Functional genomic analysis of sulfur and flavour-related yeast metabolism in wine fermentation with and without fining agents pectin and carrageenan

A thesis presented for the degree of Doctor of Philosophy by

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Statement of Authentication

This thesis is submitted in fulfilment of the requirements for the degree of Doctor of Philosophy at the University of Western Sydney, School of Science and Health. The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in full or in part, for a degree at this or any other institution.

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Refereed Journal Papers

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Abbreviations and Acronyms	Definition
µL	Microlitre
aa-dUTP	5'-(3-aminoallyl)-2'-deoxyuridine-5'-triphosphate
AAT	Alcohol acetyltransferase
ANOVA	Analysis of Variance
ANZFA	Australia New Zealand Food Authority
AWRI	Australian Wine Research Institute
BSE	Bovine spongiform encephalitis
BY4743	A laboratory *Saccharomyces cerevisiae* strain derived from S288C, with the genotype MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/MET15 lys2Δ0/LYS2 ura3Δ0/ura3Δ0
cDNA	Complementary deoxyribonucleic acid
cDNA	Complementary DNA
DAP	Di-ammonium phosphate
DE	Degree of esterification
DEPC	Diethylpyrocarbonate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
DTT	Dithiothreitol
EAT	Ethanol acetyltransferase
EDTA	Ethylene diamine tetraacetic acid
FDR	False Discovery Rate
GC	Gas chromatography
GC	Gas chromatography
GC-MS	Gas chromatography – mass spectrometry
GC-MS SPME	Solid phase microextraction gas chromatography – mass spectrometry
GC-O	Gas chromatography – olfactometry

GEO	Gene Expression Omnibus
H_2S	Hydrogen sulfide
HM	High methoxyl
IAT	Isoamyl alcohol acetyltransferase
IR	Infrared spectroscopy
kL	Kilolitres
LC	Liquid chromatography
LC	Liquid chromatography
LMA	Amidated low methoxyl
LOWESS	Locally weighted scatterplot smoothing
min	Minutes
MIPS	Munich Information Center for Protein Sequences
mL	Millilitres
mRNA	Messenger RNA
mRNA	Messenger Ribonucleic acid
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
NCR	Nitrogen catabolite repression
ng/µL	Nanograms per microlitre
NMR	Nuclear magnetic resonance
OAV	Olfactory activity values
^oC	Degrees Celsius
OD_{xxx}	Optical Density at XXX nm
ORF	Open Reading Frames
PC1	First principal component
PC2	Second principal component
PC3	Third principal component
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
pmol/µL	Picomole per microlitre
ppb	Parts per billion (generally µg/L)
ppm	Parts per million (generally mg/L)

qPCR	Quantitative Real-Time Polymerase Chain Reaction
RNA	Ribonucleic acid
rpm	Revolutions per minute
S. bayanus	*Saccharomyces bayanus*
S. cerevisiae	*Saccharomyces cerevisiae*
S^{2-}	Sulfide
SDS	Sodium Dodecyl Sulfate
SGD	Saccharomyces Genome Database
SO_2	Sulfur dioxide
SPME	Solid phase microextraction
SSC buffer	Salt and Sodium Citrate buffer
TAN	Total assimilable nitrogen
Tris base	Tris (hydroxymethyl)-aminomethane
UNSW	The University of New South Wales
UPLC	Ultra-performance liquid chromatography
UWS	The University of Western Sydney
v/v	Volume per Volume
w/v	Weight per Volume
YAN	Yeast assimilabe nitrogen
YEPD	Yeast extract / peptone / dextrose
YG-S98	Yeast Genome S98

Saccharomyces cerevisiae / *Saccharomyces bayanus* genes are referred to by a three letter mnemonic, followed by a three-digit number. Gene names are italicised, dominant alleles capitalised and recessive ones given in lower case. Deletion is indicated by the Δ symbol as a suffix. Protein gene products are named according to the gene name, with the first letter capitalised and suffix –p.

Abstract

Since the first vineyard was planted in Sydney more than 200 years ago, the Australian wine industry, propelled by technological advances in yeast biology, viticulture and the fermentation process, has been transformed into a multi-billion dollar economy for export and domestic consumption. Flavour, flavour stability and clarity are key characteristics of white wines. The wine flavour profile is primarily governed by secondary metabolites from fermenting yeast (*Saccharomyces cerevisiae*) and most of the flavoursome metabolites result from nitrogen metabolism, with many of the flavour compounds derived from amino acids. In another aspect, sulfur-containing compounds, such as hydrogen sulfide (H_2S) and sulfur dioxide (SO_2) are metabolic by-products which can have a serious impact on fermented beverages. Clarity is one of the major constraints in the wine industry, since most of the methods available to avoid clarity problems such as hazing involve expensive or time-consuming procedures that often lead to loss of positive flavour compounds. In this project, the molecular mechanisms underlying H_2S and SO_2 metabolism were investigated, and a new clarity-enhancing measure was explored and its effect on flavour profile analysed. The experimental approaches included anaerobic fermentation in both laboratory and pilot-scale fermenters, yeast cDNA gene expression microarray technology and solid phase microextraction gas chromatography - mass spectrometry (GC-MS SPME) for gene expression and volatile flavour profiling over time and between treatments.

At the start of this PhD project, there were limited options in terms of yeast genome-wide differential gene expression microarray platforms. Due to the cost restraint and the need to outsource the one-colour Affymetrix chips, optimisation of glass-based two-colour cDNA microarray technology was carried out in-house. Many aspects of the protocol were examined such as cDNA method, dye type and hybridisation condition. As a result, however, only limited improvements were achieved. Since then, the outsourced more reliable one-colour Affymetrix chips became cheaper, and were therefore used as the microarray platform for subsequent experiments. The issue of replicate design was investigated in order to clarify whether biological triplicate microarray data were necessary if similar results were achievable in duplicates with lower costs. To verify this, microarray data that had been obtained

in biological triplicate was analysed in a duplicate manner and the generated lists of significant genes were compared. Comparison of ANOVA analyses of the triplicate dataset and each of the three possible duplicate datasets showed R^2 values of at least 0.95, indicating high correlation between these sets. Out of those genes that were significantly up- or down-regulated, three-quarters of the genes were common between the triplicate gene list and each of the duplicate sets, with the genes that weren't common being those with only minor differential gene expression. This suggests that yeast microarray experiments can be carried out in biological duplicates, saving a third of the costs without significantly changing the results. However, a major disadvantage is that p-value generation requires triplicate data. The analysis also demonstrated that technical replicates were unnecessary.

H_2S is an essential sulfur-containing compound found in wine. Growing conditions containing cysteine resulted in elevated H_2S and SO_2 production, however, when a nitrogen source was added, H_2S was decreased. To elucidate the underlying molecular mechanism, microarray analysis was performed with a set of conditions including cysteine only, nitrogen (in the form of ammonium sulfate) only, combination of cysteine and nitrogen, and a control with neither cysteine nor nitrogen. The data analysis suggests that nitrogen catabolite repression (NCR) may be responsible for both the reduction in H_2S quantities when the rich nitrogen source, ammonium, is present, but also could be linked with why yeast release H_2S when grown in cysteine-rich media.

Due to consumer preferences, wine clarity is a major concern for winemakers. Many methods have been developed to combat colloidal instability caused by an excess of proteins, phenolic compounds and metal ions which results in 'cloudy' wine. These methods utilise a range of materials such as bentonite, isinglass and egg or milk products. However, there are disadvantages associated with their application, as they can be expensive, time-consuming and most undesirably, they can strip the wine of flavour compounds. In this study, the macromolecules, pectin and carrageenan were investigated for their potential as fining agents in improving clarity and flavour stability. Both pectin and carrageenan are natural products from citrus or marine plants and are currently used extensively in the food industry. Initial experiments found that pre-treatment with these two compounds not only improved the clarity of the final wine but also the flavour of the final wine product revealed by GC-MS SPME. This was further confirmed using 20 L Chardonnay fermentations to determine

the effects of pectin (1 g/L) and carrageenan (0.15 g/L) on flavour and the gene expression of the wine yeast strain (QA23). Gene expression data revealed expression of genes encoding for amino acid permeases were up-regulated, which could lead to higher amino acid uptake and in turn cause higher levels of many flavour compounds. While the adverse-tasting higher alcohols were also increased in the treated wine, the levels remained below their flavour threshold. The three key flavour compounds that were greatly elevated were ethyl hexanoate, ethyl octanoate and isoamyl acetate, all of which impart fruity characteristics. However, this fermentation was only done in singlet due to the high cost involved. Further validation experiments followed using laboratory-scale (2 L) fermentations with frozen Chardonnay grape juice in triplicate of a control, pectin only, carrageenan only and a combination of pectin and carrageenan, respectively, to further understand the mechanisms using gene expression microarrays.

A significant increase in H_2S production in the pectin-treated fermentations was an intriguing finding. In comparison, the carrageenan treatment did not produce any detectable H_2S. The combined pectin and carrageenan treatment resulted in a similar amount of H_2S as the carrageenan only treatment, indicating that the carrageenan counter-acted the effect of pectin on H_2S metabolism. In terms of sulfur dioxide (SO_2), carrageenan decreased in concentration towards the end of the fermentation, which was also evident in the pectin and carrageenan treatment whereas the amounts of SO_2 in the control and pectin treatments remained unchanged. The genes from the sulfur pathway were down-regulated in the presence of pectin at 48 h, but this pathway was unaffected by the other treatments.

Chapter 1: General introduction

1.1. Project overview

Since the first vineyards were planted in Australia early in the 1800s, the Australian wine industry has been transformed into a multi-billion dollar economy, exporting to more than 100 countries around the world. The underpinning technical factors that drive the industry are innovation in viticulture, advances in yeast biology and process improvements in wine production. On the other side, the business and marketing aspects, it is brand management and compliance with the Australia New Zealand Food Authority (ANZFA) and international wine making regulators which govern the global success and acceptance of wine products. Winemaking is continually under review, as are all matters in the fast moving food and beverage industries. The emergence of bovine spongiform encephalitis (BSE), commonly known as mad cow's disease (MacLachlan, 2012), highlighted the sensitivity of the business to much broader agricultural concerns than ever before realised. The retail sector became overly aware, even paranoid, about the use of animal products in winemaking for clarification, and demanded transparent labelling be adhered to. This, of course, had always been lobbied for by vegans and vegetarian lobbyists but the BSE scare thrust this issue into the spotlight where it could no longer be ignored.

New developments in yeast genetics and metabolism have always influenced winemaking processes but the new labelling regulations and the reluctance of most winemakers to detail animal residues on labels accelerated an urgent search for replacements that had no labelling requirements. This was the time when these studies commenced. The question became whether animal products such as gelatine, fish isinglass, albumin and other protein products, even bovine blood, could be eliminated or replaced with plant-based products. Important considerations for the replacement include whether the additive could be flavour neutral or preferably improve the flavour of the wine or flavour management, and ideally create physically stable wine and even improve the yield of the wine.

This research was initiated by a commercial problem and a commercially relevant application was sought based on yeast genetics. At the time, there was a study that had been well advanced at Foster's Group Limited, regarding the use of pectin and carrageenan as fining agents for the heat stabilisation of white wines in place of

gelatine, isinglass or the more heavy handed and unpopular use of bentonite and diatomaceous earths. As discussed later in section 1.3.4, bentonite has remained the most commonly used clarification aid, despite its disadvantages, due to the lack of a more suitable alternative. This project evolved in parallel with the study at Foster's, and looked at the effects of these alternative treatments on flavour creation during white wine fermentations; Foster's went on to patent the use of pectin and carrageenan for white wine physical stabilisation by reducing the concentration of calcium ions in white wines. The additives can also contribute to heat stability. In the meantime, a genetics approach to consider the effects of modified wine juice composition on yeast gene expression and thereby flavour outcomes was developed.

Many flavour compounds contribute to the flavour profile of a given wine, and a great number of these flavour compounds are derived from the metabolism of *Saccharomyces cerevisiae* (yeast) during fermentation. One of the most important factors relating to the flavour profile is the nitrogen metabolism since many of the flavour compounds originate from amino acids. In addition, sulfur-containing compounds, such as hydrogen sulfide (H_2S) and sulfur dioxide (SO_2) are important metabolic by-products because of their impact on fermented beverages. As mentioned, clarity is a major constraint in the white wine industry and measures have been developed to tackle the problem. However, they can be expensive or time-consuming and, introduce flavour (taint) defects (Waters et al., 2005). The holy grail of winemakers is to make white wine of high quality with no introduced defects, as clear as mountain stream water, with exceptional flavour and physical stability.

This project aims to gain a greater understanding of yeast nitrogen and sulfur metabolism, especially in regards to H_2S and SO_2 production as well as to investigate two new fining agents, pectin and carrageenan for their potential to enhance wine clarity and their effects on wine flavours. Molecular mechanisms for H_2S and SO_2 production were investigated by analysis of yeast cultures in varying nitrogen and sulfur conditions using the genome-wide gene expression profiling technology, cDNA microarray transcriptomics. The effects of pectin and carrageenan on clarity and flavours were studied via initial wine fermentations using pilot plant fermenters at Fosters Australia in Melbourne. This was then followed up with a laboratory scale fermentation setup at UWS in Campbelltown, NSW. Detailed characterisation was then carried out on wine samples including clarity tests, sensory panel assessments and flavour analysis using gas-chromatography-coupled mass spectrometry (GC-MS),

and on yeast samples for genome-wide gene expression profiling using cDNA microarray transcriptomics. Although this sounds straightforward, there have been many logistical problems that had to be sorted out because of the transfer of grape juice across state borders and the need to keep the grape juice frozen to eliminate spoilage.

In the following sections of this introductory chapter, I will discuss the winemaking process, the sensory flavour wheel and the major effectors of wine flavour and the use of fining agents in winemaking. In addition, these sections will cover sulfur and nitrogen metabolism within the context of general yeast metabolism, gene expression, the use of functional genomics which is part of my investigations, and finally a review of my aims for this project.

1.2. Wine

Wine, an alcoholic and flavourful beverage, is traditionally made via the fermentation of grape juice by yeast and has been an important part of human need and culture (Joffe, 1998). Wine consumption around the world is increasing year by year along with the population growth and the rise of affluent, urban Asian markets. This in turn has led to more wine production. In Australia alone, 1.6 million tonnes of crushed grapes were fermented and nearly 1.14 billion litres of wine were produced in the 2009-2010 financial year. Most of the wine, approximately 788 million litres (worth $2.2 billion), was exported to the rest of the world, and the remainder was consumed domestically. In the 2009-2010 financial year, white wine sold in the Australian domestic market rose 3.3% to 219.5 million litres out of the total wine sales of 470.8 million litres (Australian Bureau of Statistics, 2010). Australia is consistently in the top ten wine producing countries of the world. Wine is produced all around the country, with the main areas being the Barossa Valley in South Australia, the Hunter Valley and the Riverina in NSW. As the Australian wine industry is worth $4.3 billion (Australian Bureau of Statistics, 2010), it is important to maintain and promote its growth. To achieve this, active research and development is a necessity. The Australian Wine Research Institute (AWRI) in Adelaide has been a driving force in winemaking innovation. In fact, the Australian industry demonstrated to the rest of the world how quality wine could be made in a marginal country. The mobility of winemakers and their regular contact with winemakers in other countries

has accelerated the spread of new knowledge. While Australia may have been well ahead of others in the 70s and 80s and even later, that advantage has been reduced and other countries are now far more efficient at manufacturing quality wines – including South America, South Africa, China and the UK.

There is a large variety of wines, which result from differences in yeast strains used in fermentation and grape varieties. However, wines can be broadly split into two main types, characterised by their colour; red and white. The differences arise mainly due to the grape varieties used, namely red and black grapes for red wine and green grapes for white wine, although white wine can also be made from the darker varieties by removing the skins prior to fermentation. Red wine contains more polyphenols, such as tannins, than white wine, because these phenolics are contained in grape skins. Unlike white wine fermentation, these remain in the juice during the entire fermentation for red wine production. Polyphenols are thought to provide red wine with additional health benefits such as the prevention of heart disease and atherosclerosis. *Prima facie* evidence as well as scientific research found that the French population has a lower prevalence of coronary heart disease despite the French diet often being rich in saturated fat. This phenomenon is commonly termed the "French Paradox." It is believed that dietary saturated fat was counteracted by a moderate consumption of red wine in French culture (Renaud and De Lorgeril, 1992; Criqui and Ringel, 1994; de Leiris and Boucher, 2008). Previous research has also revealed a J-curve relationship between the consumption of wine and the risk of heart disease, in that heavy drinkers (more than 100 g of alcohol per day) have a high risk but moderate drinkers (up to 55 g of alcohol per day) actually have a lower risk than those who don't drink any wine at all (Renaud et al., 1999). A similar correlation pattern was also found between heavy drinkers and the increased lung cancer risk (Freudenheim et al., 2005) whilst moderate wine consumption improves lung function, with white wine having a stronger correlation (Schünemann et al., 2002). However, these benefits conferred by moderate wine consumption did not extend to other alcoholic beverages (Prescott et al., 1999). Alternatively, certain scientific evidences suggested that the increased folate intake due to higher fruit and vegetable consumption is the cause of the French Paradox (Parodi, 1997) or that the perceived health benefits are actually due to moderate wine drinkers having a higher social status (Mortensen et al., 2001). Nonetheless, the benefit of moderate wine drinking has become popularised within the general public and, therefore, wine consumption

has increased based on the actual or perceived related health benefits (Dodd and Morse, 1994).

1.3. Wine fermentation

The overall winemaking process is schematically illustrated in Figure 1.1. This includes selection of grape variety, crushing, use of filtered (white wine) or unfiltered juice (red wine), yeast fermentation, clarification and fining, maturation and bottling. These steps are outlined in the next few sections.

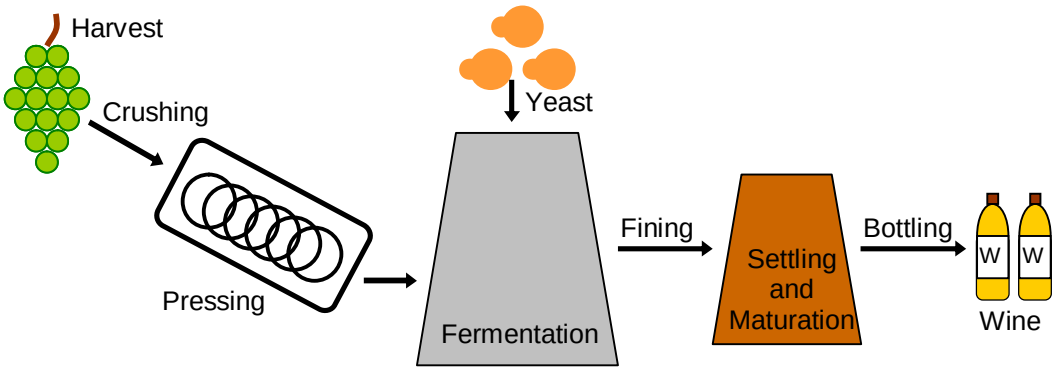


Figure 1.1. Schematic diagram of the overall current white winemaking process.

1.3.1. Grape vines

The base ingredient of wine is grape juice from crushed grapes. Over 70% of the grapes grown in the world are crushed for wine fermentation. The grapes used for wine are most commonly from *Vitis vinifera* species and contain approximately 24% sugar (by weight) when they are harvested. The grape widely grown in Australia for white wine production is the Chardonnay variety. Originally from Asia and made popular in Burgundy, France, it was first brought to Australia in 1832 by James Busby but did not become popular until the 1950s. It is now the main white wine variety produced in Australia due to its climatic adaptability. As Chardonnay wine is very popular and clarity is a critical issue for this type of wine, this juice was used in this study. Chardonnay wines have a wide range of flavour characteristics depending on the method of fermentation but are generally fruity wines.

1.3.2. Crushing

After the grapes have been harvested, they are crushed prior to fermentation. Originally this was done manually by treading on the grapes in a barrel to break open the grapes and release the juice. Nowadays this is achieved using mechanical methods. White wine is produced when yeast ferments the sugars in grape juice (also called must) which is free from skin and seeds, while red wine is made from the juice in the presence of grape skins and seeds, which allows colour, tannins and other compounds to leach out into the wine during the fermentation; the latter step is referred to as maceration.

1.3.3. Yeast fermentation

Once the grapes have been crushed and the stems removed, the next step is the yeast fermentation. Originally, the yeast (*S. cerevisiae* or other strains such as *Saccharomyces bayanus*) came from the skins of grapes being fermented (Bisson, 2004) and is still sometimes used to add complexity to wine (Varela et al., 2009), but these days selected pure or mixed wine yeast cultures are added to ensure that the flavours are consistently desirable for consumers. This practice also practically eliminates the possibility of an acetic bacterial contamination in the fermentation. This kind of contamination can lead to wine spoilage, usually turning it into vinegar if conditions allow. This can also happen after the wine has been bottled, especially in the case of red wine, as discussed in the review by Bartowsky and Henschke (2008). During fermentation, the yeast utilise the sugar and other compounds from the grape juice to produce the ethanol, carbon dioxide and flavour compounds that make up the wine.

Yeast are not only used in the fermentation industries, but also as an scientific model for pure research into higher organisms. Technological advances in this field as well as the industrial field have led to an increase in the potential manipulation and understanding of yeast genetics and therefore yeast themselves (Borneman et al., 2007). Such technology has been used to analyse the transcriptome, proteome and metabolome of different yeast strains or yeast strains under different growth conditions, enabling researchers to understand what is happening by changing the yeast strain or growth condition. This knowledge has been used by winemakers and researchers for the development of yeast strains and fermentation conditions that

improve winemaking. Such developments have included the concentration of nitrogen required in the must and indicators for a stuck fermentation (Bell and Henschke, 2005; Vilanova et al., 2007). A stuck fermentation occurs when unfavourable conditions cause the fermentation to stop prematurely. Since nitrogen deficiency in the grape juice is one of the common causes of stuck fermentations, the monitoring of such can help prevent the problem. AWRI have published the genome sequence of five wine strains, AWRI 1631, AWRI 796, QA23, VL3 and VIN13 since 2008 (Chambers and Pretorius, 2010).

1.3.4. Clarification and fining

While wine flavour is of utmost importance, the clarity of the wine, especially for white wines, is also extremely critical. Wine can haze or become cloudy for a variety of reasons, including an excess of protein or calcium and inappropriate storage conditions, which take place once the consumer has purchased the wine. Although wine haze generally does not have a detrimental effect on flavour but rather only appearance, consumers prefer to drink clear wine partly because of the usual link they make between cloudiness of solutions and bacterial contamination. To avoid colloidal instability, winemakers clarify and fine wine using agents such as bentonite, milk products and egg white (Rankine and Emerson, 1963; Lambri et al., 2012).

Many of the fining agents, bentonite for example, have a negative impact on the wine and its production process. Bentonite, as mentioned earlier, is a clay and, as such, is physically difficult to remove from the wine as well, thereby reducing the volume of wine. More importantly, not only does bentonite remove proteins from the wine but also indiscriminately removes flavour compounds as well, therefore, reducing the flavour profile of the resulting wine. However, bentonite has still been the leading choice for winemakers since its initial suggestion in 1934 (Blade and Boulton, 1988) due to its effectiveness in clarifying wine and its long history of use. Most of the other fining agents are animal-derived and are thus unsuitable for the increasing vegan and environmentally-conscious consumer market, as mentioned earlier. In addition, some consumers have allergic reactions to the sub-trace amounts of these substances in the wine, despite being present in levels below detection by tests (Vassilopoulou et al., 2011). Some work has been conducted to counter the negative effects of fining agents and to investigate non-animal-based products,

however, bentonite, in conjunction with milk and egg products, is still the leading fining agent in the wine industry because of its effectiveness at preventing haze. The clarification and fining agents, pectin and carrageenan which were studied in this project, are further discussed in Section 1.8.

1.4. Alcohol in wine

Apart from water, alcohol is the main component of fermented beverages, including wine. Generally, wine contains 10-15% (v/v) alcohol. It is the alcoholic nature of fermented beverages that accounts for their popularity; however, it is also responsible for their negative side-effects. One of the obvious advantages for the high alcohol content is its inhibition of microbial growth, allowing foods and drink to be stored for much longer. As described in Section 1.2, there are also health implications associated with drinking wine; positive for moderate drinkers and negative for heavy drinkers. There are many health and behavioural disadvantages to drinking alcoholic beverages; however, these are generally limited to drinking in excess.

There are several methods of determining the alcohol content of wine. One of them is based on the difference in refractive index between ethanol and water. This is a quick and easy method with an accuracy level of ± 0.5% volume, utilising a refractometer and correcting for the temperature of the wine. A hydrometer is also commonly used to determine the specific gravity of the wine sample, which allows for the determination of ethanol content by differences in densities. Another method with similar accuracy is the ebulliometer method, which determines the level of alcohol in a wine sample by measuring the boiling points (alcohol boils at 78.4 ^{o}C while water boils at 100 ^{o}C). Other methods include capillary electrophoresis (Collins et al., 1997), spectrophotometer measurement after dilution with a potassium dichromate solution (Magrí et al., 1997), GC-MS (Stackler and Christensen, 1976) and amperometric biosensors (Esti et al., 2003).

1.5. Volatile flavour compounds in wine

As mentioned previously, wine flavours vary with grape varieties, yeast strains and the type of fermentation. Wine generally contains 85-90% water, 10-15% alcohol, 0.4-1% glycerol, 0.5-1.5% acid, with volatile flavour compounds accounting for less

than 1% of the volume of the wine. These volatile flavour compounds make up a large and important part of the organoleptic properties of wine, which includes taste, aroma, mouthfeel and colour. Many of these compounds have a low sensory threshold, which means that their effect can be very pronounced with relevantly low content involved. The term "sensory threshold" for a given flavour compound, is defined as the concentration at which 50% of people can detect the presence of the flavour (Santos et al., 2010). A compound present at concentrations below its threshold concentration is generally not detected by taste buds. For this very reason, bad-tasting compounds might not spoil the flavour of a wine if present at below-threshold concentrations. Conversely, it also means that relatively small increases above the threshold for desirable flavour compounds can have a significant difference in the flavour of a wine. In reality, the composition of wine flavour compounds is more complex and the threshold level of a given compound can change considerably depending on what other flavours are present in the wine, due to compounds' interactions. Such a combinatory effect is described as the "matrix effect" (Ebeler and Thorngate, 2009). Thus, the same flavour compound can be experienced positively at one concentration (generally close to the flavour threshold) in one wine but negatively in another wine due to the presence or absence of other compounds.

Wine flavours can be described and reported in a number of ways, but one conventional method is to use a flavour wheel established according to the flavour profile obtained from a trained sensory panel, like the one shown in Figure 1.2 (Swiegers et al., 2005a; Arroyo et al., 2009). The sensory panel approach can be more accurate in terms of assessing overall flavours which are governed by combinations among different volatile flavour compounds. However, the amounts and attributed individual flavours for each volatile can not be differentiated, and compounds below the flavour threshold are certainly not detected. Therefore, to complement such a deficiency, GC-MS profiling is necessary for flavour analysis in addition to a sensory panel assessment. The various types of wine flavour compounds are described in the following sections, which are broadly categorised as esters, higher alcohols, carbonyl compounds, volatile acids and volatile sulfur compounds.

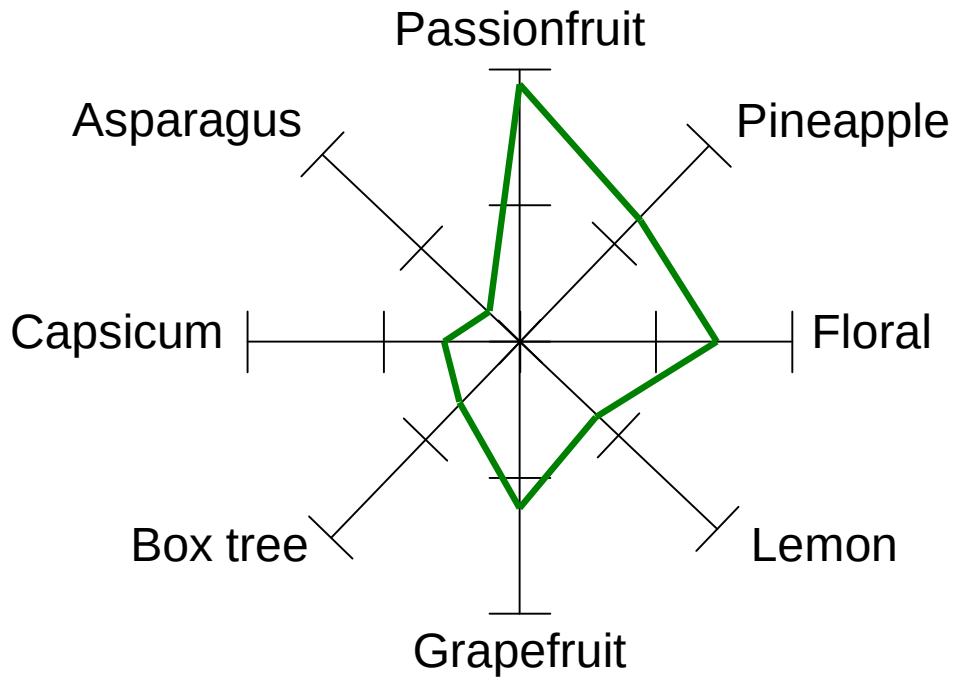
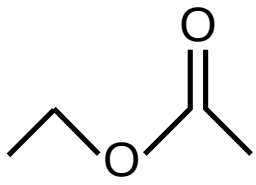
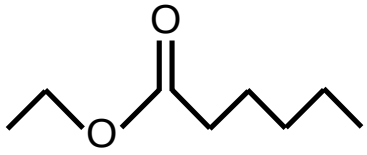
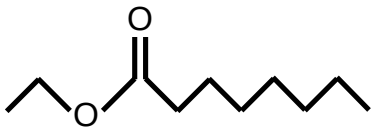
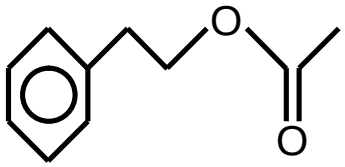
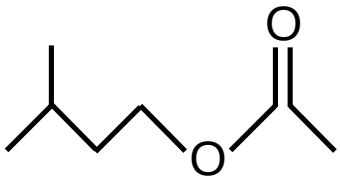


Figure 1.2. A spider wheel of some flavours in a theoretical wine, showing some of its flavour characteristics, as judged by a human sensory panel. Adapted from Swiegers et al. (2005b).

1.5.1. Esters

Esters are a group of compounds with the structure of R-COO-R′ where R and R′ are various side chains. They are generally produced by the reaction between a carboxylic acid and an alcohol, a process called esterification. There are two main types of ester – ethyl esters (where R' is $–CH_2CH_3$) and acetate esters (where R is $–CH_3$). Examples of these include isoamyl acetate, phenyl ethyl acetate and, the most abundant ester in wine, ethyl acetate. These are described in Table 1.1. While esters are found in wine at very low concentrations, they also have low flavour thresholds, making them important elements of the flavour profile. Esters generally impart pleasant fruity and floral characteristics. Ester production is influenced by numerous different factors, e.g. yeast strain, temperature, must clarity, winemaking methods, grape skin contact, SO_2 levels and amino acids concentration. Fermentation at a higher temperature can result in an increase of ester production (Saerens et al., 2008b).

Table 1.1. Characteristics of important esters in wine.

	Flavour	Flavour threshold (mg/L)[1]	Structure
Ethyl acetate	Fruity, pineapple, sometimes nail polish	7.5	
Ethyl hexanoate	Fruity, green apple	0.005	
Ethyl octanoate	Fruity, pear, sweet soap	0.002	
Phenylethyl acetate	Floral, roses, fruity, honey	0.25	
Isoamyl acetate	Banana, pear	0.03	

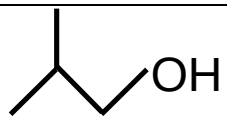
[1](Moreno et al., 2005)

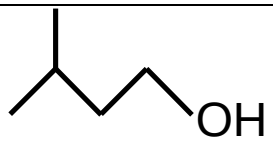
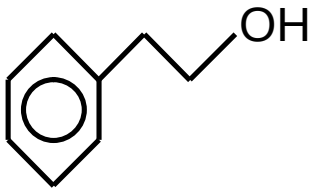
1.5.2. Higher alcohols

Higher alcohols are compounds with an -OH alcohol group and more than two carbons. They are produced by yeast and found in wine at high concentrations compared to other flavour compounds. Examples include isoamyl alcohol, propanol, isobutanol and phenylethyl alcohol, as shown in Table 1.2. When present at optimal concentrations (below 300 mg/L), higher alcohols add a beneficial complexity and fruity flavour to wine (Rapp and Versini, 1995). However, if they exceed 400 mg/L,

they can have negative consequences (Rapp and Versini, 1995), such as giving the wine a strong, pungent flavour (Swiegers et al., 2005b). As with esters, the production of higher alcohols by yeast is increased at higher fermentation temperatures (Landaud et al., 2001). High nitrogen concentrations could decrease higher alcohol production (Hernández-Orte et al., 2005), particularly for phenylethyl alcohol, methionol and isoamyl alcohol where the decrease can be close to 60%. Clarification of the grape must by filteration prior to fermentation reduces higher alcohols, most notably isoamyl alcohol (Ancín et al., 1996). Although leucine is thought to be the precursor amino acid of isoamyl alcohol, they do not seem to be directly linked in fermented filtered must (Ancín et al., 1996). In fact, studies with labelled amino acids show that greater proportions of higher alcohols are synthesised from sugar via α-keto acids (Chen, 1978). These α-keto acids are the building blocks of higher alcohols and are derived from both amino acid catabolism and glucose anabolism (Reazin et al., 1973; Suomalainen and Lehtonen, 1978; Lilly et al., 2006b). When media is amino acid deficient, yeast will generally synthesise amino acids from glycolysis-derived α-keto acids, which are then used for the production of higher alcohols in nitrogen-poor conditions (Ugliano and Henschke, 2009a).

Table 1.2. Characteristics of important higher alcohols in wine.

	Flavour	Flavour threshold (mg/L)[1]	Structure
n-Propanol	Pungent, harsh	830	
Isobutanol	Fusel, spirituous	40	

Isoamyl alcohol	Fusel, cheese (can be fruity)	30	
Phenylethyl alcohol	Floral, roses	10	

[1](Moreno et al., 2005)

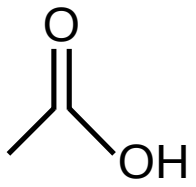
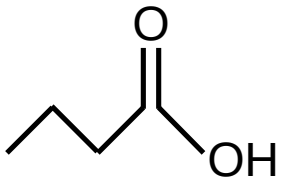
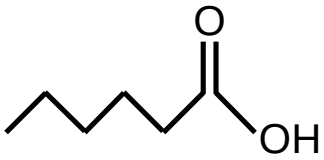
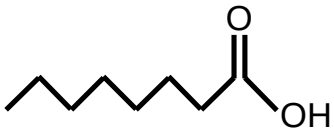
1.5.3. Carbonyl compounds

Carbonyl compounds contain a carbon double-bonded to an oxygen atom such as aldehydes and ketones. While esters technically fall into this category, they are considered separately in the wine industry due to their desirable characteristics in contrast with the carbonyl compounds which are often oxidised to off-flavour molecules. The main carbonyl compound found in wine is acetaldehyde, which is the precursor for ethanol. It is found in large quantities during fermentation. The amount of acetaldehyde decreases towards the end of fermentation, because of its conversion into ethanol. The addition of sulfur dioxide to must can result in a higher accumulation of this intermediate compound because of the strong affinity between these two compounds (Herrero et al., 2003). Another important carbonyl compound is diacetyl, which contributes a buttery or butterscotch flavour at low concentrations (1-4 mg/L), but is undesirable at high concentrations (Swiegers et al., 2005b).

1.5.4. Volatile acids

The major volatile acid in wine is acetic acid, making up approximately 90% of the volatile acids in wine. Others include hexanoic acid, octanoic acid and decanoic acid, shown in Table 1.3, which are produced as a result of fatty acid metabolism in yeast. These compounds generally have unpleasant flavours, such as cheese, rancidity and sweat although octanoic acid and decanoic acid can sometimes have hints of faint fruity aromas (Morris, 1935; Swiegers et al., 2005b).

Table 1.3. Characteristics of important volatile acids in wine.

	Flavour	Flavour threshold (mg/L)[1]	Structure
Acetic acid	Vinegar-like	280[2]	
Butanoic acid	Cheese, rancidity, sweat	10	
Hexanoic acid	Sweat, hint of fruity/grass	3	
Octanoic acid	Sweat, cheese	8.8	
Decanoic acid	Rancidity, fatty	15	

[1](Moreno et al., 2005), [2](Swiegers et al., 2005b)

1.5.5. Volatile sulfur compounds

There are a number of sulfur compounds found in wine, some with positive characteristics, and others with negative ones. Most of these have low flavour thresholds. However, at concentrations slightly above their flavour threshold, they

often impact positive or neutral aromas, with negative traits being detected when present at high concentrations (Duan et al., 2004; Miracle et al., 2005). The non-volatile precursors of volatile sulfur compounds are converted by yeast to form those thiols found in the wine. Many of these precursor compounds are bound with cysteine in the grapes, which is released during yeast metabolism (Swiegers and Pretorius, 2007).

Some of the desirable sulfur compounds are 4-mercapto-4-methylpentan-2-one (4MMP) with a flavour threshold in wine of 3.3 ppb, 3-mercaptohexan-1-ol (3MH, threshold 60 ppb) and 3-mercaptohexyl acetate (3MHA, threshold 2-4 ppb) which impart tropical fruit and passionfruit flavours to the product when present in small amounts. However, these can be perceived as cat's urine if present at higher concentrations.

The sulfur compounds that contribute negatively to wine include diethyl sulfide (rubbery), dimethyl disulfide (cabbage, onion), diethyl disulfide (garlic, burnt rubber), dimethyl sulfide (sulfurous cabbage) (Hansen et al., 2002), methyl mercaptan (rotten cabbage), methionol (meaty, onion) (Miracle et al., 2005) and hydrogen sulfide (H_2S) (rotten egg). In this study, the primary sulfur compound being investigated was H_2S due to its intense rotten egg odour and very low threshold. With the flavour threshold of only 10 ppb (Burdock, 2002; Swiegers et al., 2005), H_2S is highly undesirable and numerous research initiatives have been carried out to understand the metabolic mechanisms of the compound's production in the course of wine fermentation. Research has been carried out, for example in terms of varying vitamin and amino acid levels (Eschenbruch and Bonish, 1976), nitrogen availability (Jiranek et al., 1995; Gardner et al., 2002; Ugliano et al., 2009a), the effect H_2S production has on flavour production, such as an increase in n-propanol production (Giudici et al., 1993) and which genes are involved in this process (Linderholm et al., 2008). This last study utilised a yeast deletion library grown on BiGGY agar (Bismuth Glucose Glyine Yeast agar) which is used to distinguish strains with different H_2S production by the colour of the colony caused by bismuth sulfide precipitation. These results were not correlated to the results found in wine studies, however, but suggest that *MET17*, *CYS4*, *HOM2*, *HOM6* and *SER33* are involved (Linderholm et al., 2008). Due to the importance of controlling H_2S in wine, an additive that could reduce these levels while improving or maintaining overall wine flavour would be beneficial to the wine industry.

Another extremely important sulfur-containing compound is sulfur dioxide (SO_2), which is metabolically closely linked to H_2S but has a very different role in wine. SO_2 acts as a preservative and has many benefits when present in wine up to its relatively-high flavour threshold of 25 ppm, which is 400 times higher than that of H_2S (Landaud et al., 2008). Below this threshold, SO_2 does not have a negative effect on the flavour and aroma. Rather, it gives the wine or beer antioxidant protection by scavenging reactive oxygen species (ROS) and foul-tasting carbonyl compounds. This promotes wine and beer stability (Hansen and Kielland-Brandt, 1996a; Duan et al., 2004). Although it is produced via yeast metabolism, winemakers often add SO_2 to the wine prior to bottling. Wine oxidation produces a molecule called acetaldehyde that has a bruised apple odour. This molecule binds to SO_2 neutralising this affect on the wine (Usseglio-Tomasset, 1992; Osborne et al., 2000). For these reasons, SO_2 is considered to be a favourable molecule to have in wine, and is closely monitored during wine fermentation. Due to its close link to H_2S, SO_2 will also be investigated in this study.

As described above, the wine flavour profile is inextricably linked to the starting grape varieties, yeast metabolism and interactions between various flavour components. As yeast are responsible for much of the flavour and alcohol in wine, it has always been a subject of intensive research at genetic and molecular levels (Backhus et al., 2001; Bisson, 2004; Abbott et al., 2009).

1.5.6. Grape flavour compounds

The flavour profile of a wine is made up of compounds that are derived from yeast and grapes. The grape flavour compounds include terpenes, norisoprenoids, phenolics and methoxypyrazines. Many of these compounds are bound while in the grape and may be metabolised by yeast to form volatile compounds, which increases the complexity of a wine.

The terpenes found in grapes lend a characteristic floral aroma to whole grapes as well as creating a similar flavour in wine (Ebeler, 2001). When two of the same terpenes combine, they form a monoterpene compound. These complex terpenes are abundant in grapes and add to the floral aroma. Norisoprenoids, another form of terpenes present in grapes, contribute to a wine's complex aroma, including grassy, pineapple, lime and honey (Ebeler, 2001). The low pH of wine combined with yeast

enzymes convert the terpene glycosides in must to free volatile terpenes in wine (Ebeler and Thorngate, 2009).

Phenolic compounds are primarily found in the seeds and skins of the grapes, therefore, are in higher concentrations in red wine than in white wine, where skins are discarded prior to fermentation. The phenolic content of white wines is low, approximately 100 – 250 mg/L gallic acid equivalent, while red wine contains 1,000 – 3,500 mg/L gallic acid equivalent. While some of the phenolics have positive aroma characteristics, many are considered off-flavours, such as ethyl-phenols, which contribute a medicinal or barnyard flavour to wine (Dubois, 1983; Swiegers et al., 2005). A common class of phenolic compounds is wine tannins, primarily found in red wine, which are modified proanthocyanidins (Swiegers et al., 2005).

Methoxypyrazines are described as having a vegetable aroma, contributing to a wine's varietal characteristic (Allen et al., 1991). Grape maturity has a large impact on the levels of these compounds. Therefore, early harvest grapes are sometimes fermented together with late harvest grapes to get the ideal level of methoxypyrazines in wine.

Yeast fermentation has a large impact on the final form of many of these grape-derived flavour compounds, and thus has a large effect on the final flavour profile of the wine produced. For instance, many of these compounds are bound in grapes but become released by yeast activity and achieve their volatile potential. An example of this is seen with volatile thiols, the precursors of which are bound to cysteine, and are released following the pitching of yeast into the grape must (Tominaga et al., 1998; Swiegers and Pretorius, 2005).

1.6. Saccharomyces cerevisiae

S. cerevisiae is a species of budding yeast within the fungi family. Its ability to ferment sugars into ethanol and carbon dioxide forms a fundamental basis for the alcoholic beverage and baking industries (Bamforth, 2000). Long before humans knew what yeast was, its functionality was empirically utilised in order to make fermented foods and beverages. *S. cerevisiae* has, thus, been used as a workhorse microorganism to make wine, beer, bread and other fermented food and drink products for many thousands of years (Campbell-Platt, 1994; Hansen and Kielland-Brandt, 1996c).

Most wine yeast strains are thought to have originated in Mesopotamia before spreading to the rest of the world via human migration (Legras et al., 2007). People living in the Middle East around 8,000 BC are thought to have been the first to begin controlled food production, rather than sourcing their food from the wild (Campbell-Platt, 1994; Cornell, 2000). This would have given them more time to consider alternatives to what they were eating, to find new processing techniques to decrease food spoilage and improve the taste of some foods. The first evidence of alcoholic fermentation used to produce beer and wine using barley and grapes was thought to date to around 4,000 BC in lower Mesopotamia by the Sumerians. Recent archaeological findings demonstrated that fermented alcoholic drinks were brewed by Chinese villagers as far back as 7,000 BC (McGovern et al., 2004). At the time, dirty drinking water and the short lifespan of many foods made beer, wine, yoghurt and other fermented foods a more feasible option. Their high ethanol or acid content prevented contamination by other bacteria and fungi, making the food or drink less perishable (Campbell-Platt, 1994). This, as well as the improved taste and the relaxing effect of the alcohol content, popularised alcoholic beverages.

Even though fermented products such as beer and wine have been consumed by humans for at least 6,000 years, the working organism, yeast, was not observed through a microscope until 1680 by Antonie van Leeuwenhoek (Schierbeek, 1953). Only in 1857 Louis Pasteur demonstrated that living yeast caused fermentation (Pasteur, 1860).

Industrial strains of *S. cerevisiae*, such as the brewing and wine strains, have been isolated from the natural environment, with favourable traits retained and unfavourable traits eliminated via genetic breeding. Industrial strains are polyploid, having more than two copies of each gene (Panchal et al., 1984). In contrast, laboratory strains used in experimental studies around the world have either one copy (haploid) or two copies (diploid) of each chromosome (Sherman, 2002). The genomic complexity of industrial strains makes them more robust by enabling them to cope with the stresses of industrial beer- and winemaking processes such as the fluctuation of temperature and the higher concentration of sugar and ethanol. Interestingly, those strains such as BY4743 employed in this project have actually originated from the industrial strains. The genetic simplicity of the laboratory strains, however, makes them amenable to genetic modification, such as generation of the deletion mutants used herein. The laboratory yeast strain S288C became the first eukaryote to have its

genome fully sequenced, after a worldwide effort in 1996, using the resources of 600 scientists (Goffeau et al., 1996). The genome has just over 12,000 kilobases (kb) across 16 chromosomes, arranged in 5,885 open reading frames (ORFs) which are potential genes that encode for proteins (Goffeau et al., 1996). The *S. cerevisiae* genome is comparatively compact, with a protein-encoding gene being found for each 2 kb, 15 times more frequently than the genome of humans (Goffeau et al., 1996).

Due to the importance of yeast metabolism on alcohol fermentation and wine flavour, technical innovation in the yeast industry, which is in turn driven by scientific research, plays a pivotal role in wine fermentation. An example on this front is the use of gene expression microarray technology in the discovery of genes related to the aroma profile of wine and followed with the tailored modification of wine yeast. Overexpression of five genes, *YMR210W*, *BAT1*, *AAD10*, *AAD14* and *ACS1*, demonstrated the reliability and usefulness of microarrays because of the correlation of the data, since changing these genes can be used to produce wine with desirable flavour profiles (Rossouw et al., 2008).

The industrial yeast strain used for this study is the *Saccharomyces cerevisiae* variant *bayanus* strain QA23® (from Lalvin, Lallemand, Blagnac Cedex, France). It has many advantages over other wine yeast, including its tolerance of up to 16% alcohol, its fast fermentation rate, very low assimilable nitrogen requirement, producing low levels of volatile acidity (less than the equivalence of 0.2 g/L H_2SO_4 on average) as well as low H_2S and SO_2 production. This strain primarily produces citrus fruit and pineapple aromas when used to ferment Chardonnay grape juice.

Apart from its prominent role in industrial applications, yeast is also the most studied eukaryote at the molecular level and serves as a paradigm of higher species like plants, animals and human beings in fundamental cellular studies. The completion of the whole yeast genome sequencing in 1996 has enabled numerous breakthroughs in the understanding of basic cellular and molecular processes (Dujon, 1996; Goffeau et al., 1996; Piškur and Langkjær, 2004), which greatly benefits other research fields such as medicine. The development of the complete set of gene deletion yeast mutants has further advanced both basic science and the wine industry.

1.6.1. Yeast deletion library

The yeast deletion library is a collection of yeast strains, each of which has a single gene deleted using gene disruption techniques. The ease of genetic manipulation in yeast enables a gene to be disabled by inserting a deletion cassette in the place of the regular ORF (Giaever et al., 2002). The cassette used to construct the yeast deletion library was developed by Wach et al. (1994). Apart from the transcriptional and translational control sequences, this *KanMX* cassette also comprised an ORF from the *Escherichia coli* transposon Tn903, which contained a geneticin (G418) resistance gene (Wach et al., 1994). This makes all yeast strains that have been transformed with the *KanMX* cassette (the deletion yeast strains) resistant to the antibiotic, whereas the parent strain, *S. cerevisiae* BY4743, is not viable under geneticin conditions, therefore allowing for selection. The addition of this cassette into the genome of the mutant strains makes it easier to keep them free from contamination with other microbes and the wildtype parent strain. An additional advantage of using the *KanMX* cassette is the inclusion of UPTAG and DOWNTAG sequences flanked by universal primers. These sequences, unique to each mutant, allows for its identification by sequencing or microarrays (Winzeler et al., 1999; Giaever et al., 2002).

The comprehensive deletion of single genes throughout the whole genome has only been carried out in a single organism and that is the yeast. For each of the 4,757 non-essential genes in the yeast's genome, there is now a strain that contains every other gene that the wildtype yeast would have, except for that deleted gene (Giaever et al., 2002). Due to the deletion of these genes, researchers can now investigate what would happen to a yeast cell if it did not have that gene present in its genome (Giaever et al., 2002). This allows us some insight into the function of the protein encoded by the ORF and whether the treatment interacts with a particular protein or group of proteins, by observing how the cell reacts when the protein is missing.

The advances in yeast genomics and gene deletion mutants have facilitated a myriad of studies in yeast metabolic pathways (Backhus et al., 2001; Harshman and Martínez-A, 2002; Pérez-Ortín et al., 2002; Rossignol et al., 2003; Rossignol et al., 2006; Tanaka et al., 2006). The resulting knowledge is crucial to the wine industry since most of the flavours found in wine are produced as secondary by-products of

yeast metabolism, particularly involving sugar, nitrogen, sulfur and amino acid metabolism.

1.7. Yeast metabolism

1.7.1. General metabolism

Yeast metabolism is the production line for the chemical components of wine including ethanol and flavour compounds. As shown in Figure 1.3, there are two types of metabolism depending on oxygen availability, namely aerobic respiration and anaerobic fermentation, although yeast will undergo fermentation aerobically if they are grown in high sugar conditions. Fermentation is usually inhibited by oxygen, resulting in respiration; this is known as the Pasteur Effect (Pasteur, 1860). During aerobic respiration, sugar is converted into pyruvate and NADH, which is then processed through the citric acid cycle with oxidative phosphorylation to produce CO_2 and ATP. This process yields 18 times more energy than fermentation (36 ATP molecules instead of only two) so that growth and biomass accumulation is much quicker when yeast respire (Dharmadhikari, 2001). In the absence of oxygen, or because of catabolite repression (which occurs when high sugar and oxygen are both present), anaerobic fermentation takes place (Zamora, 2009). Fermentation taking place in aerated high-sugar grape juice / media is known as the Crabtree Effect (Crabtree, 1928). During fermentation, pyruvate is converted into ethanol and oxaloacetate, releasing glycerol, ethanol, CO_2 and succinate from the cell. It is this anaerobic fermentation that produces wine from grape juice. Yeast gains a competitive advantage over other microbes associated with the starting material – grapes – due to the production of ethanol and yeasts' capacity of survival in a much higher concentration of alcohol. This competitive advantage is most likely the reason for the preference of alcohol production (fermentation) over biomass production (respiration) in aerated high-sugar environments (Dharmadhikari, 2001; Zamora, 2009). Along with the alcoholic fermentation, flavour compounds are produced as secondary metabolites, as shown in Figure 1.4.

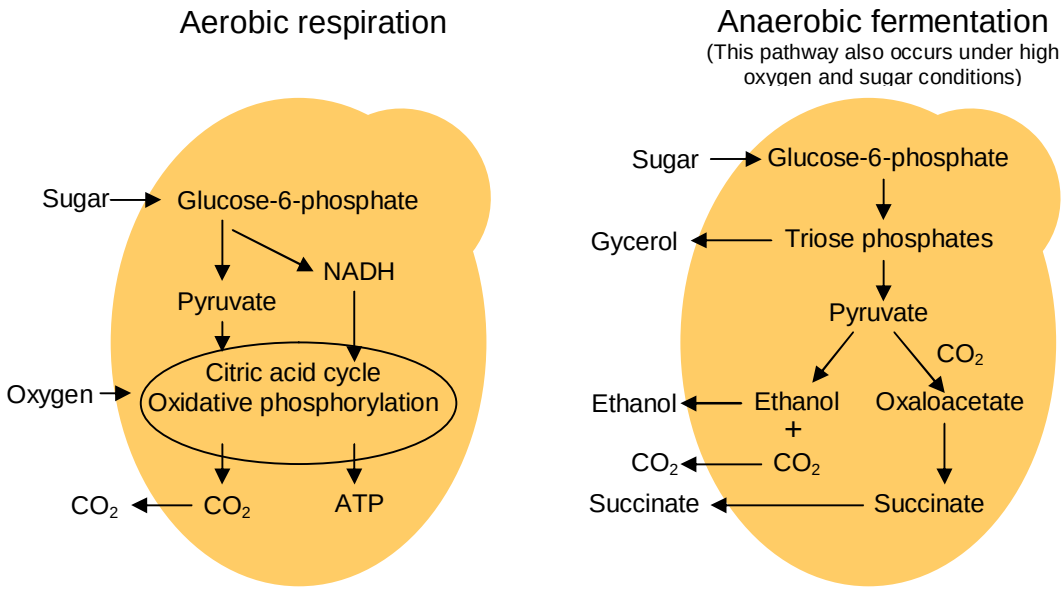


Figure 1.3. Sugar metabolism in yeast under aerobic and anaerobic conditions.

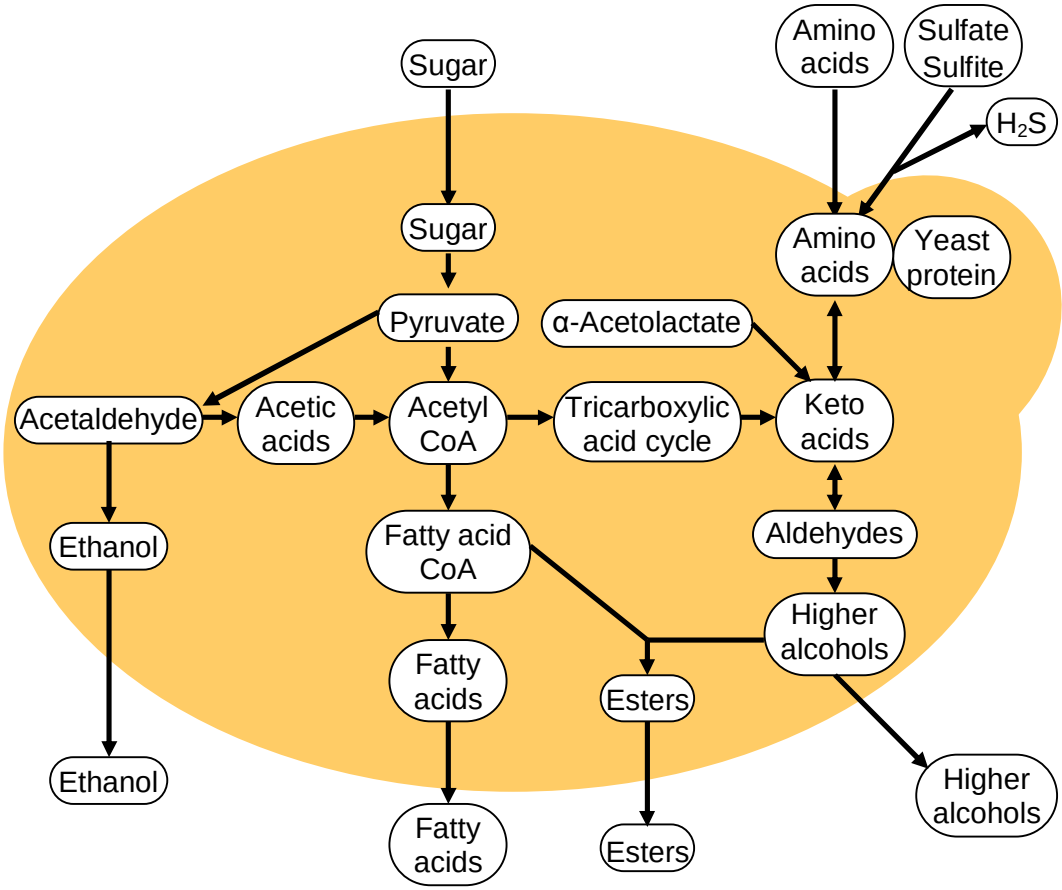


Figure 1.4. A simplified schematic of yeast metabolism involving derivation of flavour compounds from sugar, amino acids and sulfur metabolism in wine yeast. Adapted from (Swiegers et al., 2005b).

An important category of these compounds are esters, which are produced by the reaction between higher alcohols generated by nitrogen and sugar metabolism in conjunction with Acetyl-CoA or Acyl CoA from sugar and lipid metabolism (Chen, 1978; Verstrepen et al., 2003). While some volatile esters are formed through the degradation of the amino acids from grape juice or supplementation, most are produced by other mechanisms (Miller et al., 2007). The production of acetate esters are regulated by at least three acetyltransferases; alcohol acetyltransferase (AAT), ethanol acetyltransferase (EAT) and iso-amyl alcohol acetyltransferase (IAT) which interact with acetyl-CoA and higher alcohols to form the acetate ester (Pretorius, 2000). Various genes are responsible for synthesising different esters and higher alcohols, including *ATF1*, *ATF2*, *EHT1* and *IAH1*. The alcohol acetyltransferases, *ATF1* and *ATF2*, synthesises ethyl acetate and isoamyl acetate. The over-expression of *ATF1* results in a large increase in ethyl acetate, isoamyl acetate, phenylethyl acetate and ethyl hexanoate while the over-expression of *ATF2* had a lesser impact on ethyl acetate and isoamyl acetate concentrations. *IAH1* overexpression caused a decrease in ethyl acetate, isoamyl acetate, hexyl acetate and phenylethyl acetate. *EHT1* overexpression increased ethyl hexanoate, ethyl octanoate and ethyl decanoate concentrations (Lilly et al., 2006b; Rossouw et al., 2008).

Amino acids are an integral part of the flavour production pathways as shown in Figure 1.5, with amino acids coloured blue and flavour compounds coloured green, for example yeast convert valine into isobutanol and valine indirectly influences propanol. Lucine and isoleucine directly contribute to isoamyl alcohol production. While most of the flavour compounds measured in this study are included in this schematic, there are other compounds mentioned later. There are ten major genes that are involved in flavour production, which are *ADH1*, *BAP2*, *BAT1*, *BAT2*, *ILV5*, *ATF1*, *ATF2*, *IAH1*, *EHT1* and *EEB1* (Saerens et al., 2008a).

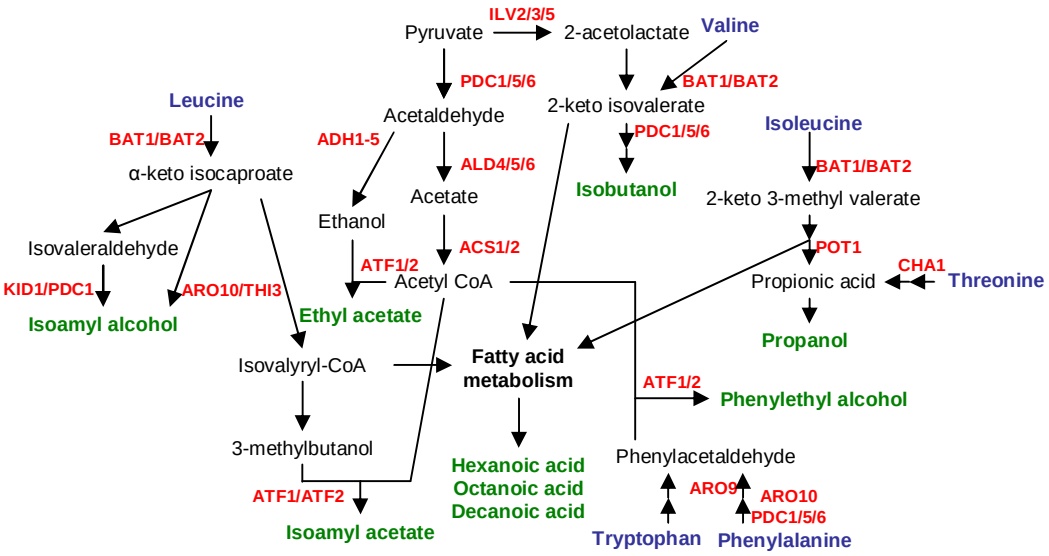


Figure 1.5. Simple schematic of the production of flavour compounds by yeast metabolism. As shown in the diagram, amino acids are integral to flavour production. Blue indicates amino acids, green indicates flavour compounds and red indicates gene names. Adapted from Lilly et al. (2006a) and Rossouw et al. (2008).

1.7.2. Nitrogen metabolism in yeast

Understanding nitrogen metabolism is essential for understanding wine fermentations, because nitrogen metabolism is an integral part of the inter-connected networks including cell growth, alcohol fermentation, sulfur metabolism and flavour profile. The main nitrogen sources for yeast using grape juice, are ammonia, amino acids, polypeptides and other amines. Amazingly, in contrast to human beings, the yeast cell contains all the biosynthetic genes for every amino acid. From ammonium alone, each amino acid can be synthesised. Furthermore, glutamine and glutamic acid can serve as the precursors of the remaining amino acids (Avendano et al, 1997; Ter Schure et al, 1998). As a consequence, ammonia, glutamine and glutamic acid are the primary or preferred nitrogen supplies for yeast growth, while other nitrogen sources are secondary. Yeast cells have an intrinsic ability, acquired through their long evolutionary history, to sense the quality and amount of the nitrogen sources in their environment.

When the preferred ammonia, glutamine and glutamic acid are in excess, the yeast cell only utilises these compounds for amino acid biosynthesis and protein translation. At the same time, the cell represses the expression of the genes required for the

metabolism of the secondary nitrogen sources. This process is called nitrogen catabolite repression (NCR), which is depicted in Figure 1.6a. The process is regulated via the TOR (target of rapamycin) pathway, by phosphorylating the GATA transcription factor, Gln3p. Once phosphorylated, the transcription factor resides in the cytoplasm by binding to Ure2p, which serves as an anchor, rendering it inactive (Bertram et al, 2000). Consequently, the preferred nitrogen sources are used in cell cycle and cell proliferation. On the other hand, under deficiency of the preferred nitrogen source, NCR sensitive genes are no longer repressed. This de-repression process is mediated through the GATA transcription factors, such as Gln3p and Dal80p (Coffman et al., 1997; Cunningham et al, 2000; Georis et al, 2009a, Georis et al, 2009b). Gln3p is the positive, and, Dal80p the negative regulators (Figure 1.6b). During growth on poor nitrogen sources, Gln3p is translocated from the cytoplasm to the nucleus where it binds to GATA sequences in promoters of NCR sensitive genes, such as *GAP1*, *PUT4*, *GDH1* and *GLN1* (Ter Schure et al, 1998). Such a process involves dephosphorylation of Gln3p and importation of Gln3p into the nucleus (Bertram et al, 2000).

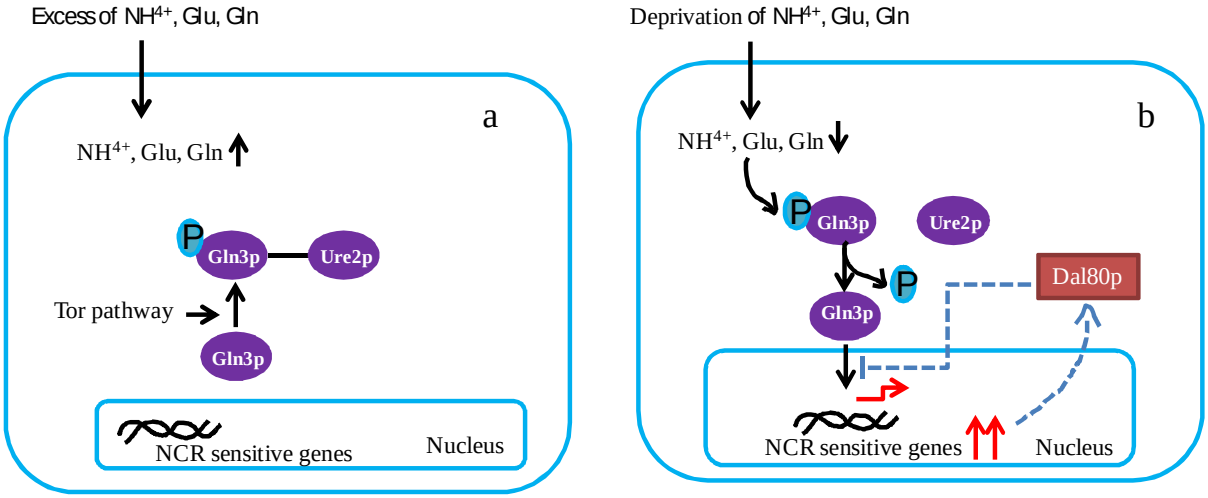


Figure 1.6. Schematic diagram of nitrogen catabolite repression (NCR) and the de-repression process. (a) With abundance of the preferred nitrogen source, the transcription factor, Gln3p, remains phosphorylated and anchored in the cytosol by Ure2p. As a result, the expression of the genes required for the secondary nitrogen sources is repressed. This process is called NCR. (b) Under deficiency of the preferred nitrogen source, the NCR is de-repressed and Gln3p is dephosphorylated and translocated to the nucleus, which activates the NCR-sensitive genes. The level of such activation is further controlled by the negative regulator, Dal80p, as indicated by the broken lines. Glu denotes glutamine and Gln, glutamic acid.

Ultimately, the knowledge of nitrogen metabolism may be used in practice, that is, winemaking. The key point is knowing how to manipulate nitrogen metabolism and its inter-connected network and knowing what constitutes a desirable wine. Or in commercial terms, wines that consumers prefer. For instance, nutrient supplementation is a commonly used practice to enrich nutritionally suboptimal grape juice to favour preferred aroma compounds or reduce others regarded as defects (Winter et al, 2011). Such nutrient additives, allowed by wine regulatory authorities in many countries, include the preferred nitrogen source, usually added as diammonium phosphate (DAP). The concentrations of esters and higher alcohols, which impart fruity and fusel aromas are greatly influenced by nitrogen availability (Bell and Henschke, 2005; Ugliano et al, 2008). A previous study also demonstrated that nitrogen is a critical modulator of volatile sulfur compounds such as H_2S, a volatile with a rotten egg odour (Rauhut, 1993). Part of this project is focused on the effects of ammonium and cysteine on the concentrations of H_2S and SO_2.

1.7.3. Amino acid metabolism in yeast

Wine yeast need to import and use external amino acids for protein translation and catabolic metabolism, under both anaerobic and aerobic conditions. Proline, stands apart as the only amino acid which can only be assimilated under aerobic conditions (Boulton et al, 1996a). Amino acids are known for their roles as the building blocks of proteins, the basic functioning and regulatory molecules for cellular structure and metabolism. They are also the precursors for flavour compounds as described in Section 1.5; for example, the amino acid phenylalanine is the precursor of phenylethyl alcohol (Dickinson et al, 2003). Amino acids can be divided into several groups within yeast metabolism, according to the precursors in their biosynthetic pathways; for instance, glutamate, glutamine, proline and arginine are synthesised from alpha-ketoglutarate; valine, alanine, leucine from pyruvate; aspartate, asparagine, methionine, threonine, isoleucine, lysine from alpha-ketoglutarate; serine, glycine, csyteine from 3-phosphoglycerate; phenylalanine, tyrosine, tryptophan from phosphoenolpyruvate and erthrose-4-phosphate; and, histidine from ribose 5-phosphate families. Grapes contain many amino acids, especially proline, arginine, alanine, glutamate, glutamine, serine and threonine. Arginine and proline are generally in the highest abundance; one could imagine that it is a pity that proline can

only be taken up in aerobic conditions (Duteutre et al, 1971). Ammonium (usually from DAP) is the preferred nitrogen source for yeast as compared to arginine, perhaps understandably as arginine is uniquely positioned for other important processes such as the production and metabolism of ornithine, urea, glutamate and ammonium as well as general amino acid biosynthesis. In fact nitrogen application to grape vines prior to harvest promotes grape arginine levels. Bell and Henschke (2005) reported and proposed that this may be more beneficial than DAP supplementation.

Amino acid uptake is facilitated by amino acid permeases. Gap1p is a general amino acid permease, which is usually expressed when amino acid levels are low, so that yeast can allow as many amino acids as possible to enter the cell (Rubio-Texeira and Kaiser, 2006). Gap1p regulation occurs via the localisation of the protein – Gap1p is found in the plasma membrane when amino acids are in low concentrations, and in the vacuole when amino acids are abundant. There are many permeases that are specific to individual amino acids, such as those listed in Table 1.4. Yeast utilise various amino acid permeases that are involved with the uptake of one or more amino acids to ensure the correct amino acid concentration in the cell.

Table 1.4. List of the most prominent amino acid permeases.

Gene name	Description	Reference
AGP1	General permease for most uncharged amino acids, particularly asparagine and glutamine	Schreve et al, 1998; Regenberg et al, 1999
AGP3	Low-affinity amino acid permease, uptakes amino acids as a nitrogen source in nitrogen-poor conditions, induced under sulfur limited conditions	Schreve and Garrett, 2004
BAP2	High-affinity leucine permease, uptake of leucine, isoleucine and valine	Grausland et al, 1995
BAP3	Uptake of cysteine, leucine, isoleucine and valine	Regenberg et al, 1999
GNP1	Uptake of glutamine (expressed regardless of the available nitrogen source)	Zhu et al, 1996

TAT2	Permease of phenylalanine, tryptophan and tyrosine	Regenberg et al, 1999
PUT4	Permease for alanine, glycine and proline	Regenberg et al, 1999

As illustrated previously with the NCR pathway, proper manipulation of amino acid uptake can improve the usage of amino acids like proline, arginine and isoleucine, and can increase fermentation efficiency, which of course is very commercially important. Inactivation of the gene, from an industrial yeast strain involved in negative regulation, *URE2*, the anchor for Gln3p within the cytoplasm, leads to the increased use of secondary amino acids and high fermentation efficiency in terms of alcohol production (Salmon and Barre, 1998).

1.7.4. Sulfur metabolism in yeast

Saccharomyces cerevisiae has a highly effective sulfate reductive pathway in which sulfate is reduced stepwise to sulfite and subsequently to H_2S (see Figure 1.7 and subsequent text). The reduced sulfur is used to synthesise important organic metabolites, most notably methionine and cysteine, which are required for cell growth and metabolism. So, on the one hand, sulfate reduction can be considered an electron sink, a redox buffer that can contribute very significant to redox balance. Under redox stress, one can imagine that high levels of H_2S can be released during yeast fermentations. In addition, under some conditions SO_2 can accumulate if redox stress is not a factor and if sulfur demand for biosynthesis is low. As described in Section 1.5.5, H_2S and SO_2 are highly relevant sulfur-containing compounds to winemaking. Winemakers want to avoid detrimental levels of H_2S while preferring to increase the beneficial SO_2 content (up to its flavour threshold). While SO_2 can be added to the ferments to boost the antioxidant capacity of wine and safeguard flavour, H_2S cannot be readily removed. In fact, H_2S is part of the sulfur metabolism in the synthesis of sulfur-containing amino acids such as cysteine and methionine, and other compounds like homocysteine and glutathione. Copper sulfate has been used in winemaking to remove excess H_2S. 'Copper guns' are still in practice in some breweries and probably in the odd winery as well, however, copper sulfate is more commonly used (Ugliano et al., 2011). Even silver salts have been used to remove reduced sulfur compounds. However, these approaches lead to negative consequences, namely the

simultaneous removal of beneficial thiols from wine (Swiegers and Pretorius, 2007). So the 'sledge hammer' approach to deal with H_2S is just not sophisticated enough especially in today's aspirational markets. Grape must contains low levels of organic sulfur compounds but high levels of inorganic sulfur compounds. This allows yeast to synthesise organic sulfur compounds from the inorganic ones, but the balance between different thiols that is needed to achieve a desired sensory result rests on this complicated interplay between anabolic and catabolic networks.

H_2S and SO_2 formation from sulfate is described in Figure 1.7. Sulfate (SO_4^{2-}) enters the yeast cell, where it is converted into sulfite (SO_3^{2-}) (in equilibrium with SO_2) and sulfite (S^{2-}) (i.e. H_2S). The combination of H_2S and *O*-acetyl homoserine, catalysed by *O*-acetyl homoserine-*O*-acetyl serine sulfhydrylase (Met17p) encoded by *MET17*, form homocysteine, which is the precursor of methionine and cysteine (via cystathionine), as shown in Figure 1.7 (Hansen and Kielland-Brandt, 1996a; Duan et al, 2004; Linderholm et al, 2008; Rauhut, 2009). In previous works, such as Duan et al (2004) and Ono et al (1996), *O*-acetyl serine was said to combine with H_2S in the formation of cysteine with the aid of Met17p. However, this has since been shown to only occur *in vitro* (Linderholm, et al, 2008; Rauhut, 2009). The earlier view was that Met17p acted as both an *O*-acetyl homoserine sulfhydrylase and an *O*-acetyl serine sulfhydrylase in yeast cells. In fact, Met17p facilitates the combination of H_2S and *O*-acetyl homoserine to form homocysteine, and does not enable cysteine formation directly from *O*-acetyl serine. Clearly Met17p performs both functions *in vitro*, but *in vivo* it only acts as *O*-acetyl homoserine sulfhydrylase, that is the combination of H_2S and *O*-acetyl homoserine to create homocysteine, which indirectly leads to cysteine rather than the direct method seen *in vitro*.

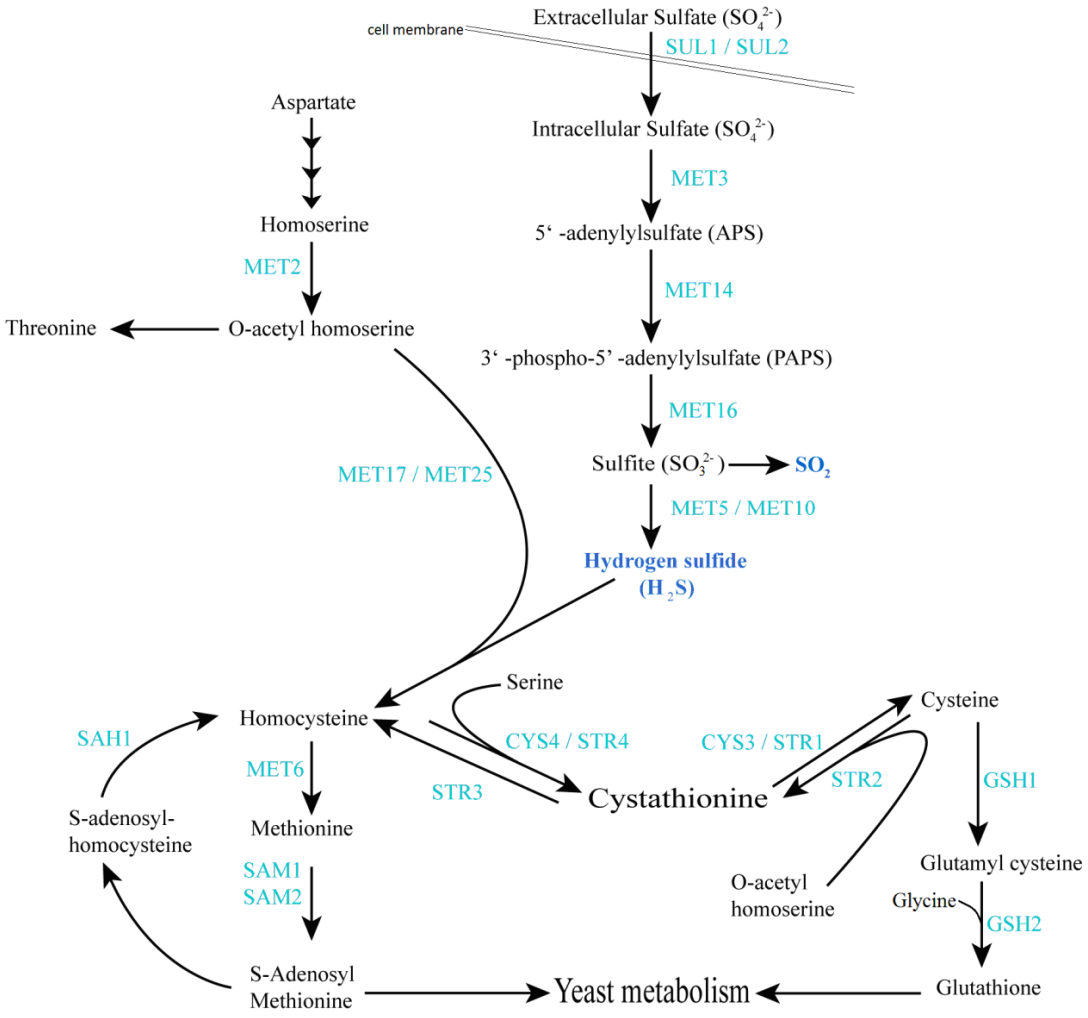


Figure 1.7. The sulfur pathway, adapted from Duan et al. (2004), Linderholm et al. (2008) and Rauhut (2009). The names of genes are in light blue, while H_2S and SO_2 are in bolded dark blue.

Glutathione is produced from cysteine. These three molecules, i.e. cysteine, methionine and the tri-peptide glutathione are very important for yeast survival. Cysteine is involved in the formation of disulfide bonds that are required for the tertiary and quaternary structures of functional proteins. Methionine is the start codon of all protein synthesis. Methionine limitation would cripple yeast metabolism. Glutathione is required to maintain a reduced intracellular environment for normal cellular metabolism (Grant and Dawes, 1996). Therefore, cutting off or cutting back on sulfate runs the risk of stalling metabolism and debilitating the yeast (Hansen and Kielland-Brandt, 1996a; Duan et al, 2004). It is the sort of risk that most wine makers would not be prepared to make. Interestingly though, H_2S stimulation happened when

glutathione was added to synthetic medium despite apparently unchanged sulfur gene expression (Winter et al, 2011). Complex networks require careful consideration.

As shown in the biochemical pathway illustrated in Figure 1.7, *O*-acetyl homoserine is derived from aspartate within the nitrogen metabolism pathways. Therefore, if grape must contains low nitrogen levels, H_2S can not be converted into methionine and cysteine but instead accumulates in the cell as free H_2S and can diffuse into the wine (Vos and Gray, 1979). Figure 1.7 shows H_2S combining with *O*-acetyl homoserine to form homocysteine (and in turn methionine and cysteine). Hence, nitrogen availability in must seems crucial to the amount of H_2S produced, and therefore the addition of DAP into nitrogen deficient grape must can control the amount of H_2S produced (Vos and Gray, 1979; Ugliano et al, 2009b).

In addition to nitrogen levels, sulfur metabolism can also be altered by environmental conditions or yeast variants with genetic distinction (Hansen and Kielland-Brandt, 1996a; Hansen and Kielland-Brandt, 1996b; Duan et al, 2004). Hansen and Kielland-Brandt (1996a) found that inactivating the *MET2* gene increases sulfite levels in beer. The *MET2* gene in *S. cerevisiae* encodes for the protein homoserine *O*-acetyl transferase, a catalyst involved in the conversion of homoserine into *O*-acetyl homoserine, which leads to homocysteine, in the presence of H_2S. Therefore, without this gene, *MET2*, there is no demand for H_2S which leads to the build-up of sulfide and sulfite. The accumulation of these sulfur compounds was most significant when all of the copies of the *MET2* gene were knocked out; however, the presence of only one *MET2* gene still resulted in significant sulfur compound accumulation (Hansen and Kielland-Brandt, 1996a). Hansen and Kielland-Brandt (1996b) later discovered that SO_2 levels and the stability of flavour in the resultant beer increases when *MET10*, a gene that encodes for the α-subunit of the sulfite reductase enzyme, was inactivated. Duan et al (2004) found that H_2S and SO_2 levels increased when cysteine and methionine were added to their growth media. With the addition of nitrogen, however, H_2S concentrations decreased, and SO_2 levels slightly increased, a desirable outcome in brewing fermentations (Duan et al, 2004). This inverse relationship between H_2S and nitrogen has also been reported by Vos and Gray (1979).

1.8. Wine clarification and fining

While wine flavour is of utmost importance, the clarity of the wine, especially for white wines, is also extremely relevant to consumers and winemakers, as discussed in Section 1.3.4. Despite hazing only being a cosmetic issue as opposed to safety or wine flavour, consumers demand clear wine because it looks more appealing. The public associate cloudiness with bacterial or other contamination making hazed wine unappealing to consume. Wine hazing, a problem mainly in white wines, can be caused by colloidal instability resulting from excess levels of proteins in the wine, contributed by the presence of calcium, brought on by increased temperatures. This can occur, for example, where a wine bottle has been left in a consumers' car for too long. Therefore, removing excess protein from the wine before they are sold is a viable solution. To achieve this, winemakers clarify and fine wine using filtration, bentonite, milk products, egg white, etc. However, many of these fining agents have negative impacts on the wine and its production process. For example, bentonite is a clay and, as such, is difficult to remove from the wine after fining. It not only removes proteins from the wine but also indiscriminately removes flavour compounds resulting in an altered wine flavour profile. Most of the other fining agents are animal-derived and, therefore, unsuitable for the increasing vegan and environmentally-conscious market. Despite the disadvantages, due to the effectiveness of haze-avoidance and the lack of suitable alternatives, bentonite has been the leading choice for winemakers since its initial suggestion in 1934 (Blade and Boulton, 1988) with egg and milk products also commonly used.

It has been suggested by Cabello-Pasini et al. (2005) as well as one of the largest wine companies in the world, Fosters Australia (Personal Communication, Prof Peter Rogers, Fosters Australia, 2007), that the polysaccharides pectin and carrageenan could be used to clarify and fine wine. These two anionic (negatively-charged) polysaccharides are plant-derived from fruit and seaweed, respectively. In addition, they are commonly used in the food industry already as additives, carrageenan is added to chocolate flavoured milk drinks and used to thicken or improve mouthfeel of food and drinks. Carrageenan has also been used within the beer brewing industry to precipitate proteins from the wort (Ryder and Power, 1995).

1.8.1. Pectin (E440)

Pectin is a hetero-polysaccharide, found in the cell walls of higher terrestrial plants such as citrus. It can be quite abundant, with 30% of citrus peel being pectin. Pectin contains methylated esters of polygalacturonic acid, consisting of chains of 300 to 1,000 D-galacturonic acid units via 1α→4 linkages, as shown in Figure 1.8. It has an aldehyde group at C1 and a carboxylic acid or methyl ester group at C6.

Pectin is used to gel, thicken and stabilise foods (similar to carrageenan's use) and is also used for acid stability. It is commonly used as the gelling ingredient for fruit preserves, jellies and jam. Pectin is also able to precipitate calcium and it is because of this property that it is employed for fining in wine fermentation because calcium promotes protein precipitation, leading to wine haze. Commercial pectin is produced in Denmark, Germany and Brazil by CP Kelco (under the brand name GENU®). There are three types – high methoxyl (HM), amidated low methoxyl (LMA) and conventional low methoxyl (LMC). The raw ingredients for pectin are generally citrus peel, water and acid. Alkali (NH_3) is also added to make LMA. Pectin is precipitated with alcohol and standardised using sucrose. Since pectin gels in the presence of calcium and other cations, pectin is standardised to calcium responses with added sugars.

The gelling property of pectin is mainly affected by the degree of esterification (DE). This relates to how many methyl ester units (-$COOCH_3$) the structure contains for every carboxyl group (-COOH). For example, three –$COOCH_3$ to every 2 –COOH units equates to 60% DE (which is also called a DE-60 pectin). The calcium reactive pectin used in this study is a low-methyl ester (LM) with a DE of less than 50%.

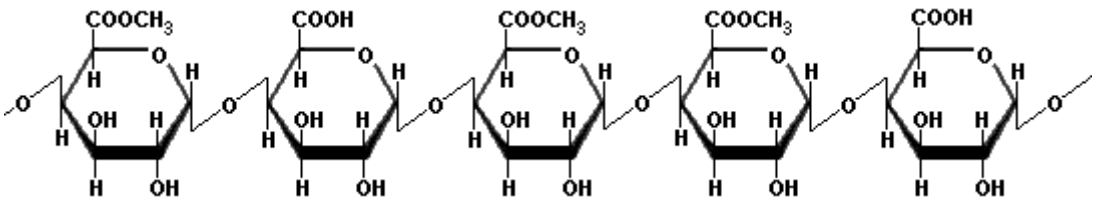


Figure 1.8. Structure of pectin, a polymer of α-galacturonic acid containing a variable number of methyl ester groups.

1.8.2. Carrageenan (E407 (a))

Carrageenan is a high molecular weight linear sulfated polysaccharide, found in red seaweeds and is made up of repeating units of galactose and 3,6 anhydrogalactose (3,6-AG) both sulfated and non-sulfated using alternating 1-3 and beta 1-4 glycosidic linkages, as depicted in Figure 1.9. Carrageenan differs from agar because it contains sulfate groups (-OSO_3-) instead of some of the hydroxyl groups. Carrageenan, like pectin, is commonly used in the food industry for thickening, suspending and gelling food products. For example, it is found in most chocolate flavoured milk drinks, such as those from the Oak™ brand, listed as a vegetable gum. The carrageenan used for wine fining in this study is the iota-dominated cold water soluble type. It has been used in beer production as a clarifier, to remove haze-causing proteins.

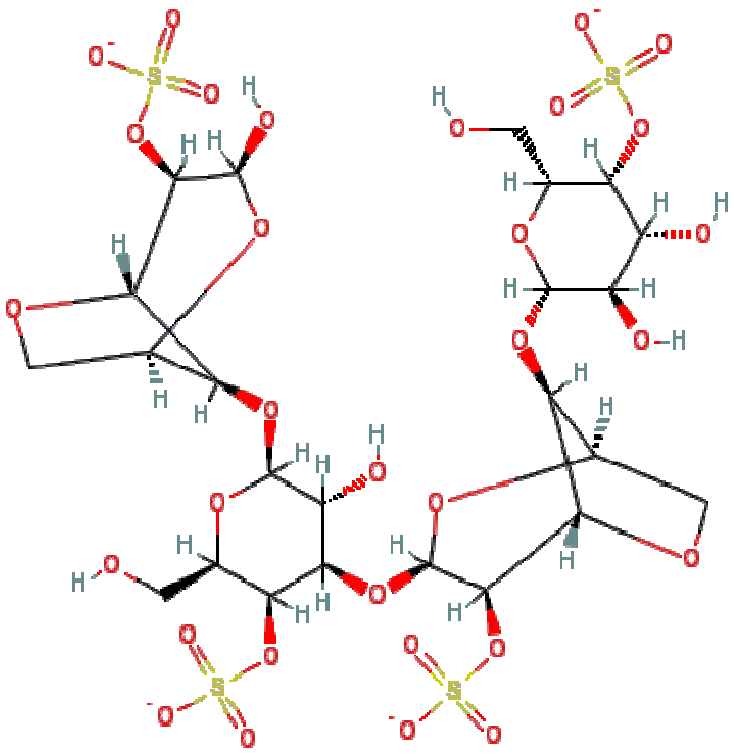


Figure 1.9. Diagram showing the structure of carrageenan.

It is important to determine what effect the added compounds are having on the wine in terms of both clarity and flavour but an additional test is to determine why such compounds (in this case, pectin and carrageenan) are having the effect they are having on the wine. This can be done using functional genomic analyses such as microarrays which allows for the measurement of differential gene expression in the wine yeast.

1.9. Functional genomic analyses

Genomics is the study of the genome of an organism, particularly related to the sequencing, transcription and translation of the genome into protein. The first sequenced genome was of a bacteriophage by Fred Sanger in 1977 (Sanger et al., 1978), with yeast being the first eukaryotic organism to have its genome sequenced in 1996 (Goffeau et al., 1996). Due to major technological advances, sequencing has become less expensive and quicker, thus genome sequencing projects are becoming more frequent, with the human genome completely sequenced by 2007 (approximately 6 billion base pairs). A major benefit of this knowledge is the introduction of the field of functional genomics, including transcriptomic analysis, whereby the genome-wide expression can be analysed under various conditions. The limitation of most genomic projects involves bioinformatics and computer capacity, which has greatly increased in the past few decades. A popular sequencer at the moment is the SOLiD system from Applied Biosystems which can sequence 60 gigabases during a run (Pandey et al., 2008). The number of genomes that have been sequenced has greatly increased in the last few years, including humans (Lander et al., 2001; Venter et al., 2001), *S. cerevisiae* (Goffeau et al., 1996), *Drosophila melanogaster* (Adams et al., 2000) and mice (Eppig et al., 2005) among many other species.

There is a variety of functional genomics platforms available to the scientific community that are able to provide details about changes taking place within a cell at the molecular level. This allows for the comparison of treated samples to controls. Some of these involve phenotypic analysis (the study of physical changes), transcriptomics (the study of gene expression), proteomics (measurement of proteins), metabolomics (metabolites; small-molecules produced via metabolism), glycomics (the study of sugars) and lipidomics (the study of lipids). Incorporating the

information obtained from each of these 'omics' enables a more thorough understanding of the influences of a particular stimulus.

1.9.1. Transcriptomics: cDNA microarrays

Genes within a genome are transcribed into messenger RNA (mRNA) and then translated into functional proteins (Schena et al., 1995). Analysing the mRNA transcripts can reflect what is happening in a cell. Complementary DNA (cDNA) microarray technology facilitates this type of analysis. cDNA microarrays are a popular method for gene expression analysis, as they are holistic, high throughput and informative (Epstein and Butow, 2000; Hegde et al., 2000; Hughes and Shoemaker, 2001). It enables the measurement of the expression of thousands of genes when comparing two conditions – such as presence versus absence of a drug or different environmental conditions. This can rapidly indicate which genes have increased or decreased in expression in response to the altered environmental conditions the cell faces. The differentially expressed genes would provide clues for understanding the biological processes. A major advantage of cDNA microarray technology is its genome-wide nature, whereby all genes and pathways are investigated at the same time, rather than using directed research techniques, which require prior knowledge to focus experiments (Kothapalli et al., 2002). This lack of bias between genes in microarray analysis arises because the gene expression profiles of a gene from a single sample are treated under the same condition on the same microarray slide or chip. RNA is extracted from a sample of treated cells and is then in turn converted into cDNA, labelled with a fluorescent dye and the labelled cDNA hybridised onto a slide before the slide is scanned.

When microarrays were first introduced to the scientific community, they largely used two-colour fluorescent labels. That is, two cDNA samples were labelled with different dyes and hybridised on the same chip. Today, one-colour microarrays are the most common method, which has many advantages over the previous two-colour system. A single sample is hybridised to a single chip and thus allows for more combinations between samples to be made without having to hybridise the same sample onto multiple chips (Duggan et al., 1999; Harshman and Martínez-A, 2002; Jaluria et al., 2007).

Due to the vast amount of data that is produced from microarray experiments, bioinformatic programs are used to create lists of genes that have differential gene expression compared to another chip (one-colour system) or to the other channel on the same chip (two-colour system). They also produce p-values and other statistical data to identify which of the gene expression changes are likely to be due to the treatment rather than to chance. The benefits of using bioinformatics are the speed of analysis, the ability of programs to recognise patterns within the data, lack of bias and calculations of statistical significance. However, the disadvantages involved are cost and that the quality of the results is based on the quality of the program and the programmers who wrote the program. If information or patterns exist that are not expected by the programmers, they may be missed (Brazma and Vilo, 2000; Kothapalli et al., 2002).

1.9.2. Metabolites: Volatile flavour compound detection

There are a wide range of flavour compounds, many with vastly differing properties and, as such, there are many ways of detecting and quantifying these compounds. The earliest technique used a sensory panel, which utilised trained experts to identify the presence of flavour compounds in a sample by smelling and tasting wine samples. Quantification protocols using equipment such as gas chromatography – mass spectrometry (GC-MS) have been developed to more accurately identify and quantify flavour compounds than a standard sensory panel. However, there are disadvantages to this change. Compound interactions create different tastes than the single compounds alone, which a human nose can identify but an instrument simply detects the compounds that have been empirically determined to be responsible for such aromas and flavours. These protocols, however, have led to unbiased assessments and allow for actual compound quantification, even those below the flavour threshold. Research into these methods began in 1942 (Rapp, 1998) by Henning and Villforth, who suggested that esters were an integral aspect of a wine's flavour by compiling a list of compounds in wine (Amerine and Joslyn, 1970).

Since the 1950s, many instrumental methods have been tested, including liquid chromatography (LC), gas chromatography (GC), infrared spectroscopy (IR) and nuclear magnetic resonance (NMR). GC has now been combined with mass spectrometry (GC-MS) to become the industry standard in flavour detection and

quantification (Boutou and Chatonnet, 2007). Sometimes, a middle-ground option is used, which is gas chromatography-olfactometry (GC-O) where the volatiles are separated by GC and then the flavours are detected by a trained human subject to identify the smell of a particular compound or to identify the isolated compound (Pons et al., 2008).

GC-MS determines what sensory compounds are in a liquid sample by separating the compounds using GC and then detecting the compound using MS. It is commonly used in many different fields such as the detection of drugs and forensics. GC-MS was developed by Roland Gohlke and Fred McLafferty in the 1950s (Gohlke and McLafferty, 1993), but has improved in sensitivity since then. Separation via the GC occurs when a sample is eluted through a capillary column with varying properties depending on what attributes desired compounds have, with differences being in column length, diameter, phase, etc. When a compound elutes from the GC column, it is processed by the MS which ionizes the molecules into fragments for detection, allowing for accurate identification of compounds. This occurs by comparing the detected mass to charge ratios to those in a library of known values or directly comparing to standards run through the same machine (Boutou and Chatonnet, 2007). This project will utilise headspace solid-phase microextraction (SPME) with GC-MS, similar to that used in Wang et al. (2004), using the library comparison method.

1.9.3. Amino acid analysis for wine samples

As outlined in Section 1.7.3, amino acids are not only the source of nitrogen for yeast metabolism but also the metabolic intermediate in flavour production. The AccQ Waters amino acid system is suitable for amino acid identification and quantification. It uses ultra-performance liquid chromatography (UPLC) to separate and detect amino acids. Derivatisation uses the Waters AccQ-Fluor reagent (6-aminoquinolyl-N-hydrozysuccinimidyl carbamate; ACQ), which converts primary and secondary amino acids to stable, fluorescent derivatives. The by-product, 6-aminoquinoline, does not interfere with this system and as it does not co-elute with any amino acid, its peak on the chromatogram is used to demonstrate derivatisation efficiency. Separation of the amino acids occurs via a solvent gradient through a mass trak “physiological” column and detection is at 260 nm (UV). The details of this method can be found on the Waters website at www.waters.com.

1.9.4. Phenotypic screening

Another method of determining what is happening within a cell as a result of a particular stimulus is phenotypic screening. This is where yeast is grown under different conditions or different yeast deletant library mutants are grown in the same growth condition to determine what physical differences or different growth patterns might arise. An example of this is the H_2S membrane assay in this project where a single mutant can be grown in each well of a 96-well microtitre plate and a silver nitrate infused membrane is placed on top of the plate, with H_2S production of all the mutants in the plate monitored. Growth is simultaneously monitored using the microtitre plate reader and by 'frogging' the microtitre plates out onto agar plates, to ensure that the strains are growing at a normal rate.

1.10. Project aims

With the emergence of frontier technologies such as transcriptomics following the sequencing of yeast genome, yeast metabolism is being dissected in an increasing pace at molecular level. Sulfur-containing compounds, such as hydrogen sulfide (H_2S) and sulfur dioxide (SO_2) are important metabolites underpinned by both nitrogen and sulfur metabolism. A better understanding of their metabolism through transcriptomics would lead to new fermentation innovations for increasing desirable SO_2 and lessening off-flavour H_2S. Additionally, as the wine industry lacks an effective method to alleviate clarity problems, two new fining agents, pectin and carrageenan, are investigated for their ability to enhance wine clarity and their effects on wine flavour in the hope that developing a possible method to overcome clarity problems for white wine production. Therefore, the aims of this project were as follows:

- To establish a transcriptomic method of analysis through optimisation of two-colour generic cDNA microarray protocols and, in addition, to determine the veracity of singlet, duplicate and triplicate datasets with one-colour Affymetrix microarrays.
- To delineate the effects of cysteine, nitrogen (in the form of ammonium sulfate) and cysteine plus nitrogen on yeast H_2S metabolism using cDNA microarray transcriptomics.

- To investigate the effect of pectin and carrageenan on wine clarity and flavour using fermentations on industrial and laboratory scales.
- To gain insights into the effect of pectin and carrageenan on wine yeast metabolism by functional genomics analyses.

Chapter 2: Materials and Methods

2.1. Materials

2.1.1. General reagents

The common chemicals and organic solvents used in this study were of analytical grade or higher and were obtained from commercial suppliers including Sigma-Aldrich (St Louis, MO, USA) and Merck (Whitehouse Station, NJ, USA). Type 1 ultrapure deionised water (Sartorius Stedim Biotech, Aubagne, France) or Milli-Q deionised water (Millipore Corporation, Billerica, MA, USA) was used to make up all media and buffers.

2.1.2. Laboratory yeast strains

The diploid laboratory strain of yeast used in this study was BY4743. Its genotype is *MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/LYS2 met15Δ0/MET15 ura3Δ0/ura3Δ0*. The genotype of the yeast deletion library is *MATa/α orfΔ::kanMX4/orfΔ::kanMX4 his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/LYS2 met15Δ0/MET15 ura3Δ0/ura3Δ0* (Winzeler et al., 1999). BY4743 was derived from the S288C-originated BY4742 and BY4741 haploid strains. The 'patriarch' S288C strain is an early parental laboratory yeast strain used in the sequencing project (Mortimer and Johnston, 1986; Brachmann et al., 1998). A homozygous diploid *S. cerevisiae* yeast deletion library with 4,757 deletion mutants derived from BY4743 was purchased from EUROSCARF (European *Saccharomyces cerevisiae* archive for functional analysis; Frankfurt, Germany). Each of the deletion strains has a single ORF knocked out (Giaever et al., 2002).

2.1.3. Industrial yeast strain

The industrial wine strain Lalvin QA23 (*Saccharomyces bayanus*) was obtained from Winequip (Melbourne, VIC, Australia). This strain was commercialised by Lallemand (Blagnac Cedex, France) after isolation in Portugal and has many advantages over other wine strains, including its high alcohol tolerance (16%), fast fermentation rate, very low assimilable nitrogen requirement, low volatile acidity

production (generally less than the equivalence of 0.2 g/L H_2SO_4) and low H_2S and SO_2 production as described by the manufacturer. It is commonly used to ferment Chardonnay grape juice, usually giving rise to citrus fruit and pineapple aromas.

2.1.4. Yeast growth media

BY4743 and its mutants were cultured in liquid minimal medium, which consists of 20 g/L D-glucose, 1.7 g/L yeast nitrogen base with neither ammonium sulfate nor amino acids, and 5 g/L ammonium sulfate, supplemented with 20 mg/L uracil, 10 mg/L adenine and the following amino acids: 50 mg/L L-arginine, 80 mg/L L-aspartic acid, 20 mg/L L-histidine HCl, 100 mg/L L-leucine, 50 mg/L L-lysine HCl, 20 mg/L L-methionine, 50 mg/L L-phenylalanine, 100 mg/L L-threonine, 50 mg/L L-tryptophan, 50 mg/L L-tyrosine, 140 mg/L L-valine, and 50 mg/L isoleucine.

Variations of the minimal media were also used, with differing concentrations of ammonium sulfate (between 0 and 30 g/L total ammonium sulfate) and the addition of cysteine (between 0 and 100 ppm total cysteine). Stock cysteine solutions were freshly prepared by filter-sterilisation using a sterile pyrogen-free 25 µm, 75 psi cellulose acetate disposable syringe filter unit (Advantec MFS, Inc, Dublin, CA, USA).

Yeast strains were temporarily maintained by streaking on Yeast Extract / Peptone / Dextrose (YEPD) agar plates, which consist of 10 g/L yeast extract, 20 g/L bacteriological peptone, 20 g/L D-dextrose (glucose) and 20 g/L agar. These plates were stored at 4 ^{o}C. Fresh yeast cells were prepared when necessary by re-streaking from the storage plates onto new YEPD agar plates followed by incubation at 30 ^{o}C for 48 h. Long-term stocks of the yeast strains were kept at –80 ^{o}C in YEPD containing 15% glycerol (v/v).

2.1.5. Synthetic Grape Juice Media

The synthetic grape juice medium used for fermentation in this study was developed by Dr Simon Schimdt et al. at the Australian Wine Research Institute (AWRI, Adelaide, SA, Australia) to emulate Chardonnay grape juice (Personal Communication, Dr Simon Schimdt, 2010). It consisted of numerous chemicals, supplemented by trace element, vitamin and amino acid / nitrogen stock solutions as described below. The medium was made up in 4 L batches and distributed evenly

between four fermenters at a time (making up a single replicate). Sufficient dry components of Table 2.1 to make up 4 L of the synthetic grape juice medium were added to 2.8 L water with mixing. The stock solutions (trace elements, vitamins and amino acid / nitrogen mixes) as shown in Table 2.1 were then added. The pH was adjusted to 3.5 with 1 M potassium hydroxide (KOH) before deionised water was added to a final volume of 4 L. The synthetic grape juice was filter-sterilised through a 0.45 µm cellulose acetate membrane filter (Whatman, Maidstone, Kent, UK) via vacuum filtration using a reusable Bottle-top Filter unit PSF (Nalgene, Rochester, NY, USA), with 500 mL filtered through an individual filter before replacement. After filtration into twelve 2 L fermenters, lipid and sterol stocks (2 mL each) were finally added.

Table 2.1. Synthetic grape juice media recipe (per 4 L batch of media)

Component	**Weight (g) / Vol (mL)**	**Source**
Glucose	400	Sigma
Fructose	400	Sigma
Citric acid	0.8	Merck
Malic acid	12	Aldrich
KH Tartrate	10	Aldrich
KH_2PO_4	12	BDH
$MgSO_4 \cdot 7H_2O$	6	BDH
$CaCl_2 \cdot 2H_2O$	1.6	ChemSupply
H_3BO_3	0.16	Aldrich
Trace element stock	4 mL	(Stock 1 below)
Vitamin stock	4 mL	(Stock 2 below)
Nitrogen mix	80 mL	(Stock 3 below)
Lipid stock	2 mL per fermenter (2 L)	(Stock 4 below)
Sterol stock	2 mL per fermenter (2 L)	(Stock 5 below)

Table 2.2. Stock #1: The components for the trace element stock for synthetic grape juice media (1000 ×).

Trace element	Weight (g)	Source
$MnSO_4•H_2O$	3.5	Univar
$ZnCl_2$	1	Sigma
$FeSO_4•7H_2O$	6	BDH
$CuSO_4•5H_2O$	1.5	BDH
KIO_3	0.01	Univar
$Co(NO_3)_2•6H_2O$	0.03	Aldrich
$Na_2MoO_4•2H_2O$	0.025	Unilab
LiCl	0.1	Sigma
$NiSO_4•6H_2O$	0.05	Sigma
RbCl	0.7	Sigma

The trace element stock components in Table 2.2 were dissolved in 800 mL deionised water, the pH was adjusted with concentrated HCl (Sigma) to 1.5. The total volume was then made up to 1 L with water. After autoclaving, the 1000 × trace elements stock was stored at 4 ^oC.

Table 2.3. Stock #2: The components for the vitamin stock for synthetic grape juice media (1000 ×).

Vitamin	Weight (g)	Source
Thiamine HCl	0.5	Sigma
Riboflavin	0.2	Sigma
Pyridoxine HCl	1	Sigma
Calcium D-pantothenate	1	Sigma
Nicotinic acid	1	Sigma
Myo-inositol	10	Sigma
Biotin	0.05	Sigma
Folic acid	0.05	Sigma
4-amino benzoic acid	0.05	Sigma

The vitamin stock components in Table 2.3 were also dissolved in 800 mL deionised water, the pH was adjusted with KOH to pH 7 and the volume was then made up to 1 L. This stock was sterilised by vacuum filtration as described above using 0.45 µm cellulose acetate membrane filters, prior to being stored at 4 ^{o}C. The stock was used within a week.

Table 2.4. Stock #3: The amino acid components for the amino acid / nitrogen stock for synthetic grape juice media (50 ×)

Amino acid	**Weight (g)**	**Source**
Alanine	10.542	Sigma
α-amino butyrate	7.229	Sigma
Arginine	27.108	Sigma
Asparagine	0.422	Sigma
Aspartic acid	3.012	Sigma
Citruline	0.422	Sigma
Glutamic acid	6.024	Sigma
Glutamine	8.434	Sigma
Glycine	0.422	Sigma
Histidine	1.205	Sigma
Isoleucine	1.205	Sigma
Leucine	1.205	Sigma
Lysine	0.422	Sigma
Methionine	0.422	Sigma
Ornithine	0.422	Sigma
Phenylalanine	0.843	Sigma
Serine	5.422	Sigma
Threonine	6.024	Sigma
Tryptophan	0.422	Sigma
Tyrosine	0.422	Sigma
Valine	2.108	Sigma
Cysteine	1.205	Sigma
Proline	65.060	Sigma

The amino acids listed in Table 2.4 were dissolved in 800 mL deionised water and the pH adjusted to 2.5 with concentrated HCl. Once completely dissolved, 27.8 g of 28% ammonium hydroxide (Sigma) was added to the solution. The pH was then adjusted to 3.5 with concentrated HCl and the solution was made up to a total volume of 1 L.

According to the calculation by AWRI, the final yeast assimilable nitrogen (YAN – ammonia and free alpha amino acids) of this medium is approximately 250 mg N/L (mg/L equivalence of Nitrogen) and total assimilable nitrogen (TAN) excluding proline (proline is excluded because under anaerobic conditions yeast cannot metabolise proline) is 440 mg N/L. Analytical measurement of the medium made up by AWRI using this protocol showed that the YAN was 246 mg N/L, ammonia was 91 mg/L and the α-amino content was 171 mg/L, which was comparable with the theoretical calculation.

Table 2.5. Stock #4: The components for the lipid stock for synthetic grape juice media (1000 ×).

Lipid	Weight (g)	Source
Palmitic acid (C16:0)	0.2	Sigma
Oleic acid (C18:1)	0.1	Sigma
Linoleic acid (C18:2)	0.3	Sigma
Linolenic acid (C18:3)	0.05	Sigma

Each of the lipids in Table 2.5 was weighed out into absolute ethanol (Sigma) in 1.5 mL eppendorf tubes, with the palmitic acid warmed to 30 ^{o}C for 5 min to dissolve it. The four dissolved lipids were then combined and absolute ethanol added to a final volume of 100 mL to make the 1000 × lipid stock.

Stock #5 was the 1000 × sterol stock which was made by dissolving 0.1 g β-sitosterol in 100 mL absolute ethanol.

2.1.6. Fermenter setup for the lab-scale wine fermentations

The lab-scale wine fermentations were carried out in 2 L Schott bottles (Boeco, Hamburg, Germany), with pour rings removed from the bottles in order for the fermentation caps to be set up. The fermentation caps were made up of a three-port cap with one port blocked off and the other two capped with a blue port with silicone tubing running through. The point of connection was wrapped with plumbers tape (Bunnings, Australia) prior to the blue port being attached to the three-port cap to ensure an airtight seal. One of the blue ports was connected to long tubing that would reach to nearly the bottom of the Schott bottle for sample collection with a 50 mL syringe on the outer end of the tubing. A stopcock valve was located between the blue port and the syringe to allow sample to be removed without allowing air to enter the system. The other blue port was set up the same way except that the internal tubing was a lot shorter so that it reached only the headspace and a H_2S detection tube (see Section 2.3.2 for a description of these tubes) replacing the syringe. A silicone O-ring was inserted between the three-port cap and the Schott bottle in place of the removed pour ring. A photograph of this fermentation setup is shown in Figure 2.1.

The Schott bottles, O-rings, tubing and the three-port cap with plumbers tape, tubing and blue ports were sterilised by autoclaving at 121 ^{o}C for 15 min. The stopcock valves and syringes were supplied sterile.

This fermentation setup was tested before use to determine whether it was anaerobic and airtight. One test was conducted by attempting to withdraw liquid through a syringe via tubing in the completed setup. If the system is airtight, such an attempt should be impossible. The other test was performed by pushing air through the setup with the other valve closed when the entire setup was under water. There should be no bubbles produced if the setup is airtight. The fermentation setup in this study passed both tests.

Figure 2.1. Photograph of the lab-scale wine fermentation setup before inoculation, showing the H_2S detection tube outlet on the left and the sampling tubing on the right.

2.1.7. Commercial wine

For comparison purposes, commercial un-oaked Chardonnay wines were used in some of the assays described in this study. Four such commercial wines were purchased from Dan Murphy's at Macarthur Square (NSW, Australia) in April 2010 for wines 1 to 3 and in November 2010 for wine 4.

Commercial wine #1 (codes 7534 and B used in the analysis) was a 750 mL unoaked Chardonnay from Somerton in 2009. The label stated that it had "*an inviting*

bouquet of ripe peaches and apricots. The palate is rich and generous, smooth and creamy with a crisp lively finish." It was produced in South-Eastern Australia with an alcohol content of 13.0% (approx. 7.7 standard drinks with each standard drink containing 10 g of alcohol). Preservative 220 (SO_2) was added and the wine was produced with the aid of milk products. It was produced by Miranda Wines Pty Ltd, Old Wentworth Road, Merbein, Vic 3505, Australia.

Commercial wine #2 (codes 8825 and A) was a 750 mL Southern Western Australian unwooded Chardonnay from Goundry. It received the gold medal at the Hobart International Wine Show in 2008 (class 6). The alcohol content was 13.5% (approx. 8 standard drinks). Preservative 220 was added and traces of the fining agents which included egg and / or milk products may remain. This wine was produced by Goudrey Wines, location 10460, Vasse Highway, Nannup, WA 6275, Australia. The label stated that this wine was a "*mouth-watering fruit-driven wine. Flavours of pineapple, peach and fresh melon will melt in your mouth, while the wine's natural crispness will leave you wanting more. Made in a fresh and vibrant style without the use of oak.*"

Commercial wine #3 (codes 9017 and C) was a 750 mL South Australian unwooded Chardonnay from the Yalumba Y series from 2008. This wine had an alcohol content of 12.5% (approx. 7.4 standard drinks). Preservative 220 was added and the wine contained milk products. It was vintaged by Yalumba, Eden Valley Road, Angaston, SA 5353, Australia. Its label stated that this wine "*has rich aromas of melon, grapefruit and honey. Fresh tropical fruit flavours of peach, pineapple and fig gave this wine texture and palate weight. A crisp, citrus acidity brings balance and zest to this fruit driven Chardonnay.*"

Commercial wine #4, used as a control in the real grape juice lab-scale fermentation, was also a 750 mL South Australian unwooded Chardonnay from the Yalumba Y series as was commercial wine #2, but from the following year, 2009. The other characteristics were the same as the 2008 wine, although the label stated that the flavours included "*grapefruit and pineapple*".

2.1.8. Pectin and carrageenan

GENU® pectins were derived from citrus peel or sugar beet pulp, extracted in hot acidified water. The type used in this study (TS1580) was a low methylester (LM) pectin which forms a gel in the presence of calcium and other divalent cations (degree of esterification (DE) lower than 50), as described in Section 1.8.1.

Carrageenan used in this study was the CSW-2 cold water soluble type. Carrageenan was derived from the *Rhodophyceae* red seaweed family, extracted using hot water under neutral or alkaline conditions. The type used was iota carrageenan, as described in Section 1.8.2.

Both the carrageenan and pectin were sourced from CP Kelco (Atlanta, Georgia, USA) via Fosters Australia (Melbourne, Victoria, Australia). They were added at a concentration of 0.15 g/L for carrageenan and 1 g/L for pectin. These concentrations were found to be ideal by the Fosters research group (Personal communication, Dr David Duan, Fosters Australia, 2007).

2.2. Yeast culture

2.2.1. BY4743 and its mutant cultures

Yeast cells in exponential phase, typically at OD_{600} 1.0, were used in all assays. This was achieved by inoculating a colony of yeast into 1 mL sterile water. The measured OD_{600} was used to calculate how much of this yeast solution should be added into media. The calculation was made for a 19 h, 120 mL minimal media BY4743 culture, using the empirical formula below.

Volume required = 62.832 / OD_{600} of the yeast suspension

For an example of a yeast suspension of OD_{600} 1.5, using the above formulae, 41.9 μL of the suspension is needed for inoculating 120 mL minimal medium in a flask. It is expected that after an incubation of 19 h at 30 ^{o}C with shaking at 150 rpm, the OD_{600} of this culture should reach 1.0. Adjustments were made for different culture volumes and strains. The empirical practice provided a good control in experimental planning and implementation.

2.2.2. Industrial wine yeast in fermentation

Dried *Saccharomyces bayanus* (*S. bayanus*) strain QA23, the industrial wine yeast, as described in Section 1.3.3, was used for the pilot-scale industrial fermentation at Fosters Australia (Melbourne, VIC, Australia) and both of the lab-scale fermentations at the University of Western Sydney. The yeast was mixed with ten times the volume of 35 ^{o}C sterile deionised water, stirred and allowed to hydrate for approximately 20 min. The pilot-scale fermentation required 31.12 g of yeast which was re-hydrated in 320 mL water (35 ^{o}C). This was equally split between the fermenters, resulting in the desired concentration of 0.4 g/L yeast (dry weight). For both of the lab-scale fermentations, 20 g of QA23 yeast was added to 200 mL water and 8 mL of this yeast mixture was added to each of the 2 L fermenters, resulting in the same final concentration of 0.4 g/L yeast (dry weight).

2.3. Detection of sulfur compounds

2.3.1. The membrane assay H_2S detection using silver nitrate

Relative H_2S production was analysed using a slightly modified version of the membrane overlay method developed by Duan et al. (2004). The reaction in this assay between H_2S gas and the silver nitrate in the membrane results in silver sulfide which is black, so the reaction can be seen on the membrane by the presence of spots, with the intensity of the colour related to the amount of H_2S produced. Cultures were added to wells in a microtitre plate, with the addition of filter-sterilised cysteine (100 ppm) into medium to induce H_2S production where necessary to increase the sensitivity of the assay. The differential rates of H_2S formation detected by the membrane assay were not caused by varying yeast growth, as demonstrated by monitoring yeast growth spectrometrically, which was found to be constant among treatments.

A 7.5 × 11.5 cm piece of Whatman® 3MM Chromatography paper was infused with approximately 3.6 mL of freshly prepared 20% (w/v) silver nitrate ($AgNO_3$). The membrane was blotted to remove excess liquid and laid over the microtitre plate wells. After sealing with a lid, the plates were wrapped in aluminium foil in order to protect the reaction from light and incubated statically at 30 ^{o}C. After 12 and 24 h of incubation, the results were observed and photographs of the membranes were taken

of the paper using a digital camera. Spots were quantitated using ImageJ (Image Processing and Analysis in Java), available online at http://imagej.nih.gov/ij/, where the intensity of spots were compared to the background (Abràmoff et al., 2004).

2.3.2. Headspace H_2S detection

H_2S production in the headspace of the wine fermentations was determined using a H_2S detection tube with a range of 25 – 2000 ppm (Airmet Scientific, Nunawading, VIC, Australia). The sealed ends of the tubes were firstly snapped off using tweezers and then connected to the top of each fermenter via the shorter length of tubing, allowing H_2S from the headspace to react with the silver nitrate in the H_2S detection tube. The top of each tube was supported to ensure the tube was vertical. The more H_2S that was produced during fermentation, the higher the dark colour change rose in the tube. Each tube had markings with corresponding H_2S ppm levels. The tubing was connected to the fermenter with a one-way stopcock valve so that when the colour change had reached the top of a tube, the valve could be closed and the tube replaced without loss of H_2S and without allowing air to enter the fermentation directly.

2.3.3. Sulfite Kit Test

SO_2 levels were determined using the photometric sulfite test kit (Merck, Darmstadt, Germany). The volumes required to assay the samples were reduced by a factor of 8 to increase the number of samples that could be tested. The pH of the samples was adjusted to between 6.5 and 7.5 with 1 M NaOH, prior to measurement. The reagent for testing nine samples was prepared by thoroughly dissolving 1 microspoon of reagent SO_3-1 in reagent SO_3-2 (3 mL) by shaking and then diluting in deionised water (5 mL). Each pH-adjusted sample (200 µL) was combined with reagent (800 µL) in a cuvette and incubated at room temperature for 2 min before the absorbance was measured at 412 nm, with sodium metabisulfite (SMBS; $Na_2S_2O_5$, Aldrich, St Louis, MO, USA) as the standard. A range of SMBS concentrations was used to establish the standard curve. The standard curve allowed SO_2 levels (in ppm) to be determined by multiplying the absorbance by 0.032, with a R^2 value of 0.9962.

2.4. Microarray gene expression profiling technology

Microarray technology can determine the gene expression of a large number of genes, if not all, in a particular organism under a certain treatment. For example, *S. cerevisiae* cells grown in low and high nitrogen conditions can be directly compared to each other, with the cDNA of each condition processed in parallel through hybridisation to the comprehensive set of gene probes derived from 6,250 ORFs. However, it is important to get accurate information from the microarray chips, with as few as possible non-hybridised spots, to ensure data can be collected about each gene and a low background with high reproducibility.

There are a number of steps in the protocol to prepare samples for gene expression analysis using microarray technology. These include extraction of RNA, conversion of the RNA into cDNA, labelling the cDNA with fluorescent dyes and hybridising the labelled cDNA onto microarray slides. These steps for both two-colour microarrays and one-colour Affymetrix microarrays are outlined in the following sections, while sample preparation is outlined in relevant sections. First, the steps involving extraction and purification of RNA are described, followed by the protocols for the two-colour and then one-colour microarrays.

2.5. Extraction and purification of RNA

2.5.1. Total RNA isolation

Total RNA was isolated from the cells using the TRIzol Reagent® (Invitrogen, USA) using a modified version of the method developed by Simms et al. (1993).

TRIzol Reagent® (1 mL) was added to frozen yeast pellets. This was transferred to a tube with 0.75 g of 425-600 µm glass beads (Sigma). The mixture was then homogenised using a Bead Mill for 2 min at 4 ^{o}C and incubated in ice for 5 min. Chloroform (200 µL) was added to the lysed cells and the tube was then shaken vigorously for 15 sec and incubated at room temperature for 3 min. The phases were separated by centrifuging at 12,000 × *g* for 15 min at 4 ^{o}C. The clear top layer (400 µL) was carefully transferred to a fresh microfuge tube for RNA precipitation.

Isopropyl alcohol (500 µL) was added to the clear supernatant and the tubes inverted once to mix. This was then incubated at room temperature for 10 min and centrifuged at 12,000 × *g* for 10 min at 4 ^{o}C. The pellets were washed with 75%

ethanol (1 mL) and centrifuged at 7,500 × *g* for 5 min at 4 ^{o}C. The supernatant was carefully removed, the tubes re-centrifuged and the residual ethanol pipetted off before the pellets were air dried for 20 min. The RNA pellets were then resuspended in RNase-free water (30 - 200 µL depending on expected concentration). The concentration, quality and integrity of RNA were determined by NanoDrop and Bioanalyzer as per Section 2.5.3.

2.5.2. RNA clean-up

RNA samples were further purified using the QIAGEN RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Each RNA sample, with no more than 100 µg of RNA made up to a final volume of 100 µL with deionised water, was mixed with lysis buffer RLT (350 µL; proprietary composition but containing guanidine thiocyanate) and 100% ethanol (250 µL). This was added to the RNeasy column and centrifuged for 15 sec at 8,000 × *g* at room temperature. The RNA was washed twice on-column with wash buffer RPE (500 µL each; proprietary composition but diluted with 4 volumes of 100% ethanol prior to use) before the column was dried to remove excess solvent. The RNA was eluted with RNase-free water (50 µL and then a further 30 µL). The concentration, quality and integrity of RNA were determined using the NanoDrop and Bioanalyzer as described in Section 2.5.3.

2.5.3. Assessment of quantity and quality of nucleic acids

The concentration and purity of nucleic acid (DNA, RNA and cDNA) were assessed using a NanoDrop® Spectrophotometer ND-1000 machine obtained from BioLab (Mulgrave, VIC, Australia). The NanoDrop spectrophotometer measures the absorbance of the nucleic acid sample continuously between wavelengths of 220 and 350 nm. With this information, the software determines the concentration of the nucleic acid as well as the purity of the samples. The levels of nucleic acid / protein contamination is determined by the ratio between the absorbances at 260 nm and 280 nm (the 260/280 ratio) and levels of sugar / solvent contamination is determined by the ratio between the absorbances at 260 nm and 230 nm (the 260/230 ratio). Samples with both of these ratios above 1.8 and a smooth absorption curve are considered to be of high purity and samples having a 260/230 ratio above 1.5 considered acceptable.

The integrity of the RNA samples was analysed using an RNA 6000 Nano LabChip® on a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA), following the manufacturer's instructions. Samples are considered to have intact RNA if the sample has distinct 18S and 28S peaks within the chromatogram, without unexpected peaks, such as those associated with DNA.

2.6. Two-colour custom microarrays

Multiple steps were involved in the two-colour cDNA microarray protocol following the cell harvest, snap-freeze, total RNA isolation, RNA clean-up and quantitative/qualitative assessments as described above. These steps include conversion and labelling of the purified RNA into cDNA with cyanine or Alexa fluor dyes, hybridisation onto the microarray slides, data acquisition and analysis.

2.6.1. cDNA conversion method 1: Non-kit method

First strand buffer (8 µL), anchored oligo dT primer (1.5 µL; Invitrogen, Melbourne, Australia), 0.1 M DTT (4 µL), total RNA (20 µg) and RNase-free water (to a total volume of 32.2 µL) were combined in a 0.2 mL RNase-free PCR tube. These samples were incubated in a GeneAmp PCR System 2700 PCR machine (Applied Biosystems, Melbourne, Australia) or a heating block at 65 ^{o}C for 5 min and then at 42 ^{o}C for 5 min. While at 42 ^{o}C, 10 mM d(ACG)TP (2 µL), 2.5 mM dTTP (2.6 µL), 10 mM aa-dUTP (1.35 µL) and Superscript III Reverse Transcriptase (2 µL) were added. The samples were incubated at 42 ^{o}C for a further 2.5 h.

To hydrolyse the unconverted RNA, 0.5 M EDTA (5 µL) and 0.25 M NaOH (10 µL) were added and incubated at 65 ^{o}C for 20 min, and then 0.2 M acetic acid (15 µL) was added. The cDNA was then purified from unused dNTPs and hydrolysed RNA using Qiagen QiaQuick® PCR purification columns as described in Section 2.6.5. The samples were concentrated to 1 – 2 µL by vacuum centrifugation before the concentration and purity of cDNA samples were tested using a NanoDrop spectrophotometer (see Section 2.5.3).

2.6.2. cDNA conversion method 2: Invitrogen kit-based method

Rather than using separate reagents as in method 1, this method was based on the Invitrogen SuperScript™ Plus indirect cDNA labelling system (Invitrogen). RNA (20 µg) and RNase-free water (to a total volume of 16 µL) were combined in a microcentrifuge tube. After the addition of 2 µL anchored oligo dT_{20} primer to each tube, they were incubated at 70 °C for 5 min in a heating block and then on ice for 1.5 to 2 min. After the incubation but with the tubes still on ice, first strand buffer (6 µL; 250 mM Tris HCl, pH 8.3, 375 M KCl and 15 mM $MgCl_2$), 0.1 M DTT (1.5 µL), dNTP mix (1.5 µL), RNase OUT (1 µL) and SuperScript III Reverse Transcriptase (2 µL) were added to each tube as part of a master mix. These tubes were incubated at 42 °C for 3 h.

To hydrolyse the unconverted RNA, 1 M NaOH (15 µL) was added and incubated at 70 °C for 10 min. 1 M HCl (15 µL) was added. The solutions were purified to remove unused dNTPs and hydrolysed RNA using Qiagen QiaQuick® PCR purification columns as described in Section 2.6.5. The samples were concentrated to 1 – 2 µL by vacuum centrifugation before the concentration and purity of samples were tested using a NanoDrop spectrophotometer, as described in Section 2.5.3.

2.6.3. Cyanine dye coupling

From this point on, all work (including slide washing) was carried out in the dark. Limited light was provided by a fluorescent light in an adjacent room. The reason for this was to prevent the cyanine dyes from degradation, as they are sensitive to UV and ozone levels in the atmosphere. The cyanine dyes, Cy3 and Cy5, were resuspended separately using DMSO (18.5 µL for 1 × strength or 10 µL for 2 × strength). The relevant cyanine dyes (Cy3 or Cy5; 2 µL each) were coupled to the cDNA in the presence of 0.1 M $NaHCO_3$, pH 9 (9 µL) and incubated at room temperature in the dark for 45 min. The labelled cDNA probes were then purified using Qiagen QiaQuick® PCR purification columns as described in Section 2.6.5 to remove the uncoupled dye. The samples were concentrated to approximately 3 – 5 µL by vacuum centrifugation.

2.6.4. Alexa fluor dye coupling

Alexa fluor dye coupling was also used in this project. As was the case with the cyanine dye, all consecutive steps were carried out in the dark with a fluorescent light in the adjacent room. The cDNA pellets were resuspended in 2 × coupling buffer (5 µL) provided in the Invitrogen SuperScript Plus kit. The Alexa fluor dyes, Alexa fluor 555 (AF 555) and Alexa fluor 647 (AF 647), were resuspended separately in DMSO (2 µL each). The appropriate alexa fluor dye was added to the cDNA and incubated at room temperature in the dark for 2 h. The labelled cDNA probes were then purified using Qiagen QiaQuick® PCR purification columns as described in Section 2.6.5 to remove uncoupled dye. The samples were finally concentrated to approximately 3 – 5 µL by vacuum centrifugation.

2.6.5. Qiagen DNA purification clean-up

Purification of cDNA is necessary at several steps during the microarray protocol. It was performed using a QIAGEN QIAquick PCR Purification kit (Qiagen, Hilden, Germany) to remove free nucleotides, primers and unbound fluorescent dyes. The cDNA solution was diluted in binding buffer PB (350 µL; propriety composition, obtained separated from Qiagen, rather than the supplied buffer PBI due to possible interference with later processes). This mixture was added to the column and centrifuged at 13,200 × *g* for 1 min at room temperature. The DNA was washed twice on-column using 700 µL 75% ethanol before being air dried to remove excess ethanol. The DNA was eluted in 50 µL RNase-free water (Invitrogen), followed by a further 30 µL.

2.6.6. Microarray slide blocking

S. cerevisiae microarray slides were obtained from the Ramaciotti Centre for Gene Function Analysis at the University of New South Wales (Sydney, NSW, Australia). Slides were Schott Nexterion® Slide A+ with an amino-link coating (Schott, Mainz, Germany) and spotted with 50-mer oligonucleotides probes for 6,250 yeast ORFs (Version MWGSc6K; MWG Biotech, Ebersburg, Germany) in duplicate. Microarray slides were baked at 120 ^{o}C for 30 min prior to delivery. The slides were blocked during the 2.5 h of cDNA conversion in Section 2.6.1. This was carried out by

dipping the slides (in a metal rack) in 0.1% SDS at 95 ^{o}C for 1 min with constant shaking, then dipping them immediately in 5% ethanol for 1 min with constant shaking, and finally dipping them in de-ionised water at room temperature for 1 min with constant shaking. The slides were then centrifuged at 2,000 × *g* for 20 sec and stored in a dark dust-free box until the samples were ready to be hybridised within 10 h. The glass coverslips were washed in 100% ethanol, using gloves and Kimwipes (Kimberly-Clark Corporation, Irving, TX, USA).

2.6.7. Sample hybridisation

DIG-Easy hyb buffer (50 µL; propriety composition, Roche, Mannheim, Germany), yeast tRNA (2.65 µL, Ambion, Austin, TX, USA) and herring sperm DNA (2.5 µL, Invitrogen) were added into each tube of labelled cDNA. The solution in each Cy5 or Alexa Fluor 647 tube was mixed with the corresponding Cy3 or Alexa Fluor 555 tube. The mixture was then incubated at 65 ^{o}C for 5 min and centrifuged at 16,110 × *g* for 5 min. With the coverslips placed on the slides such that the ridges were facing the slide, the labelled cDNA probe solution was applied through the gap between the coverslip and the corresponding slide. Slide numbers were recorded and the slides were hybridised overnight at 37 ^{o}C in a rocking hybridisation incubator (Grant Boekel HIS25, BioLab) held on ridges above a Kimwipe soaked in 2 × SSC buffer (prepared from 20 × SSC stock containing 3 M sodium chloride and 0.3 M tri-sodium citrate, adjusted to pH 7.0 and sterilised by autoclaving at 121 ^{o}C for 15 min) inside a light proof box in order to create a humid environment. Alternatively, an a-Hyb hybridisation station (Miltenyi Biotec) at a pump rate of 1 mL/min was used to circulate the hybridisation mixture over the chip. Approximately double the volume was used for this method, in order to ensure enough fluid to cover the chip.

2.6.8. Slide washing method A: 1 × strength

Following hybridisation, the slides were washed by immersing them in 1 × SSC buffer at room temperature in individual 50 mL centrifuge tubes until the coverslips dislodged. The slides were then washed in 1 × SSC, 0.1% SDS for 15 min at 50 ^{o}C three times. The residual SDS was removed by rinsing three times in 1 × SSC at 50 ^{o}C for 15 min each time, then once in 0.2 × SSC at room temperature for 15 min. The washed slides were centrifuged at 2,000 × g for at least 20 sec to dry and stored in

50 mL centrifuge tubes in a double layer of aluminium foil for transporting (approximately 1.5 h duration) to the Ramaciotti Centre at UNSW for scanning.

2.6.9. Slide washing method B: 2 × strength

As described above, following hybridisation, the slides were briefly agitated in 2 × SSC buffer, 0.2% SDS at room temperature in a glass container until the coverslips fell off. The slides were then placed in a metal rack and washed in this solution for a further 10 min at room temperature with gentle rocking using a rocking hybridisation incubator. The metal rack was first transferred into a fresh glass container containing 1 × SSC and the slides were washed for 10 min, then they were washed in 0.2 × SSC at room temperature for a further 10 min with gentle rocking. The washed slides were tapped onto Kimwipes to get rid of excess liquid and then centrifuged at 2,000 × g for 20 sec to dry and stored in the same manner, as described previously, prior to scanning.

2.6.10. Scanning and acquisition of data

The microarray slides were scanned at 635 nm (Cy5 / AF 647) and 532 nm (Cy3 / AF 555) with a GenePix® 4000B scanner from Axon Instruments (Molecular Devices, Sunnyvale, CA, USA) at the Ramaciotti Centre within UNSW. This was performed within 2 h following completion of the final wash. The images were analysed using GenePix® Pro 6.0 software, as described in Section 2.8.1.

2.7. One-colour Affymetrix microarrays

Cell pellets from prepared samples were snap-frozen and stored at -80 ^{o}C until required. Total RNA was isolated using the TRIzol method outlined in Section 2.5.1, purified using Qiagen RNeasy columns as described in Section 2.5.2 and analysed using the NanoDrop and Bioanalyzer as described in Section 2.5.3. Pure intact RNA (50 µL of a 50 ng/µL solution) was transported to the Ramaciotti Centre (UNSW) for outsourced processing with the Yeast 2.0 Affymetrix microarray chips using the FS450_0003 fluidics protocol (available from www.affymetrix.com) for hybridisation, washing and scanning. Once processed, the data were sent to the Garvin Institute of Medical Research (Sydney, NSW, Australia) before being accessed electronically at

UWS, in the .cel file format and a pre-normalised .txt file. Only the .cel files were used for further analysis with Partek, as described in Section 2.8.3.

2.8. Bioinformatics analysis

The vast amounts of data that are generated by high-throughput experiments such as microarray analysis require various programs to analyse the data. The ones used are outlined below.

2.8.1. GenePix

Two-colour microarray images were analysed using GenePix Pro 6.0 microarray and acquisition software. This program aligned the features (spots) on the slide with the genes in the appropriate array list file (MWGSc6Kv4_GP.gal). The alignments were manually checked and altered where necessary. Some spots were flagged and not included in further analysis if the background was too high or if the intensity from a neighbouring spot had falsely registered as belonging to that spot. These anomalies were mostly automatically marked, but manually checking was performed. The program allowed for the conversion of the data from the scanned image into quantitative numerical data for further analyses. Bioinformatic analysis of these data was carried out using the programs described below.

2.8.2. GeneSpring

Pre-analysis was performed using GneePix Pro 6.0, as described above, before the data was imported into GeneSpring. Firstly, normalisation was performed on the data using the LOWESS method within the GeneSpring GX 7.3.1 (Agilent Technologies) analysis software package. The genes whose expression ratio (treatment / control) was significantly different (fold-change of 2 or more) were identified based on Welch's analysis of one-way ANOVA where the variances were not assumed to be equal and the level of significance was set to 0.05.

2.8.3. Partek Gene Expression Analysis

The one-colour Affymetrix microarray data, in the form of .cel files, were imported into the Partek® Genomics Suite 6.5 (Partek Incorporated, St Louis, MO,

USA). A new column was added with the attributes of the files, which was classed as a "categorical factor" before Analysis of Variance (ANOVA) was carried out. Only the files involved in each individual ANOVA was inputted into the program, so that normalisation would only occur on these files. For datasets with at least three replicates, false discovery rate (FDR) and volcano plot analyses were carried out to identify significant genes with a fold change above 1.5 or 2 and a p-value above the figure generated by the FDR analysis. The replicate analysis, as described in Chapter 3, involved several different choices of fold change for comparison purposes.

2.8.4. FunSpec (Functional Specification)

Lists of significant genes identified from the previous step were inputted into the FunSpec program, available online at http://funspec.med.utoronto.ca/ (Robinson et al., 2002). The program classifies the genes in the dataset into groups based on the function of the genes and returns a p-value for each group, based on the likelihood that the result was not by chance. The functional specifications are derived from GO Molecular Function, GO Biological Process and MIPS Functional Classification databases.

2.8.5. Saccharomyces Genome Database (SGD)

SGD is an online database, with information about the genome of *S. cerevisiae*. Available at www.yeastgenome.org (Cherry et al., 1998; Dwight et al., 2002), it is a free resource, which includes information about each gene. SGD was used to discover the purpose of each gene, along with other data about the genes and pathways of yeast.

2.9. Ammonium sulfate and cysteine sample preparation

Overnight BY4743 cultures prepared as outlined in Section 2.2.1 (OD_{600} of 1.0) were spun down at 4,000 × *g* for 5 min at 20 ^{o}C and the supernatant discarded. The pellets from 20 mL for each sample were resuspended in 100 mL of one of four varieties of minimal media – 30 g/L ammonium sulfate and 100 ppm cysteine, 30 g/L ammonium sulfate and 0 ppm cysteine, 0 g/L ammonium sulfate and 100 ppm cysteine and 0 g/L ammonium sulfate and 0 ppm cysteine. Minimal media containing

0 and 30 g/L ammonium sulfate was prepared prior to the experiment while 220 µL of the freshly dissolved and filtered cysteine stock or water was added to 110 mL of the media within 1 h of use. Once resuspended, 200 µL aliquots were taken in triplicate for the membrane assay to monitor H_2S before the samples were incubated at 30 ^oC with shaking at 150 rpm. Once the OD_{600} of the main cultures had reached 1.0, the samples were split into two 50 mL tubes which were centrifuged at 4,000 × *g* for 5 min at 20 ^oC. The supernatant was removed and the pellets spun again. The residual media was thoroughly removed before the cell pellets were snap frozen in liquid nitrogen and stored at -80 ^oC for RNA isolation, as outlined in Section 2.5.1. The supernatant was stored for later analysis. The biological triplicates for this experiment were prepared independently to ensure accuracy.

2.10. Wine fermentation

2.10.1. Pilot-scale wine fermentation with grape juice

Four 20 L fermentations (two red and two white wines) were run at the pilot-scale fermentation plant at the Abbotsford pilot-scale facility at Carlton and United Breweries, Fosters Group (Melbourne, VIC, Australia). The grape juices used were Tumbarumba Sauvignon Blanc, combining the free run juice and pressings (for the white wine) and Cab Sauvignon (from Karadoc for the red wine), which was frozen at -20 ^oC until required (sourced from the Great Western winery region, VIC, Australia). The white grape juice had a Baumé level of 12 (21% or 210 g/L sugars), pH 3.25, total acidity of 7.77 g/L, 14 ppm free SO_2 (41 ppm total SO_2), yeast assimilable nitrogen (YAN, the amount of total nitrogen including alpha nitrogen and ammonia) of 239 mg N/L (in the normal range for grape juice) and contained 4.6 g/L malate. The red grape juice had 44 ppm free SO_2 (150 ppm total SO_2), pH 3.66, 12.6 Baumé (22.05% or 220.5 g/L sugars) and 4.7 g/L total acidity.

Table 2.6. Composition of white and red grape juice used in the pilot-scale fermentation.

	White grape juice	Red grape juice
Baumé (% sugar)	12 (21% sugar)	12.6 (22% sugar)
pH	3.25	3.66
Total acidity (g/L)	7.77	4.7
Free SO_2 (ppm)	14	44
Total SO_2 (ppm)	41	150
Yeast assimilible nitrogen (YAN) (mg N/L)	239	Not determined
Malate (g/L)	4.6	Not determined

Pectin and carrageenan were added to half of the defrosted grape juice (20 L) and then mixed for 15 min with a mechanical stirrer in a plastic bucket. The juice was then transferred into the 25 L fermenters without letting it settle and the untreated juice was added to the remaining fermenters. Three days after addition, the grape juice was inoculated with the yeast, *S. bayanus* wine strain QA23 re-hydrated as described in Section 2.2.2 to a final concentration of 0.4 g/L yeast. Inoculation of all four of the fermenters took 10 min. The temperature of the pilot-scale fermentation facility was found to be between 16 and 20 ^{o}C during the study, averaging 18.7 ^{o}C.

Samples of the fermented grape juice / wine were taken twice a day for the first four days and then daily for the remainder of the 10 day fermentation. At each timepoint, two batches of approximately 50 mL of grape juice / wine was removed from each fermenter using a 60 mL syringe and long hose, which was rinsed clean with hot water between fermenters. These tubes were centrifuged for 3 min at 4 ^{o}C at 2,000 × *g* for the first 7 samples (including the 72 h sample) and 1,500 × *g* for the remaining samples (from 90 h onwards). The two supernatants from each fermenter were pooled and stored at -20 ^{o}C until processed for volatile flavour compound analysis. The yeast pellets were re-suspended in the residual liquid and pooled into a 2 mL screw-capped tube. This was centrifuged at 1,500 × *g* for 3 min at 4 ^{o}C, supernatant discarded, re-centrifuged at 1,500 × *g* for 3 min at 4 ^{o}C and the residual supernatant discarded. RNA isolation then proceeded as described in Section 2.5.1,

except that the samples were homogenised in the Bead Mill for 2 min, followed by 2 min on ice and then a further 2 min in the Bead Mill. The clear RNA layer after chloroform addition was, in fact, yellowish (for the white wine samples) and pinkish red (for the red wine samples) instead of a clear liquid phase because of the wine colour.

The first sample (0 h) was analysed slightly differently, with 40 mL of the juice centrifuged initially, followed by a further 20 – 50 mL added to this approximately 10 min later because the yeast pellets were not large enough. The samples were homogenized using the Bead Mill for only 2 min, which was increased to 4 min for all subsequent samples to ensure the cells were thoroughly broken open. After homogenization, 1 mL of TRIzol was used for the 0 h sample RNA isolation, but in order to increase the volume of air inside the tubes to assist with the homogenization of the cells, this was reduced to 800 µL for all the following timepoints. In addition, the samples from the 72 h to 120 h treated red wine fermentations were processed individually instead of pooling the two lots of samples due to the large pellet sizes for this sample.

Two additional lots of approximately 50 mL were sampled from each of the treated and untreated red wine fermenters, with two water washes (50 mL each time) included for these extra samples before the yeast pellets were combined and the RNA isolated as normal, in order to test whether any of the colouration of the red wine samples could be removed.

Once sampled, the supernatant of each sample was frozen at -20 ^{o}C until analysis with GC-MS, alcohol content and haze testing, by Fosters Australia using their standard methods similar to those outlined in Section 2.11.

2.10.2. Lab-scale wine fermentation with synthetic grape juice media

Lab-scale fermentations were carried out with the fermenters set up as described in Section 2.1.6, the synthetic grape juice medium as described in Section 2.1.5 and 0.4 g/L of the wine yeast strain QA23 (Section 2.2.2). Samples were taken at frequent intervals (0, 6, 12, 24, 37, 50, 61, 74, 99, 109, 122, 135, 147, 157, 170, 194, 218, 241, 268, 289 and 341 h) over a two week period, using a syringe through the tubing at the top of the fermenter. The wine samples were analysed as described in Section 2.11.

Once the two week fermentation was complete, the wine was centrifuged at 10,000 × *g* for 4 min at 20 ^{o}C in 50 mL batches. The supernatant was then filtered through glass wool using gravity filtration to remove particulate matter.

2.10.3. Lab-scale wine fermentation with grape juice

Chardonnay grape juice was sourced from the Australian Wine Research Institute (AWRI, Adelaide, SA, Australia) and shipped frozen to UWS before being completely thawed prior to use. The fermentations were carried out in an air-conditioned room with the temperature found to be 17 – 20 ^{o}C throughout the fermentation, averaging 18.7 ^{o}C. Four lots of 500 mL grape juice was aliquoted into each of 12 × 2 L Schott bottles. Pectin (2 g per bottle) and carrageenan (0.3 g per bottle) was added into the grape juice such that there were three replicates for pectin only, carrageenan only and pectin and carrageenan combined, respectively. Three bottles of grape juice with nothing added were used as the controls. All bottles were shaken vigorously before the fermentation caps were put on the bottles. Once the setup was complete, the fermenters were inoculated with re-hydrated QA23 yeast to a final concentration of 0.4 g/L yeast, as described in Section 2.2.2. The fermentations then proceeded over two weeks approximately, with samples taken at frequent timepoints (0, 12, 24, 36, 48, 60, 74, 85, 96, 109, 120, 149, 172, 219, 244, 266, 293, 336 and 375 h), by removing 50 mL from each fermentation vessel using a 50 mL syringe via the tubing in the fermentation setup. The sample was then transferred into a 50 mL centrifuge tube and centrifuged at 1,500 × *g* for 5 min at room temperature. The pellet was re-spun to remove any remaining supernatant before being snap-frozen in liquid nitrogen and stored at -80 ^{o}C. The initial supernatant was re-centrifuged at 6,000 × *g* for 5 min with the resultant supernatant stored for later analysis, as described in Section 2.11.

2.11. Wine analysis

In order to determine what effects the pectin and carrageenan have on actual wine, the supernatant from each sample was tested for various parameters. Some of the tests were conducted only with the final timepoint, such as the haze and sensory panel tests, whilst various timepoints during the two weeks were analysed for sugar, amino acid, protein and volatile metabolite levels, as described in this section.

2.11.1. Sugar

Wine samples were stored at -20 ^{o}C until the sugar test was carried out. Any samples that contained more than 4 g/L total sugar was first diluted up to 60 times to ensure they were in the linear range of this assay. Sugar levels were determined using the glucose / fructose (Gluc/Fru) UV method kit from Randox Laboratories (Crumlin, Co Antrim, UK). Buffer R1 (800 µL) was combined with 8 µL of sample in a cuvette, mixed by inverting while holding parafilm over the cuvette top. The absorbance of this solution was measured at 340 nm (A_1). Eight µL of glucose enzyme (hexokinase and glucose-6-phosphate dihydrogenase) R2 was added, mixed as above and incubated for 10 min at room temperature before the absorbance was re-measured (A_2). Four µL of fructose enzyme (phosphogluccose isomerase) R3 was added, mixed as above, incubated for a further 10 min and the absorbance re-measured (A_3). The concentrations of glucose and fructose of each solution was determined using the formulae on the next page.

$$[\text{Glucose (g/L)}] = (A_{2\,(\text{sample})} - A_{1\,(\text{sample})}) - (A_{2\,(\text{blank})} - A_{1\,(\text{blank})}) \times 2.917$$

$$[\text{Fructose (g/L)}] = (A_{3\,(\text{sample})} - A_{2\,(\text{sample})}) - (A_{3\,(\text{blank})} - A_{2\,(\text{blank})}) \times 2.930$$

2.11.2. Protein

The protein concentration of wine samples was determined using a slightly modified Bradford reagent (Bradford, 1976; Waters et al., 1991). The reagent was made up by dissolving Coomassie Brilliant Blue G-250 (0.2 g; Amresco, Solon, OH, USA) in 50 mL 96% ethanol and 100 mL 85% phosphoric acid. Deionised water was added to make the volume up to 1 L and thoroughly mixed. This mixture was filtered through 1MM filter paper (approximately 100 mL per sheet) and the reagent was stored at 4 ^{o}C until use. The sample (50 µL) and Bradford reagent (1.5 mL) were combined in a cuvette of 10 mm width and 3 mL capacity , incubated at room temperature for 45 min, before the absorbance was measured at 595 nm, using bovine serum albumin (BSA; Sigma) as a standard.

2.11.3. Haze test

The absorbance of each sample was measured at 430, 540 and 650 nm beforehand. Five mL of each sample was added to a glass test-tube and sealed. The samples were

incubated at 80 ^{o}C for 7.5 h, then at 4 ^{o}C for 12 h before the absorbance was measured again at the same wavelengths. The difference between the absorbance readings before and after the heat / cool treatment was compared. The greater the difference, the more likely the wine is to form a haze after bottling.

2.11.4. Sensory panel

Sensory panel analysis was carried out using the final wine supernatants from the two lab-scale fermentations. Both panels consisted of academics and students within the science research groups at UWS Campbelltown, none of whom had received wine tasting training. Participants did not eat, drink or smoke during the 30 min preceding the test. The timing of the tests was staggered to allow for the testing to run smoothly. Coffee beans and water were available to all participants in case they needed to cleanse their sense of smell or palate between samples.

The second fermentation set was made with synthetic grape juice media using commercially supplied chemicals as described in Section 2.1.5. Some ingredients could be hazardous to health. To ensure the safety of the participants of the sensory panel, this test was by smell only. Twenty participants were involved in this panel, all of whom volunteered. Codes were given to each sample to avoid bias. This consisted of five random digits preceded by an identifying digit, being 1 for the 10% ethanol control, 2 for the non-treated control wine, 4 for the pectin/carrageenan treated wine, 8 for carrageenan treated wine, 9 for pectin treated wine and 0 for the commercial wine (coded 9017 in Section 2.1.7). Each participant smelled one biological replicate, with six people smelling set A and seven each for sets B and C. White wine glasses (obtained from Big W, Campbelltown, NSW, Australia) were used for the samples.

The second lab-scale fermentation from the real grape juice was safe for human consumption. All glassware was purchased brand-new for the specific use in this experiment in order to avoid any hazards for sensory testing later on. The samples were centrifuged to remove all yeast and other particles. The wine samples were tested in a similar way to the one described above, however, participants were asked to drink the wine after smelling it. Twenty seven participants were asked to assess the fruitiness, sweetness and overall pleasantness of the smell and taste of each wine, with one participant limited to smelling the wine due to the need to drive after the test.

2.11.5. Amino acid analysis

The amino acid content of the wine samples was determined by the Australian Proteome Analysis Facility (APAF) at Macquarie University (Sydney, NSW, Australia). The supernatants from the wine fermentation were stored at -70 ^{o}C until analysis. The samples were centrifuged through a 10 kDa molecular weight cut-off filter at 2,112 × *g* for 1 h at 8 ^{o}C. This filtrate was mixed with an equal volume of the internal standard Norvaline (Sigma) and then analysed using the Waters AccQ-Tag Ultra chemistry on a Waters Acquity Ultra Performance LC mass trak "physiological" 2.1 × 150 mm column (Waters, Milford, MA, USA). This utilised the AccQ-Fluor reagent which derivatises primary and secondary amino acids. Sample (10 µL), buffer (70 µL) and the AccQ-Fluor reagent (20 µL) were combined in a LC-MS vial, vortexed, incubated at room temperature for 1 min and incubated 55 ^{o}C for 10 min before being separated on the column. Two µL was injected into the machine, using a flow rate of 0.6 mL/min with a gradient of 20 min at 60 ^{o}C and 11,000 – 13,800 psi prior to detection at 260 nm UV. The raw data was normalised using the internal standard and amino acid concentrations were calculated by APAF using the free amino acid molecular weights. Tryptophan and cysteine could not be measured using this protocol.

2.12. Volatile metabolic profiling

2.12.1. Qualitative GC-MS standard and sample preparation

Three drops of ethyl hexanoate (≥ 99% purity, Sigma) and two drops of methyl nonanoate (≥ 98% purity, Sigma) (20 to 40 mg each) were added to 50% ethanol (5 mL) and made up to a volume of 100 mL in a volumetric flask using absolute ethanol. This was accurately diluted one in ten using a glass pipette and a volumetric flask with absolute ethanol. Twenty microlitres of this was added to deionised water (5 mL) in a 20 mL screwtop GC-MS vial, 2-2.5 g NaCl added and the vial tightly sealed immediately on addition. The standards were then placed into the rack for analysis. Standards were included at the beginning and end of each run as well as between samples such that no more than six samples were run in a row between standards.

Sample (5 mL) was alioquoted into a GC-MS vial before 2-2.5 g NaCl was added. The vial was tightly closed immediately on addition and the sample was then ready for analysis. Samples were analysed within a 12 h period of processing.

2.12.2. Qualitative Headspace GC-MS protocol

The SPME GC-MS volatile metabolite profiling protocol was modified from the standard protocol used by Fosters Australia and optimised for the local GC-MS by Sergio Baipas, so that in-house profiling of the two lab-scale fermentations could be carried out. This was essential as volatiles can be compromised by storage. This protocol utilised an SGE BP20 60 m × 0.25 mm × 0.25 μm column using an Agilent 7890 series Gas Chromatograph equipped with COMBIPAL robot, Agilent 5975 series MS detector and headspace injector capability and Enhanced MSD Chemstation software (Version E.02.00493) with a Supelco 50/30 μm DVB Carboxen / PDMS Stableflex (grey) fibre.

The sealed vial containing the sample was placed by the robot into a 60 ^{o}C heating block and agitated at 500 rpm to promote volatile release while the fibre pierced the vial seal and penetrated 22 mm into the vial to expose the fibre to the volatiles. This fibre exposure time lasted 40 min and overlapped with the sample separation of the previous injection. The volatiles were then desorbed at 240 ^{o}C over 2 min penetrating 54 mm into the injector port. The volatiles were eluted onto the GC column for splitless separation over 50 min with a 2 mL/min flow rate (consisting of 50 ^{o}C for 2 min, 5 ^{o}C/min ramp to 220 ^{o}C, 220 ^{o}C for 10 min, 20 ^{o}C/min ramp to 260 ^{o}C which was held for 2 min). The resulting chromatograms were analysed using the accompanying software. A cut-off area of 50 million was used to eliminate noise.

Chapter 3: Optimisation of cDNA microarray conditions for functional genomic analysis of yeast sulfur and flavour compound metabolism

3.1. Introduction

Yeast sulfur and flavour metabolites, as previously described in Chapter 1, are critical to wine quality. In order to fully understand their metabolism at the gene expression level, a high-throughput methodology for determining genome-wide expression, namely cDNA microarray transcriptomics, was used in this study. Since the technology involves a large number of parameters in numerous steps which could affect the final result, an optimised procedure must be established first. Consequently, the objective of this chapter is to optimise and establish the cDNA microarray protocol including RNA purification, cDNA synthesis, fluorescent dye labelling, hybridisation and washing conditions as well as determining the appropriate quantity of replicates.

At the start of this study, there were two options for conducting cDNA microarray analysis - the in-house analysis by hybridisation of labelled cDNA samples onto two-colour yeast genome oligo-printed glass slides or the outsourced service by sending RNA samples to a microarray processing centre such as the Ramaciotti Centre at the University of New South Wales for cDNA labelling and hybridisation onto one-colour Affymetrix® yeast chips. The advantages of the in-house option were its provision of a learning experience in broad molecular biology and using this complex technology in particular, the freedom in controlling experimental conditions, and the low cost. However, these advantages could be, seriously challenged by the large number of parameters, time-consuming nature of optimisation and quality control of each required step of analysis. To gain experience in this comprehensive functional genomic technology and determine the best option for transcriptomic analysis in a range of experimental conditions for yeast sulfur and flavour compound metabolism, both in-house and outsourced cDNA microarrays were explored in this chapter.

Affymetrix GeneChip® microarray chips have been available since the early 1990s. The earliest version of the Affymetrix Yeast Expression GeneChip series was the Ye6100. Its dataset was derived from the data available as of September 1996

from the Saccharomyces Genome Database (SGD). The second version (Yeast Genome S98; YG-S98) was developed using open reading frames identified by SGD as of December 1998 as well as those identified by MIPS (Munich Information Center for Protein Sequences) and other databases. The most recent array (GeneChip Yeast Genome 2.0 Array) was released in 2005 and represented 5,841 *S. cerevisiae* genes (out of a possible 5,845) and 5,031 *Schizosaccharomyces pombe* transcripts (out of 5,031), derived from databases in 2004. This last one was the chip used in this study. These two species diverged from each other more than 500 million years ago and are therefore the most genetically diverse yeast species that scientists study. Each transcript is detected by 11 oligonucleotide pairs, which makes the results more reliable.

My specific objectives of this chapter are as follows:

- To optimise the protocol for two-colour cDNA microarrays.
- To analyse the common elements in gene lists generated from biological and technical replicates in one-colour Affymetrix cDNA microarrays.

3.2. Summary of two-colour cDNA microarray methodology

The methodology for two-colour cDNA microarrays involves many steps, such as sample preparation, RNA isolation and purification, cDNA synthesis, fluorescent labelling, hybridisation to chips, washing and scanning, as seen in Figure 3.1. Many parameters exist within these steps that could be optimised. A series of 13 batches of two-colour microarrays were run, with between two and nine slides in each run, totalling 64 microarrys. Between each batch, various parameters were changed in an attempt to improve the quality of the microarray slides produced. A summary of the methodological properties of these microarray batches is shown in Table 3.1.

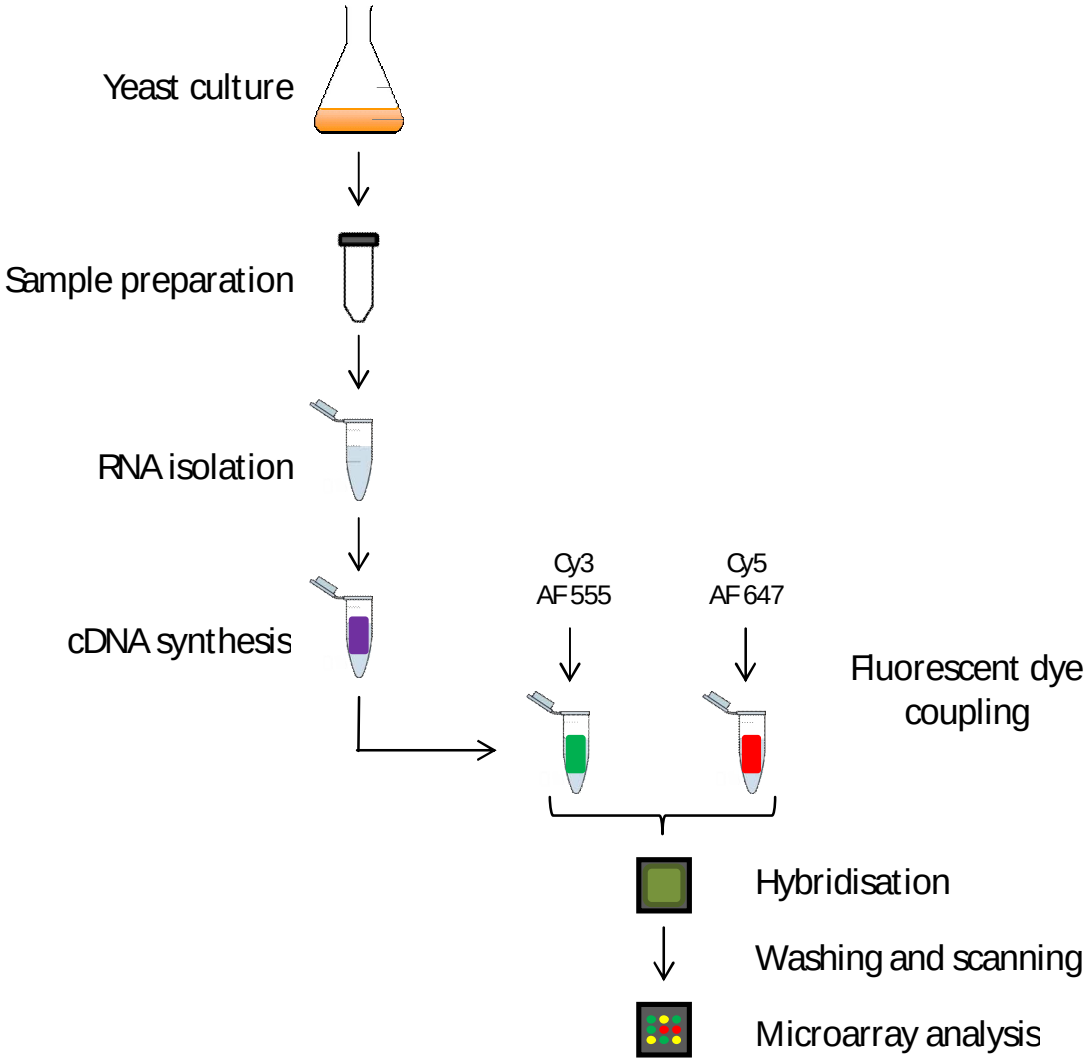


Figure 3.1. Schematic of the overall two-colour microarray process.

Table 3.1. Summary of the 13 batches of two-colour microarrays.

Batch	No. of slides	cDNA synthesis method	Coupling method	Hybridisation Method	Wash	Quality
1	9	Non-kit using 42 ^{o}C	Cy3 / Cy5 [1×]	Static	[1×] 7×15 min	Low
2	9	Non-kit using 42 ^{o}C	Cy3 / Cy5 [1×]	Static	[1×] 7×15 min	Low
3	9	Non-kit using 50 ^{o}C	Cy3 / Cy5 [1×]	Static	[1×] 7×15 min	Low
4	4	Invitrogen kit using 46 ^{o}C with acetic acid	Alexa fluor 555/647	Static	[2×] 3×15 min, 4×1 min	High (samples from another student)
5	2	Invitrogen kit using 46 ^{o}C with HCl	Alexa fluor 555/647	Static	[2×] 3×10 min	Medium
6	2	Invitrogen kit using 46 ^{o}C with acetic acid	Alexa fluor 555/647	Static	[2×] 3×10 min	High (used in Chapter 4)
7	8	Invitrogen kit using 46 ^{o}C with acetic acid or HCl	Alexa fluor 555/647	Static & a-hyb	[2×] 3×5 min	High (used in Chapter 4)
8	5	Invitrogen kit using 46 ^{o}C with HCl	Alexa fluor 555/647	Static & a-hyb	[2×] 3×10 min	High (used in Gauci et al. (2009) article)
9	4	Invitrogen kit using 50 ^{o}C with HCl	Alexa fluor 555/647	a-hyb	[2×] 3×10 min	Low (wine samples)
10	4	Non-kit using 50 ^{o}C	Cy3 / Cy5 [2×]	a-hyb	[2×] 3×10 min	High (used in Gauci et al)
11	2	Non-kit using 50 ^{o}C	Cy3 / Cy5 [2×]	a-hyb	[2×] 3×10 min	Low (wine samples)
12	3	Invitrogen kit using 46 ^{o}C with HCl	Alexa fluor 555/647	a-hyb	[2×] 3×10 min	Medium
13	3	Invitrogen kit using 46 ^{o}C with HCl	Alexa fluor 555/647	a-hyb	[2×] 3×10 min	Medium

Note: The non-kit cDNA synthesis method is outlined in Section 2.6.1, Chapter 2. The Invitrogen kit used was the Invitrogen SuperScript Plus indirect cDNA labelling system, outlined in Section 2.6.2, Chapter 2. The temperatures listed in the table are those used during the 2.5 h extension period. To hydrolyse the unconverted RNA, two methods were used for the Invitrogen kit – 1 M NaOH and 1 M HCl as in the protocol (listed in the table as "with HCl") or 0.25 M NaOH and 0.2 M acetic acid instead (listed in the table as "with acetic acid"). The cyanine (Cy3 and Cy5) coupling method is outlined in Section 2.6.3, Chapter 2 where [1×] or [2×] refers to the strength of the cyanine dye, with [2×] using approx. half the volume of DMSO to dissolve the dye as used in the [1×] preparation. The Alexa fluor (555/647) coupling method is outlined in Section 2.6.4, Chapter 2. Sample hybridisation, described in Section 2.6.7, was of two types, either static (under a coverslip) or with circulation using the a-Hyb hybridisation station. Slide washing was carried out as described in Section 2.6.8 for [1×] and in Section 2.6.9 for [2×] with wash times outlined in the table above. The complete raw microarray dataset for the published zinc-related microarrays from Gauci et al. (2009), in which I am a co-author, is available online at the Gene Expression Omnibus (GEO) database (Accession number: GSE11878).

3.3. Slide quality

3.3.1. Hybridisation rate

In terms of the quality of two-colour cDNA microarray slides, a major question that first needs to be addressed is what is actually meant by "quality". The most important factor is the hybridisation rate, which is the rate at which the coupled cDNA attaches to the probes on the microarray slides, or in other words how many gene probes have data. Figure 3.2 shows a single block within two microarrays, one with a low hybridisation rate and one with a high hybridisation rate. Only the second microarray contains usable data.

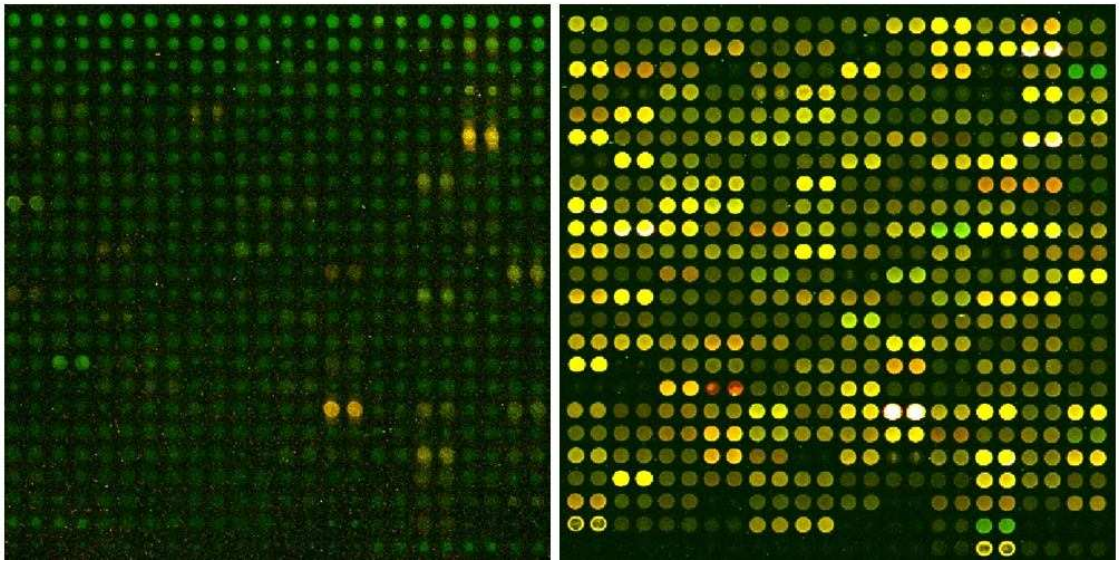


Figure 3.2. Illustration of low (left) and high (right) hybridisation rates of two-colour cDNA microarray slides. The image has been enlarged to show a single block of each microarray (out of 24 blocks each). The slide on the left is from batch 2 while the right hand slide is from batch 6.

3.3.2. Slide appearance

The appearance of the slide is an important factor in the quality of two-colour cDNA microarrays. Two-colour microarrays tend to have a bias towards the green dye, however, this is generally even across the slide and is compensated for during the bioinformatic normalisation process. Problems with normalisation arise, however, when background noise is uneven such as in the left and centre panels of Figure 3.3, where patterns can be clearly seen against the background. The first of these has

massive red spots covering large sections of the microarray blocks and the second has a “smudge” across most of the bottom half of the slide. These cause two problems – firstly, the background is uneven causing inaccurate results to be reported by bioinformatic programs, and secondly, data from those spots that are “underneath” this interference are obscured.

Good quality spots have uniform fluorescence and a circular spot that does not interfere with the surrounding spots. In the right hand panel of Figure 3.3, bad quality spots are shown. Where spots appear to have “tails,” some of the fluorescence from these spots have bled into the surrounding area, sometimes including covering the surrounding spots, meaning that these spots cannot be accurately analysed. Another issue is the “doughnut effect” where the fluorescence is uneven across the spot, usually having fluorescence only on the outside of the spot. This makes accurate analysis difficult.

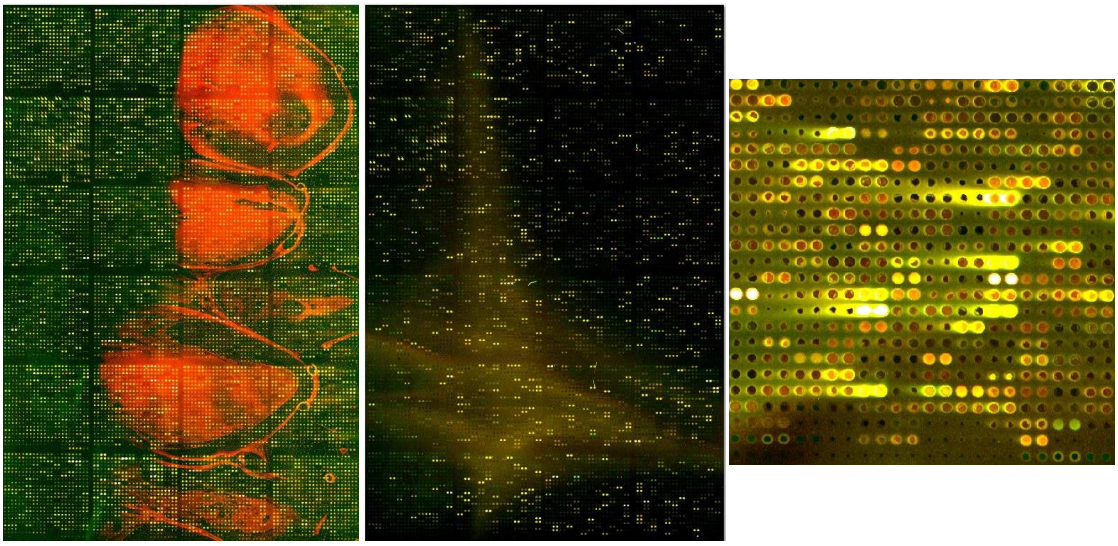


Figure 3.3. Examples of two-colour cDNA microarray slides with poor background quality (left and middle) and poor spot quality (right). The first two panels show the entire microarray slide with uneven background caused by incorrect binding or insufficient slide washing. The third panel shows two separate issues with spot quality, namely spots bleeding into the surrounding features and the “doughnut” effect where the spot fluorescence is only seen on the edges of the spots and not in the centre.

3.4. Preparation of labelled probe hybridisation mixture

3.4.1. Yeast strains

The cDNA microarrays using glass slides were designed using the genome of the laboratory yeast strains. Information from our industrial partners indicated that the industrial strains had lower hybridisation rates than that obtained for laboratory strains. The microarray probes are specifically designed for laboratory strains and this could account for this difference. Alternatively, the industrial strains being polyploidy or more robust could require fewer genes to be expressed, especially under conditions that may be considered "stressful" for laboratory yeast strains. Batch 3 contained microarrays for both industrial brewing strain A and laboratory strain BY4743. Other than yeast strain, these samples were prepared and analysed in an identical manner, thus allowing the comparison of strain type on the quality of the microarray data, particularly hybridisation rate.

An example of the industrial strain microarrays and the laboratory strain microarrays can be seen in Figure 3.4, which show that the quality of the two images are very similar, despite showing very low hybridisation rates. This shows that strain type is unlikely to be an important factor in a successful microarray experiment, despite what industrial sources have suggested.

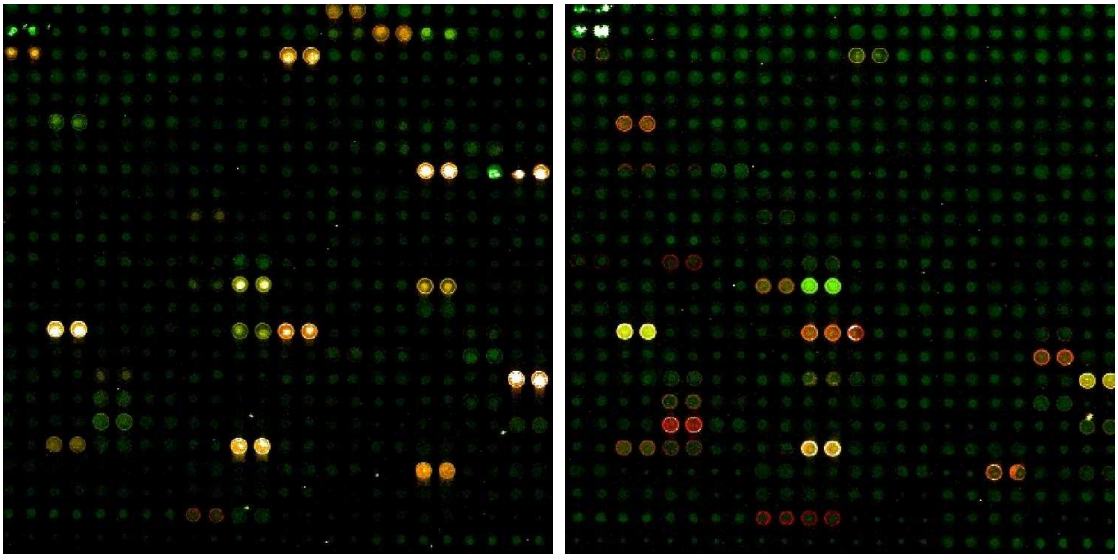


Figure 3.4. Images of two-colour microarrays using laboratory strain BY4743 (left) and industrial brewing strains A vs O (right) show similarly low hybridisation rates. These arrays are part of batch 3.

3.4.2. The effect of residual medium on RNA quality

Once samples were prepared, the yeast cells were snap-frozen in liquid nitrogen ready for RNA isolation. The last step prior to snap-freezing is the removal of the media from the yeast cells. The samples were centrifuged and the medium discarded. These were then centrifuged again and the residual medium (100 to 200 μL) discarded by pipette. In order to test whether this second step is necessary, two samples were prepared, identical in all ways except that the residual media was only removed in one of the samples. RNA was isolated from both samples as normal and compared on the Bioanalyzer. The Bioanalyzer analyses the RNA for DNA contamination as well as RNA intactness. A clean chromatograph generally shows three sharp peaks, showing 5S, 18S and 28S (from left to right) ribosomal RNA without a broad peak around 50 sec that would indicate DNA contamination. The Bioanalyzer chromatograph in Figure 3.5 shows the normal sample without residual medium in red and the sample containing the residual medium is shown in blue. The 5S peak (around 26 seconds) is similar between the two samples; however, the 18S and 28S peaks (around 42 and 46 seconds, respectively) are much lower in the RNA sample that contained the residual media. This shows how important discarding all the residual media is in order to get good quality intact RNA.

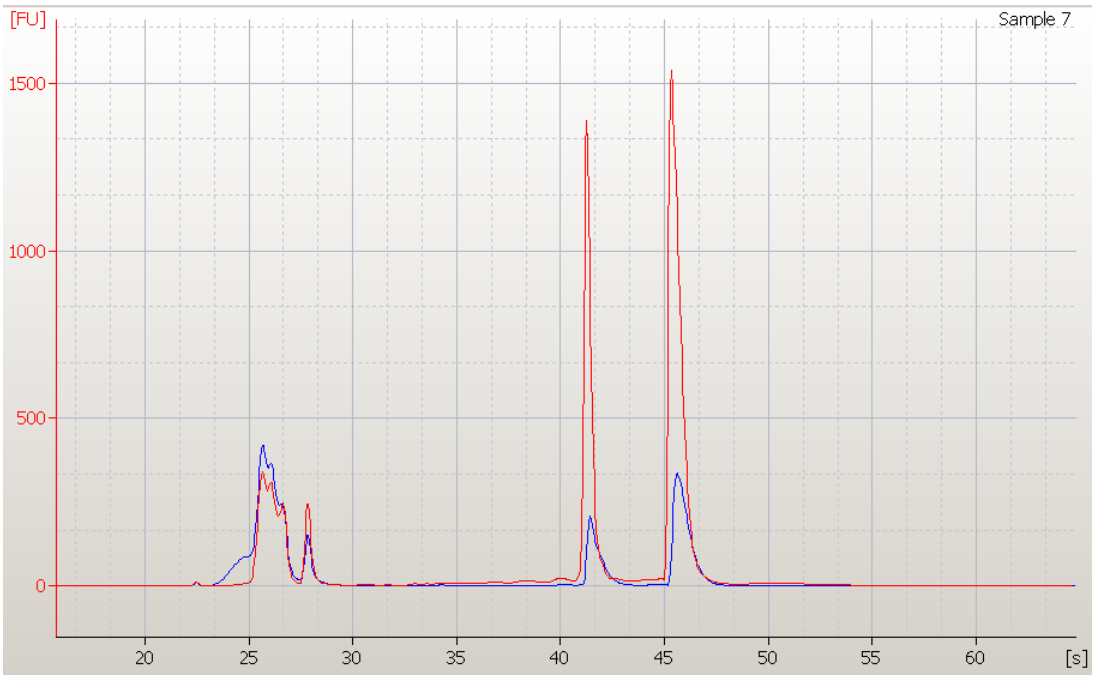


Figure 3.5. Bioanalyzer trace of total RNA isolated from yeast cells with (blue) and without (red) residual media. The three main peaks at 26, 42 and 46 sec represent the 5S, 18S and 28S ribosomal RNA peaks, respectively.

3.4.3. cDNA synthesis

Increased amounts of cDNA should result in a higher level of coupled cDNA in each reaction for hybridisation onto a slide, which should produce higher signal intensities on a microarray slide. Therefore, cDNA synthesis optimisation was considered. Two different cDNA synthesis methods were used, each with slight variations. These included a non-kit version using individual components as well as a kit-based method using the 'Invitrogen SuperScript Plus indirect cDNA labelling' system.

Variations to these methods included an increase in the temperature for the cDNA extension step. While the non-kit protocol called for this step to be at 42 ^{o}C, other protocols using SuperScript III reverse transcriptase advised the use of 50 ^{o}C while 42 ^{o}C was more commonly used for SuperScript II reverse transcriptase. The Invitrogen kit method used a temperature of 46 ^{o}C. A temperature of 50 ^{o}C was trialled for the extension step for both of these methods.

There was a large variation in the cDNA concentrations between batches and within the same cDNA synthesis batch. cDNA concentrations throughout the entire project from the non-kit method at 42 ^{o}C resulted in 2.9 to 156.8 while at 50 ^{o}C, the cDNA ranged from 11.2 to 201.3 μg/μL. The lower temperature averaged 49.2 μg/μL (n=40) and the higher temperature averaged 76.6 μg/μL (n=70). While the spread of these cDNA concentrations greatly varied, the minimum, maximum and average showed that increasing the temperature to 50 ^{o}C for the extension process was advisable. The main question was whether this increased temperature would have an effect on the microarray quality. Batch 2 used 42 ^{o}C and batch 3 used 50 ^{o}C, however, they both had low microarray quality with a low hybridisation rate, indicating that the temperature of the extension step as well as the concentration of cDNA (beyond a threshold) has little effect on quality.

An important part of the quenching step to hydrolyse the unconverted RNA is the neutralisation of the pH. The original method comprising of individual components advised that equal volumes of the acid and base used in the quenching step should turn PBI buffer (supplied with the Qiagen QiaQuick® PCR purification columns) yellow. The Invitrogen method used a higher concentration of NaOH (1 M instead of 0.25 M) and HCl (1 M) instead of acetic acid (0.2 M). However, the more concentrated NaOH and HCl did not turn the solution yellow, so the less concentrated NaOH and acetic acid were trialled as well since they did result in a yellow colour when combined with the PBI buffer.

The non-kit methods were capable of obtaining higher cDNA concentrations than the Invitrogen kit methods, as shown in Table 3.2. This table also shows the effect of temperature on both the non-kit method and the Invitrogen kit method using HCl. An increase in the extension temperature from 42 ^{o}C to 50 ^{o}C for the non-kit method increased the cDNA concentrations. However, the temperature increase from 46 ^{o}C to 50 ^{o}C for the Invitrogen kit method using HCl resulted in a lower average cDNA concentration. These results showed that the best temperature for the non-kit method is 50 ^{o}C and the best temperature for the Invitrogen kit-based method was 46 ^{o}C.

Table 3.2. cDNA concentrations obtained through different synthesis methods.

	Range (µg/µL)	**Average (µg/µL)**	**Number of samples**
Invitrogen kit using 46 ^{o}C with acetic acid	2 to 47.4	19.5	32
Invitrogen kit using 46 ^{o}C with HCl	0.9 to 23.7	8.3	83
Invitrogen kit using 50 ^{o}C with HCl	1.3 to 7.8	2.5	11
Non-kit method using 42 ^{o}C	2.9 to 156.8	49.2	40
Non-kit method using 50 ^{o}C	11.2 to 201.3	76.6	70

An important aspect to consider is the labelling efficiencies of these methods, which is addressed in the following section.

3.4.4. Fluorescent dye coupling

In order to visualise the cDNA on the microarray, the cDNA is coupled with fluorescent dyes, either the cyanine dyes, Cy3 and Cy5 or the Alexa Fluor 555 or 647 dyes. Cy3 and Alexa Fluor 555 appear as a green colour while Cy5 and Alexa Fluor 647 appear red.

Figure 3.6 shows the comparison of the cDNA concentration of samples after the cDNA synthesis step and their corresponding labelled probe concentration after the labelling step, including data from samples that did not continue to the hybridisation step. This shows that while the non-kit method resulted in the highest cDNA concentration, the Invitrogen kit method resulted in the higher level of dye concentration, showing that this method enables high coupling efficiencies.

The previous section showed that, on average, the Invitrogen kit method using acetic acid as the quenching acid obtained higher concentrations of cDNA than the method using HCl, this graph shows that there is a high correlation between the cDNA and dye concentrations for the HCl method, with a 89% correlation (see Figure 3.6) compared to 5% and 11% correlation for the Invitrogen kit method with acetic acid and the non-kit method, respectively (data not shown). This result, coupled with HCl being the recommended quenching acid by Invitrogen, has led us to prefer HCl as the

quenching acid over acetic acid. There was no visible difference between the various methods in terms of microarray quality.

Our experience during this optimisation process suggests that the best microarray results can be obtained by ensuring only samples with a cDNA concentration of 10 ng/µL or higher using the Invitrogen kit method with HCl and a dye concentration of 1 pmol/µL using Alexa Fluor dyes are hybridised onto the slides. If these thresholds are not met, there is a much higher chance of the microarrays being unsuccessful.

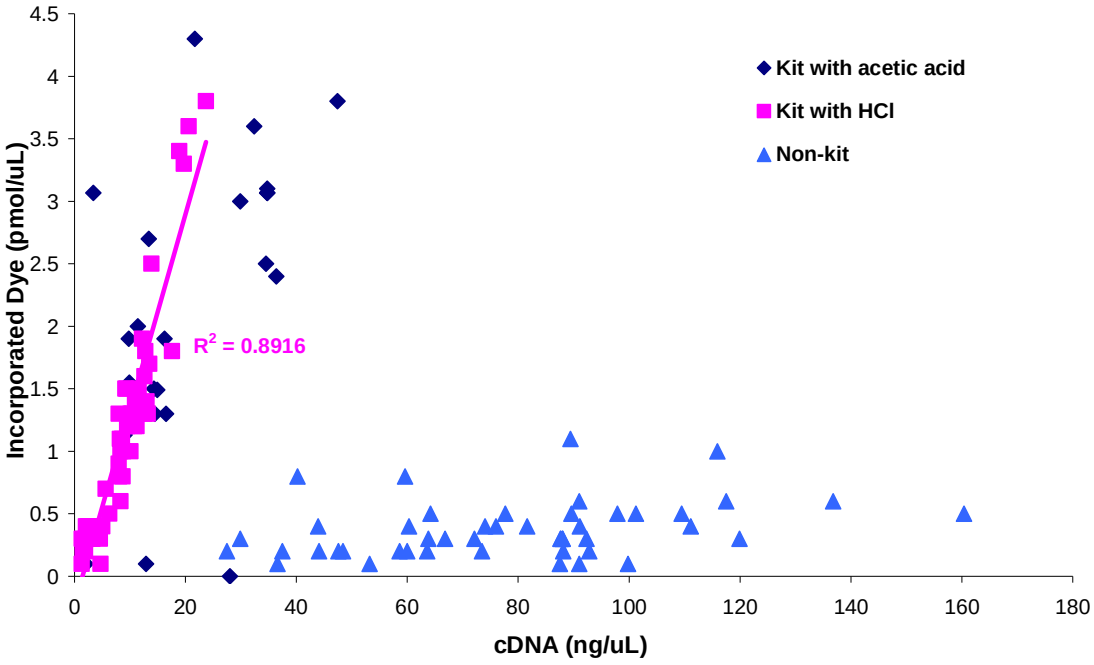


Figure 3.6. cDNA concentration vs labelled probe concentration, grouped by the cDNA synthesis method used. Legend: Samples made using the Invitrogen kit using acetic acid cDNA method are indicated by the dark blue diamonds, those made using the Invitrogen kit using HCl are shown as pink squares and the non-kit samples are represented by the light blue triangles.

3.5. Microarray slides

Once we have labelled cDNA, the next step involves the actual microarray slide. The microarray slides were purchased from the Ramaciotti Centre at the University of New South Wales and this process was out of our control. We were able to access the

printing records afterwards, which allowed us to determine the effect on the microarrays of the time that had passed between slide printing and hybridisation, of the manufacturing batch in which the slides were made and the blocking protocol used just prior to hybridisation.

Each microarray was visually examined using GenePix and given a score between 0 and 5 (5 being the best) for three different quality parameters, namely the number of spots (or hybridisation rate), the background quality and the quality of the spots.

3.5.1. Slide age

As shown in Figure 3.7, there was no correlation between the age of the slides and the background quality or the hybridisation rate, however, the only microarrays with questionable spot quality were those that were more than 18 months old. Despite this, we recommend that slides are used within six months of printing.

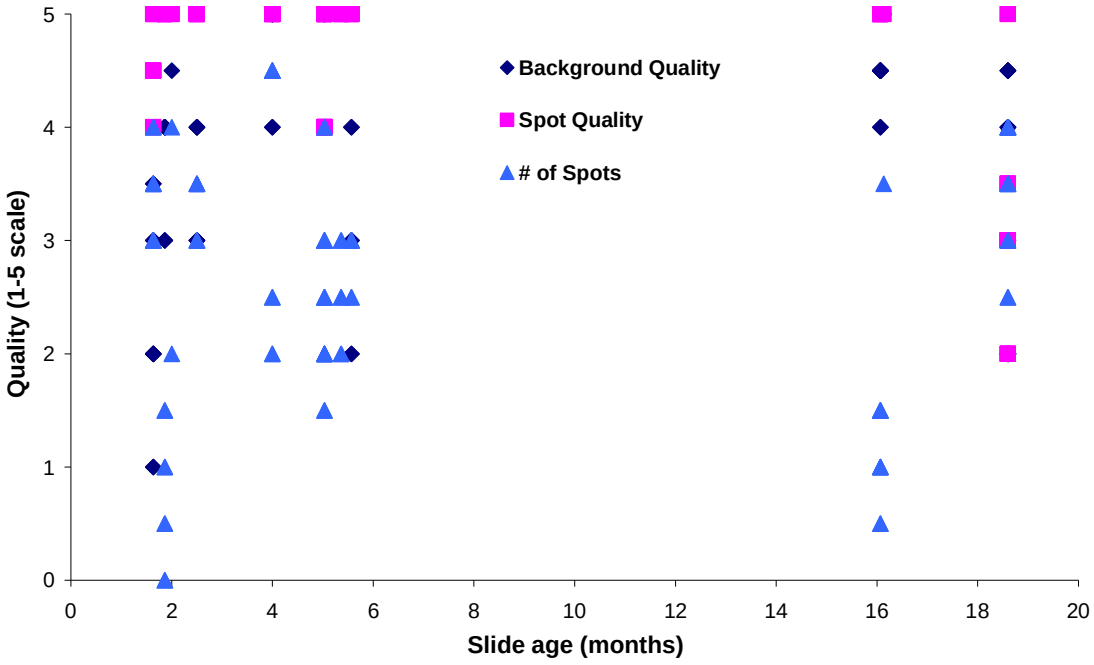


Figure 3.7. Visually determined quality ratings vs the age of the microarray slides in months. Legend inset.

3.5.2. Slide printing batch

During the duration of the optimisation process, we received microarray slides at various points, however, they all were part of four printing batches. The four slide manufacturing batches did not appear to have a large impact on the quality of the results, as shown in Figure 3.8, although batch 2 had lower spot quality, batches 1 and 4 had lower numbers of spots and batches 2 and 4 had lower background quality, although all of these had at least one slide that reached an acceptable quality level.

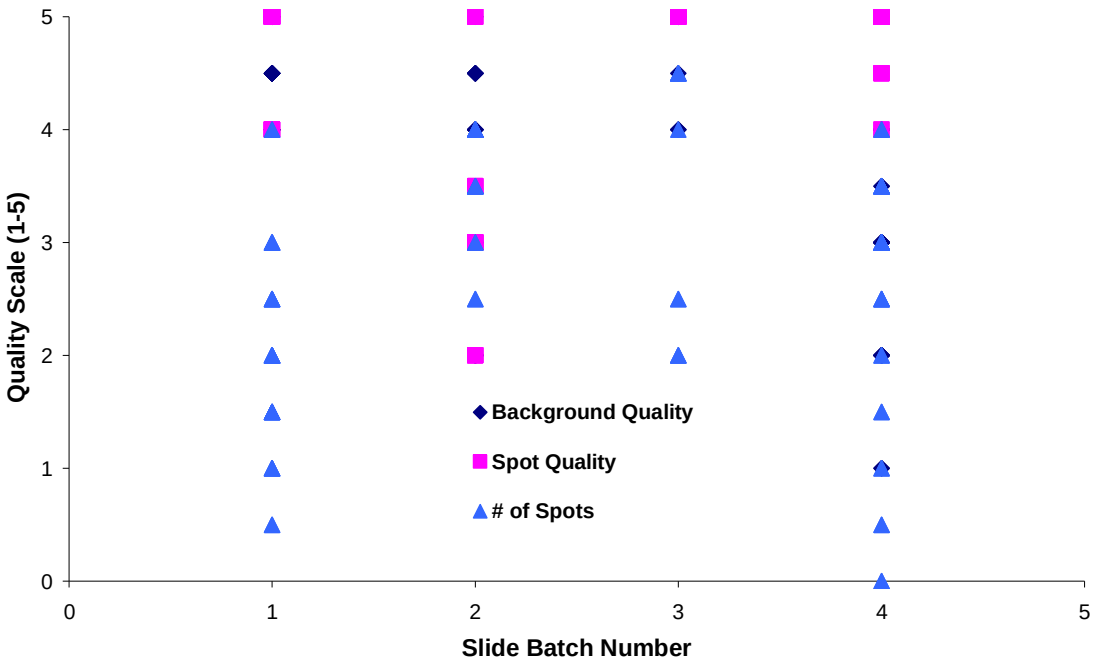


Figure 3.8. Visual determined quality ratings vs the batch of slide printing.

3.5.3. Slide blocking

After delivery of the pre-printed slides but before hybridisation, the slides were blocked, to ensure that anything that had attached during manufacture, transport and storage were removed. This involved a process of washing the slides. Hegde et al. (2000) found that if cDNA microarray slides are not used immediately after the blocking process, there is a decrease in the efficiency of hybridisation, particularly if it is left for more than an hour.

Initially, the slides were blocked a maximum of three hours in advance, but were kept in a dust-free box after blocking, which would have reduced this risk. Later, we ensured that slides were blocked no more than half an hour before hybridisation. However, the time between the blocking process and hybridisation did not appear to impact the quality of the microarrays.

Another blocking method was attempted during batch 4 of the microarrays. This was shown not to have any influence on the quality of the slides, so the original blocking method was retained.

3.6. Hybridisation and washing

The fundamental basis of microarray technology is the specific hybridisation of each probe on slide to the labelled complementary target during the hybridisation process. Considerable efforts were therefore spent on this step. cDNA samples labelled with either the Cy3 or Alexa Fluor 555 dye were combined with their corresponding samples labelled with either the Cy5 or Alexa Fluor 647 dye and then hybridised onto the slide. Once the probes were hybridised onto the microarray slides, the excess was washed off before the slides were scanned.

3.6.1. Hybridisation method

Two hybridisation methods were examined: static and fluid. The static method made use of capillary forces and a coverslip to hold the hybridisation fluid in place over the microarray slide during hybridisation. The fluid method utilised a machine called the a-hyb hybridisation station, which actively pumped the hybridisation mixture across the slide, allowing the probes more access to the slides. While this hybridisation method did not appear to improve the hybridisation rate, it did seem to reduce the chance of an uneven background.

3.6.2. Slide washing

After hybridisation overnight, the microarray slides were washed to remove unhybridised cDNA and dyes. Different wash durations and stringency were used during the optimisation, and these found that the more stringent wash cycle for a medium duration of time obtained the best results in terms of background and spot quality.

3.7. Optimised protocol

After the complete optimisation process, the protocol below was established as the one that would result in the best quality microarray slides possible. It needs to be mentioned that this protocol was also described in detail in the chapter of Materials and Methods, Chapter 2 (in the sections specified below).

1. Sample preparation: Remove all residual media from the samples prior to snap-freezing them.
2. RNA Isolation: As outlined in Section 2.5.1. RNeasy clean-up columns are recommended, as described in Section 2.5.2. Ensure that the RNA is of good quality and is intact, as described in Section 2.5.3.
3. cDNA synthesis: As outlined in Section 2.6.2 using the Invitrogen SuperScript Plus indirect cDNA labelling system. Ensure that the concentration of cDNA is above 10 ng/μL before continuing to the next step.
4. Dye coupling: As outlined in Section 2.6.4, using Alexa fluor dyes. Ensure that the dye concentration exceeds 1 pmol/μL for the relevant dye (either 555 or 647) before continuing to the next step.
5. Blocking: As described in Section 2.6.6, within an hour of hybridisation.
6. Hybridisation: Either the static or fluid protocols described in Section 2.6.7, although if the choice is available, the a-hyb hybridisation machine is the preferred choice.
7. Washing and scanning: Follow the washing protocol as described in Section 2.6.9. Within 2 h of washing, scan the slides using a GenePix scanner as described in Section 2.6.10.

3.8. Overall assessment of two-colour microarray quality

Optimisation of the two-colour microarrays led to the acquisition of reliable good-quality data for both a hydrogen sulfide project (the results of which are the subject of Chapter 4) and a zinc project (which resulted in a publication by Gauci et al (2009)). However, in order to obtain these meaningful results, many arrays had to be processed because of the occurrence of some unsuccessful ones – only six of the 11 zinc microarrays scanned were used in the publication (Gauci et al., 2009) and only eight of the 27 scanned for the H_2S experiments were usable due to the low hybridisation rates or high background seen with the remaining microarrays. In addition, six microarrays were analysed using wine samples from pilot-scale fermentations, with none of these providing good quality data.

Thus, the inconsistency of two-colour microarray quality became an issue, which led me to investigate the one-colour Affymetrix microarrays, particularly because the financial benefit of the two-colour system is eliminated by the large number of unusable microarrays produced in addition to the usable ones.

3.9. One-colour Affymetrix microarrays

The table over page, Table 3.3, shows that the one-colour Affymetrix microarrays were of high quality for each of the four batches used. When comparing this data to the data in Table 3.1, it is clear that the one-colour Affymetrix microarrays are more consistent with their high quality data. It is for this reason that we have employed the one-colour Affymetrix system in the transcriptomic gene expression profiling studies of the following chapters, despite the improvements seen using the optimised protocol and the publishable data obtained during this process.

Table 3.3. Summary of the four batches of one-colour Affymetrix microarrays.

Batch	No. of slides	Description	Notes	Quality
1	2	Pilot-scale wine fermentation (96 h)	Same RNA samples as used in Batches 9 and 11 in Table 3.1	High (used in Chapter 6)
2	12	Nitrogen and sulfur samples		High (used in Chapter 4)
3	12	Lab-scale grape juice wine fermentation (48 h)		High (used in Chapter 6)
4	12	Lab-scale grape juice wine fermentation (96 h)	1 microarray was an outlier and was discarded	High (used in Chapter 6)

3.10. Replicates for one-colour Affymetrix cDNA microarrays

Microarray experiments generate large amounts of data. In order to achieve accuracy, replicates for each treatment are carried out. There are two types of replicates – biological and technical. Biological replicates involve separately prepared samples for a treatment and technical replicates involve analysing the same sample twice or thrice, as shown in Figure 3.9. Biological replicates measure the actual differences related to the biological entity such as patients or yeast whereas technical replicates measure the differences related to the technology and experiment itself.

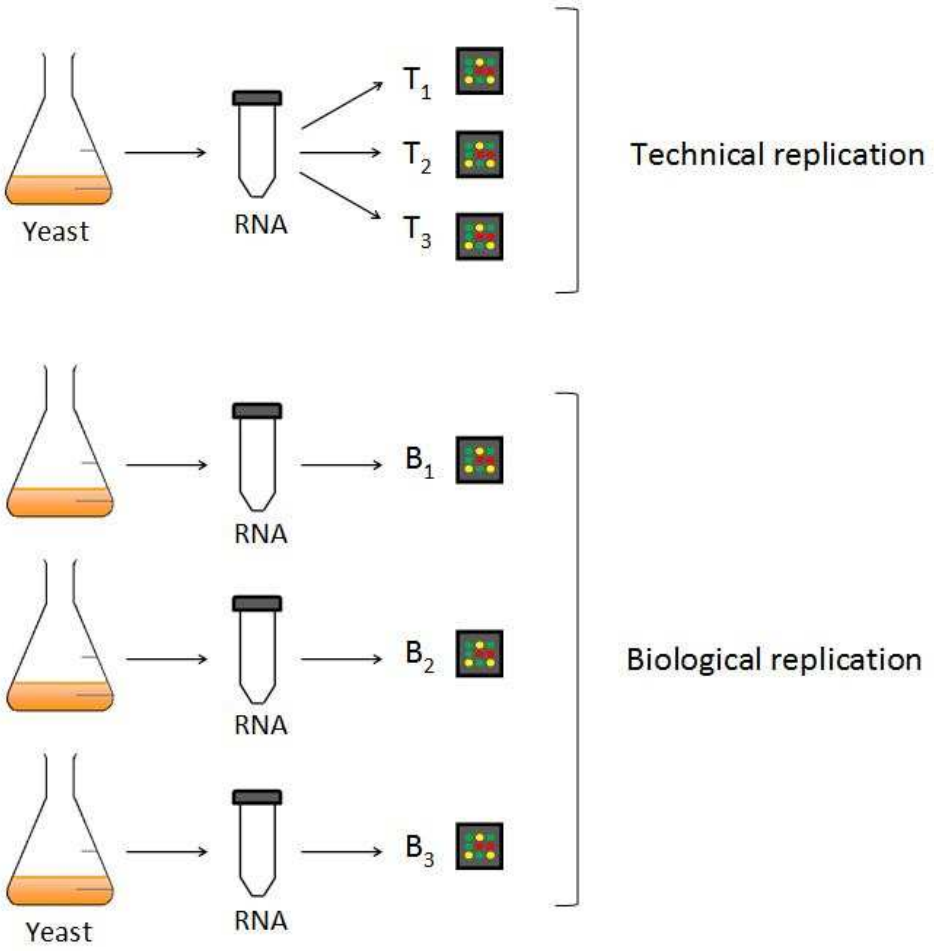


Figure 3.9. Schematic of the differences between technical and biological replications, specifically in terms of microarrays.

Three biological replicates are generally thought to be the minimum requirement for one-colour microarrays, with technical replicates not usually used. However, in some cases biological replicates are not possible to obtain, such as in the case of Chapter 6 where only a single biological replicate of a pilot-scale fermentation was possible due to equipment limitations. This work aims to determine the usefulness of replicated data within the one-colour Affymetrix microarray platform – will the same reliable data be generated from microarray data with one, two or three biological replicates? Is there any need for technical replicates? If fewer replicates generate similar data, doing so will save a third or half of the resources required, and will allow microarrays from experiments that require limited equipment such as our pilot-scale fermentation.

Most single-channel microarray experiments no longer use technical replicates, due to the high reliability found in the system. However, in terms of the generation and comparison of gene lists, no study has determined whether the use of technical replicates are in fact not necessary and do not add any extra significance to the data.

These questions will be answered by examining several datasets that exist within my research group, including a set that has technical duplicate of a biological triplicate.

3.11. Methodology for replicate comparison in one-colour Affymetrix microarray

I am more interested in the actual gene lists rather than the statistical implications of using different levels of replication within the one-colour Affymetrix microarray platform. Datasets that contained replicates were analysed as if biological or technical replication had not occurred and then the ANOVA lists of genes compared between the datasets to determine the level of differences seen from the inclusion of replication.

This examination of replicates uses the following:

- PCA plots generated through the bioinfromatic program Partek – these plots show the overall similarity between samples and whether grouping has occurred.
- ANOVA lists of gene expression fold change (through Partek) – these lists show the gene expression differences caused by the treatment.
- XY scatterplots – These graphs compare the fold change of a particular gene in one list with that of another. This is useful in determining correlation. The XY scatterplot shows the similarity of the entire dataset.
- Venn diagrams – An online Venn diagram generator called Pangloss (available at www.pangloss.com/seidel/Protocols/venn4.cgi) was used to generate numbers of common genes, etc between various lists of significant genes. Venn diagrams used for illustrative purposes in this chapter were created using Venny, an online tool (available online at http://bioinfogp.cnb.csic.es/tools/venny/index.html).
- "Commonality graph" – Another graph, here I have called it a "commonality" graph, was created to determine the fold change level where differences

between datasets can be seen. This separated the significant gene into three categories – firstly, a "common" category with genes that were significant in all of the datasets, secondly, the "false positive" category containing genes that were significant in the partial dataset but not in the complete dataset, and finally, the "false negative" category containing genes that were significant in the complete dataset but not in the partial datasets. The range of fold changes of the genes that fall into each category can then easily be seen, such as in the graph in Figure 3.14.

3.12. Comparison of bioinformatic programs Partek and GeneSpring

Firstly, I wanted to ensure that the results from different bioinformatic programs would be similar. Two of the most popular bioinformatic programs at the time were Partek and GeneSpring. The ratios (treated vs control) generated via the two programs using the same gene data were found to be almost identical with over 99.9% correlation. A comparison of lists of significantly altered genes found that only one gene different between the two lists. This indicates that the same results are obtained when using either of these two bioinformatic programs. Since the results were almost identical but Partek was easier to use with one-colour Affymetrix microarrays, Partek has been used for the remainder of the analysis for one-colour Affymetrix microarrays.

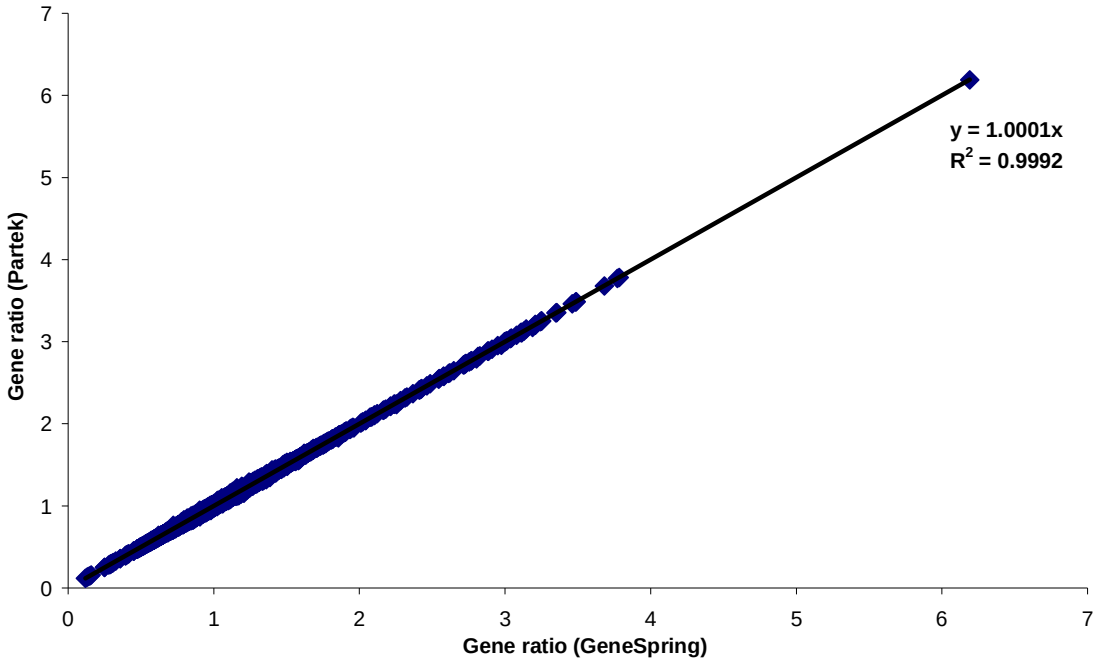


Figure 3.10. XY scatterplot of the gene fold ratios obtained through Partek (y-axis) compared to those found using GeneSpring (x-axis) with the same raw data.

3.13. Technical replication of one-colour Affymetrix microarrays

This section asks whether the accepted view of not utilising technical replicates within the one-colour Affymetrix microarray platform is warranted, or whether their inclusion adds something to the results. The dataset used for this comparison was generated by colleagues who carried out an oxidative stress experiment in biological triplicate and included technical duplicate microarrays for statistical reasons associated with comparisons with metabolomic data (O'Doherty, Wu and Higgins, personal communication, 2009). These files were then analysed as if technical replication had not occurred and then the ANOVA lists of genes compared between the three datasets (being each of the two technical replicate sets individually and also the gene list as a result of analysing both technical replicates together) to determine the level of differences, if any, that would result from the inclusion of technical replication.

Principal component analysis (PCA) illustrates the overall similarity between samples (microarrays in this case). The position of each sample in relation to the

others shows the similarity and grouping between samples. The components of difference are mapped out in 3D, where the highest level of difference (called the first principal component, or PC1) is represented on the x-axis, the second, PC2, on the y-axis and the third, PC3, on the z-axis.

The PCA plot for this dataset showed definite separation between the six control microarrays (represented by the diamonds) and the six treated microarrays (represented by the circles), as shown in Figure 3.11. This graph shows that while there is a difference between the biological replicates for each set, the technical replicates for this data practically overlap. The first set of technical replicates is represented by red shapes, hereafter called "Set A", and the other set is represented by blue shapes, hereafter called "Set B". The entire dataset with all 12 microarrays are hereafter called "Set AB."

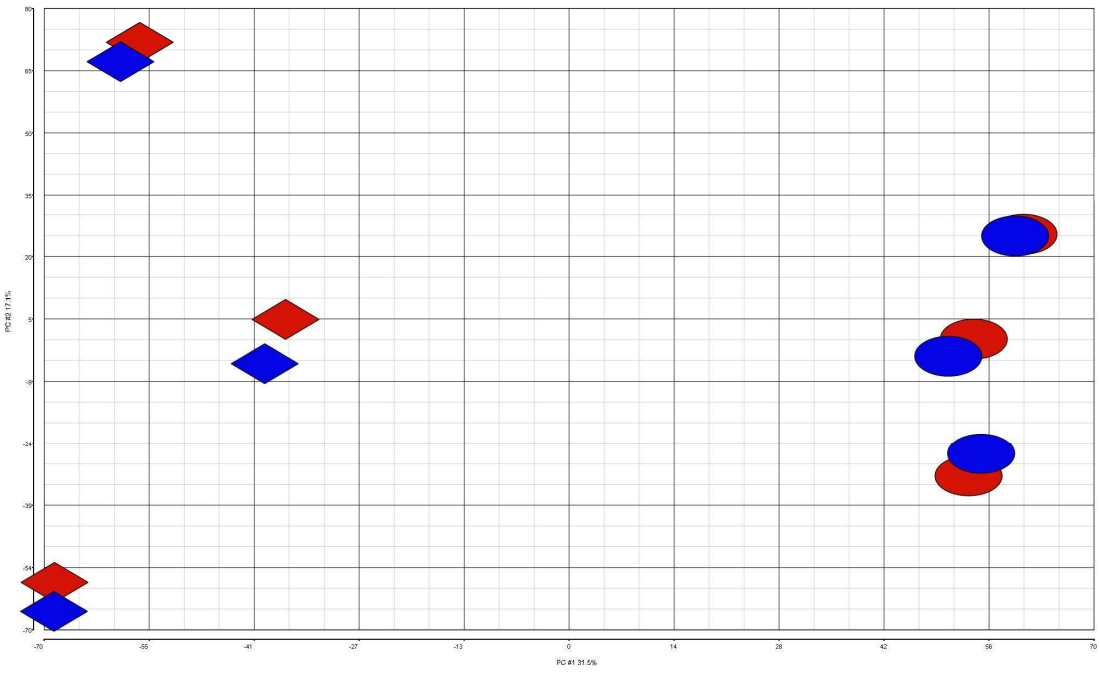


Figure 3.11. PCA plot of a microarray dataset using biological triplicates and technical duplicates. Key: Diamonds are control samples, Circles are treated samples, Red represents the first technical replicate (Set A) and blue is the second technical replicate dataset (Set B).

The degree of similarity between these technical replicates was determined by normalising the three datasets (A, B and AB) separately by RMA and using ANOVA to generate gene lists. The XY scatterplot in Figure 3.12 showed that there was a correlation of approximately 98 % between the gene expression fold change of the technical singlet datasets A and B and that of the technical duplicate dataset AB.

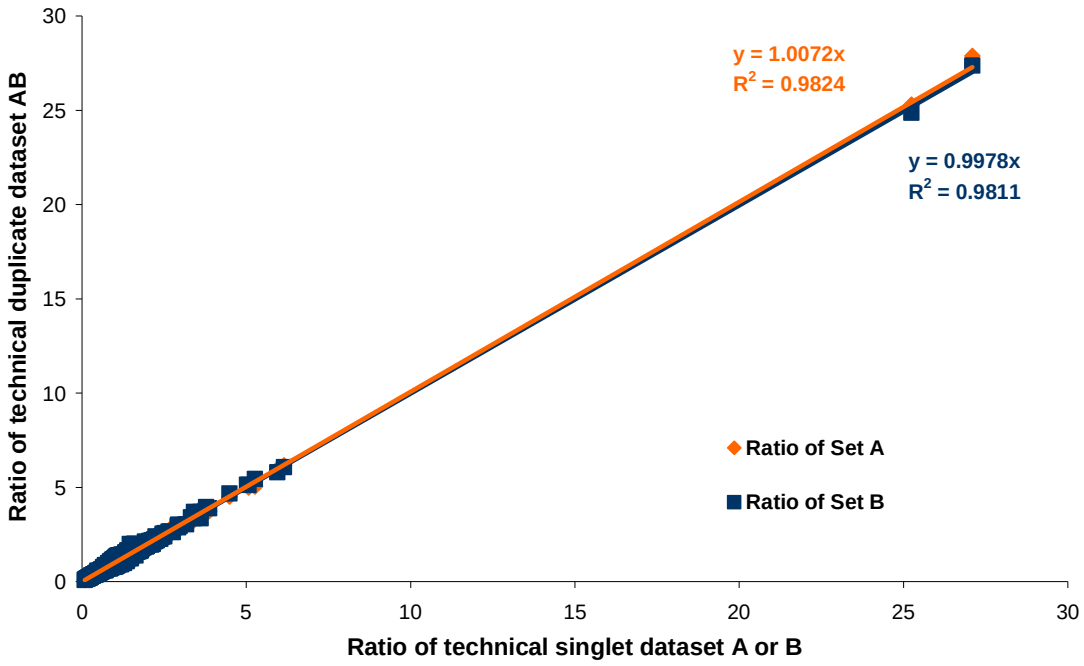


Figure 3.12. XY scatter plot comparing the ratios of technical single replicate sets A (orange) and B (blue) over the technical duplicate dataset AB.

While Figure 3.12 demonstrated a high overall correlation, the point of microarray experiments is to determine which genes are significantly changed by the treatment. Lists of significantly altered genes were generated for each dataset and compared to show the number of genes and the corresponding fold changes of these genes that fall into three different categories: common to the technical singlet (either set A or B) and the technical duplicate AB or unique to either list, as seen in Table 3.4 and Figure 3.13. The complete dataset with technical duplicate contained 124 down-regulated and 66 up-regulated genes, with fold changes ranging from to -11.8 to 27.1.

The comparison showed that 97% and 95.5% of the up-regulated genes in Set AB were also present in Sets A and B, respectively. The down-regulated genes were 96% and 96.8% present in Sets A and B, respectively. However, the question remains about the impact of the other genes, which fall into two categories – the false positives that are significant in the singlet datasets but are not significant in the duplicates, and the false negatives which are significant in the duplicates but not in the singlet dataset. If only a singlet dataset were used, the false positives would be incorrectly included in analysis and the false negatives would be incorrectly left out of the analysis. While there are not many of these genes, the important consideration is that of the fold changes of these.

Table 3.4. Number of genes and their fold changes for technical replicate analysis.

		Set "A" vs "AB"	**Set "B" vs "AB"**
Common to both	up	64 (up to 27.87)	63 (up to 27.38)
	down	119 (down to -11.58)	120 (down to -13.13)
False positives (Unique to singlet)	up	6 (up to 2.07)	2 (up to 2.12)
	down	5 (down to -2.04)	18 (down to -2.51)
False negatives (Unique to duplicate)	up	2 (up to 2.08)	3 (up to 2.16)
	down	5 (down to -2.03)	4 (down to -2.12)

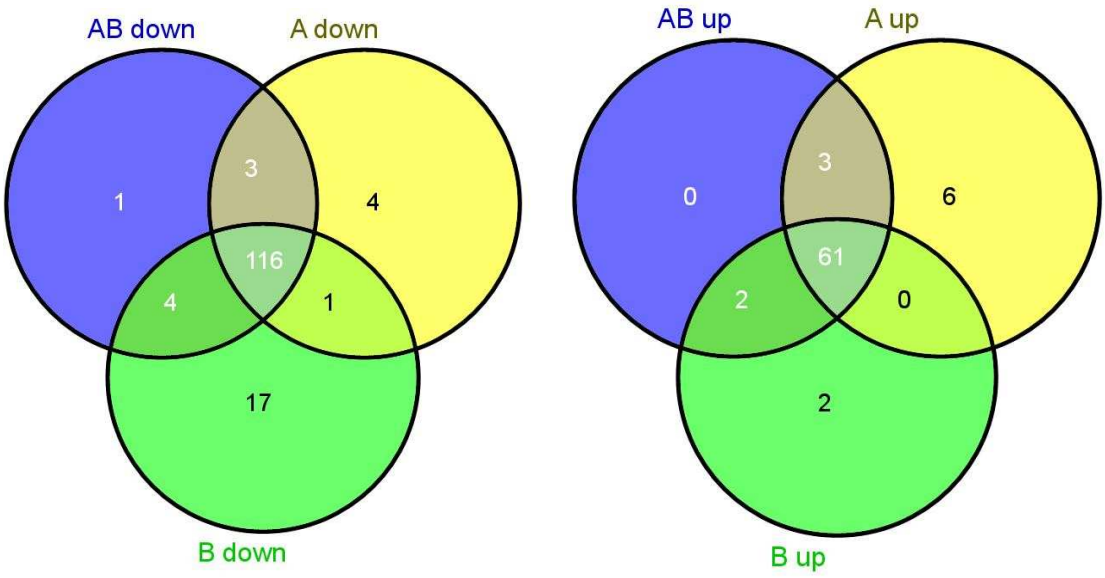


Figure 3.13. Venn diagrams showing the number of genes that are common or unique to the technical singlets A or B and the technical duplicate AB.

The "commonality" graph in Figure 3.14 showed that the non-common genes were of low fold changes, indicating that if only one technical replicate was used, only a few genes that are of low fold change would vary which are, therefore, of lower significance to the overall results of a research project.

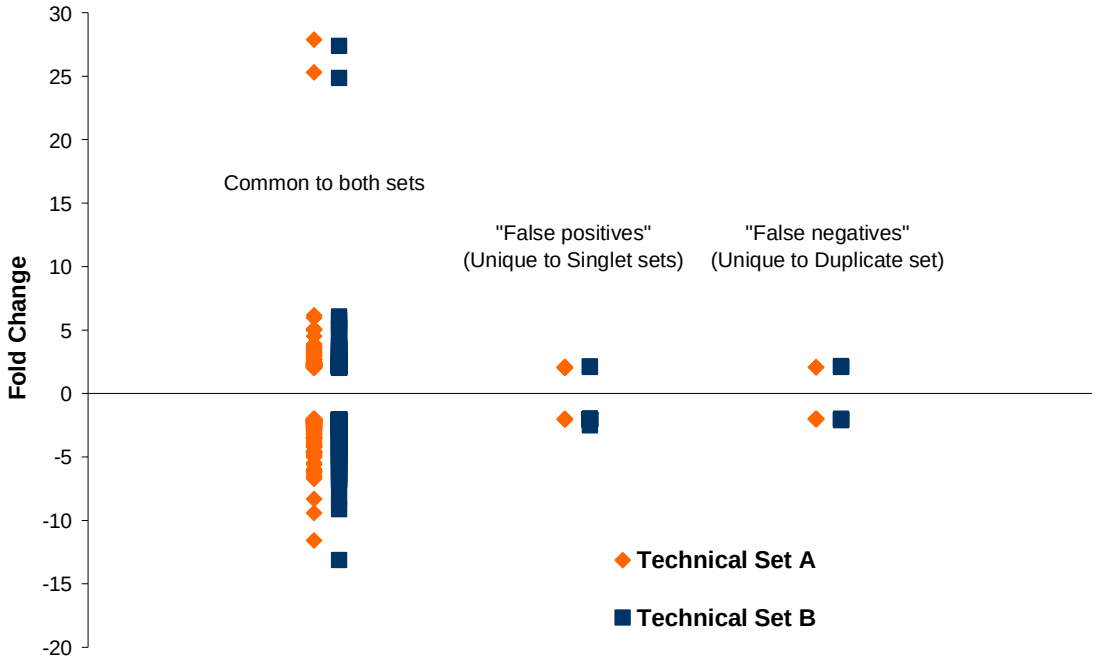


Figure 3.14. Commonality graph for technical replicates. The orange spots are those from technical singlet Set A and the blue spots represent genes from technical singlet Set B.

This indicates that the general rule of not including technical replicates has merit and will save resources without compromising on the results.

3.14. Biological replicates of one-colour Affymetrix microarray

Biologically triplicated data are generally the suggested minimum, especially for publication, within the scientific community, although some only use duplicate (Mira et al., 2009). However, especially with cultures as opposed to tissue or biopsy material, it may be possible to get very similar results with fewer biological replicates. This is relevant in situations where biological replicates are not possible due to the lack of resources.

The real impact of biological replication on the outcome of a research project was determined by comparing the three possible singlet datasets (A, B and C) to the three possible duplicate datasets (AB, AC and BC) to the triplicate dataset (ABC) of the first technical replicate of the oxidative stress experiment described above. The PCA

plot in Figure 3.15 shows the three corresponding biological replicates that make up this microarray data, with orange representing Set A, blue representing Set B and purple representing Set C. There is a distinct difference between the treated and control microarrays for the first principal component (PC1) on the x-axis, which accounts for 37.5% of the differences between the samples whereas PC2 accounts for 23.1%.

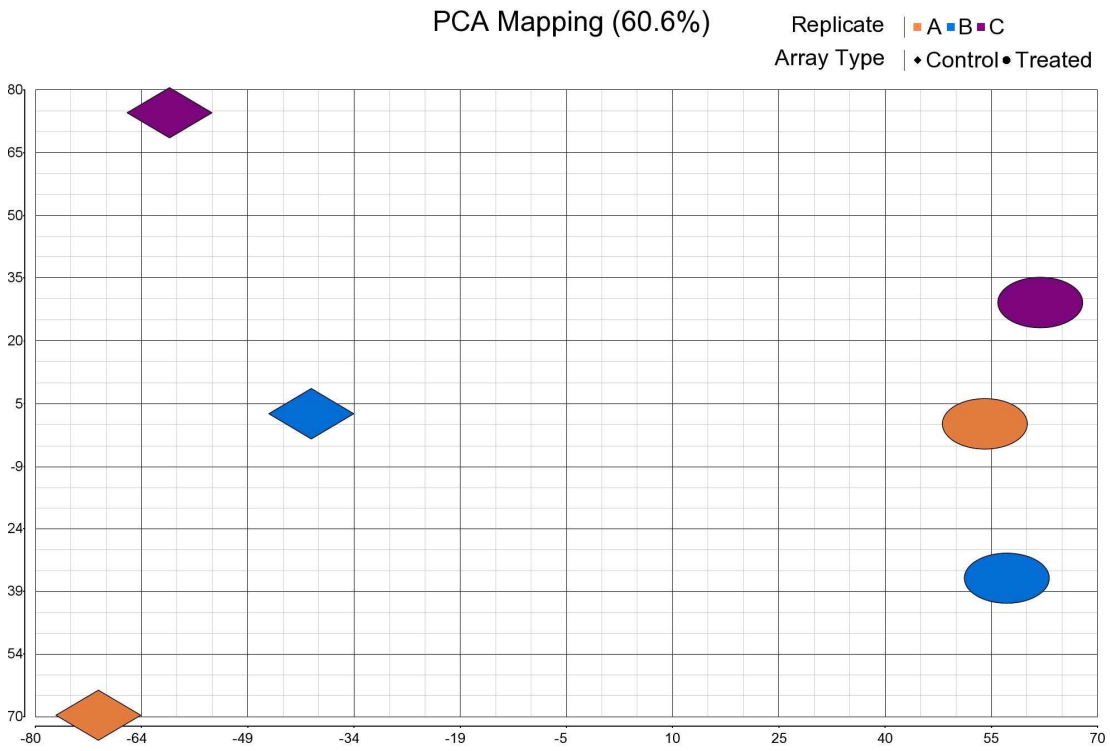


Figure 3.15. PCA plot of the biological triplicate dataset. Diamonds represent the control microarrays, circles represent the treated microarrays, orange represents Set A, blue represents Set B and purple represents Set C.

The XY scatterplot in Figure 3.16 shows that the similarity between the duplicate sets and the triplicate set is over 95.3%. The comparison with the singlet data shows that while two of these (A and C) are over 88.9%, Set B has 65% correlation. While this shows that duplicates are more closely correlated, and therefore more accurate, the data for the singlet datasets are still promising.

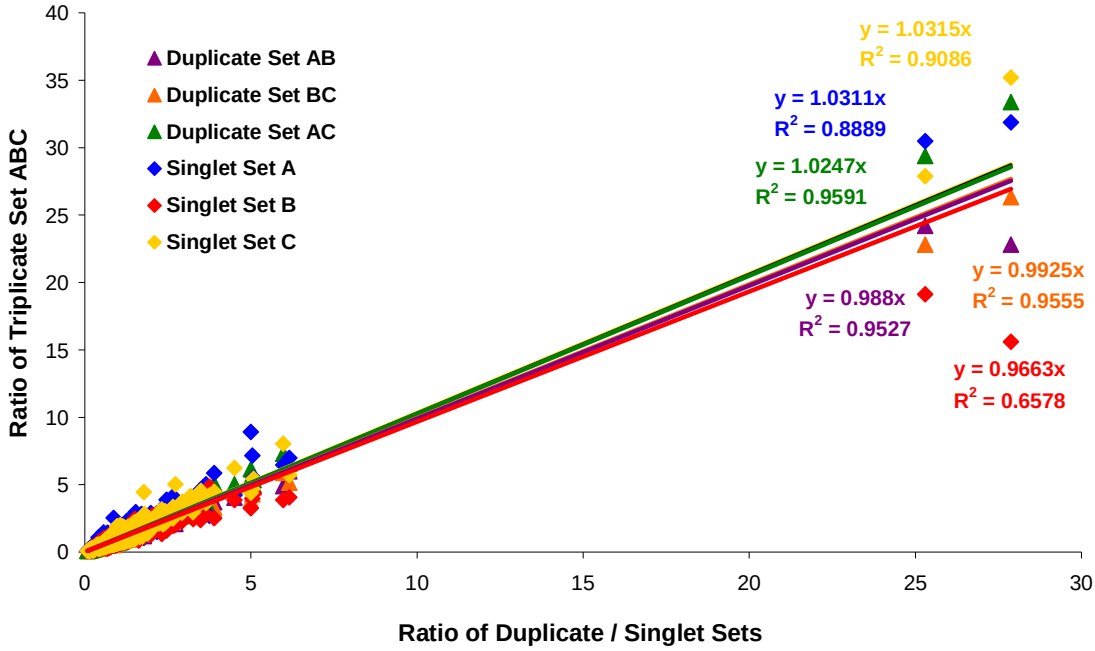


Figure 3.16. XY Scatterplot of biological replicates. See inset for the legend. The equations and R^2 correlation values for each trend line is shown in the corresponding colour.

Figure 3.17 shows the actual numbers and Figure 3.18 below shows the proportion of genes that are common or unique in each dataset. The common and false positive categories (blue plus green) that add up to 100% in the latter graph are those genes that would appear in the significant list if only duplicates or singlets had been tested. The false negative category (orange), on the other hand, are those genes that the triplicate data deemed significant while the duplicate / singlet data suggest that they are not significant, thus being missed if only duplicates or singlets were analysed.

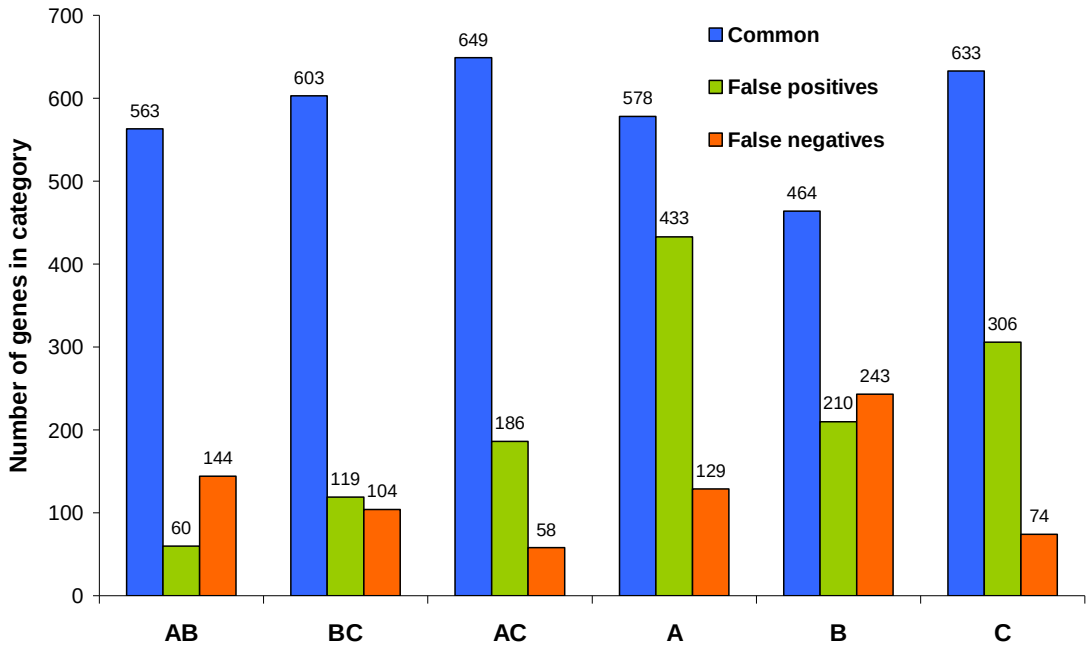


Figure 3.17. Number of genes that are common or unique to the biological replicate datasets, with a fold change cut-off of 1.5. Legend inset.

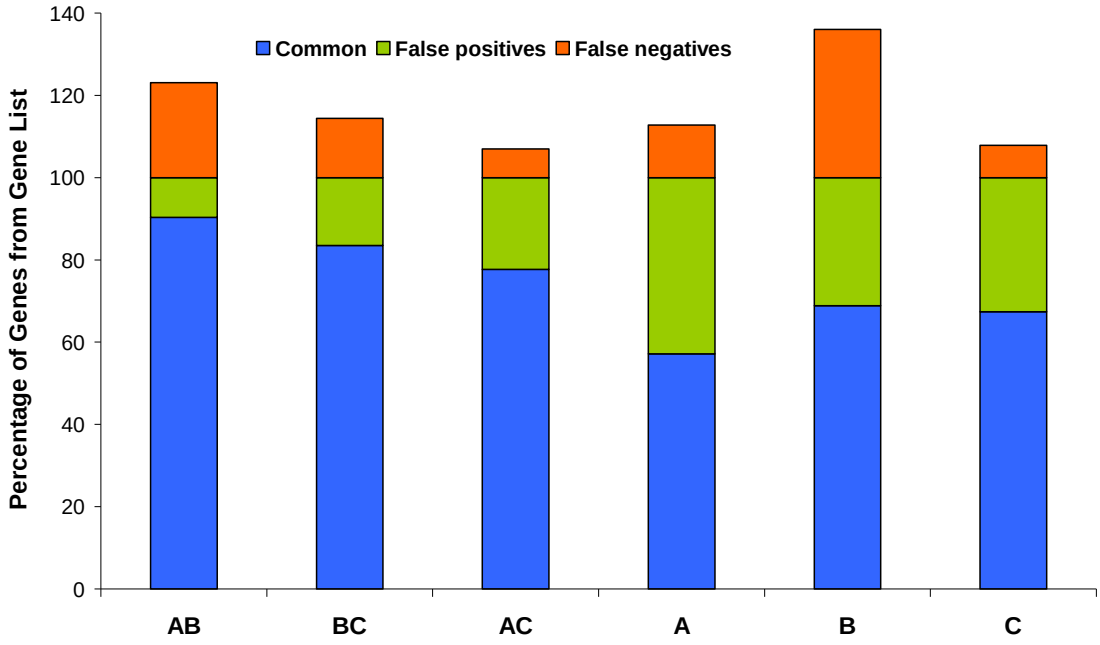


Figure 3.18. Proportion of the common, false positive and false negative genes in the biological replicate datasets, with a fold change cut-off of 1.5. Legend inset.

While the common genes are the prominent feature of the duplicate datasets, the singlet datasets contain many false negatives and false positives. This suggests that at least duplicate data is advisable. However, when looking at the fold change of these three categories in Figure 3.19, it is clear that these genes that are different (either present when they should not be or absent when they should be present) are of low fold change. This indicates that while replication is important, data from experiments where only single biological data is possible such as the pilot-scale fermentation can still be meaningful in a research study.

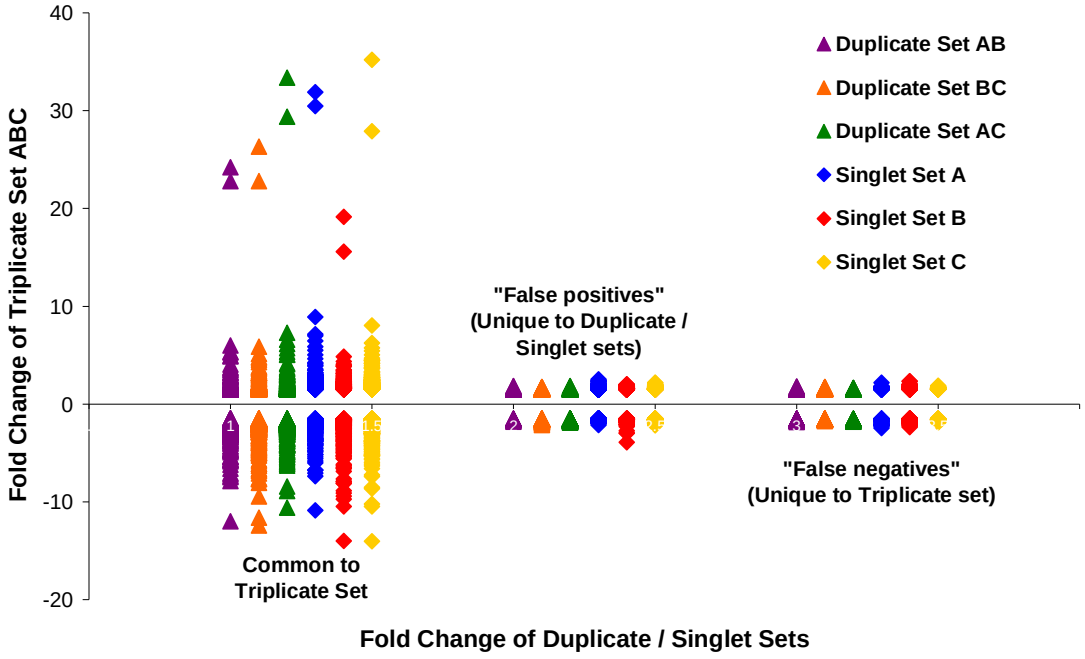


Figure 3.19. Commonality graph of biological replicates. Legend inset.

The genes that are not common are of lower fold changes and are therefore assumed to be of less significance to the research. One way to see whether this is indeed the case is via functional specification (FunSpec) analysis, which groups genes based on the function of the genes. The above analysis used a cut-off of 1.5, but for this part I used a cut-off of 2 to allow a manageable number of genes to be properly analysed. The Venn diagram in Figure 3.20 shows the grouping of the significant genes. For this exercise, the up and down regulated genes were combined.

To allow for an easy comparison, each FunSpec category in the triplicate dataset results was assigned an unique number, as shown in Table 3.5 sorted by the p-values of ABC. The number of genes in each category within the duplicate datasets is similar to that of the triplicate dataset. There were four categories in ABC that contained more than five genes. These were also the top categories in the duplicate datasets. This suggests that the duplicate dataset would result in similar molecular mechanisms within the research study.

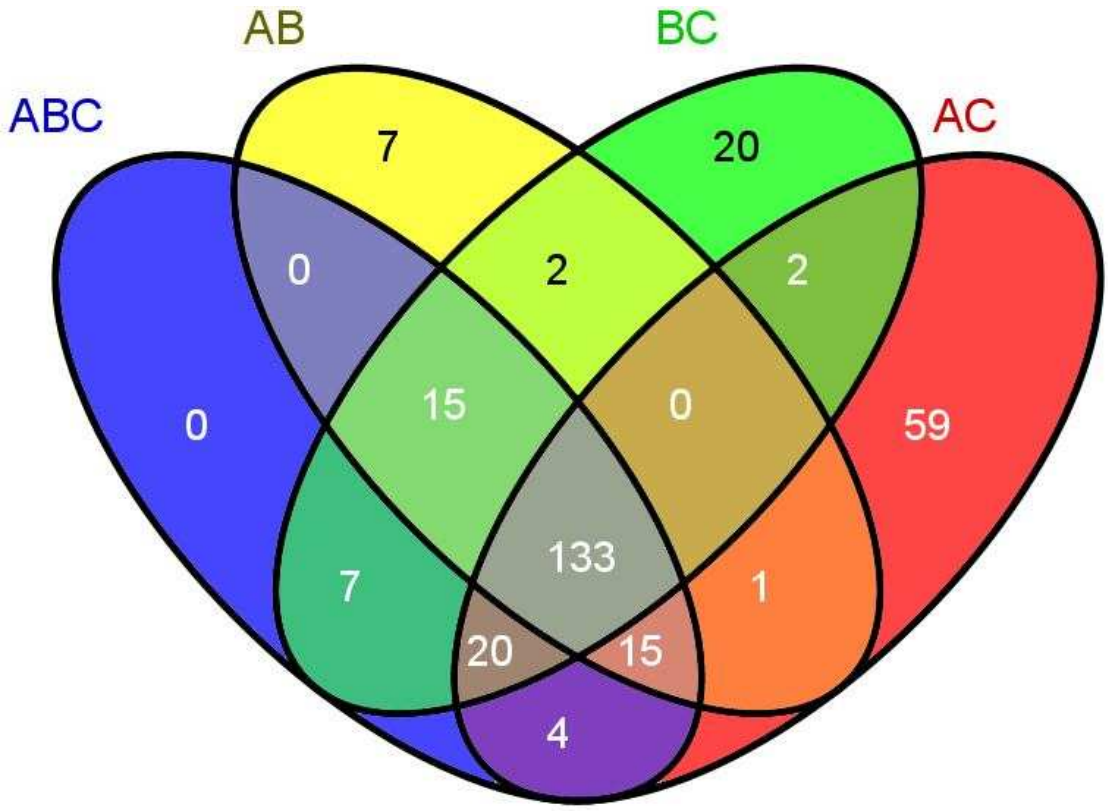


Figure 3.20. Venn diagram showing the grouping of the significant genes with a cut-off fold change of 2. The Venn diagram was generated using the Venny online tool.

Table 3.5. FunSpec analysis of the significant genes (above 2 fold change) from triplicate dataset ABC and duplicate datasets AB, BC and AC using MIPS functional classification (Robinson et al., 2002). Each category has been assigned a unique number.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
ABC	22	11	7	6	5	5	5	5	5	5	4	4	4	4	4	3	3	3	3	3	2	2	2	2
AB	21	11	7	5	5	0	5	4	5	5	4	4	4	4	4	3	3	3	3	3	2	2	2	2
BC	20	9	8	6	5	5	5	5	0	5	4	3	0	4	4	3	3	0	3	3	2	2	0	0
AC	23	9	6	5	5	0	5	4	0	0	5	4	4	4	4	3	3	3	3	3	0	2	0	0

3.15. Duplicate vs triplicate analysis in published data

After noticing the trend continued for other microarray datasets within the research group, I wanted to test whether it would also be true for that of a microarray dataset that was completely processed elsewhere and had been published. This publication tested yeast cultures under the stress condition of 5% ethanol, 60 g/L glucose compared to the normal condition of 0 % ethanol, 20 g/L glucose. While Alper et al. (2006) examined both the wildtype yeast and a mutant strain, only the wildtype yeast data was compared here (data accessible at NCBI GEO database (Edgar et al., 2002), accession GSE5185). Raw data files obtained through GEO were analysed using Partek.

The degree of similarity between the duplicate datasets, XY, YZ and XZ and the triplicate dataset XYZ, is high as seen in Figure 3.21, with correlation levels of at least 95.8%. As shown in Figure 3.22, the same trend was seen where the genes that were unique to either the duplicate set or the triplicate set were of very low fold changes, therefore being of minor significance to the overall results.

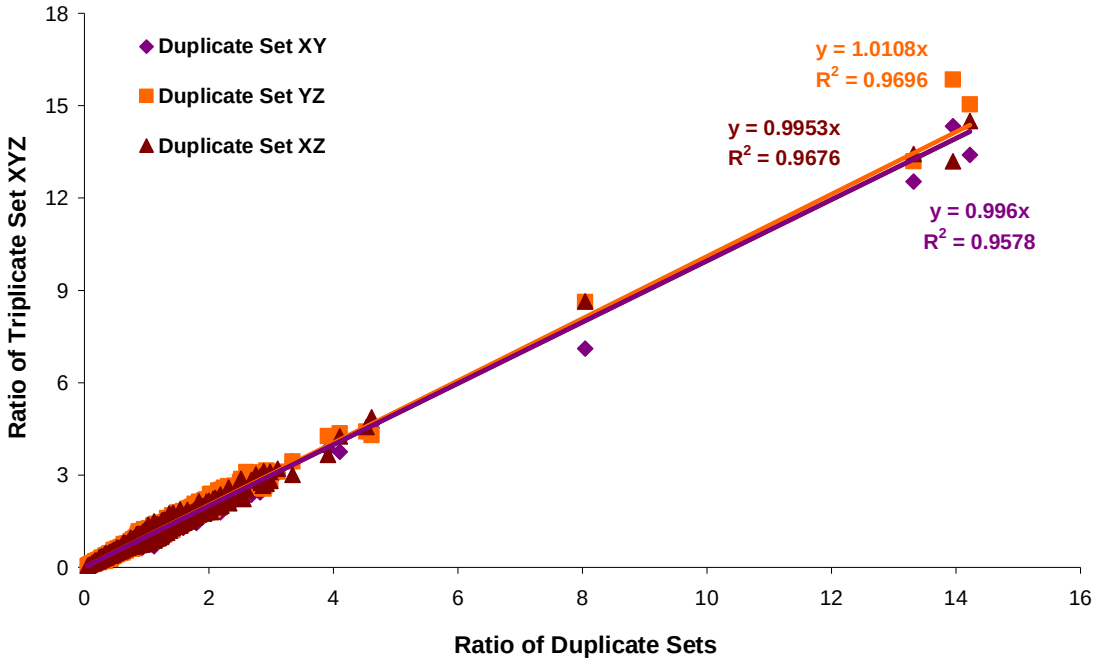


Figure 3.21. XY scatterplot for the duplicate vs triplicate dataset.

The triplicate dataset contained 190 significantly down-regulated and 67 up-regulated genes, with a total of 257 significant genes. Duplicate sets XY, YZ and XZ contained 90.3%, 97.7% and 91.1% of these genes, respectively. These genes that were not common between the sets (the false negatives and false positives) were of low fold change, illustrated in Figure 3.22, like the examples given above.

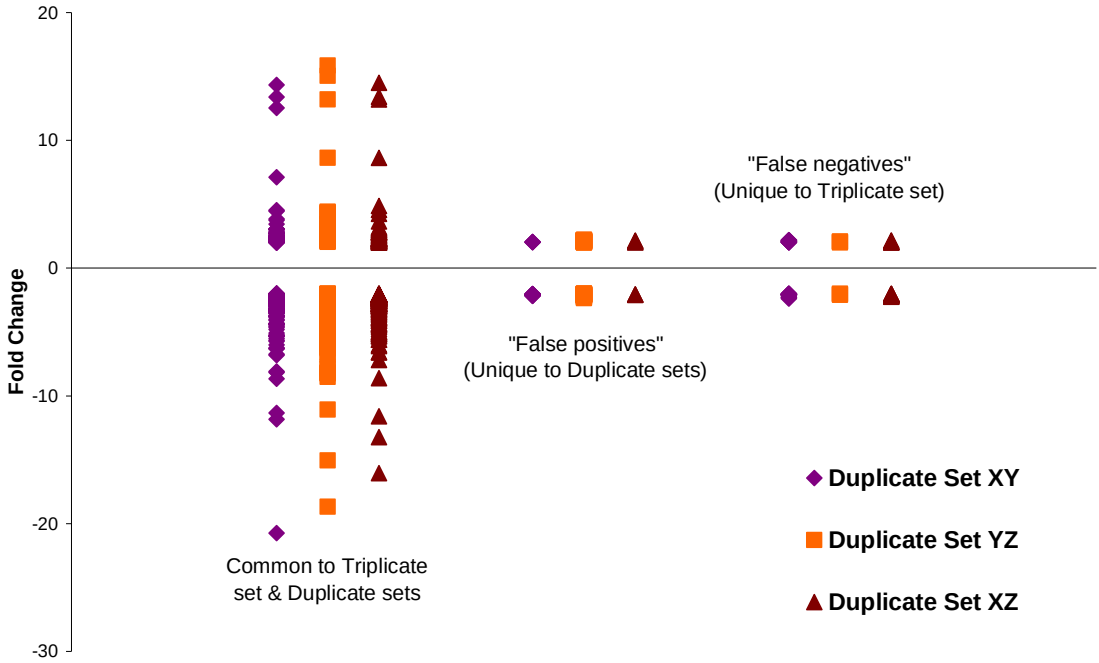


Figure 3.22. Commonality graph comparing duplicate and triplicate datasets. Legend inset.

3.16. Mouse back skin microarray replicate analysis

Yeast cell cultures are more likely to be more biologically similar to each other because of more-controllable parameters. Is it possible to extend the replicate analysis to studies that involved tissue samples rather than yeast cultures?

This question might be answered using one-colour Affymetrix data (data accessible at NCBI GEO database (Edgar et al., 2002), accession GDS2629) from a study which tested biological triplicate samples of skin from the backs of embryonic mice to examine epidermal differentiation in knock outs vs the wildtype mice (Yu et al., 2006). As before, the raw data files were analysed using Partek. Correlation between the duplicate sets and the triplicate set are 66.4%, 32.7% and 44.1% for the two duplicate sets named KM, LK and LM, respectively. Many of the significant genes above a fold change of 2 were common to duplicate and triplicate dataset. However, the commonality graph in Figure 3.23 shows that the fold change of genes unique to one group (the false positives and false negatives) are higher than seen in

the situations above, including a gene found in Set L at a fold change of 12.7 which would be considered to be extremely significant but which would not be present in the triplicated data.

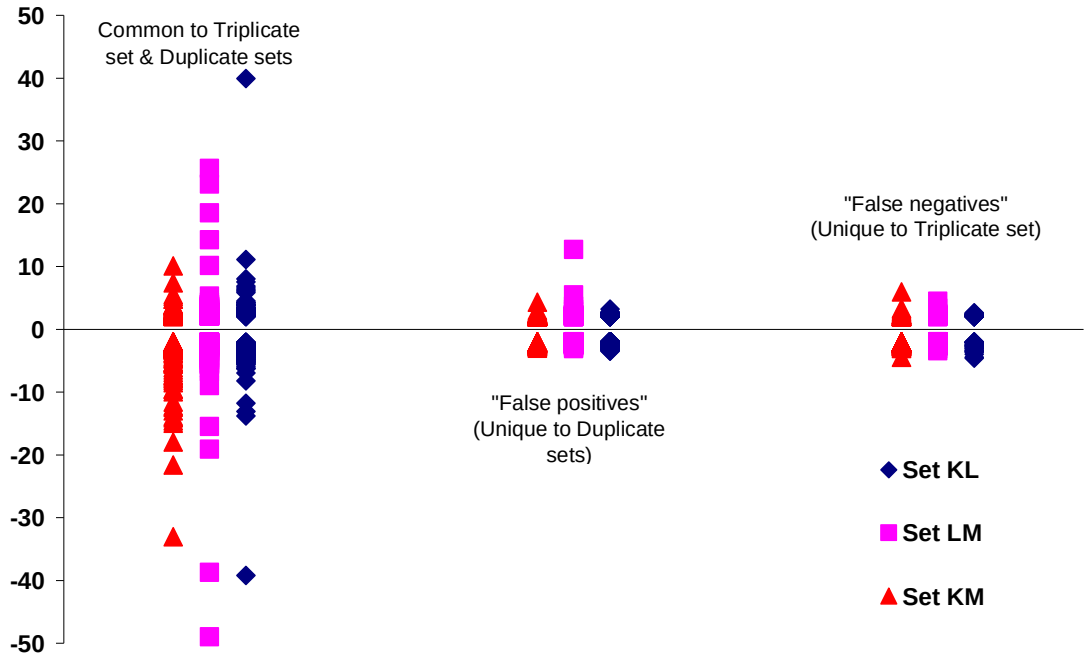


Figure 3.23. Commonality graph for a microarray experiment involving samples of the skin from the back of mice (Yu et al., 2006).

This shows that for samples involving tissue samples, there is a larger difference and triplicated data is always advisable, while for yeast culture microarrays fewer replicates, when necessary, will allow reliable accurate data, due to the inherent low variability between cultures.

3.17. Discussion

3.17.1. Two-colour microarray protocol optimisation

Gene expression analysis is very useful in understanding yeast metabolism and the molecular mechanisms involved in various treatments. cDNA microarrays analyse the expression of every gene in an organism at the same time. At the start of this research, two-colour microarrays were commonly used due to their lower cost, however sometimes resulted in low hybridisation rates or highly uneven background noise. Considerable time and efforts were spent on optimising these two-colour microarrays, as described. According to the results of 64 two-colour arrays performed, the cDNA synthesis method based on an Invitrogen kit, using an a-hyb hybridisation machine and a quicker stringent washing protocol resulted in high quality microarrays with good hybridisation rates in five of the 13 batches. However, to get these high quality data, many more slides had to be processed because of the occurrence of inconsistency. For example, only six of the 11 zinc microarrays scanned were of publication quality (Gauci et al., 2009) and only eight of the 27 H_2S microarrays scanned were able to be analysed in Chapter 4. Low hybridisation rates or an uneven background is a major issue. Important aspects to consider during the two-colour microarray process are to remove the residual medium from samples prior to snap freezing them in liquid nitrogen for RNA isolation, and to ensure optimal hybridisation by using at least 10 ng/µL cDNA and 1 pmol/µL labelled probe solutions.

The inconsistency of these microarrays led to the investigation into the one-colour Affymetrix microarray platform. Four batches of Affymetrix cDNA arrays were carried out, all of which resulted in high quality microarrays, the significance of which were described in Chapters 4 and 6.

3.17.2. Replicates of one-colour Affymetrix microarray

An important parameter in microarray experiments is the design of replicates, namely, biological replicates, where separate samples of a treatment are analysed on individual chips, and technical replicates, where the same sample is analysed more than once.

The data shown in this chapter demonstrate that technical replication in one-colour yeast Affymetrix microarrays are not necessary, due to the very high correlation between a singlet technical replicate and that of a duplicate. The few genes that were different had very low fold changes, suggesting that the added technical replicate would not provide significant benefit to the research. It is important to note that this replicate analysis is only relevant to the one-colour Affymetrix microarray platform. The two-colour microarray system discussed in the first half of this chapter has the added complexity of a requirement of a dye-swap replicate, either biological or technical, due to the differences seen between the dyes.

A very high correlation exists between biological dupliate and biological triplicate data in one-colour Affymetrix arrays. The differences between these two groups are minimal with relatively few genes varying between the lists. Those that are different have very low fold changes between the treatment and control, suggesting no effect on the overall study. The data in this chapter from different sources (Figure 3.19 and Figure 3.22) indicate that biological duplicate datasets are capable of producing very similar results while cutting the costs by a third. The downside, however, is that it makes statistical output less accurate. Therefore, where possible, biological triplicate is suggested for one-colour Affymetrix microarrays, however, duplicates would be acceptable where necessary, for example, in the case of an outlier in the data described in Chapter 6.

Is this the case for microarrays using higher organisms or more complex types of samples? While duplicate datasets from mouse tissue microarrays showed similarity with that of the triplicate datasets, those genes that are different had much larger fold changes (up to 12.7) than those from yeast cultures (Figure 3.23). Tissue samples have more biological variation than a cell culture, so a reduction in biological replicates is not wise for cDNA microarrays.

Furthermore, comparison of biological singlet data to that of triplicate data shows that the correlation was less reliable despite two of the three possible singlet datasets having good correlation of approximately 90%, with the other at 66% (Figure 3.16). The genes present in the singlet data but absent from the triplicate data are false positives, whereby they falsely suggest that the changes were caused by the treatment. While singlet data contain many genes that are common with that of triplicate data, the difference lies in the number of false positives within the data, which would confuse the results. Despite this, due to the genes that are different being of low fold

change (Figure 3.19), this study suggests that where necessary, they still could provide meaningful data. While not suggesting that researchers change to a singlet dataset methodology, this research suggests that when replication is not possible due to lack of resources or the scale of experiments, such as in the case of the pilot-scale wine fermentation of this project, singlet datasets can still give researchers an insight into the molecular mechanisms involved. This is because the genes present in the singlet dataset with high fold changes were generally found to be also present in datasets with multiple replicates as shown previously. Where possible, however, biological triplication is always preferable to ensure the accuracy of experimental data.

So much was learnt, in terms of the intricacies of cDNA microarrays, from the works of this chapter, which formed the bases of the gene expression analyses for the effects of sulfur and nitrogen (Chapter 4) and the fining agents, pectin and carrageenan (Chapter 6).

Chapter 4: Application of cDNA microarray in studying yeast sulfur pathways

4.1. Introduction

Wine's organoleptic qualities are determined by a range of flavour compounds, including alcohols, esters, monoterpenes and thiols. Unfortunately, the off-flavour hydrogen sulfide (H_2S) in the finished product is a problem often encountered in fermentation industries. H_2S is monitored during wine fermentation. As discussed in Section 1.5.5, Chapter 1, its intense rotten egg odour and very low threshold of only 10 ppb makes it highly undesirable. Closely linked to the metabolism of H_2S is sulfur dioxide (SO_2) with a much higher threshold of 25 ppm, as previously described, which is also important to winemakers due to its antioxidant properties. The production of H_2S and SO_2 as intermediate metabolites by yeast cells is inextricably related in the so-called sulfur pathway (Linderholm et al., 2008). Previous studies carried out with wine yeasts demonstrated that formation of H_2S is a response to nitrogen depletion (Jiranek et al., 1995). Consequently, research and winemaking practice have found that supplementation of di-ammonium phosphate as a source of nitrogen can reduce the production of H_2S, whilst the addition of cysteine in media or grape juice increases H_2S. Following the completion of cDNA microarray optimisation as described in the previous chapter, here, the optimised protocol was used to examine the effect of nitrogen and cysteine addition on the metabolism, particularly the sulfur pathway, of the yeast lab strain BY4743. The gene expression profiling from the two-colour microarray was supplemented with one-colour Affymetrix microarrays. It is important to keep in mind that the concentrations of other compounds in wine or media could influence how nitrogen is metabolised by yeast, and thus there must be caution in extrapolating findings into the larger winemaking field (Torrea et al., 2011).

4.2. Methodology

Relative H_2S production was analysed using a silver nitrate infused membrane over a 96-well microtitre plate, as described in Section 2.3.1, Chapter 2. This captured the H_2S being produced during yeast growth in the microtitre plate, turning the silver nitrate into silver sulfide, appearing as black spots on the membrane.

As described in Section 2.9, Chapter 2, the ammonium sulfate and / or cysteine treatments for the microarray analysis were prepared by resuspending pellets from an overnight OD_{600} 1.0 culture into the treatment media to an OD_{600} of 0.2 and grown back up to an OD_{600} of 1.0. Four media conditions were used – 30 g/L ammonium sulfate plus 100 ppm cysteine, 30 g/L ammonium sulfate only, 100 ppm cysteine only and a control without ammonium sulfate nor cysteine. The yeast was grown up in the fresh control and treatment media to an OD_{600} of 1.0 before the pellet was snap-frozen, RNA isolated and microarrays carried out, using the two-colour microarray platform as described in Section 2.6, Chapter 2 and the one-colour Affymetrix microarray platform, as described in Section 2.7, Chapter 2. Each biological replicate for this experiment was prepared independently on separate days to ensure accuracy. Concurrently, samples of these cultures were grown in microtitre plates and tested for H_2S production using the silver nitrate membrane described above, to ensure that the media was correctly prepared.

4.3. H_2S concentrations were increased by cysteine and lowered by ammonium

While the addition of cysteine increased the production of H_2S, ammonium sulfate (as a source of nitrogen) decreased its production, counteracting the effect of cysteine, as shown in Figure 4.1 on the next page. The media types used in the gene expression analysis later in this chapter demonstrated that the cysteine only media resulted in very high H_2S concentrations, the ammonium sulfate plus cysteine media resulted in very low production concentrations, with ammonium sulfate and control treatments below detection levels. It is important to note that the growth seen for these concentrations of ammonium sulfate (0 – 30 g/L) and cysteine (0 – 100 ppm) in minimal media remained constant, thereby showing that the lack of H_2S production was not due to poor growth caused by possible supplement toxicity, but rather the supplements themselves.

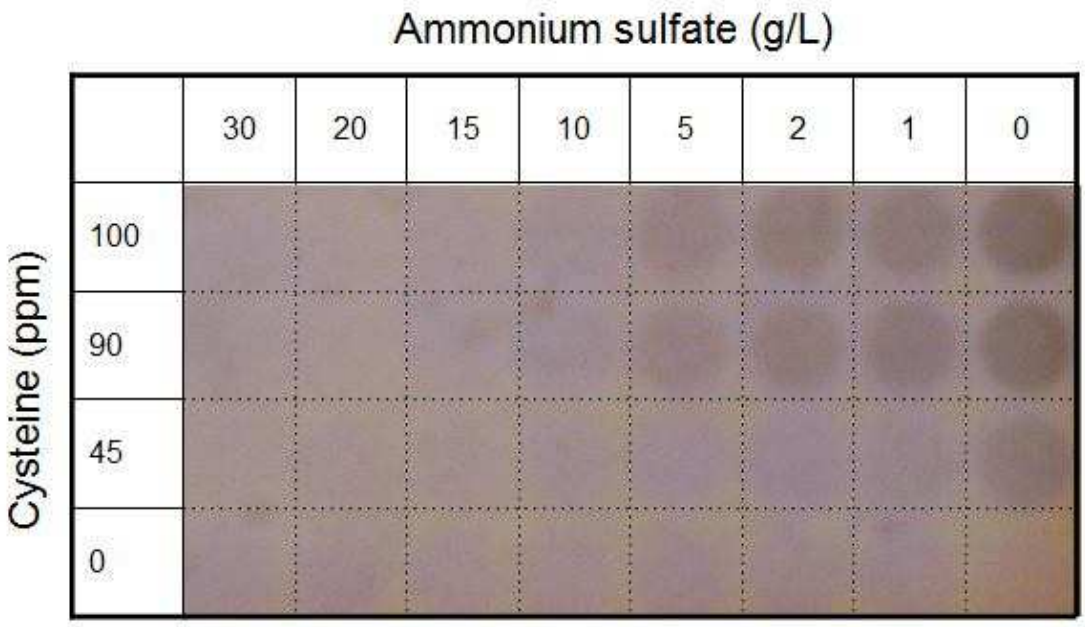


Figure 4.1. H_2S production in yeast grown in media containing a range of ammonium sulfate and cysteine concentrations, detected via a silver nitrate membrane test.

4.4. Ammonium and sulfate ions

The silver nitrate membrane test was also used to test other sources of ammonium and sulfate, such as ammonium chloride and magnesium sulfate, which showed that the ammonium ion in $(NH_4)_2SO_4$ is the cause of the effect shown in Figure 4.1, with no difference in H_2S production when magnesium sulfate was used to that of the control and no difference in H_2S production when ammonium chloride was used instead of ammonium sulfate (data not shown).

4.5. Initial gene expression analysis with two-colour microarrays

Initially, gene expression was tested using the two-colour cDNA microarrays, which was analysed using GeneSpring, as described in Section 2.8.2 of Chapter 2. Ammonium sulfate plus cysteine was compared to cysteine in biological duplicate with a technical dye-swap replicate. Cysteine vs control and ammonium sulfate plus cysteine vs control were also tested, each using a technical dye-swap replicate. Dye-swap replicates involve reciprocal labelling of samples to account for dye bias in two-

colour microarrays. Ammonium sulfate up-regulated 62 genes and down-regulated 58 genes, compared to the control. Ammonium sulfate plus cysteine up-regulated 138 genes and down-regulated 81, compared to cysteine only. Cysteine up-regulated 25 genes and down-regulated 45 genes, compared to the control.

Enrichment of the functional categories found in the significantly altered genes was determined by FunSpec (Robinson et al., 2002), as described in Section 2.8.4 of Chapter 2. This analysis, shown in Table 4.1, reveals similarity in the enrichment between ammonium sulfate vs control and ammonium sulfate plus cysteine vs cysteine, due to the presence of ammonium sulfate in both conditions. Many of the adenine requiring (*ADE*) genes, such as *ADE1*, *ADE13*, *ADE17*, *ADE4* and *ADE2* involved in the purine nucleotide biosynthesis pathway, were up-regulated in these two microarray sets. The down-regulated categories involve heavy metals, branched chain amino acids and nitrogen metabolism. Interestingly, biosynthesis of cysteine was up-regulated in the ammonium sulfate plus cysteine sample compared to cysteine despite both samples containing the same amount of cysteine, indicating that ammonium sulfate might affect this pathway, or prevent cysteine from accessing the cell. The cysteine in the media up-regulates arginine biosynthesis and the urea cycle, while down-regulating the methionine and cysteine pathways.

The ammonium sulfate media also caused down-regulation of amino acid importation, particularly indicated by *GAP1*, which is the general amino acid permease and is regulated in response to the available nitrogen source. *GAP1* gene expression was over 10 fold down-regulated for the microarrays involving ammonium sulfate whereas *GAP1* was up-regulated (+2.2) in the cysteine condition. The ammonium sulfate treatments include ammonium sulfate over the control (-10.7), as well as ammonium sulfate plus cysteine over the cysteine conditions (-12.3).

Table 4.1. Functional enrichment of the genes significantly changed by ammonium sulfate and / or cysteine treatment, as determined by MIPS Functional Classification, FunSpec (Robinson et al., 2002) using the two-colour microarray platform. Enrichment was considered at $p < 0.01$. Up-regulated categories are highlighted in red and down-regulated categories are highlighted in green.

Category	p-value	Genes in category
Ammonium sulfate plus cysteine vs cysteine (two-colour microarrays):		
Up-regulated genes:		
Ribosomal proteins	1.987e-14	*RPL19B RPS11B RPS6B RPL21A RPL41A RPL4B RPP2B RPL27B RPL30 RPS26A RPL24B RPS0A RPS20 RPL2B RPL14A RPS21A RPL10 RPS31 RPL16B RPS15 RPL18A RPL20B RPL21B RPS6A RPL5 RPL33A NIP7*
Purine nucleotide / nucleoside / nucleobase anabolism	4.762e-11	*ADE1 ADE8 ADE5,7 ADE3 MTD1 ADE13 ADE17 ADE4 ADE2 SER1*
Tetrahydrofolate-dependent C-1-transfer	5.431e-8	*SHM1 ADE8 ADE3 MTD1 SHM2 ADE17*
Degradation of glycine	1.618e-6	*GCV3 SHM1 SHM2 GCV2*
Biosynthesis of serine	3.721e-6	*SHM1 SER3 SHM2 SER1*
Glycolysis and gluconeogenesis	8.258e-6	*CDC19 TPI1 TDH3 ENO1 TDH2 FBA1 GPM1*
Sugar, glucoside, polyol and carboxylate catabolism	1.591e-5	*CDC19 TPI1 TDH3 ENO1 TDH2 FBA1 GPM1 ACO1 FUM1*
Metabolism of methionine	3.184e-5	*SAM2 MET18 MET3 SAM1 MET17*
Translation elongation	0.0005205	*EFB1 TEF2 RPP2B TEF1*
C-compound and carbohydrate metabolism	0.0007987	*CIT2 FEN1 EMI2 DLD3 HSP12 HXK1 HXK2 ADH4 ENO1 MAE1 PDC1 ADH1*
C-1 compound anabolism	0.001004	*SHM1 SHM2*
Metabolism of vitamins, cofactors, and prosthetic groups	0.001089	*GCV3 SHM1 MTD1 SHM2 GCV2*
Alcohol fermentation	0.001537	*ADH4 PDC1 ADH1*
C4-dicarboxylate transport (eg malate, succinate, fumarate)	0.001983	*OAC1 DIC1*
Biosynthesis of cysteine	0.001983	*CYS3 MET17*
Degradation of lysine	0.003265	*SHM1 SHM2*
C-1 compound catabolism	0.003265	*GCV3 GCV2*
S-adenosyl-methionine-homocysteine cycle	0.006692	*SAM2 SAM1*
Biosynthesis of leucine	0.008815	*LEU1 ILV5*
Down-regulated genes:		
Biosynthesis of arginine	2.263e-7	*ARG4 CPA2 PUT1 ARG7 CPA1*
Metabolism of urea (urea cycle)	2.52e-7	*DUR1,2 ARG4 CAR2 CAR1*
Cellular import	8.177e-7	*HXT7 HXT6 CAN1 GAP1 FRE1 HXT2 FET3 MEP2 CTR1*
Aminoadipic acid pathway	0.0001218	*LYS2 LYS21 LYS20*
Degradation of arginine	0.0001342	*CAR2 CAR1*
Metabolism of alkaloids	0.0001342	*ARO8 ARO9*
Aromate anabolism	00003997	*ARO4 ARO3*

Metabolism of the cysteine – aromatic group	0.0003997	*ARO4 ARO3*
C-compound and carbohydrate transport	0.000608	*DUR3 ESBP6 ODC2 PDR12*
Degradation of leucine	0.0007933	*ARO10 BAT2*
Degradation of glutamine	0.0007933	*CPA2 CPA1*
Homeostasis of metal ions (Na, K, Ca, etc)	0.0009291	*GGC1 IRC7 FRE1 FET3 ISU1 CTR1*
Catabolism of nitrogenous compounds	0.001312	*DAL7 DAL3*
Amino acid / amino acid derivatives transport	0.001773	*AGP1 CAN1 GAP1 ODC2*
Biosynthesis of valine	0.001953	*ILV6 BAT2*
Nitrogen, sulfur and selenium metabolism	0.003478	*IRC7 MET13 CAR2 ISU1*
Metabolism of derivatives of dehydroquinic acid, shikimic acid and chorismic acid	0.003591	*ARO4 ARO3*
Metabolism of tryptophan	0.003591	*ARO8 ARO9*
Purine nucleotide /nucleoside / nucleobase metabolism	0.004937	*DUR1,2 DAL7 DAL3*
Cytoplasmic and nuclear protein degradation	0.005087	*PRB1 LAP4 UBI4 LAP3*
Sugar transport	0.00542	*HXT7 HXT6 HXT2*
Peptide transport	0.006897	*PTR2 OPT2*
Vacuolar protein degradation	0.006897	*PRB1 LAP4*
Anion transport	0.006897	*DUR3 MEP2*
Metabolism of phenylalanine	0.009634	*ARO8 ARO9*
Alcohol fermentation	0.009634	*ADH5 ARO10*
Ammonium sulfate vs control (two-colour microarrays):		
Up-regulated genes:		
Purine nucleotide / nucleoside / nucleobase anabolism	2.897e-9	*ADE1 MTD1 ADE13 ADE17 ADE4 ADE2 SER1*
Degradation of glycine	6.875e-8	*GCV3 GCV1 SHM2 GCV2*
C-1 compound catabolism	5.71e-6	*GCV3 GCV1 GCV2*
Biosynthesis of serine	1.975e-5	*SER3 SHM2 SER1*
Metabolism of vitamins, cofactors, and prosthetic groups	2.755e-5	*GCV3 GCV1 MTD1 SHM2 GCV2*
Tetrahydrofolate-dependent C-1-transfer	0.0001556	*MTD1 SHM2 ADE17*
Lactate fermentation	0.0004193	*DLD1 DLD3*
Sulfate assimilation	0.001915	*MET3 MET14*
Alcohol fermentation	0.00519	*ADH3 ADH1*
Conjunction of sulfate	0.008481	*MET3*
Down-regulated genes:		
Biosynthesis of leucine	2.161e-7	*LEU1 BAT2 ILV5 LEU4*
Biosynthesis of valine	8.543e-6	*BAT2 ILV5 ILV2*
Inorganic chemical agent resistance (eg heavy metals)	1.487e-5	*CUP1-1 CUP1-2 FET3*
Vacuolar protein degradation	6.858e-5	*APE3 PRB1 PEP4*

Translation elongation	0.0005235	*TEF2 EFT2 EFT1*
Biosynthesis of isoleucine	0.0005763	*BAT2 ILV5*
Cellular import	0.000603	*HXT7 HXT6 CAN1 GAP1 FET3*
Metabolism of urea (urea cycle)	0.0008602	*DUR1,2 CAR2*
Heavy metal binding (Cu, Fe, Zn)	0.00159	*CUP1-1 CUP1-2*
Biosynthesis of arginine	0.004321	*PUT1 ARG7*
Amino acid / amino acid derivatives transport	0.004903	*CAN1 GAP1 ODC2*
Nutrient starvation response	0.00576	*PRB1 PEP4*
Translation	0.007387	*PAB1 ASC1*
Biotin binding	0.007724	*DUR1,2*
Metabolism of proline	0.007724	*CAR2*
Urea catabolism (not urea cycle)	0.007724	*DUR1,2*
Cysteine vs control (two-colour microarrays):		
Up-regulated genes:		
Biosynthesis of arginine	9.393e-8	*ARG4 ARG7 ARG1 CPA1*
Metabolism of urea (urea cycle)	0.0001883	*ARG4 ARG1*
C-compound and carbohydrate transport	0.0002345	*DUR3 MCH4 PDR12*
Metabolism of aspartate	0.000263	*ARG4 ARG1*
Anion transport	0.0006826	*DUR3 MEP2*
Homeostasis of anions	0.003635	*DUR3*
Cellular import	0.004029	*GAP1 FRE1 MEP2*
Homeostasis of metal ions (Na, K, Ca, etc)	0.005118	*GGC1 IRC7 FRE1*
C-compound and carbohydrate metabolism	0.007905	*ADH5 GND1 DAL7 PDR12*
Drug / toxin transport	0.008644	*QDR3 PDR12*
Heavy metal ion transport (Cu^{+}, Fe^{3+}, etc)	0.009523	*GGC1 FRE1*
Down-regulated genes:		
Metabolism of methionine	7.712e-7	*SAM2 MET18 SAM1 MET17*
Electron transport and membrane-associated energy conservation	1.641e-6	*INH1 QCR7 COX4 CYC1 QCR2*
Biosynthesis of cysteine	7.564e-5	*CYS3 MET17*
Tricarboxylic-acid pathway	0.0001774	*KGD2 MDH1 CIT1*
S-adenosyl-methionine-homocysteine cycle	0.000263	*SAM2 SAM1*
Inorganic chemical agent resistance (eg heavy metals)	0.000263	*CUP1-1 CUP1-2*
Heavy metal binding (Cu, Fe, Zn)	0.0003499	*CUP1-1 CUP1-2*
Aerobic respiration	0.002588	*QCR7 COX4 QCR2*
Sugar, glucoside, polyol and carboxylate catabolism	0.002989	*KGD2 MDH1 CIT1*
Electron transport	0.003204	*INH1 COX4 CYC1*
Catalase reaction	0.007257	*CTT1*

4.6. Further gene expression analysis using one-colour Affymetrix microarrays

The above microarray data was derived from technical dye-swap replicates with biological singlet for two of the conditions and biological duplicate for the third condition, using the two-colour microarray platform. In order to include further replication and to enable comparison of ammonium sulfate plus cysteine to the control condition, one-colour Affymetrix microarrays were performed on samples made in a similar way to that above and analysed using Partek. In this case, it was possible to compare ammonium sulfate plus cysteine to a control of nothing added instead of above where the control used was media containing cysteine.

Principal component analysis (PCA) was conducted on Affymetrix microarrays of yeast grown in ammonium sulfate, ammonium sulfate plus cysteine, cysteine and control conditions. A PCA plot illustrates the similarity between the microarrays and groups them in terms of this similarity. The PCA plot in Figure 4.2 shows that while there is a difference between the control (purple) and cysteine (green) samples, the close grouping of the ammonium sulfate plus cysteine and ammonium sulfate microarrays (red and blue, respectively) in the PCA plot shows that the addition of cysteine in the presence of ammonium sulfate does not have a significant impact on the gene expression of yeast.

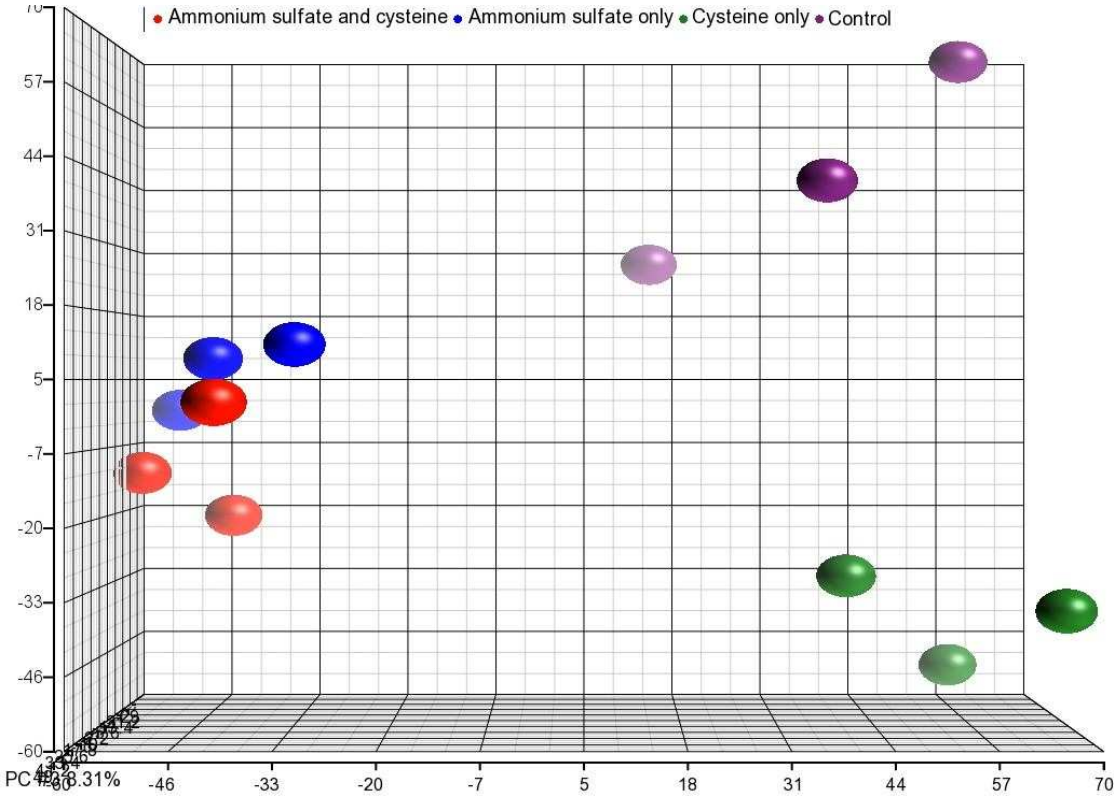


Figure 4.2. PCA plot for one-colour Affymetrix ammonium sulfate and cysteine microarrays. Legend: Red represents the ammonium sulfate plus cysteine microarrays, blue are ammonium sulfate only, green cysteine only and purple are the control microarrays.

Lists of significant genes were determined by ANOVA. Significant genes were those that had a fold change of at least 2, with genes discarded if their p-value was below the false discovery rate (FDR) p-value, which were at p-values of 0.021, 0.020 and 0.012 for ammonium sulfate plus cysteine, ammonium sulfate only and cysteine only, respectively, each compared to the control. Ammonium sulfate plus cysteine vs control contained 79 up-regulated and 169 down-regulated genes, ammonium sulfate vs control contained 52 and 130, respectively and cysteine had 53 and 63, respectively.

Enrichment of the functional categories found in the significantly altered genes, shown in Table 4.2, shows an up-regulation of arginine biosynthesis and glycine degradation in the ammonium sulfate plus cysteine samples, with down-regulation of nitrogen catabolism (with genes such as *DAL2*, *DAL3* and *DAL7*), arginine, leucine and proline degradation and stress response. Ammonium sulfate up-regulated many

genes that involved the sulfur pathway, which will be discussed in further details below, and down-regulated genes involved with nitrogen, as expected. Cysteine up-regulates genes with a nitrogen involvement, such as *DAL1*, *DAL2* and *DAL7*, which was down-regulated by ammonium sulfate. Metal ion homeostasis is also up-regulated and, not surprisingly, cysteine metabolism has been down-regulated.

Table 4.2. Functional enrichment of the genes significantly changed by ammonium sulfate and / or cysteine treatment, as determined by MIPS Functional Classification, FunSpec (Robinson et al., 2002), using the one-colour Affymetrix microarray platform. Enrichment was considered at $p < 0.01$. Up-regulated categories are highlighted in red and down-regulated categories are highlighted in green.

Category	p-value	Genes in category
Ammonium sulfate plus cysteine vs control (one-colour Affymetrix microarrays):		
Up-regulated genes:		
rRNA processing	1.109e-05	*POP8 SRD1 FAL1 RPF1 RIX1 FAF1 HCA4 REX4 RRP36 RRS1*
Metabolism of nonprotein amino acids	0.0001355	*ARG5,6 ARG3 ARG8*
Biosynthesis of arginine	0.0003154	*ARG5,6 ARG3 ARG8*
C4-dicarboxylate transport	0.0006746	*OAC1 DIC1*
Metabolism of vitamins, cofactors, and prosthetic groups	0.001104	*GCV1 PYC1 GCV2 ISU2*
C-1 compound catabolism	0.001116	*GCV1 GCV2*
Degradation of glycine	0.001663	*GCV1 GCV2*
Ribosome biogenesis	0.004799	*RIX1 DHR2 REX4 RRS1*
Biosynthesis of vitamins, cofactors, and prosthetic groups	0.006373	*BNA4 THI4 BIO2 SNO1 SNZ1*
Down-regulated genes:		
Catabolism of nitrogenous compounds	1.936e-06	*DAL1 DAL2 DAL7 DAL3*
Metabolism of energy reserves	7.149e-05	*TPS2 GLC3 GIP2 GSY1 GSC2 TSL1 GAC1 GDB1*
Anion transport	0.0001134	*MEP1 DUR3 MEP2 MEP3*
Alcohol fermentation	0.0002361	*NDE2 ARO10 NDE1 ALD4*
Metabolism of urea (urea cycle)	0.0003005	*DUR1,2 CAR2 CAR1*
Cellular import	0.0004162	*CAN1 SIT1 MEP1 HXT5 DAL5 GAP1 HXT2 MEP2 PUT4*
Peroxidase reaction	0.0005161	*GPX2 GPX1 HMX1*
Electron transport and membrane-associated energy conservation	0.0005924	*NDE2 CYC7 CYC1 SDH1 CYB2 NDI1 NDE1*
Degradation of arginine	0.0006359	*CAR2 CAR1*
Purine nucleotide / nucleoside / nucleobase	0.0008318	*DUR1,2 GUD1 DAL1 DAL7 DAL3*

metabolism		
Peptide transport	0.002258	*OPT1 PTR2 OPT2*
Nitrogen, sulfur and selenium metabolism	0.002261	*GDH2 AMD2 IRC7 OPT1 CAR2 ATO2*
Stress response	0.002569	*SSE2 HSP30 TPS2 CYC7 SLT2 XBP1 SDP1 MSN4 UBI4 TSL1 DDR2*
Aerobic respiration	0.003172	*NDE2 MBR1 SDH1 NDI1 ISF1 NDE1 CYT1*
Respiration	0.003557	*CYC7 GUT2 CYC1 CYB2 YTP1 ALD4*
Degradation of leucine	0.00369	*ARO10 BAT2*
Degradation of proline	0.00369	*PUT2 PUT1*
Amine / polyamine transport	0.00471	*DUR3 TPO1 PNS1*
Amino acid / amino acid derivatives transport	0.005257	*AGP1 CAN1 GAP1 PUT4 ODC1*
Protease inhibitor	0.006047	*YHR138C TFS1*
Ammonium sulfate vs control (one-colour Affymetrix microarrays):		
Up-regulated genes:		
Sulfate assimilation	1.685e-07	*MET10 MET3 MET5 MET14*
NAD/NADP binding	0.0001221	*SER3 MET10 GND2 MET5*
Sulfate / sulfite transport	0.0001545	*OAC1 SUL2*
Metabolism of methionine	0.0004374	*MET3 MHT1 MET17*
C-1 compound catabolism	0.0005103	*GCV1 GCV2*
Degradation of glycine	0.000762	*GCV1 GCV2*
Biosynthesis of methionine	0.000762	*MET14 MET2*
Biosynthesis of homocysteine	0.001062	*MET10 MET5*
Conjunction of sulfate	0.007269	*MET3*
Down-regulated genes:		
Catabolism of nitrogenous compounds	6.851e-07	*DAL1 DAL2 DAL7 DAL3*
Metabolism of urea (urea cycle)	2.024e-06	*DUR1,2 ARG4 CAR2 CAR1*
Anion transport	4.126e-05	*MEP1 DUR3 MEP2 MEP3*
Cellular import	5.823e-05	*CAN1 SIT1 MEP1 DAL5 GAP1 HXT2 FET3 MEP2 PUT4*
Alcohol fermentation	8.673e-05	*ADH5 NDE2 ARO10 ALD4*
Biosynthesis of arginine	8.673e-05	*ARG4 CPA2 PUT1 ARG7*
Purine nucleotide / nucleoside / nucleobase metabolism	0.0002535	*DUR1,2 GUD1 DAL1 DAL7 DAL3*
Degradation of arginine	0.0003788	*CAR2 CAR1*
Nitrogen, sulfur and selenium metabolism	0.0005928	*GDH2 AMD2 IRC7 CAR2 ATO2 GLN1*
Degradation of leucine	0.002215	*ARO10 BAT2*
Metabolism of glutamate	0.003644	*GDH2 GLN1*
Metabolism of energy reserves	0.004533	*TPS2 GLC3 GIP2 GSC2 GAC1*
Regulation of nitrogen metabolism	0.009818	*GAT1 DAL80*
Cysteine vs control (one-colour Affymetrix microarrays):		
Up-regulated genes:		
Homeostasis of metal ions (Na, K, Ca, etc)	1.121e-08	*CCC2 SIT1 IRC7 ARN2 SMF3 FRE1 FET4 COT1 FRE3 CTR1*
Sideophore-iron transport	3.619e-08	*SIT1 ARN1 ARN2 ENB1 FRE3*
Drug / toxin transport	1.019e-06	*QDR3 SIT1 ARN1 ARN2 ENB1 PDR12*
Heavy metal ion transport (Cu^{+}, Fe^{3+}, etc)	1.385e-06	*CCC2 SMF3 FRE1 FET4 COT1 CTR1*

Detoxification	6.039e-06	*QDR3 ADH5 SIT1 ARN1 ARN2 ENB1 SSU1*
Catabolism of nitrogenous compounds	6.692e-06	*DAL1 DAL2 DAL7*
Cellular import	1.325e-05	*UGA4 SIT1 DAL4 DAL5 FRE1 FET4 CTR1*
Alcohol fermentation	0.0001819	*ADH5 ARO10 ADH4*
C-compound and carbohydrate metabolism	0.0007486	*ADH5 YDR248C ARO10 ADH4 ATF2 GND1 DAL7 PDR12*
C-1 compound catabolism	0.0007715	*GCV1 GCV2*
Degradation of glycine	0.001151	*GCV1 GCV2*
Ion transport	0.001602	*FIT2 FIT3*
ABC transporters	0.001894	*VMR1 ENB1 PDR12*
Allantoin and allantoate transport	0.002123	*DAL4 DAL5*
C-compound and carbohydrate transport	0.003331	*ESBP6 MCH4 PDR12*
Metabolism of vitamins, cofactors, and prosthetic groups	0.00649	*GCV1 ARN2 GCV2*
Transport facilities	0.007376	*VHT1 ESBP6 MCH4 OPT2*
Pentose-phosphate pathway oxidative branch	0.008935	*SOL3*
Degradation of phenylalanine	0.008935	*ARO10*
Down-regulated genes:		
Electron transport and membrane-associated energy conservation	1.447e-11	*INH1 RIP1 CYC7 COX13 QCR10 CYC1 SDH2 NDE1 COX7 QCR2*
Aerobic respiration	2.724e-10	*RIP1 COX13 QCR10 MBR1 SDH2 ISF1 NDE1 COX7 CYT1 QCR2*
Electron transport	2.127e-07	*INH1 CYC7 COX13 CYC1 SDH2 NDE1 COX7 CYT1*
Oxidative stress response	0.0008672	*MXR1 CTT1 SRX1 GRE1*
Metabolism of cysteine	0.002114	*MET32 MHT1*

4.7. Sulfur pathway

The cysteine and ammonium sulfate treatments had an impact on the production of H_2S. The two-colour microarray results showed that the addition of cysteine in media results in a down-regulation of the sulfur pathway whereas the addition of ammonium sulfate results in an up-regulation. This was shown to be the case with the Affymetrix microarray results as well, as shown in Table 4.3. Ammonium sulfate in the growth media caused an up-regulation of the sulfur pathway, particularly the transition between extracellular sulfate to homocysteine.

As seen in Figure 4.3, most of the genes in the sulfur pathway are down-regulated when grown in medium containing cysteine and up-regulated when ammonium sulfate is present. There is a lessening of this effect in the ammonium sulfate plus cysteine microarrays (as seen in Table 4.3), suggesting that there is a combined effect from both compounds.

Table 4.3. Gene expression of the sulfur pathway for the ammonium sulfate and / or cysteine treatments, using data from the one-colour Affymetrix microarrays. The red highlight indicates a fold change above 2, pink 1.5 to 2, lime green -1.5 to -2 and green a fold change below -2.

	Ammonium sulfate plus cysteine vs control	Ammonium sulfate vs control	Cysteine vs control
SUL1	**-1.08**	**-1.24**	**1.05**
SUL2	**1.26**	**2.90**	**-1.12**
MET3	**2.33**	**5.57**	**-1.35**
MET14	**1.94**	**4.28**	**-1.72**
MET16	**-1.02**	**1.62**	**1.10**
SSU1	**-1.07**	**-1.33**	**2.31**
MET5	**1.63**	**3.14**	**-1.49**
MET10	**1.46**	**3.15**	**1.06**
MET17	**1.72**	**2.36**	**-1.89**
MET6	**1.40**	**1.30**	**1.05**
SAM1	**1.42**	**1.50**	**-1.75**
SAM2	**-1.14**	**-1.02**	**-1.48**
SAH1	**-1.08**	**-1.13**	**1.02**
STR1/CYS3	**-1.16**	**1.17**	**-1.82**
STR2	**-1.53**	**-1.95**	**1.99**
STR3	**1.34**	**1.09**	**-1.40**
STR4/CYS4	**-1.15**	**-1.03**	**1.05**
MET2	**2.17**	**5.01**	**-3.46**
HOM3	**-1.22**	**-1.55**	**1.36**
HOM2	**-1.08**	**-1.23**	**1.15**
HOM6	**1.06**	**1.07**	**1.06**
THR1	**-1.01**	**-1.06**	**1.08**
THR4	**1.03**	**-1.02**	**1.09**
GAP1	**-29.7**	**-35.7**	**1.4**
MUP1	**-1.1**	**1.1**	**-1.2**
MUP3	**-1.8**	**-1.7**	**-1.8**
YCT1	**1.1**	**-1.2**	**1.3**
MEP1	**-19.7**	**-18.3**	**1.4**
MEP2	**-21.9**	**-23.9**	**1.5**
MEP3	**-3.2**	**-3.1**	**1.1**

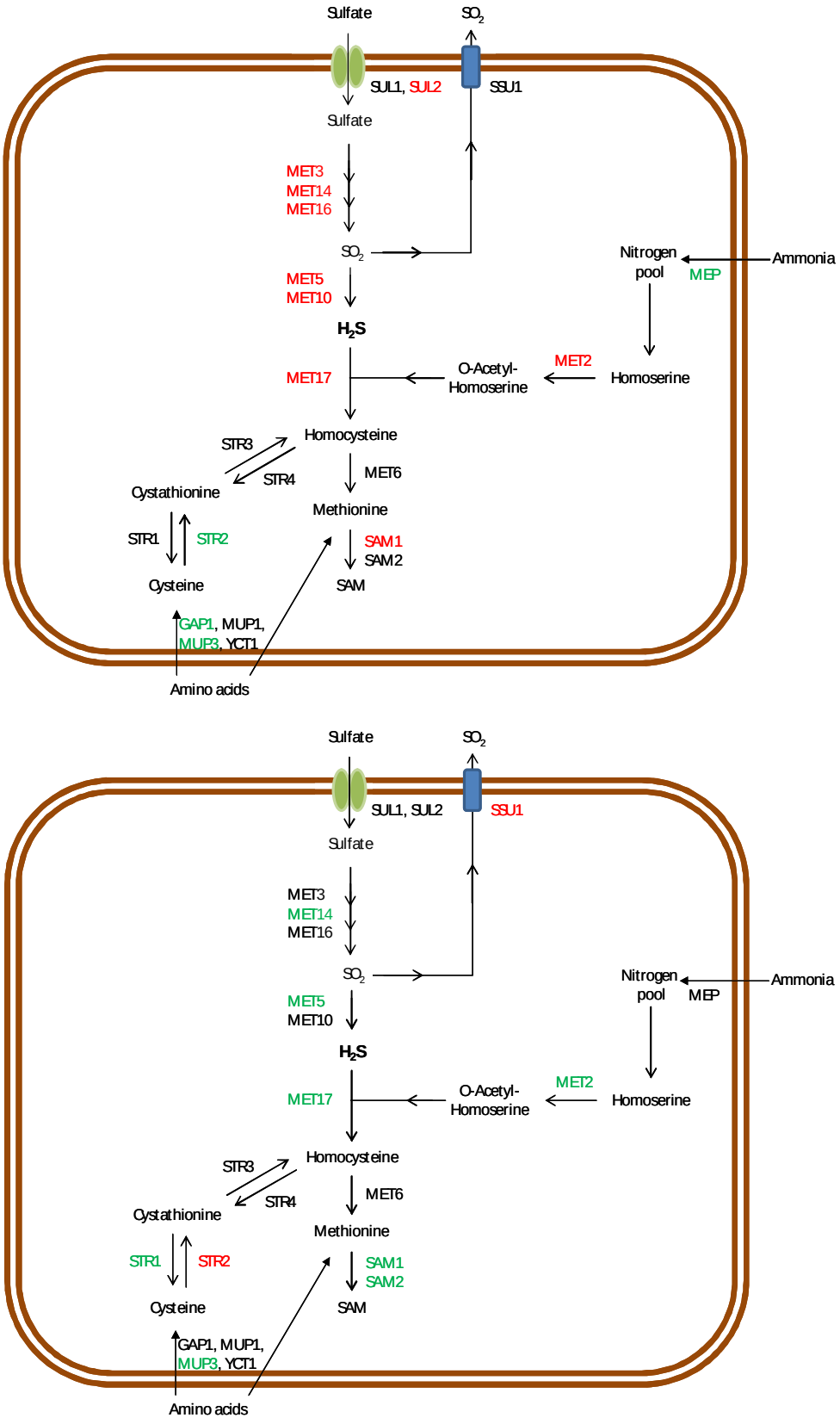


Figure 4.3. The effect of ammonium sulfate (top) and cysteine (bottom) on expression of genes in the sulfur pathway, as well as amino acid and ammonia transporters. Gene names coloured in red denote up-regulation and gene names in green indicate down-regulation, with a cut-off of 1.5 in expression.

4.8. The effect of deletion of genes involved in the sulfur pathway on the production of H_2S

The non-essential deletion yeast mutants, each of which lacked a gene in the sulfur pathway, were cultured and tested for H_2S production using the silver nitrate membrane method. *Met14Δ* produced more H_2S in media containing ammonium sulfate plus cysteine, compared to the wildtype BY4743, with medium production. *Sam1Δ*, *Sam2Δ*, *Str1Δ* and *Fre1Δ* had low quantities of H_2S detected, compared to the wildtype which was below the detection level. *Ser2Δ*, and to a lesser extent, *Ser1Δ*, produced less H_2S than the control when grown in media containing cysteine without nitrogen.

An unexpected result in this deletion strain analysis is that of *Cdc10Δ*, which was included in the analysis randomly in an attempt to fill up the microtitre plate. The mutant strain actually produced H_2S at the same rate in the ammonium sulfate plus cysteine as in the cysteine medium, whereas the wildtype BY4743 produced H_2S below the detection limit when ammonium sulfate was present in the medium. *CDC10* encodes a septin, which is required for cytokinesis and is involved in budding and can act as a barrier to membrane diffusion, although its deletion does not eliminate cytokinesis (Frazier et al., 1998; Takizawa et al., 2000). This mutant was re-tested using various nitrogen concentrations, which confirmed this result. *CDC10* might be preventing ammonium entering the cell due to the membrane diffusion role of Cdc10p.

4.9. Discussion

Hydrogen sulfide (H_2S) is an important off-flavour in wine, both because of its intense rotten egg odour and because of the difficulty of removal (Linderholm et al, 2008). Previous studies carried out with wine yeast demonstrated that formation of H_2S is a response to nitrogen depletion (Jiranek et al, 1995). Consequently, research and winemaking practice have found that supplementation of di-ammonium phosphate as a source of nitrogen can reduce the production of H_2S (Mendes-Ferreira et al, 2009), whilst the addition of cysteine in media or grape juice increases H_2S. Here, we examined the effect of nitrogen and cysteine addition on the metabolism, particularly the sulfur pathway, of the yeast lab strain BY4743. Ammonium sulfate was used in

this study because it was found that only the ammonium ion altered the level of H_2S production by yeast and that the sulfate ion had no effect in the silver nitrate membrane assay as shown in the results (Section 4.4). However, I am cognisant that the levels of other compounds in the wine or medium could change the way nitrogen interacts with the yeast metabolism. Torrea et al (2011) urged caution extrapolating these sorts of data to commercial winemaking.

Initial two-colour gene expression analysis showed that many of the adenine requiring (*ADE*) genes, such as *ADE1*, *ADE2*, *ADE4*, *ADE13* and *ADE17*, involved in the purine nucleotide biosynthesis pathway, were up-regulated in the presence of ammonium sulfate. The categories of down-regulated genes involved heavy metals and branched chain amino acids. Interestingly, cysteine biosynthesis was up-regulated in the ammonium sulfate plus cysteine sample compared to cysteine alone despite both samples containing the same amount of cysteine. This can be explained if NCR causes yeast to use the ammonium ions not only as a nitrogen source but also for amino acid synthesis; that is in preference to importing cysteine. This was further seen by the down-regulation of amino acid importation, particularly *GAP1*, which encodes the general amino acid permease and is regulated in response to the type of nitrogen source available. *GAP1* gene expression was over 10 fold down-regulated in the microarrays involving ammonium sulfate whereas *GAP1* was up-regulated (+2.2) in the cysteine only samples, due to a lack of favourable nitrogen sources. The ammonium sulfate treatments include ammonium sulfate over the control (-10.7), as well as ammonium sulfate plus cysteine over the cysteine conditions (-12.3).

Subsequent analysis using the Affymetrix one-colour microarray system in triplicate showed similar results, showing that the presence of ammonium sulfate caused the yeast cell to up-regulate amino acid biosynthesis, including the genes within the sulfur pathway. Ammonium sulfate in the growth medium caused an up-regulation of the sulfur pathway, particularly the route from extracellular sulfate to homocysteine. The presence of cysteine instead of ammonium sulfate in the medium resulted in down-regulation of this pathway, which is shown in Figure 4.3. When ammonium ion was present in the medium, the gene, *MET2*, was up-regulated by five fold. This gene encodes L-homoserine-O-acetyltransferase, important for the synthesis of O-acetyl-homoserine which is the intermediate metabolite to react with H_2S to form homocysteine (Hansen and Kielland-Brandt, 1996a). The gene involved in this down-stream reaction is *MET17*, which was also up-regulated (+2.36). Such enhanced gene

expression is likely to push the metabolism past H_2S, and on to methionine and cysteine, rather than being stuck at the H_2S step causing its accumulation. A previous study showed that nitrogen affects the sulfur pathway and the production of H_2S because nitrogen sources are involved in the production of homoserine (Bell and Henschke, 2005). My findings correlate to these findings by Bell and Henschke (2005).

Interestingly, the genes involved in the up-stream process from aspartate to homoserine were largely unaffected other than the first step which was slightly down-regulated (*HOM3*, -1.55). *MET2* is also up-regulated when ammonium sulfate and cysteine are added, but to a lesser extent (+2.17), while cysteine down-regulated this gene (-3.46), indicating that there is a combination effect here. The addition of cysteine up-regulated the SO_2 exporter, *SSU1* (+2.31) (Park and Bakalinsky, 2000; Donalies and Stahl, 2002), but had little effect on the rest of the pathway, although select genes had a low level of down-regulation. These included *STR1* (-1.82) which is responsible for the conversion of cystathionine into cysteine and *MET17* (-1.89) which is responsible for converting H_2S into homocysteine (Cherest et al, 1993). This result is important because, without an avenue for further metabolism, H_2S would accumulate and be released as seen under cysteine only conditions.

Non-essential deletion yeast mutants, each of which lacked a gene in the sulfur pathway, were grown up and tested for H_2S production using the silver nitrate membrane method. *Met14Δ* produced more H_2S in media containing ammonium sulfate plus cysteine, compared to the wild type BY4743, with medium production. *Sam1Δ*, *Sam2Δ*, *Cys3Δ* and *Fre1Δ* had low levels of H_2S detected, compared to the wildtype which was below the detection level. *Ser2Δ*, and to a lesser extent, *Ser1Δ*, produced less H_2S than the control when grown in medium containing cysteine only. These findings are in agreement with the transcriptomic data set.

An unexpected result in this deletion strain analysis was that of *Cdc10Δ*, which was included in the analysis randomly in an attempt to fill up the microtitre plate. The mutant strain actually produced H_2S at the same rate in the ammonium sulfate plus cysteine medium as in the cysteine only medium, whereas the wildtype BY4743 produced H_2S below the detection limit when ammonium sulfate was present in the medium. *CDC10* encodes a septin, which is required for cytokinesis and is involved in budding and can act as a barrier to membrane diffusion, although its deletion does not eliminate cytokinesis (Frazier et al, 1998; Takizawa et al, 2000). This mutant was

re-tested using various nitrogen concentrations, which confirmed this result. *CDC10* might be involved in preventing the ammonium entering the cell due to the membrane diffusion role of Cdc10p.

An important aspect to note here is that these experiments, using either the microtitre plate method for H_2S measurement or the flask cultures for cDNA profiling, were not carried out under anaerobic conditions. Secondly, the laboratory yeast strain BY4743 was grown to exponential phase prior to cDNA microarray analysis. This time-point must be considered when trying to relate the dataset here to wine fermentations. The cells were prepared by diluting overnight cultures of OD_{600} 1.0 five fold and then grown back up in the various media conditions up to an OD_{600} 1.0 while shaking, taking approximately five hours before being processed. In a winemaking scenario, yeast are grown in complex grape juice under anaerobic conditions with about ten times the amount of sugar than the minimum media used in the laboratory scenario, with fermentation continuing for two weeks. Therefore, it is significant to find that nitrogen supplementation still suppresses H_2S production (as shown in Figure 4.1). This demonstrates that the phenomenon discovered in wine fermentation is also true under aerobic conditions, as was also revealed in research by Stratford and Rose (1985).

As is known, nitrogen assimilation is managed via activation or repression of nitrogen catabolite repression (NCR) (Ter Schure et al., 1998; Beltran et al., 2004). In the presence of favourable nitrogen sources, such as ammonium or glutamate, NCR sensitive genes responsible for utilisation of less favourable nitrogen sources, for example other amino acids, are repressed (Bell and Henschke, 2005). The NCR regulators are *GLN1*, *GAT1*, *URE2* and *DAL80* (Coffman et al, 1995; Ter Schure et al, 1998). In response to the ammonium sulfate conditions, *GLN1*, *GAT1* and *DAL80* were down-regulated 2.3, 3 and 8 fold, respectively, while the gene expression of *URE2* remained unchanged. Conversely, in cysteine rich conditions, *DAL80* expression was shown to increase 8 fold. Most of the *DAL* and *DUR* genes behaved in this way, with down-regulation by ammonium sulfate and up-regulation in the cysteine condition. The allantoin degradation pathway was down-regulated since it is only necessary when ammonium is not present. The *MEP* family of ammonium permeases were down-regulated due to NCR (an over 20 fold decrease for *MEP2*) and up-regulated in the cysteine rich media (+2.7). *GAP1* was heavily down-regulated by the ammonium sulfate condition with an at least 10 fold reduction in expression, as

seen from both the two-colour and one-colour microarray data. However, *GAP1* (Chiva et al, 2009) was slightly up-regulated (2.2 and 1.4 in the two-colour and one-colour systems, respectively) under the cysteine condition, which is caused by the presence of amino acids (secondary) rather than ammonium or glutamate (preferred) as the nitrogen source. Other genes with an involvement with preferable nitrogen sources are *IRC7, ARG4* and *DAL7,* all of which were down-regulated in ammonium sulfate rich conditions and up-regulated in the presence of cysteine (less favourable nitrogen source). These results demonstrate how important the type of nitrogen source in the media is to the amino acid biosynthetic pathways, which in turn affect the level of H_2S production due to its close link to methionine and cysteine.

It is important to compare these results to those of published data. A transcriptomic analysis of the effect of DAP as a nitrogen source using wine yeast by Marks et al (2003) showed up-regulation of the genes involved in the assimilation of sulfate and *de novo* purine biosynthesis (*ADE* genes). These results are very similar to those presented here, despite the differences in experimental design, where Marks et al (2003) used industrial wine yeast that had reached stationary phase in a fermentation setup. Up-regulation of the sulfur pathway and purine biosynthesis and down-regulation of nitrogen related genes due to NCR were also seen by Aranda et al (2006). Gene-deletion studies by Hansen and Kielland-Brandt (1996a) showed that the lack of *MET2* increased the production of H_2S. This correlates with our studies where *MET2* increased 5-fold under nitrogen-rich conditions. *MET2* up-regulation would result in a higher level of *O*-acetyl homoserine, which sequesters H_2S. When more *O*-acetyl homoserine is produced stoichiometrically than the level of H_2S, a low level of H_2S arises. This molecular mechanism explains the inhibition of H_2S level by the ammonium in the membrane assay.

Chapter 5: Wine flavour and clarity: The effects of pectin and carrageenan

5.1. Introduction

A major problem for winemakers, particularly when producing white wines, is cloudiness or hazing. An excess amount of proteins and phenolic compounds in the wine often results in precipitation due to heat or aging, leading to cloudy wine. Whilst the hazing has little or no effect on the flavour of the wine, consumers prefer clear wine. The perception that cloudiness of liquids is associated with bacterial contamination keeps consumers away from hazy wines. As described in Section 1.3.4, Chapter 1, the measures currently taken by the Australian winemakers to combat this problem are to add bentonite or animal by-products such as egg and milk products and previously isinglass to remove the excess protein, polyphenols and other haze-initiating compounds from the wine. There are, however, some major disadvantages to these measures. For example, bentonite is a clay and, as such, is difficult to remove from the wine which can be costly to the winemaker, both in terms of raw materials and because of the added cost associated with filtration. Furthermore, the most undesirable feature of bentonite is the reduction of flavour compounds by the indiscriminate removal of positively charged compounds (Waters et al., 2005). Other alternative fining agents like egg and milk products are undesired by the increasing consumer market of vegan, vegetarian and animal-conscious consumers.

In this chapter, the fining potential of pectin and carrageenan when added to grape juice prior to fermentation was investigated. Both of these compounds are naturally available, as described in Sections 1.8.1 and 1.8.2, Chapter 1. Pectins are hetero-polysaccharides found in the cell walls of higher terrestrial plants such as citrus peel, apples and even hop cones while carrageenans are high molecular weight, linear, sulfated polysaccharides present in red seaweed. They have been used in beer making and have been suggested for use in wine, because carrageenan can have the same clarification capability under some conditions as bentonite (Cabello-Pasini et al., 2005). However, their usefulness depends ultimately on their effect on the flavour profile and in this chapter I wish to address this issue.

My specific objectives of this chapter are as follows:

- To determine whether the rate of fermentation differs due to the addition of pectin alone, carrageenan alone and pectin plus carrageenan in combination compared to a control fermentation.
- To determine whether pectin and carrageenan treatments change / improve the clarity of white wine.
- To determine whether the pectin and carrageenan treatments cause any changes in flavour profile - the esters, acids and higher alcohols of the wine.
- To determine whether pectin and carrageenan treatment affects hydrogen sulphide (H_2S) and sulfur dioxide (SO_2) in finished wine.

5.2. Methodology

Three sets of fermentations were undertaken involving pectin and carrageenan pre-treatment of grape juice. The wine yeast strain QA23 was used for these studies. The first set was a pilot-scale study (20 L fermentations) at the Fosters Group laboratories, in Melbourne, Victoria, using red and white grape juice either treated with both pectin and carrageenan pre-fermentation or left untreated (control) in singlet (two fermenters for red and two for white). The second and third sets were at lab-scale studies (2 L) carried out in triplicate with either pectin or carrageenan or pectin plus carrageenan in combination. Synthetic Chardonnay grape juice medium was used in the first lab-scale fermentation and real Chardonnay grape juice, obtained from AWRI (Adelaide, SA, Australia) for the second lab-scale set, as shown in Figure 5.1. The fermentation methodology was described in Section 2.10, Chapter 2.

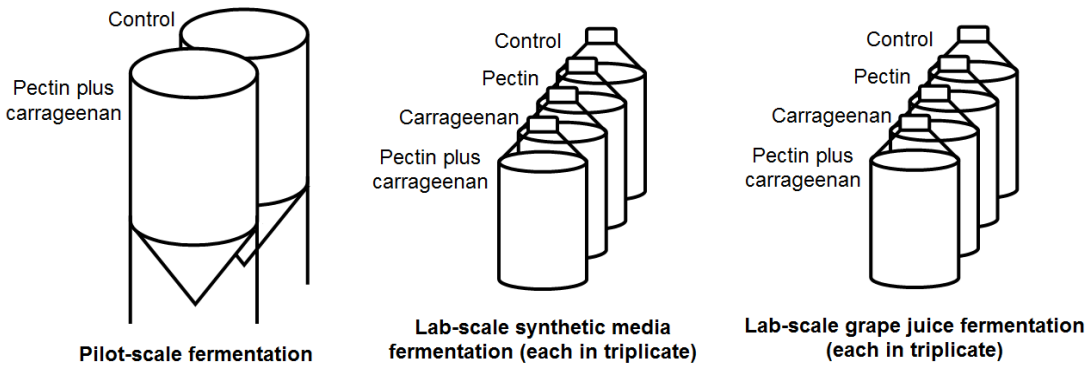


Figure 5.1. A schematic of the three fermentations that were carried out. The pilot-scale ferments were carried out using the Foster's Group pilot brewery at Abbotsford in Melbourne. The lab-scale ferments were conducted at UWS with synthetic grape juice and also for authentic grape juice.

Ferments were periodically sampled. The samples were centrifuged to obtain the yeast pellets which were then snap-frozen in liquid nitrogen and stored at -80 ^{o}C for cDNA microarray gene expression profiling analysis, which is described in Chapter 6. The supernatants were centrifuged again and the clear supernatants were analysed as described in the text. Fermentation rates are based on ethanol or sugar concentrations. The clarity of the wines was determined using absorbance, which was then converted to the EBC scale of colour. Heat stability was estimated as described in Section 2.11.3, Chapter 2. Sodium, potassium, magnesium and calcium ions were monitored during the pilot-scale fermentations. Headspace H_2S and soluble SO_2 were also determined. The assays were performed as described in Section 2.11, Chapter 2.

Volatile flavour compounds were determined using SPME GC-MS. These included ethyl acetate, ethyl hexanoate, ethyl octanoate, phenylethyl acetate, isoamyl acetate (esters), n-propanol, isobutanol, isoamyl alcohol and phenylethyl alcohol (higher alcohols) and butyric acid, hexanoic acid, octanoic acid and decanoic acid (acids). These were quantitated for the pilot-scale fermentation whereas the entire detectable profile was anlaysed for the lab fermentation against a compound library, relative to each other, as described in Section 2.12, Chapter 2.

In addition to volatile flavour analysis, an amateur sensory panel was used to assess consumer preference for the smell and taste of the pectin and carrageenan treated wines, as described in Section 2.11.4, Chapter 2. Twenty participants selected

at random were involved, each panel member ranking one biological set in order of preference. Twenty seven participants smelled the real grape juice lab-scale fermentation and ranked the wine based on perceived fruitness, sweetness and overall pleasantness, with 26 of these paricipants also tasting the wine.

5.3. The fermentation rate is unchanged by pectin and carrageenan treatment of grape juice

The fermentation rate was monitored by determining the ethanol concentration of each of the four pilot-scale fermentations at various timepoints of 24, 72, 95, 139 and 263 h over the 11-day fermentation period, as shown in Figure 5.2 for white wine and Figure 5.3 for red wine. The result showed that the final ethanol concentration was much the same in the treated and the control fermentations for both the red and the white wine ferments, namely, 13.9% and 12.6% for the white and red wine ferments, respectively. The red wine fermentation was a little slower than the white wine. The addition of pectin and carrageenan to the grape juice did not influence the fermentation rate of the wine, indicating that the fining agents were not detrimental to the yeast metabolism.

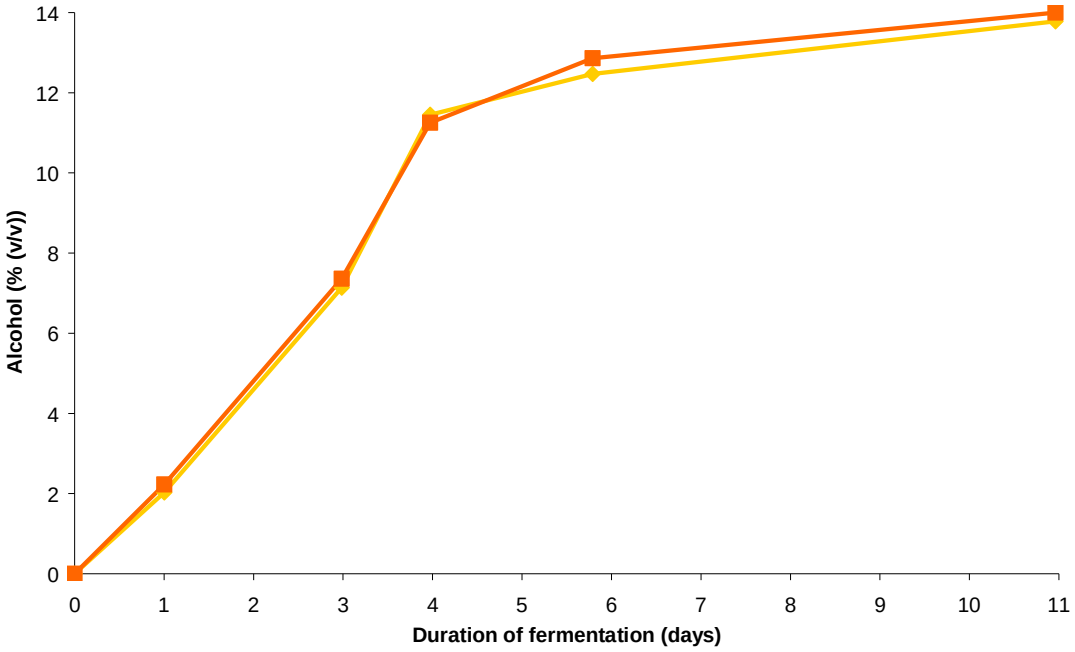


Figure 5.2. The effect of pectin plus carrageenan pre-treatment on alcohol production during white wine fermentation under pilot-scale conditions at Foster's Group in Melbourne. Yellow indicates the pectin plus carrageenan pre-treatment, while the orange line is the control.

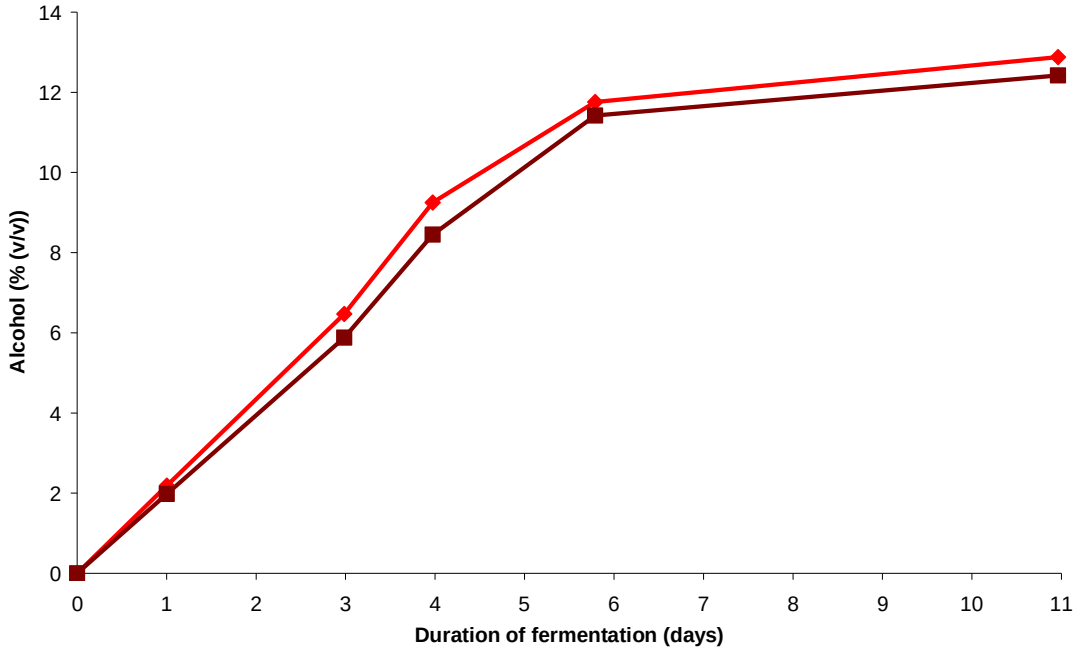


Figure 5.3. Ethanol production during red wine fermentation of a pectin plus carrageenan treated and untreated red wine, under pilot-scale conditions at the Foster's Group in Melbourne. Red indicates treated and maroon the control fermentation.

Clearly, the fermentation rate was unchanged by pectin plus carrageenan pre-treatment of grape juice, be it red or white juice during the pilot-scale fermentation at Foster's. During the lab-scale fermentation, sugar consumption was tested instead of ethanol production. In addition, a measurement of the final yeast optical density for the grape juice lab-scale fermentation was taken to compare the fermentation rates of the treatment. The sugar consumption for the synthetic grape juice media fermentations rose dramatically for the first six days and then plateaued as the sugar was consumed, as shown in Figure 5.4. This consumption was at a much more consistent rate over the fermentation for the real grape juice set, as shown below in Figure 5.5. There are some variations between the four treatments. However, they all follow a similar pattern. The pattern of consumption of glucose and fructose are similar to each other, so only total sugar consumption amounts have been shown here.

Despite the similar pattern in sugar consumption between the treatments, statistical analysis via ANOVA showed a significant increase in the residual sugar in the control fermentations compared to the pectin and / or carrageenan treatments ($p < 0.05$), as shown in Figure 5.6. There was a significantly higher amount of fructose in the control wine at the end of fermentation, compared to the pectin treated wine, as shown in Figure 5.6. The others do not have significantly altered fructose concentration. Glucose, on the other hand, is present in the control wine at a much higher rate than the pectin, carrageenan and pectin plus carrageenan wines, with significance levels of $p < 0.01$. This suggests that the control wine is sweeter than the treated wines. While this is not at concentrations that would make the control wine a sweet wine, it would be a concentration at which wine drinkers would notice (Berthels et al., 2008).

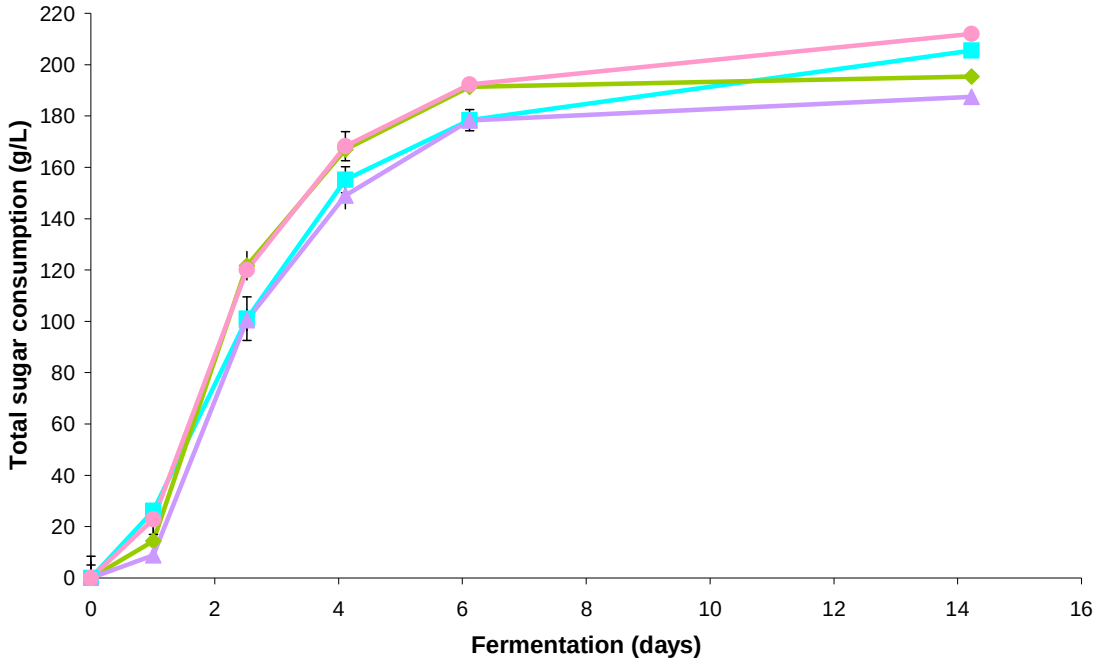


Figure 5.4. Total sugar consumed (glucose plus fructose) over the duration of the fermentation of the lab-scale synthetic media fermentations at UWS, normalised to the relevant 0 h timepoint. Key: Turquoise denotes control, lime green denotes pectin only treatment, rose denotes carrageenan only treatment and lavender denotes the pectin plus carrageenan treatment.

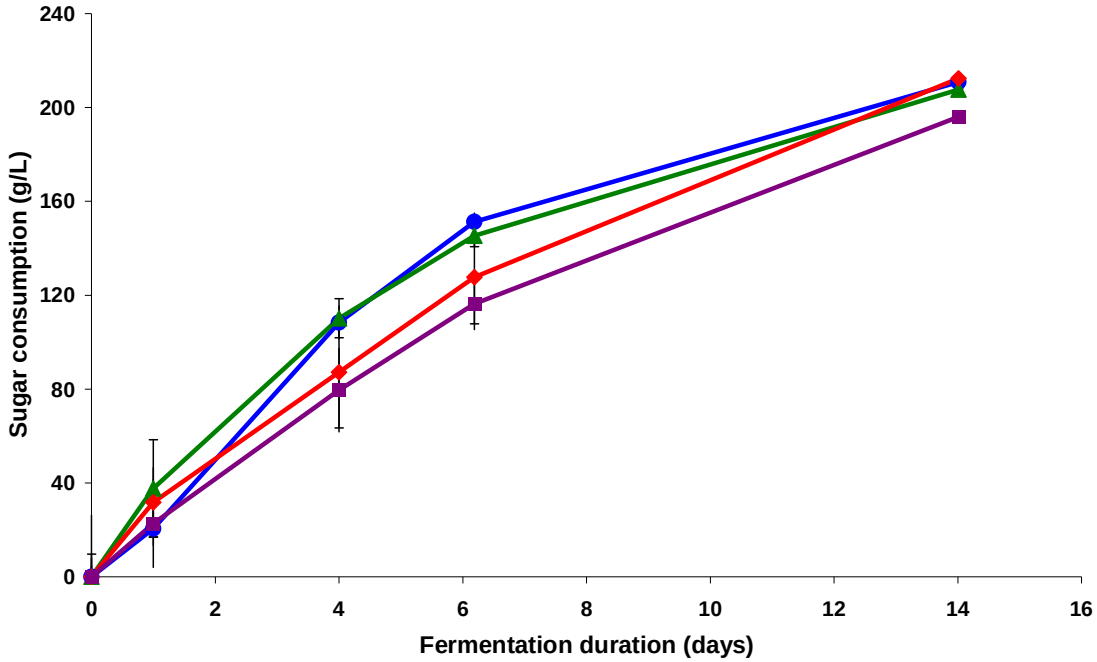


Figure 5.5. Total sugar (glucose plus fructose) consumed over the duration of the fermentations for the lab-scale grape juice wine fermentations at UWS. Key: Blue denotes control, green denotes pectin treatment, red denotes carrageenan treatment and purple denotes the pectin plus carrageenan treatment.

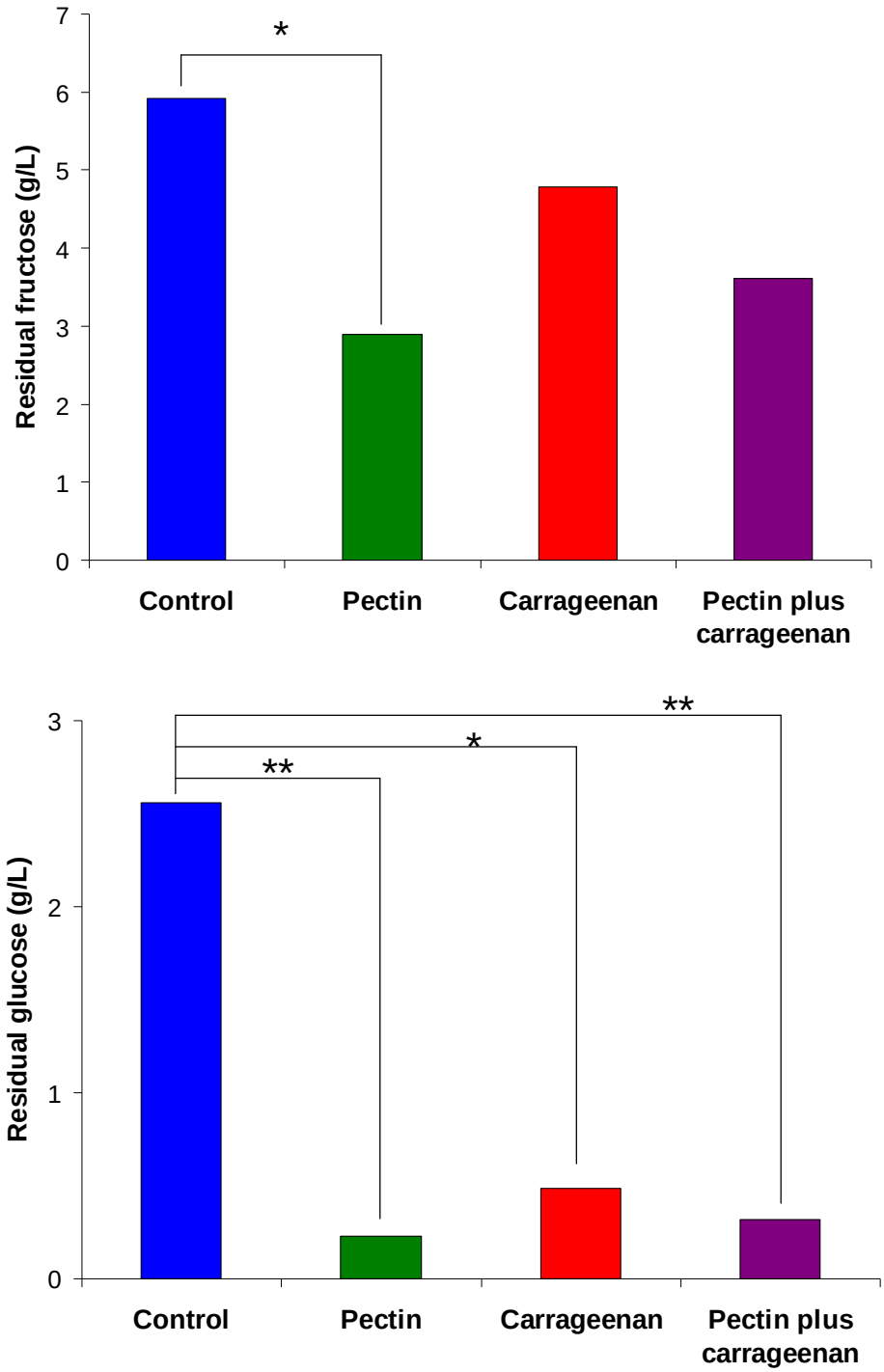


Figure 5.6. Residual fructose (top) and glucose (bottom) at the end of grape juice fermentation (336 h). Connecting bars show significance. * indicates $P < 0.05$, ** indicates $P < 0.005$.

There were differences in the glucose and fructose concentrations in the end ferments as shown in Figure 5.6; these data show that fructose and glucose are used more in the carrageenan or pectin treated fermentations. This aspect is one that I did not pursue, despite being interesting, because there were other features of the fermentation results that were more important to winemakers.

Yeast samples were recovered from these fermentations periodically and the yeast recovered by centrifugation and then snap frozen for later analysis, as described in Chapter 6. As may be seen in Figure 5.7, there is not a great deal of difference in turbidity at the end of the fermentation period. The pectin treated wine had the highest, with 12.9% more yeast than the control fermentation.

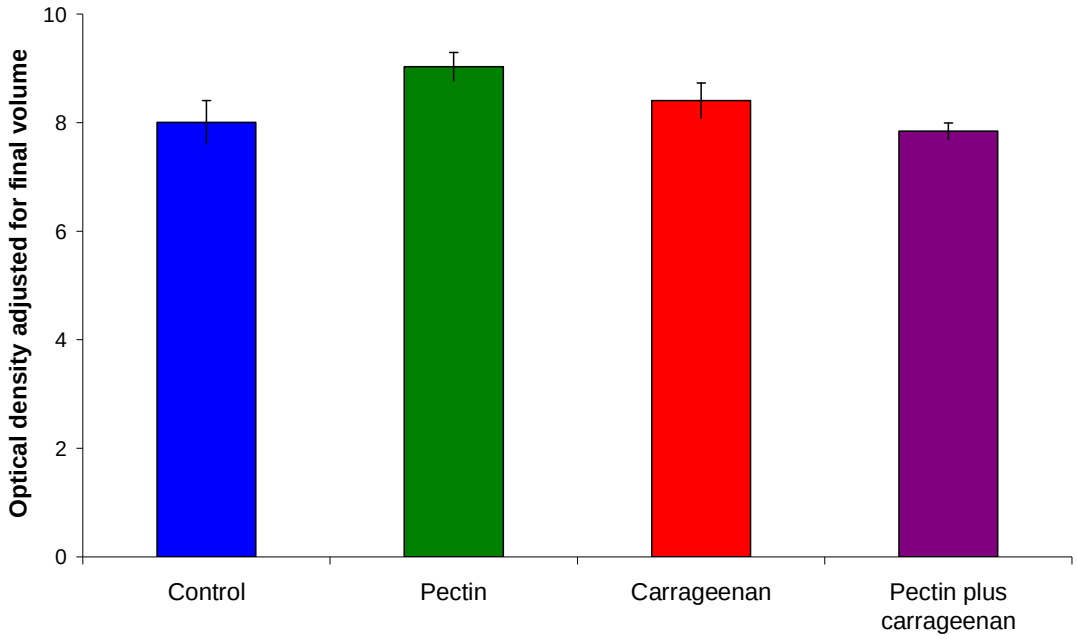


Figure 5.7. Absorbance measurements at 600 nm at the close of fermentation for the control and polysaccharide treated wine ferments at UWS. Key: Blue denotes control, green denotes pectin treatment, red denotes carrageenan treatment and purple denotes the pectin and carrageenan treatment.

5.4. The clarity and heat stability of white wine was improved by pectin and carrageenan treatment in white wine fermentation

The effect of pre-fermentation pectin and carrageenan treatment on the clarity and stability of a final white wine product was investigated, as the primary goal of this chapter. White wine samples collected from fermentations treated with pectin and carrageenan were tested for thermal stability, protein content and colour intensity. The results for each of these tests are described below.

5.4.1. Heat stability / haze test

The heat stability or haze test was described in Section 2.11.3, Chapter 2. This test mimics an extended period of storage under normal conditions. The more heat stable a wine is, the less turbidity that accumulates during this time. At the end of the first batch, the pilot-scale industrial fermentation (11^{th} day), the heat stability of the control white wine was 15.4 NTU (Nephelometric Turbidity Units) while the white wine that had been treated with pectin and carrageenan had a heat stability level of 6.62 NTU, a reduction of 57%. This indicated that the treated white wine was much more stable in the heat compared to the untreated wine, and therefore less likely to haze during storage. Statistical analysis of these results was not possible due to the use of only a single replicate. Therefore, the trend may or may not be applicable.

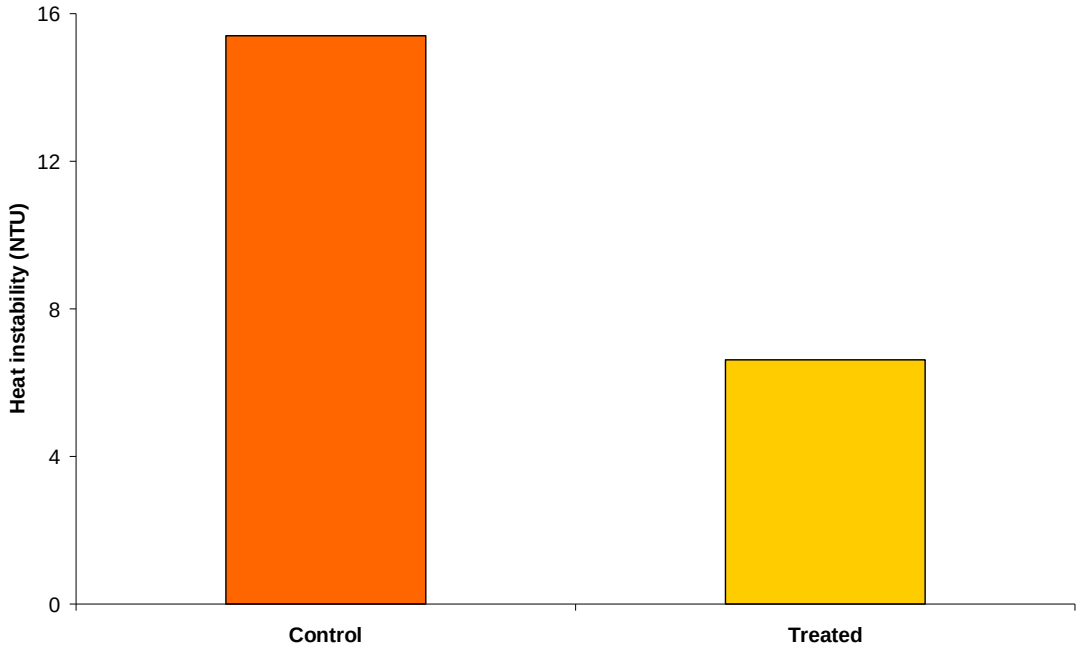


Figure 5.8. Heat stability of control (dark orange) and pectin/carrageenan treated (yellow) white wine made at Foster's Group in Melbourne.

Using the lab-scale fermentation, I obtained similar results with the authentic grape juice. The control wine had the lowest heat stability (Figure 5.9). The pectin-treated wine had the best heat stability (34% of the control or 2.93 times more effective). The carrageenan and pectin plus carrageenan treated wines had similar heat stability to each other, 46 and 47% of the control or about twice as effective. These findings indicate that both of the agents are effective in fining the wine.

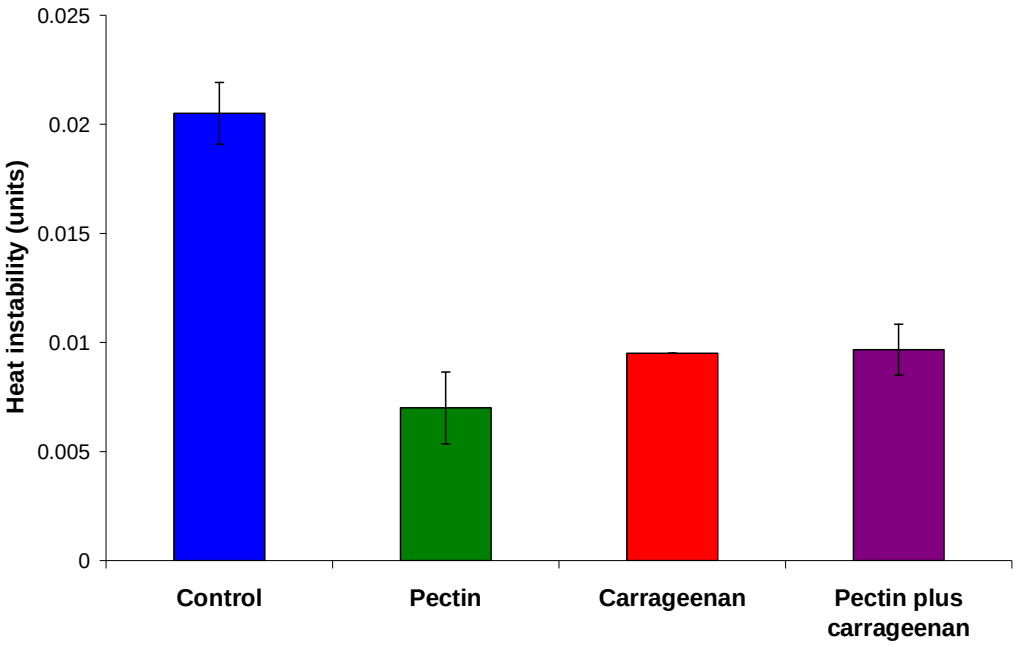


Figure 5.9. Heat instability of control (purple), pectin (red), carrageenan (green) and pectin plus carrageenan (blue) treated wine from the lab-scale real grape juice fermentations.

Heat stability tests demonstrated that the treatments indeed improved the stability and clarity of the white wines that had been treated with pectin and carrageenan.

5.4.2. Effects of pectin and carrageenan on white wine colour

An indirect way of testing wine clarity in white wine is the colour of the wine. Since the same grape juice was used for both the control and treated samples, it stands to reason that the resulting wine with a lighter colour would be clearer and have higher clarity compared to wine of darker colour. The colour of the white wine samples was tested after 96 and 264 h of fermentation, using the European Brewing Convention (EBC) scale. This means measuring absorbance at 430 nm and multiplying this value by 25 to obtain EBC units. The pectin plus carrageenan wine exhibited lower colour during fermentation and at the end (see Figure 5.10), with 32% less colour at the end of fermentation than the control samples.

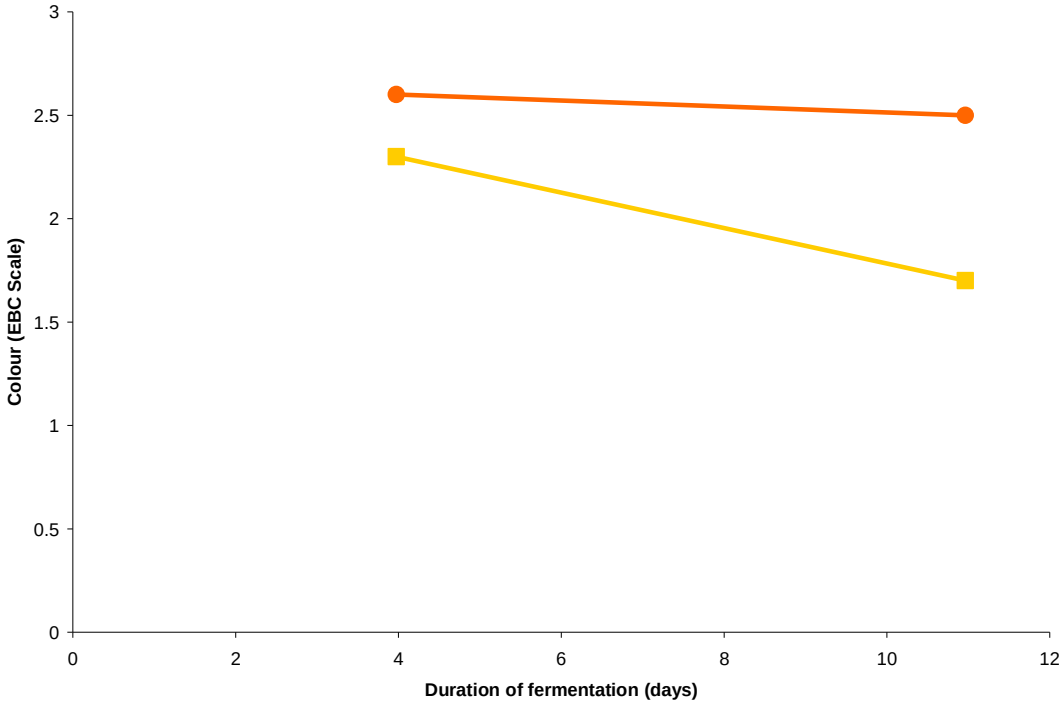


Figure 5.10. EBC "colour" of the control (orange) and pectin plus carrageenan treated (yellow) white wines after 96 and 264 h of the pilot-scale fermentation.

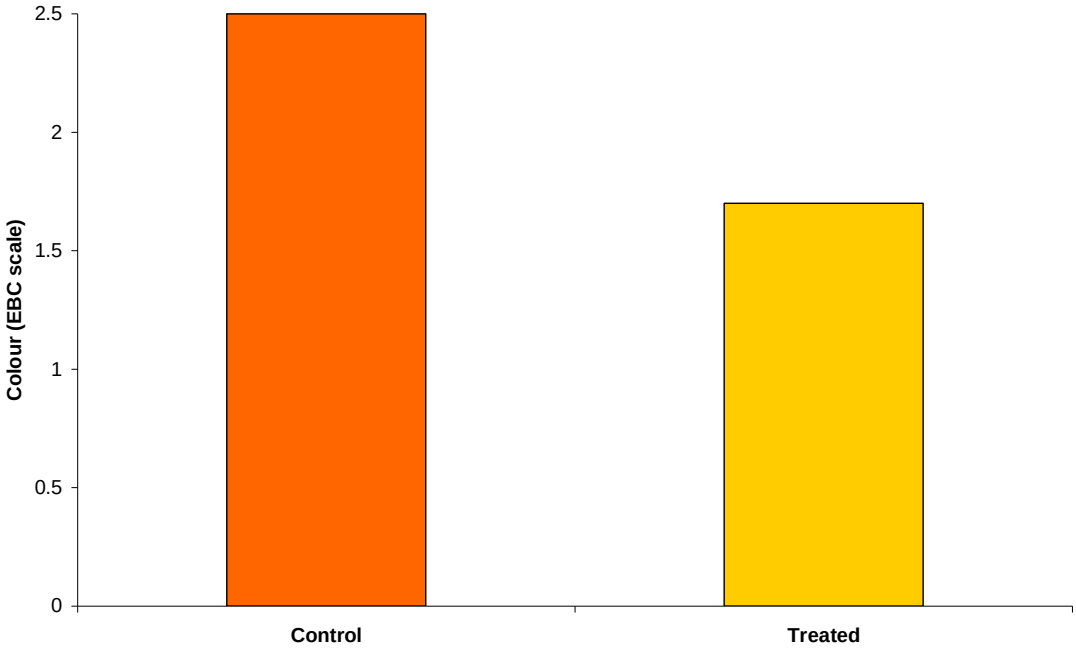


Figure 5.11. EBC "colour" of the control (orange) and pectin/carragenan treated (yellow) white wines after 264 h of the pilot-scale fermentation. The treated wine had a 32% reduction in colour compared to the control.

As with the heat stability tests, the synthetic grape juice media fermentation samples could not be tested due to the low absorbance of this media. The real grape juice fermentations were monitored, however, as shown in Figure 5.12. The four fermentations began at the same point and the carrageenan and the pectin and carrageenan treatments remained steady throughout the entire fermentation. However, after 4 days, the colour for both the control and pectin fermentations increased. The control rose sharply until day 7 (4.1 units on the EBC scale compared to the ~1.7 "baseline" of the other samples) and then slowly decreased, but ended at a much higher level than the starting point (2.9). The pectin treated wine rose with a lower peak of 2.7 units on the EBC scale around day 7 and then decreased back to the starting point around the 12th day (1.7). The increased colour of the control wine compared to the treated wines demonstrated that the pectin and carrageenan were able to prevent an increase rather than causing a decrease in the colour. The carrageenan was also able to over-ride the pectin affect, shown by the pectin peak and the lack of such a peak in the pectin and carrageenan wine.

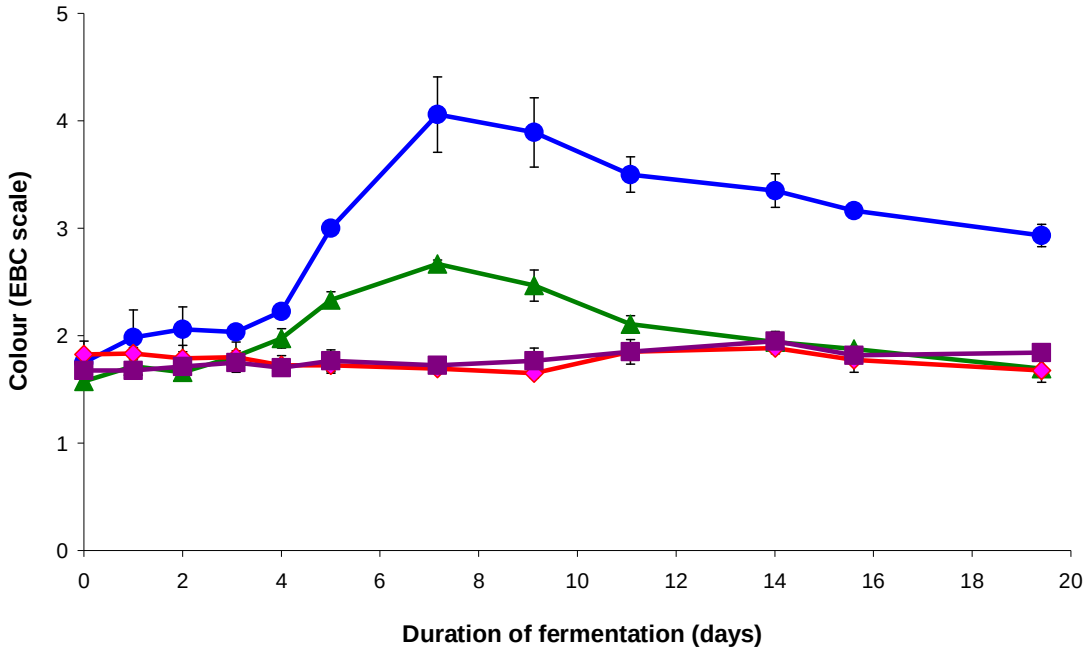


Figure 5.12. The effect of pectin and carrageenan on wine EBC "colour". The colour levels were measured throughout the time course during the grape juice lab-scale fermentation. The control is denoted in blue, pectin in green, carrageenan in red and pectin plus carrageenan is purple.

5.4.3. Protein concentration

Excessive protein concentration contributes to hazing in wine. Protein concentration was measured at the end of the synthetic grape juice media fermentation and the results are shown in Figure 5.13. The pectin treated wine contained 0.61 mg/mL protein compared to only 0.34, an 82% increase. The carrageenan treated wine contained 0.15 mg/mL, only 46% the amount of protein in the control. The pectin/carrageenan treated wine contained 0.42 mg/mL, 23% more than the control. While pectin increased the protein concentration in the wine and carrageenan reduced it, together there was a slight increase. While this seems counterintuitive since increased protein concentrations should lead to wine instability and other researchers have reported reduced protein levels (Marangon et al., 2012), the heat stability tests show that the treatments resulted in increased stability.

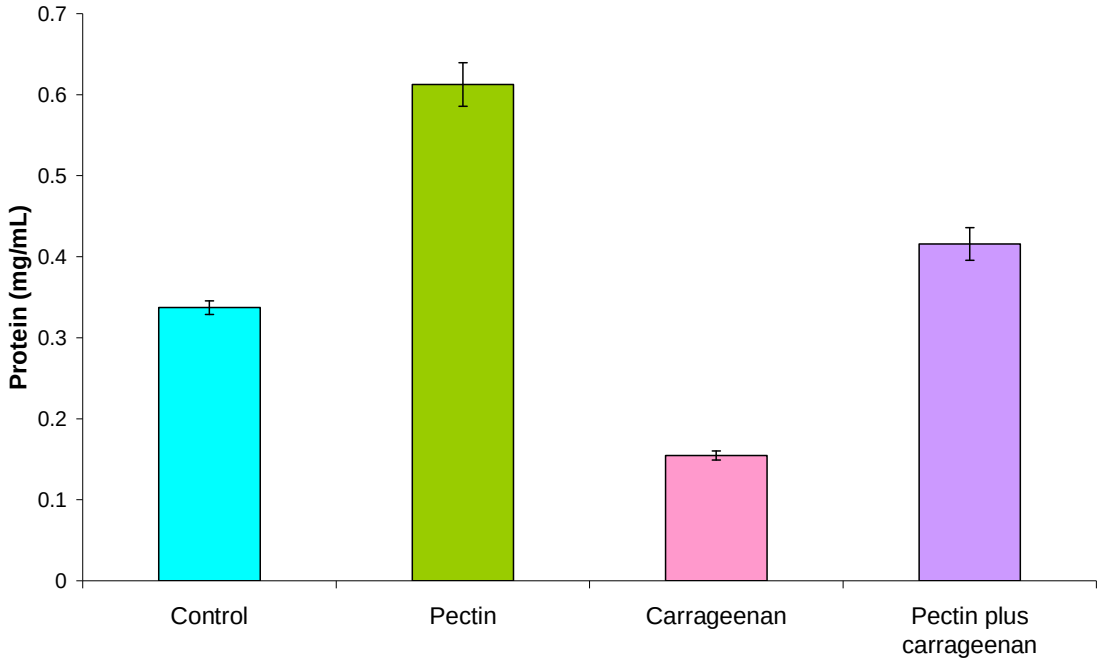


Figure 5.13. Protein concentrations after 336 hours of fermentation using synthetic grape juice media, using a modified Bradford's reagent and BSA as a standard.

5.4.4. pH levels of wine were not significantly affected by pectin and carrageenan

pH levels were monitored for the three fermentations – for the white and red wines in the pilot-scale fermentations at Fosters (Figure 5.14 and Figure 5.15, respectively), the synthetic grape juice media fermentation (Figure 5.16) and the real grape juice fermentation (Figure 5.17). The figures below all show a range of pH between 2.4 and 4, to enable easier comparisons. There was a difference between the pH of the different fermentations, with the pilot-scale red wine fermentation around 3.9 while the real grape juice fermentation at UWS recorded a pH of about 3. However, comparisons within each of the fermentation sets showed that the pH levels were not significantly different. While the two pilot-scale fermentations were only monitored at two timepoints, the UWS ones were monitored throughout the fermentation. Both of these showed a slight dip in pH at the second to third day of fermentation, followed by an increase back up to the original level.

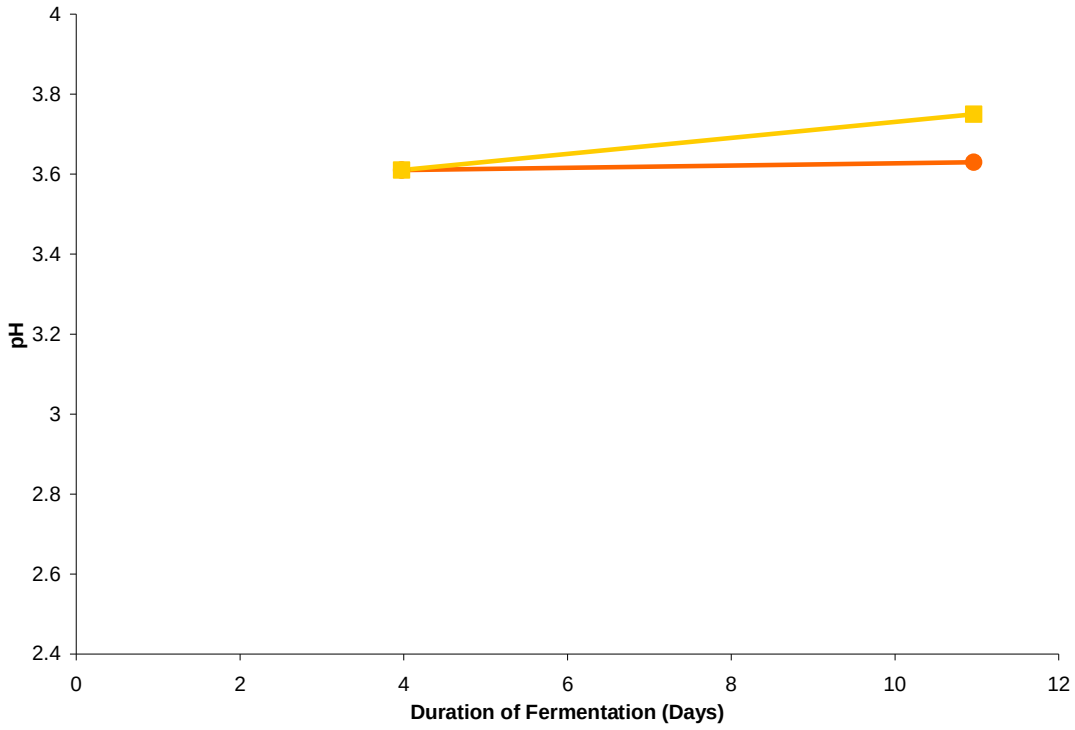


Figure 5.14. pH of the control (orange) and pectin plus carrageenan treated (yellow) white wines after 96 and 264 h of the pilot-scale fermentation at Foster's. The pH at the end of the fermentation was 3.63 and 3.75 for control and treated respectively.

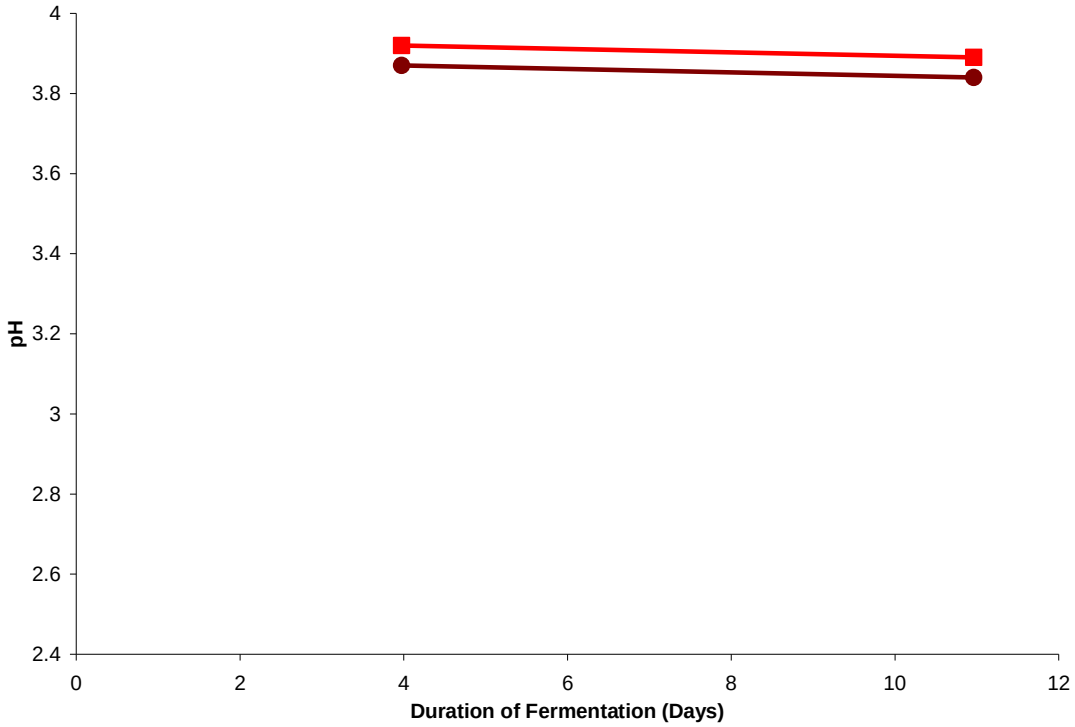


Figure 5.15. pH of the control (burgundy) and pectin plus carrageenan treated (red) red wines after 96 and 264 h of the pilot-scale fermentation at Foster's. The pH at the end of the fermentation was 3.84 and 3.89 for control and treated respectively.

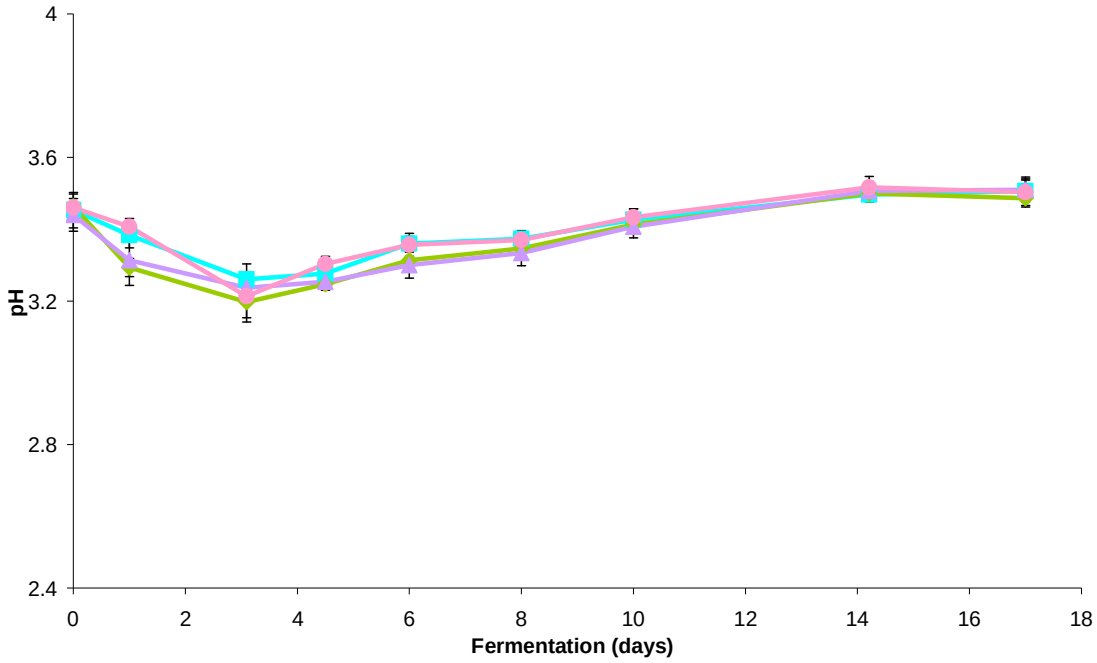


Figure 5.16. pH of triplicate control (light blue), pectin (lime green), carrageenan (pink) and pectin plus carrageenan (lavender) treated white wine monitored throughout the synthetic grape juice lab-scale fermentation.

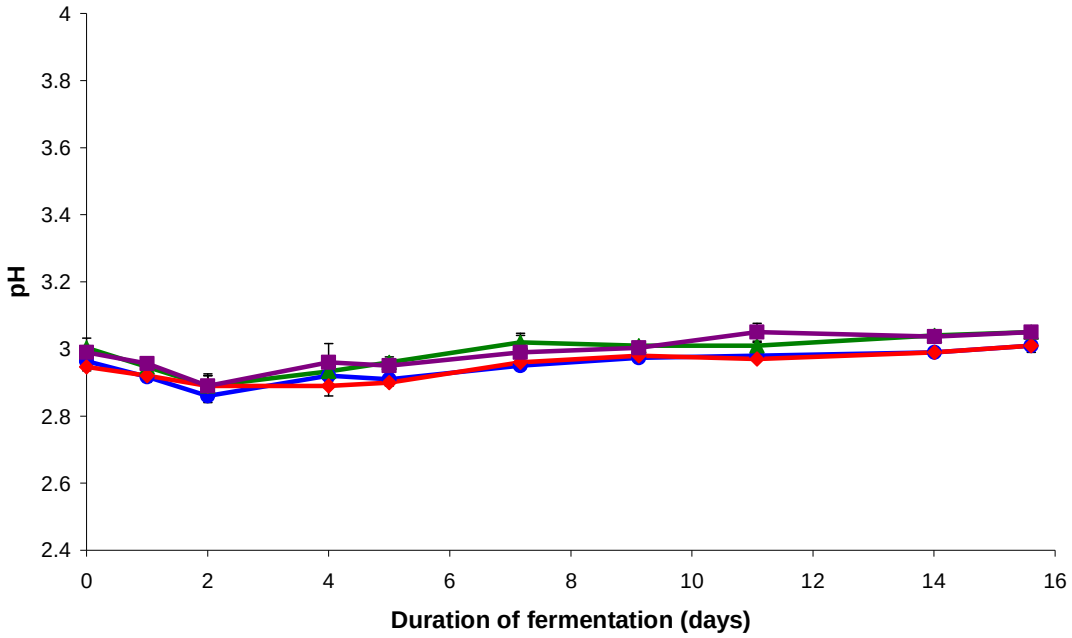


Figure 5.17. pH of triplicate control (blue), pectin (green), carrageenan (red) and pectin plus carrageenan (purple) treated white wine monitored throughout the lab-scale fermentation.

5.5. Metal ion analysis showed that pectin and carrageenan treated wine contained more sodium and less calcium than control wine

The concentration of sodium, potassium, magnesium and calcium ions was determined for the white and red wine pilot-scale fermentations only. These results are shown in the following graphs with all values normalised to the relevant control to allow for comparison. Sodium concentrations more than doubled in the pectin plus carrageenan treated wine (58 and 126 mg/L for the control and treated wines). This was also the case for the red wine, although the increase was slightly less (44 versus 84 mg/L). Potassium concentrations were above the detection limit for the white wine samples; the red wine concentrations were unchanged. Magnesium concentrations were 14% higher for the treated white wine sample but 3.8% lower in the treated red wine. Calcium concentrations decreased in the treated white and red wines, by 28% and 14%, respectively).

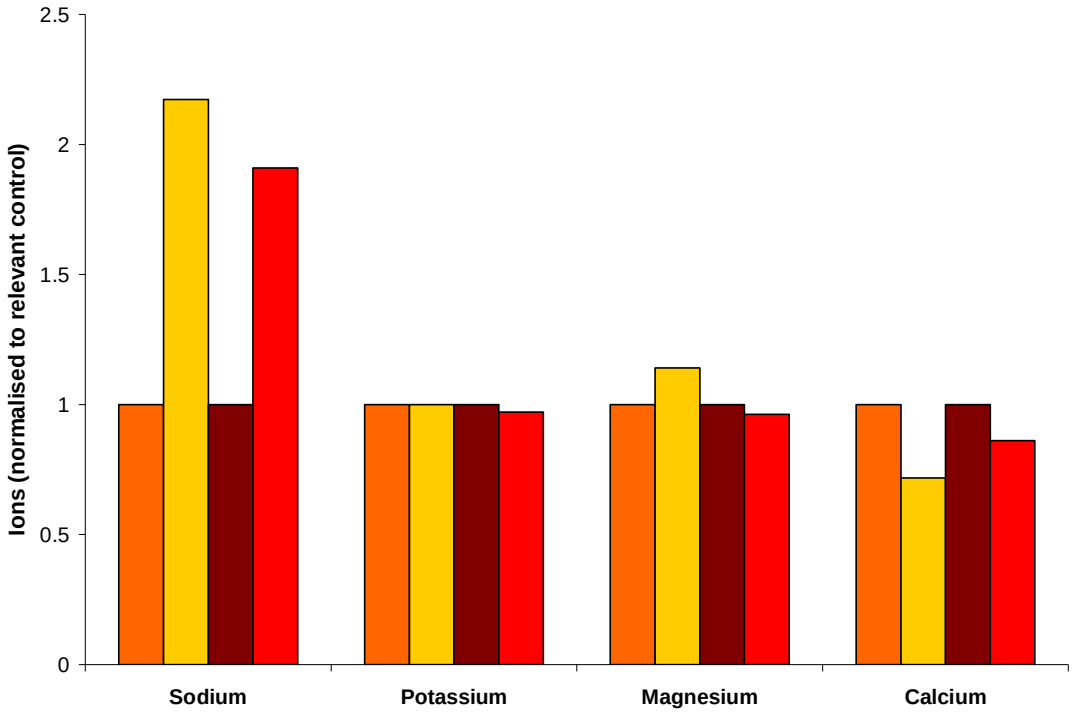


Figure 5.18. Metal ion concentrations in white and red wines, with values normalised to the corresponding control. Dark orange = White wine control; Yellow = White wine treated; Burgundy = Red wine control; Red = Red wine treated. Note: Potassium exceeded levels of detection in white wine and was normalised to 1. (n=1)

Sodium and calcium were most affected by the fining treatment (Figure 5.19 and Figure 5.20). This is because of the carryover of sodium in both pectin and carrageenan. It is interesting to note that for both wine types, the concentration of sodium increases over the time course of the fermentation. There were decreased concentrations of calcium in the treated samples, 28% and 14% lower in the white and red wines, respectively. Pectin forms a complex with calcium ions and the presence of calcium contributes to the problem of hazing by destabilising proteins. There is a very slight reduction in calcium over the course of the fermentation.

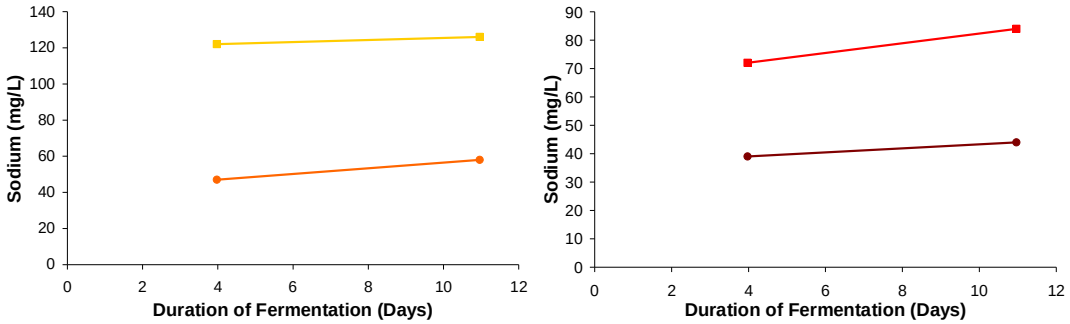


Figure 5.19. Sodium concentrations of pectin and carrageenan treated and control fermentations for pilot-scale white (left) and red (right) wine. Key: dark orange = control white wine, yellow = treated white wine, burgundy = control red wine and red = treated red wine.

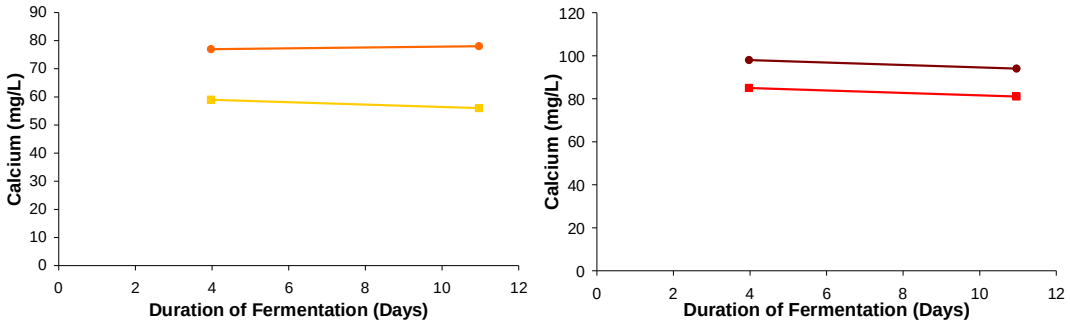


Figure 5.20. Calcium concentrations of pectin and carrageenan treated and control fermentations for pilot-scale white (left) and red (right) wine. Key: dark orange = control white wine, yellow = treated white wine, burgundy = control red wine and red = treated red wine.

Studies that were later carried out at Foster's in Melbourne by Dr David Duan, Peter Rogers and Allen Hart, using Victorian grape juice, where they achieved reduction in calcium concentrations as well. Pectin treated wine had the largest decrease, whereas pectin plus carrageenan responded like pectin only did (personal communication, 2012).

5.6. Volatile flavour compounds are increased by the pectin and carrageenan treatment in wine

5.6.1. Sensory panels

An amateur sensory panel, consisting of myself and a semi-trained Fosters wine taster, Dr David Duan, were able to observe a difference between the trial and control wine fermentations, in terms of smell and taste, from the fifth day of fermentation onwards. There was apparently a much stronger banana flavour from the treated fermentations. The wine odour was sweeter, more pleasant and occurred earlier for the treated wine.

A larger amateur sensory panel was carried out for the second fermentation, using synthetic grape juice. However, due to the chemicals used to make the media, the wine was only smelled rather than tasted. Each of the 20 panel members were asked to smell the four conditions from one of the three replicates plus two controls; a commercial un-oaked Chardonnay and a 10% ethanol solution. The solutions were given random codes to ensure that participants would remain unbiased and served in a white wine glass. Panel members were asked to rank the smell of the solutions from 0 to 5, giving each a unique rank. The control wine was given an average rank of 2 and the treated wines were 2.8 (an increase of 40%), 2.5 (25% higher) and 2.95 (47.5% higher) for pectin, carrageenan and pectin plus carrgeenan treatments, respectively (data not shown).

The third fermentation set used frozen grape juice and, therefore, the sensory panel could taste the wine. This time, all of the samples were smelled and tasted by all of the participants, instead of just a single replicate, except for one participant who smelled but did not taste the wine.

Each person rated the sweetness, fruitiness and desirability of the aroma and taste of each test wine. ANOVA significance tests determined that sweetness and desirability of the odour of the wine was not significant ($P = 0.708$ for sweetness and 0.606 for the desirability of the aroma). However, the fruity aroma as well as sweetness, fruitiness and desirability of the taste of the wine had significant differences and are graphed below in Figure 5.21.

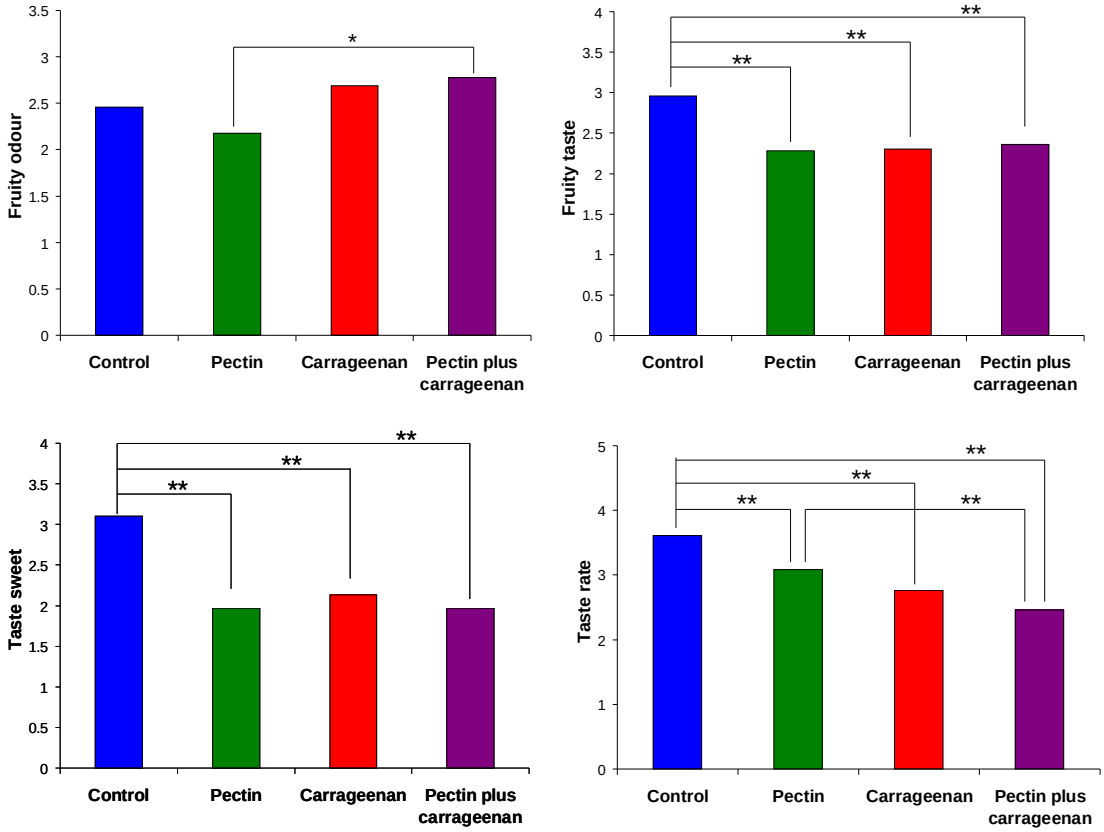


Figure 5.21. Sensory panel analysis of pectin and carrageenan treated wine. Connecting bars indicate significance. * indicates $P < 0.05$, ** indicates $P < 0.005$. Key: blue = control, green = pectin treated wine, red = carrageenan treated wine, purple = pectin and carrageenan treated wine.

Figure 5.21 shows the average rating the sensory panel gave each criteria – fruitness of the aroma, fruitiness of the taste, sweetness (taste) and overall rate when tasting the wine. Panel members were also asked for the sweetness and overall rate of the aroma of the wine, but there was no significant difference in those categories. The carrageenan and pectin plus carrageenan treated wine had more of a fruity smell than the control, with a significant difference between pectin and pectin plus carrageeenan. The control was higher than the three treatments in all of the taste tests – fruity, sweetness and overall rate, with a significant difference beteween the control and each of the treatments. While there was no significant difference between the fruitiness and sweetness of the treated wines to each other, the pectin treated wine received the highest overall rate, significantly higher than the pectin and carrageenan treated wine.

5.6.2. GC-MS volatile flavour analysis of the pilot-scale fermentation

The final timepoint is more indicative of what the actual wine will taste like for consumers – after this point, the wine is bottled and stored before purchase. At the end point of the pilot-scale fermentation at the Fosters Group in Melbourne, the overall flavour compound composition of the wine samples was higher for the treated samples than for their relevant controls, as shown in Figure 5.22. The red wine samples were consistently higher in flavour concentrations than the white wine samples, although the increase between treated compared to control is more prominent for the white wine than for the red wine. The concentration of all the flavour compounds tested (esters, acids and higher alcohols combined) was 259 mg/L for the control white wine and 352 mg/L for the treated white wine, an increase of 36%. For the red wine samples, the difference was 19% with 317 mg/L flavours detected in the controls and 379 mg/L for the treated wine.

Whilst clarity was enhanced by the pectin and carrageenan treatment, it also resulted in a higher flavour profile outcome in the white wine than in the red wine. There was a 49% increase in esters (fruity flavours), 34% increase in higher alcohols and 58% increase in volatile acids in the treated white wine compared to the control white wine. These values for the red wines were 5%, 23% and 27%, respectively. However, the white wine flavour increase of 36% suggests a possibility to winemakers, of diluting three litres of the 14% alcohol (v/v) pectin and carrageenan treated wine to get four litres of 9% alcohol (v/v) 'normal' flavoured wine, resulting in 35% more wine for a similar cost and therefore more profit for the wine industry and/or lower prices for the consumers. This could be beneficial to the wine industry because consumers desire wine with low alcohol content, due to the negative consequences of alcohol abuse, without compromising the wine's flavour (Erten and Campbell, 2001). This is also good for society so it could increase the popularity of wine companies as they can be seen to be socially conscious. A few drinks are actually beneficial to the health of moderate drinkers, as discussed in Section 1.2, Chapter 1. The flavour profile would be improved in this situation because while the dilution would reduce the higher alcohols to their normal concentration, the fruity esters and the acids would still remain at a higher concentration in the diluted wine. The acids are below the sensory detection level of humans (as explored below in Section 5.10) so that the fruity esters should be the only real difference, thereby

suggesting an improvement in the overall flavour of the white wines, although this would have to be tested by a sensory panel.

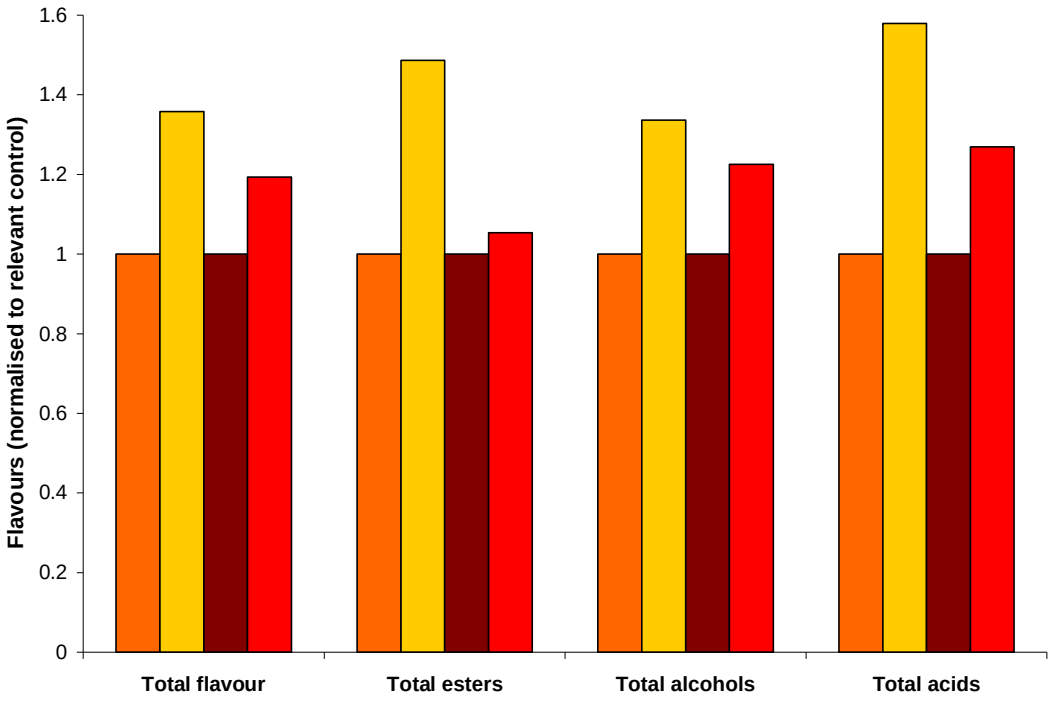


Figure 5.22. Comparative flavour levels between control and pectin plus carrageenan wines at Foster's, with values normalised to the corresponding controls. Dark orange = White wine control; Yellow = White wine treated; Burgundy = Red wine control; Red = Red wine treated. (n=1).

Table 5.1. Flavour concentrations in treated and control white and red wines in the pilot-scale fermentation at the final timepoint (in mg/L) (n=1).

	Control white wine	**Treated white wine**	**Control red wine**	**Treated red wine**
Ethyl acetate	23.1	34.1	58.72	61.42
Ethyl hexanoate	0.33	0.53	0.14	0.16
Ethyl octanoate	0.34	0.61	0.06	0.12
Phenylethyl acetate	0.23	0.28	0.24	0.6
Isoamyl acetate	1.07	1.75	0.56	0.64
Total esters	**25.07**	**37.27**	**59.72**	**62.94**
n-propanol	44.6	64.4	12.94	29.34
Isobutanol	42.7	71.2	46.62	67.86
Isoamyl alcohol	106.1	135	124.82	144.32
Phenylethyl alcohol	33.2	32.2	70.04	70.18
Total alcohols	**226.6**	**302.8**	**254.42**	**311.7**
Butyric acid	1.26	1.94	0.64	0.68
Hexanoic acid	2.14	3.03	1.08	1.24
Octanoic acid	3.22	4.99	1.24	1.68
Decanoic acid	0.8	1.76	0.38	0.64
Total acids	**7.42**	**11.72**	**3.34**	**4.24**
Total flavours	**259.09**	**351.79**	**317.48**	**378.88**

Table 5.2. Percentage change in the aroma compound concentrations between pectin and carrageenan treated white and red wines over control wine in the pilot-scale fermentation at the final timepoint.

	Change measured in the white wine samples (%)	Change measured in the red wine samples (%)
Ethyl acetate	47.6	4.6
Ethyl hexanoate	60.6	14.3
Ethyl octanoate	79.4	100.0
Phenylethyl acetate	21.7	150.0
Isoamyl acetate	63.6	14.3
Total esters	**48.7**	**5.4**
n-propanol	44.4	126.7
Isobutanol	66.7	45.6
Isoamyl alcohol	27.2	15.6
Phenylethyl alcohol	-3.0	0.2
Total alcohols	**33.6**	**22.5**
Butyric acid	54.0	6.3
Hexanoic acid	41.6	14.8
Octanoic acid	55.0	35.5
Decanoic acid	120.0	68.4
Total acids	**58.0**	**26.9**
Total flavours	**35.8**	**19.3**

5.7. Esters of the treated wine were increased in pilot-scale fermentation.

Esters are an essential part of the flavour of wine, adding primarily sweet fruity or floral aroma and flavours, as described in Section 1.5.1. They are found at very low concentrations, but due to their low flavour thresholds, they greatly impact the wine characteristics. In the pilot-scale fermentation, the esters tested were ethyl acetate, ethyl hexanoate, ethyl octanoate, phenylethyl acetate and isoamyl acetate. The

concentration of each of these five compounds was added to calculate the total ester content of the wines.

The total ester quantities increased with the treatment by 48.6% in the white wine samples, from 25.1 mg/L in the control wines to 37.3 mg/L total esters in the pectin and carrageenan treated wines. The red wine differences were lower despite a higher amount of esters in the wine. The difference was only 5.4%, 59.7 mg/L for the control wine up to 62.9 mg/L for the treated wine, however at 96 h, the treated wine contained 57% more esters than the control wine (14.0 mg/L and 32.5 mg/L for the control and treated, respectively).

The most prominent ester was ethyl acetate, which has a fruity, pineapple and slightly solvent odour, accounting for more than 90% of the esters. The percentage change in white wine for ethyl acetate was 47.6%, however, for red wine, the change was insignificant (only 4.6%). The most significant change in esters was phenylethyl acetate in red wine at a 150% increase, although this change for white wine was only 21.7%. As shown in Figure 5.23, Figure 5.24 and Figure 5.25, in all cases, the pectin and carrageenan treated wine contained more ester than the corresponding control. This can be an improvement for the wine as esters have fruity and floral characteristics, but the resultant effect on wine flavour needs to be confirmed by formal sensory evaluation tests.

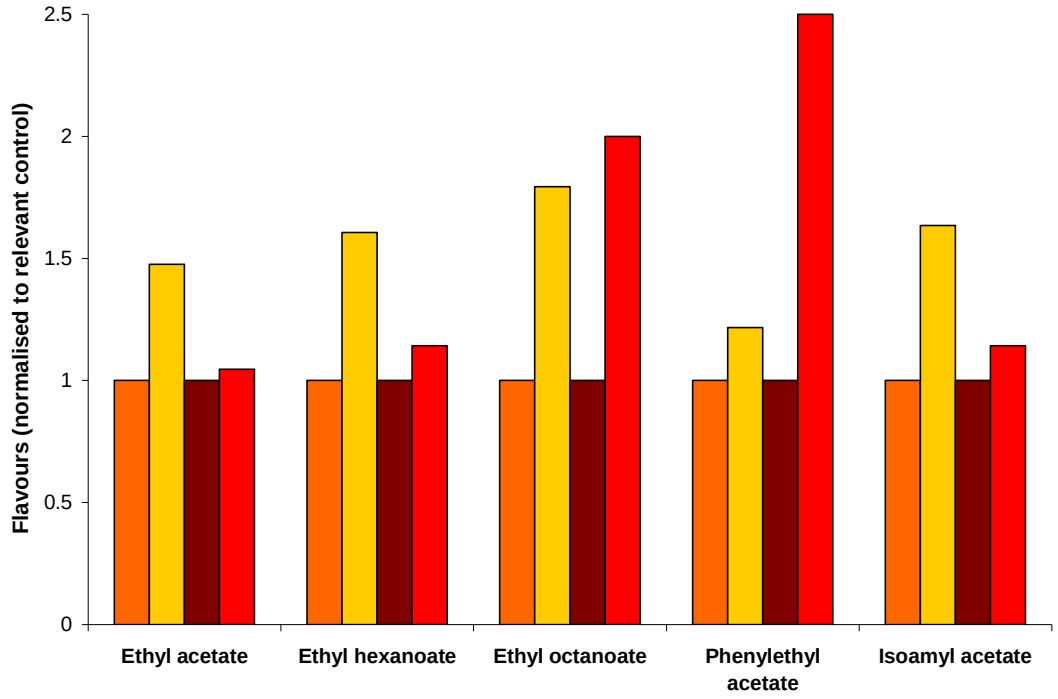


Figure 5.23. Comparative ester quantities in control and treated wines, with values normalised to the corresponding controls. Dark orange = White wine control; Yellow = White wine treated; Burgundy = Red wine control; Red = Red wine treated.

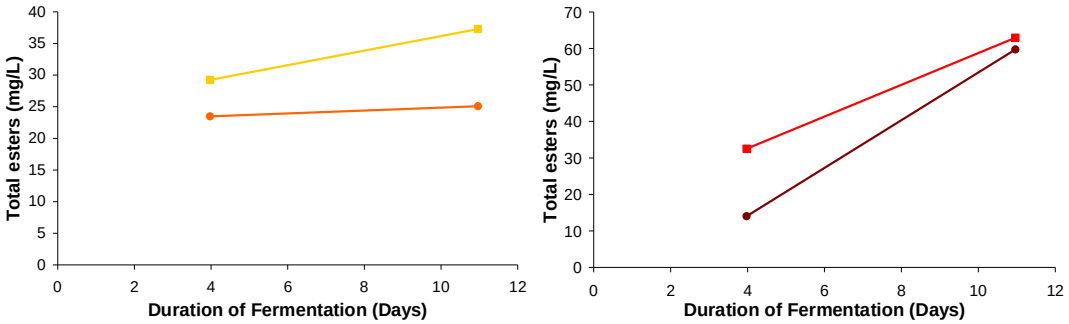


Figure 5.24. Total ester quantities in pectin and carrageenan treated and control fermentations for pilot-scale white (left) and red (right) wine. Key: dark orange = control white wine, yellow = treated white wine, burgundy = control red wine and red = treated red wine.

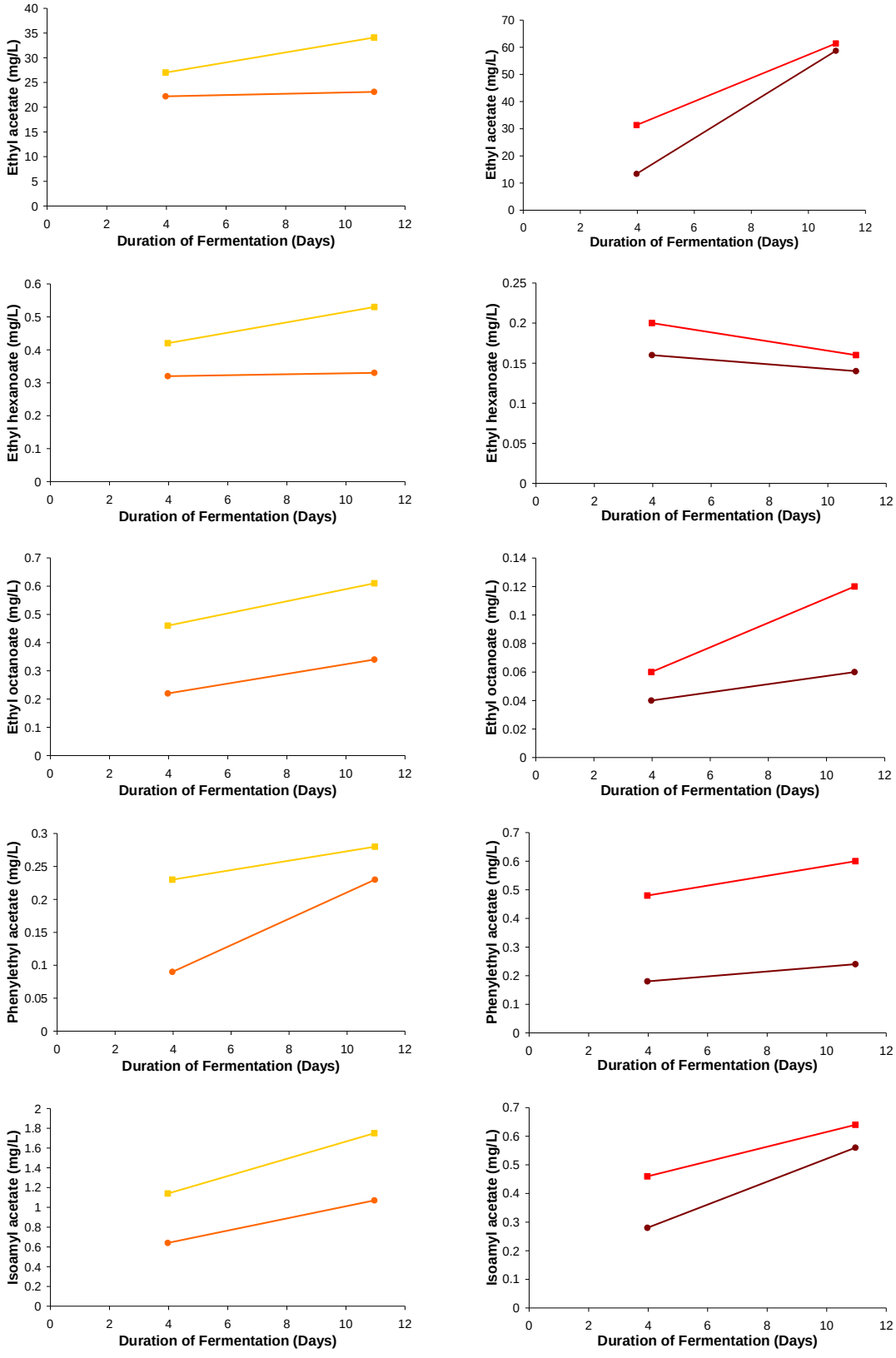


Figure 5.25. Quantities of the esters, ethyl acetate, ethyl hexanoate, ethyl octanoate, phenylethyl acetate and isoamyl acetate, in pectin and carrageenan treated and control fermentations for pilot-scale white (left) and red (right) wine. Key: dark orange = control white wine, yellow = treated white wine, burgundy = control red wine and red = treated red wine.

5.8. Higher alcohols of the treated wine were increased in pilot-scale fermentation

As discussed in Section 1.5.2, higher alcohols are found in higher concentrations than most flavour compounds. When present at optimal concentrations of 300 mg/L or less, higher alcohols add a beneficial complexity and fruity flavour to wine. This optimal concentration only applies to regular wine and not distilled wine, such as brandy, which contains greater concentrations of higher alcohols. In the pilot-scale fermentation, n-propanol, isobutanol, isoamyl alcohol and phenylethyl alcohol were measured. The pectin and carrageenan treatment increased total alcohols from 226.6 mg/L to 302.8 mg/L (33.6%) in white wine and from 254.4 mg/L to 311.7 mg/L (22.5%) in red wine. Higher alcohols made up more than 80% of the total flavour compounds.

The most prominent higher alcohol in the fermentations was isoamyl alcohol, which gives wine an unpleasant cheesy flavour when present at concentrations above its flavour threshold, however, when it is below this concentration, isoamyl alcohol can add a fruity undertone to wine. The largest increase was for n-propanol with 126.7% for red wine. All the esters increased, except for phenylethyl alcohol which was not significantly altered, with a 3% decrease in white wine and a 0.2% increase in red wine. Figure 5.26, Figure 5.27 and Figure 5.28 shows that n-propanol, isobutanol and isoamyl alcohol were increased by the treated whereas phenylethyl alcohol was unchanged at the end of the fermentation.

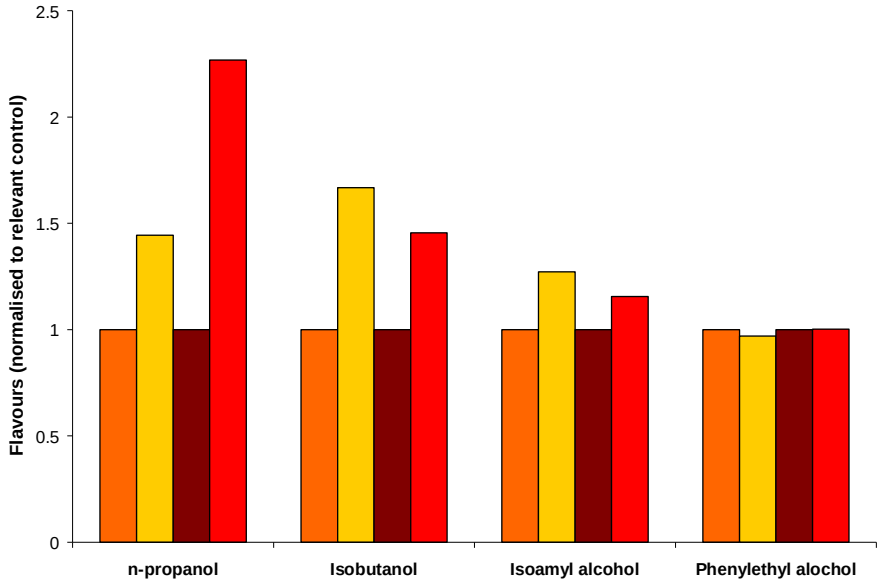


Figure 5.26. Comparative higher alcohol concentrations between control and treated wines, with values normalised to the corresponding controls. Dark orange = White wine control; Yellow = White wine treated; Burgundy = Red wine control; Red = Red wine treated.

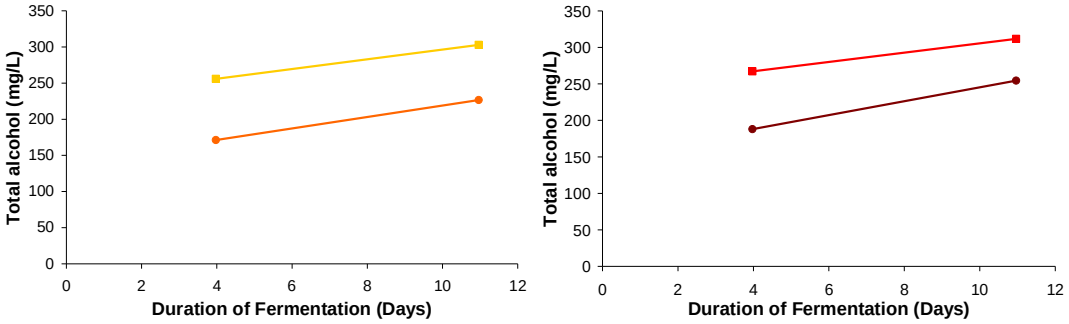


Figure 5.27. Total alcohol concentrations in pectin and carrageenan treated and control fermentations for pilot-scale white (left) and red (right) wine. Key: dark orange = control white wine, yellow = treated white wine, burgundy = control red wine and red = treated red wine.

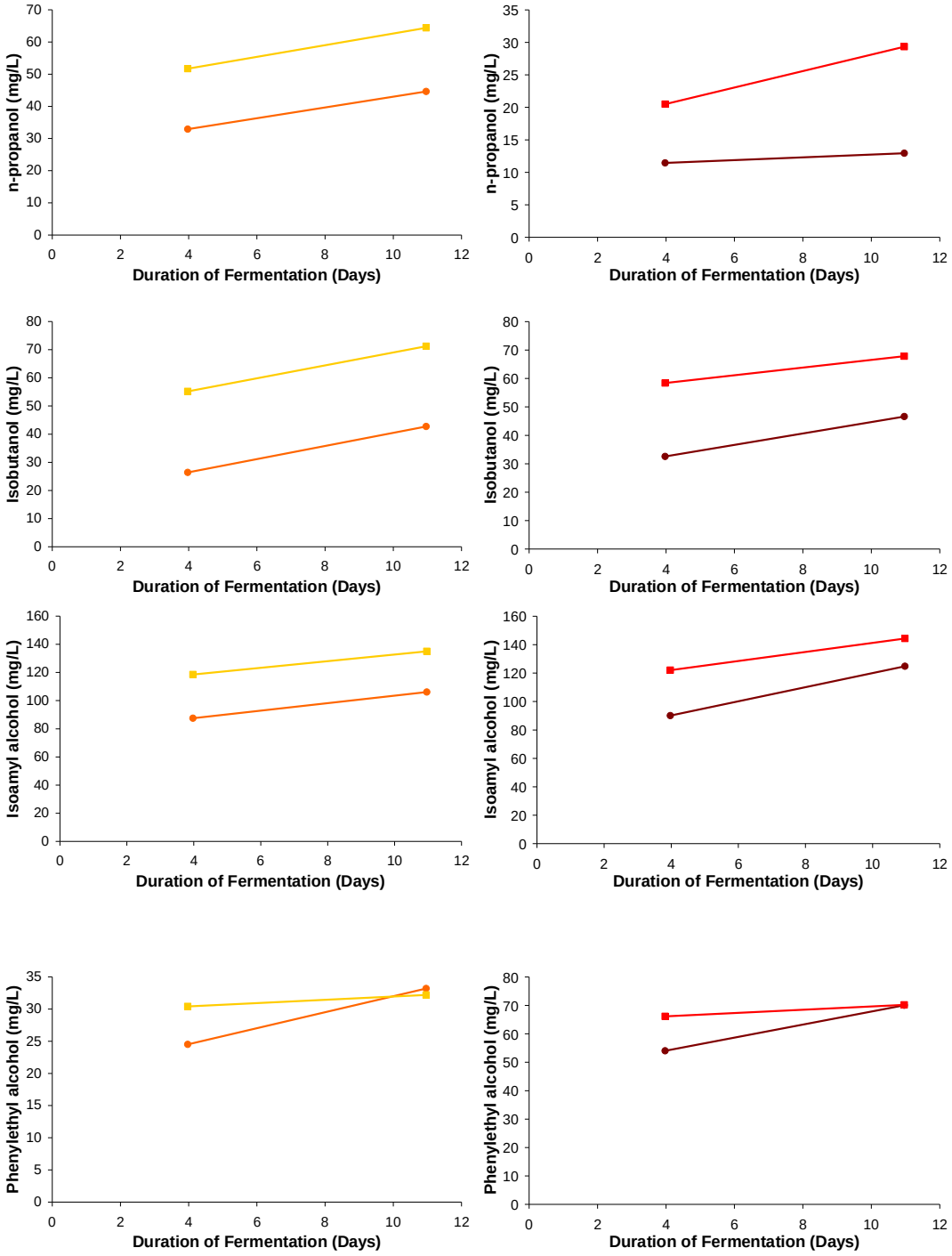


Figure 5.28. Concentrations of the alcohols, n-propanol, isobutanol, isoamyl alcohol and phenylethyl alcohol, in pectin and carrageenan treated and control fermentations for pilot-scale white (left) and red (right) wine. Key: dark orange = control white wine, yellow = treated white wine, burgundy = control red wine and red = treated red wine.

5.9. Acids of the treated wine were increased in pilot-scale fermentation

Acids are generally negative flavour compounds, as discussed in Section 1.5.4, Chapter 1. They are mostly by-products of yeast fatty acid metabolism and have unpleasant flavours. The acids that were tested were butyric acid, hexanoic acid, octanoic acid and decanoic acid.

The total acids increased from 7.42 mg/L to 11.72 mg/L in the white wine and from 3.34 mg/L to 4.24 mg/L in the red wine (increases of 58% and 27%, respectively). Decanoic acid was the flavour compound that was increased the most, with a 120% increase in white wine and 68.4% increase in red wine. The lowest increase was 6.3%, which was butyric acid in red wine, although this increase was 54% in the white wine samples. Figure 5.29, Figure 5.30 and Figure 5.31 demonstrate these increases.

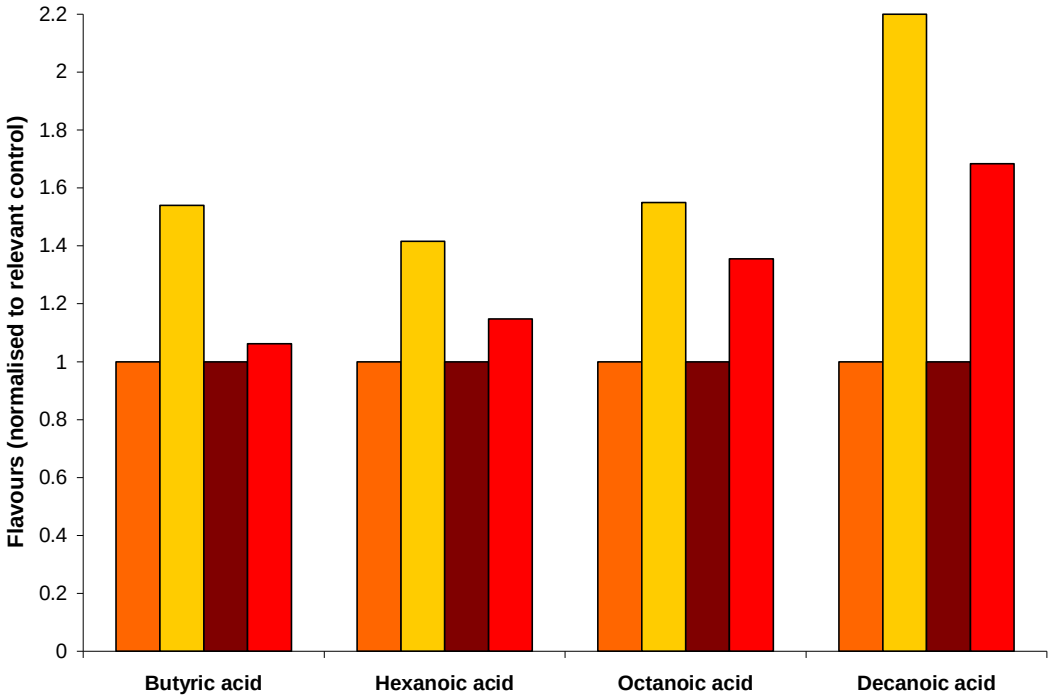


Figure 5.29. Comparative acid concentrations between control and treated wines, with values normalised to the corresponding controls. Dark orange = White wine control; Yellow = White wine treated; Burgundy = Red wine control; Red = Red wine treated.

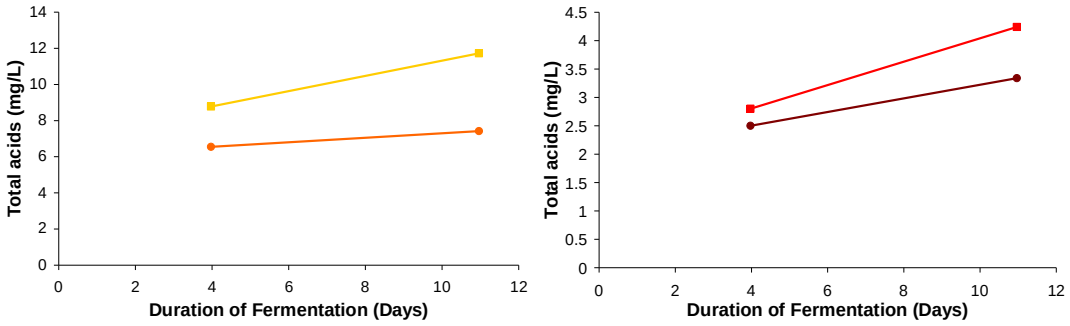
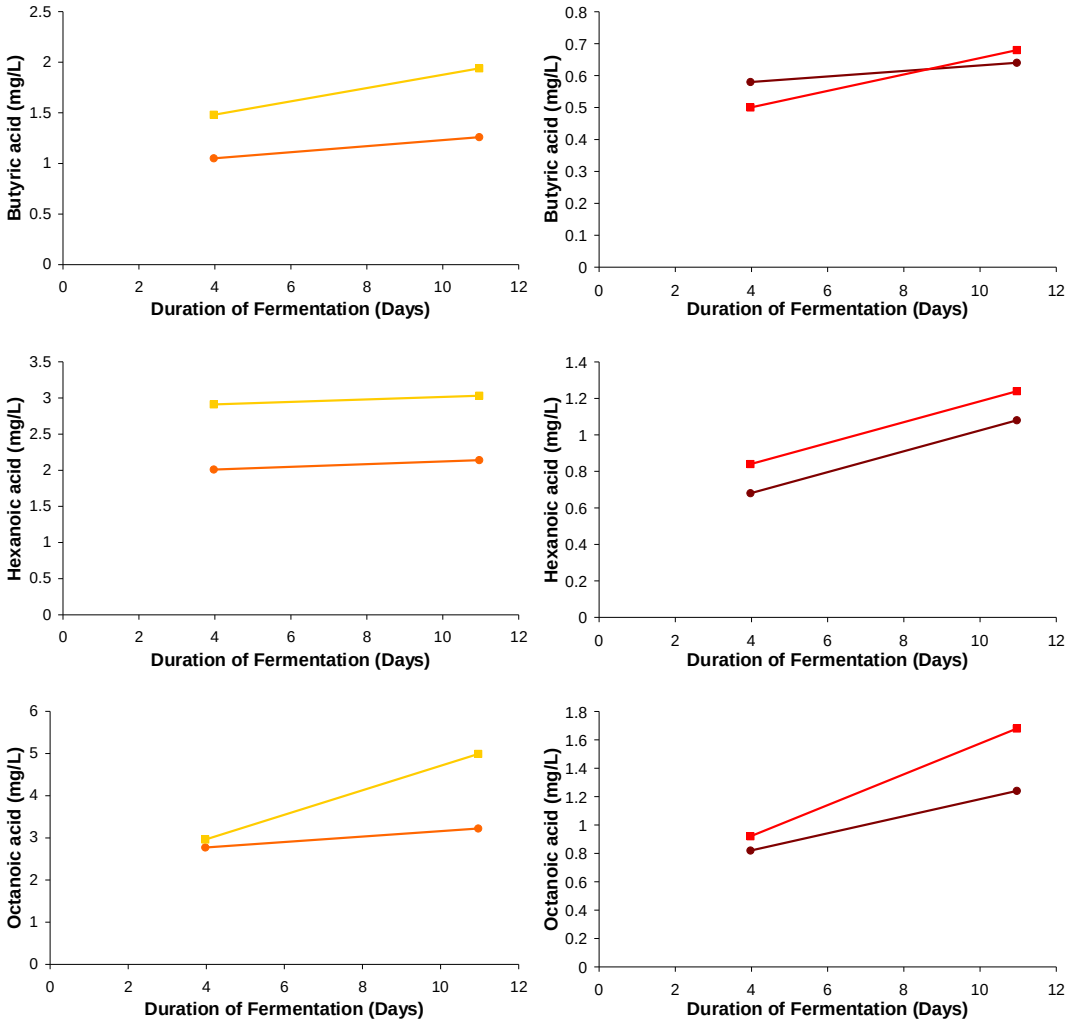


Figure 5.30. Total acid concentrations in pectin and carrageenan treated and control fermentations for pilot-scale white (left) and red (right) wine. Key: dark orange = control white wine, yellow = treated white wine, burgundy = control red wine and red = treated red wine.



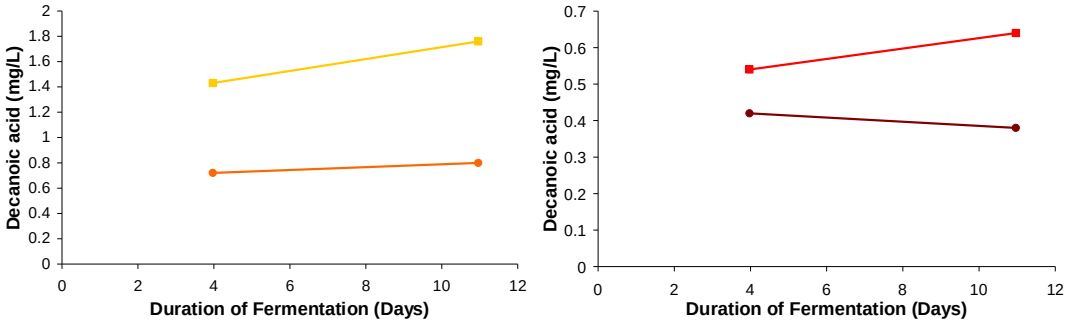


Figure 5.31. Concentrations of the acids, butyric acid, hexanoic acid, octanoic acid and decanoic acid, in pectin and carrageenan treated and control fermentations for pilot-scale white (left) and red (right) wine. Key: dark orange = control white wine, yellow = treated white wine, burgundy = control red wine and red = treated red wine.

In summary, esters, higher alcohols and acids all increased as a result of the pectin plus carrageenan treatment. Despite the increase in the negative flavours as well as the increase in the positive flavours, only the fruity positive flavours seem to come through in the flavour and odour of the final wine. The reason behind this lies in the flavour thresholds of different compounds, which is investigated in the next section.

5.10. Flavour thresholds and olfactory activity values (OAV)

Various flavour compounds are detected by humans at different concentrations. The concentration of a volatile flavour compound where 50% of the population are expected to detect the presence of the compound is called the flavour threshold. The olfactory activity value (OAV), which is the division of the flavour concentration by the flavour threshold, indicates whether a flavour would be perceived as present (OAV > 1) or absent (OAV < 1) (Du et al., 2010). Since the flavour analysis for the pilot-scale fermentation was quantitative, OAVs could be calculated for these flavour compounds, using the flavour thresholds stated in the Introduction. Table 5.3 shows that a number of the compounds would not be detected in either the treated or control wines, even though the treatment had caused an increase in the concentration of the flavour compound.

Table 5.3. Olfactory activity values (OAV) for flavour compounds in treated and control white and red wines in the pilot-scale fermentation at the final timepoint. Bold indicates OAV above 1, indicating that these compounds are detectable by humans.

	Control white wine	**Treated white wine**	**Control red wine**	**Treated red wine**
Ethyl acetate	**3.1**	**4.5**	**7.8**	**8.2**
Ethyl hexanoate	**66**	**106**	**28**	**32**
Ethyl octanoate	**170**	**305**	**30**	**60**
Phenylethyl acetate	0.92	**1.12**	0.96	**2.4**
Isoamyl acetate	**35.7**	**58.3**	**18.7**	**21.3**
n-propanol	0.05	0.08	0.02	0.04
Isobutanol	**1.07**	**1.8**	**1.17**	**1.7**
Isoamyl alcohol	**3.5**	**4.5**	**4.2**	**4.8**
Phenylethyl alcohol	**3.3**	**3.2**	**7.0**	**7.0**
Butyric acid	0.13	0.19	0.06	0.07
Hexanoic acid	0.71	**1.01**	0.36	0.4
Octanoic acid	0.37	0.57	0.14	0.19
Decanoic acid	0.05	0.12	0.03	0.04

Compounds with high OAV indicate suggest that these compounds have a larger impact on the wine flavour profile than compounds with low OAV, for example, ethyl hexanoate, ethyl octanoate and isoamyl acetate, all with OAV above 10 and with fruity, green apple, pear and banana characteristics, which were evident in the wine. The OAV is much more informative than the raw concentrations in determining the affect the flavour compound actually has on the wine itself.

These data can be visualised using radial plots illustrating the OAVs normalised to the OAV of the control for white wine (Figure 5.32) and red wine (Figure 5.33). This demonstrates that while there is an increase in the flavour compounds caused by the treatment (except for phenylethyl alcohol), some of these compounds are still below the detection level for humans. These compounds are n-propanol and all of the acids, except for hexanoic acid in the treated white wine, which is only at 1.01. These compounds have pungent, harsh, cheese, rancid, cheese and fatty characteristics,

which are undesirable in wine, however, due to the low OAV, these increases do not have any affect on the wine.

An interesting compound is phenylethyl acetate, which has floral and fruity characteristics and is undetectable in both of the control wines with OAVs of 0.92 and 0.96 but detected in the treated wines with OAVs of 1.12 and 2.4 (for the white and red wines, respectively). This flavour compound is desirable in wine, therefore, it would result in an improvement of the flavour profile in the treated wines.

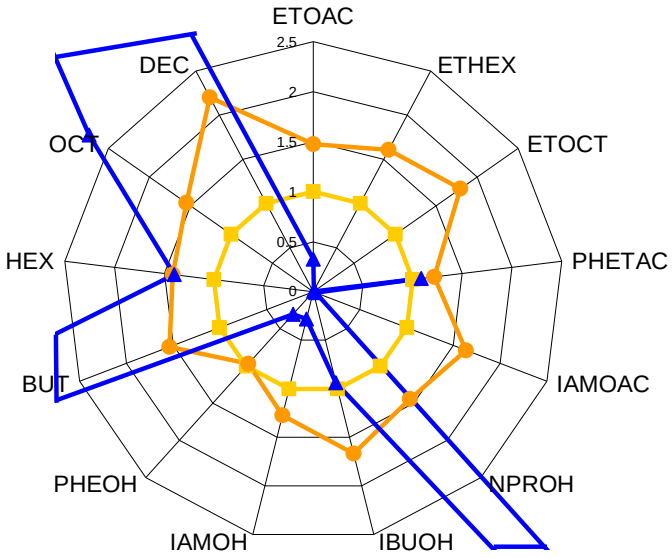


Figure 5.32. Spider graph of the treated (orange) and control (yellow) white wine compared to the flavour threshold (blue) of each flavour compound, normalised to the control.

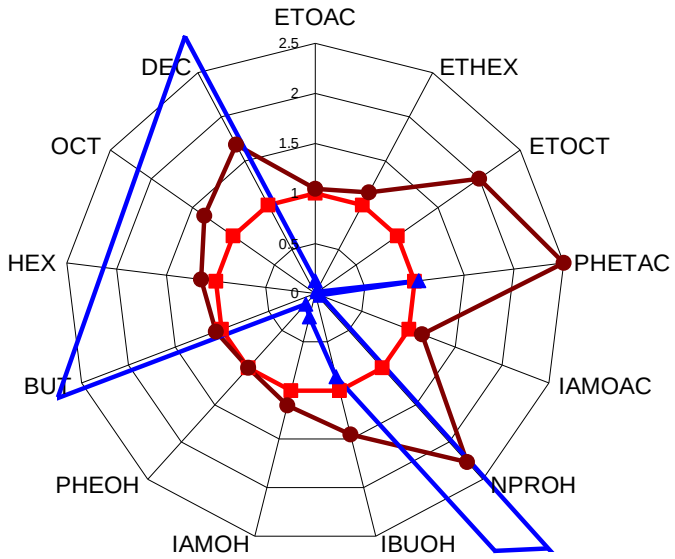


Figure 5.33. Spider graph of the treated (red) and control (maroon) red wine compared to the flavour threshold (blue) of each flavour compound, normalised to the control.

When comparing the changes in flavour compound concentrations between the treated and control wines and examining the OAVs, it is clear that the flavour profile differences caused by the pectin and carrageenan treatment are favourable in terms of intensity. Sensory evaluation would be needed to show preference. The positive esters were more concentrated in the treated wine as well as being detectable, whereas the negative acids were not detectable in the wine. The higher alcohols added a rich complexity to the wine and were not increased to the upper threshold or above. Therefore, the findings demonstrated that the pectin and carrageenan treatment in the pilot-scale fermentation is likely to have a positive effect on flavour.

5.11. Flavour profile for grape juice lab-scale fermentation

Flavour analysis of the grape juice lab-scale fermentation, analysed by GC-MS, is shown in the figures below (Figure 5.34). Unlike the pilot-scale fermentation above, the trend of the flavour profile varied with the flavour compound, instead of having a unified trend. The fruity flavours, such as ethyl acetate, isoamyl acetate and phenylethyl acetate tended to have similar or higher quantities in the treated wine compared to the control. This correlated with the higher fruitiness that the sensory panel found when smelling these samples, as shown in Figure 5.21.

While most of the flavour compounds were similar or higher in the treated wine, some flavour compounds were found at a lower concentration in the treated wine, such as hexanol and ethyl decanoate, which were similar in the pectin treated wine but lower in the carrageenan and pectin plus carrageenan wines. Many of the acids were lower in all three treated wines compared to the control.

The pectin plus carrageenan flavour profile closely resembled that of the carrageenan flavour profile. Most of the differences seen were not large differences, other than that of ethyl n-dodecanoate, which was found to be greatly reduced in the carrageenan and pectin plus carrageenan samples, being more than 80% lower than the control. The flavour of this compound can be described as fatty (Simpson and Miller, 1984).

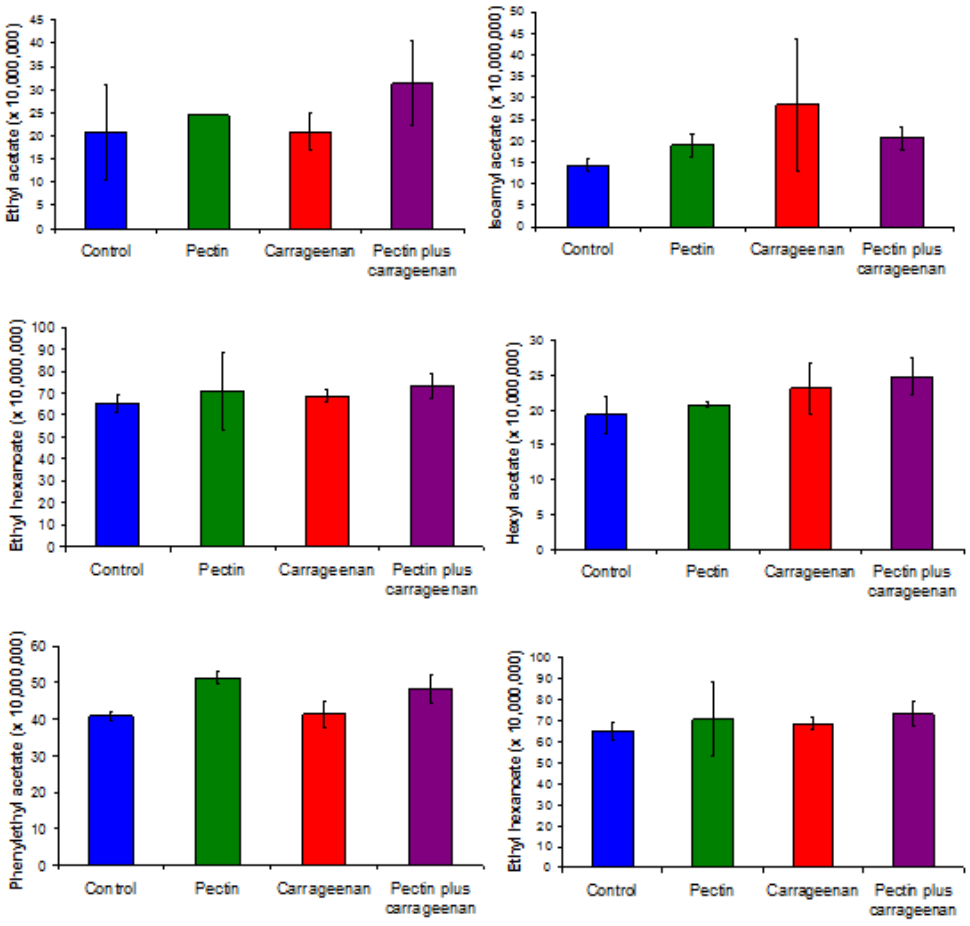
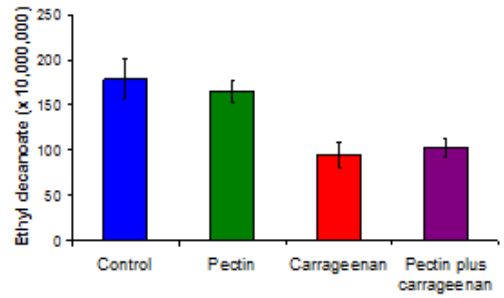
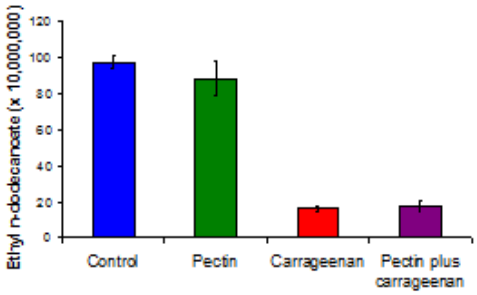
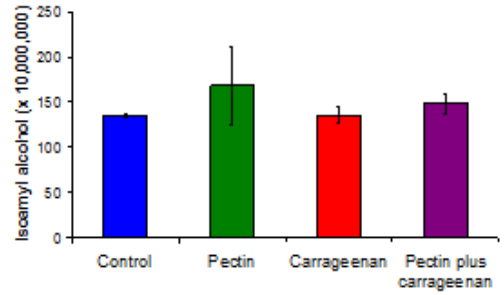
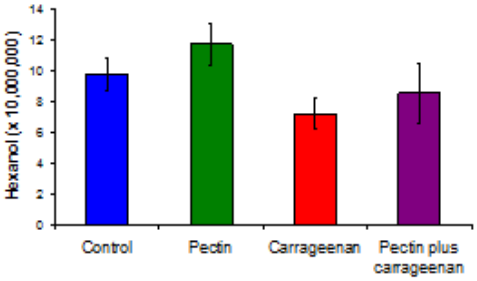


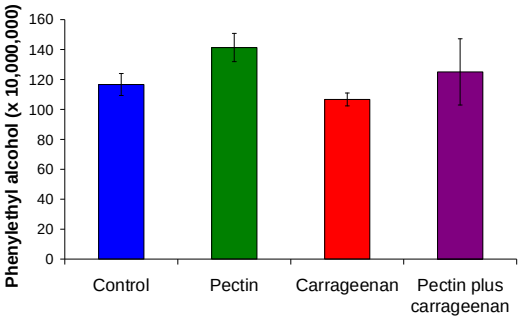
Figure 5.34. Figure continues on the following page.

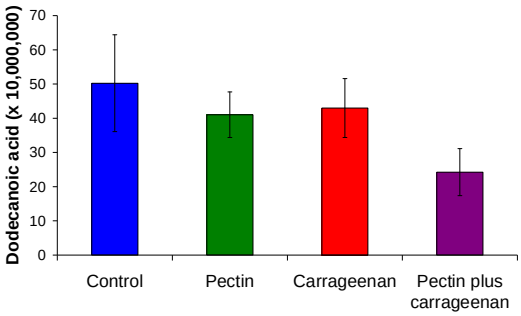

Ethyl decanoate (x 10,000,000)
250
200
150
100
50
0
Control
Pectin
Carrageenan
Pectin plus carrageenan

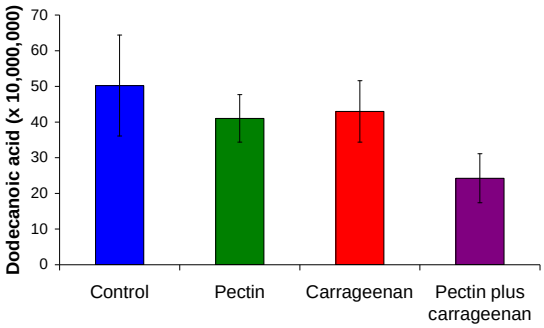

Ethyl n-dodecanoate (x 10,000,000)
120
100
80
60
40
20
0
Control
Pectin
Carrageenan
Pectin plus carrageenan

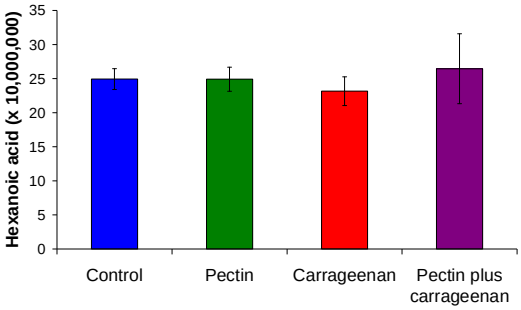

Isoamyl alcohol (x 10,000,000)
250
200
150
100
50
0
Control
Pectin
Carrageenan
Pectin plus carrageenan

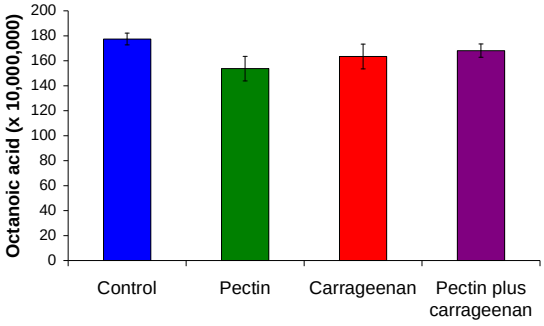

Hexanol (x 10,000,000)
14
12
10
8
6
4
2
0
Control
Pectin
Carrageenan
Pectin plus carrageenan

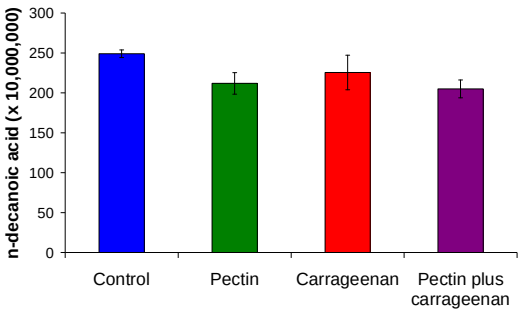

Phenylethyl alcohol (x 10,000,000)
160
140
120
100
80
60
40
20
0
Control
Pectin
Carrageenan
Pectin plus carrageenan


Dodecanoic acid (x 10,000,000)
70
60
50
40
30
20
10
0
Control
Pectin
Carrageenan
Pectin plus carrageenan


Dodecanoic acid (x 10,000,000)
70
60
50
40
30
20
10
0
Control
Pectin
Carrageenan
Pectin plus carrageenan


Hexanoic acid (x 10,000,000)
35
30
25
20
15
10
5
0
Control
Pectin
Carrageenan
Pectin plus carrageenan


Octanoic acid (x 10,000,000)
200
180
160
140
120
100
80
60
40
20
0
Control
Pectin
Carrageenan
Pectin plus carrageenan


n-decanoic acid (x 10,000,000)
300
250
200
150
100
50
0
Control
Pectin
Carrageenan
Pectin plus carrageenan

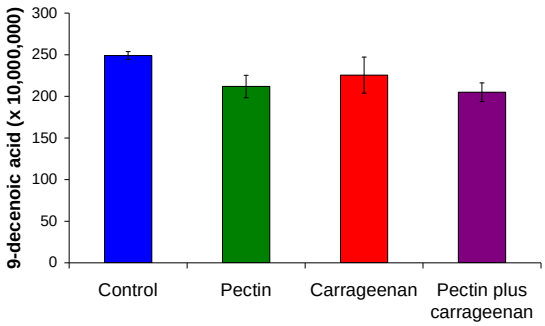


Figure 5.34. The effect of pectin and carrageenan on the flavour profile of wine, as determined by GC-MS.

5.12. Pectin and carrageenan altered H_2S and SO_2 production

H_2S and SO_2 balance is a continuing topic for winemakers (Jiranek et al., 1995), as described in Section 1.5.5, Chapter 1 and discussed in Chapter 4. These levels were measured in a study similar to our pilot-scale fermentation, and showed that free SO_2 concentrations increased approximately four-fold due to the treatment, from 21 ppm in the control to 81 ppm in the treated wine (Figure 5.35). This indicated that pectin and carrageenan were actually increasing the amount of SO_2 produced by the yeast or that the compounds were breaking down to form SO_2. However, this is contradictory to other results discovered during the lab-scale fermentations, as seen in Figure 5.37, which tested the amount of SO_2 in the liquid samples. After an initial dip that all the samples experienced, the control and pectin treated wine remained relatively constant, while after the 11^{th} day, the SO_2 decreased in the carrageenan and pectin and carrageenan treated wines to about half the concentration of the control (approximately 18 ppm compared to approximately 37 ppm). The similarity between the carrageenan and pectin/carrageenan suggests that carrageenan was solely responsible for this change.

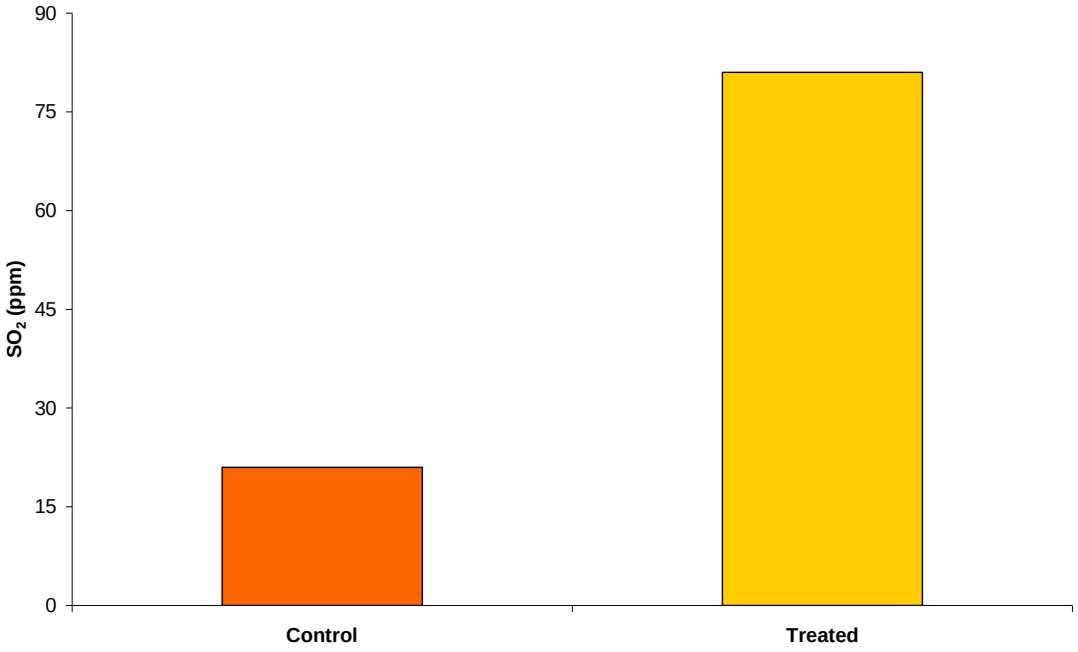


Figure 5.35. Free sulfur dioxide (ppm) in white wine (orange control, yellow treated). Data by Foster's from 2009 in the pilot plant using pectin plus carrageenan treated white grape juice.

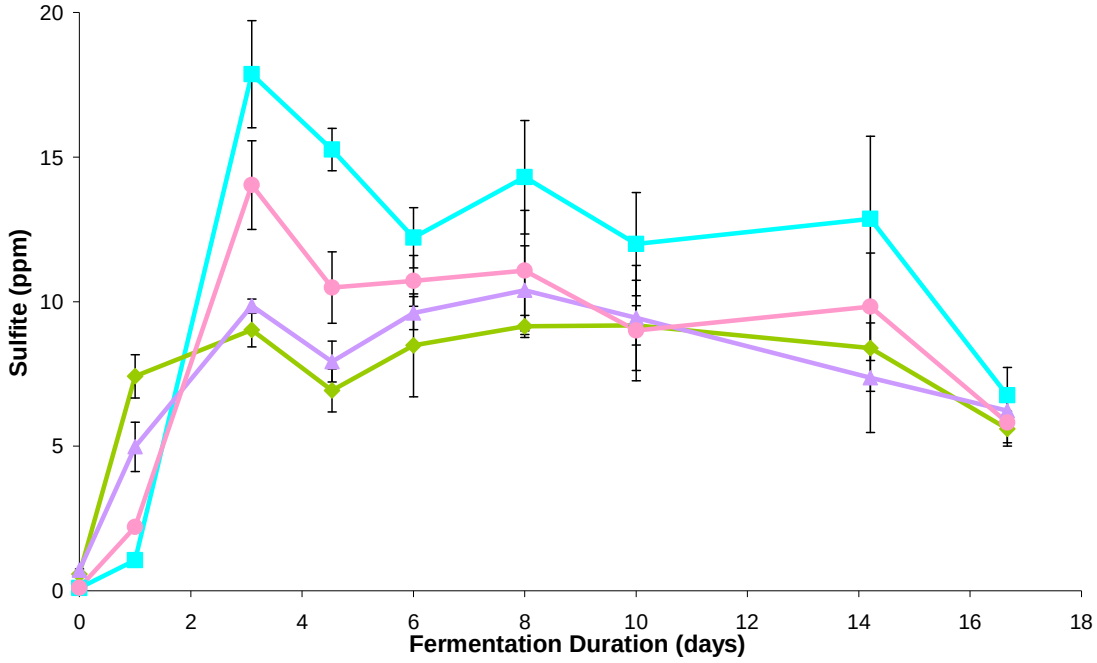


Figure 5.36. Wine SO_2 concentrations (in-liquid) for synthetic grape juice media fermentation. Standard curve with SMBS y = 0.032x (data not shown) was used to determine SO_2 (ppm). Key: Control = light blue; Pectin = lime green; Carrageenan = pink; Pectin plus carrageenan = lavender.

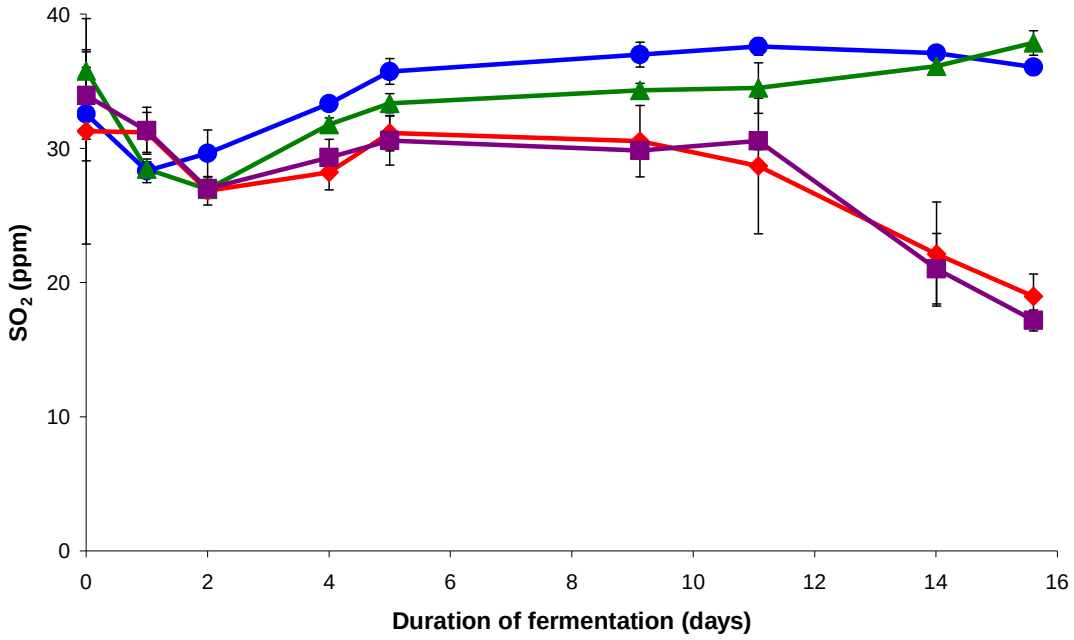


Figure 5.37. SO_2 concentrations (in-liquid) for real grape juice wine fermentation. Standard curve with SMBS indicated that the relationship was y = 0.032x (data not shown) to determine the concentration of SO_2 (ppm). Key: Control = Blue; Pectin = Green; Carrageenan = Red, Pectin plus carrageenan = purple.

The consequences for H_2S formation through the use of pectin and carrageenan are very interesting. H_2S headspace detection tubes were used for both of the lab-scale fermentations at UWS. The synthetic grape juice media fermentations resulted in no H_2S production at all. However, this was not the case for the real grape juice fermentation. As shown in Figure 5.38 below, the result seen for headspace H_2S production was quite significant. The headspace H_2S was very elevated in the pectin treated wines and reached 1,900 ppm over the duration of the fermentation. Compare this to the zero value for the carrageenan treated wine. There was also a marked decrease in headspace H_2S concentrations in the pectin plus carrageenan treated wine which had only 50 ppm compared to 495 ppm of H_2S in the control. Despite the huge increase brought about by the pectin treatment, the carrageenan was still able to suppress this phenomen to values nearly ten times less than the control. ANOVA statistical analysis showed that the differences between each of the conditions were significant ($p < 0.05$), other than between the pectin plus carrageenan and carrageenan H_2S concentrations.

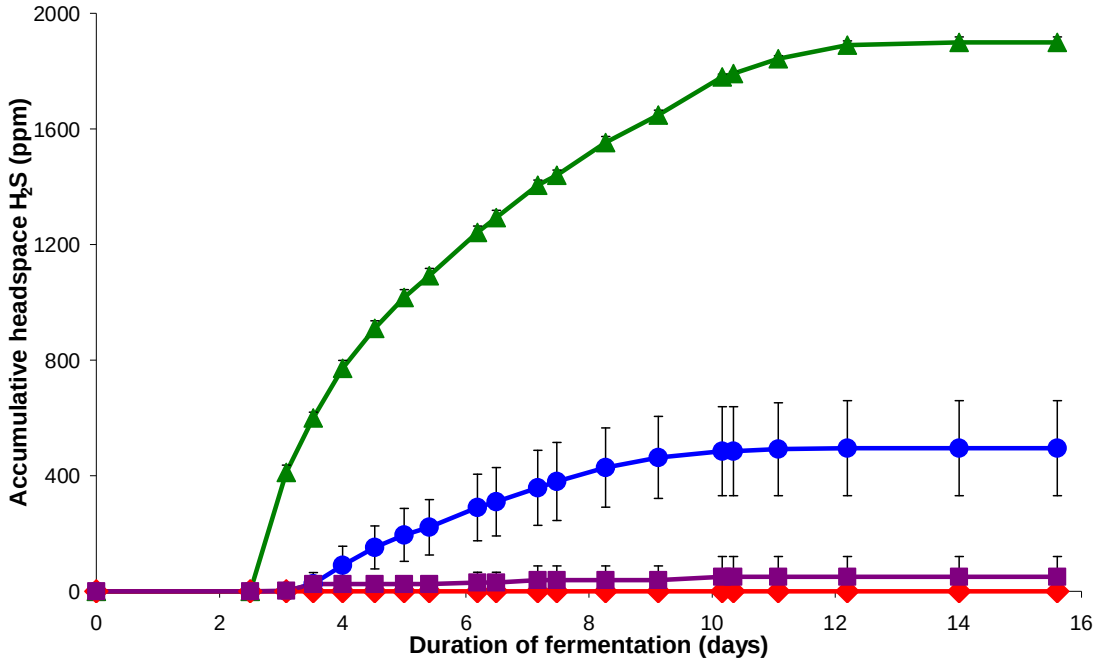


Figure 5.38. Accumulative H_2S production in the headspace of wine fermentation. Key: Control = Blue; Pectin = Green; Carrageenan = Red, Pectin plus carrageenan = purple.

Another question to consider was whether the increase of H_2S in the pectin-treated wine is at a particular point and then stabilises to follow the others or if this increase is throughout the fermentation. The latter can be seen to be the case in Figure 5.39, where the rate of production is higher at all points during the fermentation. While the rate of production is highest initially and decreases over the course of the fermentation, it is still higher at all times. This initial increase and then decrease in rate over the course can be seen for the control fermentation as well. The other two treatments did not produce enough H_2S to illustrate the same pattern.

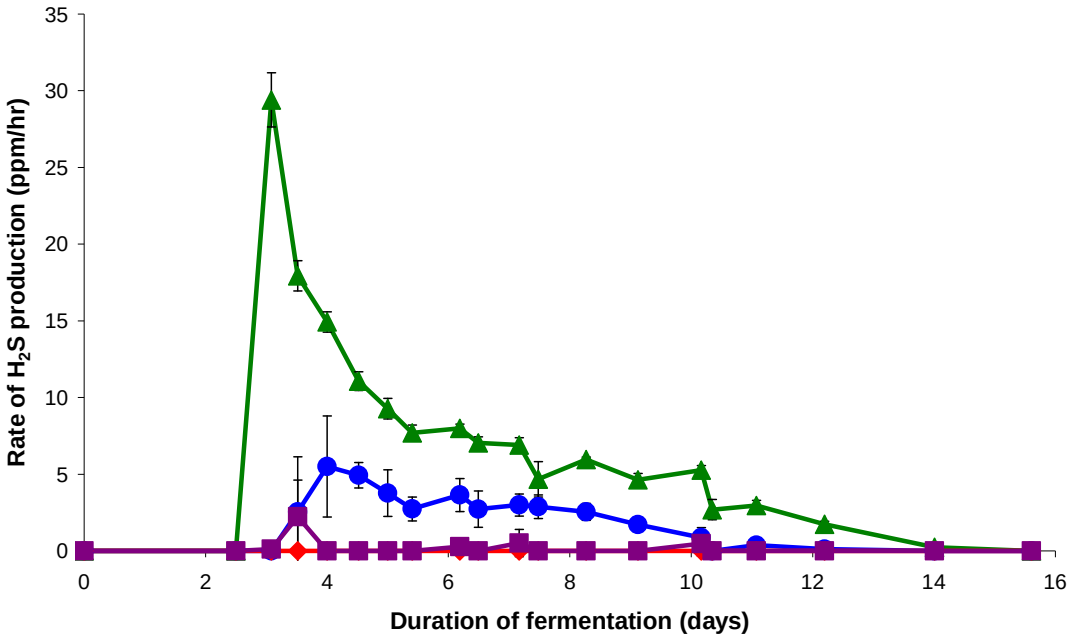


Figure 5.39. Rate of H_2S production during fermentation. Key: Control = Blue; Pectin = Green; Carrageenan = Red, Pectin plus carrageenan = purple.

5.13. Discussion

Pectin and carrageenan are both plant-based compounds, derived from citrus peel and red seaweed, respectively. They have both been used extensively in the food industry, mainly as thickening agents, and are therefore safe for inclusion in wine fermentation protocols. However, the important aspects to consider are their impact on the flavour and clarity of the wine. The results as described above are both expected and surprising. It is expected and indeed demonstrated in this study that pectin and carrageenan impacted positively on the wine clarity. However, their marked effects on the flavour of the wine are totally anew. Three fermentation sets were carried out – with the first being a pilot-scale fermentation and then, two lab-scale fermentations. The attempt that utilised a synthetic Chardonnay grape juice media in the lab-scale failed to give some insight into the effects of pectin and carrageenan on wine clarity, due to the media's absorbance being too low to be measured from the start. Therefore, frozen grape juice was sourced from South Australia and the same setup was used for the second lab-scale fermentation.

The pilot-scale fermentation showed no impact of pectin and carrageenan on the fermentation rate, but demonstrated their potential as fining agents, since any adverse

effect on fermentation would devalue their usefulness. This notion is further validated by the finding with the lab-scale fermentation, namely the residual sugar remaining at the end of the fermentation being significantly higher in the control fermentation than for the treatments, suggesting that pectin and carrageenan can actually promote fermentation.

The most important question here is the one of clarity. Pectin and carrageenan clearly improved the heat stability of the wine by over 50%, meaning this wine would be 1.75 times less likely to form a haze than the control. The treated grape juice lab-scale wines were two to three times less likely than the control wine to haze, with the pectin treated wine being less likely than the carrageenan and pectin plus carrageenan treatments. These results demonstrate that pectin and carrageenan indeed clarified the wine. The underlying rationale for their fining role was found, as expected, to be due to their sequestration of calcium by ionic interaction in the wine (Figure 5.20). Calcium, a contributor to wine hazing, is positively charged and is chelated by anionic pectin and carrageenan.

While the agents' positive effect on clarity was affirmed, their influence on wine flavour, as mentioned previously, is a complete surprise. The taste and aroma of the treated pilot-scale fermentation was much more fruity and pleasant than the control. GC-MS analysis showed that the tested flavour compounds were more abundant in the treated fermentation than the control, even those with unpleasant flavours or aromas. However, the 'unpleasant' components were below the flavour threshold, so only the pleasant fruity flavours were apparent in the treated wine, making it more pleasant to drink than the control wine. Although the grape juice lab-scale fermentation did not show such a clear-cut result as that of the pilot-scale fermentation, the effects of these potential fining agents were clearly demonstrated. Therefore, it is imperative for researchers like me to further investigate how such effects arose at the molecular level. This is exactly what I did and the findings are described in the next chapter.

The sensory panel data was not conclusive and the work here with the lab-scale fermentations was really never intended to be quantitative. The use of an amateur group, the difficulty in getting the same panel members and the level of wine appreciation was restricted. But it is worth nothing that the taste and aroma appreciation differed. Some would prefer one wine on the basis of aroma, but another on the basis of taste. In general, preference for aroma and preference for taste did not

coincide. I was also concerned that preference for sweetness may have unduly influenced the panel members.

Correlation exists between increased fruity volatile metabolites detected by GC-MS and fruity odours detected by a sensory panel. This does not correlate with a fruity taste, however. There is more at play here than thresholds. The taste profile differed from the aroma profile. While the sensory panel suggests a positive increase in the fruity aroma, the taste was negatively affected by the treatments. The lab-scale fermentation utilised a comparative approach to the GC-MS without being able to assess the actual concentrations. Some of those compounds that had a flavour or aroma that negatively affects wine but was under the threshold in the pilot-scale fermentation could be above the flavour threshold in this lab-scale fermentation and thus contributing to the flavour negatively.

As noted above, residual sugar concentrations differed between the conditions, with the control remaining sweeter than the treatments at the end of the fermentation. This correlated with the sweet taste detected by the sensory panel and could have contributed to the preference of the control over the treated samples. A correlation exists between the sweet taste detected by the sensory panel and the sugar test. The control, which was sweeter, was more liked by the sensory panel (Figure 5.6 and Figure 5.21).

H_2S concentrations were dramatically altered by the pectin and carrageenan, with four times as much headspace H_2S in the pectin treated wine than the control wine, a tenth of the headspace H_2S in the pectin plus carrageenan treated wine compared to the control wine and no detectable headspace H_2S in the carrageenan treated wine. Despite the huge increase caused by the pectin treatment, the carrageenan was able to mostly counteract this increase in the pectin plus carrageenan treated wine, containing nearly 40 times less H_2S than the pectin treated wine. This is a remarkable result especially when the structure of the polysaccharides is considered, with carrageenan and not pectin containing sulfate. Instead, perhaps the yeast metabolism is responsible for this result? This question is examined in Chapter 6 with gene expression microarrays compared at 48 and 96 h timepoints.

The potential application of pectin and carrageenan in the context of winemaking has not been investigated in too much detail to date, however, Cabello-Pasini et al (2005) and Marangon et al (2012) have studied these biopolymers in wine. Cabello-Pasini et al (2005) supported the results presented here, showing that pectin and

carrageenan were capable of reducing the probability of wine hazing. The results here indicated that pectin and carrageenan increased protein levels, however, our sample size was small and the results contradicted those of Marangon et al (2012), who demonstrated that proteins and small polypeptides were removed by pectin and carrageenan, increasing wine stability; this increase in stability was also seen in my study.

Taken together, these results demonstrated the potential of pectin and carrageenan being fining agents for winemaking. Furthermore, they are potent in flavour modulation. In order to understand their effect on yeast metabolism at the molecular level, gene expression microarrays profiling was carried out, which are described in the following chapter.

Chapter 6: Understanding the effects of pectin and carrageenan on yeast metabolism by means of gene expression profiling

6.1. Introduction

Pectin and carrageenan have an impact on the clarity, flavour and headspace hydrogen sulfide (H_2S) production of wine, as described in Chapter 5. The effect on white wine clarity is explainable because of their anionic nature and hence being capable of forming complexes with cations and positively charged proteins, leading to improved clarity and heat stability. However, the treatment led to additional unexpected outcomes, such as an increased quantity of some flavour compounds and a dramatic impact on headspace H_2S levels. These unexpected outcomes prompted the exploration in this chapter in order to decipher the molecular mechanisms of wine yeast treated with pectin and carrageenan. Here, the gene expression of wine yeast isolated from the pectin and carrageenan treated fermentation was investigated using cDNA microarray analysis at 96 hours for the pilot-scale fermentation and at 48 and 96 hours for the grape juice lab-scale fermentation under the treatments as detailed in Chapter 5.

The specific objectives of this chapter are as follows:

- To delineate the effect of pectin / carrageenan treatment in the pilot-scale fermentation on flavour compounds at a gene expression level.
- To understand molecular mechanisms whereby pectin increased and carrageenan decreased H_2S concentrations in the lab-scale fermentation.

6.2. Transcriptomic gene expression profiling

Pilot-scale (20 L) and lab-scale (2 L) white wine fermentations were set up to study the effect of pectin and carrageenan. The pilot-scale fermentation involved a comparison between the pectin plus carrageenan treatment and the control. The lab-scale fermentation compared pectin only, carrageenan only and pectin plus carrageenan treatments to a control respectively, in triplicate, as shown in Figure 6.1. Yeast samples were taken from the wine fermentations after 96 h of pilot-scale

fermentation and after 48 and 96 h of lab-scale fermentation, and RNA purification was followed. cDNA was transcribed from each isolated RNA and hybridised onto Affymetrix® yeast gene expression microarrays. These data were analysed through the Partek® bioinformatic program to determine the genes of significant fold change. Significant genes were deemed to be those with a higher than 1.5 fold change. A full list of significant genes above a cut-off of 2 can be seen in Appendix A.

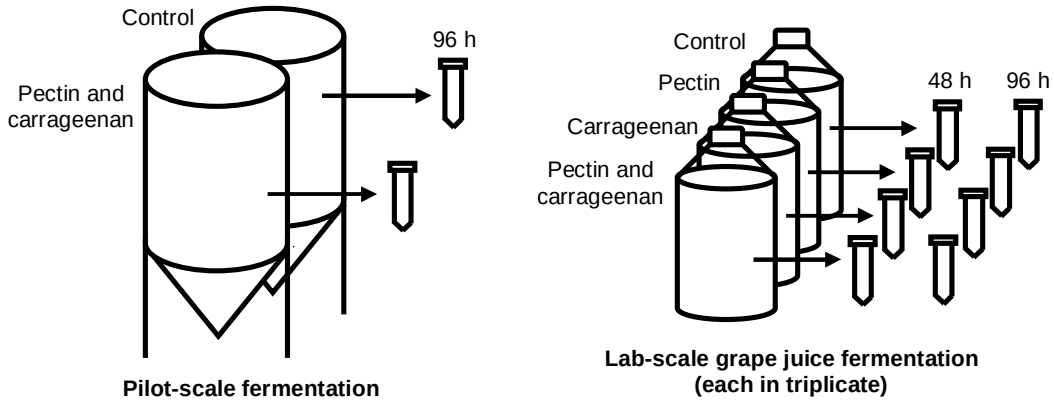


Figure 6.1. Experimental scheme of the fermentations analysed by gene expression microarrays.

6.3. Amino acid uptake was increased in pilot-scale fermentation

Transcriptomic analysis from the Affymetrix microarray data showed that 451 genes were significantly altered (above a 1.5 fold change) by the pectin and carrageenan treatment after 96 h in the pilot-scale fermentation. Of these, 252 were up-regulated, 54 of which had fold changes above 2, and 199 genes were down-regulated, of which 30 showed a fold change below -2.

Functional Specification (FunSpec) analysis, as described in Chapter 2, Section 2.8.4, using the databases GO Molecular Function, GO Biological Process and MIPS Functional Classification, showed that the molecular response of yeast to pectin and carrageenan treatment involved stimulation of amino acid uptake and repression of alternative nitrogen source catabolism, as shown in Table 6.1.

Table 6.1. Amino acid and nitrogen uptake genes significantly altered by pectin and carrageenan treatment in pilot-scale wine fermentation.

Gene	Description	Fold change
BAP2	High-affinity leucine permease, functions as a branched-chain amino acid permease involved in the uptake of leucine, isoleucine and valine.	**1.81**
BAP3	Amino acid permease involved in the uptake of cysteine, leucine, isoleucine and valine.	**2.12**
GNP1	High-affinity glutamine permease, also transports leucine, serine, threonine, cysteine, methionine and asparagine.	**2.74**
MUP1	High affinity methionine permease, also involved in cysteine uptake.	**1.99**
MUP3	Low affinity methionine permease, similar to Mup1p.	**1.65**
MMP1	High-affinity S-methylmethionine permease, required for utilisation of S-methylmethionine as a sulfur source; has similarity to S-adenosylmethionine permease Sam3p.	**2.46**
TAT2	High affinity tryptophan and tyrosine permease.	**1.51**
BTN2	v-SNARE binding protein that facilitates specific protein retrieval from a late endosome to the Golgi; modulates arginine uptake.	**7.18**
STP3	Zinc-finger protein of unknown function, possibly involved in pre-tRNA splicing and in uptake of branched-chain amino acids.	**1.53**
DUR1,2	Urea amidolyase, contains both urea carboxylase and allophanate hydrolase activities, degrades urea to CO_2 and NH_3; expression sensitive to nitrogen catabolite repression and induced by allophanate, an intermediate in allantoin degradation.	**-4.07**
DUR3	Plasma membrane transporter for both urea and polyamines, expression is highly sensitive to nitrogen catabolic repression and induced by allophanate, the last intermediate of the allantoin degradative pathway.	**-3.16**
DAL2	Allantoicase, converts allantoate to urea and ureidoglycolate in the second step of allantoin degradation; expression sensitive to nitrogen catabolite repression and induced by allophanate, an intermediate in allantoin degradation.	**-1.77**
DAL7	Malate synthase, role in allantoin degradation unknown; expression sensitive to nitrogen catabolite repression and induced by allophanate, an intermediate in allantoin degradation.	**-2.85**

Note: The FunSpec categories were amino acid transmembrane transporter activity, amino acid transport, allantoin catabolic process, urea catabolic process and catabolism of nitrogenous compounds. Gene descriptions were derived from Saccharomyces Genome Database (SGD). The fold changes highlighted in red shows that the expression is up-regulated in the treated sample and the green highlight shows down-regulation.

On the basis of the gene expression at 96 h, there was a broad increase of the amino acid transporter gene expression. *BAP2* (+1.81 fold increase) encodes a branched-chain amino acid permease involved in the uptake of the branched-chain amino acids like leucine, isoleucine and valine (Grauslund et al., 1995). *BAP3* (+2.12) encodes an amino acid permease with a similar function as Bap2p but which also involved in the uptake of cysteine (Regenberg et al., 1999). *GNP1* (+2.74) is a gene for the high-affinity glutamine permease, which can transport leucine, serine, threonine, cysteine, methionine and asparagine (Zhu et al., 1996; Regenberg et al., 1999). *MUP1* (+1.99) encodes a high affinity methionine permease, which is involved in cysteine uptake as well (Isnard et al., 1996; Kosugi et al., 2001). *MUP3* (+1.65) is a gene that encodes a low affinity methionine permease with similar function to Mup1p (Isnard et al., 1996). *MMP1* (+2.46) is a gene for high affinity S-methylmethionine permease, which is required for utilisation of S-methylmethionine as a sulfur source and is similar to the S-adenosylmethionine permease, Sam3p (Rouillon et al., 1999). *TAT2* (+1.51) encodes a high affinity permease of tryptophan and tyrosine (Schmidt et al., 1994). *BTN2* (+7.18) encodes for a v-SNARE binding protein that also modulates arginine uptake (Chattopadhyay and Pearce, 2002). *STP3* (+1.53) encodes for a zinc-finger protein with unknown function, which is possibly involved in the uptake of branched-chain amino acids (Abdel-Sater et al., 2004).

The increase of these transporter genes for amino acids suggests the elevated uptake activity of the yeast cells under pectin and carrageenan treatment, which could explain the enchanced levels of flavour compounds (Chapter 5, Section 5.6), since the branched amino acids are precursors for such compounds (Dickinson et al., 1998; Dickinson et al., 2000; Hazelwood et al., 2008). For example, valine is the amino acid precursor for isobutanol which was increased in the treated wine by 67% and leucine is the precursor of isoamyl acetate (increased by 64%) and isoamyl alcohol (increased by 27%). The branched-chain amino acids are involved in the Ehrlich pathway (Hazelwood et al., 2008), whereby the amino acids are metabolised to form higher alcohols and then esters, as shown in Table 6.2 and Figure 6.2. The example of leucine is shown in Figure 6.3, demonstrating how an increased uptake of this amino acid can account for the increase of isoamyl acetate and isoamyl alcohol.

Table 6.2. Amino acids that involved in the Ehrlich pathway and the resulting higher alcohols and esters produced.

Amino acid	Higher alcohol	Ester
Leucine	Isoamyl alcohol	Isoamyl acetate
Isoleucine	Active amyl alcohol	Active amyl acetate
Valine	Isobutanol	Isobutyl acetate
Phenylalanine	Phenylethanol	
Tyrosine	Tyrosol	
Trpytophan	Tryptophol	
Methionine	Methionol	

References: Lilly et al. (2006a), Lee et al. (2011), Hazelwood et al. (2008).

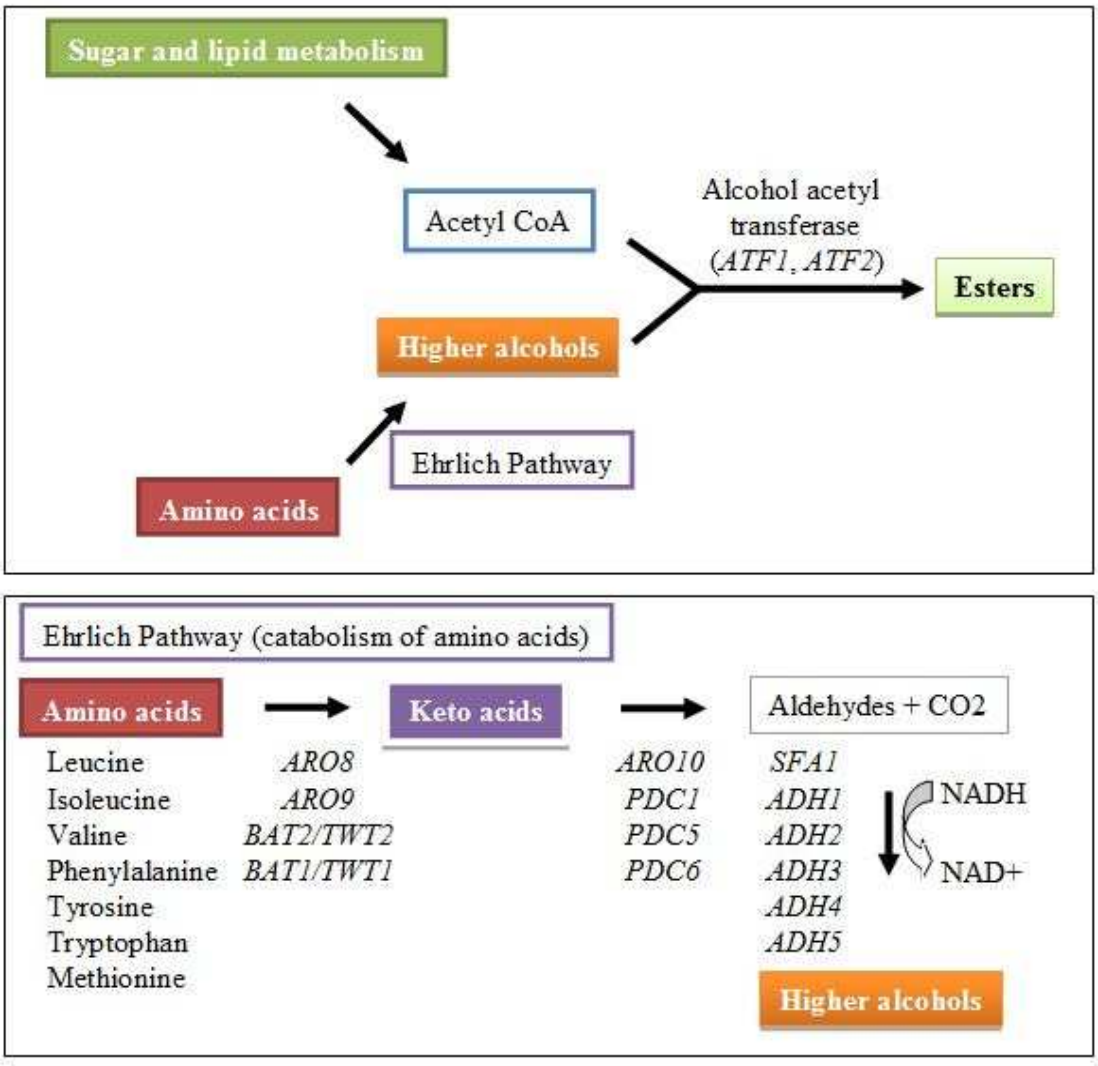


Figure 6.2. Amino acids are the precursors for higher alcohols and esters, through the Ehrlich pathway.

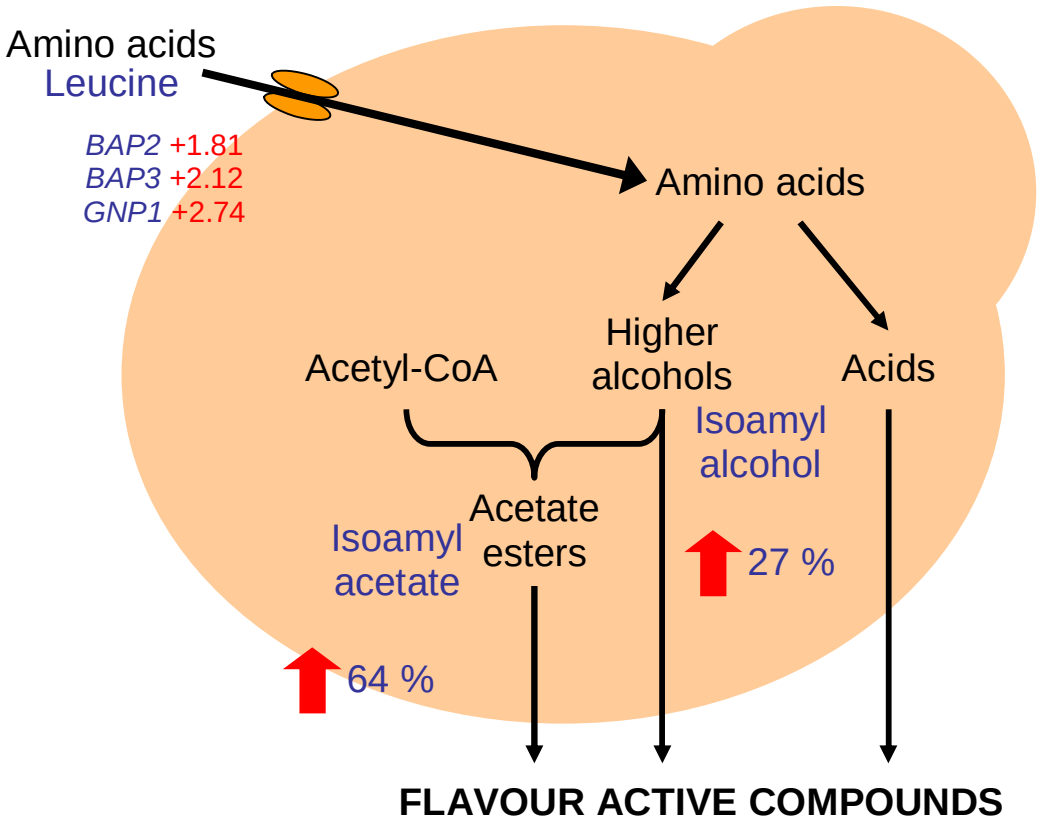


Figure 6.3. Schematic of how an amino acid (leucine in this case) affects flavour production.

6.4. Grape juice lab-scale fermentation

The grape juice lab-scale (2 L) fermentation involved pectin only, carrageenan only and pectin plus carrageenan treatments compared to control fermentations, in triplicate. Transcriptomic analysis of the Affymetrix microarrays was conducted on the wine yeast from these fermentations isolated at 48 and 96 h. Firstly, principal component analysis (PCA) was conducted on these Affymetrix microarray data using Partek and the results are shown in Figure 6.4. All of the datasets were grouped reasonably, except for an outlier of carrageenan at 96 h, which was excluded from the bioinformatic analysis.

The PCA plot is useful to investigate the similarity between various samples (in this case, each point represents a single microarray). The position of each sample in relation to the others shows how similar or different it is to the others. The components of difference are mapped out in 3D, where the highest level of difference

seen is represented on the x-axis, then on the y-axis and the third level is represented on the z-axis. Distances between samples on the x-axis represent more difference between samples than distances on the other axes. In the plot below, PC1 (x-axis) represents 52.6% of the difference, PC2 (y-axis) accounts for 12.8% and PC3 for 9.62%. The spheres in the PCA represent the 48 h timepoints whereas the diamonds represent the 96 h microarrays. The colours represent the various fermentation treatments, with pectin only, carrageenan only, pectin plus carrageenan and control represented by purple, red, green and blue, respectively. The triplicates are seen grouped together, except for a 96 h carrageenan microarray, which was grouped with the 48 h microarrays and was thus excluded from any down-stream analysis. While the 48 h timepoints are not as tightly grouped as the 96 h counterparts, there is little difference on the x-axis, indicating that these microarrays are similar to each other. It appears that the carrageenan only and pectin plus carrageenan treatments are the most similar out of the treatments, with overlap at 48 h.

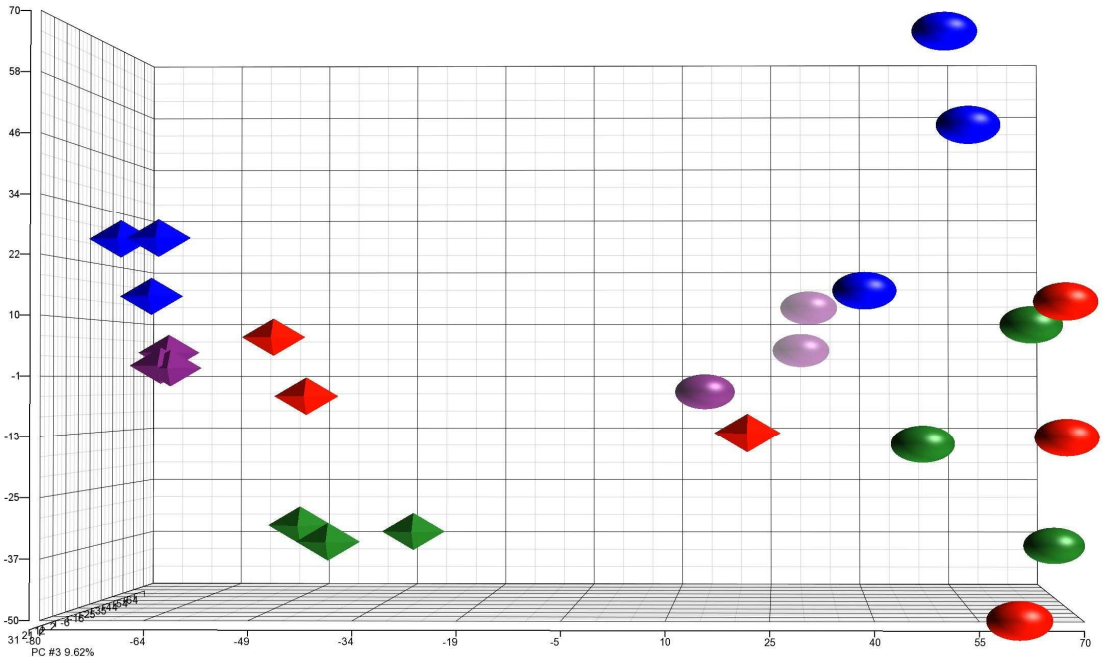


Figure 6.4. PCA plot of microarrays after 48 and 96 h of grape juice lab-scale fermentation.

Note: The outlying microarray of the third replicate of the carrageenan treated sample at 96 hours was excluded (the right most diamond in the figure above). Legend: Spheres = 48 h, diamonds = 96 h; red = carrageenan only, blue = control, green = pectin, purple = pectin plus carrageenan.

The number of significant genes in the microarray data is tabulated in Table 6.3 below, showing the number of genes found in each dataset both above 1.5 and above 2 fold changes. There is a larger amount of variation between the treatment and the control in the 48 h samples with a smaller number at 96 h. The exception to this is the pectin and carrageenan treatment, which had more differentially expressed genes at 96 h than at 48 h.

When looking at the carrageenan treatment, many of these genes were common with the pectin plus carrageenan treatment, for example 23 of the 37 down-regulated genes at 96 h were common with the pectin plus carrageenan down-regulated genes (62%). The majority of the up-regulated genes in the carrageenan treatment (75 out of 94) were also up-regulated in the pectin plus carrageenan treated gene list (80%). Ninety-nine of the 148 up-regulated genes at 48 h were common with pectin plus carrageenan (67%) and 64 of the 199 down-regulated genes were common (32%).

In contrast, the comparison between the pectin and the pectin plus carrageenan datasets showed some opposite findings. Whilst 14 of the 17 up-regulated pectin genes were up-regulated in both treatments, pectin and pectin plus carrageenan at 96 h (82%), the down-regulated comparison showed six common genes and an additional two genes that were up-regulated in the pectin plus carrageenan dataset. At 48 h, 95 of the 302 up-regulated pectin genes were also up-regulated in the pectin plus carrageeenan samples. However, eight genes were down-regulated. The down-regulated genes at 48 h showed that 16 of the 206 genes were commonly down-regulated in pectin and pectin plus carrageenan datasets, however, one gene was up-regulated in pectin plus carrageenan. Venn diagrams for these datasets at both 48 and 96 h are presented in Figure 6.5.

Table 6.3. Number of significant genes for each treatment.

	PC 48 hr	Pectin 48 hr	Carra 48 hr	PC 96 hr	Pectin 96 hr	Carra 96 hr
> 2	21	59	19	60	0	30
> 1.5	161	302	148	284	17	94
< -1.5	111	206	199	181	23	37
< -2	14	29	48	9	0	3

Note: PC denotes pectin plus carrageenan treatment, Carra denotes carrageenan treatment.

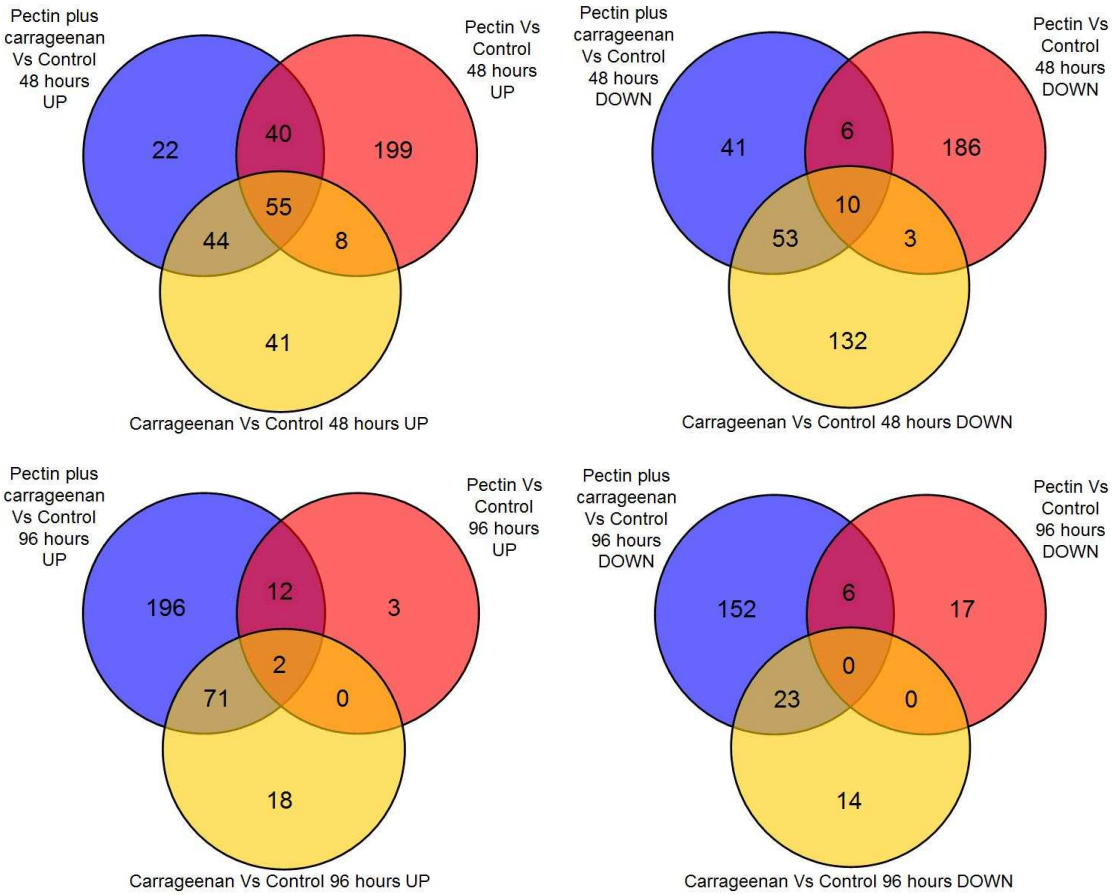


Figure 6.5. Venn diagrams showing the common genes between the three treatments, pectin (red), carrageenan (yellow) and pectin plus carrageenan (blue) vs the control at 48 h (top half) and 96 h (bottom half), with up-regulation on the left and down-regulation on the right. The analysis was facilitated by the web-based tool Pangloss, found at www.pangloss.com/seidel/Protocols/venn.cgi.

Enrichment of the functional categories found in the significantly altered gene dataset was examined in Table 6.4, Table 6.5 and Table 6.6 for pectin plus carrageenan, pectin, and carrageenan vs the control, respectively for 48 and 96 h. Some involvement of rRNA and protein processing was found in both of the pectin plus carrageenan up-regulated datasets. At 96 h, the pectin plus carrageenan dataset also had genes that were involved with polysaccharide and branched-chain amino acid metabolism. Iron uptake, metabolism relating to energy, glutamate degradation was down-regulated, with a large portion of unclassified proteins in the pectin plus carrageenan 48 h dataset.

Metabolism relating to energy was up-regulated in the pectin vs control dataset at 48 h. Down-regulated genes for the pectin vs control dataset at 48 h fall primarily into sulfate and sulfur-containing amino acid metabolism (as discussed later in detail) as well as purine nucleotide anabolism (mainly the *ADE* genes). In addition, there were some amino acid transport genes down-regulated, which was unexpected since these genes were up-regulated in the pilot-scale fermentation, although some of these genes were up-regulated in some other conditions, such as *BAP3* which had fold changes of +1.98 and +2.68 in the pectin plus carrageenan and carrageenan datasets at 48 h despite a fold change of -1.89 for the pectin dataset at 48 h. Iron uptake was again down-regulated in the pectin vs control 48 h dataset. Metabolism of nitrogen was down-regulated at 96 h.

As with the pectin plus carrageenan dataset, rRNA involvement was present in the carrageenan vs control dataset at 48 h. Iron uptake was down-regulated at 48 h, as was the case in the other two treatments. Homeostasis of metal ions was also down-regulated, with copper and iron mainly involved.

Table 6.4. Functional enrichment of the genes significantly changed by pectin plus carrageenan treatment at 48 and 96 h, as determined by MIPS Functional Classification, FunSpec (Robinson et al., 2002). Enrichment was considered as $p < 0.01$. Up-regulated categories are highlighted in red and down-regulated categories are highlighted in green.

Category	p-value	Genes in category
48 hours (pectin plus carrageenan vs control)		
Up-regulated genes at 48 h:		
rRNA processing	7.658e-10	*ENP1 RSA4 NOP1 UTP6 DBP3 UTP22 NSR1 IMP3 DBP8 UTP10 REX2 ERB1 UTP15 HAS1NOP2 DBP2 POP3 UTP23 NOP58 NOC4*
RNA binding	0.001036	*ENP1 SRO9 UTP6 UTP22 NSR1 IMP3 SNP1 UTP10 UTP15 NAF1 VTS1 NOC4*
Ribosome biogenesis	0.002946	*RSA4 ARX1 IMP3 RRB1 NOG1 NOC4*
Cytoskeleton / structural proteins	0.003707	*SMY2 ATC1 SPR6 PAC10 GIC1 AUR1 ICY2 CLN2*
rRNA modification	0.007033	*NOP1 IMP3 NOP58*
Down-regulated genes at 48 h:		
Siderophore-iron transport	7.025e-7	*FTR1 ARN1 FET3 FRE4 ENB1*
Metabolism of energy reserves	2.736e-5	*GLC3 GIP2 GSY1 IMA1 PIG2 GLG1 GSY2*
Glycogen anabolism	3.929e-5	*GSY1 GLG1 GSY2*
Proton driven symporter	0.0007579	*MAL31 UGA4*
Aerobic respiration	0.001399	*ETR1 NDE2 COX20 AAC1 MRPL22 NCA2*
Degradation of glutamate	0.0015	*UGA2 GAD1*
Antiporter	0.005087	*SFC1 AAC1*
Amino acid / amino acid derivatives transport	0.00564	*UGA4 MMP1 PUT4 DIP5*
Unclassified proteins	0.008084	YBR182C-A YBR285W *FMP16 RGI1* YGR053C YGR174W-A YGR204C-A *SPG1 AIM17* YIL029C *OM45* YIL165C *FMP33* YJL163C YJR005C-A YKR075C YLR053C *RKM5* YLR177W YLR312C *ART10 BLS1 EIS1* YMR105W-A *SPG4* YNL115C YNL144C YNL195C *AIM39 DCS2* YOR186W YPL119C-A *UIP4*
C-2 compound and organic acid catabolism	0.008539	*SYM1 ALD4*
96 hours (pectin plus carrageenan vs control)		
Up-regulated genes at 96 h:		
Ribosome biogenesis	<1e-14	*MAK16 RSA4 TSR1 NOP14 ARX1 NSA2 LOC1 CGR1 NOP7 IMP3 RIX1 URB2 MRT4 RIX7 RLP24 RRB1 NOP15 NOG2 NOC2 YTM1 RRP12 NOG1 NOP53 NOC4*
rRNA processing	<1e-14	*ENP1 RRP7 SPB1 PWP2 RSA4 NOP1 TSR1 NOP14 NHP2 RRP8 RRP1 UTP4 ESF1 UTP6 CGR1 DBP3 UTP22 NOP7 UTP8 NSR1 RRP3 IMP3 UTP9 RIX1 HCA4 UTP10 MRT4 URB1 UTP11 EBP2 NOC3 DIP2 CBF5 NOP56 UTP13 IFH1 UTP21 UTP14 ERB1 UTP15 RNT1 HAS1 DBP2 RRP36 NOP58 RRP12 NAN1 NOP53 NIP7 MRD1 RRP15 NOC4*
RNA binding	6.134e-13	*ENP1 SRO9 PWP2 LHP1 NOP14 NHP2 UTP4 ESF1 UTP6 LOC1 UTP22 UTP8 NSR1 SSF1 IMP3 NMD3 UTP9 UTP10 UTP11 DIP2 UTP13 UTP21 RPL6B UTP14 UTP15 RNT1 NOP13 BRX1 TRM11 VTS1 NAN1 MRD1 NOC4*

Ribosomal proteins	6.725e-7	*RRP7 NOP1 RPL13A MAK21 RLI1 RPS17B RPL27B RPL12A RPS26B RPL22B DBP3 RPL9A NSR1 SSF1 NMD3 RPS22A RPL43B RPL40B RPS28B RPL38 RPS29A RPL6B RPS18B RPS10B BRX1 RPS7A NIP7 RRP15*
rRNA synthesis	1.558e-6	*RPB5 RPC17 RRN7 RPA34 RPC25 RRB1 RPA49 RPA43 RPA190 RPA135 RPC40 RPO26*
rRNA modification	3.821e-6	*SPB1 NOP1 NHP2 IMP3 CBF5 NOP56 NOP58*
Biogenesis of cellular components	1.324e-5	*MAK21 CGR1 RRB1 NOC2*
Metabolism of the pyruvate family (alanine, isoleucine, leucine, valine) and D-alanine	0.001666	*ILV1 MAE1*
Polysaccharide metabolism	0.002638	*CHS2 SMI1 GAS3 SCW10 GAS1 SUN4*
tRNA synthesis	0.00405	*RPB5 LHP1 RPC17 RPC25 RPC40 RPO26*
Translation initiation	0.00526	*RPG1 RLI1 TIF4631 SUI2 NIP1 PRT1*
Transcription	0.007815	*SRO9* YCR087C-A *NMD3 SRP40 NOP13*
Cytokinesis	0.008029	*CHS2 PWP2 SCW11 DSE2 CTS1 HOF1 NOP15 DSE4*
Down-regulated genes at 96 h:		
Sugar, glucoside, polyol and carboxylate anabolism	0.0002641	*GAL7 TKL2 TPS1 INO1 XYL2 ATH1*
Metabolism of urea (urea cycle)	0.0003393	*DUR1,2 CAR2 CAR1*
Metabolism of secondary monosaccharides	0.0006905	*INO1 XYL2*
Degradation of arginine	0.0006905	*CAR2 CAR1*
Siderophore-iron transport	0.00332	*ARN1 FET3 ENB1*
Metabolism of energy reserves	0.003347	*TPS1 GLC3 GIP2 PIG2 GLG1 ATH1*
Sugar, glucoside, polyol and carboxylate catabolism	0.005276	*GAL7 TKL2 TPS1 XKS1 XYL2 ZWF1 ATH1*
Catabolism of nitrogenous compounds	0.006552	*DAL2 DAL7*
Modification by phosphorylation, dephosphorylation, autophosphorylation	0.009821	*PRR2 GIP2 PKP2 SIP2 SAP4 TPK1 PTK2 KKQ8 YPK2 MEK1 RAD53*

Table 6.5. Functional enrichment of the genes significantly changed by pectin treatment at 48 h and 96 h, as determined by MIPS Functional Classification, FunSpec (Robinson et al., 2002). Enrichment was considered as $p < 0.01$. Up-regulated categories are highlighted in red and down-regulated categories are highlighted in green.

Category	p-value	Genes in category
48 hours (pectin vs control)		
Up-regulated genes at 48 h:		
Tricarboxylic-acid pathway	4.41e-5	*GDH3 CIT2* YJL045W *MDH1 SDH1 ACO1 YMR118C FUM1*
Degradation of glutamate	0.0003347	*GDH3 UGA2 GAD1*
Metabolism of nonprotein amino acids	0.000645	*ARG3 CAT2 ALD3 ALD2*
Accessory proteins of electron transport and membrane-associated energy conservation	0.001963	*RAV1 CYT1*
Oxidative stress response	0.002697	*UGA2 TSA2 SCH9 SKN7 GTT1 FMP46 GAD1 AFT2*
Sugar, glucoside, polyol and carboxylate catabolism	0.009294	*TKL2 SUC2 RPE1 MDH1 SDH1 XYL2 ACO1 PGM2 FUM1*
C-compound and carbohydrate metabolism	0.009906	*BDH2 CIT2 BSC1 EHD3 ARO10 SCS2 MIG1 UGA1 IRC24* YJL045W *RGT1 ECM38 ALD3 ALD2 FKS3 LAT1 MLS1 IRC15*
Down-regulated genes at 48 h:		
Sulfate assimilation	6.684e-13	*MET8 MET10 MET3 MET5 MET14 MET1 MET22 MET16*
Purine nucleotide / nucleoside / nucleobase anabolism	1.595e-11	*ADE1 HIS4 ADE8 ADE5,7 ADE6 IMD2 MTD1 ADE13 ADE17 ADE12 ADE2 SER1*
Metabolism of methionine	1.826e-6	*MET32 MET3 MET1 MHT1 MET17 SAM4 MET16*
Metabolism of cysteine	2.909e-6	*MET32 YLL058W MHT1 YNL247W SAM4*
Nitrogen, sulfur and selenium metabolism	4.248e-6	*STR3 YHR112C FMO1 NIT1 BNA3 OPT1 YLL058W ALT1 MET17 CAR2*
Degradation of glycine	1.216e-5	*GCV3 GCV1 SHM2 GCV2*
Biosynthesis of serine	2.769e-5	*SER2 SER33 SHM2 SER1*
Biosynthesis of homocysteine	2.769e-5	*MET10 STR3 YHR112C MET5*
C-1 compound catabolism	0.0002696	*GCV3 GCV1 GCV2*
Metabolism of vitamins, cofactors and prosthetic groups	0.0002856	*GCV3 PYC2 GCV1 MTD1 SHM2 PCD1 GCV2*
Tetrahydofolate-dependent C-1-transfer	0.0004893	*ADE8 MTD1 SHM2 ADE17*
Biosynthesis of methionine	0.000527	*MET14 MET2 MET22*
Biosynthesis of valine	0.000527	*ILV6 ILV3 BAT2*
NAD/NADP binding	0.0006882	*MET8 MET10 SER33 LYS12 MET5 MTD1*
Transcription elongation	0.002265	*PAF1 RTF1 MFT1 TOP1 CTR9*
Amino acid/amino acid derivatives transport	0.00229	*AGP1 BAP3 MUP1 HIP1 MMP1 DIP5*
Sulfate/sulfite transport	0.002738	*SUL1 SUL2*
Homeostasis of sulfate	0.002738	*SUL1 SUL2*
Chromosome condensation	0.004033	*MCD1 ECO1 CTF18 TOP1*
Extension / polymerisation activity	0.00501	*POL4 SLD2 CDC45 RFC3 TOP1*
Siderophore-iron transport	0.005059	*ARN1 FET3 ENB1*

Amino acid metabolism	0.006299	*MET8 SUL1 MUP1 ALT1 SUL2*
Spindle pole body / centrosome and microtubule cycle	0.008644	*SPC19 CLB1 DAD2 CIK1 BBP1*
Biosynthesis of isoleucine	0.008762	*ILV3 BAT2*
Chromosome segregation / division	0.0089	*MTW1 SPC19 DAD2 CSM3 CTR9 SGO1*
Metabolism of porphyrins	0.00978	*MET8 MET1 FET3*
96 hours (pectin vs control)		
Up-regulated genes at 96 h:		
(no significant categories)	>0.01	(no significant genes)
Down-regulated genes at 96 h:		
Catabolism of nitrogenous compounds	0.0001153	*DAL2 DAL7*
Regulation of nitrogen, sulfur and selenium metabolism	0.00257	*DCG1 CAR1*
Homeostasis of anions	0.003483	*DUR3*
Degradation of arginine	0.006955	*CAR1*

Table 6.6. Functional enrichment of the genes significantly changed by carrageenan treatment at 48 and 96 h, as determined by MIPS Functional Classification, FunSpec (Robinson et al., 2002). Enrichment was considered as $p < 0.01$. Up-regulated categories are highlighted in red and down-regulated categories are highlighted in green.

Category	p-value	Genes in category
48 hours (carrageenan vs control)		
Up-regulated genes at 48 h:		
rRNA processing	1.192e-5	*ENP1 RSA4 NOP1 UTP6 DBP3 NSR1 IMP3 REX2 ERB1 HAS1 NOP2 DBP2 NOP58 NOC4*
Degradation of threonine	3.675e-5	*CHA1 GLY1 ILV1*
Ion channels	0.001004	*AQY2* YLL053C *AQY1*
rRNA modification	0.006028	*NOP1 IMP3 NOP58*
Metabolism of urea	0.006331	*ARG3 ARG1*
Down-regulated genes at 48 h:		
Siderophore-iron transport	5.048e-7	*FTR1 ARN1 FET3 ATX1 FRE4 ENB1*
Metabolism of energy reserves	5.513e-7	*GLC3 GIP2 GSY1 IMA1 PIG2 GLG1 GSY2 TSL1 PGM2 GPH1 GDB1*
Development of asco-basidio- or zygospore	1.703e-6	*GIP1 YSW1 DOA4 SPR28 DIT2 SHC1 SPO74 SPR3 PFS1 SPO75 OSW2 CDA1 TEP1 SPR1 SSP2 OSW1 SPS4 SMA1*
Catabolism of nitrogenous compounds	3.68e-6	*DAL1 DAL2 DAL7 DAL3*
Glycogen anabolism	0.0002465	*GSY1 GLG1 GSY2*
Allantoin and allantoate transport	0.001292	*DAL4 YCT1 THI73*
Purin nucleotide / nucleoside / nucleobase metabolism	0.001704	*DUR1,2 YBR284W DAL1 DAL7 DAL3*
Polysaccharide metabolism	0.003444	*GLC3 CDA1 GAS4 GPH1 GDB1*
Degradation of proline	0.005057	*PUT2 PUT1*
Degradation of glutamate	0.005057	*UGA2 GAD1*
Protease inhibitor	0.008263	*PAI3 PBI2*
Homeostatis of metal ions (Na, K, Ca, etc)	0.008389	*CCC2 FTR1 COX17 SMF3 CTR3 FET3 ATX1 FRE4*
ABC transporters	0.008819	*VMR1 NFT1 YKR104W ENB1*

96 hours (carrageenan vs control)		
Up-regulated genes at 96 h:		
C-compound and carbohydrate metabolism	0.0004874	*PHO5 FEN1 BSC1 EXG2 SCW11* YGL039W *HXK2 ATF2 DSE2 CSI2*
Polysaccharide metabolism	0.0009451	*CHS2 GAS3 SCW10 SUN4*
Cytokinesis	0.001993	*CHS2 SCW11 DSE2 HOF1 DSE4*
Enzymatic activity regulation	0.003015	*CLB1 CLN1 PCL1 CLN2 CLB2*
Cell wall	0.005461	*PSA1 EXG2 DSE1 DSE2 CIS3 GAS3* YMR317W *CSI2*
Ion channels	0.006735	*AQY2* YLL053C
Deoxyribonucleotide metabolism	0.009717	*RNR1 RNR3*
Down-regulated genes at 96 h:		
Stress response	0.00193	*SSA3 HSP30 XBP1 ALD3 DDR2*
Amino / polyamine transport	0.002665	*UGA4 PNS1*
Degradation of serine	0.005604	*CHA1*

6.4.1. The sulfur pathway and headspace hydrogen sulfide

The real grape juice lab-scale wine fermentation showed a remarkable difference in headspace H_2S between the treatments, whereby pectin dramatically increased the concentration above the control while the carrageenan treated fermentation resulted in concentrations below detection, as shown in Figure 5.38, Chapter 5. The pectin and carrageenan treatment resulted in a very low quantity of headspace H_2S. The gene expression data here were explored to gain insights into the understanding of these remarkable observations. As shown in Table 6.7, the sulfur pathway was largely unaffected by most of the conditions at both timepoints, except that at 48 h, the pectin treatment caused this pathway to be down-regulated. As seen in Figure 6.6, the down-regulated genes seem to funnel towards homocysteine, the compound in the pathway after H_2S.

As shown in Figure 6.6, extracellular sulfate is brought into the yeast cell by the high affinity sulfate permeases, *SUL1* and *SUL2* (Cherest et al., 1997). These two genes were down-regulated 1.5 and 3.3 fold in the pectin treated ferment compared to the control at 48 h. This demonstrated that the pathway was suppressed from the start. Further down-regulation by pectin were exhibited with ATP sulfurylase encoded by *MET3* (-5.4) which converts sulfate into 5′-adenylylsulfate, the first step of the sulfur pathway (Cherest et al., 1985). The transcription of *MET3* is strongly repressed by methionine through the transcription factors, Met4p, Met31p and Met32p (with fold changes of -1.1, 1.0 and -4.4 under pectin treatment). Met14p (-5.7), adenylylsulfate kinase, is responsible for the next step with conversion to 3′-phospho-5′-

adenylylsulfate. This is combined with reduced thioredoxin by Met16p (-3.2) to form adenosine-3′,5′-bisphosphate and free SO_2 (Schwenn et al., 1988), the latter being transported out of the cell by Ssu1p (-1.3), a plasma membrane sulfite pump (Park and Bakalinsky, 2000). *MET5* (-3.6) encodes for a sulfite reductase beta subunit and *MET10* (-1.9) encodes for the alpha subunit of sulfite reductase, which together convert SO_2 into H_2S. *MET17* encodes for O-acetyl homoserine sulfhydrylase, which combines O-acetyl homoserine and H_2S to form homocysteine, which then goes on to form methionine and cysteine, the sulfur-containing amino acids. The down-regulation of the sulfur pathway by the pectin treatment seems to end at this point with homocysteine production, which is also the case with *STR3* (-2.7) which encodes for peroxisomal cystathionine beta-lyase which converts cystathionine into homocysteine. The reverse reaction involved *STR4* which was unchanged (-1.3).

Table 6.7. Gene expression data for the sulfur assimilation pathway under treatment of the fining agents.

	P&C vs Control (48 hr)	Pectin vs Control (48 hr)	Carra vs Control (48 hr)	P&C vs Control (96 hr)	Pectin vs Control (96 hr)	Carra vs Control (96 hr)
SUL1	-1.38	-1.51	-1.08	-1.71	1.05	-1.45
SUL2	-1.49	-3.32	-1.16	-1.29	-1.37	1.02
MET3	-1.15	-5.43	1.19	-1.05	1.01	1.01
MET14	-1.11	-5.74	1.13	1.12	-1.04	1.04
MET16	-1.01	-3.16	1.27	1.15	1.02	1.09
SSU1	1.03	1.02	-1.03	-1.06	1.01	1.01
MET5	-1.11	-3.57	1.17	-1.11	1.10	-1.03
MET10	1.05	-1.94	1.31	1.19	-1.06	1.14
MET17	-1.12	-2.25	1.00	1.06	-1.07	1.12
MET6	-1.07	-1.08	-1.06	1.04	-1.02	1.06
SAM1	1.40	1.16	1.63	1.85	1.09	1.45
SAM2	1.09	1.18	1.04	1.05	1.02	1.01
SAH1	1.14	1.16	1.09	1.03	-1.01	-1.00
STR1	1.36	-1.34	1.46	1.57	-1.01	1.25
STR2	-1.19	-1.18	-1.19	-1.04	-1.03	-1.04
STR3	-1.45	-2.73	-1.25	-1.09	-1.04	-1.01
STR4	1.04	-1.25	1.11	1.29	1.08	1.16
MET2	-1.42	-2.48	-1.20	-1.06	-1.09	-1.00
HOM3	1.43	-1.09	1.40	1.51	1.24	1.17
HOM2	1.01	-1.27	1.02	1.15	1.07	1.14
HOM6	-1.04	-1.20	1.02	1.04	1.07	1.01
THR1	1.22	-1.35	1.27	1.01	-1.04	1.09
THR4	1.72	1.63	1.76	1.21	1.06	1.03

Note: P&C denotes pectin plus carrageenan treatment, Carra denotes carrageenan treatment. Green indicates a gene expression lower than -1.5 and red indicates a gene expression above 1.5.

Table 6.8. Annotation of the genes involved in the sulfur pathway.

Gene	Description	Fold change
SUL1	High affinity sulfate permease.	-1.51
SUL2	High affinity sulfate permease.	-3.32
MET3	ATP sulfurylase, catalyses the primary step of intracellular sulfate activation, essential for assimilatory reduction of sulfate to sulfide.	-5.43
MET14	Adenylylsulfate kinase, required for sulfate assimilation.	-5.74
MET16	3'-phosphadenylsulfate reductase, reduces 3'-phosphoadenylyl sulfate to adenosine-3',5'-bisphosphate and free sulfite using reduced thioredoxin as cosubstrate.	-3.16
SSU1	Plasma membrane sulfite pump involved in sulfite metabolism and required for efficient sulfite efflux.	1.02
MET5	Sulfite reductase beta subunit.	-3.57
MET10	Subunit alpha of assimilatory sulfite reductase, which converts sulfite into sulfide.	-1.94
MET17	Methionine and cysteine synthase (O-acetyl homoserine-O-acetyl serine sulfhydrylase).	-2.25
MET6	Cobalamin-independent methionine synthase, involved in methionine biosynthesis and regeneration.	-1.08
SAM1	S-adenosylmethionine synthetase, catalyses transfer of the adenosyl group of ATP to the sulfur atom of methionine.	1.16
SAM2	S-adenosylmethionine synthetase, catalyses transfer of the adenosyl group of ATP to the sulfur atom of methionine (Sam1p and Sam2p are differentially regulated isozymes).	1.18
SAH1	S-adenosyl-L-homocysteine hydrolase, catabolises S-adenosyl-L-homocysteine which is formed after donation of the activated methyl group of S-adenosyl-L-methionine (AdoMet) to an acceptor.	1.16
STR1	Cystathionine gamma-lyase, catalyses one of the two reactions involved in the transsulfuration pathway that yields cysteine from homocysteine with the intermediary formation of cystathionine.	-1.34
STR2	Cystathionine gamma-synthase, converts cysteine into cystathionine.	-1.18
STR3	Peroxisomal cystathionine beta-lyase, converts cystathionine into homocysteine.	-2.73
STR4	Cystathionine beta-synthase, catalyses synthesis of cystathionine from serine and homocysteine, the first committed step in cysteine biosynthesis; responsible for H_2S generation.	-1.25
MET2	L-homoserine-O-acetyltransferase, catalyses the conversion of homoserine to O-acetyl homoserine which is the first step of the methionine biosynthetic pathway.	-2.48
HOM3	Aspartate kinase, catalyses the first step in the common pathway for methionine and threonine biosynthesis.	-1.09
HOM2	Aspartic beta semi-aldehyde dehydrogenase, catalyses the second step in the common pathway for methionine and	-1.27

	threonine biosynthesis.	
HOM6	Homoserine dehydrogenase, catalyses the third step in the common pathway for methionine and threonine biosynthesis.	-1.20
THR1	Homoserine kinase, conserved protein required for threonine biosynthesis.	-1.35
THR4	Threonine synthase, conserved protein that catalyses the formation of threonine from O-phosphohomoserine.	1.63

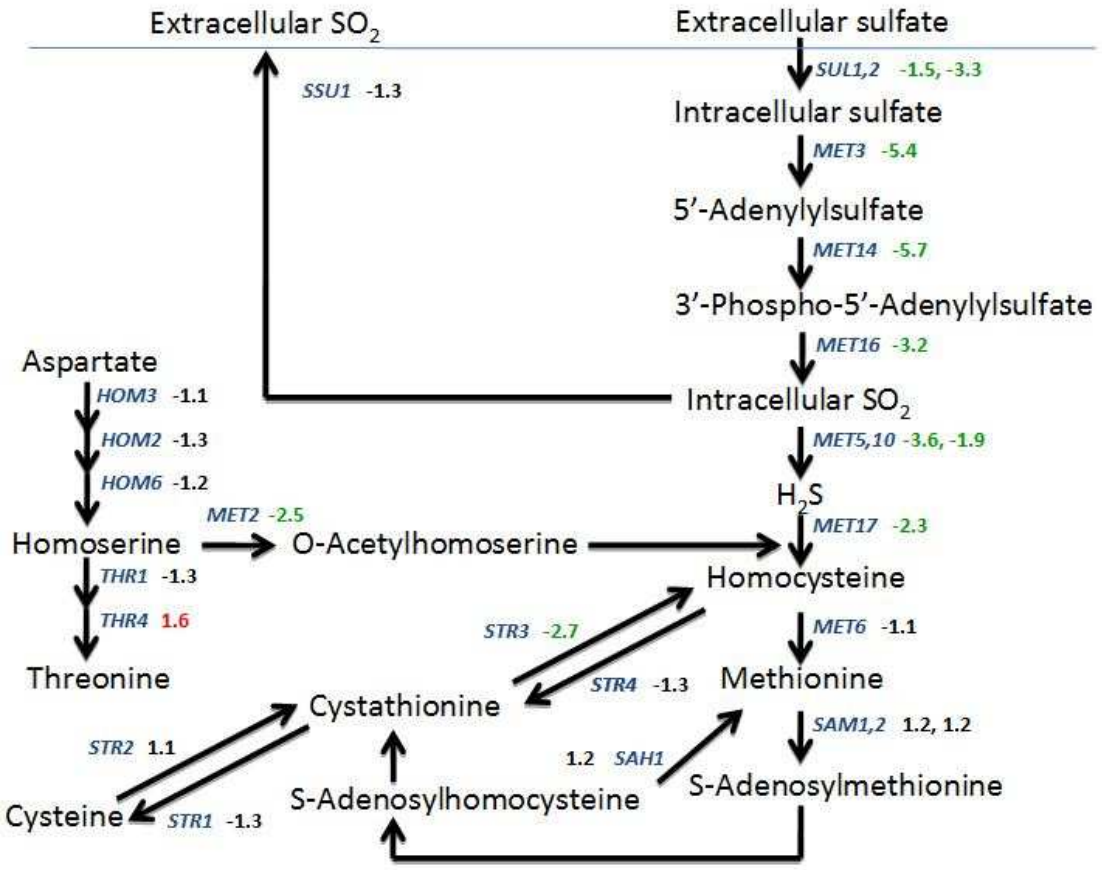


Figure 6.6. Yeast sulfur pathway showing the gene expression data for pectin vs control at 48 h. Note: blue indicates the gene name with the numbers beside them denoting the gene expression fold change for pectin vs control at 48 h, green being down-regulated and red being up-regulated.

6.5. Discussion

Following the outset of this chapter, a fundamental understanding of the effects of pectin and carrageenan on yeast metabolism was pursued through transcriptomic gene profiling. The acquired datasets as described above revealed significant insights. In Chapter 5, pectin and carrageenan showed an impact on wine clarity and flavour as well as on the production of hydrogen sulfide (H_2S). While, as mentioned previously, the effect on wine clarity is most likely the result of interaction between the pectin / carrageenan and the cations and positively charged proteins, the initial thinking that their influences on wine flavour compounds and H_2S are due to their modulation on yeast metabolism was confirmed by the findings in this chapter, namely the differences of their transcriptome under each treatment.

Acetate esters and ethyl esters, produced by yeast during fermentation, are important flavour compounds in wine and other fermented beverages (Verstrepen et al., 2003; Swiegers et al., 2005b; Saerens et al., 2008a). That the amino acid permeases were up-regulated in the treated pilot-scale fermentation (Table 6.1 and, for the specific example of leucine, Figure 6.3) provides an important clue for its enhanced flavour compounds, because these branched amino acids are precursors for higher alcohols and esters (Hazelwood et al., 2008). This was not the case for the lab-scale fermentations, however, with a few of these genes down-regulated. Amino acid concentrations were assessed during the wine fermentation relative to each other, however, they were used up so quickly that results were difficult to analyse, although it does seem that pectin used up the amino acids quicker and carrageenan slower than the control. Nonetheless, this leads to a harder question – how pectin and carrageenan incur such a beneficial phenomenon?

Similarly, the transcriptomic analysis unravelled that the sulfur pathway was down-regulated in the pectin vs control microarray at 48 h, but largely unchanged for the other conditions at the 96 h timepoint. This finding points to the possibility that H_2S levels could be already elevated under pectin treatment prior to the 48 h timepoint, resulting in the down-regulation via negative feedback loop of the pathway. The benign variation in gene expression for pectin plus carrageenan treatment and carrageenan alone might explain their inhibitive effect on H_2S levels. But, more experiments are definitely needed to shed more light on these potential agents' effect on yeast fermentation.

From the gene expression data, one thing is certain, that is, pectin and carrageenan affects yeast metabolism. To understand this and the question asked a moment ago, we need to look at them from the basics. Marangon et al (2012) showed that pectin and carrageenan reduce the amount of protein and small polypeptides available as a nitrogen source, leading the yeast to up-regulate their amino acid permease activity, which could explain the results seen in the pilot-scale fermentation microarrays. Lower nitrogen availability has been shown to induce the sulfur pathway, which may be the case here with the pectin and carrageenan treated samples where this pathway is down-regulated in pectin samples.

How exactly are pectin and carrageenan impacting the yeast gene expression? These compounds are too large and complex to be directly metabolised by the yeast. This means that they must be somehow indirectly impacting the yeast metabolism. Both pectin and carrageenan are anionic so ionic interactions are possible with cations of interest. This raises the question of which cations would bind to pectin and carrageenan. The principal cations are probably metal ions such as calcium and zinc, and in addition amino acids and proteins that carry a positive charge should bind. Most wine proteins have a positive overall charge at the acidic pH of wine (Waters et al., 2005). While most amino acids are neutral, arginine, histidine and lysine are positively charged and thus would bind with pectin and carrageenan.

The other method of sequestration could involve the cross-linking of pectin and carrageenan, which is how they form gels in solution. Calcium is involved in this process as a counter-ion. Some components, such as metal ions, proteins, carbohydrates and amino acids may be sequestered by pectin and carrageenan. Evidence suggesting that this is possible comes from their possibility as drug delivery agents (Ashford et al., 1993; Murano, 2000).

The differences between the primary structure of pectin and carrageenan could result in different sequestration abilities and therefore could explain the differences seen in the results in Chapter 5. Yeast cells detect the presence or absence of various compounds, which adjusts their gene expression so that the yeast adapt to their growth media and utilise the available resources suitably. If pectin and carrageenan are sequestering various compounds within the grape juice within a cross-linking gelling framework as well as binding cations, this could explain how they are having such an impact on the yeast gene expression without being directly metabolised.

Metal ions impact the fermentation rate, stability, clarity and flavour amongst other characteristics of wine, either in a positive or a negative way. Zinc, for example, has a positive impact on the fermentation rate, yeast biomass and alcohol production. This positive impact is due to the yeast's requirements for zinc and fermentations can become "stuck" when there is insufficient zinc in the must (Gauci et al., 2009; Tariba, 2011). Calcium and potassium can result in clarity issues in bottled wine, due to the precipitation of tartaric salts. Copper and iron are also involved in haze formation and wine discolouration. As well as haze, a wine's flavour profile is affected by the presence of metal ions; potassium and manganese have a positive impact on white wine flavour, while sodium, selenium and zinc have a negative impact (Tariba, 2011). A limit of 60 mg/L sodium (stoichiometrically exceeding chloride concentration) has been imposed by the Office International de la Vigne et du Vin.

Calcium binds with pectins (and is found as a counter-ion of pectin compounds in the cell wall) as well as contributing to the iron-phosphate hazing phenomenon (Aceto, 2003). High calcium concentrations also play a role in suppressing fermentation, most likely by hindering magnesium uptake (Birch et al., 2003), which is an important cofactor for many enzymatic reactions in yeast. Increasing the magnesium : calcium ratio (as would be the case with the reduction of calcium ions) resulted in increased fermentation rates and yeast growth (Birch et al., 2003). They also found that pre-conditioning yeast cells with magnesium resulted in a higher rate of ethanol production despite not influencing yeast growth. Fermentations with higher magnesium concentrations enhance ethanol production as well as final wine quality whereas higher calcium concentrations result in a more acidic wine (Birch et al., 2003).

Pectin and carrageenan could be indirectly affecting the yeast gene expression through limiting the nutrient availability of the fermentation, thus, leading to a change in flavour compound and H_2S production. Availability of nutrients in individual fermentations and differences seen between lab-scale and pilot-scale fermentors themselves could account for differences seen between these two fermentations. The effect of pectin and carrageenan on nutrient availability could be tested by determining the effect of nutrient supplementation experiments. The effect of certain nutrients on the flavour and other aspects of wine could give us insights into which nutrients could be influenced by pectin and carrageenan.

It is entirely possible that pectin and carrageenan could be used commercially to clarify grape juice and control the amount of H_2S in wine ferments.

Chapter 7: General discussion

What constitutes a good white wine? Clarity and flavour and the overall impression of aroma and taste compounds are major determinants of white wine quality. Of these, wine flavour is the most mysterious facet due to its complexity. It is considered to be a function of a range of parameters including grape variety, yeast strain, supplements in fermentation, élevage and aging, all of which can be exploited for making a better or unique wine. For winemakers and molecular biologists, it is the yeasts and supplements that their efforts have been focusing upon. In this study, I began by attempting to establish a transcriptomic cDNA microarray gene profiling methodology, then moved to the application of the technology to understand the yeast's sulfur metabolism. The usefulness of pectin and carrageenan in wine fining was thoroughly assessed and their effects on yeast fermentation and production of aroma compounds were dissected at the gene expression level. A number of discoveries and unexpected findings were described in the previous chapters, and they are discussed together below.

7.1. Understanding yeast fermentation through gene expression profiling

Polypoid industrial *S. cerevisiae* plays a major role in oenology. Unlike laboratory yeast strains which have haploid or diploid yeast genome containing only around 6,000 protein-encoding genes (Goffeau et al., 1996), industrial wine yeasts are highly specialised in improved fermentative efficiency, stress resistance, production of metabolites and in particular aroma compounds (Rainieri and Pretorius, 2000; Ugliano et al., 2009a). Increasingly, the science of transcriptomics has been employed to correlate the gene function of wine yeasts to phenotypic attributes (Rossouw et al., 2009). When this project started in 2007, cDNA microarray in Australia was still not fully established as a robust high throughput functional genomic technology.

7.2. Yeast metabolism and winemaking

Yeast metabolism is inextricably linked to wine aroma. Apart from nitrogen and phosphorus metabolism, the sulfate in the medium is assimilated in the sulfur pathway, leading to the synthesis of the key amino acids, cysteine and methionine. Along with this line of metabolism, an unwanted intermediate, H_2S, is generated. The addition of cysteine into media increases the release of H_2S, while the addition of nitrogen sources such as ammonium sulfate or DAP results in lower H_2S amounts (Spiropoulos et al., 2000). Chapter 4 investigated this phenonomen using a laboratory setting, which showed the same effect as seen in wine fermentations under anaerobic conditions. The gene expression of yeast under these conditions was examined using microarrays. It was found that increased ammonium availability caused a strong down-regulation of nitrogen catabolite repression (NCR), together with a concurrent up-regulation of the sulfur pathway. Expression of sulfur related genes, such as *MET17*, *MET5*, *MET3* and *SAM1*, and the likely increased availability of homoserine, allowed H_2S to continue through to the final sulfur metabolism products, cysteine and methionine.

In stark contrast, treatment with cysteine resulted in repression of the sulfur pathway and up-regulation of genes under NCR regulatory control, likely causing the H_2S intermediate to accumulate and ultimately be released. This suggests that H_2S production is dependent on the available nitrogen source, ie a preferred nitrogen one such as ammonium or a poor nitrogen source such as most of the amino acids, excluding glutamate. This is supported by the work carried out by Jiranek et al (1995), which suggests that poor nitrogen sources are less effective in providing yeast with cellular nitrogen for producing sufficient *O*-acetyl homoserine to sequester H_2S. The NCR appeared to also be involved when DAP was added into the grape juice under fermentation conditions (Marks et al., 2003).

The central carbon metabolism is of course the backbone of the whole yeast fermentation, resulting in alcohol, carbon dioxide, fatty acids and amino acids production. Fatty acid co-enzyme A and higher alcohols then combine to produce important esters, which are the most desirable flavour compounds. Any supplements to the fermentation medium should bring about improvements without a negative impact on flavours. This should be the case for H_2S reduction measures and for wine fining agents as well. As outlined in Figure 7.1, this study focused on the effects of

pectin and carrageenan, either alone or in combination when applied in fermentation. The findings described in the previous chapters revealed the unexpected aspects of the fining polysaccharides, namely their impacts on yeast metabolism.

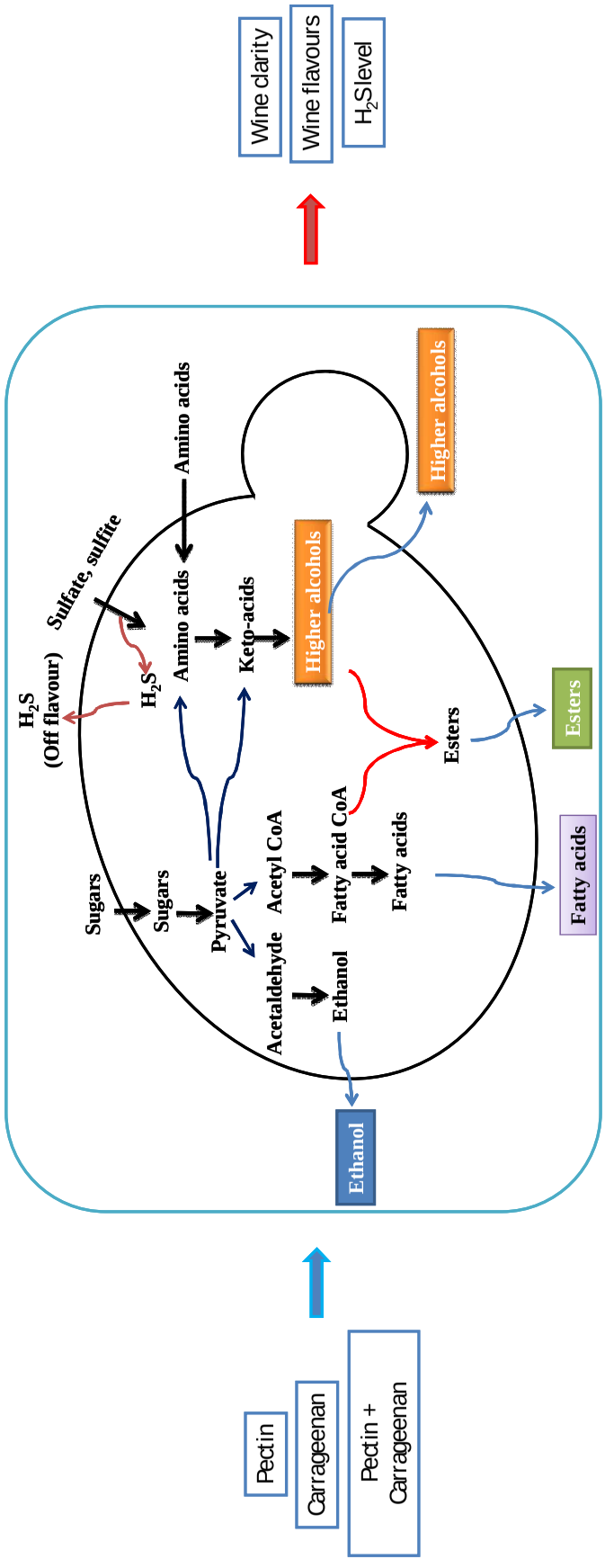


Figure 7.1. Yeast metabolism is affected by pectin and carrageenan.

Pectin and carrageenan both exhibit potential as wine fining agents, as shown in Chapter 5. Although, it is their other attributes that arouse significant scientific curiosity, which is behind the gene expression analysis in Chapter 6. How did pectin cause a dramatic surge in H_2S production in the middle of fermentation? The gene expression profile at 48 h showed a complete down-regulation of the sulfur assimilation pathway. This suggests several scenarios. Firstly, pectin affected the sulfur metabolism well before the testing time point – 48 h. The amount of intracellular H_2S was already elevated, which then triggered a negative feedback loop, leading to the down-regulation of genes. Alternatively, this gene expression could show us that the same situation as that above with cysteine is occurring – where due to down-regulation, the sulfur pathway, which leads to accumulation and then release of H_2S. The non-event or no effect on the pathway by carrageenan and carrageenan plus pectin treatments demonstrated that carrageenan has a different impact on yeast metabolism relative to pectin. Specifically, carrageenan resulted in a decrease in H_2S release (Figure 5.38, Chapter 5). The precise molecular mechanism for this observed reduction of H_2S by carrageenan remains to be answered.

7.3. Pectin and Carrageenan as fining agents and much more

Pectin and carrageenan are polysaccharides, with pectins being the most abundant carbohydrate in the primary cell wall and middle lamellae of terrestrial plants whereas carrageenan is dominantly in seaweeds (Caffall and Mohnen, 2009). Their primary biological function is to maintain cell integrity and form a defence barrier, similar to mammalian epidermis. Their effects on a variety of biological systems from bacteria to humans have been investigated in the past decade because of their application as food additives. Carrageenan was found to have a range of biological activities other than its original role in seaweed, from anticoagulant/antithrombotic, antiviral, immuno-inflammatory, antilipidemic and antioxidant activities, to their potential for therapeutic application (Jiao et al., 2011).

The primary biological function of pectin is to cross-link cellulose and hemicellulose fibres, providing rigidity to the plant cell wall, although pectin also has a role in cell signalling (Ridley et al., 2001). What is known in regard to the structure and function of pectin and carrageenan provides an important foundation for understanding the discoveries in this project. The anionic nature of these two

biopolymers allow the formation of a complex with positively charged proteins and ions such as calcium (Ca^{2+}) in wine. This directly leads to an enhanced wine clarity.

In the past few years wine districts that experienced hot, long summers produced sparkling white wines that were susceptible to post bottling haze formation. This has been the experience of Foster's wine arm (now called Treasury Wines). Product recall is damaging and expensive to those concerned, especially when you consider that at least half the wine sold in Australia is white wine. High calcium concentrations seem to have arisen during the sustained hot and dry weather experienced in most wine districts in Australia over recent years. In order to cope with heat stress, plants use various mechanisms. These include the changes in membrane fluidity and hence makeup, scavenging of reactive oxygen species, production of anti-oxidants, the accumulation of particular protein kinases, and significantly for this discussion the activation of the Ca-dependent protein kinase cascade. It seems that the changes in membrane fluidity trigger the influx of Ca^{2+} (Wahid et al., 2007).

Other effects of pectin and carrageenan on yeast metabolism observed in this study, have prompted the notion that these compounds are potentially more than just fining agents. The reduction of H_2S by carrageenan, for example, demonstrated an exciting promise for its role in wine fermentation, since any reduction of H_2S would clearly beneficial. Secondly, the overall enhancement of the flavour profile of wine is highly significant, whereby an overall increase of 35% across the different flavour categories – acids, esters and higher alcohols – was seen (Chapter 5, Section 5.6). Importantly, the flavour compounds associated with undesirable flavours remained at concentrations below their flavour threshold, for the pilot-scale fermentation. Some variations in the flavour profile were seen in the lab-scale fermentation, which were confirmed by a decrease in desirability, as determined by a sensory panel. Differences between the fermentations could have led to these variations in flavour, possibly attributable to the scale and equipment used, an unknown flavour compound negatively influencing the treated wine, the available nutrients in the grape juice, the concentrations of undesirable flavours observed in the lab-scale being above the flavour threshold, and finally the high amount of sugar in the control, which correlates with the sensory panel results.

By using transcriptomic gene profiling technology, their effects on yeast metabolism after 48 and 96 h of wine fermentation were unravelled (Chapter 6).

Specific synthetic pathways are responsible for the formation of wine aroma compounds. The profiles of esters, higher alcohols, and organic acids have a strong impact on wine quality. The aroma balance of these compounds is often used as an organoleptic fingerprint for specific wines (Saerens et al., 2008b). The higher amounts of isoamyl alcohol and isobutanol in pectin plus carrageenan treated pilot-scale ferment are likely derived from amino acid metabolism via Ehrlich pathway activity that was first proposed a century ago (Hazelwood et al., 2008) while isobutanol and phenyl alcohol can be produced from valine and phenylalanine respectively (Etschmann et al., 2002).

S. cerevisiae degrade the aromatic amino acids (tyrosine, phenylalanine, and tryptophan) and branched-chain amino acids (valine, leucine, and isoleucine) and thiol aminoacid (methionine) via the Ehrlich pathway. This pathway is comprised of the following steps: 1) deamination of the amino acid to the corresponding alpha-keto acid catalysed by amino acid aminotransferase; 2) decarboxylation of the resulting alpha-keto acid to the respective aldehyde by decarboxylase; and, 3) reduction of the aldehyde by alcohol dehydrogenase to form the corresponding long chain or complex alcohol, known as a fusel alcohol or higher alcohol. Higher alcohols are important flavor and aroma compounds in yeast-fermented food products and beverages.

7.4. Where to from here – Are carrageenan and pectin acceptable commercial finings agents?

Pectin is a natural molecule found in plant cells. Grape cell walls contain about 30% pectin (Mojsov et al., 2011). Exploitation of this pectin source would benefit winemakers. Indeed, attempts to release grape pectin with pectinases demonstrated that pectolytic treatment can break the physical barrier of grape skin and increase not only wine clarity but also the yield per ton of grapes obtained (Mojsov et al., 2011).

Furthermore, pectinase exists in grapes, and is actually involved in the ripening process. Can pectinases be used to release grape pectin rather than supplementing citrus pectin instead? The answer to this is yes, but not using grape pectinases because they are inactive under the pH and SO_2 conditions associated with winemaking. Fungal pectinases, in contrast, are resistant to the harsh winemaking conditions (Canal-Llaubènes, 1993). This study reinforced the role of pectin in wine clarity. The

additional findings are its role in flavour enhancement and its application together with carrageenan.

The fining of wine can be performed pre- or post-fermentation. This study shows that grape juice treatment (pre-fermentation fining) may be a more convenient, simpler way to stabilize wine than current practice, which treats wine after the fermentation is complete (Pocock and Waters, 2006). Bentonite by itself has a very limited ability to remove calcium from wine, when added either pre- or post-fermentation. However, pectin is a very effective agent for calcium removal (Waters et al., 2005). In combination with carrageenan it appears to be slightly more effective. Carrageenan removes protein yet has almost no negative effects on volatile levels. The amounts of these two agents used are relatively very minute for having a fining effect. This should provide plenty of scope for wine maker versatility. So it seems that this could be a real commercial approach to stabilising white wines. The work of Marangon et al. (2012) supported the findings of this study since they found that protein levels could be reduced without the detrimental reduction in flavours.

In answer to the question of commercial application that was posed at the start, yes, it does seem that treatment with these polysaccharides could be used commercially. Both carrageenan and pectins are cheap and simple calculations indicate that cost should not be a hindrance to uptake, although whether this process will achieve accreditation for wine making by ANZFA remains unknown. With more trials and studies, there is no doubt that the fining capacity of pectin and carrageenan will be considered commercially. After all, pectin and carageenan are natural products with wide applications in other areas of food processing (Willats et al., 2006). In fact the production of pectins is booming and for some pectin producers the problem is getting enough raw materials to satisfy demand.

Is carrageenan a tool to manipulate H_2S and flavour? My research has shown that pectin increases hydrogen sulfide production; carrageenan eliminates H_2S production (below detection levels), and that pectin plus carrageenan together decrease hydrogen sulfide production. Winemakers may choose to remove H_2S by simple treatment of grape juice with carrageenan. This does not appear to reduce the fermentation rate, the end production of alcohol and at the same time possibly offers greater aroma control. However, we must bear in mind that full industrial production is several steps removed from the laboratory studies carried out here.

The work of Marangon et al. (2012) which was carried out with industrial partners, the AWRI and at the instigation of the Foster's Group, showed that pectin and carrageenan do remove protein from white wine, in addition to partially stabilising the wine, almost to within target values below 2 NTU. This was also observed in laboratory trials carried out at the Foster's group with wine made in their pilot fermentation plant. Similar outcomes were obtained from work carried out with Foster's research winemaker. Although the bentonite required after this to achieve acceptable stability was greatly reduced, it would not be surprising if the ratio of carrageenan to pectin has a determining effect on the distribution of esters in wine. This is something that I did not have time to investigate further.

What is the effect of carrageenan dampening H_2S production? Is it possible that in the end carrageenan can be the arbitrator and hence be used by winemakers to 'trim' flavor outcomes? Flavour control is something of a holy grail for researchers in the art (Swiegers et al., 2006) and if asked to look into the future of polysaccharides I believe they could be very important tools.

Therefore, taken together, the data suggest that pectin and carrageenan treatment do not compromise yeast activity, they can increase ester formation, they definitely can affect H_2S production and there is the exciting prospect of being able to use this approach commercially to manipulate flavour.

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Appendix A: Pectin and carrageenan gene expression

		Pilot	48 h Lab scale			96 h Lab scale		
			Pectin + carrageenan	Pectin only	Carrageenan only	Pectin + carrageenan	Pectin only	Carrageenan only
Ensembl	Gene Sym	Fold-Chan	Fold-Change	Fold-Chang	Fold-Change	Fold-Change	Fold-Chang	Fold-Change
YCR104W /	PAU15 ///	2.68436	4.72062	1.24613	5.65004	2.52789	-1.18059	1.64836
YLR461W /	PAU4	2.68798	1.9156	1.25918	2.05613	3.57487	1.01259	2.77027
YNR057C	BIO4	-2.15639	-1.12062	-1.28244	-3.5953	2.07148	1.17356	2.35852
YNR058W	BIO3	-3.24901	1.1355	1.11747	-2.70429	2.28649	1.1875	2.47823
YAL065C	---	1.21821	2.24079	1.59524	1.23462	2.60673	1.20653	2.28492
YAL068C	---	2.26553	1.98197	1.23857	2.33405	2.53018	1.0472	1.9544
YAL068C //	---	2.01062	1.26534	1.12234	1.27164	2.18363	1.06755	2.24576
YBR301W	DAN3	2.83888	1.2712	1.15809	1.31724	3.63298	1.28893	3.78299
YCL069W /	VBA3	-1.16582	-2.31348	-2.41982	-3.10995	-1.23235	-1.0291	-1.3455
YDL037C	BSC1	-1.2998	2.08067	2.08226	1.88813	3.03206	1.56729	1.77364
YDL210W	UGA4	-1.11613	-2.06131	5.06239	-2.19081	-1.07139	-1.0685	-1.65649
YDR508C	GNP1	2.73908	2.30546	-1.16534	5.87672	1.30587	1.03013	1.03559
YGR236C	---	2.74558	-1.6224	-1.13046	-1.04615	-2.40338	-1.00197	-2.04599
YHR033W	---	2.20905	-1.12611	1.21178	1.28912	-2.66944	-1.32313	-2.33123
YIL176C ///	PAU1	2.01926	1.76415	1.21298	1.80264	2.74041	1.09986	2.376
YJL121C	RPE1	1.16185	2.0624	2.45642	2.23314	1.12573	1.08882	-1.03838
YJR005C-A	---	-1.31548	-2.05157	-2.04873	-2.63307	-1.8605	-1.2761	-1.47774
YKR013W	PRY2	1.0082	1.48301	1.0852	2.08512	2.81914	-1.17217	2.2018
YLL064C //	PAU6	2.04936	1.72221	1.27373	1.81938	3.13626	-1.04815	2.6563
YLR063W	---	1.15883	2.47703	2.69373	1.93541	2.04451	1.28059	1.27844
YMR058W	FET3	1.20799	-3.56766	-1.56513	-2.45824	-2.0097	-1.29622	1.07719
YMR170C	ALD2	-1.77253	-2.12278	3.4381	-2.66731	1.00567	1.066	-1.22109
YMR310C	---	1.53064	2.08756	2.22516	2.07686	1.11117	1.03188	-1.0258
YMR317W	---	-1.18087	3.08175	-1.0715	4.28729	1.5713	-1.01353	2.38934
YNL300W	---	1.33308	1.55318	-1.26789	2.16284	3.69563	-1.07118	2.3661
YOR010C	TIR2	1.10093	1.87587	1.71178	2.00778	3.46757	1.29527	3.8308
YOR247W	SRL1	1.15806	2.18977	2.19326	1.86834	3.41401	1.13563	1.97507
YPL256C	CLN2	-1.16964	1.95311	2.16324	1.7307	3.7018	1.19704	2.22489
YAL018C	---	-1.33269	1.27575	3.04914	-2.40273	-1.03272	-1.00786	-1.08018
YBR006W	UGA2	-1.49806	-2.09464	1.66652	-2.48149	-1.19738	-1.08152	-1.04504
YBR040W	FIG1	-2.65341	-1.04283	1.19886	-2.22737	-1.05026	1.0018	-1.07717
YBR162C	TOS1	-1.04593	1.22158	-1.06371	1.33472	2.39909	1.12845	2.39946
YBR208C	DUR1,2	-4.07401	1.01209	-2.00181	-1.78215	-1.5875	-1.45037	1.33932
YBR299W /	MAL12 ///	1.41927	2.08063	2.15055	1.53148	1.27795	-1.03115	1.18198
YCL064C	CHA1	-3.88051	1.43645	-1.13703	3.2861	-1.01202	1.42821	-1.57062
YDL038C	---	-1.26	1.98463	2.02749	1.80252	2.00404	1.3898	1.61623
YDL127W	PCL2	1.38762	2.01021	1.92524	2.26945	1.47833	1.20312	1.01555
YDL214C	PRR2	1.29573	-2.09962	1.0313	-2.30764	-1.62053	-1.25787	-1.17785
YDR046C	BAP3	2.11516	1.98243	-1.8877	2.68408	1.00243	-1.03713	1.00734
YDR146C	SWI5	-1.19685	-1.21479	-1.29778	1.02494	2.0549	-1.16378	2.01734
YDR222W	---	-1.63674	1.78383	1.40412	1.57748	4.18638	1.42317	3.88125
YDR242W	AMD2	-1.53374	-1.84554	3.70495	-2.15401	-1.03272	1.15632	-1.36197
YEL049W	PAU2	1.77557	1.14513	1.10484	1.14002	2.24814	1.08214	2.43892
YER145C	FTR1	1.54297	-2.36089	-1.2286	-2.03277	-1.37946	-1.01227	-1.06405
YFL051C	---	1.36004	2.69309	1.76393	2.11687	1.9918	1.66876	-1.03631
YGL138C	---	-2.04018	-1.33668	1.83636	-4.41825	-1.01561	1.15091	-1.04123
YGR213C	RTA1	1.39932	2.78321	1.25939	2.51007	1.65276	1.15	1.23261
YHL040C	ARN1	-1.0277	-2.12096	-1.92331	-2.11744	-1.79721	-1.1586	-1.10272
YHR143W	DSE2	1.15896	1.28396	1.4234	1.18855	2.67102	1.27715	2.53983
YHR213W-/	---	1.12997	2.19396	1.55178	1.71429	2.07612	1.6596	1.03239
YIL158W	---	-1.61965	-1.1455	-1.49062	1.07886	3.175	-1.19983	2.56546
YIR031C	DAL7	-2.85372	-1.34145	-1.64153	-2.88782	-1.70273	-1.73825	1.16034
YJL133C-A	---	2.26169	1.47279	4.56418	1.11213	-1.32399	1.09163	-1.36226
YJR154W	---	-2.29936	-1.08375	-1.1388	-2.62966	1.10316	-1.10126	1.33955

YLL061W	MMP1	2.46389	-1.8122	-3.49913	-1.05784	-1.08686	-1.19578	1.11116
YLR037C	DAN2	1.25378	1.23907	1.17273	1.19028	2.05902	-1.01018	2.4967
YLR073C	---	1.63061	2.39537	1.82205	1.97042	2.16486	1.57987	1.43449
YLR197W	SIK1	2.15461	1.30599	-1.39961	1.33405	2.31394	-1.12543	1.21803
YLR307W	CDA1	-2.03408	-1.05889	1.46228	-2.85351	-1.05835	-1.06123	1.00886
YLR372W	SUR4	1.17755	1.17105	-1.37528	1.23565	3.49289	1.36362	2.57155
YMR042W	ARG80	1.69589	2.13309	2.39126	1.74905	1.56938	1.17691	1.02138
YMR118C	---	-1.47032	-2.06692	1.97462	-2.28388	-1.61691	-1.13048	-1.29578
YMR244W	---	2.1017	1.43278	1.9087	1.01548	2.16365	1.50825	1.53806
YMR305C	SCW10	1.22073	1.22896	-1.24212	1.54711	2.27238	-1.07163	2.11957
YNL112W	DBP2	1.12631	1.82029	2.18833	1.73663	2.05776	1.5614	1.39682
YNL289W	PCL1	-1.61046	1.41386	1.08047	1.81493	4.13672	-1.23485	2.45658
YNR056C	BIO5	-1.73536	-1.22464	-1.32438	-4.40821	1.72092	1.15553	2.40729
YNR067C	DSE4	-1.55035	1.09742	1.16236	1.27416	2.1002	-1.05742	2.38718
YOL007C	CSI2	-1.14026	1.30337	-1.2153	1.67302	3.38848	-1.07976	2.07133
YOL111C	MDY2	1.19397	1.72596	2.06605	2.18033	-1.12776	-1.01479	-1.12313
YOR011W	AUS1	-1.08794	1.50343	1.1265	1.36248	2.13444	1.16852	2.55584
YOR255W	OSW1	-1.26532	1.13255	2.24004	-3.57409	-1.14477	-1.13781	-1.06197
YOR359W	VTS1	1.25655	2.02189	2.1587	1.69698	1.76166	1.3646	1.38974
YPL033C	---	-2.23854	-1.18842	1.60174	-3.80008	1.26036	1.1478	1.11323
YPL130W	SPO19	-2.10427	1.07946	1.28833	-2.10543	1.02519	1.03495	-1.13873
YPR119W	CLB2	-1.3252	-1.21414	-1.24945	-1.04586	2.37767	-1.11347	2.24332
YAL040C	CLN3	1.02958	1.48266	2.54838	1.2464	1.26492	1.13709	1.24332
YAR015W	ADE1	-1.31395	-1.42127	-2.56627	-1.10166	-1.09089	1.00601	1.04867
YAR020C	PAU7	1.76469	1.26688	1.16232	1.34768	1.82407	-1.07695	2.24721
YAR068W /	---	1.49712	1.51964	1.20708	2.29826	1.2381	1.10837	1.02113
YBL029W	---	-1.53536	1.3849	2.02544	1.06586	1.21619	1.1784	-1.00893
YBL059C-A	---	-2.24038	-1.21968	1.24558	-1.42607	1.04619	-1.08788	-1.01494
YBL082C //	ALG3	1.35398	1.50252	2.03117	1.73048	1.05257	-1.04446	-1.00768
YBR047W	---	-2.16945	-1.19535	-1.20524	-1.20868	-1.15915	-1.10754	-1.08974
YBR072W	HSP26	-1.07635	-1.56595	1.1426	-2.30434	-1.53777	-1.16661	-1.09796
YBR076W	ECM8	-1.43616	-1.46319	1.09499	-2.75003	-1.55884	-1.09344	-1.32381
YBR213W	MET8	1.31801	-1.45944	-2.11805	-1.1059	1.10243	1.07355	1.0968
YBR233W-/	DAD3	-2.11054	-1.21116	-1.39349	-1.09533	1.3754	-1.03877	1.0599
YBR238C	---	-1.08096	1.7614	2.05251	1.43688	1.47617	1.39344	1.09288
YBR241C	---	1.67553	-1.06021	2.06326	-1.147	-1.24895	-1.1275	-1.14476
YBR247C	ENP1	1.29376	2.01723	1.77175	1.6998	1.58168	1.16119	1.16745
YCL002C	---	2.41189	1.00528	1.0249	1.03056	1.11849	1.22272	-1.00495
YCL025C	AGP1	-1.0569	-1.03346	-2.69742	-1.0405	1.68048	-1.0049	1.19733
YCL063W	VAC17	-1.09075	1.40229	-1.00878	1.6941	2.18819	1.06994	1.78295
YCR021C	HSP30	2.61444	-1.41057	-1.06944	-1.59301	-1.10896	-1.22453	-1.51316
YCR034W	FEN1	1.37084	1.43455	-1.20918	1.65379	2.11561	1.23875	1.67831
YCR046C	IMG1	1.43153	1.37152	2.42945	1.19938	-1.21775	-1.07099	-1.067
YCR090C	---	-2.06136	1.05568	-1.04037	-1.02273	1.26568	1.00084	1.09065
YDL055C	PSA1	-1.16378	-1.02829	-1.08543	-1.01028	2.0333	1.27959	1.66368
YDL058W	USO1	1.0239	-1.08202	-2.00743	-1.01628	1.35322	-1.97717	-1.20181
YDL059C	RAD59	-1.02009	-1.5099	-3.49719	-1.17444	-1.07677	-1.11458	1.03644
YDL086W	---	1.30939	1.24639	2.18876	1.32503	-1.05167	-1.00468	-1.14569
YDL114W	---	-1.52509	-1.20518	1.31337	-2.23786	-1.41456	-1.12216	-1.20987
YDL121C	---	-2.03574	1.19085	1.00732	1.059	1.35977	1.12226	1.05658
YDL141W	BPL1	-1.09236	1.47096	2.16741	-1.42704	1.14367	1.03073	1.28373
YDL148C	NOP14	1.77118	1.29714	-1.05158	1.19775	2.07852	-1.02585	1.16967
YDL205C	HEM3	-2.25533	-1.27822	-1.322	-1.1222	-1.34909	-1.1225	-1.06656
YDL222C	FMP45	3.25293	-1.03778	1.39585	1.3581	-1.95904	-1.03024	-1.96873
YDR019C	GCV1	-1.44246	-1.57501	-2.56637	-1.15765	-1.12791	1.06835	1.02699
YDR044W	HEM13	-1.07207	1.23726	1.26122	1.23976	1.48259	-1.1829	2.07045
YDR156W	RPA14	1.48291	1.95061	2.26873	1.77829	-1.04313	-1.03684	-1.03841
YDR218C	SPR28	1.17968	-1.01829	1.33865	-2.19466	1.31635	-1.08582	1.11003
YDR247W	VHS1	1.23514	1.31086	2.15812	1.19918	-1.1144	1.07456	-1.23628
YDR253C	MET32	1.61775	-1.46096	-4.36671	1.05456	-1.0096	-1.15404	-1.37753
YDR315C	IPK1	-2.05079	-1.3199	-1.05946	-1.35248	1.0418	-1.00422	-1.02193
YDR357C	---	-2.16377	-1.24228	1.01898	-1.38515	1.14137	-1.04763	1

YDR380W	ARO10	-1.10192	1.21051	5.22458	-1.03374	1.09995	1.12323	-1.19857
YDR453C	TSA2	1.18296	1.85241	3.23406	1.5157	-1.34435	-1.12581	-1.05376
YEL069C	HXT13	2.76652	-1.10506	1.21914	-1.30943	-1.42993	-1.12952	-1.14844
YEL069C //	HXT13 /// H	2.84453	1.15449	1.07052	1.03666	1.04745	-1.05551	1.073
YER103W	SSA4	3.50548	1.01664	1.29079	-1.09656	-1.0303	-1.00034	-1.13293
YER106W	MAM1	-1.04513	-1.18023	-1.03194	-2.29095	1.14359	-1.18938	-1.07009
YER126C	NSA2	1.75246	1.36095	-1.04493	1.34432	2.2691	1.23638	1.55794
YER153C	PET122	2.07738	1.16704	1.04494	1.10438	1.01087	-1.07142	1.12649
YER156C	---	1.25402	1.97383	1.70989	2.30222	1.28473	-1.05881	1.07562
YER158C	---	-1.08932	1.06111	2.01598	-1.02397	-1.21887	1.13468	-1.16839
YFL012W	---	-1.64302	-1.01607	1.43387	-2.50044	1.36063	1.06915	1.18766
YFL031W	HAC1	2.19124	-1.11244	-1.067	-1.25659	-1.16446	1.04525	-1.01209
YFR017C	---	1.64204	-1.005	2.45573	-1.19761	-1.85014	-1.31563	-1.34034
YFR023W	PES4	-1.39443	-1.00016	1.65608	-2.71184	-1.10562	-1.00728	-1.08017
YFR032C	---	-1.03336	1.80433	2.10236	-1.00099	1.13414	1.07113	1.02982
YFR057W	---	-1.65934	1.06808	1.42652	-2.18588	1.28143	1.09545	-1.08338
YGL028C	SCW11	-1.21989	1.16625	1.29833	1.17536	2.14691	1.17042	1.85748
YGL126W	SCS3	2.56194	1.34001	1.63831	-1.15475	1.08114	1.08151	1.0024
YGL147C	RPL9A	-1.42295	1.1218	-1.63476	1.22267	2.26493	1.24643	1.4312
YGL159W	---	-2.08793	-1.06122	1.0965	-1.05566	1.07016	-1.01167	1.04779
YGL170C	SPO74	-1.26004	-1.06486	2.40457	-1.74351	-1.1319	1.10759	1.01154
YGL184C	STR3	-1.11621	-1.44648	-2.72509	-1.25214	-1.09482	-1.03942	-1.01305
YGL255W	ZRT1	-1.27409	1.98212	2.02096	1.23689	1.94021	1.11796	1.51896
YGL256W	ADH4	-1.33117	2.60277	1.36968	1.46728	1.23128	1.18329	1.12103
YGR055W	MUP1	1.98958	1.26911	-1.75328	2.39491	1.69784	1.12251	1.45472
YGR059W	SPR3	-1.12471	-1.30376	-1.09451	-2.26027	-1.10469	-1.25532	-1.0955
YGR063C	SPT4	2.56913	-1.10657	-1.12722	-1.0311	1.11848	1.21296	-1.02696
YGR065C	VHT1	-1.41376	-1.0316	1.20707	-3.03993	1.38266	1.03421	1.5059
YGR109C	CLB6	1.13194	1.11381	-1.17561	1.37793	2.41388	-1.01674	1.42374
YGR142W	BTN2	7.18208	1.16246	-1.1591	-1.08428	1.43046	-1.3847	1.18833
YGR154C	GTO1	-1.19267	-1.42088	-2.02195	-1.24056	-1.3846	1.02865	-1.57662
YGR204C-A	---	2.62658	-1.5727	1.06804	-1.59037	-1.06617	-1.04089	-1.07781
YGR243W	---	2.17406	-1.4165	-1.17658	-1.19995	1.07095	1.04741	-1.06154
YGR248W	SOL4	1.35468	1.28208	2.30395	1.10959	-1.7637	-1.21412	-1.54442
YGR273C	---	1.04593	1.07088	1.70865	-2.71474	1.70858	-1.05605	1.54027
YGR280C	PXR1	2.03408	1.18184	-1.23146	1.17457	1.05528	-1.33214	-1.09814
YHL016C	DUR3	-3.16024	-1.1102	-1.13206	-1.94917	-1.46322	-1.78786	1.16021
YHL026C	---	1.34209	2.12161	1.452	1.86509	1.00383	-1.25347	1.72738
YHR037W	PUT2	-1.29484	-1.20032	1.44689	-2.02646	1.05414	1.05674	1.00147
YHR041C	SRB2	2.14499	1.45806	1.44705	1.37512	1.38086	1.1869	1.18661
YHR061C	GIC1	1.36776	1.58699	1.19644	1.75914	2.67576	1.15754	1.93934
YHR092C	HXT4	1.02691	-2.30853	1.13165	-1.88702	-1.25161	-1.27001	1.30833
YHR126C	---	1.09271	1.3804	1.49268	-1.6917	2.57888	1.5513	-1.05168
YHR137W	ARO9	1.20297	1.08773	7.22201	-1.03995	-1.20403	-1.0384	-1.15151
YHR139C	SPS100	1.01002	-1.28455	-1.04085	-1.00288	-1.63027	1.35169	-2.32217
YHR205W	SCH9	1.19549	1.38963	2.46837	1.38758	1.21685	1.08902	-1.0379
YIL074C	SER33	1.03074	-1.18978	-3.02289	1.05837	-1.02228	1.01376	1.11474
YIL122W	POG1	1.19301	1.3347	2.31257	-1.01827	-1.22236	-1.02295	-1.05823
YIL162W	SUC2	1.15225	-1.02396	2.27606	-1.73474	1.11724	-1.14628	1.2867
YIR014W	---	1	-1.48663	-1.24337	-1.21381	-2.08392	-1.34001	-1.38262
YIR030C	DCG1	-2.2726	-1.22942	-1.20473	-1.62081	-1.295	-1.53534	1.1327
YIR032C	DAL3	1.29124	-1.49252	-1.39343	-2.50755	-1.34036	-1.1919	1.03266
YJL003W	COX16	-2.11269	-1.28718	1.11195	-1.34601	-1.12816	-1.114	-1.04453
YJL038C	---	-1.16006	-1.30562	1.01536	-2.74457	-1.31543	-1.29545	-1.06361
YJL088W	ARG3	1.59872	1.50965	2.54846	1.78325	1.84527	1.90958	-1.07219
YJL158C	CIS3	1.08779	1.23917	1.12536	1.12591	2.17246	-1.01491	1.88809
YJL161W	---	-1.30757	-1.62854	1.43844	-2.2293	-1.55622	-1.17072	-1.18198
YJL190C	RPS22A	1.1701	1.05877	-1.31676	1.12458	2.34994	1.3455	1.51425
YJL212C	OPT1	1.45958	-1.32929	-2.99155	-1.05486	1.03402	1.17678	-1.40517
YJR004C	SAG1	2.07168	1.09602	1.53749	-1.02186	1.04401	1.1738	-1.04523
YJR010W	MET3	1.1644	-1.15018	-5.42646	1.18538	-1.0513	1.01486	1.00847
YJR070C	LIA1	1.16327	1.81508	1.14124	1.70068	2.06301	1.31045	1.4674

YJR112W	NNF1	-1.14862	2.09064	1.61886	1.95832	1.29321	1.08794	-1.01243
YJR137C	ECM17	1.01435	-1.11331	-3.56805	1.1724	-1.106	1.10311	-1.03322
YJR155W	AAD10	-1.18956	-1.28118	-1.00264	-3.47768	-1.60259	-1.03604	-1.46094
YKL001C	MET14	-1.2837	-1.11191	-5.73773	1.12518	1.12334	-1.04423	1.04132
YKL068W-A	---	1.31561	-1.42139	-5.15518	-1.2029	1.78322	1.43233	1.14758
YKL081W	TEF4	1.02437	1.70228	1.27757	1.74577	2.04698	1.36015	1.46377
YKL082C	RRP14	2.65452	1.12375	-1.61324	1.18158	1.6074	-1.78653	-1.23681
YKL086W	SRX1	2.35826	-1.15658	1.20869	-1.25697	-1.22449	-1.12384	-1.39788
YKL099C	UTP11	2.01932	1.23657	-1.05016	1.23081	1.50016	-1.10678	1.16482
YKL164C	PIR1	1.2349	1.61465	2.11834	1.53945	1.43088	1.02043	1.26184
YKR080W	MTD1	-1.31652	-1.24117	-2.16875	-1.06916	-1.15189	-1.06206	-1.06527
YKR092C	SRP40	-1.01941	1.82669	2.11103	1.60628	1.65695	1.27305	1.07191
YLL010C	PSR1	-1.0659	1.69303	2.40998	1.45802	1.18701	1.29181	-1.04501
YLL055W	YCT1	1.06779	-1.79352	-2.58538	-1.70515	-1.14869	1.01032	-1.00823
YLL062C	MHT1	1.34861	-1.31907	-3.50685	1.04434	-1.16333	-1.12697	-1.05203
YLR053C	---	-1.79101	-1.84134	-1.21021	-2.03792	-1.10416	-1.01166	-1.25163
YLR092W	SUL2	1.29265	-1.48596	-3.32348	-1.16243	-1.28643	-1.36572	1.02122
YLR094C	GIS3	2.06109	-1.21674	1.0563	-1.13127	-1.13193	-1.03213	1.03248
YLR136C	TIS11	-1.05043	-1.64101	-1.42462	-2.01685	-1.49841	-1.0507	-1.08154
YLR142W	PUT1	-1.88435	-1.23802	1.39606	-3.976	-1.0173	1.00541	-1.05884
YLR154C-H	---	2.40245	-1.06807	1.10167	1.02043	-1.18577	1.29228	-1.14902
YLR162W-A	---	3.82048	-1.33147	-1.13523	-1.40684	-1.83656	-1.10893	1.04399
YLR245C	CDD1	1.20095	1.58932	2.0844	1.30807	1.35228	1.19022	1.0555
YLR258W	GSY2	1.01404	-1.53927	1.42557	-2.17257	-1.46936	-1.44543	1.03876
YLR281C	---	2.31621	-1.04523	1.32263	-1.12164	1.11416	1.33616	1.00343
YLR301W	---	-2.18545	-1.07446	-1.42231	1.00099	1.37778	1.00205	1.3073
YLR303W	MET17	1.04911	-1.11942	-2.2546	1.00432	1.05563	-1.06876	1.12103
YLR307C-A	---	-2.05526	-1.38618	-1.0419	-1.35465	1.51643	1.6595	-1.33438
YLR308W	CDA2	-1.62436	1.0582	2.04883	-1.42503	-1.00336	1.25371	1.01586
YLR312C	---	-1.12983	-2.00938	1.09084	-1.80299	-1.41278	1.04627	-1.29891
YLR356W	---	1.38212	1.36525	2.06667	1.35679	1.00518	1.06801	-1.03824
YLR364W	---	1.40082	-1.07511	-3.87013	1.28104	1.16978	1.25552	-1.01337
YLR456W	---	-2.10725	1.02893	-1.0704	-1.04303	1.25805	1.11835	1.01678
YML007C-A	---	2.07068	1.19401	1.39912	-1.00022	1.21468	1.25361	-1.2062
YML022W	APT1	2.97681	1.25146	-1.06767	1.26399	1.65305	1.29758	1.20151
YML027W	YOX1	1.23195	1.29719	1.46095	1.70729	2.38704	-1.18264	1.95802
YML123C	PHO84	-1.0327	-1.39166	-2.68097	1.36338	1.25573	1.01821	1.21237
YMR011W	HXT2	3.10831	-1.2446	1.00388	1.6433	-1.41822	1.48771	-1.01977
YMR104C	YPK2	2.12365	-1.19968	1.1703	-1.40402	-1.6101	-1.11835	-1.22624
YMR169C	ALD3	1.26278	-1.10764	2.46471	-1.13336	-1.49168	1.03983	-1.53325
YMR175W-	---	-1.11038	-1.24401	1.30061	-1.08863	-2.18448	-1.37274	-1.43383
YMR194C-B	---	2.72122	-1.00092	1.31852	-1.23021	-1.43951	-1.02265	-1.07863
YMR215W	GAS3	1.00548	1.17451	-1.25712	1.48474	2.50374	1.14546	1.84114
YMR290C	HAS1	1.42779	1.57591	1.31096	1.66753	2.17863	1.30626	1.4251
YMR292W	GOT1	2.42787	-1.00709	1.02717	1.04805	1.23278	1.06675	1.03443
YMR323W	ERR1 /// E	-1.19908	-1.36991	1.31965	-1.17578	-2.0029	-1.37052	-1.25151
YNL042W-B	---	3.34009	-1.10855	-1.01809	-1.01385	-1.26539	1.6888	-1.06783
YNL066W	SUN4	1.20673	1.53093	1.49143	1.52252	2.24321	1.13577	1.83361
YNL074C	MLF3	1.00733	1.30058	2.26983	1.36264	-1.34437	-1.02428	-1.2468
YNL093W	YPT53	1.09293	-2.06867	-1.53542	-1.92735	-1.69396	-1.03129	-1.38333
YNL110C	NOP15	2.32227	1.3118	-1.11334	1.21465	1.69083	-1.16782	1.16826
YNL141W	AAH1	1.14716	1.61286	2.15078	1.72264	1.36496	1.32321	1.02686
YNL230C	ELA1	1.07462	1.95621	2.51163	1.3574	1.11378	1.12257	-1.12431
YNL237W	YTP1	1.05104	-2.09244	-1.10609	-1.77231	-1.77613	-1.11268	-1.29949
YNL248C	RPA49	1.27944	1.34693	-1.05991	1.30421	2.0963	1.3338	1.54293
YNL269W	BSC4	1.50548	1.02034	2.35252	-1.08031	-1.02751	1.12414	-1.29591
YNL270C	ALP1	1.45663	-1.11608	2.08844	-1.27023	-1.03573	1.23035	-1.12356
YNL277W	MET2	1.29369	-1.42312	-2.47556	-1.2032	-1.06253	-1.08712	-1.00334
YNL318C	HXT14	-1.36583	1.01275	1.60175	-2.83317	-1.16341	-1.04617	-1.06188
YNR014W	---	2.01276	-1.31636	-1.24269	-1.0355	-1.81515	-1.26236	-1.5231
YNR062C	---	2.18515	1.1368	1.16087	1.14388	1.17111	1.20187	1.0679
YNR071C	---	1.45944	-1.20975	1.29366	-2.16055	-1.12636	-1.21164	1.10474

YNR072W	HXT17	3.04107	-1.20945	-1.15942	-1.23311	-1.23926	-1.20523	1.01522
YOL013W-A	---	2.48844	1.01398	-1.0834	1.26449	1.10882	1.43661	1.06016
YOL014W	---	1.30685	1.17785	-1.13137	1.11844	2.27956	1.1676	1.71766
YOL110W	SHR5	2.86974	1.04787	1.24584	-1.06153	1.1851	1.22755	1.02288
YOL132W	GAS4	1.07728	1.01042	1.42617	-2.56973	-1.06838	1.02529	-1.01282
YOR042W	CUE5	-1.5117	1.49552	2.15481	1.50215	1.02623	-1.05859	-1.07631
YOR044W	---	-2.14254	-1.12214	1.03427	-1.13676	1.1962	1.09563	1.02499
YOR072W-A	---	2.12637	-1.09193	1.08344	1.02778	1.17788	1.04643	-1.10252
YOR161C	PNS1	3.32706	-1.24785	1.2468	1.01631	-1.4566	-1.00179	-1.55034
YOR184W	SER1	-1.19411	-1.12464	-2.12712	1.09756	1.0789	1.00129	1.11722
YOR214C	---	-1.16097	-1.11106	1.17156	-2.36083	-1.04314	1.04416	1.0253
YOR306C	MCH5	1.28241	-1.08048	1.86956	-2.69565	1.06386	1.24294	-1.02706
YOR340C	RPA43	1.24318	1.53242	-1.02967	1.40104	2.11569	1.11052	1.5107
YOR348C	PUT4	1.4253	-1.8267	2.56938	-1.26264	-1.2654	1.01857	-1.27485
YOR382W	FIT2	1.82736	-1.03529	1.44498	1.17507	-2.30721	-1.1869	-1.70012
YOR384W	FRE5	-1.18687	-1.01544	-1.16935	-1.04306	-2.14029	-1.38773	-1.70414
YOR387C	---	-3.73235	-1.17993	-1.35837	-1.01996	-1.07552	-1.01028	1.08242
YPL036W	PMA2	1.4022	1.14861	1.38868	1.36503	2.20513	1.52731	1.11356
YPL068C	---	1.20329	1.80929	2.1981	1.71385	1.27824	1.08667	1.1233
YPL201C	YIG1	1.22061	-1.59133	-1.22928	-1.09432	-2.38125	-1.2165	-1.46212
YPL225W	---	-2.03164	-1.08572	1.0711	-1.15125	1.07618	1.08274	1.05204
YPL252C	YAH1	1.13281	1.52792	2.07086	-1.04035	1.04433	-1.01143	1.1165
YPL262W	FUM1	-1.17078	1.02877	2.09584	-1.08168	-1.25601	-1.00922	-1.12011
YPR009W	SUT2	-1.11241	1.28488	-1.13241	1.59563	2.02261	1.73577	1.20145
YPR144C	NOC4	1.22118	1.93557	2.03219	1.63052	1.80594	1.33493	1.36828
YPR159C-A	---	2.14338	-1.09254	1.14645	-1.35035	-1.10795	-1.004	-1.08994
YPR167C	MET16	-1.0992	-1.01135	-3.16218	1.27187	1.14953	1.02487	1.09059
YPR194C	OPT2	-2.32885	-1.48059	1.07754	-1.95633	-1.02181	1.06864	-1.00509