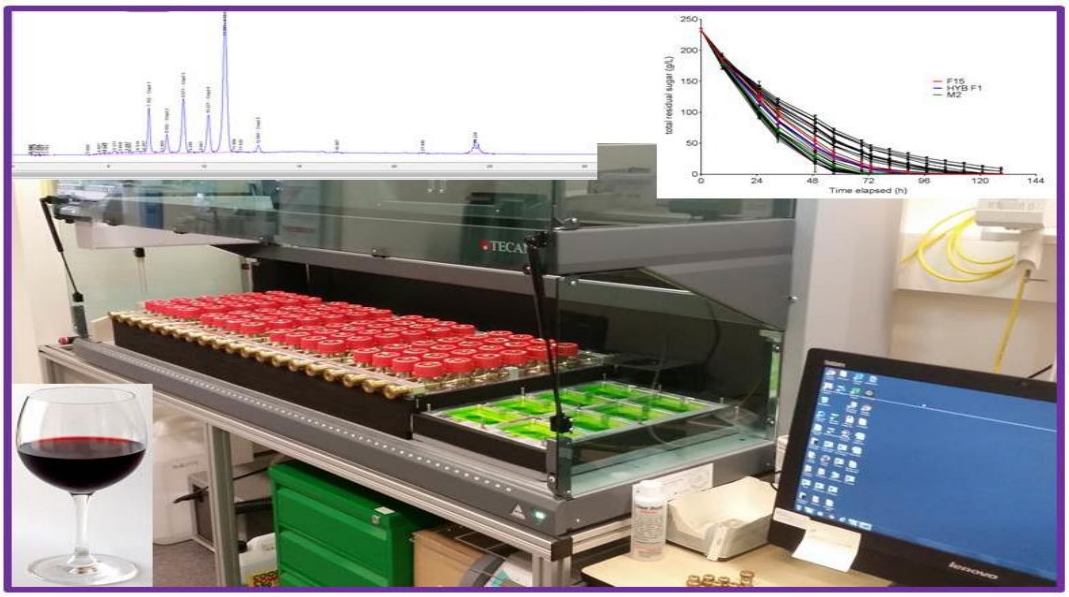
Australian Government

Australian Grape and Wine Authority

THE UNIVERSITY of ADELAIDE

Mapping of genes responsible for yeast-derived modulation of colour in model red wine



FINAL REPORT to

AUSTRALIAN GRAPE AND WINE AUTHORITY

Project Number: **UA 1405**

Principal Investigator: **Dr Michelle Walker**

Research Organisation: **University of Adelaide**

Date: **30 June 2015**

Disclaimer

UA 1405 Project Title: 'Mapping of genes responsible for yeast-derived modulation of colour in model red wine'

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Abbreviations

$A520_{nm}$	Absorbance at 520nm
CDGJM	Chemically Defined Grape Juice Medium
CIELab	A colour space defined by the Commission Internationale de l'Eclairage
L*	CIELab value for degree of lightness of colour
a*	CIELab value for red/green colouration
b*	CIELab value for blue/yellow colouration
h*	Hue or shade of colour
FAN	Free Amino Nitrogen
HPLC	High Performance Liquid Chromatography
QTL	Quantitative Trait Loci
SO_2	Sulfur dioxide

Abstract

Yeast influences the development of red wine colour during winemaking and its stability during bottling and storage. Thus, understanding the yeast genetic mechanisms regulating such phenomena will contribute to consistent wine quality. A sequenced set of 96 progeny derived from a genetic cross of two commercial wine strains was used to unravel the genetic basis of yeast-derived colour modulation of model red wine. Colour differences have been genetically linked by Quantitative Trait Loci (QTL) mapping to regions of chromosomes 8 and 15. Further work is required to characterise the identified genes which may modulate wine colour through the adsorption of red pigment on yeast biomass.

Executive Summary

This project sought to genetically map the genes responsible for yeast-derived modulation of colour in a model red wine. A set of 96 hybrid wine yeast derived from a genetic cross between two commercial wine strains (Enoferm M2 and Zymaflore F15) were evaluated for fermentation performance in synthetic red juice, and their effect on the resultant 'wine'. The biochemical and colour data were collected by Drs Michelle Walker and Tommaso Liccioli (University of Adelaide) and correlated to the genome sequences of the 96 wine strains by Dr Miguel Roncoroni and Professor Richard Gardner (University of Auckland).

The key findings are listed below:

- The genomes of the 96 hybrid wine yeast have been previously sequenced, and the allelic variations in the corresponding genes, originating from the two parents, Enoferm M and Zymaflore F15 identified (Roncoroni 2014)
- Laboratory-scale (100 mL) fermentations were conducted in batches of 96 (totalling ~350) using an automated fermentation platform which allowed continual on-line monitoring of fermentation progress
- Spectrophotometric determination of CIELab coordinates (L*, a*, b*), was used to define the colour in clarified fermented juice and re-suspended yeast biomass. Colour differences were calculated mathematically between the parental strains and hybrids, and the data 'mapped' to the 96 genomes
- Two QTLs, associated with specific regions of chromosome 15 and chromosome 8 were identified as being strongly correlated with variation in wine colour and pigmented yeast biomass. The region on chromosome 15 was linked to L*, a* and b*, whilst that of chromosome 8, to L* and b*
- A minor QTL on chromosome 6 was also identified, specifically linked to b* for only yeast biomass
- Strong correlations between visual determination of colour (CIELab) and physical and biochemical determination of colour using the Somers Method
- Computational 'mapping' of the biochemical data (anthocyanins, organic acids, glycerol, ethanol) to the 96 genomes is in progress
- A manuscript on QTL mapping of traits related to wine making is in preparation (Roncoroni et al). Colour data generated as part of this project will contribute to the results disseminated in the paper
- These findings related to colour modulation will be used as support of new funding applications to further investigate several genes identified in this preliminary study as affecting colour in model red wine through possible adsorption on yeast cells

Background

Selective intra-specific breeding between different wine yeast has the potential to enhance the genetic variability between strains and introduce new traits into existing strains, and as such, provides a powerful tool for researchers to generate novel wine yeast with distinct fermentation attributes. This technique has yet to be exploited to its full potential as the genetic basis of only a number of fermentation traits have been identified (Ambroset et al 2011, Brice el al 2014, Huang et al 2014, Zimmer et al 2014). Such studies rely on genetic crosses between two strains of *Saccharomyces cerevisiae* with different phenotypic attributes. Allelic differences between the parental strains are mapped in the hybrid genomes (through sequencing), which are correlated to the measurable phenotypes.

Wine colour is the first sensorial attribute perceived by consumers and it can significantly influence their overall impression of wine quality. Modulating wine colour is therefore as important as managing wine flavour, aroma, mouthfeel and taste. Being able to specifically manipulate wine colour is best achieved with an understanding of the mechanisms that impact this attribute. Previous studies have revealed that colour development in red wines is a very dynamic process, involving the extraction of anthocyanins from grape skins and their transformation to more stable pigmented polymers (Fulcrand et al 2004). This process is influenced by chemical and microbial factors; the interaction of which affects colour development and stability in young and aged red wines ((Ribéreau-Gayon et al 2006).

Colour modulation during red wine fermentation is known to vary between different wine yeast (Bartowsky et al 2004; Medina et al 2005), with a number of mechanisms proposed through which individual strains may contribute to the colour characteristics of wine, both during colour extraction and stabilisation periods. The release of cell-wall derived polysaccharides into the must (Escot et al 2001), adsorption to yeast lees or cell walls (Escot et al 2001; Morata et al 2003) as well as metabolites such as acetaldehyde (Romero and Bakker, 2000a) and pyruvic acid (Fulcrand et al 1998, Romero and Bakker, 2000b) have been shown to affect colour development and stability.

Our aim was to expand on this previous research and develop a detailed understanding of the relative importance of these mechanisms by which yeast affect red wine colour. This study is the first to genetically map the genes responsible for such colour differences, by investigating the wine colour and pigmented yeast biomass (lees) during small-scale fermentation of a synthetic red grape juice. To achieve this target, the project made use of 96 second generation hybrids (originating from a genetic cross between two commercial yeast strains – Enoferm M2 and Zymaflore F15) whose genomes have been sequenced. Computation of the colour data from fermentation trials against a full genetic map of the collection, would allow the identification of the genes (and nucleotide differences) between the strains, responsible for the observed colour pigmentation in a synthetic red wine. Those hybrid progeny shown to improve colour (density/anthocyanin and phenolic content/polymerisation) have a direct positive impact on perception of wine quality. In addition to the colour evaluation, aliquots of the supernatant throughout the fermentations were frozen, for later metabolite analysis (residual sugar, and nitrogen (enzymatic/spectrophotometric analysis), organic acids, glycerol and ethanol (by HPLC analysis)). This data has been collected and analysed, and is currently being correlated to the genome sequences of the 96 hybrid yeast, to identify those traits specifically related to fermentation performance.

The identification of specific DNA regions to colour enhancing-properties in wine yeast, demonstrated that QTL mapping is a powerful tool in identifying the genetic basis of any

quantifiable phenotype, which is yet to be realised. The colour data (CIELab values) has been computed in collaboration with researchers at the University of Auckland, and so far we have been able to identify two specific regions of chromosome 15 and chromosome 8, which appear to be strongly correlated with different wine colour. Further experiments were undertaken to corroborate this data, through measurement of biochemical parameters related to colour, specifically anthocyanin and phenolic content, SO_2 resistant pigments, etc. The collation of the results is in progress.

The outcomes of this project will contribute to the identification of new 'industry ready' candidate strains. Furthermore, through the selective introduction of newly mapped desirable traits, such as cold tolerance, it is now possible into commercial strains already favoured by winemakers to generate new 'second generation' strains. Moreover, the project's outcomes will contribute to the understanding of the genetic basis of colour modulation during winemaking: once confirmed, our results will lead to further and more detailed characterisation of the genes included in those areas of the yeast genome.

Project Aims and Performance Targets

The original project aimed to evaluate a unique collection of 96 strains for their impact on fermentation activity and on red wine colour, to identify the metabolic pathways influencing these phenomena. The collection of F2 progeny from a genetic cross (Enoferm M2 and Zymaflore F15) have been genetically sequenced, to be able to construct a genetic linkage map of QTLs and identify genes responsible for colour related traits.
This is an alternative approach to our current research, whereby genome-wide screens of 1000's of lab yeast mutants (single gene deletions or genes over-expressed) for fermentation are undertaken.

The project objectives in the original proposal were to:

1. Evaluate yeast effects on red wine colour using a synthetic juice. Using 96 strains cultured in triplicate 140 mL fermentations, with our automated robotic platform

2. Quantify and correlate fermentation and phenotype data (colour: CIELab and Somers measures, sugar and nitrogen) to the annotated 96 genomes and identify which allelic combinations are contributing to any colour-influencing traits.

3. Publish findings in academic and industry journals

4. Use findings to direct further research which would be subject to additional funding to expand our breeding program and strain commercialisation

A variation to the original application was approved by AGWA to enable the additional measurement of the colour matrix via HPLC to support the CIELab colour measurements. Simultaneously, other major metabolites of fermentation (organic acids, glycerol, and ethanol) were quantified by HPLC analysis, which could be correlated to genotype using the QTL approach.

This research supports selective breeding as a paradigm to generate microbial strains tailored to meet current wine production and consumer preference e.g. improved colour in wine, in particular in low alcohol wine. New hybrid strains are widely accepted in our industry. Application of robust screening techniques and targeted (QTL associated) breeding will increase current potential of intra-specific crosses in generating desirable genetic variability, which will aid identification of new 'industry-ready' candidate strains.

Methods

A collection of 96 hybrid wine yeast was gifted by Dr Miguel Roncoroni and Professor Richard Gardner for use in this study to genetically map the genes responsible for yeast-derived colour modulation of model red wine.

The 96 F2 recombinant progeny were dissected from an F1 hybrid resulting from a cross between two homozygous wine strains, Enoferm M2 and Zymaflore F15. The genomes of all 96 strains and the wine yeast were sequenced; the allelic variations between M2 and F15 identified, allowing the genotyping of the 96 recombinant progeny at these loci (Roncoroni, 2014). This collection has been used to identify many Quantitative Trait Loci (QTLs), linked to traits related to winemaking. One example is hydrogen sulfide production where the F15 alleles of *MET2* and *MET5* both increased H_2S production (Roncoroni, 2014, Huang et al 2014).

In the current project, fermentations were conducted with all 96 strains, together with the parents and F1 hybrid, and the resultant model red wine analysed for differences in red pigmentation in the wines. The physical data was correlated with the annotated genomes to allow for the identification of QTLs - chromosomal regions or individual genes responsible for these differences. The QTL analysis for colour as well as other fermentation traits such as sugar and nitrogen consumption was performed by Dr Roncoroni using R/QTL software (Broman et al 2003) on the University of Auckland computer server.

The logistical challenge of this project was to conduct triplicate fermentations of 96 strains and their parents (324 in total) in traditional flasks (~100 mL) fitted with airlocks. This was overcome through the use of a robotic platform designed and developed by Dr Liccioli as part of another AGWA-funded project (UA1302: *Fit for purpose yeast and lactic acid bacteria*). The robotic platform allowed for the automated management of small scale fermentations (up to 140 ml), by remote access using a laptop or mobile phone. Up to 768 culture samples (8 plates; 96 samples per plate) can be collected and refrigerated (-10^{o}C) at a given time, allowing for accurate and reproducible high throughput data acquisition. The 96 strains and their parents were evaluated in four fermentation batches: Experiment 1 (12 ferment triplicates) and Experiments 2 to 4, each comprising 32 ferment triplicates.

For this project, chemically defined grape juice medium or CDGJM (Henschke and Jiranek, 1993, Walker et al 2014) containing 230 g/L total sugar as equimolar glucose and fructose, 300 mg/L N as FAN and 5% (v/v) GrapEX grape skin tannin (Tarac technologies, GSKINEX) was used to simulate red grape juice. Fermentations were conducted at 28ºC (stirring 300 r.p.m.) for a total of 124 hours. Colour differences were evaluated using two methods. Clarified end of ferment ('wine') samples were analysed for density, hue, SO_2 resistant pigments, total anthocyanins and total phenolics, following the method of Iland et al (2000) adapted to 96 well format (methods outlined in Jiranek, 2009). Analysis was performed on thawed samples that had been previously clarified and frozen. Colour differences were also determined on fresh 1 mL clarified samples, using CIELab measurements (http://en.wikipedia.org/wiki/Lab_color_space). A Cintra 4040 spectrophotometer (GBC Scientific Equipment) with an integrated apparatus dedicated to the accurate determination of the CIELab values (D Taylor lab) was used as an efficient, accurate and direct method to determine colour differences in wines.

The investigation was not only limited to visual differences of the pigmentation of the wines, but the chemical composition of some key elements of wine quality was also measured. High

Performance Liquid Chromatography (HPLC) was used to measure anthocyanin composition (as outlined in Jiranek, 2009) and other key metabolites, including organic acids, glycerol, acetaldehyde and ethanol (Walker et al 2003). Samples were frozen at -20°C for later enzymatic analysis of residual glucose and fructose (Bergmeyer et al 1983), residual free amino acids (Dukes et al 1998, Jiranek 2013) and total sulfite (kit sourced from Megazyme, K-TSULPH); the original methods modified to suit a 96-well plate format. For each experiment (1-4), data was analysed using Microsoft Excel 2010 and GraphPad Prism version 6.007. The average values from triplicate data were plotted as XY graphs, and linear regression was used to fit a straight line through both datasets (95% confidence interval) with a correlation R^2 value.

The generation of such sets of data will allow more in-depth computation, with the potential for further investigation of genetic mechanisms related to red colour and other winemaking attributes.

Results/Discussion

QTL mapping of colour in 96 F2 wine hybrids

The project objective was to evaluate yeast effects on colour in model red wine. A sequenced set of 96 recombinant F2 progeny dissected from the F1 generation of a cross between Enoferm M2 and Zymaflore F15 (Roncoroni 2014). 8366 mutations were identified between the parent strains (allelic variations), and the progeny were genotyped at these loci. These loci are being used as genetic markers to link quantitative phenotypes to them (Roncoroni 2014). This project aims to identify QTLs linked to the retention of pigmented compounds in the culture supernatant *vs* adhering to the biomass in model wine fermentations containing a polyphenol extract from grapes.

The 96 hybrid wine strains were compared with the original F1 hybrid (M2 x F15) and the parent strains in laboratory scale (100 mL) fermentations using our automated robotic platform or Tee-bot. Four fermentation batches were conducted: Experiment 1 (12 ferment triplicates) and Experiments 2 to 4, each comprising 32 ferment triplicates. A spectral measurement for wine colour was chosen, whereby the colour measurements based on the absorbance of anthocyanin pigments in the visible and ultraviolet spectrum (380-780 nm) was used. The method was simple and did not require any sample preparation making it suitable for high-throughput analysis of wine colour. The following Colour data (CIELab values L* (degree of lightness), a* (red/green colour) and b* (blue/yellow colour)) which give a visual measure of colour differences were determined on both fresh culture supernatant (referred to as 'wine') and pigmented yeast biomass, suspended in water. Normalisation of the data and the construction of genetic linkage maps were performed by Dr Roncoroni.

Two regions of the genome were identified as being highly correlated to the measured colour values (L*, a* and b*), alluding to the contribution of these regions to the final colour composition of the red model wine. **Figures 1 and 2** show the correlation between the physical data (LOD score) plotted against the individual chromosomes for the respective 'wine' colour and yeast biomass. The LOD or logarithm of the odds score is a statistical test to compare the likelihood that two loci are linked. A LOD score greater than 3 means that the chances are a thousand to one, and therefore two genes are inherited together or genetically linked (http://www.genome.gov/Glossary/index.cfm?id=531). Chromosomes 8 and 15 were identified for the colouration of both 'wine' and pigmented yeast biomass, with chromosome 15 was correlated with L*, a* and b* and chromosome 8 was correlated with L* and b*. A minor QTL was also identified on chromosome 6, which correlated only to b* for the yeast biomass (data not shown).

Several genes were highlighted which include genes responsible for the synthesis of cell-wall components, which may conceivably be involved in different degrees of binding of polyphenol pigments from the fermentation medium to the yeast cell wall (thereby removing them from the culture supernatant). More work is required to characterise these putative candidate genes, a task which is not straightforward, given that closely sequence-related paralogs also exist for one of the genes and the region in question is poorly resolved in the genome sequence for the two strains as well as other wine yeast (Miguel Roncoroni, *pers. commun*).

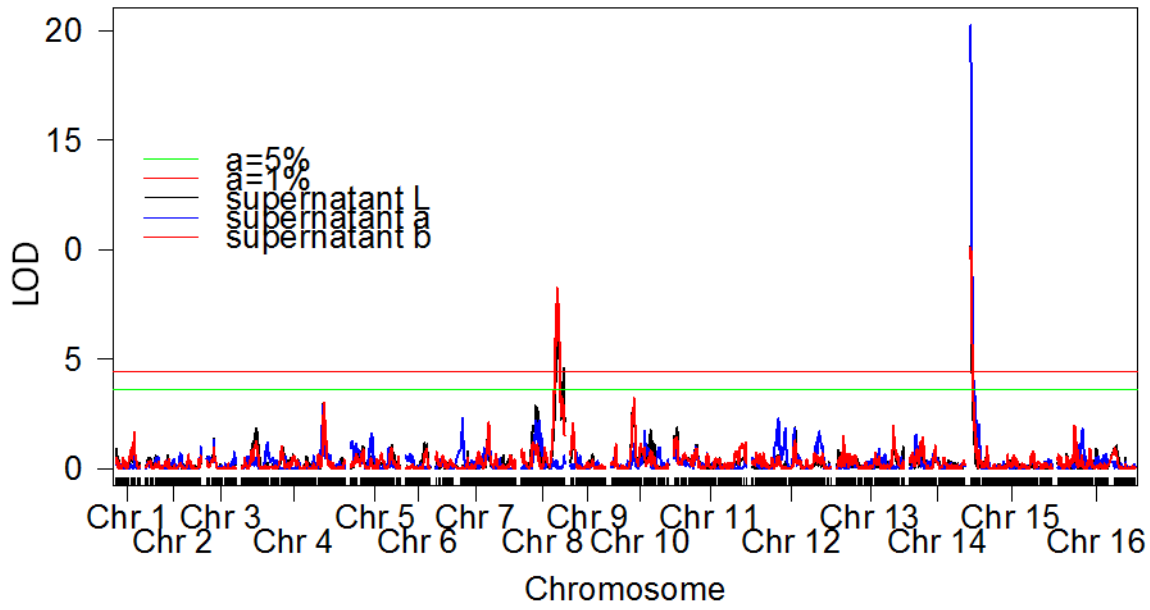


Figure 1. Wine colour maps to regions of chromosomes 8 and 15 in M2xF15 cross
LOD scores for wine colour in M2xF15 progeny, following fermentation at 28 °C, are plotted along the 16 yeast chromosomes. The green horizontal line indicates the 5% significance cut-off, whilst the red horizontal line, the 1% significance cut-off. The two major peaks on chromosomes 8 and 15 are shown. Chromosome 8 is linked to L* and b* whilst chromosome 15 is linked to L*, a* and b*.

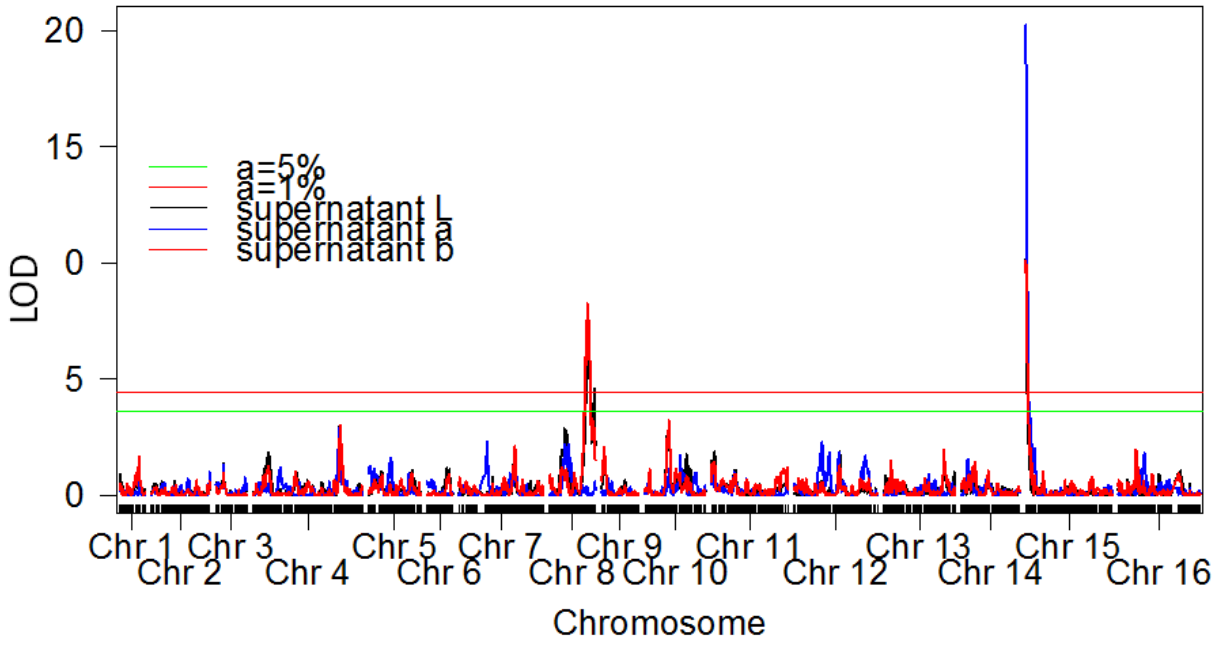


Figure 2. Yeast biomass colour maps to regions of chromosomes 8 and 15 in M2xF15 cross

LOD scores for biomass colour in M2xF15 progeny, following fermentation at 28°C, are plotted along the 16 yeast chromosomes. The green horizontal line indicates the 5% significance cut-off, whilst the red horizontal line, the 1% significance cut-off. The two major peaks on chromosomes 8 and 15 are shown. Chromosome 8 is linked to L* and b* whilst chromosome 15 is linked to L*, a* and b*.

CIELab measurement of colour was shown to be highly reliable, with the existence of differences clearly apparent between the strains as determined from spectral measurement of the individual model wines. High resolution mapping data of the identified regions is not included in this report, as the data will contribute to an extensive paper on QTL mapping of genes of oenological importance in commercial wine making yeast, which is in preparation. The published article will be forwarded to AGWA in due course.

CIELab and its relationship to biochemical measurements related to wine colour

The CIELab approach to measure colour was demonstrated to be fast and cost effective, requiring no sample preparation and minimal use of consumables. However, it does not provide information with regards to the physical and chemical parameters that are responsible for observed colour differences. These differences are most likely the result of the interaction of multiple factors including wine pH, anthocyanin and phenolic content, as well as effect of metabolites such as acetaldehyde, pyruvic acid and sulfur dioxide. In order to identify and understand the myriad of mechanisms and potential genes that are responsible, it was important to gather additional biochemical data specific to wine colour. In the context of this project, a correlation between the visual CIELab colour data and a biochemical parameter/compound was sought to strengthen the QTL mapping data associated with the measurable visual colour differences. The presence of a strong correlation between the

CIELab colour data and a biochemical parameter would allude to the likelihood of the latter having an important role in defining the final wine colour.

Analysis of density, hue (h*), SO_2 resistant pigments, total anthocyanins and total phenolics, etc was undertaken on thawed samples, following the original Somers Method (Iland et al 2000). This method, whilst potentially informative, is both labour intensive and time consuming when performed on hundreds of samples (in triplicate). While CIELab was conducted on 324 fresh wine samples, clarified supernatant had to be frozen while waiting for processing, which could have altered the composition of the wines, as some precipitation (presumably of tartaric acid, and colour compounds) was observed in the samples. Individual samples were thawed once only and used for analysis. Parameters measured included anthocyanins and acetaldehyde (by HPLC), total sulfite (enzymatic), and key metabolites (HPLC) and residual sugar and nitrogen. Plotting of the data against the genomes, is underway, to identify potential new QTLs to be included in the mentioned peer-reviewed article.

The individual biochemical parameters measured by the Somers method were determined at wine pH, to allow for direct comparison to the CIELab data. This is because the red colour exhibited by free anthocyanins is pH dependent, with reduced wine pH causing a shift in equilibrium to the red coloured form, resulting in an intensified red wine colour. Thus, at natural pH the overall colour and intensity of the wine is affected by processes which alter the wine pH (e.g. altered acidity). Whilst the correlations between parameters would have been better if the samples were fresh, as precipitation would not be evident, good correlations were observed for the following:

- L* versus $A520_{nm}$ (in absence of SO_2) – **Additional Figure 1**
- L* versus $A520_{nm}$ (in presence of acetaldehyde) – **Additional Figure 2**
- $A520_{nm}$ (in presence of acetaldehyde versus $A520_{nm}$ (in absence of SO_2) – **Additional Figure 3**
- CIE h* versus density (in absence of SO_2) – **Additional Figure 4**

The correlation of data in three of the four experiments was found to be consistent, with the exception of Experiment 1 (see **Additional Figures 1 to 4**). The correlation between L* and absorbance at 520 nm ($A520_{nm}$) in the absence of excess SO_2 (**Additional Figure 1**) is not surprising given that it gives an arbitrary estimate of the concentration of both free anthocyanins and red coloured oligomeric and polymeric compounds under natural wine pH and SO_2 conditions. The addition of excess acetaldehyde allows the estimation of these red coloured compounds present under the same pH and but devoid of any bleaching effects of SO_2 (Iland et al 2004). Acetaldehyde binds to anthocyanins to form red coloured oligomeric pigments and free SO_2. The bleaching effect on red colour by SO_2 is dependent on the level of free SO_2 and the extent of reaction with the anthocyanins. **Additional Figure 3** alludes to this relationship in that a direct correlation is observed between the two parameters. The absorbance at 520 nm in the presence of excess SO_2 provides an estimate of the red coloured pigments resistant to bleaching by SO_2. The shade of wine colour or Hue (h*) as calculated from CIELab values a* (red/green) and b* (blue/yellow) was shown to be strongly correlated to the Somers determination of density at the same wine pH and SO_2 level ($A_{520nm} + A_{420nm}$) While density describes the intensity of the colour, rather than the shade or tint of colour, it is likely that these measures are related as shown in **Additional Figure 4**.

Interestingly, under the experimental conditions no strong correlation was found between CIELab L* and direct measurement of anthocyanins by HPLC (e.g. malvidin-3-glucoside) as

shown in **Figure 3**. This was unexpected given that malividin-3-glucoside is the major monomeric anthocyanin in the model red wine (data not shown) and that as shown in **Figure 4** the malvidin-3-glucoside content of the wines varied between the strains, which would be envisaged to influence the colour. The basis of these differences is yet to be defined, it is likely that the other biochemical characteristics such as pH (Iland et al 2004) and release of macromolecules and metabolites by yeast (Escot et al 2001, Eglington et al 2004), interaction of red coloured pigmented compounds with yeast metabolites (Fulcrand et al 1998, Romero and Bakker, 2000) or adsorption on yeast biomass (Morata et al 2003) play a more important role in defining the colour of the final wine.

This project has generated an extensive amount of data related to colour in model red wine, as well as fermentation performance. This report only details some of the research findings, with others yet to be analysed. The results have shown that the choice of the strain employed in oenological fermentations can contribute significantly to red wine colour alongside other recognised winemaking parameters such as grape variety and fermentation management. The automated fermentation system and the model red wine not only is useful to understanding how individual strains modulated the colour of red wine but also provides a rapid, reproducible and inexpensive model to test large numbers of new strains for the capacity to affect colour.

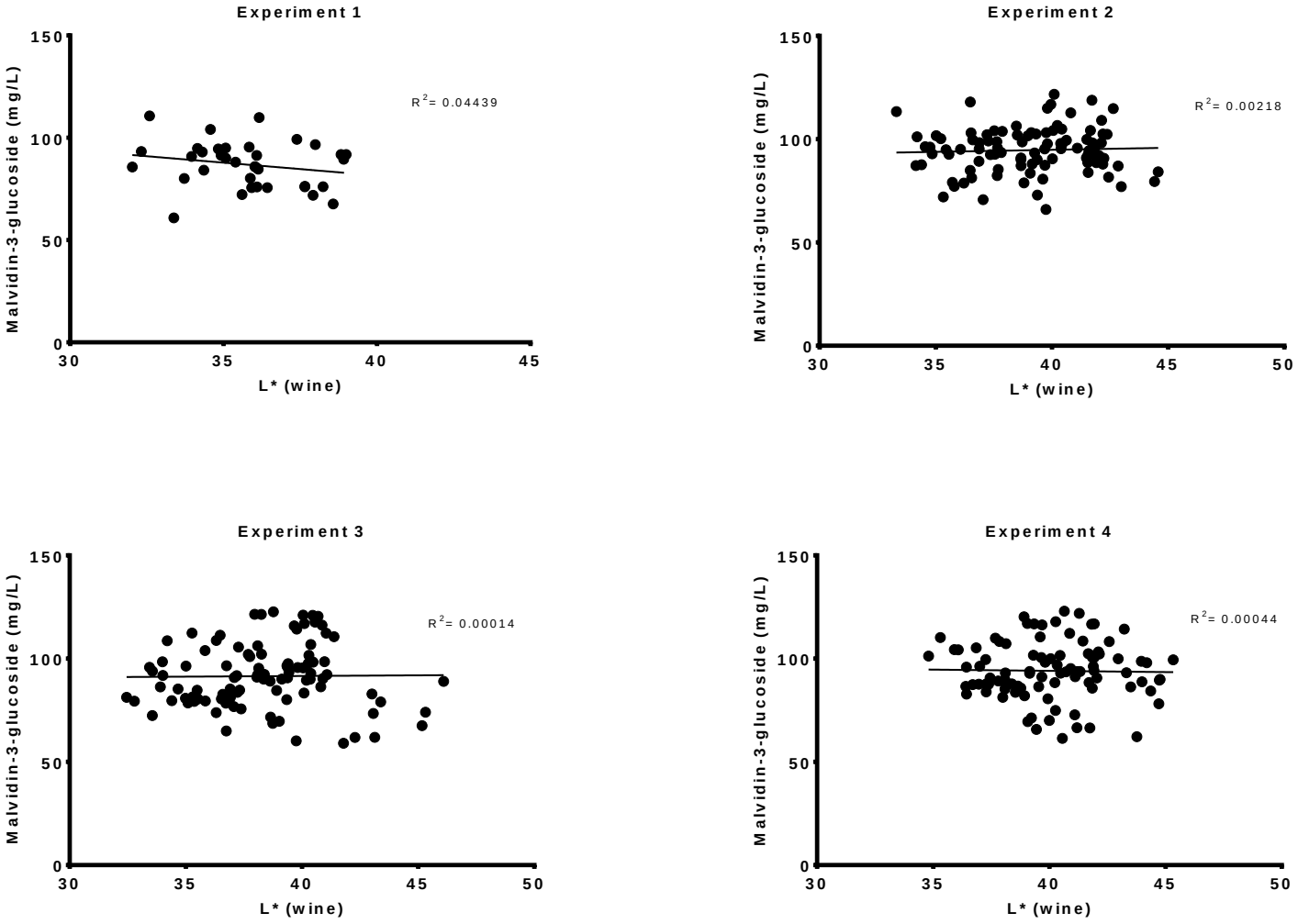


Figure 3. CIELab colour value L* of wine versus malvidin-3-glucoside content as determined by HPLC.

Graph represents data from some of the 96 F2 recombinant progeny when compared to the original wine yeast Enoferm M2 and Zymaflore F15, and the F1 hybrid. Arrow bars represent standard deviation of the mean (±SD), number of replicates per strain n = 3.

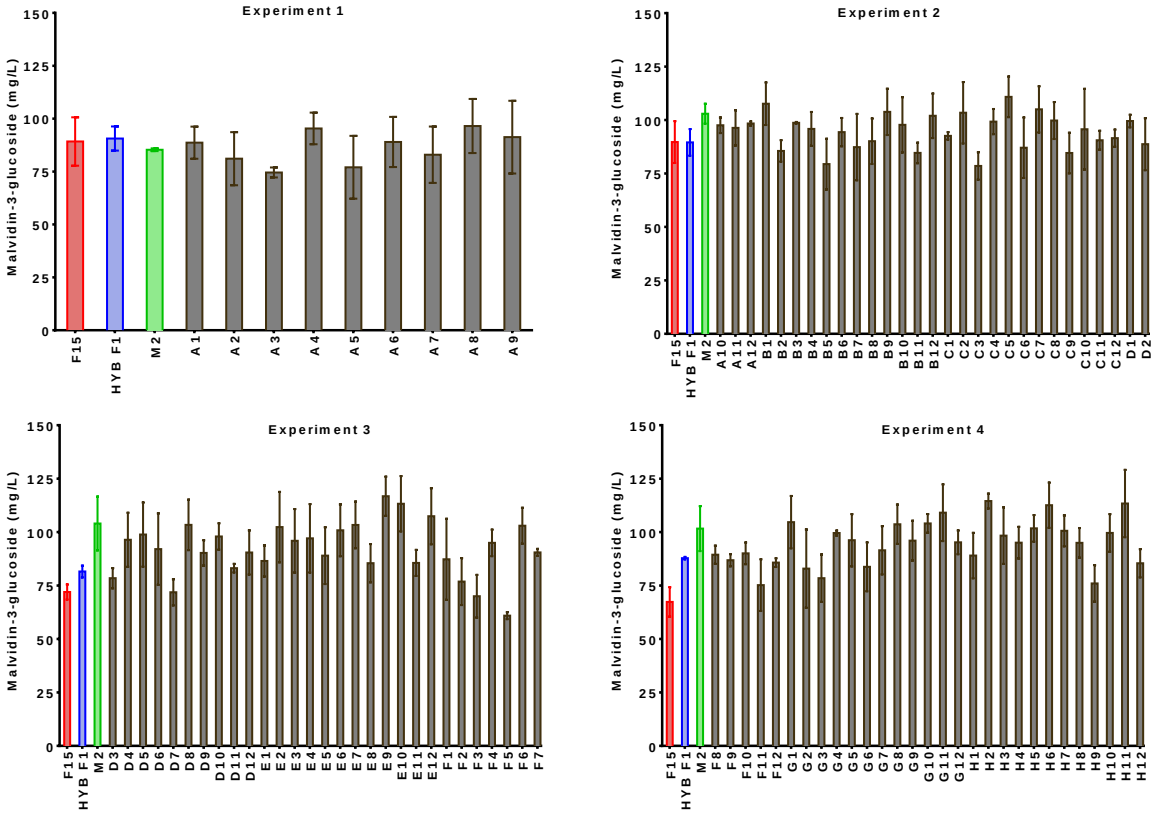


Figure 4. Malvidin-3-glucoside content of individual 'wine' samples as determined by HPLC.

Malvidin-3-glucoside content (mg/L) was measured on thawed clarified culture supernatants or 'wine' using HPLC. Arrow bars represent standard deviation of the mean (±SD), number of replicates per strain n = 3.

Outcome/Conclusion

The project performed well against the planned objectives stated in the original application with the main objective of mapping the genes behind yeast modulation of wine colour being achieved. Two QTLs on Chromosome 8 and 15 linked to CIELab values L*, a* and b* associated with colouration of the wine and pigmentation of the yeast biomass, were identified, together with a minor one on chromosome 6 (biomass b*).

This is the first large-scale application of the automated fermentation platform, whose development was supported by AGWA. The evaluation of the 96 recombinant progeny for their colour affecting capacities (a total of 324 fermentations) has proven the reliability and accuracy of the 'Tee-bot', and confirmed it to be central to the undertaking of high-throughput fermentation screening exercises.

A grant variation to allow the employment of Ms Kendall Corbin to complete the HPLC analysis of anthocyanins and key metabolites was a cost-effective exercise given the number of samples that were analyzed. As a result, a large quantity of data was generated from the one experiment, which is still to be fully analysed and correlated to the genomes of the 96 recombinant progeny for QTL analysis.

The evaluation of the 96 progeny was undertaken in a series of fermentation experiments, whereby samples were frozen, with the exception of CIELab determination, for later analysis by HPLC and the Somers Method. Several improvements could be made to the project with regards to sample preparation and the analysis of the 'wine' samples. The original Somers Method was chosen to allow for a direct comparison at wine pH and SO_2 levels (Iland et al 2000). This method gives a measure of colour as well as phenolic composition and anthocyanin equilibria. In order to improve consistency and minimise the effect of freezing, namely precipitation of pigmented compounds and tartaric acid which can influence pH, it would be better to perform the analysis on fresh samples, or within a 24 hour timeframe if refrigerated. A modified version of the Somers Method has been developed (Mercurio et al 2007), whereby the wine pH and alcohol concentration are standardised prior to the initial analysis. It would be useful to compare both methods, to see whether standardising the pH and ethanol content would influence the correlations made to the CIELab data. Both the Somers Method and HPLC analyses are time consuming in terms of processing and analysis time. In order to coordinate sampling (terminal ferment samples) by the Tee-bot and HPLC analysis, an automated sampler is being sought to allow for high-throughput sampling (96-well plate format), which would improve both consistency of results and reduce sample preparation time.

This sequenced set of 96 recombinant progeny has been made available to selected members of the research community to undertake a comprehensive QTL analysis of metabolic traits important to winemaking, which will increase the understanding behind different fermentation characteristics. The ability to evaluate these sets of strains quickly and cost-effectively as detailed herein will enable the high throughput screening required for such an undertaking. With the wider adoption of QTL analysis, the wine sector will benefit through the application in 'marker assisted' breeding programs similar to those used in cereal production. While this particular set of wine yeast is currently for academic research, it is anticipated that those wine strains shown to be highly improved could be readily adopted by industry, or that new traits (which have genetic markers) could be introduced into existing commercial yeast.

This project focused on wine colour, which is the first sensorial attribute perceived by consumers and therefore can significantly influence their overall impression of wine quality. The generation of novel yeast strains which can improve colour will directly impact on perception of wine quality. If this is valid for premium/icon wines, yeast contribution to colour could be even more important for commercial wines. Typically, for economic and logistical reasons, selection of grapes and colour extraction during fermentation of commercial wines cannot be carried out with the same attention used for top quality wines. This could result in lighter coloured wines, decreasing the product value which may represent the economic base/strength of a winery.

The use of yeast strains with improved colour extraction and stabilisation properties will make a significant contribution to reducing the environmental impact. Less time will be required during winemaking to obtain optimal colour and therefore requiring lower inputs in terms of energy, additives and labour. Moreover, quality enhancement of grapes typically classified with a lower grade could be enhanced. Considering that winemaking will always require an energy input, it is logical to try to obtain the best economical profit in return.

Enabling the production of higher value wines will increase the sustainability and profitability of the whole wine industry. This will contribute to securing employment in the wine and allied sectors, which are often located in regional areas of Australia. Refining tools for winemaking (i.e. yeast contributing to improved colour) will also facilitate production of novel wine styles for new domestic and international markets (i.e. low alcohol wine).

Recommendations

This project has the potential to benefit the wine sector through Program 3: Improving products and processes, in particular Subprogram 3.2 Germplasm (yeast and bacterial) and Subprogram 3.1 (Objective measures of quality and assessment systems). The project has yielded a large amount of outputs related to understanding the genetic basis of yeast-derived modulation of wine colour, together with other fermentation-related traits.

We propose that these studies should be extended through funding opportunities to support one or two PhD or MPhil scholarships to undertake follow-up projects:

Project 1: To identify and characterise the yeast genes affecting wine colour
Three QTLs have been identified as regions of the genome that are linked to colour values L*, a* and b* in both model wine and yeast biomass. This new project would identify and characterise the genes that have a potential role in affecting wine colour through the binding of polyphenol pigments from the fermentation medium to the yeast cell wall (thereby removing them from wine). Hemizygotes (WT/Δ) would be constructed for each gene in the commercial yeast M2 and F15, to test for dominance of the genetic trait. Reciprocal allele swaps would be constructed in both genetic backgrounds, to determine which gene is responsible for the phenotype.

Project 2: QTL mapping of genes responsible for yeast-derived modification of wine colour
The research potential of this set of sequenced 96 recombinant wine yeast progeny is yet to be captured. A repeat of the experiment using actual grape must (different red grape varieties) would verify the current findings using a synthetic red juice with added grape polyphenols. Genes encoding anthocyanin-degrading enzymes (glycosidases, anthocyanases, oxidase, etc) have the potential to be identified using a more complex colour matrix. The use of actual grape must (different red grape varieties) would verify the current findings using a synthetic red juice with added grape polyphenols as well as other as yet unidentified genes.

Project 3: High-throughput screening of new improved yeast
This project has demonstrated the reliability and accuracy of the automated fermentation platform (Tee-bot) for high-throughput screening, which could be utilised in the evaluation of new improved yeast (and bacteria), as natural isolates, improved strains, or intra-specific hybrids. This project would be suited to an Honours candidate studying oenology undertaking a six month research program.

QTL analysis of hybrid collections not only provides a resource of 'industry ready' non-recombinant yeast, but can identify possible genetic targets for exploitation in the development of strains tailored for fermentation robustness of specific compositional contributions to wine.

Appendix 1: Communication

Communication and dissemination of findings from this research will be achieved through publications, conferences and seminar presentations. An outline of the work has also been provided to international academics visiting the Waite Campus.
It is anticipated that the research findings will be reported in an academic peer-reviewed article and in industry relevant literature (1 article).

Symposium Presentation:
10th International Symposium of Enology of Bordeaux, June 29 to July 1, 2015. "Generation and characterisation of improved wine microbes". V. Jiranek, Liccioli, T., Betteridge, A., Sumby, K. Walker, M., Gardner, J. and J. Sundstrom

Appendix 2: Intellectual Property

The sequenced set of 96 recombinant F2 progeny dissected from the F1 generation of a cross between the winemaking strains Enoferm M2 and Zymaflore F15, is the intellectual property of the University of Auckland. The collection was gifted to us for the purposes of academic research only, whereby information would be shared between the University of Adelaide (Professor Jiranek's group) and the University of Auckland (Professor Richard Gardner).

Key, valuable IP centres on the identification of specific Quantitative Trait Loci (QTLs), linked to the retention of pigmented compounds in the culture supernatant *vs* adhering to the biomass in model wine fermentations containing a polyphenol extract from grapes. Several candidate regions have been highlighted including those responsible for the synthesis of cell-wall components. Further work is however required to more fully characterise these genes to the level where IP protection might be sought.

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Appendix 4: Staff

The following staff contributed to this project:

The University of Adelaide
Dr Michelle Walker (Project Supervisor)
Dr Tommaso Liccioli (Chief Investigator)

Staff:
Ms Kendall Corbin

Collaborators:
Professor Richard Gardner, University of Auckland
Dr Miguel Roncoroni, KU Leuven (formerly University of Auckland)

Appendix 5: Other Relevant Material

Technical Specification of GrapEX from Tarac technologies

taractechnologies

GrapEX

Technical Specification

PRODUCT NAME: **Grape Skin Tannin** **PRODUCT CODE:** **GSKINEX**

Description

GrapEX™, GskinEX - Grape Skin Tannin is a complex of natural tannins from red wine grape skin (Vitis Vinifera).
GskinEX is recommended for enhancing the characteristics of red wine.

Specification

Physical Characteristics:	Liquid ruby red colour with natural grape characteristics and flavour.

Spectral Analysis:	
Colour density (Abs = 520nm + 420nm @ pH 3.0)	280 - 320 AU

Chemical Analysis:	
pH	<3.3
Density (g/ml @ 20° C)	1.10 ± 0.05
Volatile Acidity (g/L)	< 5
Reducing Sugars (g/L)	< 25
Sulphur Dioxide (total - mg/L)	< 500
Malic Acid (g/L)	< 5
Ethanol (% v/v)	< 1
Copper (mg/L)	< 5
Iron (mg/L)	< 50
Heavy Metals (As, Cd, Hg, Pb - mg/L)	< 10
Pesticides Residues	< MRL's

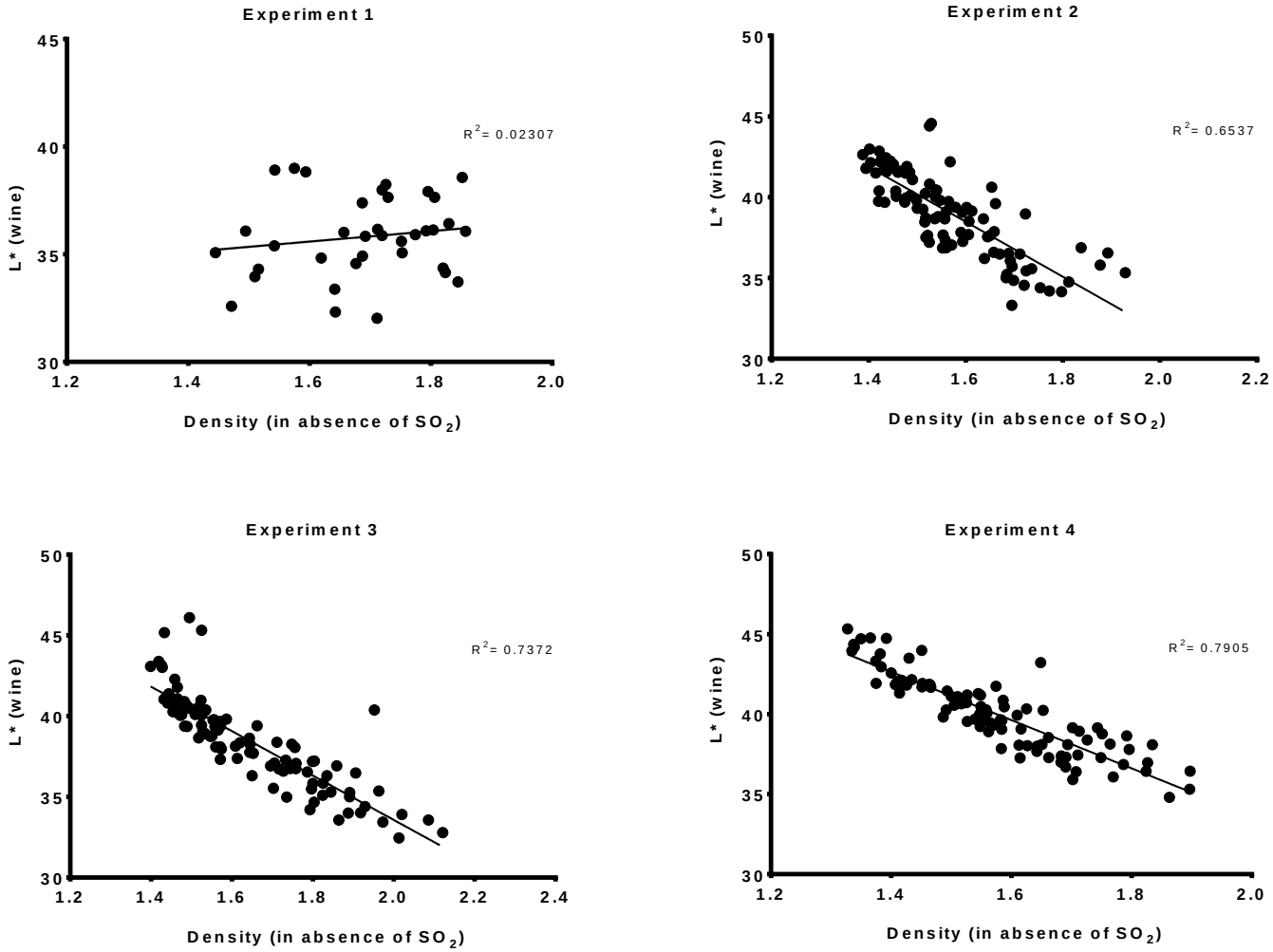
NOTE: Specification may alter due to seasonal variation in materials.

Microbiological Analysis:	
Total Plate Count (cfu/mL)	< 1000
Yeast (cfu/mL)	< 100
Mould (cfu/mL)	< 100

Storage

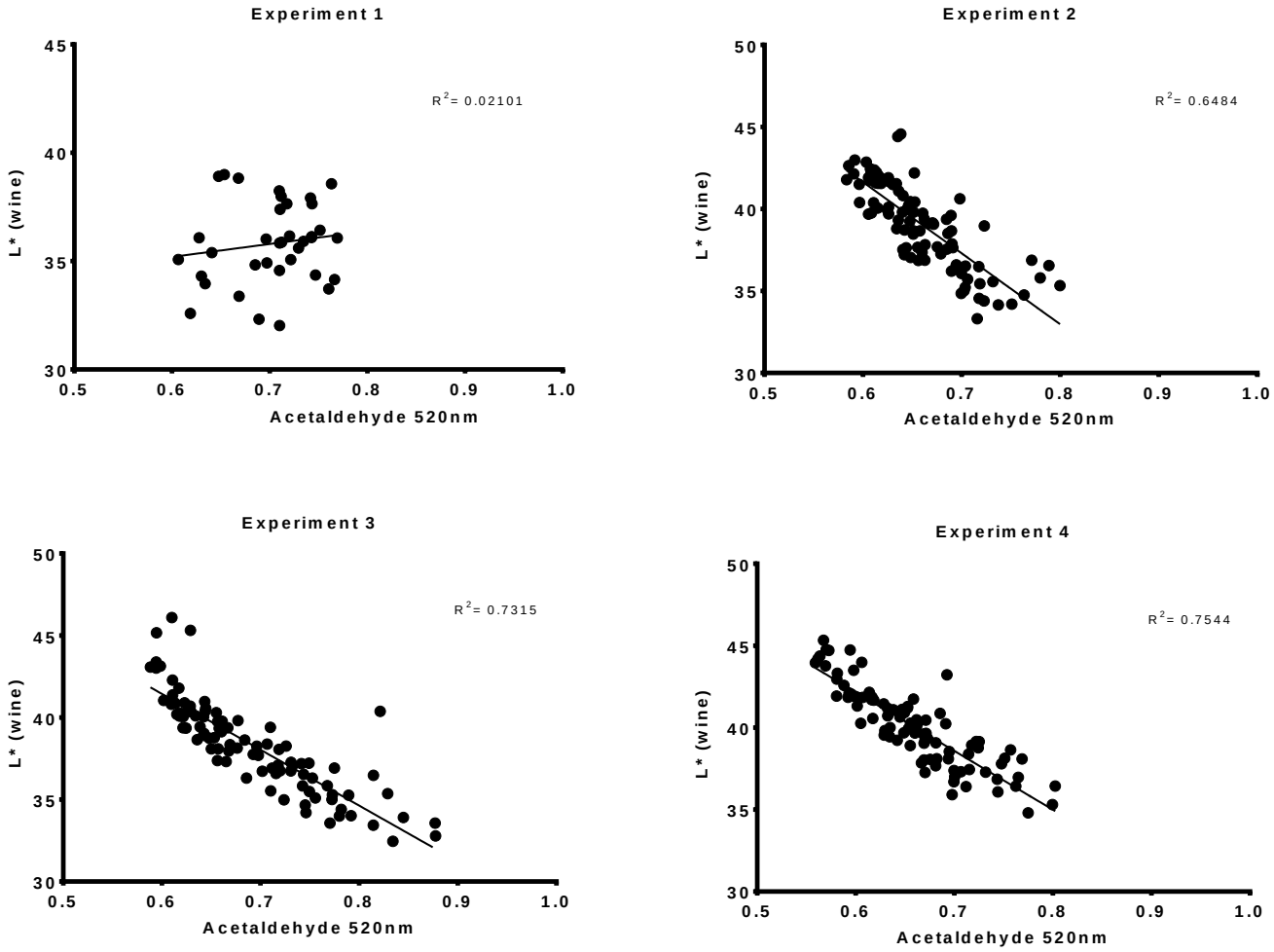
Store at 1 to 5°C, protected from light. Under these conditions this product maintains its specification for 12 months.

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South Australia, 5355
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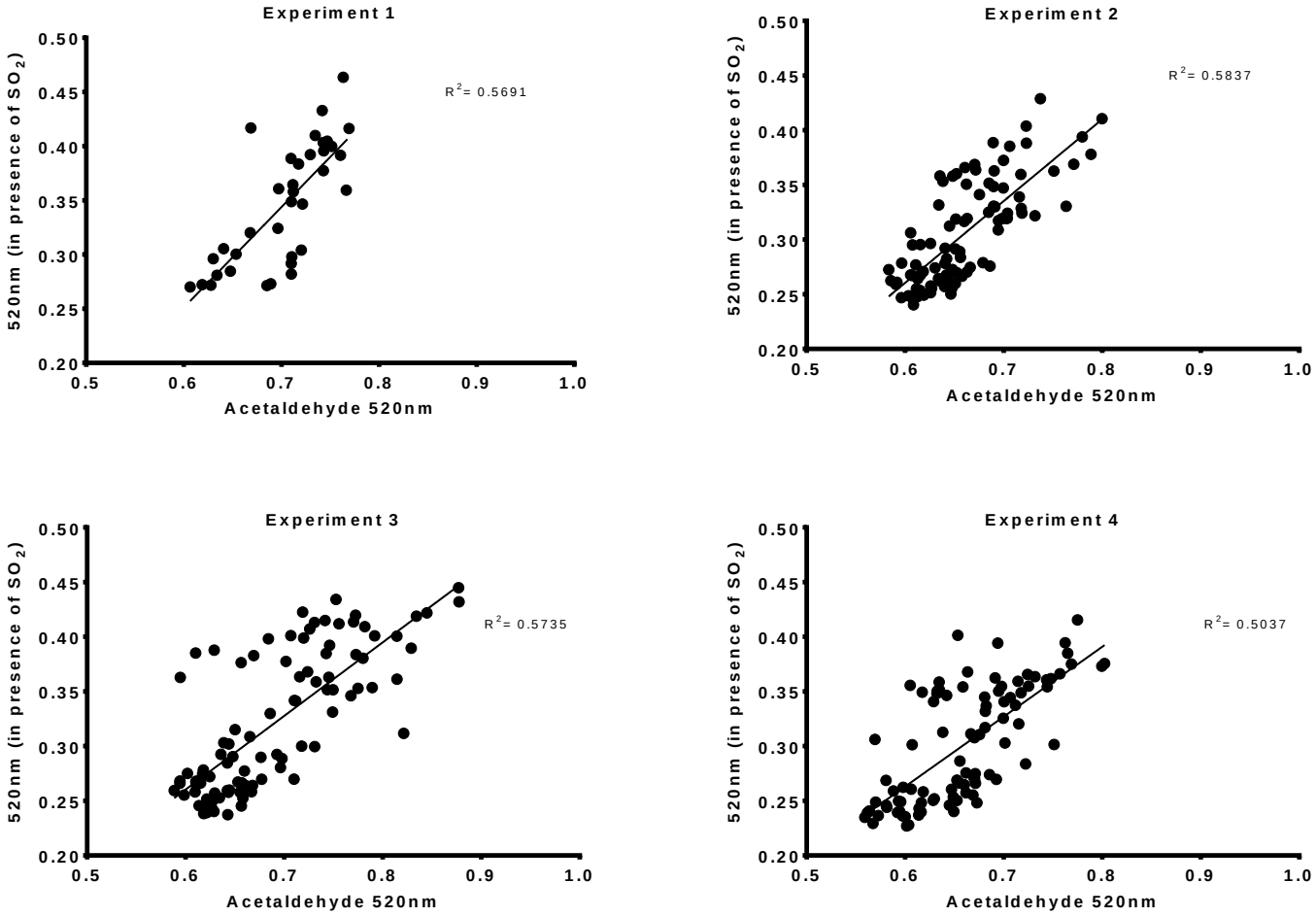
Additional Figure 1. Graphical plot of CIELab colour value L* of wine versus absorbance of wine at 520 nm ($A520_{nm}$) in the absence SO_2 as determined by Somers Method

CIELab was determined on fresh clarified culture supernatant ('wine) using a Cintra 4040 spectrophotometer. Somers method was used to measure the absorbance of the clarified culture ('wine') at 520 nm in the absence of added sulfur dioxide (SO_2) (Iland et al 2000). Triplicate determinations were made per fermentation replicate for the Somers method (n=9), whilst single CIELab determinations were performed on the each of the replicate samples per strain (n=3).

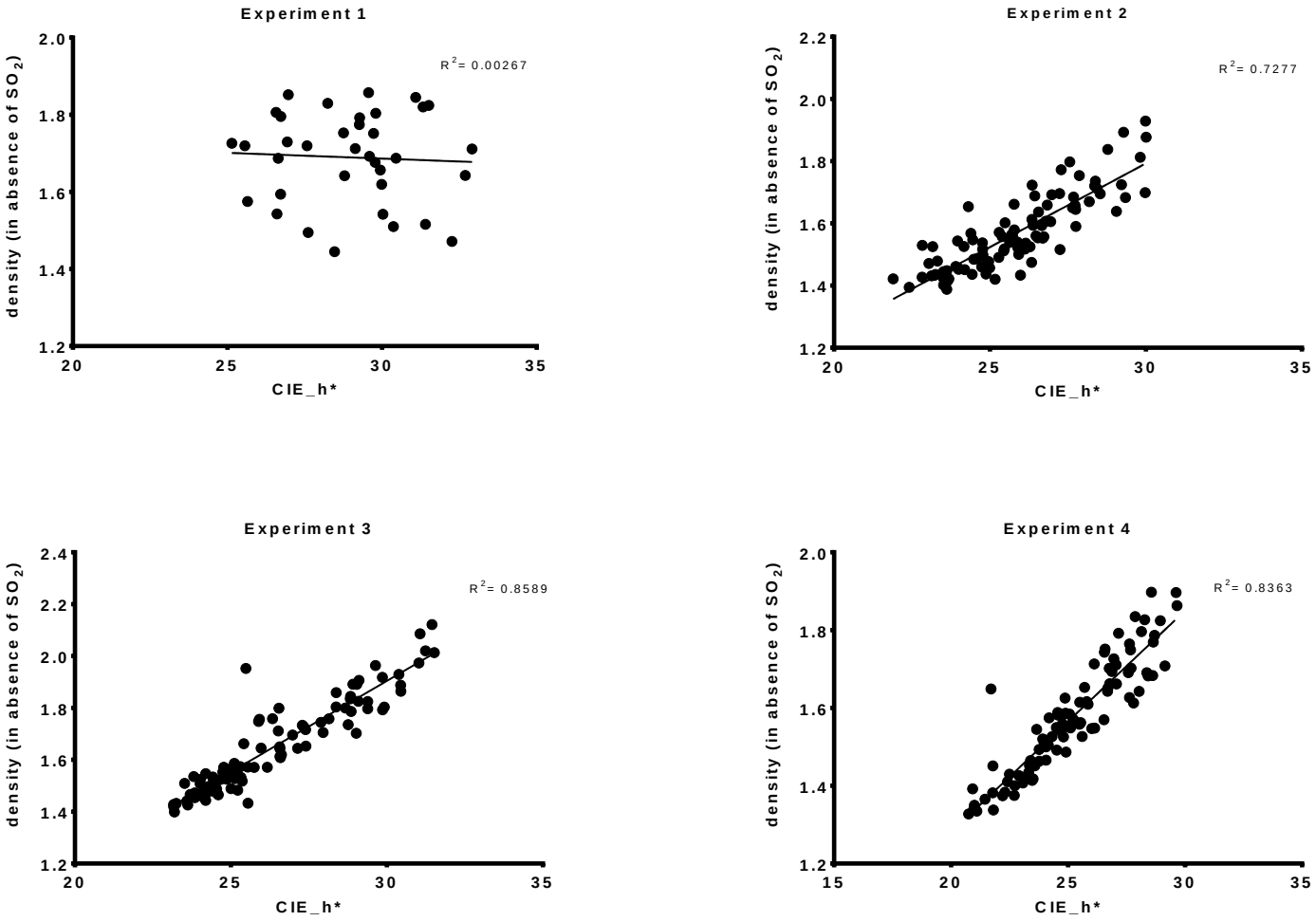


Additional Figure 2. Graphical plot of CIELab colour value L* of wine versus absorbance of wine at 520 nm ($A520_{nm}$) in the presence of acetaldehyde as determined by Somers Method

CIELab was determined on fresh clarified culture supernatant ('wine) using a Cintra 4040 spectrophotometer. Somers method was used to measure separately the absorbance of the thawed 'wine' at 520 nm in the presence of added acetaldehyde (Iland et al 2000). Triplicate determinations were made per fermentation replicate for the Somers method (n=9), whilst single CIELab determinations were performed on the each of the replicate samples per strain (n=3).



Additional Figure 3. Graphical plot of absorbance of wine at 520 nm ($A520_{nm}$) in the presence of acetaldehyde versus in presence of SO_2 as determined by Somers Method
Somers method was used to measure separately the absorbance of the thawed 'wine' at (i) 520 nm (with excess SO_2) and (ii) with excess acetaldehyde (Iland et al 2004).
Triplicate determinations were made per fermentation replicate for the Somers method (n=9), whilst single CIELab determinations were performed on the each of the replicate samples per strain (n=3).



Additional Figure 4. Graphical plot of absorbance of density (in the absence of SO_2) determined by Somers method versus in Hue (h*) calculated from CIELab a* and b* values

Somers method was used to measure the absorbance of at 520 nm (red coloured pigments) and 420 nm (yellow/brown pigments) using thawed 'wine' samples. Density was calculated as (A_{520nm} + A_{420nm}). Hue (h*) was calculated from CIELab values a* and b* in Excel as DEGREES(ATAN (a*/b*)

Triplicate determinations were made per fermentation replicate for the Somers method (n=9), whilst single CIELab determinations were performed on the each of the replicate samples per strain (n=3).

Appendix 6: Budget R**econciliation**

End of Project Financial Statement submitted online via AGWA's Clarity Investment Management System