermentation rate coupled with low uptake of amino acids and a slower rate of TCA cycle could be an important factor contributing to higher concentration of certain aroma compounds in co-ferments with PKKR1 compared to mono-ferments with Saccharomyces yeast. However, to discern whether this phenomenon is due to interaction between Saccharomyces yeast and PKKR1; experimental ferments (mono and co-ferments of both the yeast) with isotope labelled amino acids (ones that were identified as biomarkers in this study) or grape sugars need to be performed, followed by analyses of extracellular metabolites and aroma concentrations (Vivanco et al., 2011).

Pichia kluyveri PKKR1 on its own is unable to ferment wine at the same rate as VL3, however, it is able to persist in relatively high cell numbers (both mono and co-ferment) for a reasonable duration (at least 16 days) in a semi-anaerobic environment (figure 5.6b). This suggests at an alternate
mechanism of nutrition and respiration, this was also observed by Anfang (2010) when determining
the survivability and nutrition of PKKR1 during fermentation.

However, PKKR1 would have been under stress from lack of oxygen and increasing ethanol
concentration, one strategy of redox balance during stress is the enzymatic reduction of sulphate and
release of hydrogen sulphide (H\textsubscript{2}S), thus, explaining the increase in sulphur relay mechanism
observed for co-ferments (Figure 5.12). Interestingly, H\textsubscript{2}S is thought to play a role in population
signalling serving to co-ordinate metabolic activity of individual cells (Linderholm et al., 2010).

Additionally, Glutamic acid was present at 4.6 folds greater while glutamine was 0.5 folds greater in
co-ferment compared to VL3-only ferment (table 5). Glutamic acid is an important pre-cursor
metabolite for many central carbon pathways such as Histidine metabolism, Nitrogen metabolism,
Glutathione metabolism, Porphyrin metabolism all of which were down regulated in co-ferment
(figure 5.12). These metabolites are related to the production of aroma compounds such as fusel
alcohol during fermentation, therefore, in the future these aroma compounds need to be monitored as
well to understand the interaction between VL3 and PKKR1 (Styger et al., 2011).

Recently, Glutathione and its cysteine conjugates were implicated to play an important role in
enabling yeast express varietal aroma in Sauvignon Blanc (Roland et al., 2010a; Subileau et al.,
2008a). Glutamic acid is an intermediate in Glutathione metabolism, it was found in greater
abundance in co-ferment compared to VL3-only ferment. This coupled with lower activity of
Glutathione pathway in co-ferments suggest at a similar mechanism for 3MHA production in co-
ferment to one described by Roland et al. (2010a). Therefore these findings are encouraging to
continue further research into the extracellular metabolite profiles of co-ferments to discern the
evolution of thiols during fermentation.

Another study found that in Candida nitratophila, a low ratio of 0.1 of Glutamine: Glutamate was an
indicator of nitrogen stress in the organism (Flynn and Gallon, 1990). In this study, (based on the data
from table 5.5) ratio of Glutamine: Glutamate is 0.1 in co-ferments indicating at nitrogen stress, this is
to be expected during fermentation (Bell and Henschke, 2005). Perhaps in the future ratio of
Glutamine: Glutamate could be monitored regularly and used as an indicator of fermentation progress
in multi-starter or co-ferments and prevent sluggish or stuck ferments.
5.7 Conclusion

The global analysis of mono and co-ferments of VL3 and PKKR1 yielded in the identification of 237 yeast proteins and 83 extracellular metabolites in wine. The null hypothesis that there are differences between the overall protein profiles of mono and co-ferments could not be disproved using only VL3 proteome; a model co-fermentation calculation (mVP) was also unable to disprove the null hypothesis. Identification of any potential proteomic biomarkers was not possible from this study, this could have been due to the fact that the proteome of VL3 is as yet incomplete and the proteome of PKKR1 is unavailable.

On the other hand the metabolite profile of the two ferments showed significant differences thus allowing disproving the null hypothesis. The population dynamics of the conducted co-ferment varied from that observed by Anfang et al. (2009) and could have been a factor contributing to the lack of significant increase in 3MHA observed in this study in comparison to that observed in the literature.

Three potential metabolite biomarkers were identified; Leucine, Isoleucine and Glutamic acid. Significant increase in sulphur relay mechanism in co-ferments coupled with other characteristics such as decreased fermentation rates, reduced 3MH levels but increased 3MHA levels allowed for speculation that the thiol 3MHA/3MH could be involved in a redox-sensitive signalling mechanism similar to H₂S redox- balance system to alleviate stress (Linderholm et al., 2010).
General Conclusion
6.1 General background

New Zealand has had a relatively short history in commercial winemaking compared to continental Europe (Robinson, 2006). It was the year 1979, when Montana first released its Sauvignon Blanc from Marlborough and commercially established that variety (Cooper, 2002; Cooper and McDermott, 1996). Since then New Zealand Sauvignon Blanc has taken the wine world by storm (Cooper, 2002; Cooper and McDermott, 1996). It is due to the characteristic blend of herbaceous, capsicum and gooseberry aromas together with tropical fruit notes of passionfruit and pineapple, that makes this wine variety a major contender in the New Zealand export wine market (Robinson, 2006). Due to its popularity, there is direct competition from similar style wines from other countries. Therefore, to ensure the reputation of a high quality original product in the international wine market, it is important to understand the key factors responsible for the distinctive character of New Zealand Sauvignon Blanc.

Since 2004, there has been a research initiative to understand key factors responsible for the characteristics of New Zealand Sauvignon Blanc. The Sauvignon Blanc programme is funded by the New Zealand Winegrowers association along with Foundation for Research, Science and Technology (FRST) and later by Ministry of Science and Innovation (MSI) to support research to understand the nature and origin of key aroma compounds along with ways to control and manipulate these aromas from various perspectives (1.4.2 general introduction). My PhD was funded by the Sauvignon Blanc project.

In 1998, Tominaga et al. (1998a, 1998b) found that certain volatile sulphur compounds called thiols were responsible for the characteristic aroma of French Sauvignon Blanc. The commonly perceived aromas of passionfruit and grapefruit were attributed to thiols 3-mercaptohexanol (3MH) and mercaptohexyl acetate (3MHA) (Tominaga et al., 1998b). These volatile thiols were absent in juice and were produced during fermentation due to yeast activity (1998a). In 2006, Nicolau et al. (2006) found that the concentration of these volatile thiols in New Zealand Sauvignon Blanc (especially Marlborough) was significantly higher when compared to Sauvignon Blanc from other countries (France, USA, Australia and South Africa). Sensory analyses by Lund et al. (2009) showed that it was 3MH and 3MHA (along with methoxypyrazines) that contributed to the distinct aroma perceived in a New Zealand Sauvignon Blanc.

Volatile thiols are liberated during fermentation by yeast activity (Tominaga et al., 1998b). Investigation into the potential of thiol liberation by different commercial isolates of S. cerevisiae showed significant differences between them (Murat et al., 2001a; Swiegers et al., 2006b). However,
little was understood regarding the potential of natural _Saccharomyces_ yeast and other non-
_Saccharomyces_ yeast to release thiols during fermentation.

Industrialisation of winemaking has led to a lack of diversity in winemaking techniques. This has
diminished further exploration of aroma potential of both grape variety and yeasts available to
ferment them. Recently, co-fermentation and multi-yeast starter cultures have become a popular way
to introduce uniqueness into wine (Ciani *et al.*, 2010). Thus unearthing the potential of yeast isolates
can provide winemakers with tools to control wine aroma. Goddard *et al.* (2010) found a unique
population of _S. cerevisiae_ and other non-Saccharomyces yeast in New Zealand. However, little was
known regarding the aroma potential (ability to release thiols during fermentation) of these isolates.
As part of her doctoral research Nicole Anfang examined the ability of natural _Saccharomyces_ and
non-Saccharomyces yeast to release thiols during fermentation thus underscoring the importance of
natural isolates in aroma of New Zealand Sauvignon Blanc.

In 2009, Anfang *et al.* (2009) showed that non-Saccharomyces yeast _P. kluyveri_ (PKKR1) when co-
fermented with commercial wine yeast _S. cerevisiae_ VL3 was responsible for a 2 fold increase in
levels of 3MHA in Sauvignon Blanc when compared to fermentation by VL3 on its own. This
increase in 3MHA levels was found to be due to a synergistic interaction between PKKR1 and VL3
(Anfang *et al.*, 2009). However, the mechanism behind this interaction is unknown.

More than 90 per cent of New Zealand Sauvignon Blanc is made using commercial yeast isolates (M.
Brajkovich MW). Little is known regarding the aroma potential of New Zealand natural yeast isolates
when fermenting Sauvignon Blanc (Anfang *et al.*, 2009). Thus, a part of my research was to identify
yeast involved in a un-inoculated fermentation of New Zealand Sauvignon Blanc.

The main objectives of this thesis were:

To examine the identity and population dynamics of yeast isolates involved in the un-inoculated
fermentation of Marlborough Sauvignon Blanc (chapter 2).

To examine the fermentation kinetics and aroma potential of _Saccharomyces_ and non-Saccharomyces
yeast isolated from the un-inoculated fermentation of Marlborough Sauvignon Blanc (chapter 3 and
4).

To examine the mechanism of interaction between PKKR1 and VL3 during Sauvignon Blanc
fermentation utilising systems biology tools (chapter 5).
6.2 Nature and identity of yeast isolates involved in the fermentation of
2008 Kumeu River Sauvignon Blanc

Kumeu River winery located in west Auckland with head winemaker Mr M. Brajkovich (Master of Wine) is one of the few world-class wineries in New Zealand which produce wine from natural or un-inoculated ferments; this was an ideal place to monitor natural yeast isolates with commercial fermentation potential. In 2008, I sampled and monitored the commercial un-inoculated fermentation of Sauvignon Blanc from Kumeu River winery (section 2.8.1 for methods, section 2.9.2, 2.9.3, figure 2.5 and table 2.6). This led to the identification of 907 isolates as *S. bayanus*; the dominating yeast present in the un-inoculated ferment. Furthermore, ITS-RFLP and microsatellite analyses of randomly selected *S. bayanus* isolates showed them to be not related to any known commercial yeast. Microsatellite analyses showed that there was potentially a vast diversity within these isolates, and also enabled in the identification of natural hybrids between *S. bayanus* and *S. cerevisiae* (see appendix 2 and section 2.9.4 and figure 2.8). Goddard *et al.* (2010) also found a vast diversity of *S. cerevisiae* when monitoring a un-inoculated New Zealand Chardonnay ferment.

This is the first in-depth study of natural isolates in a Sauvignon Blanc, other studies have insufficient data to enable the understanding of population dynamics of natural yeast isolates in fermentation (Lopandic *et al.*, 2008; Pramateftaki *et al.*, 2000; Torija *et al.*, 2001). In-depth analyses of the population of yeast would allow for further exploration of unique aromas in established wine varieties. Furthermore, unique yeast species or hybrids can be identified which could have commercial implications. Also, tracking the changes in population of all the yeasts identified during fermentation allows for understanding and further testing of the mechanisms involved in influencing these dynamics.

In Kumeu River un-inoculated ferment of Marlborough Sauvignon Blanc, *S. bayanus* came to dominate and carry out fermentation. However why was *S. cerevisiae* which was also identified early on during fermentation unable to do the same? A hypothesis was proposed that a lower temperature condition (below 14°C) in the initial stages of fermentation allowed for a distinct growth advantage to the cryotolerant *S. bayanus* allowing it to dominate and outnumber *S. cerevisiae* isolates. Upon testing the growth rates of randomly selected *S. bayanus* and *S. cerevisiae* isolates from Kumeu River Sauvignon Blanc; it was found *S. bayanus* isolates on average had a significantly higher growth rate compared to *S. cerevisiae* at lower temperature (12.8°C). (Section 2.9.5.3; figure 2.11).
Change in population of \textit{S. bayanus} and \textit{S. cerevisiae} between days 1 and 7 of Kumeu River fermentation was predicted by calculating their Malthusian fitness ($m$) and applying it in the equation provided by Hartl and Clark (1997). This model was temperature dependent and was only able to predict the initial population dynamics between these two species. According to the model, the observed change in population dynamics 1:46 (\textit{S. cerevisiae}: \textit{S. bayanus}) would take 4.68 days at 12.8°C when compared to 7 days it took during fermentation. This model was able to predict the temperature advantage of \textit{S. bayanus} over \textit{S. cerevisiae} in a limited capacity; while not accounting for the presence of other non-Saccharomyces yeast and other oenological factors. Interestingly, \textit{S. bayanus} isolates identified in un-inoculated ferments of red wine from St. Georgen region in Austria (cold climate) was unable to dominate the fermentation process (Lopandic et al., 2008). Therefore, it will be necessary to separately test the specific oenological conditions required by \textit{S. bayanus} to dominate different types of wine. However, these cryotolerant \textit{S. bayanus} isolates could prove useful in other studies focusing on low temperature fermentation and reducing acetic acid in wine while not compromising aroma (Eglinton et al., 2000).

Volatile thiols; 3MH, 3MHA and 4MMP were also monitored during fermentation (figure 2.12). The levels of 3MH (3330 ng/mL ± 79 ng/mL) and 3MHA (211 ng/L ± 10.5 ng/L) were lower in Kumeu River Sauvignon Blanc compared to other New Zealand Sauvignon Blanc; the concentration of thiol 4MMP (135 ng/L ±5 ng/L) was extremely high. Although 3MH and 3MHA are important varietal aromas, 4MMP is also capable of influencing the aroma profile with distinct aroma of boxwood and blueberries. Thus 4MMP can add an extra dimension to the aroma of New Zealand Sauvignon Blanc. This result was in line with the observations made by Dubourdieu et al. (2006) who showed that Sauvignon Blanc made using \textit{S. bayanus} was capable of producing relatively high levels of 4MMP when compared to \textit{S. cerevisiae}.

Kumeu River Sauvignon Blanc of 2008 was the first commercial un-inoculated ferment monitored which had \textit{S. bayanus} as the dominant fermenting yeast. It was hypothesised that at low temperatures such as those observed in the initial period of this ferment, \textit{S. bayanus} had a growth advantage over \textit{S. cerevisiae} which allowed it to be dominant yeast. This hypothesis was supported by a significant growth rate observed in \textit{S. bayanus} isolates compared to \textit{S. cerevisiae} isolates at 12.8°C. A population dynamics model (temperature dependent) using the growth rate of these two species was able to predict the change in the observed population dynamics in a limited way. Thus in a limited capacity, the growth advantage of \textit{S. bayanus} over \textit{S. cerevisiae} was proved to be an important factor in the population dynamics of these species in Kumeu River Sauvignon Blanc. Finally the capacity of \textit{S. bayanus} to produce 4MMP can be used to enhance the characteristic aroma of New Zealand Sauvignon Blanc.
6.3 Fermentation kinetics of 20 isolates of *S. bayanus* from a natural population

From chapter 2 it can be seen that 907 isolates were identified as *S. bayanus* from Kumeu River Sauvignon Blanc. Microsatellite profiling of 20 of these *S. bayanus* isolates followed by phylogenetic analyses showed a vast diversity (figure 2.8). The isolates of *S. bayanus* provided a vast potential of fermentation capabilities. However, they were identified from un-inoculated ferment which was initially not controlled for temperature, therefore to gauge their fermentation potential they were fermented at a lowest (12.8°C) and highest fermentation temperatures (20°C) recorded during the Kumeu River fermentation. Traditionally, *S. bayanus* isolates were considered not capable of fermenting wine but it was shown that *S. bayanus* are indeed capable of fermenting at various temperatures (Masneuf-Pomarède *et al.*, 2006).

A scatterplot of the maximum fermentation rates of these isolates clearly showed the differences within these isolates in their ability to ferment at the two different temperatures (figure 3.5). A student’s t-test showed that there was a significant (P-value = 4.26E⁻¹⁸) difference between the Vmax values of the isolates at each temperature. While some isolates performed better at 12.8 °C some did so at 20 °C. Furthermore, there were significant differences in the Vmax of isolates within each temperature (Section 3.5.2). Another criterion that needed to be examined was the time required to reach the maximum fermentation kinetics of each isolates at the different temperatures. This information was important in understanding the ability of certain isolates to dominate over others during un-inoculated fermentation. Fisher’s exact test was used to test whether the distribution of these isolates based on the time taken to achieve Vmax at 12.8 and 20°C were significantly different. Upon performing the test, a P-value of 2.133E⁻⁰⁹ was obtained; this shows that there is a significant difference in the time taken by the 20 isolates to achieve Vmax between 12.8 and 20 °C.

These results indicate that perhaps not all *S. bayanus* isolates were involved in fermentation, however they were able to survive throughout the fermentation process. Further research into the survival of these isolates and their interaction with other *Saccharomyces* yeast in a un-inoculated fermentation can provide information regarding the natural selection process shaping the biodiversity of *S. bayanus*. Furthermore, these isolates can be used in a survey of geographical distribution of *S. bayanus* in New Zealand, a similar survey for *S. cerevisiae* distribution is being carried out (Gayevskiy and Goddard, 2012).

Based on the fermentation rates at 12.8°C and 20°C and the time required to reach this rate, one *S. bayanus* isolate SBJ1d was selected for further comparisons with commercial *S. cerevisiae* VL3. S.
*Saccharomyces* VL3 is routinely used to ferment New Zealand Sauvignon Blanc and is able to produce 4MMP unlike other commercial *S. cerevisiae* (Roncoroni *et al.*, 2011).

### 6.4 Design of full-factorial experiment to elucidate the impact of yeast interaction on New Zealand Sauvignon Blanc fermentation and their impact on aroma

The main aim of my thesis was to identify and understand yeast interaction in New Zealand Sauvignon Blanc ferment and their impact on varietal aroma of wine. I achieved this by designing and performing a full-factorial experiment (Box *et al.*, 2005). Full factorial experiments are routinely used to test microbial capabilities in the food industry (Mertens *et al.*, 2012). When examining the impact of oenological factors (temperature, yeast species and YAN) on varietal aroma (thiols and any other important aroma) a two-step process was utilised. Firstly, fermentations were performed at a range of the oenological factors being examined (Figure 6.1); this was followed by monitoring fermentation kinetics and concentrations of the varietal aroma in these ferments.

![Figure 6.1: Layout of the full factorial experiment to identify potentially important oenological factors contributing to a distinctive style of New Zealand Sauvignon Blanc.](image)

Since the primary aim was to identify yeast capable of influencing a distinct aroma in Sauvignon Blanc, specific comparisons between different ferments (mono and co-ferments) were made using the available data-set (fermentation kinetics and aroma concentration). These involved the testing of
various null hypotheses to identify any difference in the fermentation and aroma potential of these ferments. The null hypotheses are outlined below;

1) Test the null hypothesis that there is no difference in fermentation kinetics and aroma concentration between *S. bayanus* and *S. cerevisiae* when fermented in a range of oenological environments (temperature, Yeast Available Nitrogen, presence of a non-*Saccharomyces* yeast (PKKR1)) under controlled conditions

2) Test the null hypothesis that there is no difference in the fermentation kinetics and aroma concentration between *S. bayanus*-only and *S. bayanus* with 3 different non-*Saccharomyces* yeast (from Kumeu River Sauvignon Blanc) in a range of oenological environments under controlled conditions

6.4.1 Comparison between *S. bayanus* (SBJ1d) and *S. cerevisiae* (VL3) ferments conducted at a range of oenological conditions

In this research SBJ1d was compared against only one commercial *S. cerevisiae* (VL3). Only *S. cerevisiae* VL3 was chosen as the onus of my PhD was on yeast interaction (*Saccharomyces* and non-*Saccharomyces*), and VL3 is known to produce 4MMP and have a specific interaction with a non-*Saccharomyces* yeast and was therefore used in the large multi factorial experiment (Anfang *et al.*, 2009) (chapter 3 and chapter 4). However, in the future, when determining the ability of SBJ1d to ferment commercially, it will be necessary to compare it between several *S. cerevisiae* isolates in different wine varieties in order to identify its overall fermentation potential.

*S. bayanus*-only ferments were able to ferment quicker and had a greater Vmax compared to *S. cerevisiae*-only ferments under similar conditions (see section 3.5.3). As seen in figure 3.7, *S. bayanus*-only ferment had a significantly greater Vmax compared to *S. cerevisiae*-only ferments at four different oenological conditions (12.8⁰C+Y1, 12.8⁰C +Y2, 20⁰C+ Y1 and 20⁰C+Y2). These results were partially contradicting research conducted by Tosi *et al.* (2009) who showed no significant difference between the maximum rate of ethanol production between the selected *S. cerevisiae* and *S. bayanus* isolates. Ethanol production can be compared with weight loss measurements (lost as CO₂) as they were shown to be directly proportional (Bely *et al.*, 1990). An important caveat to this study is that these results are only valid for the specific strains of *S. bayanus* and *S. cerevisiae* used.

Analyses of concentration of varietal thiols (3MH, 3MHA and 4MMP) produced by *S. bayanus*-only and *S. cerevisiae*-only ferments led to identification of key characteristics of the Sauvignon Blanc produced by these ferments. Irrespective of the oenological condition of the ferments, *S. bayanus*-only
ferments had significantly greater levels of thiol 4MMP (greater than 2-fold). Although, Lund et al. (2009) suggested that the New Zealand Sauvignon Blanc was distinct due to high levels of thiols 3MH and 3MHA along with methoxypyrazines, high levels of 4MMP is capable of introducing complex notes of boxwood and blueberries to wine. High levels of 4MMP is not desired in French Sauvignon Blanc as it can sometimes introduce odours such as ‘cat’s pee’ into the finished wine or mask varietal aroma influence, however, the opinion on the quality of wine with this aroma in New Zealand Sauvignon Blanc is mixed (Masneuf-Pomarède et al., 2010; Serra et al., 2005). The levels of other thiols 3MH and 3MHA were also higher in S. bayanus-only ferments and addition of DAP (i.e. Y2 juice) had mixed impact on their concentrations (for full details see section 4.7.3). Overall, S. bayanus SBJ1d was able to produce significantly higher concentrations thiols when compared to S. cerevisiae (VL3)-only ferments.

Analyses of other volatile aroma compounds showed that the major differences in this category between S. bayanus-only and S. cerevisiae-only ferments were due to concentration of higher alcohols and their acetates (section 4.7.3.1). These compounds were significantly influenced by the temperature, YAN and yeast species used in fermentation. At lower temperature of 12.8°C isoamyl alcohol, isobutyl acetate and isoamyl acetate were present at a significantly higher concentration (greater than 2-fold) in S. cerevisiae-only ferments. However, phenyl-ethyl-ethanol (PEE) and beta-phenyl-ethyl acetate (BPEA) concentrations were higher (greater than 2-fold) in S. bayanus-only ferments (table 4.7). However at 20°C, the concentration of higher alcohols and their acetates were significantly higher (greater than 2-fold) in S. bayanus-only ferments (table 4.8). Thus, different oenological conditions had different impact on the final aroma composition (table 4.9). Little is known regarding the influence of other volatile aromas in New Zealand Sauvignon Blanc, better understanding of these aromas can provide a unique opportunity to enhance the characteristics of New Zealand Sauvignon Blanc. Sauvignon Blanc from Hawkes Bay region tends to be less influenced by thiols compared to Sauvignon Blanc from Marlborough (Lund et al., 2009; Nicolau et al., 2006). Perhaps utilising S. bayanus to ferment juice from Hawkes Bay region could potentially enhance the influence of higher alcohols and 4MMP, thereby adding new characters to the resulting wine. Also, fermenting other white wine such as Pinot Gris and Gewürztraminer using SBJ1d could enhance their floral qualities.

6.4.1.1 Comparison between SBJ1d and PKKR1 and VL3 and PKKR1 co-ferments conducted at a range of oenological conditions

New Zealand Sauvignon Blanc (Marlborough in particular) has higher concentration of varietal thiol 3MHA (greater than 2-fold) when fermented by S. cerevisiae (VL3) and P. kluyveri (PKKR1) co-ferment compared to S. cerevisiae (VL3)-only fermentation (Anfang et al., 2009). This synergistic interaction is yeast species specific and had to have a specific initial inoculum (1:9, Saccharomyces to
non-*Saccharomyces* ratio) which determined to be crucial for the increased production of 3MHA indicating an interaction between the two species (Anfang et al., 2009). Therefore as part of my full-factorial experiment, I conducted co-ferments of *S. bayanus* (SBJ1d) and PKKR1 as suggested by Anfang et al. (2009) at different oenological conditions and compared it co-ferments of VL3 and PKKR1 to identify any possible interaction between PKKR1 and SBJ1d. In addition to comparing the differences between the two co-ferments, I also compared the fermentation kinetics and aroma profiles of *S. bayanus* co-ferment with *S. bayanus*-only mono-ferment to identify any possible interaction between *S. bayanus* (SBJ1d) and PKKR1.

The presence of PKKR1 at 12.8°C did not significantly alter the fermentation kinetics when compared to mono-ferments of SB and VL3. Whereas, at a higher temperature of 20°C, the presence of PKKR1 significantly lowered the average Vmax of both the co-ferments in comparison to the respective mono-ferments (see section 3.5.4). However, *S. cerevisiae* (VL3) co-ferments had a significantly lower Vmax values compared to *S. bayanus* (SBJ1d) co-ferments, thus suggesting that the *S. bayanus* (SBJ1d) had a lower impact on its rate of fermentation due to the presence of PKKR1 under the tested conditions.

The concentration of volatile thiols 4MMP and 3MH were significantly higher in SB+PKKR1 co-ferment compared to VL3+PKKR1 co-ferments (table 4.10 and 4.11). Addition of YAN and change in temperature had a mixed impact on the thiol levels produced by the co-ferments (section 4.7.3.2).

Importantly, the SB+PKKR1 co-ferment had a significantly (P-value < 2.12 e\(^{-16}\)) higher concentration of 4MMP (3.62 fold) compared to *S. bayanus*–only ferment despite lower rate of fermentation thus indicating a potential synergistic interaction (to produce higher levels of 4MMP) between the two species. However, it cannot be confirmed from this data-set as population sizes of these two species during fermentation were not enumerated to preclude an additive effect.

Furthermore, there was no difference in the levels of 3MHA produced by VL3+PKKR1 compared to *S. cerevisiae*-only ferments. Therefore it can be deduced that the increased 3MHA levels observed in VL3+PKKR1 co-ferments by Anfang et al. (2009) is specific at the defined oenological condition. Thus, the interaction between VL3 and PKKR1 needs to be examined at different oenological conditions to identify the exact parameters required to enhance 3MHA levels in Sauvignon Blanc. Another option is to explore this interaction and its influence on the aroma of other white wine varieties.

Analyses of other aroma compounds showed that higher alcohols and their acetates were significantly higher in SB+PKKR1 compared to VL3+PKKR1 (refer table 4.10 and 4.11). Additionally, SB+PKKR1 co-ferments had a significantly (both comparisons with P-value < 2.26e\(^{-16}\)) higher concentration of higher alcohols (greater than 2-fold) isoamyl alcohol and isobutyl alcohol when
compared to *S. bayanus*-only ferments irrespective of the different temperature and YAN conditions. Also there was a 25-fold increase in the concentration of isoamyl acetate in the SB+PKKR1 co-ferment compared to *S. bayanus*-only ferment at 12.8°C. While it was present only in slightly higher levels in co-ferment compared to *S. bayanus*-only ferment at 20°C. These changes in aroma concentration of 4MMP, isoamyl alcohol, isobutyl alcohol and isoamyl acetate was not observed when comparing VL3+PKKR1 co-ferment and *S. cerevisiae*-only ferments. Whilst, this full-factorial experiment was useful in examining the interaction between the different yeast species, it had limitations in its applicability to commercial winemaking due to the unnaturally high YAN concentration of Y2 juice. Perhaps as a next step, a full-factorial experiment with a panel of Sauvignon Blanc juice from different locations/vintages as factor can be conducted to further elucidate yeast interaction and their broad application in commercial winemaking.

These results suggest that any interaction between *S. bayanus* (SBJ1d) and PKKR1 could involve different mechanisms when compared to interactions between *S. cerevisiae* (VL3) and PKKR1. However, a holistic approach is required to elucidate the exact nature of these mechanisms.

### 6.4.2 Comparison between *S. bayanus* (SBJ1d)-only ferment with its selected non-*Saccharomyces* yeast co-ferments conducted at a range of oenological conditions

As part of the full factorial experiment, *S. bayanus* (SBJ1d) was co-fermented with PKKR1 and *T. delbrueckii* (SB+TD) and *P. anomala* (SB+PA) which were two non-*Saccharomyces* yeast randomly selected from those identified in 2008 Kumeu River Sauvignon Blanc. This was done to explore any interaction of non-*Saccharomyces* yeast with *S. bayanus* in Kumeu River Sauvignon Blanc. The initial inoculum ratio of *Saccharomyces* to non-*Saccharomyces* yeast was maintained at 1:9 (respectively) for the three different co-ferments as suggested by Anfang *et al.* (2009) for co-ferment between *S. cerevisiae* (VL3) and PKKR1, this ratio imitates the initial population of yeasts in an unsterile juice (Fleet, 2003).

Upon examining the fermentation kinetics by comparing Vmax values of the different co-ferments, it could be seen that there was no significant difference between the Vmax values of *S. bayanus*-only and the three co-ferments (section 3.5.5, table 3.6). However, addition of DAP (Y2 juice) at both temperatures (12.8°C and 20°C) resulted in a significantly lowered Vmax values of co-ferments especially SB+PA and SB+ TD.

Analyses of varietal thiols showed that all the co-ferments in this study had 2-fold or greater concentration of 4MMP compared to *S. bayanus*-only ferment. However this fold difference was observed only at 12.8°C and Y1 juice condition. Addition of DAP (Y2 juice) and increasing the
ferment temperature to 20°C diminished the difference in 4MMP levels (see section 4.7.3.3, tables 4.13, 4.14, 4.15 and 4.16).

Additionally, higher alcohols and their acetates (except PEE) were found in significantly higher concentrations (greater than 2-fold) at 12.8°C and Y1 juice condition in *S. bayanus* co-ferments with PKK1 and *T. delbrueckii*. However, *S. bayanus*-only ferment had greater concentration of these compounds compared to co-ferment with *P. anomala* under the same oenological condition.

Increasing the YAN (Y2 juice) at 12.8°C seem to favour increased concentration (greater than 2-fold) of these aroma compounds in all the three co-ferments compared to *S. bayanus*-only ferment.

However, increase in temperature (20°C) diminished the differences between the co-ferments and *S. bayanus*-only ferment; this finding was in line with studies which suggest a negative influence of temperature on aroma (Ribéreau-Gayon et al., 2006a). The importance of low temperature is understood for the survival of non-*Saccharomyces* yeast, however, impact of nitrogen sources is yet to be completely understood (Charoenchai et al., 1998; Ciani and Picciotti, 1995).

Acetylation ratios of Isoamyl alcohol to Isoamyl acetate, PEE to BPEA and 3MH to 3MHA provided valuable insights into the differences in aroma production between *S. bayanus* and *S. cerevisiae* (VL3) and the impact of the different non-*Saccharomyces* yeast (see table 4.18). However, aroma producing capacity of the various yeast are strain dependent (Rojas et al., 2001; Swiegers et al., 2009).

Thus, non-*Saccharomyces* yeast from this study can be used to co-ferment *S. bayanus* (SBJ1d) to increase levels of 4MMP and higher alcohols and their acetates provided the fermentation is carried out at a low temperature. These results suggest that in low temperature fermentation, the non-*Saccharomyces* yeast are actively involved in shaping the aroma and flavour of Sauvignon Blanc and not merely surviving. However, to obtain a complete picture of non-*Saccharomyces* and *S. bayanus* interactions in Kumeu River Sauvignon Blanc fermentation, a pre-selection process of the different yeast is a necessary step before the design of a full-factorial or a fractional factorial experimental design (Mertens et al., 2012).

Analysis of responses from the full factorial experiment unravelled the fermentation and aroma potential of *S. bayanus* (SBJ1d). Observing the 4MMP levels in Kumeu River Sauvignon Blanc and that of trial wine produced using SBJ1d, it can be seen that levels of 4MMP produced by SBJ1d is lower. Perhaps the 4MMP levels in Kumeu River Sauvignon Blanc was higher due to presence of other *S. bayanus* isolates capable of producing more 4MMP compared to SBJ1d or it could have been due to the interaction of various yeast present in the ferment. Thus, the library of yeasts from Kumeu River Sauvignon Blanc provides an immense potential to enhance the aroma of Sauvignon Blanc.

The full factorial experiment design produced a large and cumbersome data-set. However, it was a necessary first step to identifying fermentation potential of candidate yeast.
A variety of analysis could be performed on this large data-set however, analyses was limited to testing the above mentioned null hypotheses. From this dataset, the oenological conditions responsible for the production of lowest and highest concentration of all the aromas analysed are shown below in table 6.1.

From this study it can also be shown that oenological conditions such as yeast used in fermentation, temperature, YAN have a major impact on the final aroma of New Zealand Sauvignon Blanc. Therefore impact of other oenological conditions such as Brix and pH on varietal aroma concentration of New Zealand Sauvignon Blanc should be further investigated. Detailed knowledge of the impact of the range of oenological conditions is necessary to manipulate the aroma concentration and drive a distinctive style of New Zealand Sauvignon Blanc.
### Responses

<table>
<thead>
<tr>
<th>Aroma compound</th>
<th>Highest value (mg/L)</th>
<th>± SEM (n=6)</th>
<th>Factors involved</th>
<th>Lowest value (mg/L)</th>
<th>± SEM (n=6)</th>
<th>Factors involved</th>
</tr>
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<tbody>
<tr>
<td>isobutanol</td>
<td>112.561</td>
<td>± 1.483</td>
<td>VL3 + PKKR1, 12.8°C and Y2</td>
<td>0.000</td>
<td>± 0.000</td>
<td>SB+ PA, 12.8°C and Y1</td>
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<td>isobutyl acetate</td>
<td>0.442</td>
<td>± 0.010</td>
<td>SB+ PKKR1, 12.8°C and Y2</td>
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<td>± 0.001</td>
<td>VL3 + PKKR1, 20°C and Y2</td>
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<td>isoamyl alcohol</td>
<td>212.859</td>
<td>± 6.792</td>
<td>SB+ PKKR1, 12.8°C and Y1</td>
<td>0.000</td>
<td>± 0.000</td>
<td>SB+ PA, 12.8°C and Y1</td>
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<td>isoamyl acetate</td>
<td>140.788</td>
<td>± 1.015</td>
<td>VL3 + PKKR1, 12.8°C and Y2</td>
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<td>± 0.000</td>
<td>VL3 + PKKR1, 20°C and Y2</td>
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<tr>
<td>PEE</td>
<td>193.944</td>
<td>± 3.927</td>
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<td>± 0.000</td>
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<td>BPEA</td>
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<td>VL3, 12.8°C and Y1</td>
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<td>VL3, 12.8°C and Y1</td>
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<td>± 0.026</td>
<td>SB+ PKKR1, 20°C and Y2</td>
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<td>hexyl acetate</td>
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<td>decanoic acid</td>
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<td>± 0.064</td>
<td>SB+ TD, 20°C and Y2</td>
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<td>± 0.110</td>
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<td>ethyl decanoate</td>
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<td>ethyl lactate</td>
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<td>SB+ PA, 20°C and Y1</td>
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### Thios

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<th>Factors involved</th>
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<td>4MMP</td>
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<td>± 11.005</td>
<td>SB+ TD, 12.8°C and Y1</td>
<td>0.000</td>
<td>± 0.000</td>
<td>VL3, 20°C and Y2</td>
</tr>
<tr>
<td>3MH</td>
<td>4229.659</td>
<td>± 217.311</td>
<td>SB, 20°C and Y2</td>
<td>1295.405</td>
<td>± 18.414</td>
<td>VL3 + PKKR1, 20°C and Y2</td>
</tr>
<tr>
<td>3MHA</td>
<td>254.351</td>
<td>± 3.239</td>
<td>SB, 20°C and Y2</td>
<td>164.706</td>
<td>± 1.630</td>
<td>VL3, 12.8°C and Y1</td>
</tr>
</tbody>
</table>

Table 6.1: The factors responsible for the highest and lowest values of fermentation kinetics and aroma concentrations observed in the data-set from full-factorial experiment.
6.5 Analyses of interaction between *S. cerevisiae* (VL3) and *P. kluyveri* (PKKR1)

Apart from identifying and quantifying the impact of yeast interaction on aroma of New Zealand Sauvignon Blanc, this thesis also focused on the systemic analyses of interaction between two yeast species. Indeed, the structure and function of a biological community is driven by interactions between its constituent species (Siepielski and McPeek, 2010). However, to date only a few species interactions have been monitored in-depth (Jessup *et al.*, 2004). The caveat to monitoring species interaction lies in the complexity of a biological community; on the contrary, using a simplified controlled laboratory niche construct to understand species interaction seems inadequate. Therefore an ecological model which is an ideal intermediary is necessary to pursue in-depth study of species interaction in a community (refer chapter 1). Co-fermentation of sterile grape juice (in this study Sauvignon Blanc) via a specific inoculum of two yeast species under controlled condition was proposed as an ideal model to study species (yeast) interaction in a natural environment.

6.5.1 Interaction between *S. cerevisiae* (VL3) and *P. kluyveri* (PKKR1)

The co-ferment of Sauvignon Blanc using VL3 and PKKR1 as prescribed by Anfang *et al.* (2009) was an ideal starting point for the in-depth study of the mechanism of interaction between these two species. This synergistic interaction between VL3 and PKKR1 could be easily monitored via population dynamics and the concentration of aromatic thiol 3MHA in mono and co-ferments.

The caveat of this co-fermentation interaction between PKKR1 and VL3 is that it is impacted by oenological conditions. From chapter 3 (section 3.5.1), it can be seen that by altering the oxygen availability of the co-ferment, the population dynamics of the two yeast species was significantly altered. Furthermore, from section 4.7.3.1 and 4.7.3.2, it can be seen that at temperature 12.8°C and 20°C there was no difference in concentration of 3MHA between VL3-only and VL3+PKKR1 co-ferments. Thus it was assumed that a fermenting temperature of 15°C was crucial to observe synergistic interaction between these two species.

One of the aims of this thesis was to identify potential biomarkers for monitoring the interaction between VL3 and PKKR1 in co-ferment through a holistic approach using systems biology tools such as iTRAQ protein analyses and metabolic footprinting. To achieve this, co-ferment of VL3 and PKKR1 along with the respective mono-ferments were carried out under controlled conditions as prescribed by (Anfang *et al.*, 2009). A sampling protocol was set-up to gather proteomic and metabolomics data-set from mono and co-ferments (refer section 5.6.2), it was based on the proportion of VL3 and PKKR1 at different time-points during co-fermentation. The isobaric labelling
of samples in the different iTRAQ runs were based on a Latin squares experimental design (refer section 5.6.3.1, table 5.1) (Mertens et al., 2012). Furthermore, null hypothesis stating that there is no difference in VL3-only ferments compared to VL3+PKKR1 co-ferments was tested separately on proteomic and extracellular metabolite data to identify potentially new biomarkers for monitoring the interaction between VL3 and PKKR1.

6.5.2 Analyses of iTRAQ data-set to identify potential biomarkers for the interaction between *S. cerevisiae* (VL3) and *P. kluverri* (PKKR1)

The complete genome and proteome of VL3 was published by Borneman et al. (2011), however there is little information regarding *P. kluverri* genome and proteome. Therefore, only the protein sequence of VL3 was utilised in obtaining the overall raw data-set from the four different iTRAQ runs (refer section 5.6.3.2). The initial annotation and clean-up of the raw peak data was an important step in obtaining relevant data-set before normalisation to eliminate false positive discoveries (Becker and Bern, 2011; Jüllig et al., 2007; Karp et al., 2010). Alternatively, a combined data-base of all the *S. cerevisiae* sequence information could have been used to annotate the iTRAQ protein sequences. That could have potentially identified more proteins; however, it could have increased the non-unique false positives and introduced inaccurate sequences into the data-set.

An important consideration in the annotation and clean-up of the raw peak data was the individual contribution of VL3 and PKKR1 in the protein profile of VL3+PKKR1; this was impossible to differentiate given the lack of *P. kluverri* sequence information. Another caveat was that the removal of iTRAQ samples with a high cell population of PKKR1 (e.g.: initial time-point samples of VL3+PKKR1) would have severely reduced the overall data-set. However, it has been reported that there is a degree of redundancy (about 130 homologous genes) in the genome of hemiascomyote yeasts (both VL3 and PKKR1) (Llorente et al., 2000). Therefore to overcome the limitations mentioned above, a decision was made to obtain only the profiles of proteins with a 99% sequence homology to the VL3 proteome. Thus the data-set lacked information regarding the individual contribution of the different yeasts in protein profiles of the different samples. However, this strategy would have undoubtedly removed unique PKKR1 protein sequences from the entire data-set. Perhaps, these as yet unidentified PKKR1 proteins play an important role in driving the interaction between VL3 and PKKR1. It is also important to note that enrichment and isobaric tagging of proteins can exclude certain class of proteins (membrane proteins) which could also have played an important role in yeast interaction (Wang et al., 2008). However, there are always limitations to be considered when performing any global analyses.

The next step in the analyses of protein profiles of VL3-only and VL3+PKKR1 co-ferments was data normalisation of the iTRAQ data-set. Various methods are available for normalisation of iTRAQ
proteomic data (Becker and Bern, 2011; Bolstad et al., 2003; Jüllig et al., 2007; Karp et al., 2010). However, due to large differences in protein abundance values of the different samples, the entire data-set was normalised using Quantile normalisation followed by log2 transformation (Bolstad et al., 2003). Quantile normalisation is a technique which fits the data in all the samples to a common distribution with similar statistical properties this method is robust to outliers (Bolstad et al., 2003). Other normalisation techniques like geometric mean standardization are more susceptible to outliers and were unable normalise the data-set or introduced bias towards proteins with high abundance values (Jüllig et al., 2007). While, these techniques are useful in targeted analyses of key protein with relatively high abundances, Quantile normalisation was robust for global analyses of any important protein despite its protein abundance.

From the iTRAQ data-set, 237 proteins were identified overall; of which 67 were present in all the four runs (refer section 5.6.3.5). Based on the two-way ANOVA results (ferment-type and time-point as factors) there was no difference in the overall protein profile between VL3-only and VL3+PKKR1 co-ferments. A pair-wise t-test of individual proteins from VL3-only and VL3+PKKR1 samples identified 4 proteins that were significantly higher in VL3+PKKR1 co-ferment and one in VL3-only ferment. However, none of these proteins showed a greater than two-fold change in abundance between the different samples and were therefore not considered to be biomarkers (Becker and Bern, 2011) (refer section 5.3.6.6 and 5.3.6.7 and table 5.3).

Additionally, a theoretical proteomic profile mVP was developed based on a calculation similar to that used by Anfang et al. (2009) to test the null hypothesis that there is no difference in mVP (an additive protein profile obtained from VL3-only and PKKR1-only samples) and VL3+PKKR1 co-ferment (refer section 5.3.6.9). The two-way ANOVA produced a statistically insignificant q-value. These results suggested that there was no difference in VL3-only and VL3+PKKR1 co-ferments and failed to identify a potential biomarker for the interaction between VL3 and PKKR1. Based on the limitations discussed above, the full potential of the raw proteomic data-set was not explored. However, in the future with sequence information from PKKR1 this data-set will prove to be a more valuable tool in identifying changes in proteome of the two different yeast species during co-fermentation.

6.5.3 Analyses of extracellular metabolite data-set to identify potential biomarkers for the interaction between S. cerevisiae (VL3) and P. kluveri (PKKR1)

The structure and chemical property of metabolites is not dependent on the species studied, this conferred an advantage when analysing the extracellular metabolite data-set (Villas-Bôas and Bruheim, 2007). However, the chemical property of certain metabolites (hydrophobic) excluded them from the sample due to the derivitization technique utilised in this study (Villas-Bôas et al., 2005).
Also, the annotation of raw metabolite data-set obtained was limited by the number of reference compounds in the reference-ion library maintained at the School of Biological Sciences. However unlike the proteomic profile, the presence or absence or the proportion of either yeast did not limit in the identification of extracellular metabolites (refer section 5.6.4). The annotation, clean-up of false positives and secondary peak data, normalisation of the data-set using a control, by internal standard (D4 Alanine) and by cell population of the samples were performed according to the protocol developed by (Aggio et al., 2011). Overall, 83 different metabolites were identified from the entire data-set of which 63 were present in all samples.

A two-way ANOVA showed that the factor ferment-type (P-value = 0.02) was statistically significant, thus it was possible to differentiate between the extracellular metabolite profiles of VL3-only and VL3+PKKR1 co-ferments and disprove the null hypothesis. A pair-wise t-test of the individual metabolites identified 15 metabolites as being significantly different between VL3-only and VL3+PKKR1 co-ferments. Of which, Leucine (2.88 fold), Glutamic acid (4.6 fold) and Isoleucine (-4.5 fold) could be considered as potential biomarkers of the interaction between VL3 and PKKR1 by comparing VL3+PKKR1 with VL3-only ferments (refer section 5.6.4.4). These amino acids are key to the production of aromatic esters in wine (Swiegers et al., 2005). Also, from chapter 4, it was seen that esters and their acetates were important in distinguishing between VL3 and VL3+PKKR1 ferments. Hence it can be hypothesised that by studying the usage of the biomarkers in the two ferments (VL3-only and VL3+PKRR1), we can gain a better understanding of the interaction between VL3 and PKKR1. In the further, experimental mono and co-ferments of VL3 and PKKR1 substituted with isotope labelled Leucine, Glutamic acid and Isoleucine followed by targeted metabolite analysis (esters and thiols) could provide insights into the mechanism of interaction between these two yeasts (Vivanco et al., 2011).

A two-way ANOVA of extracellular metabolite profile of theoretical co-ferment model (mVP) with VL3+PKKR1 co-ferment yielded a P-value of $3.863e^{-12}$ for the ferment-type factor. This disproved the null hypothesis that there is no difference in the extracellular metabolite profile of VL3+PKKR1 co-ferment and the additive model of the two mono-ferments (mVP) (refer section 5.6.4.6). However, analysis of 3MHA levels in VL3-only and VL3+PKKR1 co-ferments did not show a two-fold increase as observed by Anfang et al. (2009) (refer section 5.6.6).

Pathway activity profiling (PAPi) is one among the numerous techniques which help in the visualization of metabolomic data into biological activity. In this study, it allowed in the identification of 34 metabolic pathways that were significantly different between the two ferments (refer section 5.6.7). This method is based on calculating the Activity Score of the different metabolic pathways in different samples (Aggio et al., 2010). Activity scores are dependent on the number of metabolites identified for each pathway along with their abundance; accumulation of metabolites upstream of a
pathway suggests low activity of that pathway in a particular ferment and is shown as negative fold change. Whereas, accumulation of metabolites downstream of a pathway suggests higher activity for that pathway in a particular ferment and is shown as positive fold-change (refer figure 5.12) (Aggio et al., 2010).

It is known that nitrogen is a limiting nutrient during alcoholic fermentation, perhaps the yeasts involved are either communicating nitrogen limitation or sharing amino acids between each other resulting in elevated 3MHA in Sauvignon Blanc produced from VL3+PKKR1 co-ferments (Bell and Henschke, 2005; Linderholm et al., 2010; Roland et al., 2010c; Torrea et al., 2011). Another useful indicator of nitrogen stress in co-ferment is the Glutamine: Glutamate ratio which could be used to monitor the progress of fermentation (Flynn and Gallon, 1990). However, this requires further targeted experiments with isotope labelled metabolites (Vivanco et al., 2011).

Thus in conclusion, upon testing the null hypothesis that there is no difference in proteomic or extracellular metabolite profile between VL3-only and VL3+PKKR1 co-ferment, I obtained mixed results. The extracellular metabolite comparisons between the two ferments do not agree with findings from the proteomic data. There are many reasons for this discrepancy; primarily, the difference in the biological nature of the two ‘omes contributed significantly in the interpretation and normalisation of the respective data-set (Wang et al., 2008). Also the limitation on the availability of sequence and metabolite information could have had a major impact on the outcome of the null hypothesis test (Duportet et al., 2012). Until, the availability of complete genome information of P. kluyveri, metabolomic analyses is a more viable option in identifying biomarkers involved in the interaction between VL3 and PKKR1.

6.6 Future direction

This thesis was one of the first studies to conduct an in-depth examination of a commercial uninoculated fermentation. The isolation of S. bayanus yeast from 2008 Kumeu River Sauvignon Blanc has provided new insights into the growth and competition of yeast in grape juice. The S. bayanus yeast from this study is capable of producing aroma in similar if not greater quantities in New Zealand Sauvignon Blanc when compared to S. cerevisiae VL3.

Full-factorial experiments provide valuable information when determining the fermentation potential of a yeast species. The ferment and aroma potential of the yeast isolated from 2008 Kumeu River Sauvignon Blanc is tremendous and encourages future use in other wine varieties such as Pinot Gris.
and Gewürztraminer. Also, these yeasts can be used by winemakers to drive a distinctive style of New Zealand Sauvignon Blanc both between and within vintages.

A holistic approach in understanding the mechanism of interaction between *S. cerevisiae* (VL3) and *P. kluyveri* (PKKR1) in co-ferment of Sauvignon Blanc using global proteomic and extracellular metabolomic techniques provided valuable insights. The metabolomics analyses yielded in the identification of three potential biomarkers and 34 different metabolic pathways as being important in distinguishing VL3-only and VL3+PKKR1 co-ferments. This information coupled with identification of the significance of aromatic esters and ester acetates allowed for hypothesising that by conducting controlled ferments with isotope labelled biomarkers followed by targeted metabolite analyses would allow for gaining a better understanding of the interaction between VL3 and PKKR1.

Finally, the co-ferment model for studying mechanism of yeast species interaction using systems biology is viable. Although, at this time, due to the unavailability of sequence and protein information for a majority of non-*Saccharomyces* yeast, targeted metabolite analyses is the main tool to provide insights into species interaction. Alternatively, co-ferment model can be used on two *Saccharomyces* yeast coupled with global analyses of proteins and metabolites to understand the mechanism of interaction between them.
Appendices
Appendix 1: Standard curves for quantification of 3MH, 3MHA and 4MMP in Sauvignon Blanc

To quantify the aromatic thiols via GCMS, a standard curve was utilized. The standard curves were obtained by analysing a range of pre-determined concentrations of 3MH (~450 ng/L to 4500 ng/L) and 3MHA (~100 ng/L to 1000 ng/L) and 4MMP (~25 ng/L to 350 ng/L) with a known concentration of duterated internal standards (25 nM) (section 2.8 for method). Thiols were then quantified from different samples by extrapolation from the standard curve. Figure A1 shows the standard curve and the equation used to obtained the concentration of the three thiols.

Figure A1: Standard curves for thiols 4MMP, 3MHA and 3MH. The linear equations (y) for each thiol is provided, as is the correlation co-efficient ($R^2$).
Appendix 2: Microsatellite profiles of *S. bayanus* and *S. cerevisiae* isolates identified in Kumeu River Sauvignon Blanc 2008

Microsatellite profiles for *S. bayanus* and *S. cerevisiae* isolates were obtained and analysed according to the methods described in 2.8.6. Table A 2a shows the various alleles used in distinguishing between the 20 *S. bayanus* isolates, also included is the profile of S6U a commercial *S. bayanus* isolate. Table A2b shows the microsatellite profiles of *S. cerevisiae*.

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Table A 2a: Microsatellite profile of *S. bayanus* isolates identified in 2008 Kumeu River Sauvignon Blanc. The profile of S6U is shaded grey, Green colour shading indicates primers with Hex and Blue shading indicates primers with FAM fluorescent label.
### Table A2b: Microsatellite profile of *S. cerevisiae* isolates identified in 2008 Kumeu River Sauvignon Blanc. 
Green shade indicates primers with Hex, Yellow with Ned and Blue with FAM fluorescent labels.
Appendix 3: Fermentation kinetics of 20 *S. bayanus* isolates identified in 2008 Kumeu River Sauvignon Blanc at 12.8°C and 20°C

Figure A3: Average rate of fermentation for the 20 *S. bayanus* isolates identified in 2008 Kumeu River Sauvignon Blanc at (a) 12.8°C and (b) 20°C (n=3), yellow square marker points denotes fermentation kinetics of isolate SBJ1d.
Appendix 4: Weight loss measurements recorded for the different mono and co-ferments at specific oenological conditions as part of a full-factorial experiment

Figure A4: weight loss of *S. bayanus SBJ1d*-only ( ), *S. bayanus* + *P. kluyveri* PKKR1 ( ), *S. bayanus* + *P. anomal* ( ) and *S. bayanus* + *T. delbrueckii* ( ), *S. cerevisiae* VL3-only ( ) and *S. cerevisiae*VL3+ *P. kluyveri* PKKR1 ( ) ferments at different oenological conditions. (a) 12.8°C +Y1, (b) 12.8°C +Y2, (c) 20°C +Y1 and (d) 20°C +Y2, weight loss was measured in triplicates (n=3) ±SEM.
Appendix 5: Fermentation kinetics of the mono and co-ferments with *S. cerevisiae* (VL3) and *P. kluyveri* (PKKR1) monitored during the sampling for global proteomic and extracellular metabolite analyses.

Figure A5: Fermentation kinetics of *S. cerevisiae* (VL3)-only ( ), *P. kluyveri* (PKKR1)-only ( ) and *S. cerevisiae* (VL3) + *P. kluyveri* (PKKR1) ( ) co-ferment.
Appendix 6: Generic R codes to perform statistical analyses

The R codes written below are generic, various samples and factors were read, normalised, visualised and analysed using these codes. All data was uploaded as comma delimited (CSV) files onto R using Microsoft Excel 2007 or 2010. These commands are applicable in R2.12, R2.13 and R 2.15 versions.

- Load the data into R:
  
  `data=read.csv(file.choose())`

- Check the structure of the data, looking for factors and variables:
  
  `str(data)`

- Convert variables that should be factors into factors:
  
  `as.factor()`

- Log transform the data (to base 10)
  
  `log10(data$value)`

- Log transform the data (to base 2)
  
  `log2(data$value)`

- Log transform the data (to natural log)
  
  `ln(data$value)`

- Display a boxplot of the data, split by levels of a single factor:
  
  `boxplot(data$value~data$factor1)`

- Display a scatterplot of the data, comparing values at two levels of a single factor:
  
  `plot(data$value[data$factor1 == “A”], data$value[data$factor1 == “B”])`

- Perform a Student’s t-test between two levels of a factor:
  
  `t.test(data$value[data$factor1 == “A”], data$value[data$factor1 == “B”])`

- Perform a single factor analysis of variance on values with a single factor with more than two levels:
  
  `anova(aov(data$value~data$factor2))`

- Perform a 2-factor analysis of variance on values, calculating interaction between all the factors:
  
  `anova(aov(data$value~data$factor1*factor2))`
• Perform a 3-factor analysis of variance on values, calculating interaction between all the factors:
  `anova(aov(data$value~data$factor1*factor2*factor3))`

• Perform Tukey’s HSD-corrected T-tests on all the factors and interactions in the analysis of variance:
  `TukeyHSD(aov(data$value~ data$factor1*factor2*factor3))`

• Perform a Principal Component Analysis with scaling as different variables differ by several orders of magnitude:
  `princomp(data, cor = TRUE)`

• Produce a summary of the Principal Component Analysis:
  `summary(princomp(data, cor = TRUE))`

• Calculate the loadings of the Principal Component Analysis:
  `loadings(princomp(data, cor = TRUE))`

• Plot the first two Principal Components for each datapoint:
  `biplot(princomp(data, cor = TRUE))`

• Load the “preprocessCore” library, which contains the Quantile-Quantile normalisation script:
  `library(preprocessCore)`

• Convert the data into a matrix and perform Quantile-Quantile normalisation:
  `normalised_data= normalize.quantiles(as.matrix(x$data))`

• Display a Quantile-Quantile plot of the data:
  `qqplot(normalised_data)`

• Add a 45 degree line to the plot using the formulae of y=1x+0:
  `abline(0,1)`

• Perform a Fisher’s Exact Test on a 2 X 2 table with two factors, both with levels “A” and “B”:
  `fisher.test(as.matrix(x_a,a,x_a,b,x_b,a,x_b,b))`
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