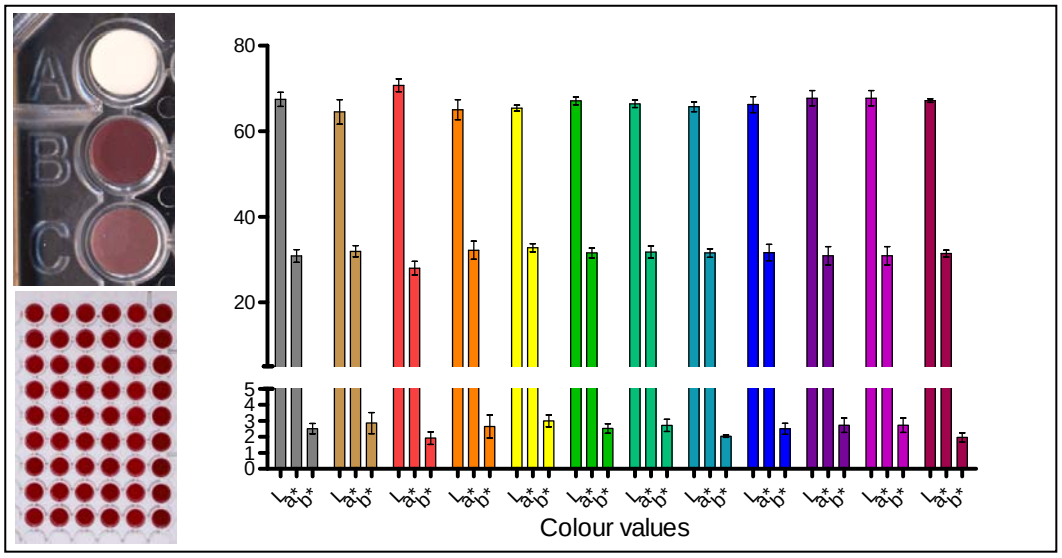
THE UNIVERSITY OF ADELAIDE
AUSTRALIA

Australian Government
Grape and Wine Research and Development Corporation

THE EFFECT OF WINE YEAST ON COLOUR EXTRACTION AND STABILISATION IN RED WINE



FINAL REPORT to

GRAPE AND WINE RESEARCH & DEVELOPMENT CORPORATION

Project Number: **UA 05/06**

Principal Investigator: **Assoc. Prof. Vladimir Jiranek**

Research Organisation: **The University of Adelaide**

Date: **30 December 2009**

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Abbreviations
AGRF: Australian Genome Research Facility
CDGJM: Chemically defined grape juice media
DAP: Diammonium phosphate
HPLC: High performance liquid chromatography
Mal-3-G: Malvidin-3-glucoside
Mal-C-G: Malvidin-6-coumarylglucoside
Pet-3-G: Petunidin-3-glucoside
Peo-3-G: Peonidin-3-glucoside
Peo-C-G: Peonidin-6-coumarylglucoside
SD: Standard deviation
TA: Titratable acidity
YEPD: Yeast extract (1%), peptone (2%), dextrose or glucose (2%)
WCD: Wine colour density
WCH: Wine colour hue

Abstract:
Colour is an important initial cue to the consumer in terms of the perception of quality in red wines. Previous work has revealed that choice of wine yeast can have a strong influence on the colour of wine. This project, through the activities of a PhD student, sought to confirm this effect and begin to more fully define the basis for this phenomenon. Two vintages of data were generated from small-scale winemaking activities (1 kg berry trials) and reiterated the marked impact of yeast strain on wine colour as determined through measures such as colour density, hue, total phenolics and total red pigments. Results in the subsequent vintage were not always consistent implying strong influences from grape and vintage as well. The impracticality of 1 kg fermentations with grapes necessitated a simplified system. Micro- and lab-scale fermentations were therefore used to explore the impact for yeast on the colour of a defined medium supplemented with a grape skin extract. With this approach 84 commercial strains were examined with their wine colour being expressed in CIELab colour units. Again several strain-related differences were apparent which may be of benefit in strain selection.

Trials with grape skin extracts and/or wine were intended to separate any fermentation effects from the influence of strain on colour. Specifically they were used to examine the extent of pigment binding to biomass. Clear differences were seen with biomass showing large reductions in the Mal-3-glu content of the wine/skin preparations. The significance of such reductions is yet to be fully defined. It is likely that marked visual impacts will be limited to lighter styles of red (or *blanc de noi*r) wines.

The final phase of work to be undertaken involved the screening of the homozygous diploid lab yeast deletion library. Key findings include the identification of some 300 genes which are essential for completion of high sugar fermentations. Further, several hundred genes were found to impact colour (positively or negatively). Their role in this phenomenon is the subject of further research aimed at producing candidate genes for the generation of strains with distinct and novel contributions to wine colour.

Background:
Colour is a critical sensory and hence quality attribute of red wine. 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 on 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. This process is influenced by several factors, both chemical and microbiological. We seek to gain a greater understanding of these factors and their interaction as a basis for colour development and stability in young and aged red wines. Currently, unrelated studies have implicated yeast metabolites, biomass and some hydrolytic enzymes as modulators of colour development. We seek 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. Ultimately, this information will be used to produce strains with *multiple* colour-enhancing properties which result from enhancements to two of more of the following: 1) increased liberation of anthocyanins from grape skins by hydrolytic enzymes, 2) reduced degradation of anthocyanins, 3) increased formation of metabolites which favour pigmented polymer formation and 4) reduced loss of pigments through binding to yeast lees.

The aims of the project were:
1. To characterised and optimise release/extraction of anthocyanins through yeast enzymes (pectinases, etc).
2. Identify and characterise biochemical reactions that are essential for the formation of stable anthocyanin-derived pigments.
3. Identify and characterise yeast catabolic processes that are responsible for anthocyanin degradation (glycosidases, oxidases, etc).
4. Quantify and characterise yeast interactions, which may contribute to removal of pigments (anthocyanins and pigmented polymers) through binding to yeast lees.
5. Develop ranking for key parameters according to magnitude of influence over colour.
6. Identify critical variables for predictable modulation of colour in red wine fermentations.

The majority of this work was to be conducted by a PhD student. However after a 6-month delay in recruitment and 12 months of candidature, the student withdrew. The remaining funding and project time frame were not conducive to recruitment of a second PhD student. As such the remainder of the work was undertaken by research associates or PDFs seconded from other projects. This arrangement meant that some aspects of the project could not be pursued as originally intended.

Project Aims and Performance Targets:
NB. Outputs have been renumbered consecutively to simplify reference to these in throughout the report.

Outputs and Performance Targets 2005-06

Outputs	Performance Targets
1. Project resources.	Technical Officer appointed. Yeasts and purified anthocyanins and pigmented polymers in hand. Analytical methods for yeast metabolites, and phenolic reactants and end-products set-up.
2. Initial data on role of yeast metabolites and biomass.	Ranking of yeast strains according to colour of young red wine. Colour-scavenging potential of different strains examined through model ferments.
3. Completion of the PhD *Structured Component*.	Attendance at formal sessions for commencing PhD students, literature review and research proposal submitted and one seminar presentation made.

Outputs and Performance Targets 2006-07

Outputs	**Performance Targets**
4. Information on stable pigment formation.	Use models of pigment evolution to further define mechanisms involved.
5. Yeast for increased anthocyanin release.	Construction/isolation and evaluation of a strain designed to influence release of anthocyanins from grapes.
6. Strains with and without degradative enzymes.	Over- & non-producers of anthocyanin-degrading enzymes (glycosidases, anthocyanases, oxidase, etc) evaluated.
7. Methods to identify yeast genes that impact on pigment adsorption to biomass.	Yeast libraries screening initiated to identify gene(s) that modulate pigment absorption to yeast biomass.
8. Findings disseminated.	1 – 3 manuscripts published or presentations delivered which describe key findings from the project to date.

Outputs and Performance Targets 2007-08

Outputs	**Performance Targets**
9. Identity established of yeast genes that impact on pigment adsorption to biomass.	Yeast libraries screened and gene(s) identified that modulate pigment absorption to yeast biomass. Over- and non-producer strains constructed or isolated.
10. Identity of key parameters for colour modulation.	Relative importance of critical parameters defined through laboratory and experimental winemaking trials.
11. Informed use of colour modifying parameters.	Trials designed and initiated to examine effects of combined strategies for modulating wine colour.
12. Findings disseminated.	1 – 3 manuscripts published or presentations delivered which describe key findings from the project to date.
13. Submitted PhD thesis.	Thesis prepared by the PhD candidate submitted for examination.

Methods:

Yeast strains

Yeast strains were grown in YEPD media (1% yeast extract, 2% Bacto-peptone, 2% D-glucose), which was solidified with 2% Bacto-agar when necessary. Yeast strains used in the study are shown in Table 1.

PCR amplification of β-glucosidase genes from non-*Saccharomyces *yeast

Genomic DNA was extracted from 10 ml cultures of yeast grown overnight in YEPD, according to Adams et al., (1987). Species identification was performed by ITS PCR (Guillamón et al., 1998); the products sequenced by Australian Genome Research Facility (Brisbane). Primers used in the amplification of β-glucosidase genes *EXG2* and *BGL2* (coding sequence only) from *Pichia anomala*, *Dekkera bruxellensis*, *Dekkera anomala* and *Hanseniaspora osmophila* are shown in Table 2. The following sequences (including accession numbers) were used in the design of the primers - *P. anomala EXG1* (EMBL:AJ002195); *EXG2* (EMBL:AJ222862); *P. stipitis BGL2* (EMBLCDS:ABN65244); *Dekkera bruxellensis EXG2* (*Dekkera bruxellensis* Random Genomic Library survey clones bb58h02.r (EMBL:EI021578), bb20a08r (EMBL:EI024371), bb43d09.f (EMBL:EI017864), bb14e03.f (EMBL:EI012049), bb46b04.f (EMBL:EI021851)); *Dekkera bruxellensis BGL2* (*Dekkera bruxellensis* Random Genomic Library survey clones bb50f05.r (EMBL:EI013642), bb06h05.f (EMBL:EI017514)) and *Hanseniaspora uvarum BGL* (EMBLCDS:AAY59870). PCR reactions were optimised for each set of primers, using DyNAzyme EXT DNA polymerase (Finnzymes), and the correct sized PCR products were isolated from 1% agarose gels (in Tris-acetate buffer) and purified using the Wizard SV Gel and PCR clean-up system (Promega). PCR fragments were cloned using the pGEM®T Easy Vector system (Promega) and sequenced (AGRF, Brisbane) to confirm identity. RNA was isolated from *Dekkera* yeast using the RNeasy mini prep (Quiagen) as per manufacturer's instructions. cDNA was made from 2 μg RNA, using superscript 3 reverse transcriptase (Invitrogen) as per manufacturer's instructions. Second strand synthesis was performed with Pfu Ultra II DNA polymerase (Promega) using gene-specific primers and oligo-dT or random hexamers (Invitrogen). Resultant bands were excised and purified and 20 ng of the product was sequenced. Sequence analysis was performed using the NCBI-BLAST 2 nucleotide tool, available from the EBI website (EMBL-EBI) and Bioedit (version 7.0.0).

Table 1. Yeast strains used during the study

Yeast	ID or genetic background	Source
Non-*Saccharomyces* Yeast		
Pichia anomala [a]	11207	CBS
Hanseniaspora osmophila [a]	10590	CBS
Dekkera anomala [a]	CBS 77	CBS
Dekkera bruxellensis [a]	AWRI 1499	CBS
Wine Yeast		
AWRI 1503 [b,c,d]		AB Mauri
AWRI 350 [b,c]		AB Mauri
AWRI 796 [b,c,d]		AB Mauri
AWRI R2 [b,c]		AB Mauri
BP 725 [b,c,d]		AB Mauri
La Riojana [b,c,d]		
PDM [b,c,d]		AB Mauri
Primeur [b,c]		AB Mauri
Techno FD 260 [b,c,d]		
Techno FD 337 [b,c,d]		
Techno FD Intec [b,c]		
UCD 522 [b,c,d]		AB Mauri
Maurivin B [b,c,d,e]		AB Mauri
EC1118 [e]		Lallemand
Fermirouge 7303 [e]		DSM
Syrah [e]		Enoferm
QA23 [e]		Lallemand
Laboratory or recombinant wine yeast		
isoC9-D Δura3 [f]	Haploid auxotroph of L2056 (MATα, Δho, ura3::KanMX)	This lab
isoC9-D Δura3 (pJC1) [f]	isoC9-D Δura3 transformed with plasmid pJC1	This lab
isoC9-D Δura3 (pJC1PAEXG2) [f]	isoC9-D Δura3 transformed with plasmid pJC1PAEXG2	This lab
Yeast deletion library (BY7473) [g]	MATa/α, his3Δ1/his3Δ1, leu2Δ0 /leu2Δ0, lys2Δ0/LYS2, MET15/met15Δ0, ura3Δ0/ura3Δ0	Applied Biosystems

[a] Non-*Saccharomyces* yeast used as sources of genomic DNA for the PCR amplification of β-glucosidase genes. [b] Wine strains used to determine yeast strain influence on colour parameters of wine derived from enriched CDGJM. [c] Wine strains used to determine yeast strain influence on colour parameters of experimental wine (2006 vintage). [d] Wine strains used to determine yeast strain influence on colour parameters of experimental wine (2007 vintage). [e] Wine strains used to determine effect of pigment adsorption of yeast biomass on red colour of commercial Shiraz and Pinot Noir wine styles. [f] Recombinant wine strains used in heterologous expression of β-glucosidase gene *EXG2* from *Pichia anomala*. [g] Homozygous diploid yeast gene deletion library used for screening of genes which influence red colour during micro-fermentation of enriched CDGJM.

Table 2. Primers used in the PCR amplification of β-glucosidase genes from non-*Saccharomyces* yeast

Name	**organism**	**Gene**	**Direction**	**Application**	**sequence**
Pammy	*Pichia*	PA*EXG2*	Fwd	Gene specific amplification full ORF	ATGCTTATTTCAACTTTTATCATTTCTTC
Philip	*Pichia*	PA*EXG2*	Rev	Gene specific amplification full ORF	CCCCTGGGAGCCCTATAATG
Pirjo	*Pichia*	PA*EXG2*	Fwd	Gene specific amplification + Restriction site BglII full ORF	GCCGAGATCTATGCTTATTTCAACTTTTATCATTTCTTC
Pekka	*Pichia*	PAEXG2	Rev	Gene specific amplification + Restriction site XhoI full ORF	GCGCTCGAGCCCCTGGGAGCCCTATAATG
Peter	*Pichia*	PA*BGL2*	Rev	homology to *Pichia stipitus* full ORF	CTATAAACCTAACCAGAAGTAAGTCTT
Pandora	*Pichia*	PA*BGL2*	Fwd	homology to *Pichia stipitus* full ORF	ATGACTCCAAGTGTTAAACAACC
Debbie	*Dekkera*	*EXG2*	Fwd	Gene specific amplification incomplete	GGAACGGAAGCATCCTG
Dennis	*Dekkera*	*EXG2*	Rev	Gene specific amplification incomplete	CAACTCCCACACAACTCAG
Derek	*Dekkera*	*EXG2*	Fwd	5’ RACE PCR	GGTCTTAACATGGTTAGGATTCC
Delilah	*Dekkera*	*EXG2*	Rev	3’ RACE PCR	GAGCAGCAGCAGCAAACG
Danna	*Dekkera*	*BGL2*	Fwd	Gene specific amplification, incomplete	GATCACCTCTTCAACTCCACATATTG
Dante	*Dekkera*	*BGL2*	Rev	Gene specific amplification, incomplete	GAGCGGACCCCTTTGAATG
Davinna	*Dekkera*	*BGL2*	Fwd	5’ RACE PCR	CACACCAAAGCAAATAGAAG
Dagon	*Dekkera*	*BGL2*	Rev	3’ RACE PCR	CTTCTCTTCTGTTGTCAATTG
Hannah	*Hanseniaspora*	*BGL2*	Fwd	3’ RACE PCR	CCACATGGGTAGAGTTG
Harriet	*Hanseniaspora*	*BGL2*	Fwd	Inverse PCR	GTTTACGGTGAAGATATCTAC
Henry	*Hanseniaspora*	*BGL2*	Rev	Inverse PCR	CAACTCTACCCATGTGG

Expression of* EXG2 *gene from* Pichia anomala *into haploid wine yeast strain isoC9 Δ ura3

Primers designed with restriction enzyme recognition sites (shown in Table 2) were used to amplify the full length coding sequence of PA*EXG2* gene (1284 bases). The PCR product was digested with XhoI and BglII, isolated on a 1% gel (in Tris acetate buffer) and purified. Plasmid pJC1 (Martin et al., 2003), was similarly digested prior to ligation with the PCR fragment. The ligation mixture was used to transform *Escherichia coli* DH5α (Ausubel 1994). Plasmid DNA (pJC1PAEXG2) from *E. coli* was made using the Wizard *Plus* SV plasmid miniprep kit (Promega), and confirmed by DNA sequencing. Haploid wine yeast strain isoC9-DΔura3 was transformed by treatment with lithium acetate (Gietz, 1992) and plated on minimal drop out (lacking uracil) media (Gutherie and Fink, 1991). Transformants were grown on the same media before fermentation as described in Section 1.12. β-glucosidase activity was measured by hydrolysis of arbutin (hydroquinone-β-D-glucopyranoside; Sigma) as described by Strauss et al. (2001). Alternatively, yeast were grown in YEPD or YM (0.3% [w/v] malt extract, 0.3% [w/v] yeast extract, 0.5% [w/v] bactopeptone, 1% glucose, pH5.5) prior to colourimetric determination of β-glucosidase activity using the substrate p-nitrophenyl-β-D-glucopyranoside as described by Grimaldi et al. (2000).

Small-scale (1 kg) berry vinifications

Shiraz berries were hand harvested from the Adelaide Hills region (2006 vintage) and Waite vineyard (2007 vintage) at optimal ripeness and cooled to 0°C. To maintain a constant juice to skin ratio, intact berries were manually de-stemmed and randomised by mixing as a monolayer. Replicates (1 kg) were distributed into 1.5 L lidded, plastic containers. Sulfur dioxide was added to 50 mg L^{-1} and diammonium phosphate to 200 mg L^{-1}. Based on analysis of the fruit (Table 3) an acid adjustment (2 g L^{-1} tartaric acid) was made to the 2007 must. Replicates were exposed to an equal amount of crushing with a sterile potato masher (60 presses), and allowed to warm (approx. 3 hrs) to the fermentation temperature of 25°C.

Inoculum cultures were prepared using a variation of a method described previously (Soden *et al.*, 2000). Single colonies, freshly grown on YEPD agar plates, were inoculated into liquid YEPD and grown overnight at 30°C with agitation (160 rpm). A 5 mL volume of this broth culture was then added to 50 mL of starter culture medium consisting of DAP-ameliorated grape juice diluted 1:2 with sterile ultra-pure water. Starter cultures were grown at 30°C with agitation (160 rpm) to a cell density of at least 2 x 10^8 cells mL^{-1}. Crushed, de-stemmed, 25°C musts were then inoculated to 1 x 10^6 cells mL^{-1}.

	2006	**2007**
Titratable Acidity	6.75 g/L	7.40 g/L
pH	3.24	3.78
Baumé	12.9°	16.4°

Table 3: Analysis of must from 2006 and 2007 vintages.

Caps were plunged twice daily and 1 mL samples collected, centrifuged and the supernatants frozen for later analysis. After 56 h, free run juice was collected in sterile 500 mL Schott® bottles to leave a minimum of ullage and bottles were loosely fitted with lids. Replicates were homogenised by inversion prior to regular sampling. Upon achieving dryness (< 2.5 g L^{-1} residual sugar by Clinitest® tablets), wine was cold settled, racked from gross lees and 50 mg

L^{-1} of SO_2 added. Samples were centrifuged and the supernatants frozen for storage and subsequent analysis.

Micro-fermentations of chemically defined grape juice media

Overnight cultures were prepared from singles colonies as described above. Chemically Defined Grape Juice Media (CDGJM) (Henschke and Jiranek 1993; McBryde *et al.*, 2006) was enriched with 5 g L^{-1} of Polyphenol Powder from Red Grapes (TPPR, Oeno Prod, France. Appendix 4.2) and filter sterilised (0.22 μm). HPLC analysis of the filtered, enriched CDGJM confirmed malvidin-3-glucoside (M3G) levels to be equivalent to the maximal amount expected to be extracted from red grapes under normal oenological conditions (approximately 400 mg L^{-1}; Waterhouse and Zimman 2004). Filtration also served to remove greater than 95% of the larger pigmented polymers present in the polyphenol preparation (data not shown). 1 mL of the enriched CDGJM was inoculated in triplicate with 5 μL of overnight culture of the respective strain in deep 96 well trays (Axygen, PDW20C), sealed with pre-cut adhesive polyethylene film pierced once per well with a sterile needle. Trays were incubated in a sealed, humidified plastic container for 100 hours at 25°C, with micro-orbital agitation. Trays were then centrifuged (2,464 x *g*, 10 min) and 200 μL of cleared supernatant assayed for colour intensity.

Statistical analysis

One-way ANOVA with Tukey's Multiple comparison post test was performed using GraphPad Prism v. 5.00 (GraphPad Software, San Diego California USA). A significance level of $p = 0.05$ was used throughout.

Colour analysis by spectrophotometric methods

Wine samples were analyzed for density, hue, SO_2-resistant pigments, total anthocyanins and total phenolics following the method of Iland *et al.,* (2000), which was modified for use with 96 well UV transparent microtitre plates. Plates were read at various wavelengths using a μQuant Microplate spectrophotometer (Bio-Tek Instruments). Samples (50 μL) for total anthocyanin and total phenolic determinations were added to 1 M HCl (5 mL) and incubated for a minimum of 3 h at room temperature before aliquots (300 μL) were transferred to 96-well microtitre plates and read at 520 nm (total anthocyanins) and 280 nm (total phenolics). Density and hue was calculated from absorbance values of neat wine (150 μL aliquots in 96 well microtitre plates) read at 420 and 520 nm. SO_2-resistant pigments were estimated by adding 30 μL SO_2 solution (25% w/v) to 1 mL of wine, mixed and 150 μL aliquots transferred to 96 well microtitre plates and read at 520 nm.

Colour analysis – spectrophotometric (high throughput screen) method

Fermentation samples were assayed by previous reported methods (Iland *et al*., 2004) modified for a 96 well plate format. Measurements were carried out at wine pH using flat bottom 96 well UV plates (Cat: 3635, Corning, sourced by DKSH Australia). Colour intensity at 420 and 520 nm was measured using 150 μL (WCD and WCH). SO_2 resistant pigments were determined by adding 5 μL of 25% (w/v) sodium metabisulphite solution (freshly made) to 150 μL, mixed 5 min before reading at 420 and 520 nm. Total phenolics: 280 nm (HCl) and total anthocyanins: 520 nm (HCl) were determined after 3 hour incubation (in dark, ambient temperature) of a 15 μL sample in 150 μL 1 M HCl. Gallic acid standards (0 – 0.04 mg L^{-1}) were used for total phenolics determinations, for quantification as gallic acid equivalent units. Effects of SO_2 on colour were eliminated by incubation of the sample (100 μL) with 10 μL of 10% (w/v) acetaldehyde, prior to reading at 420 and 520 nm.

Wine measurements were also conducted at standard pH by 1:10 dilution of sample (15 μL with 135 μL citrate buffer pH 3.2 (Medina *et al*., 2005).

CIElab determination of colour adapted for high throughput screening

Measurements were modified for 96 well plates, and used for high throughput screening of the yeast homozygous diploid deletion library. CIELab measurements were obtained using a D65 illuminant, a 10° standard observer and by measuring the absorbance (converted to transmission) of the wine at 5 nm intervals over the visible spectrum from 380 nm to 780 nm using a μQuant Microplate spectrophotometer (Bio-Tek Instruments). CIELab determinations were performed directly on 75 μL wine (fermented supernatants), whilst Adobe Photoshop software was used to determine L*, a*, b* values from scanned images of wells containing 200 μL washed yeast biomass. Chroma (C*) and Hue (H°) were determined from CIELab values. ΔE defines the spatial distance between two colours, and is calculated from L*, a*, b* using $\sqrt{((L1^*-L2^*)^2 + (a1^*-a2^*)^2 + (b1^*-b2^*)^2)}$. ΔC and ΔH were calculated from C* and H° values of the query sample minus the control sample. Liquid handling of samples was performed using a Corbett CAS3800 robot (Corbett Robotics, Sydney).

Colour compound analysis by HPLC

Anthocyanins in wine samples were separated and quantified by linear gradient elution (Schmid *et al*., 2007) using an Agilent 1100 HPLC system (Agilent Technologies) fitted with a Phenomonex Synergi Hydro-RP column (150 x 2 mm). Solvent A consisted of 1% (v/v) acetonitrile, 1.5% (v/v) phosphoric acid in water. Solvent B was made up of 20% solvent A and 80% acetonitrile. Samples (20 μL) were injected onto the column and eluted at 0.4 mL/min using the following linear gradient: 0 min (14.5% B), 18 min (27.5% B), 20 min (27.5% B), 21 min (50.5% B), 22 min (50.5% B), 26 min (100% B), and 28 min (100% B). A photodiode array detector (520 nm) was used to detect the anthocyanin compounds (Wu and Prior 2005) and quantified as malvidin-3-glucoside equivalents. Individual compounds (Table 4) were identified from their elution time with respect to malvidin-3-glucoside and their UV–Vis spectra (scanning from 220 to 700 nm) which were recorded for all peaks.

Anthocyanin	Abbreviation
Malvidin-3-glucoside	Mal-3-G
Delphinidin-3-glucoside	Del-3-G
Cyanidin-3-glucoside	Cy-3-G
Petunidin-3-glucoside	Pet-3-G
Peonidin-3-glucoside	Peo-3-G
Malvidin-acetylglucoside	Mal-A-G
Delphinidin-acetylglucoside	Del-A-G
Cyanidin-acetylglucoside	Cy-A-G
Petunidin-acetylglucoside	Pet-A-G
Peonidin-acetylglucoside	Peo-A-G
Malvidin-6-coumarylglucoside	Mal-C-G
Delphinidin-6-coumarylglucoside	Del-C-G
Cyanidin-6-coumarylglucoside	Cy-C-G
Petunidin-6-coumarylglucoside	Pet-C-G
Peonidin-6-coumarylglucoside	Peo-C-G
Malvidin-6-caffeoylglucoside	Mal-Caff-G
Delphinidin-6-caffeoylglucoside	Del-Caff-G
Cyanidin-6-caffeoylglucoside	Cy-Caff-G
Petunidin-6-caffeoylglucoside	Pet-Caff-G
Peonidin-6-caffeoylglucoside	Peo-Caff-G

Table 4. Summary of grape anthocyanins analysed for by HPLC in 2006 and 2007 Shiraz wines made using different yeast strains. Included are abbreviations of the compounds used throughout the results tables.

Measurement of residual sugar

Fermentation progress for small scale berry vinification and 100 mL fermentations was determined by measurement of residual sugar by refractive index using a digital refractrometer (30GS, Mettler Toledo, Melbourne), and from the colorimetric reaction of reducing sugars with $CuSO_4$/NaOH (Clinitest® tablets, Bayer). Residual glucose and fructose were quantified using the spectrophotometric method described by Bergmeyer (1983) modified to suit a 96-well plate (P-96-300F-PS, Axygen). The total volume of the reaction was reduced from 1.62 mL to 125 µL. Three times the concentration of the reaction buffer was used to provide NADP and ATP to excess, and to ensure accurate pipetting of the enzymes, 10 µL of 1:10 dilutions in H_2O were used (as opposed to 1 µL). Absorbance at 340 nm was measured using a µQuant Microplate spectrophotometer (Bio-Tek Instruments). The concentration of fructose and glucose was suitably diluted and determined against a set of identically diluted standards spanning the linear dynamic range of the reaction (0-0.08 g L^{-1}).

Screening of yeast homozygous diploid deletion library for red colour modulation

Media preparation

CDGJM containing 200 g L^{-1} equimolar glucose:fructose and 450 mg N L^{-1} FAN was prepared according to Henschke and Jiranek (1993). Polyphenol extract (Cat: Tppr, OenoProd, Sarl) was dissolved in 100% ethanol (3g powder in 5 mL, and left 24 h in the dark) before adding aspectically to the sterile media. As such, the larger pigment polymers were not removed from the grape polyphenol extract. The enriched CDGJM media was stored in the dark for a maximum of 24 h (4°) before use.

Micro-fermentations

The library (in 384 well plates) was replicated into 96 deep well plates (600 µL YEPD) and incubated 24-48 h at 28°C. Ferments were performed in duplicate in enriched CDGJM using 96 deep well plates covered with breathable sealing film (Cat: AXYBF-400, Axygen), and placed in a sealed plastic container. After 180 h at 28°C, samples were taken for residual sugar determination (measure of fermentation completion) and CIElab determination (measure of yeast impact on colour) using clarified ferment supernatants. Pigment adsorption on biomass was determined as follows: cells were resuspended by agitation on an orbital shaker, and 200 µL of sample was transferred to a sterile flat bottom 96 well plate. The plates were centrifuged and supernatants removed by inversion and blotting on filter paper. Cells were resuspended in 200 µL sterile water and allowed to settle. Images of the yeast biomass were scanned and CIElab colour values were determined manually using Adobe Photoshop.

Laboratory scale (100 mL) fermentations

Fermentations were performed (Walker et al., 2003) using the Multi-scale Fermentation Facility (University of Adelaide), whereby fermentation progress was monitored by cumulative weight loss of media due to CO_2 evolution (Bely et al., 1990). After 180 hours, samples were clarified by centrifugation (5 min, 16,000 x *g*) prior to HPLC analysis of anthocyanins and metabolites, and colour determination as described in sections 1.8 - 1.10.

Colour pigment adsorption by the yeast biomass

100 mL cultures of yeast were grown overnight in YEPD at 30°C. The cells were centrifuged at 5,000 rpm, 5 min and washed once in sterile saline solution (0.85% sodium chloride) prior to cell counting. 10 mL aliquots of 3 x 10^8 cells mL^{-1} were placed in 10 mL

plastic screw-capped tubes (Starstedt) and centrifuged as before. The supernatant was carefully removed and replaced with either 10 mL of University of Adelaide 2006 Adelaide Hills Shiraz or Taylors 2008 Adelaide Hills Pinot Noir wine and incubated in the dark. Five yeast strains and a control (no yeast addition) were tested in triplicate over incubation times of 24, 48 and 120 h. Colour determination of the wines was as described in Sections 1.8 and 1.9, whilst anthocyanin content was determined by HPLC (Section 1.10). Pigment colouration of the biomass was determined as described in Section 1.12.2 following a 9-fold concentration of the cells (5.4×10^8 cells in 0.2 mL were used for the assay). For the initial (0 h) biomass sample, colour values derived from an equal amount of cells (5.4×10^8 cells in 0.2 mL) resuspended in sterile MQ water were used. Photographic images of the wines and yeast biomass were taken for reference.

Results and Discussion (by Outputs):

Output 1: Project resources - Technical Officer appointed. Yeasts and purified anthocyanins and pigmented polymers in hand. Analytical methods for yeast metabolites, and phenolic reactants and end-products set-up.

Status summary: The performance targets for this Output were achieved.

1.1 Project Resources

A PhD student was appointed, after some delay, in late 2005. A collection of yeast strains and relevant phenolics was sourced from industry suppliers. HPLC methods for key fermentation metabolites and phenolics were also established.

Output 2: Initial data on role of yeast metabolites and biomass - Ranking of yeast strains according to colour of young red wine. Colour-scavenging potential of different strains examined through model ferments.

Status summary: The performance targets for this Output were largely achieved. Data from two vintages of 1 kg berry fermentations were collected. While strain-related differences were observed in many parameters, the data highlighted the complexity of the interaction between strains and grapes, prompting work with a simplified system in the latter part of the project.

2.1. Microvinification of CDGJM containing grape polyphenol extract

In an attempt to simplify and accelerate the determination of the potential contribution of commercially available wine yeast strain to the colour of red wines, we developed a micro-fermentation assay, the results from which could be compared with the data presented herein for the small scale berry (1 kg) vinifications later. In all cases, the inclusion of wine yeast in the assay resulted in an increased colour density value (Fig. 1), with Techno FD Intec and AWRI 350 increasing colour density by approximately 22% respectively compared to the control.

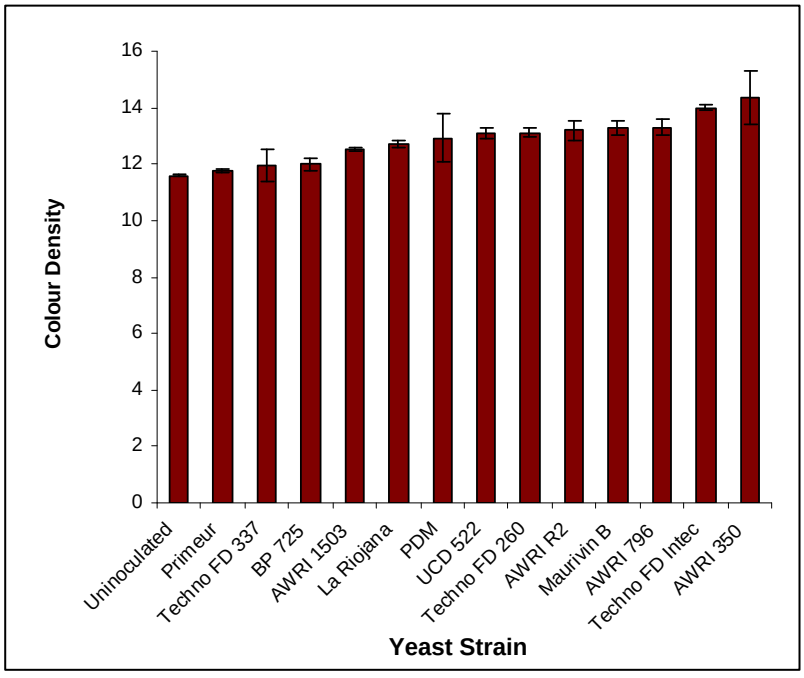


Figure 1. Colour density of polyphenol-enriched CDGJM fermented by different commercial strains. Values represent means of individual absorbance readings of triplicate fermentations. Standard error ranges are shown.

2.2. Vintage 2006 – small scale (1 kg) berry vinifications

To evaluate the effect of yeast strain on colour development in real wine, Shiraz must was fermented, in duplicate, with the same selection of yeast strains as above. Analyses of these wines included spectrophotometric analysis after fermentation (May 2006) and prolonged frozen storage (17 months). HPLC determination of individual anthocyanin colour compounds was also included for stored samples.

Wine colour density (a measure of colour intensity) measurements taken from fresh wines (2006) ranged from approximately 11 to 15 (Fig. 2a), corresponding to a maximum variance of 27%. Single factor analysis of variance (ANOVA, $p = 0.05$) demonstrated the variance to be statistically significant, with AWRI R2, AWRI 796, UCD 552 and AWRI 350 yeasts contributing to the greatest densities. Colour density values of the same wines after frozen storage (Fig. 2b) were similar to those obtained earlier for each yeast strain. Overall, values ranged from approximately 12 to 17 with AWRI R2, AWRI 796, UCD 552 and AWRI 350 retaining the highest densities. However, due to the large variances observed within some of the replicates, no significant difference could be established between the strains (ANOVA, $p = 0.05$). This variance was demonstrated to correlate poorly to the pH of the finished wines ($R^2 = 0.4356$, data not shown), suggesting the perceived differences were not solely a function of the equilibria established of the different anthocyanin isoforms in each case.

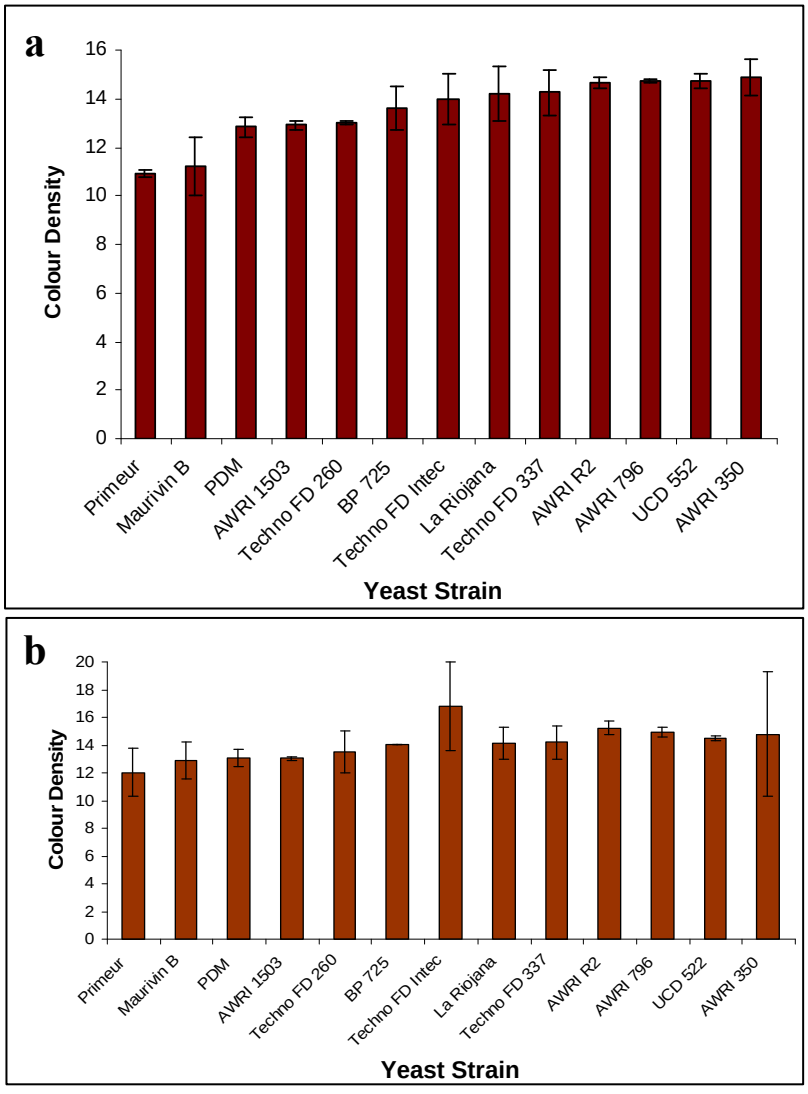


Figure 2. Colour density of wines from the 2006 vintage and determined after fermentation (a) and after 17 months frozen storage (b). Wines were produced by small-scale vinification using different commercial strains. Values represent means of absorbance readings of duplicate fermentations. Standard error ranges are shown.

Wine colour hue (an indication of tint or shade) for the stored samples ranged between 0.41 and 0.48 (Fig. 3) and was significantly affected by the choice of yeast strain (ANOVA, p = 0.05). The wine produced by Primeur had the highest hue value (0.48), followed by Maurivin B (0.47). Further statistical analysis (Tukey's Multiple comparison, p = 0.05) showed that the Primeur wine was only significantly different to the wines produced by Techno FD 337, PDM, BP 725 and AWRI 769.

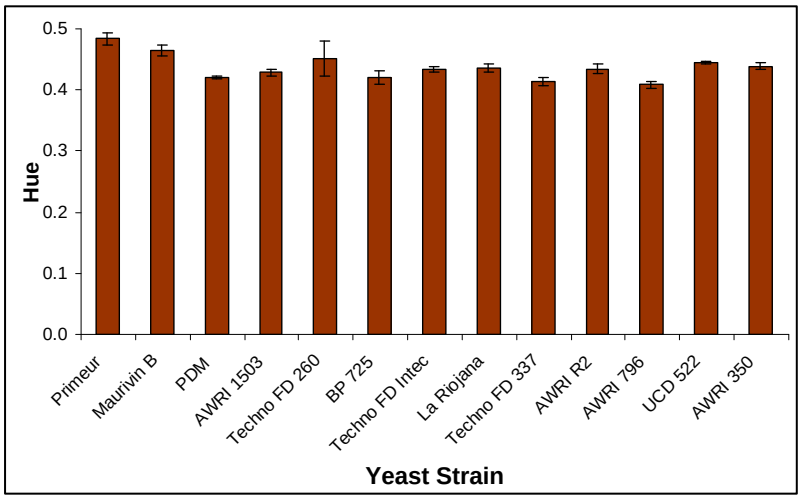


Figure 3. Hue values of wines from the 2006 vintage determined after being stored frozen for 17 months. Values represent means of duplicate fermentations. Standard error ranges are shown.

Total phenolics measurements of fresh wines ranged from 42.9 to 50.8 units (Fig. 4a), corresponding to a maximum variance of 15.5%. ANOVA did not suggest statistical significance across the strains, although individual t-tests between selected strains (eg. UCD 522 and AWRI 1503) could be used to confirm differences beyond the 95% level of confidence. When this analysis was repeated on the same stored wine samples, values were found to be lower, ranging from 9.2 to 31.4 units (Fig. 4b) along with a much greater variation in standard error of the duplicates. Subsequently no statistical differences were found across strains in this re-analysis (ANOVA, p = 0.05).

Values determined for total red pigments for the fresh wine samples ranged from 38.4 to 46.8 units (Fig. 5a), corresponding to a maximum variance of 17.9%. ANOVA did not return a statistically significant variance for the entire set of strains, but consideration of individual pairings within the set (eg UCD 522 and AWRI 796) would suggest significant difference between the range of pigment profiles. Analysis of the stored samples returned slightly higher values of total red pigments ranging from 57.2 to 78.0 units (Fig. 5b), with AWRI R2, La Riojana, UCD 522 and Primeur still retaining high total red pigments. Additionally, significant differences were now evident between strains (ANOVA, p = 0.05), especially the total red pigment of the wine produced by AWRI R2 which was significantly higher than wines made by 7 of the other strains (Tukey's Multiple comparison, p = 0.05).

Across all the wines made by the yeast strains about 13 – 18% of pigments were resistant to SO_2 bleaching (data not shown) but there was no statistical evidence that this difference could be attributed to the influence of yeast strain (ANOVA, p = 0.05).

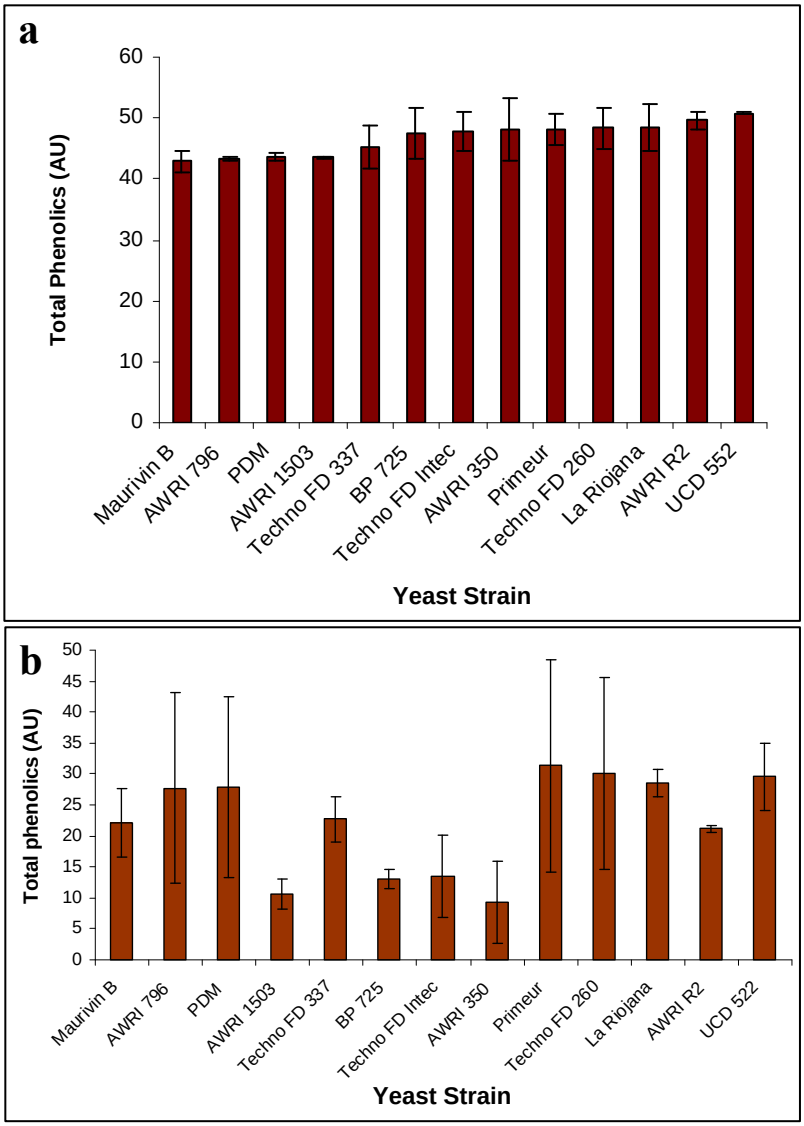


Figure 4. Total phenolics of wines from the 2006 vintage made using difference yeast strains and determined after fermentation (a) and after 17 months frozen storage (b). Values represent means of duplicate fermentations. Standard error ranges are shown.

Grape anthocyanins were quantified and expressed as malvidin-3-glucoside equivalents. Only those compounds which could be confidently identified by their retention time and UV-Vis spectrum are included in the results. Furthermore data is presented as individual grape anthocyanins detected, total of each group of anthocyanins and total anthocyanins. For the 2006 vintage only the frozen samples were analysed, and of the 22 compounds (Table 4) in total, only 11 were confidently identified and quantified (Table 5). Of the anthocyanins detected, malvidin-3-glucoside was present at the highest concentration in all strains followed by malvidin-acetylglucoside. Significantly higher concentrations of these two compounds were found in the wine produced by UCD 522 compared to the wines from the other strains (ANOVA, $p = 0.05$). No correlation was found suggesting that if a yeast strain produced a high concentration of one group of anthocyanins it would also produce higher concentrations of the other anthocyanin groups. For example UCD 522 was found to have the highest concentration of total-3-glucoside but had a significantly lower concentration of total-acetylglucosides. The data also suggests that yeast strain has a significant effect on total anthocyanin concentration (ANOVA, $p = 0.05$), however, pairing analysis (Tukey's Multiple comparison, $p = 0.05$) of UCD 522 suggests differences were limited to Maurivin B, PDM, AWRI 1503 and AWRI 769.

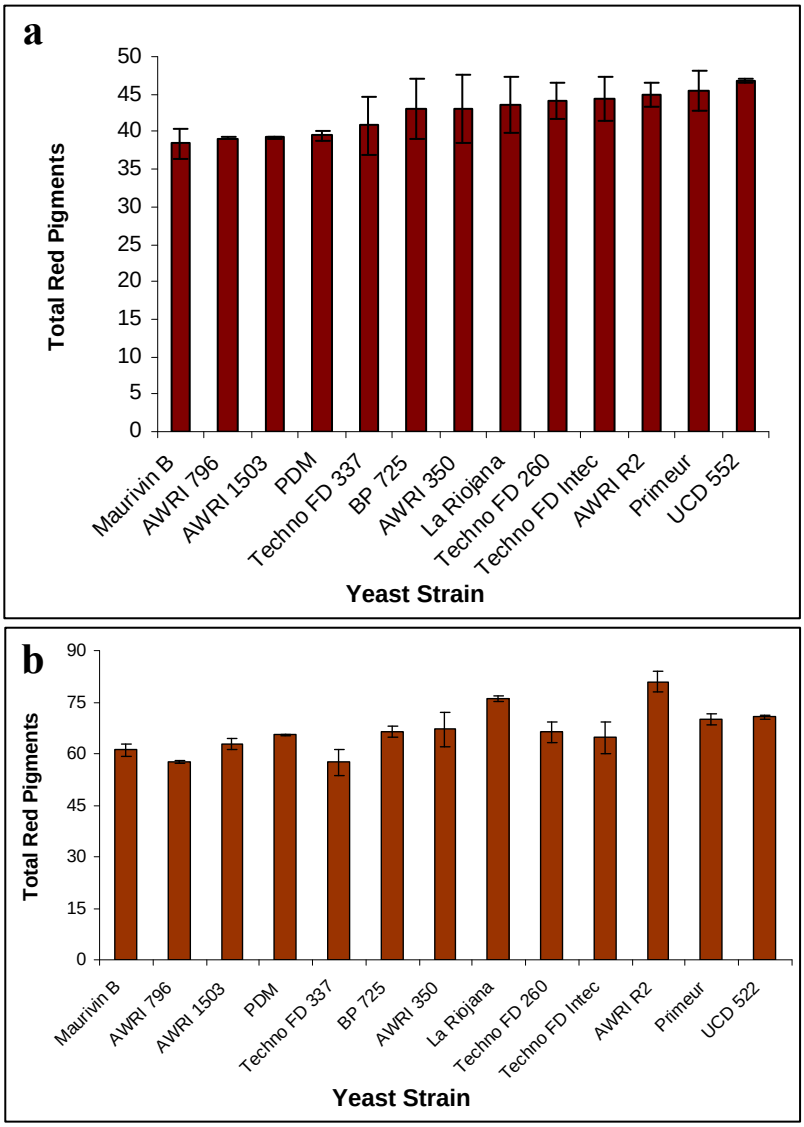


Figure 5. Total red pigments of wines from the 2006 vintage made using difference yeast strains and determined after fermentation (a) and after 17 months frozen storage (b). Values represent means of duplicate fermentations. Standard error ranges are shown.

Table 5. Identification and quantification of grape anthocyanins by HPLC of 2006 Shiraz wines fermented by various the wine yeast strains. Values are averages of duplicate fermentations along with their standard errors.

* only a single fermentation was available for analysis.

	FD Intec*	Mauri B		FD 260		AWRI 350		FD 337		Primeur		La Riojana		AWRI R2		PDM		AWRI 1503		BP 725		AWRI 769		UCD 522	
Del-3-G	27.4	14.5	0.15	19.2	1.31	22.3	1.53	22.6	1.43	24.1	0.68	22.7	2.40	25.2	1.50	18.8	0.71	20.3	0.88	22.2	0.57	18.4	2.24	25.9	0.99
Pet-3-G	38.0	27.4	0.22	33.6	1.93	35.6	2.06	33.4	3.23	35.3	1.52	35.7	2.44	38.4	2.12	30.5	0.72	30.8	0.56	34.7	0.38	29.6	3.10	40.2	1.28
Peo-3-G	21.8	8.2	0.01	14.1	0.77	16.4	0.09	21.5	0.80	23.7	0.89	19.8	0.88	22.0	0.32	16.0	0.59	16.4	0.23	18.6	0.88	13.9	1.45	21.2	0.37
Mal-3-G	212.0	189.4	4.95	219.3	4.38	219.0	2.46	210.6	11.59	217.0	4.62	224.1	7.36	229.9	9.04	207.5	0.82	201.6	1.00	223.3	3.40	197.9	14.07	246.0	7.95
Vitisin-B	0.0	0.0	0.00	7.7	0.07	8.0	0.87	0.0	0.00	7.4	1.16	5.4	0.11	9.0	0.05	0.0	0.00	8.0	0.24	6.1	0.25	7.8	0.62	9.2	0.56
Pet-A-G	10.8	8.5	0.11	9.9	0.77	10.8	0.56	9.2	1.10	9.3	0.33	9.8	0.62	10.8	0.69	8.9	0.20	8.8	0.38	9.8	0.34	8.8	0.57	11.8	0.51
Peo-A-G	12.0	10.1	0.06	12.1	0.93	12.7	0.06	12.0	0.92	12.1	0.65	12.1	0.90	13.3	0.40	10.9	0.03	10.2	0.04	11.8	0.57	10.0	0.72	14.0	0.44
Mal-A-G	57.3	51.1	1.26	61.5	1.28	59.8	0.39	58.5	3.11	58.8	0.57	61.4	1.71	62.8	2.85	57.9	0.47	54.8	0.41	60.7	0.30	53.9	3.07	66.8	2.26
Mal-Caff-G	4.6	5.5	0.21	5.5	0.15	5.3	0.06	5.5	0.38	6.1	0.26	6.5	0.29	5.3	0.23	5.1	0.11	4.6	0.08	5.8	0.31	5.3	0.44	5.3	0.26
Peo-C-G	11.7	12.0	0.24	12.0	0.28	12.0	0.07	12.2	1.39	13.0	0.96	14.3	0.74	12.4	0.31	10.8	0.05	9.6	0.13	12.0	0.11	10.8	0.88	12.1	0.49
Mal-C-G	19.7	28.2	0.22	24.6	1.69	23.9	0.59	24.6	2.86	38.6	5.56	36.8	4.85	24.4	0.07	23.1	0.67	18.9	0.12	25.0	1.67	21.2	0.89	25.2	0.52
Tot-3-G	299.2	239.4	5.3	286.2	8.4	293.3	6.1	288.0	17.0	300.1	7.7	302.4	13.1	315.6	13.0	272.8	2.8	269.0	2.7	298.7	5.2	259.8	20.8	239.0	10.6
Tot-A-G	80.1	69.8	1.4	83.5	3.0	83.3	1.0	79.7	5.1	80.2	1.6	83.3	3.2	86.8	3.9	77.7	0.7	73.7	0.8	82.3	1.2	72.7	4.4	68.3	3.2
Tot-Caff-G	4.6	5.5	0.2	5.5	0.1	5.3	0.1	5.5	0.4	6.1	0.3	6.5	0.3	5.3	0.2	5.1	0.1	4.6	0.1	5.8	0.3	5.3	0.4	4.9	0.3
Tot-C-G	31.3	40.2	0.5	36.6	2.0	35.8	0.7	36.8	4.3	51.5	6.5	51.1	5.6	36.8	0.4	33.9	0.7	28.5	0.2	37.0	1.8	31.9	1.8	30.2	1.0
Tot Anth	415.3	355.0	7.4	419.4	13.6	425.6	8.7	410.0	26.8	445.4	17.2	448.7	22.3	453.5	17.6	389.5	4.4	383.8	4.1	429.9	8.8	377.6	28.0	477.8	15.6

Precipitable tannin determinations exhibited a maximum variance of 19.6% (Fig. 6) corresponding to a range from 89.2 to 110.9 mg /L as epicatechin equivalents. Statistical analysis did not suggest significance within this data set.

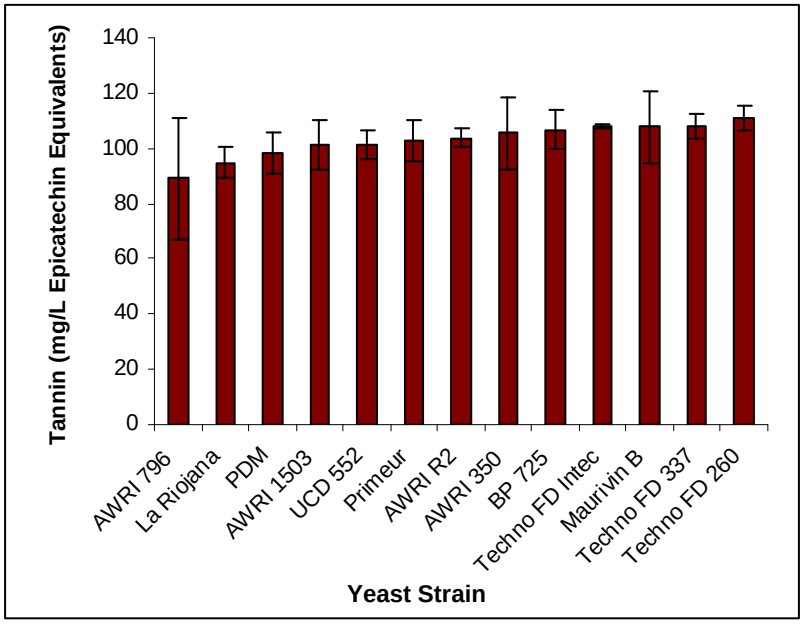


Figure 6. Methyl cellulose precipitable tannin determinations of 2006 wines produced by small-scale vinification using different commercial strains. Values represent the means of duplicate fermentations. Standard error ranges are shown.

In order to generate an overview of the colour modulating abilities of the strains examined, the measures for each strain were expressed as a percentage of the lowest value seen for the given parameter. A cumulative value for each strain was therefore determined by summing the relative percentages seen for that strain across each of four measures (Fig. 2 – 5), thereby yielding a value of at least 400% and up to 488% (Fig. 7). While somewhat artificial, this measure provides an indication that strains Maurivin B, PDM, AWRI 1503 tended to yield wines which lower values across the 4 measures investigated, while strains AWRI 350, AWRI R2 and UCD 522 produced higher values.

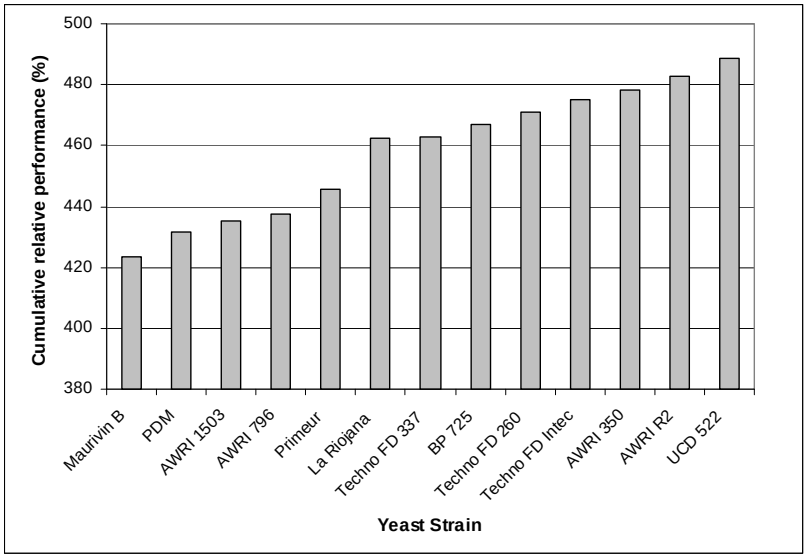


Figure 7. Relative performance of individual yeast strains, expressed as a sum of the relative colour outcome (%) across the four analyses performed (colour density, total red pigments, total phenolics, precipitable tannins) on Shiraz wines produced using different commercial strains.

2.3. Vintage 2007- small scale (1 kg) berry vinifications

The trial conducted in 2006 using supplemented CDGJM and the application of strains to grape must revealed, that yeast strain selection could have a significant influence on wine

colour development throughout fermentation. As such, strains of interest (Table 1) were identified (due to their potential for colour enhancement as shown above) and used to ferment Shiraz must from the 2007 vintage (in triplicate). For this vintage only fresh end of fermentation samples were analysed for colour.

Colour density values of wines (Fig. 8) ranged from approximately 14.8 (Maurivin B) to close to 18 (PDM, UCD 522) units, however significant differences were largely limited to these extremes. While the absolute values were about 4 units higher than seen for the 2006 vintage, the range of values was similar. Importantly, the values obtained in 2007 were not well correlated to those seen in 2006, suggesting a strong vintage or grape effect. Hue values (Fig. 9) ranged from 0.65 (UCD 522) to 0.77 (Maurivin B) and thereby were much higher compared to wines from the 2006 vintage (between 0.4 and 0.5). This outcome suggests that although the 2007 wines were similar in colour density to 2006, they were slightly darker.

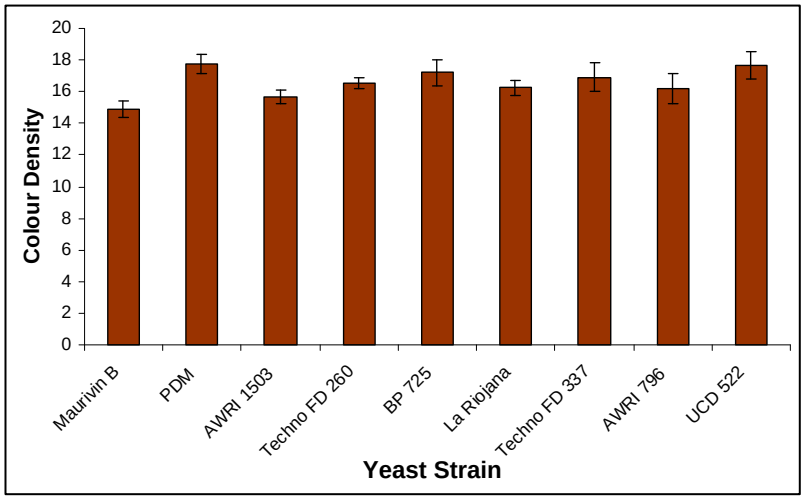


Figure 8. Colour density determinations of wine produced in 2007 by small-scale vinification using different commercial strains. Values represent means of absorbance readings of triplicate fermentations. Standard error ranges are shown.

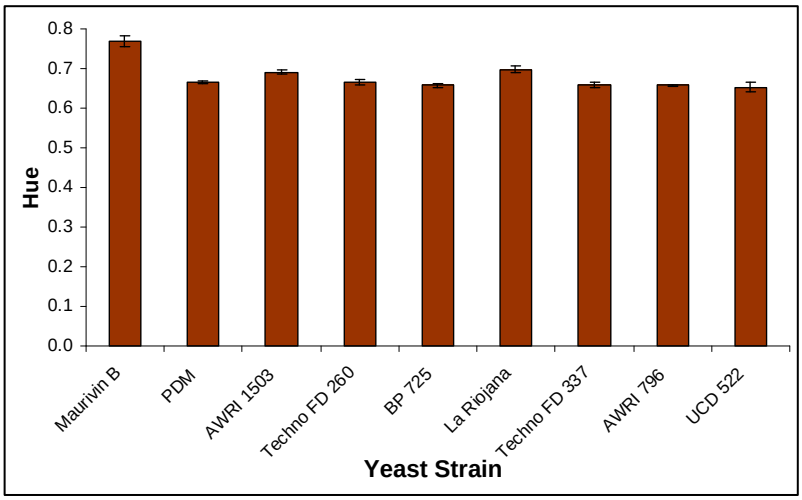


Figure 9. Hue values of 2007 vintage wines produced by small-scale vinification using different commercial strains. Values represent means of triplicate fermentations. Standard error ranges are shown.

Only a small proportion of pigments were resistant to SO_2 bleaching (3.3 to 4.3%, Fig. 10), making the colour of these wines very susceptible to pH changes and/or oxidation. The low concentration of these pigments was most likely a property of the must rather than an influence of the yeast strain, as 2006 results are about 4 to 5 times higher (data not shown) and more representative of concentrations found in red wines.

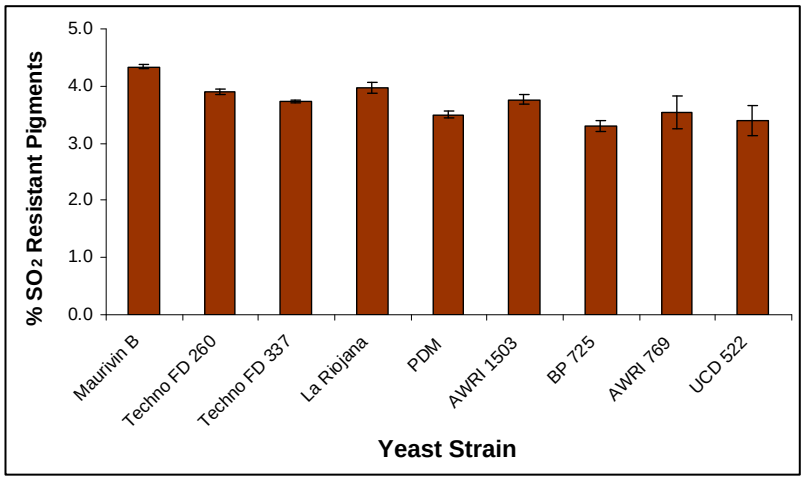


Figure 10. SO_2 resistant pigments present in 2007 vintage wines produced by small-scale vinification using different commercial strains. Values represent means of triplicate fermentations. Standard error ranges are shown.

Total phenolics (Fig. 11) ranged from 42 (Techno FD 260) to 64.4 (Maurivin B) with large variances between replicates but yeast strain effects were not significant (ANOVA, p = 0.05). Nor were there any significant differences in total anthocyanins (Fig. 12), which were present in concentrations comparable to the 2006 vintage.

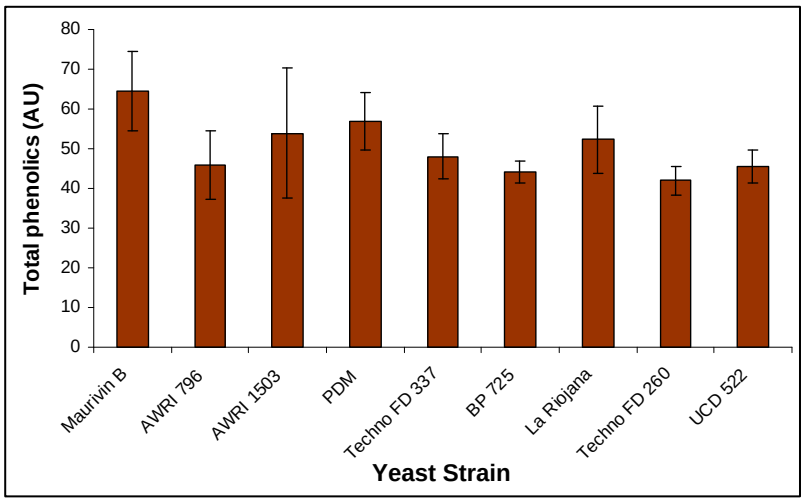


Figure 11. Total phenolics present in 2007 vintage wines produced by small-scale vinification using different commercial strains. Values represent means of triplicate fermentations. Standard error ranges are shown.

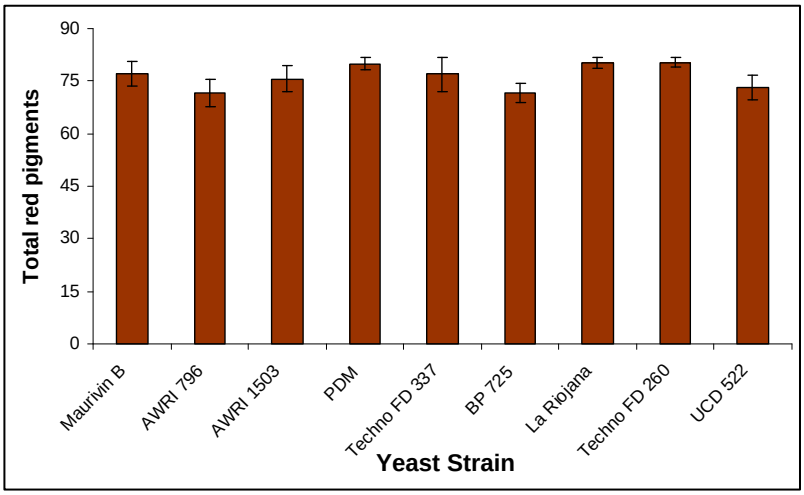


Figure 12. Total red pigments of 2007 vintage wines produced by small-scale vinification using different commercial strains. Values represent means of triplicate fermentations. Standard error ranges are shown.

Of the 22 grape anthocyanins analysed for by HPLC, 15 could be confidently identified (Table 6). The additional compound identified in these wines compared to the 2006 wines belonged mainly to the –caffeoylglucoside and –coumarylglucoside groups. Although more anthocyanins were identified, total anthocyanin levels were lower than those detected in the previous vintage wines, once again reflecting a difference in must properties rather than the effect of wine yeast strain. However, significant differences were found in the concentration of total anthocyanins suggesting, as previously, that yeast strain does have an affect on anthocyanin content of wines. For the 2007 wines La Riojana, Techno FD 260 and PDM had the greatest effect on total anthocyanin concentration.

2.4. Discussion

Using small-scale vinification methodologies, 13 winemaking strains of *Saccharomyces cerevisiae* strains were screened for their effect on the colour parameters of young Shiraz wines in 2006, and 9 strains in 2007. The existence of differences was clearly apparent from visual inspection of the wines (data not shown) and confirmed in subsequent analyses. Colour intensities were comparable to those seen for commercial wines (Iland *et al.*, 2000; Louw *et al.*, 2006). A number of the strains demonstrated colour effects in alignment with expectations based on anecdotal industrial reports. Thus strains PDM, UCD 522 and AWRI R2 have been reported as poor performers in terms of colour extraction and/or retention, whilst strains BP 725, Maurivin B and AWRI 796 have been reported to result in deeper coloured wines (pers. comm., A. Heinrich, Mauri Yeast Australia, 2006). The present findings corroborate the suggestion that PDM, and UCD 522 contribute poorly to colour density, as seen in the 2006 wine but proved to be of the highest contributors for the 2007 wines, and that contrary to expectations, UCD 522 and AWRI R2 both result in wines of greater colour. Of the three strains designated as contributing to high colour, only AWRI 796 conformed.

Wines were also assayed for total phenolic content (Fig. 4 and 11). Marked and statistically significant strain differences were observed for 2006 wines but not the 2007 wines. Total red pigments (Fig. 5 and 12) the 2006 wines were only significantly different after analysis of fresh wines. After 17 months of frozen storage these differences were no longer evident. Nor were there any significant contribution to total red pigments of the 2007 wines by yeast strain. This was further confirmed by analysis of anthocyanins by HPLC, where high levels of total anthocyanins correlated well with spectrophotometric red pigment determinations. However, high total anthocyanin concentrations in the wines (as determined by HPLC) did not correlate to higher colour density values (eg. 2006 vintage, UCD 522 or 2007 vintage, La Riojana). A much more tenable link was evident to hue values, with wines having higher total anthocyanin concentrations also having higher hue values. The basis for such differences is yet to be defined and includes several likely explanations: the release of macromolecules and metabolites by the yeast cells (Escot *et al.*, 2001; Eglington *et al.*, 2004; Romero & Bakker 2000a; Romero & Bakker 2000b Drinkine *et al.*, 2005; Fulcrand *et al.*, 1998), or else their direct interaction with or internalisation of pigmented compounds.

In addition, the temperature and time on skins are also likely to have an impact. Even so it must be stressed that no attempt was made to detect or eliminate temperature differences between fermentations, which could arise through difference in fermentation vigour. Similarly, wines were racked at the arbitrarily chosen time of 56 h rather than at a particular stage of attenuation and thus ethanol content. In both cases it was reasoned that any differences in fermentation kinetics that could lead to differences in these parameters, were inherent features of the strains and might be the basis for the observed influence on colour.

Table 6. Identification and quantification by HPLC of grape anthocyanins in 2007 Shiraz wines fermented by various wine yeast strains. Values are averages of triplicate fermentations along with their standard errors.

	Maurivin B		FD 260		FD 337		La Riojana		PDM		AWRI 1503		BP 725		AWRI 796		VCD 522	
Del-3-G	10.2	0.50	13.7	0.43	15.3	1.08	15.7	0.31	15.1	0.34	13.6	0.73	13.6	0.98	14.4	0.33	14.8	0.97
Pet-3-G	23.1	0.88	27.5	1.02	28.1	1.54	29.4	0.65	29.6	1.01	26.7	0.55	24.8	1.17	29.1	1.56	25.6	1.77
Peo-3-G	17.6	1.28	27.8	1.79	33.2	1.55	34.3	0.68	32.4	0.35	31.5	1.18	25.9	1.69	31.1	0.37	28.8	3.93
Mal-3-G	202.8	5.53	213.5	1.27	206.4	5.37	214.3	2.31	208.1	2.35	202.4	5.56	185.7	2.28	197.1	3.41	186.2	9.82
Vitisin-B	4.9	0.14	5.5	0.12	6.3	0.19	5.0	0.10	6.0	0.19	5.1	0.13	4.2	0.66	5.5	0.14	5.5	0.12
Del-A-G	2.2	0.21	2.8	0.24	2.6	0.28	2.9	0.13	2.8	0.08	2.3	0.24	1.3	0.65	2.4	0.07	2.5	0.25
Pet-A-G	8.6	0.36	9.6	0.31	8.8	0.76	9.7	0.14	9.8	0.14	8.7	0.66	8.6	0.58	9.4	0.24	7.4	0.74
Peo-A-G	11.9	0.36	14.5	0.38	13.0	0.58	13.6	0.30	13.9	0.23	12.9	0.53	10.6	0.40	13.6	0.14	11.5	1.23
Mal-A-G	64.7	2.16	73.3	1.06	66.9	2.23	68.7	0.96	69.8	1.48	66.8	2.67	60.5	0.46	66.3	1.53	60.2	3.75
Del-Caff-G	1.5	0.22	2.3	0.27	2.1	0.07	2.1	0.17	2.3	0.16	1.9	0.32	1.6	0.06	1.8	0.11	1.4	0.74
Peo-Caff-G	1.2	0.03	1.8	0.04	1.6	0.04	1.6	0.07	1.6	0.07	1.5	0.08	1.4	0.06	1.6	0.05	1.5	0.10
Mal-Caff-G	7.6	0.25	9.6	0.14	8.5	0.21	8.7	0.23	8.5	0.36	8.2	0.45	7.4	0.13	8.2	0.34	7.7	0.53
Del-C-G	2.6	0.17	2.7	0.28	2.5	0.27	2.8	0.11	2.6	0.15	2.4	0.33	2.1	0.08	2.5	0.06	2.0	0.29
Peo-C-G	5.3	0.35	5.5	0.48	4.9	0.56	5.4	0.08	5.2	0.25	4.6	0.50	3.9	0.29	4.9	0.30	4.1	0.47
Mal-C-G	19.1	1.35	19.7	1.58	17.6	1.82	19.5	0.10	18.4	1.02	17.1	1.92	14.1	0.98	16.9	1.00	14.5	1.57
Total -3-G	253.7	8.2	282.6	4.5	283.1	9.5	293.7	4.0	285.2	4.1	274.2	8.0	250.0	6.1	271.7	5.7	255.4	16.5
Total -A-G	87.4	3.1	100.2	2.0	91.3	3.9	94.9	1.5	96.3	1.9	90.6	4.1	81.0	2.1	91.7	2.0	81.6	6.0
Total -Caff-G	10.3	0.5	13.7	0.5	12.2	0.3	12.3	0.5	12.5	0.6	11.7	0.8	10.4	0.3	11.6	0.5	10.6	1.4
Total -C-G	27.0	1.9	27.9	2.3	25.0	2.6	27.7	0.3	26.1	1.4	24.2	2.8	20.1	1.3	24.2	1.4	20.6	2.3
Total Anth	383.3	13.8	429.8	9.4	417.8	16.5	433.7	6.3	426.0	8.2	405.7	15.9	365.9	10.5	404.6	9.6	373.7	26.3

A sense of the overall contribution to wine colour by the yeast strains examined was sought through an average relative ranking based on measurements from the 2006 vintage (Fig. 7). In this way a distinct pattern emerges for a number of the strains. For instance, UCD 522 produced a wine that consistently displayed high values for all the colour parameters measured. In contrast, the wine produced by strain PDM was in the lower quarter of these measurements. Still other strains, such as AWRI 350 were seen to vary widely in their relative ranking. From the results seen for the 2007 vintage it is already apparent that this relative ranking does not hold true for all strains in all winemaking conditions. Further work is required.

Given the apparent complexity of the yeast:grape interaction which leads to differences in various aspects of wine colour, further trials of the sort described above were postponed in favour of attempts to dissect the basis of this interaction. A simpler model system was therefore sought.

Apart from the seasonal restrictions of using fresh grape must to investigate yeast and red wine colour interactions, such fermentations can also be time-consuming. Medina *et al.* (2005) reported a semi-defined red grape juice medium to investigate one aspect of the influence of yeast on colour, that is the removal of anthocyanins. Whilst these workers provide no data linking the applicability of this approach to actual fermentations of red grapes, their use of anthocyanin extracts at least allowed work to be performed outside of vintage. Our attempt to simplify such protocols further led to the use of a commercially available grape extract and a reduced incubation time in a multi-well plate format conducive to high-throughput screening. It is worth noting that in contrast to the report of Medina and co-workers (2005), colour intensity values derived from our micro-fermentation system were greater for the inoculated media (Fig. 1).

When comparing the colour intensity values obtained for each strain through the micro-fermentations and from the small-scale fermentation system, no strong correlation was seen. Again the marked differences in the conditions of each fermentation system used in this study make this fact unsurprising. Even so, the list of strains producing the extreme colour values in one fermentation system largely matched that for the other system. This fact offered some endorsement (particularly within the confines of this limited-term project) for the use of micro-fermentations for large scale screening exercises such as those examining libraries of natural isolates from vineyards or wineries. The identification of high or low colour producing strains rather than an accurate determination of absolute values for various colour measures is precisely what such a rapid screening system would be used for in such a situation.

Thus the work undertaken towards Output 2 has modestly expanded on the knowledge around the influence of yeast strain on the colour of young red wines. The results have also reaffirmed the fact 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 and cap management. A greater understanding of the abilities of individual strains to modulate the colour of red wine (and ability to affect other properties) will provide winemakers with a tool which aids in the production of wines to a desired style. The identification of further strain-based tools will benefit from access to a rapid, reproducible and inexpensive model to test large numbers of strains for their colouring-affecting

capacities. The micro-fermentation system evaluated here was selected for use to this end.

Output 3: Completion of the PhD Structured Component - Attendance at formal sessions for commencing PhD students, literature review and research proposal submitted and one seminar presentation made.

Status summary: The performance targets for this Output were achieved.

3.1. PhD structured component.

The PhD student had successfully completed thes Structured Component, however, as stated elsewhere, he then chose to withdraw to take up full-time work. Objectives planned for the remainder of the project were then revised as detailed in variation requests dated 15 June 2007 and 17 July 2009.

Output 4: Information on stable pigment formation - Use models of pigment evolution to further define mechanisms involved.

Status summary: With loss of the PhD student and the need to utilise research associates on short-term contracts or else researchers seconded from other projects, the expertise for this Output was not available. Consequently the output was postponed and ultimately not completed as other aspects of the project became too time-intensive.

4.1. Information on stable pigment formation.

Given the delays in the project to date and the quantum of funds remaining it was clear that extensive work on the development of models of pigment evolution was likely to be beyond the scope of the project. As such this work was held in reserve while the more readily achievable Outputs (based on the expertise and facilities that were available) were pursued.

Outputs 5 and 6: Yeast for increased anthocyanin release - Construction/isolation and evaluation of a strain designed to influence release of anthocyanins from grapes.

Strains with and without degradative enzymes - Over- & non-producers of anthocyanin-degrading enzymes (glycosidases, anthocyanases, oxidase, etc) evaluated.

Status summary: The performance targets for these Outputs were partly achieved. Recombinant strains (or constructs for these) which could produce significant extracellular enzyme activity could not be sourced from other research groups at all or in a timely manner. Some attempt was made to regenerate these within the project, an activity for which time had not originally been allocated. As such few strains and opportunities to examine these remained. Putative BLG2 (β-glucosidase) clones were produced, but

their identity was not supported by sequencing. EXG2 (β-1,3-glucanase) was successfully cloned, however, extracellular activity was minimal and no significant effect on colour of anthocyanin supplemented defined media was seen.

*5&6.1. PCR amplification of genes encoding hydrolytic enzymes from non-***Saccharomyces** *yeast*

Yeasts possessing hydrolytic enzymes with the potential to influence either the breakdown of grape cell walls, and hence pigment release into the fermentation, or else the stability of the anthocyanins themselves were sought. These were to be used as extreme examples which would demonstrate the possible impact of yeasts with analogous activities on red wine colour. Several examples of cloned β-glucosidase genes already existed (Fig. 13). Given that resources would not allow a search for entirely novel activities, the intention was to source recombinant strains and or constructs from other research groups based on examples of such genes. In general, however, such requests were not fulfilled. Rather than abandon this work entirely, a limited effort was made to regenerate equivalent strains within the group. Thus the non-*Saccharomyces* yeast *Pichia anomala*, *Dekkera* species and *Hanseniaspora osmophila* were chosen as sources of β-glucosidase genes, as the DNA sequences were readily available on the EMBL database (www.http://www.ebi.ac.uk/) and there were no previous reports on these particular proteins with regard to vinification. Complete gene sequences are available for *EXG1* (EMBL:AJ002195) and EXG2 (EMBL:AJ222862) encoding for two related exo-1,3-beta-glucanases from *P. anomala*. Primers Pirjo and Pekka (Table 2) were designed to allow the cloning of the *EXG2* coding sequence behind the constitutive phosphoglycerate kinase (PGK) promoter in the multi-copy plasmid pJC1 (Martin et al. 2003) to give the recombinant plasmid pJC1PAEXG2. Cloning of the *BGL2* gene (encoding glucan-1,3-beta-glucanase) from *P. anomala* required primers to be designed to the sequence from *P. stipitis* (EMBLCDS:ABN65244) as there was no sequence information for the *BGL2* gene from *P. anomala*. PCR attempts with primers Peter and Pandora (Table 2) using Pfu Ultra II DNA polymerase under various conditions resulted in the amplification of three PCR products, which when sequenced did not show sequence homology to *P. stipitus BGL2* (data not shown). These findings may reflect the sequence variation of *BGL2* genes between different *Pichia* species, in particular at the N- and C- termini of the proteins.

The construction of new (degenerate) primers designed to homologous regions of the protein would allow amplification of the gene sequences using inverse PCR (Martin and Mohn, 1999). There were no sequences available for β-glucosidase genes from the yeast *H. osmophila*, however a partial sequence encoding 59 amino acids of β-glucosidase from *H. uvarum* (EMBLCDS:AAY59870) was used for the design of primers Harry, Henry and Harriet (Table 2) for use in inverse PCR and/or 3'RACE PCR. Partial sequences with homology to *EXG1* and *BGL2* were identified from the genomic library sequences of *Dekkera bruxellensis* using BLAST software (http://www.ebi.ac.uk/Tools/blast). Although the sequences were incomplete, over 1000 bases of DNA sequence was available for primers to be designed with 100% homology to appropriate regions near the 3' and 5' ends of the sequence(s). Both genes have around 70% homology to *Pichia*, or *Candida BGL* genes at the DNA level, which is greater than average 54% homology (nucleotide) observed for most *Dekkera* genes (Woolfit et al., 2007). Primers designed with homology to the *D. bruxellensis* sequences were used for the amplification of the *BGL2* and *EXG2* genes as PCR fragments, which were cloned and sequenced to confirm identity. Clone

DEK16 was identified as *BGL2*, the *D. anomala* DNA sharing 94% identity with the *D. bruxellensis* genomic library clone bb50f05.r (inferred *BGL2* gene alignment). Clones DEK11, DEK33, DEK34 and DEK36 shared ~97% homology to the inferred *D. bruxellensis EXG2* alignment in genomic library clones bb50f05.r, bb20a08.r and bb58h02.r. Attempts at RACE PCR resulted in the amplification of multiple bands using gene specific primers (Table 2) and oligo-dT, which had no sequence similarities to *Dekkera EXG2* or *BGL2*.

5&6.2. Heterologous expression of PAEXG2 in a haploid wine yeast

The isogenic haploid wine strain isoC9-DΔura3 was transformed with the plasmid pJC1PAEXG2, encoding *P. anomala* exo-1,3-beta-glucanase, *EXG2*. Six transformants were tested for β-glucosidase activity after 24h growth in minimal drop out media containing 2% glucose. Only minimal activity (0.06 - 0.08 μM PNP min^{-1} for 1 $x10^7$ cells) as detected by hydrolysis of *p*-nitrophenol (PNP; yellow colour) from the colourless substrate *p*-nitrophenyl-β-D-glucopyranoside. Quadruplicate fermentations of 100 ml of enriched CDGJM (see Methods) were performed in the Multi-scale Fermentation Facility. The yeast culture was initially grown in minimal media lacking uracil, instead of YEPD, for plasmid maintenance in the cells. Plasmid stability during fermentation was encouraged as CDGJM lacked uracil (data not shown). The fermentation profiles of yeast strains transformed with the control plasmid pJC1 or the recombinant plasmid pJCPAEXG2 were comparable (Figure 14A). Colour of the fermented media was defined in terms of CIElab values (Fig. 14B). Supernatants from all inoculated flasks were of lower L and b* values and higher a* values than the control, but the inoculated flasks did not differ significantly from each other (ANOVA, $p = 0.05$). Similar findings were made in repeated experiments (data not shown).

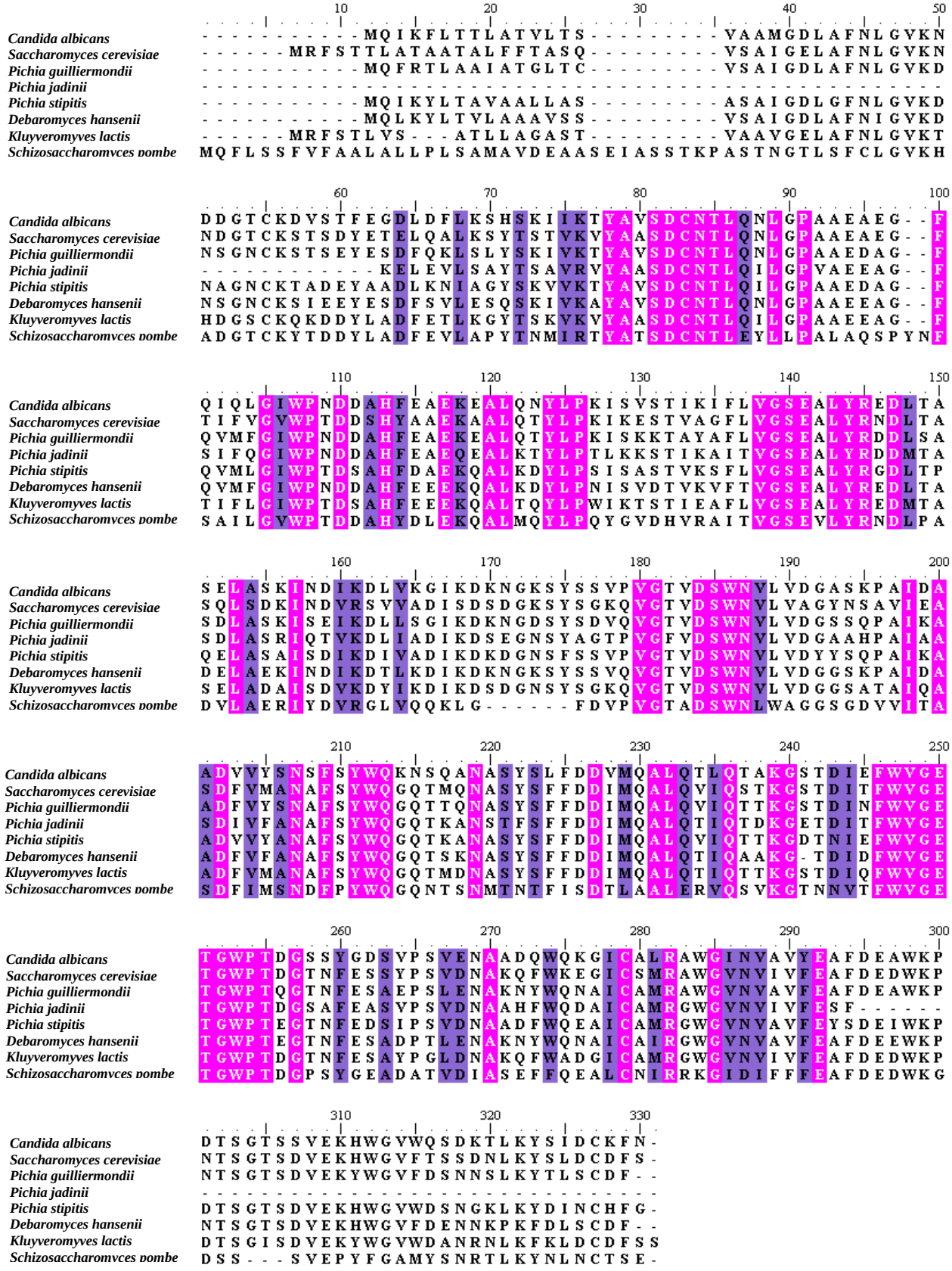


Figure 13. Sequence alignment of *BGL2* proteins from several yeasts 100% homology is shown in pink and similar amino acids in purple

Although the plasmid construct was confirmed by DNA sequencing, and the codon usage of the *PAEXG2* gene was such that translation should not have been impaired in *S. cerevisiae, PAEXG2* RNA transcription as well as protein expression need to be verified by Northern blot analysis using a *PAEXG2* specific probe, and by SDS PAGE respectively. Western blotting is not feasible as there are no antibodies to the *P. anomala* exo-1,3-beta-glucanase. No other constructs/strains resulting in reliably detectable and significant activity were able to be generated in time to be considered in this study.

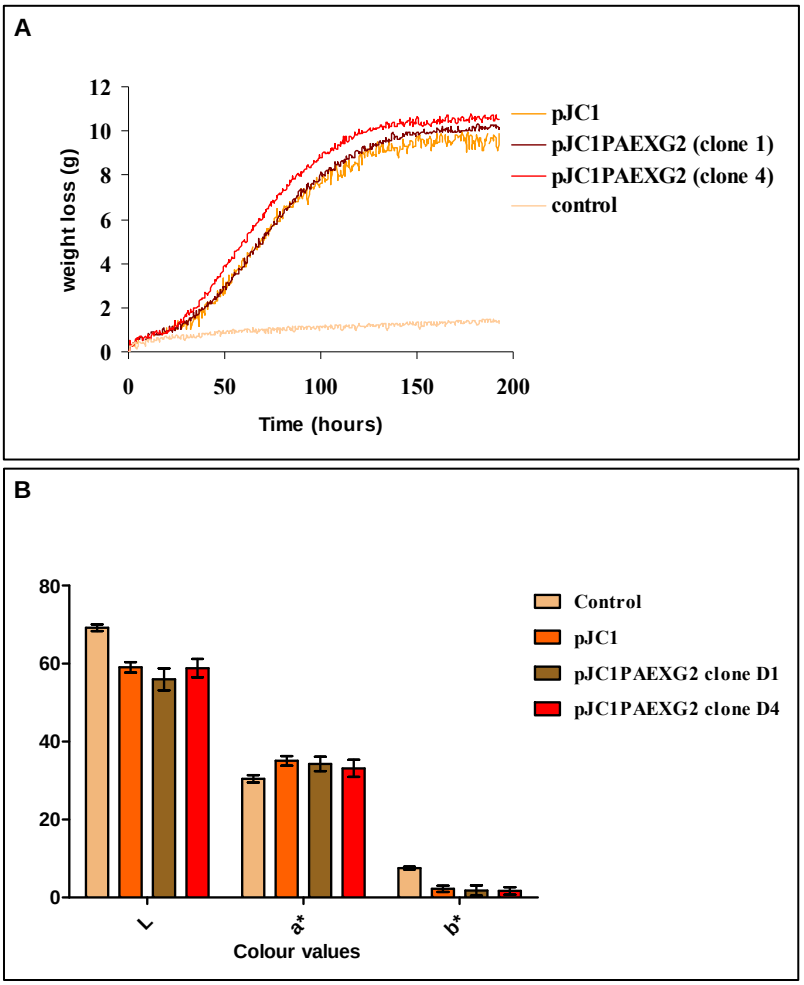


Figure 14. Fermentation of enriched CDGJM by a haploid wine yeast strain transformed with the *P. anomala EXG2* gene. Panel A: Fermentation profile of isoC9-D Δura3 transformed with control plasmid (pJC1), two clones of isoC9-D Δura3 transformed with recombinant plasmid pJC1PAEXG2 (clones 1 and 4), and an uninoculated flask (control). B: CIElab measurement of colour of fermented samples (after 192 h). L refers to lightness, a* represents red-green colour, and b* represents blue-yellow colour. Error bars represents standard deviation of the mean 24 values

5&6.3. Discussion

This phase of the project required access to recombinant strains or else the constructs used to generate such strains possessing significant extracellular degradative enzymes. The intention being to define the extent to which such strains/enzymes might increase the release of pigments from grape material or else degrade anthocyanins present in wines. This objective arose out of previous reports (e.g. Medina et al., 2005) or our own work (Final Report on GWRDC project UA 01/04) demonstrating the ability of enzymes such as β-glucosidases to degrade anthocyanins.

Sourcing the required strains or constructs so as to avoid the need to reproduce these in the group was largely unsuccessful. However, rather than abandon this phase of work entirely and given we had excellent molecular biology expertise in the group, we undertook a limited attempt to generate some of the required strains. The original

project plan did not allow time for anything more extensive. To increase the likelihood that cloned enzymes would be functional in a wine yeast background, enzymes originating from yeast rather than e.g. those sourced by our own group from lactic acid bacteria (see Project UA05/01) were used. Unfortunately, despite cloning of a number of putative *BGL2* and *EXG2* sequences, only the latter warranted transformation into yeast followed by limited fermentation trails. That these failed to alter the colour of the supplemented CDGJM during fermentation is likely due to the low levels of enzymatic activity detected in the recombinant strains. Had time allowed, further characterisation would have included confirmation that the foreign gene was being expressed sufficiently and that the product was functional. Further work is clearly required to define the importance of analogous enzymes in winemaking. Similarly, the likelihood that such enzymes might also arise from the other microflora of the wine cannot be overlooked and is part of an ongoing effort (UA 05/01).

Output 7: Methods to identify yeast genes that impact on pigment adsorption to biomass - Yeast libraries screening initiated to identify gene(s) that modulate pigment absorption to yeast biomass.

Status summary: The performance targets for this Output were achieved.

7.1. Colour pigment adsorption by the yeast biomass

Of the proposed mechanisms by which yeast might affect the colour of red wines, absorption to biomass appeared to be one whose investigation might be straightforward. That yeast bound pigments was apparent from examination of biomass from fermentations of anthocyanin supplemented media or even simple experiments where the biomass was incubated in wine or anthocyanin preparations (Fig. 15). To explore the basis for and variability in such pigment binding, we sought to simplify the approach used in Sections 2.2 and 2.3. Primarily and in the first instance we aimed to eliminate differences between strains arising out of fermentation differences, thus biomass was added to finished wines and incubated for several days before colour analysis was undertaken.



Figure 15. Visual examination of the absorption of pigments by yeast. A strain of *Saccharomyces cerevisiae* was incubated in unsupplemented CDGJM (A) or else media to which anthocyanins where added (B, C) and the washed biomass pelleted in a flat-bottom, multi-well plate for photography.

Five commercial yeast strains were used to quantify the removal of pigments (anthocyanins and pigmented polymers) through binding to yeast lees. The yeast were incubated with wine for over 120 h, with samples being removed at 24 h, 48 h and 120 h (see Method). Two wines were tested; University of Adelaide 2006 Adelaide

Hills Shiraz (a medium-bodied red wine) and Taylors 2008 Adelaide Hills Pinot Noir (a lighter red wine). Colour determinations were performed on the wine samples as described. Pigmentation of the biomass occurred after a few minutes incubation with the yeast cells. The stability of the two wines in the absence of yeast during the course of experiment differed. The Mal-3-G levels decreased by 7% in the Shiraz wine during the 120 h incubation, whilst there was a 21% decrease in Mal-3-G levels in the Pinot Noir for the same time period (data not shown). Figure 16 shows the percentage loss of Mal-3-G, presumably due to pigment adsorption onto the yeast biomass with extended time. A positive relationship between decreasing levels of Mal-3-G over time is observed for both Shiraz and Pinot wine, with up to ~21% colour loss after 120 h incubation. At 24 h, colour loss was greater in the Shiraz wine (ranging from 12.7% for EC1118 to 16.8% for QA23) compared to the Pinot Noir wine (ranging from 4.9% for QA23 to 11.3% for EC1118).

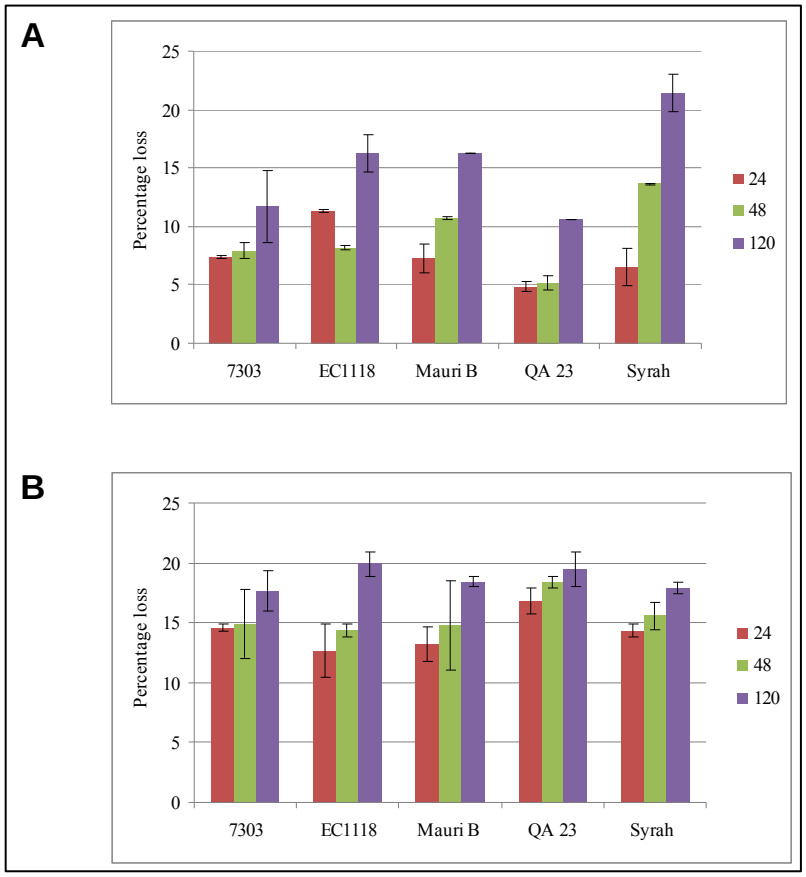


Figure 16. The calculated percentage loss of malvidin-3-glucoside in wine samples, following extended incubation with yeast cell biomass. A. Pinot Noir wine. B. Shiraz Wine. Percentage loss was calculated using Mal-3-G values of the wine sample (without yeast) and the wine sample (with yeast) for a given incubation time. Error bars represent the standard deviation of the mean of three replicates per sample.

The trend observed with Mal-3-G was not replicated for other measured anthocyanins: Peo-3-G, Pet-3-G, Mal-C-G, and Peo-C-G (data not shown). These results may account for the lack of a statistically significant decrease in total red pigments (Fig. 17) as measured according to Iland et al. (2006). Even so a trend toward a reduction in TRP was consistently seen across all strains and both wines.

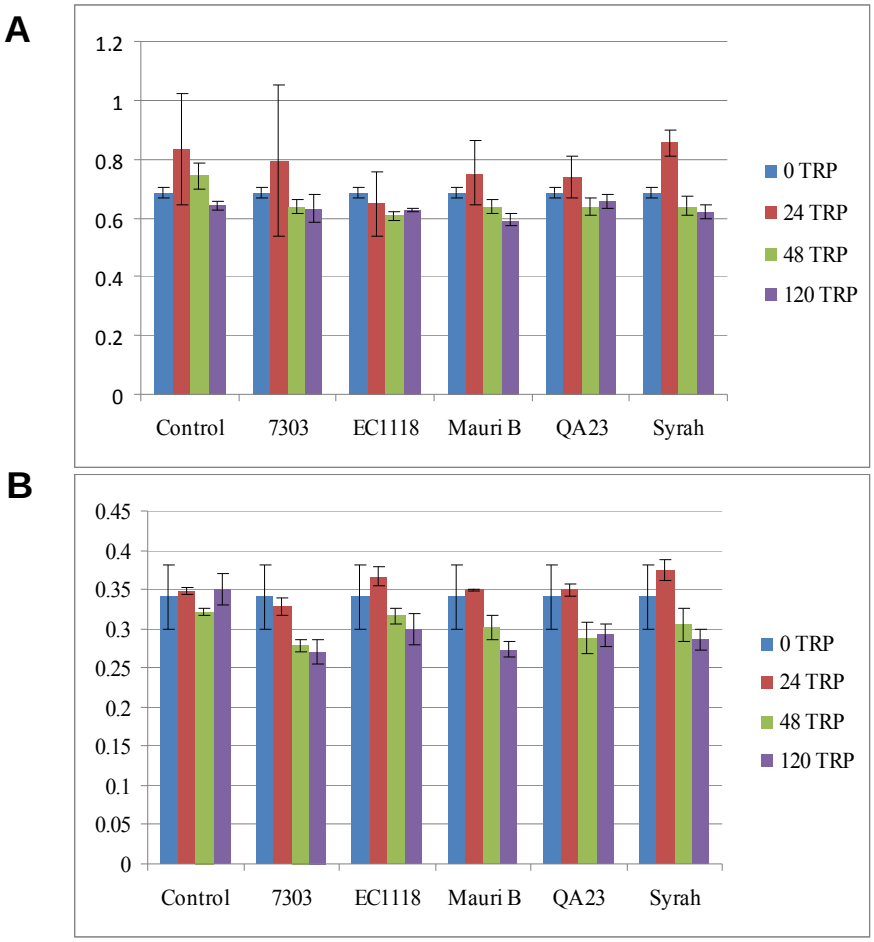


Figure 17. Effect on total red pigments (TRP) in the wine following pigment adsorption on yeast biomass. A. Pinot Noir wine. B. Shiraz wine. Error bars represent standard deviation of three replicate measurements.

To gauge the importance of the absorption of pigments by yeast biomass, the colour of the wine was also measured using CIElab (Fig. 18). Although the L value (or degree of lightness) was statistically different to the control (by ANOVA), in real terms the colour difference observed from the control was only minor, even when the biomass became visibly darker. The implication of these findings is that absorption of pigments to yeast biomass, while significant and apparently more specific in terms of Mal-3-glu, may only result in a detectable (visual) change in red wine colour in some cases. Further wines need to be considered. There does however remain a strain dependent difference in terms of the extent of the interaction of yeast biomass with red wine colour, with EC1118 achieving high colouration of its biomass in each wine examined here (Fig 18B and D).

7.2. Screening of yeast homozygous diploid deletion library to identify genes which have a potential influence on red wine colour

Yeast have different effects on the red colour of model wine (from enriched CDGJM) and experimental wine (small scale berry vinification) as detailed in sections 2.2 to 2.3. The findings using the model wine were supported by a survey of 84 commercial strains using CIElab measurements of wine colour after 7 days fermentation (see Appendix 4.1). The complexity of this interaction between yeast and wine during vinification includes i) enzyme degradation, ii) colour extraction, iii) colour stabilization, and iv) pigment adsorption by the yeast biomass (or lees). An approach utilising a yeast gene deletion

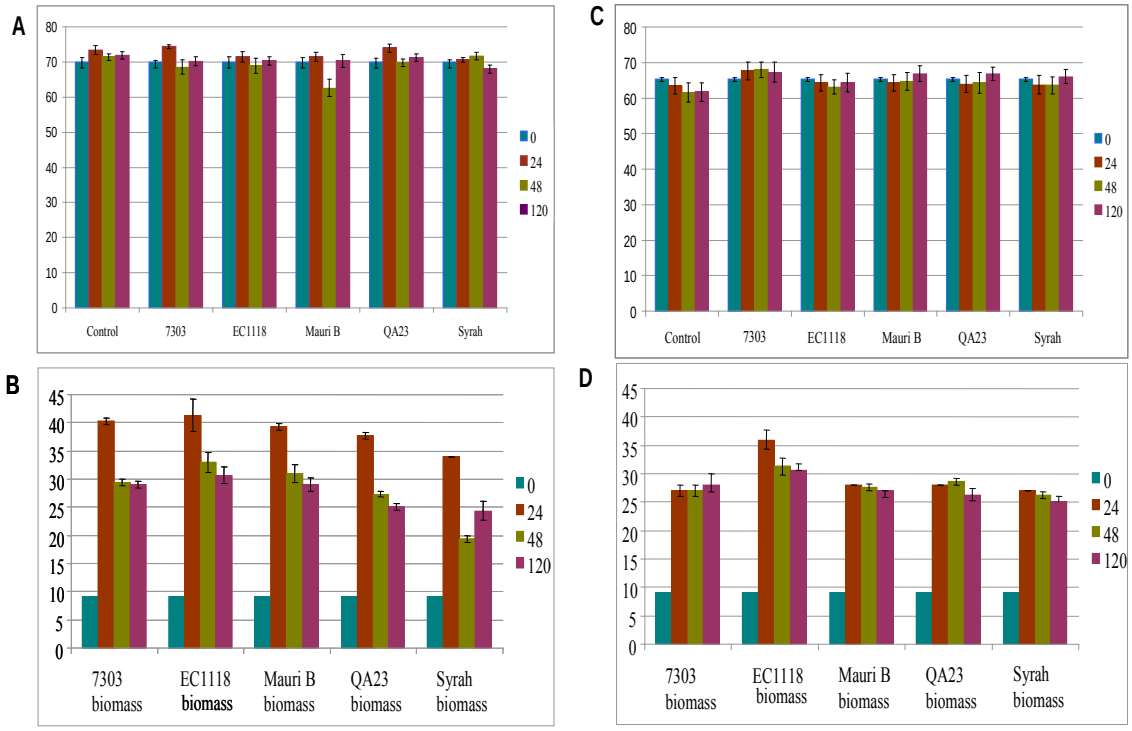


Figure 18. Red colour of Pinot Noir (A, B) and Shiraz (C, D) wines (A, C) and recovered yeast biomass (B, D) following pigment adsorption to added yeast biomass over incubation times of up to 120 h. Controls (blanks) were yeast-free aliquots incubated under the same conditions. Colour was expressed in CIElab units, but only L values are shown. In terms of the biomass, L values were determined using Adobe Photoshop software on photographic images of yeast biomass which had been washed and transferred to flat-bottom multi-well plates (as depicted in Fig 15). Error bars represent standard deviation of three replicate measurements.

library was therefore used to try to dissect this complex mechanism(s) by highlighting which genes (and therefore products or processes) might be involved. Of particular interest were those deletions which could lead to improved wine quality (e.g. through stabilisation of anthocyanins), and conversely, those resulting in decreased wine colour which may be useful in e.g. the production of rosé or blanc de noir wines.

Wine yeasts are typically diploid and prototrophic and no wine yeast deletion library is currently available. Consequently, a homozygous diploid deletion library in a laboratory yeast background was chosen. Details are found on the *Saccharomyces* Genome Deletion Project consortium's website (http://www _sequence.stanford.edu/group/ yeast_deletion_project/deletions3.html). The library contains 5271 clones which include all non-essential genes, but excludes the essential genes (1155 genes). It should be noted that 80% of the yeast ORFs are non-essential, and that there is large redundancy in the yeast genome (10%). To date, the consortium has disrupted 90% of the yeast genome. By ORF class, this represents 92% verified, 88% uncharacterized and 65% dubious ORFs which have been disrupted.

The library screen was conducted under fermentation conditions in which the lab strain BY4743 would complete in a 180 h timeframe. Specifically the medium contained 200 g L^{-1} total of equimolar glucose and fructose, and 400 mg L^{-1} FAN, with incubation occurring at 30°C. A total of 3 g L^{-1} polyphenol extract was included

in the enriched CDGJM for the experiment. This amount resulted in comparable CIElab colour measurements to those found in several Australian Shiraz wines (data not shown). The commercial polyphenol extract used is prepared from fresh grape skins which are macerated in water in the presence of sulfur dioxide (pers. comm., Amé de Saint Charles, OenoProd, see Appendix 4.2). The fermentations were performed in duplicate and were analysed for colour using CIElab values determined from the absorbance of anthocyanin compounds in the wine over the range 380 – 780 nm (described in Methods). The data was manually collated using Excel 2007 and GraphPad Prism 5 software for the following colour determinants: ΔE (the 3D spatial difference between the host and deletant strains for L, a* and b* values), ΔC (difference in C* between the host and deletant strains), and ΔH (difference in H° between the host and deletant strains). Residual sugar was determined for each clone to identify those clones which did not complete fermentation (<2.5 g L^{-1}).

The library was stored in 384 welled plates. For each such plate, duplicate 4 x 96 well plates were prepared, fermentations conducted, the colour data averaged and the statistical variation (± SD) was determined. Those clones which did not complete fermentation were classified into five groups depending on the amount of residual sugar at the end of 7 days: 2.5 - 20g L^{-1} (260 clones), 20 - 50 g L^{-1} (34 clones), 50 - 100g L^{-1} (21 clones), 100 - 180 g L^{-1} (16 clones), and >180 g L^{-1} (5 clones). The distribution of the clones with regard to the specified colour determinants (ΔE, ΔC, and ΔH) was plotted and those clones which were outside the confidence interval (CI) equal to 95% and 5% were selected. For those clones identified using ΔC and ΔH, there was either a positive or negative effect on colour. The positive effect was determined as an increase in chroma (C* or degree of colour saturation) and/or increase in hue (resulting in a colour change towards an orange-brown colouration). A negative effect on colour was either a calculated decrease in chroma and/or decreased hue (resulting in a colour change towards a pink-purple colouration). In the case of those clones identified to have ΔE values which represent 10% of the population (ΔE greater than 3.25), the L a* b* C* H° values were graphed to enable the clones to be put into 6 groups based on whether the colour values either increased or decreased (L a* b* C* H° values) and/or whether a*, b* and H° were unaffected. These 6 groups were further consolidated into three groups based on L, a* and b* values, thus increasing the number of clones in a particular group. Finally, the clones identified to have an effect the colour of the model wine in the library screen were compared to those identified not to complete fermentation, and the latter omitted. Interestingly, 31% of the (99 out of 319) clones which did not complete fermentation were shown to have an effect on colour.

Manual collation of the data resulted in the following colour groups: (i) ΔC negative value (181 clones); (ii) ΔC positive value (213 clones); (iii) ΔH negative value (194 clones); (iv) ΔH positive value (184 clones); (v) ΔE increased L value (125 clones) and (vi) ΔE decreased L value (53 clones). At the time of writing, the data analysis was near complete, but lacked a comparison to the biomass colouration of the identified clones to the host strain BY4743. The library screen predominantly resulted in clones which produced lighter coloured model wine than that produced by the host strain BY4743. Gene ontology (GO) software GO FINDER was used to look for numerical enrichment of genes (clones) based on independent vocabularies or GO terms - biological process, molecular function or cellular component. The program

used a Hypergeometric statistical algorithm and a False Rate of Discovery (FDR) function (http://genome-www.stanford.edu/Saccharomyces/) in the determination of the enrichment of genes at a p value of <0.01. In cases where there was no significant enrichment of genes, the stringency of the analysis was decreased to a cutoff p value of <0.1. The software program GO SLIMMAPPER (http://genome-www.stanford.edu/Saccharomyces/) categorized the genes (clones) into the GO terms, but did not include statistical determination. In addition, the annotation of individual genes (clones) was performed manually to determine whether there were any single genes which could have a potential affect on wine colour. This was necessary in cases where no gene enrichment was found for GO terms, at a cutoff p value of <0.1 (CI of 90%), for example in the case of the groups ΔC positive value (213 clones) and ΔH positive value (184 clones).

Gene ontology analysis of the data in which the clones (gene deletions) did not complete fermentation (319 clones or 6% of the library) demonstrated gene associations for the following GO terms as shown in Figure 20. These were deletions that either affected ion homeostasis, cellular pH, or cellular biosynthetic processes, macromolecule biosynthetic processes, autophagy and transcription. Further, only enrichment of the terms Ion Homeostasis and cellular pH was associated with the 50 clones which did not completely catabolise fructose (20 - 50 g L^{-1}). No biological process was common to the 26 clones (having <2.5 - 20 g L^{-1} residual sugar), although there was an enrichment of the following GO terms Histone Deacetylation (11.5%) and Protein Amino Acid Deacetylation (11.5%) at a cutoff of $p < 0.1$. When those clones which failed to complete fermentation were dissected into different groups based on the residual sugar content, it was of interest to note the following: for those clones with 2.5 - 50 g L^{-1} residual sugar (34 clones), the parent GO terms were ion homeostasis and cellular pH. Whilst the 21 clones with 50 - 100 g L^{-1} residual sugar had only enrichment of GO terms for primary metabolic process (85.7%) and mRNA 3'-end processing (14.3%) at a cutoff of $p < 0.05$. The 20 clones which struggled to complete fermentation (100 - 200 g L^{-1}) were enriched for cellular macromolecular complex subunit organization (30% of the listed genes at $p < 0.1$). All 21 genes encoded proteins which were associated with membrane bounded organelles.

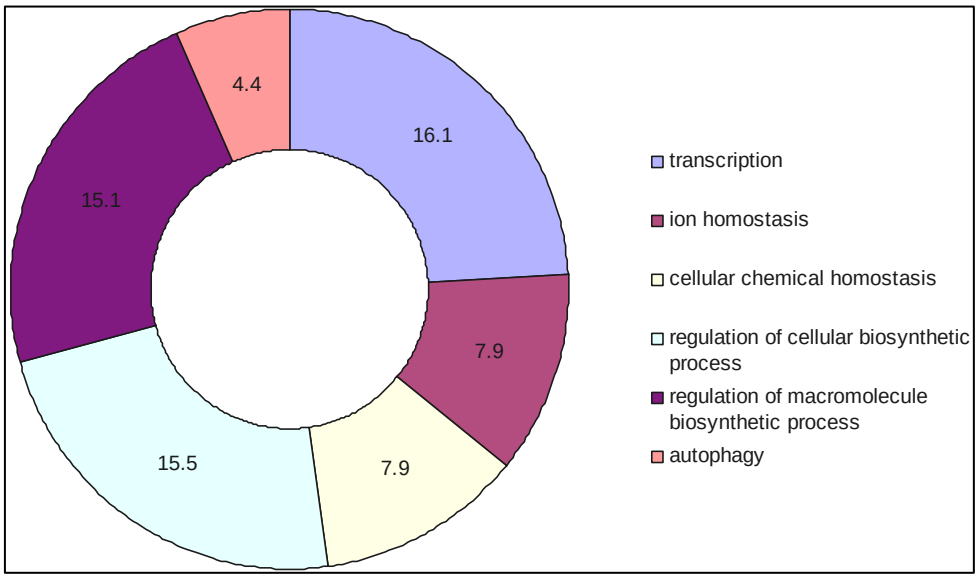


Figure 20. Gene ontology (GO) analysis of genes identified in clones (with single gene deletions) which did not complete fermentation (>2.5g -200g L^{-1}). Values represent % enrichment of genes for a particular GO term

The analysis of the colour data was not straightforward. In the case of those 125 clones which resulted in a significant increase in the CIElab L value (corresponding to a lighter wine colour), the only parent GO term association was that for ubiquitin-dependent protein catabolic process via the multivesicular body sorting pathway (4.8%), the gene products of which are associated with the endosome. However, 30% of the genes were directly annotated to the root term 'biological process unknown'. If the gene set is divided into the subtypes described above, for example the 18 clones which had a marked increase in L, decrease in a* but no change in b* or H°, there is a numerical enrichment of genes associated with glycerol transport (2 out of 18 genes ; 11%). However, there is no annotation at $p < 0.01$ for molecular function or cellular component. No association was found for biological process, molecular function or cellular component ($p < 0.1$) for the 53 clones which resulted in a decrease in the CIElab L value (corresponding to a darker wine colour). Interestingly, 18 out of 53 input genes (34%) were directly annotated to root term 'biological process unknown', 19 out of 53 (36%) input genes are directly annotated to root term 'molecular function unknown' and 13 out of 53 input (25%) genes are directly annotated to root term 'cellular component unknown'.

GO analysis of the subset of genes identified from the ΔC (+ve value) calculations revealed no annotation to the three independent root terms ($p < 0.1$). Again there was a large proportion of the clones of unknown biological process (31%), unknown molecular function (~40%), and unknown cellular component (24%). Similar results were found with the subset of genes identified from the ΔH (+ve value) calculations, with 29% of unknown biological process and 21% of unknown cellular component. The exception was three genes (*YPS5*/YGL259W, *BAR1*/YIL015W, *YPS6*/YIR039C) which encoded proteins with aspartic-type endopeptidase activity and aspartic-type peptidase activity ($p < 0.1$). These sets of genes resulted in either increased chroma or degree of colour saturatedness, and/or a change in the tint towards an orange-brown colour. Manually curated annotation is currently underway.

In the case of the gene set identified as those clones which resulted in a decrease in colour saturation (lighter colour) and/or decrease in hue (colour tint change towards pink-purple), genes were annotated to certain biological processes (p-value cutoff <0.01). For the set labelled ΔC (positive value), genes were shown to be enriched for the ubiquitin-dependent protein catabolic process via the multivesicular body sorting pathway (5%), vacuolar transport (8.3%) and intralumenal vesicle formation (2.2%). The gene products are part of the endosome, including the ESCRTIII complex (an endosomal sorting complex required for transport), which is associated with the endosome membrane. The ESCRTIII complex is required for sorting and/or concentration of multivesicular body (MVB) cargoes. The large number of genes found in the genome which are not characterised, is reflected in the 30% of genes identified in this study whose function is unknown. With the gene set labelled ΔH (positive value), the large number of genes of unknown function (39%) and unknown biological process (28%) was apparent at the p-value cutoff <0.01. However, a number of genes were annotated to astral microtubules (2.1%) and aster (2.1%) which participates in the cell cytoskeleton. It is unclear the function of these particular gene associations in relation to the library screen and more importantly to wine colour.

The data mining of the ~200 genes which have an affect on wine colour is ongoing. The time consuming manual curation of these genes is required because of the

difficulty in finding any meaningful gene associations, for example anthocyanin degradation and metabolism (see Appendix 4.3 and 4.4), and cell wall synthesis (Appendix 4.5). Further, it has not been possible within the time frame to screen the selected strains both as micro-fermentations and 100 ml scale laboratory scale fermentations, prior to small scale berry vinifications. Trialing of these recombinant strains would be in our PC1 laboratory (maximum size as 1L bottles in the fermentation facility).

7.3. Discussion

This phase of the project allowed establishment of a simple method for quantitation of the extent of pigment binding by yeast biomass. By incubating biomass with finished wines, it was possible to exclude the influence of fermentation on this process. Within this limited study, addition of yeast biomass to wines tended to reduce various measures related to colour or red pigments, but not to a significant degree. Yeast biomass was seen to simultaneously become heavily coloured. Marked removal of Mal-3-glu from the wine was observed, a pattern not repeated for other glucosides. It is likely that in the backdrop of the strongly pigmented wines used here, the importance of pigment binding to yeast biomass is small, yet in the case of lighter wines, where any pigment loss may be important, the role of yeast biomass is likely to be more significant. Further experimentation will confirm this.

Our investigation of the influence of various mutations on the colour outcome of the fermentation of a pigment-supplemented medium, is the first example of the use of the yeast deletion library in this way. While there were some compromises associated with the use of the lab yeast deletion library, some useful data have nonetheless been generated. We have identified a group of over 300 genes which are essential to fermentation completion in juice-like media. In addition, the remaining genes have been assigned to groups according to their impact on the colour of the fermented medium. Apart from the ~30% of mutants which affect genes of unknown function, many other candidate genes have been highlighted. No particularly obvious gene groups were highlighted by our preliminary analysis. As a result further work must confirm the impact of the selected mutants as well as utilise published information on gene function in order to determine the possible role of these genes (or their products) in wine colour.

A full list of the identified colour-influencing genes will be published and/or posted online once these have been finalised and their roles confirmed.

Output 8: Findings disseminated – 1 to 3 manuscripts published or presentations delivered which describe key findings from the project to date.

Status summary: The performance targets for this Output were achieved. The PhD student made a seminar presentation on the topic; Dr Walker made a presentation to the Yeast: Products and Discovery meeting at the Waite Campus as well as the School of Agriculture, Food and Wine Research Day. A further poster presentation was made by Dr Walker at the 23rd International Conference on Yeast Genetics and Molecular Biology in Manchester.

Output 9: Identity established of yeast genes that impact on pigment adsorption to biomass - Yeast libraries screened and gene(s) identified that modulate pigment absorption to yeast biomass. Over- and non-producer strains constructed or isolated.

Status summary: The performance targets for this Output were partly achieved. The screening of the deletion library has been completed. The involvement of the several hundred individual mutants (and hence genes) which have been highlighted in this way now needs to be confirmed in larger-scale trials. Until this in done, the construction or isolated of over- and non-producer strains will not occur.

Output 10: Identity of key parameters for colour modulation - Relative importance of critical parameters defined through laboratory and experimental winemaking trials.

Output 11: Informed use of colour modifying parameters - Trials designed and initiated to examine effects of combined strategies for modulating wine colour.

Output 12: Findings disseminated – 1 to 3 manuscripts published or presentations delivered which describe key findings from the project to date.

Status summary: The performance targets for these Outputs were not achieved or only partly achieved. The work undertaken served to highlight the complexity of the yeast:colour interaction, and it was not possible to dissect it to the extent intended. While very good progress were eventually made, albeit in a very piecemeal fashion, the final stage trials, protocol development and dissemination of the findings could not occur in time.

Output 13: Submitted PhD thesis - Thesis prepared by the PhD candidate submitted for examination.

Status summary: The performance targets for this Output were not achieved as the PhD student withdrew hence these are no-longer relevant.

Outcome/Conclusion:
Methodologies useful for the conduct of research in this field were developed, while pursuit of the project confirmed that choice of yeast strain for primary fermentation could influence the colour properties of the finished wine. The effect was however not entirely consistent for yeast strains, suggesting a grape and/or vintage effect also. Such findings highlighted the complexity of the interaction of yeast with grapes or anthocyanins and tannins. As a result the level of understanding, and in turn control of winemaking outcome, that was sought was not possible within the project. Nevertheless, the results that were generated, particularly around the screening of the yeast deletion library through anthocyanin-enriched fermentation (a marked achievement in itself), form an excellent resource and basis for further research.

Recommendations:
Expertise in bioinformatics should be enlisted to extract the maximum data from the library screen in a bid to shed more light on the yeast:pigment interaction. Given the complexity of this interaction, a useful strategy by which to explore specific areas of this mechanism (e.g. enzymatic degradation, yeast metabolite production, pigment binding to biomass, etc) might best be pursued via a researcher (e.g. a PhD student) dedicated to each topic.

In the absence of a comprehensive understanding of the phenomenon under investigation, the inclusion of selected yeast strains in future red winemaking activities by the Jiranek group will provide a fuller database from which to extrapolate the colour influencing tendencies of commercially available strains. Such information will be able to be immediately and readily taken by the wine sector.

Appendix 1: Communication

Oral presentations were delivered to present key findings of the screening of the yeast homozygous diploid deletion library (Output 1) which was recently completed. These presentations were at two local conferences (School of Agriculture, Food and Wine Research Day, Adelaide, 5th November 2009; 3rd Yeast Products and Discovery Conference, Adelaide, 2nd – 4th December 2009). A poster presentation was given at an international yeast genetics conference in July 2009 (see below).

In 2010, it is anticipated that the research findings from Output 1 will be reported both in academic peer-reviewed journals (2 papers) and in industry relevant literature (1 article).

Links to our research group's publications on work funded by the GWDRC will be available for access on the new School of Agriculture, Food and Wine website (under construction).

Poster Presentation:

Michelle E. Walker, Tommaso Liccioli, Tristan Warren, Alana Capaldo, Frank Schmid, and Vladimir Jiranek (2009) The influence of wine yeast on colour extraction and stabilisation in red wine. 23rd International Conference on Yeast Genetics and Molecular Biology, Manchester, UK (19-24 July, 2009)

Oral Presentations:

Michelle E.Walker, Tommaso Liccioli, Frank Schmid, and Vladimir Jiranek (2009) Identification of genes with the potential to influence red wine colour. School of Agriculture, Food and Wine Research Day, Adelaide (6th Dec, 2009)

Michelle E.Walker, Jennie M. Gardner, Tommaso Liccioli, Frank Schmid, Colin McBryde, and Vladimir Jiranek (2009) Progress towards improvement of industrial yeast for the wine industry. 3rd Yeast Products and Discovery Conference, Adelaide (2nd-4th Dec, 2009)

Appendix 2: Intellectual Property

Key, valuable IP centres on the identification of mutant which have a marked influence on wine colour. Further work is however required to more fully characterise these mutants/genes to the level where IP protection might be sought.

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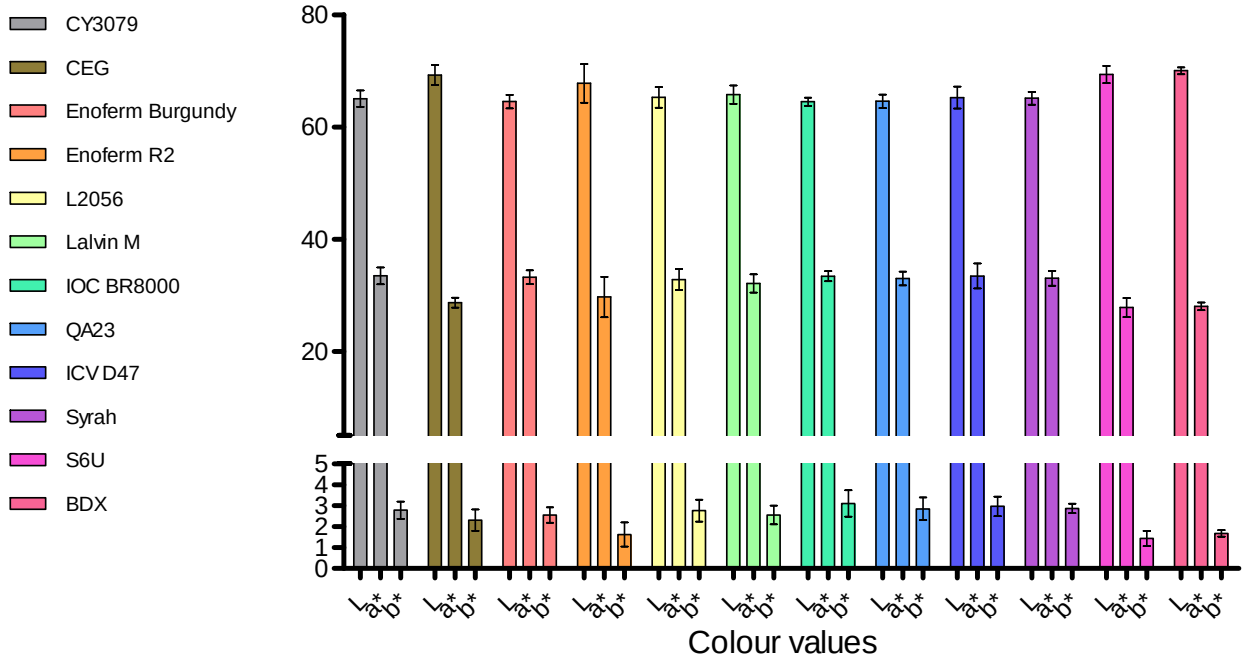
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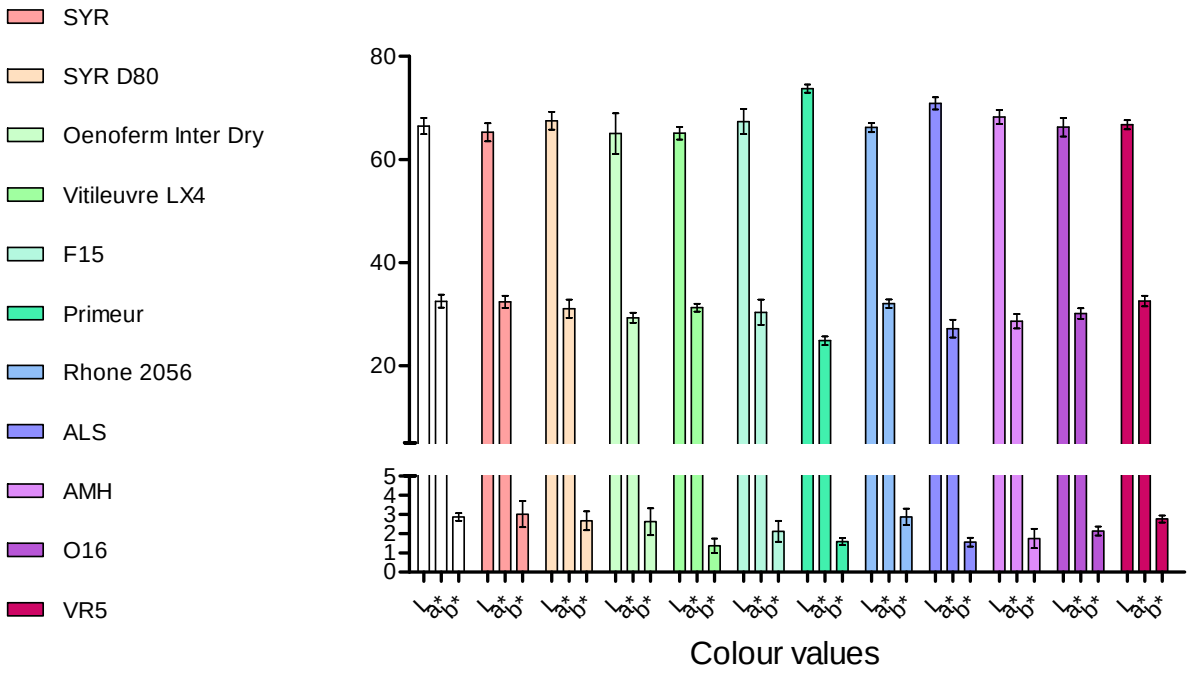
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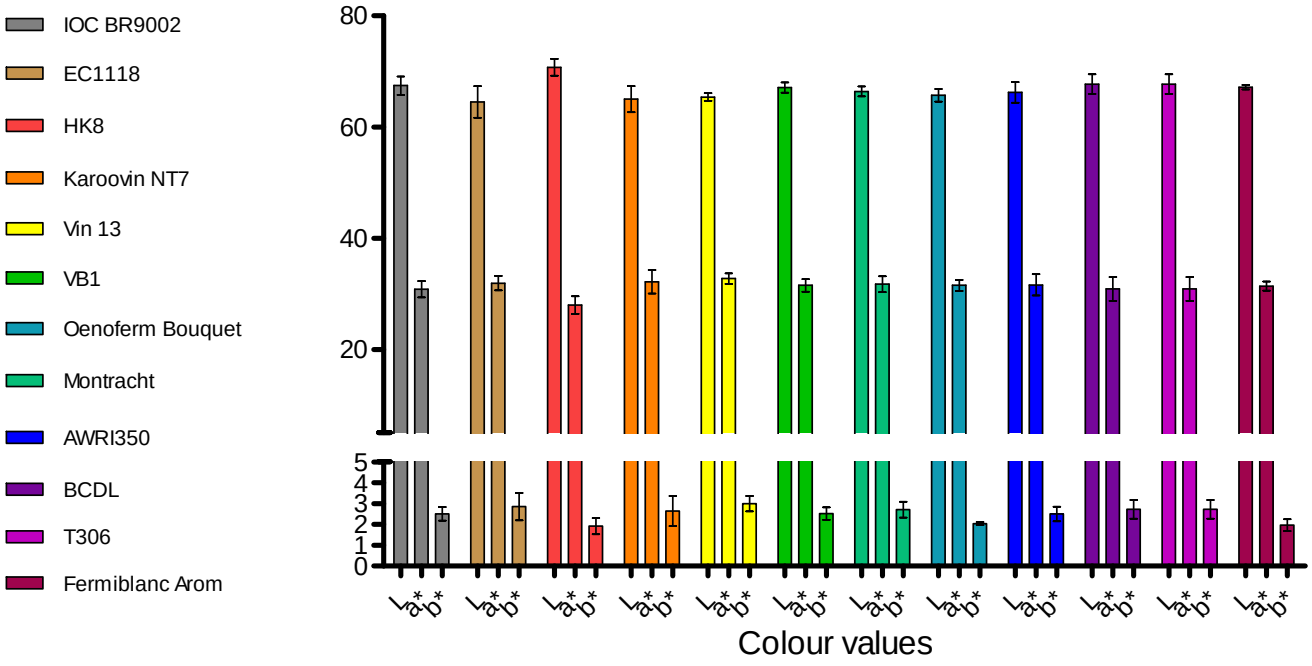
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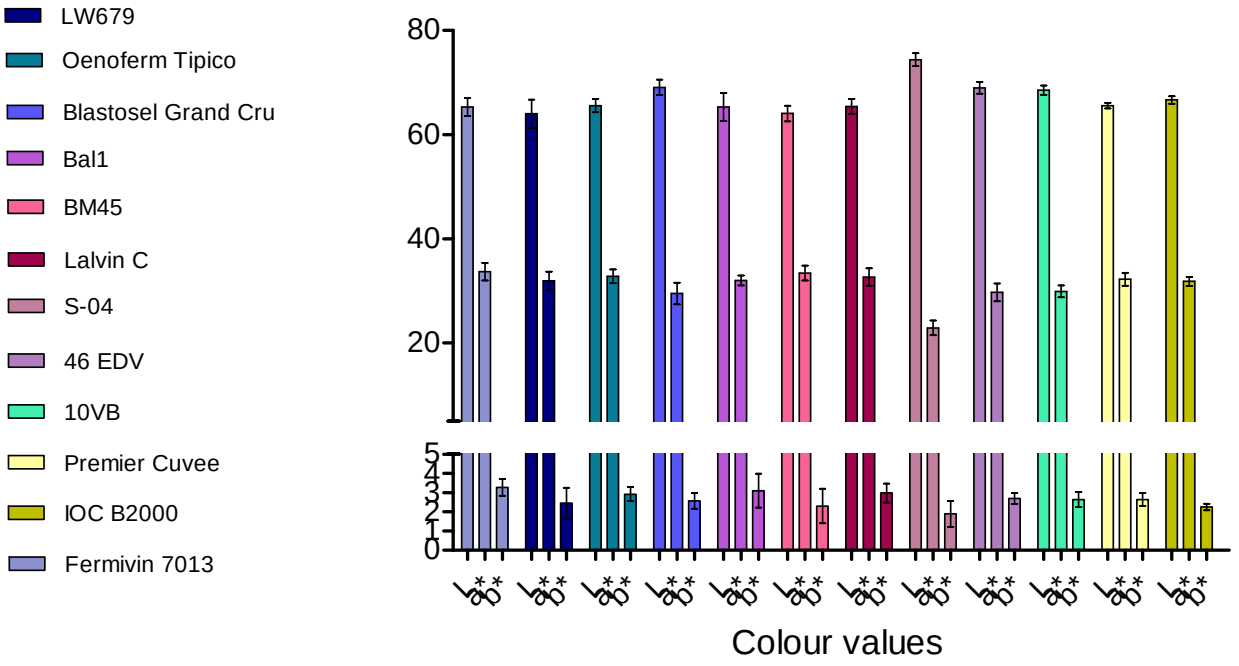
Appendix 4: Data pertinent to the Final Report

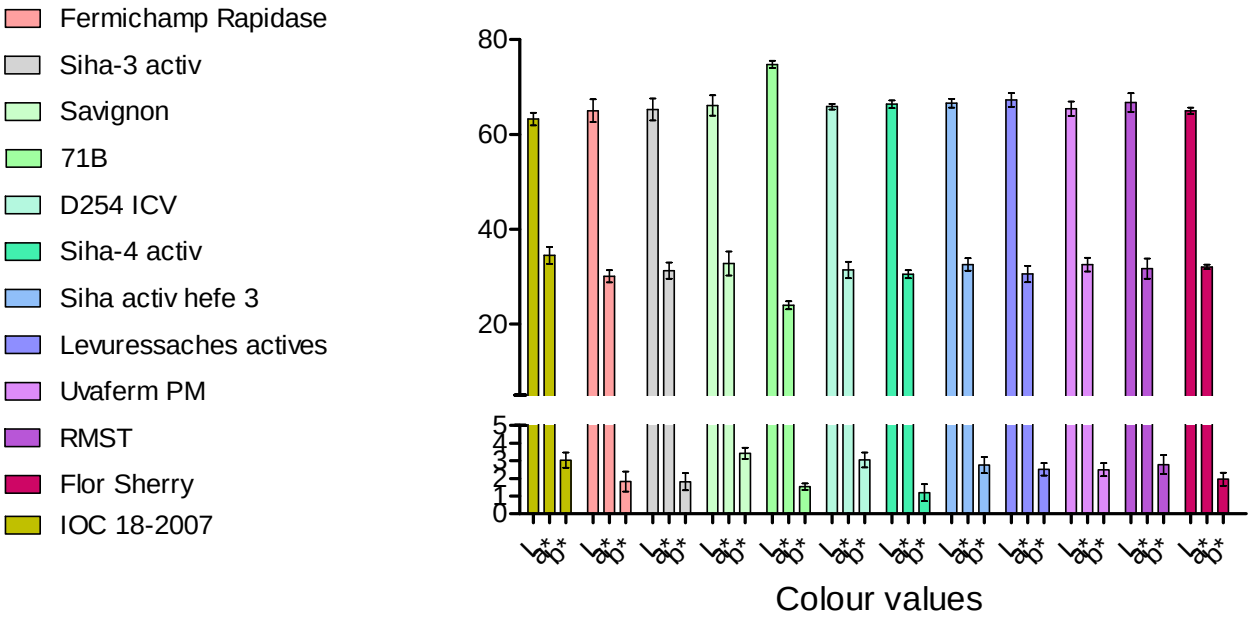
Appendix 4.1. CIELab colour values of model wines from 84 commercial strains which have undergone micro-fermentation for 7 days at 30°C in enriched CDGJM. Ferments were performed as described in Methods (Micro-fermentations of chemically defined grape juice media).

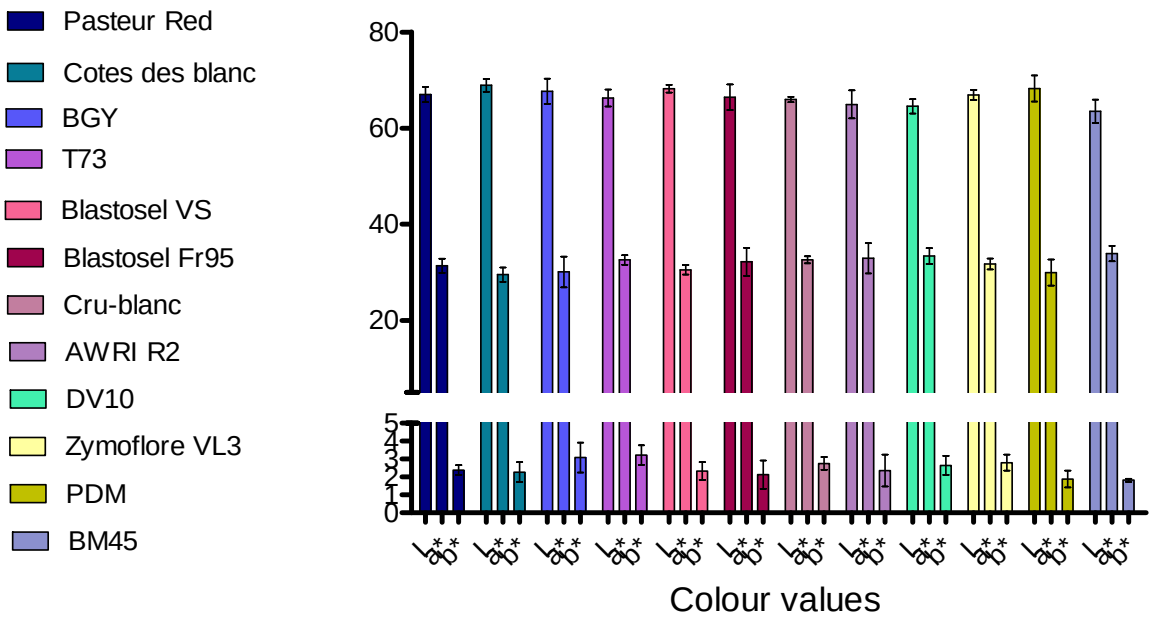


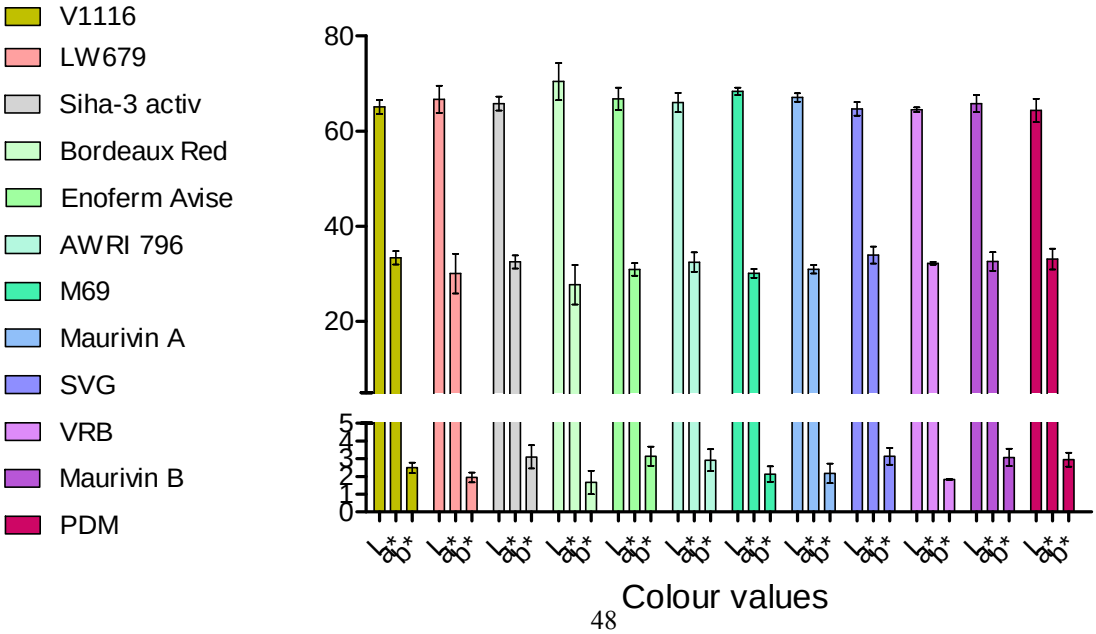



IOC BR9002
EC1118
HK8
Karoovin NT7
Vin 13
VB1
Oenoferm Bouquet
Montracht
AWRI350
BCDL
T306
Fermiblanc Arom
80
60
40
20
5
4
3
2
1
0
L* a* b*
Colour values


LW679
Oenoferm Tipico
Blastosel Grand Cru
Bal1
BM45
Lalvin C
S-04
46 EDV
10VB
Premier Cuvee
IOC B2000
Fermivin 7013
80
60
40
20
5
4
3
2
1
0
L* a* b*
Colour values


Fermichamp Rapidase
Siha-3 activ
Savignon
71B
D254 ICV
Siha-4 activ
Siha activ hefe 3
Levuressaches actives
Uvaferm PM
RMST
Flor Sherry
IOC 18-2007
80
60
40
20
5
4
3
2
1
0
L*a*b*
Colour values


Pasteur Red
Cotes des blanc
BGY
T73
Blastosel VS
Blastosel Fr95
Cru-blanc
AWRI R2
DV10
Zymoflore VL3
PDM
BM45
80
60
40
20
5
4
3
2
1
0
L*a*b*
Colour values


V1116
LW679
Siha-3 activ
Bordeaux Red
Enoferm Avise
AWRI 796
M69
Maurivin A
SVG
VRB
Maurivin B
PDM
80
60
40
20
5
4
3
2
1
0
L*a*b*
Colour values

Appendix 4.2. Grape Polyphenol extract (Cat: Tppr, OenoProd, Sarl))

Oep OenoProd

GRAPES TANNIN

TPPR
OENO PROD Wine Tannins

Description

Colored powder extracted from red grapes skin (Vitis Vinifera) which contains polyphenols, such as anthocyanins and procyanidins (OPC), with a guaranteed content.

The extraction and purification process will preserve its characteristics.

Applications

Vinification :

- Maceration :	1 à 20 gr/hl
- Protection against oxidation :	1 à 10 gr/hl
- Improving structure :	1 à 15 gr/hl
- Color stability, Clarification :	1 à 10 gr/hl

Specifications

• Aspect :	Fine powder
• Color :	Dark Red to Purple
• Smell :	Characteristic (grape)
• Taste :	Astringent
• pH :	2,0 / 4,0
• Color strength :	> 20 UC

Total Polyphenols **(in equivalent catechin)**

DO 280 nm	**> 900**
Polyphenols:	**> 55 %**
Anthocyanins:	***> 7 %***
Ashes :	**< 8 %**

Microbiology

Total viable organisms :	< 1 000 cfu / g
Yeasts, mould :	< 100 cfu / g
Total coliforms :	None

Heavy Metals

Pb :	< 10 ppm
Hg :	< 1 ppm
As :	< 3 ppm
Cd :	< 1 ppm

Pesticides Not Detected

Composition

D.O 280	1320
Condensed Tannins	+ 15%*
Tannins oligomers (proanthocyanidins)	+ 72%*
Catechin + Epicatechin	+ 5%*
Anthocyanins	+ 8%*

Advantages

- Stable powder, with guaranteed content in active principles (French Paradox).
- Very good polyphenols – price ratio.
- No solvent.

Packaging

500 Gr sachet in 25 Kg Cardboard Box.

Storage

- Protected from moisture and light at 10-25°C.
- Kept unopened in the original packaging, the product maintains all its properties for a period of 18 months.

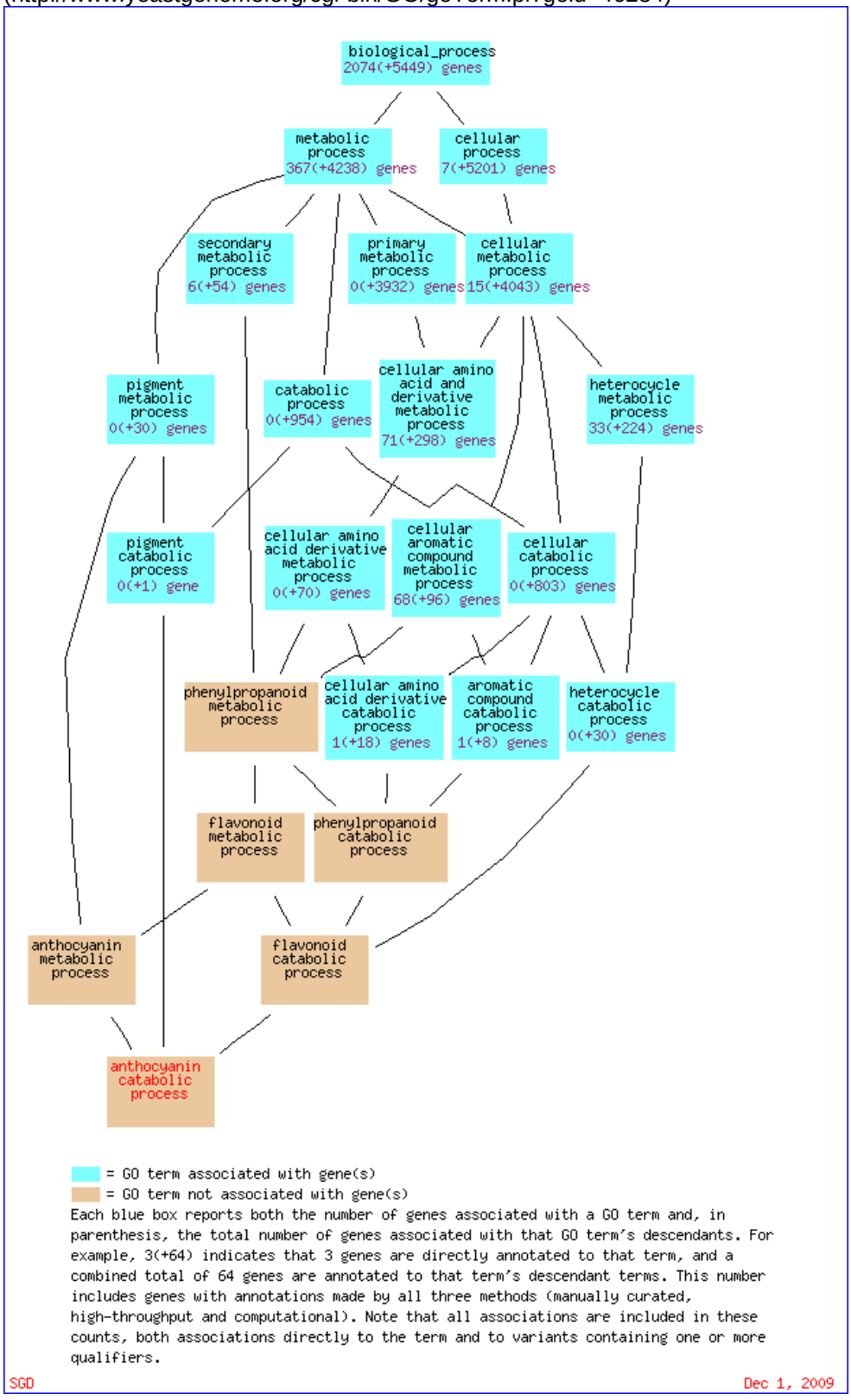
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It is for the users to check the condition of use.

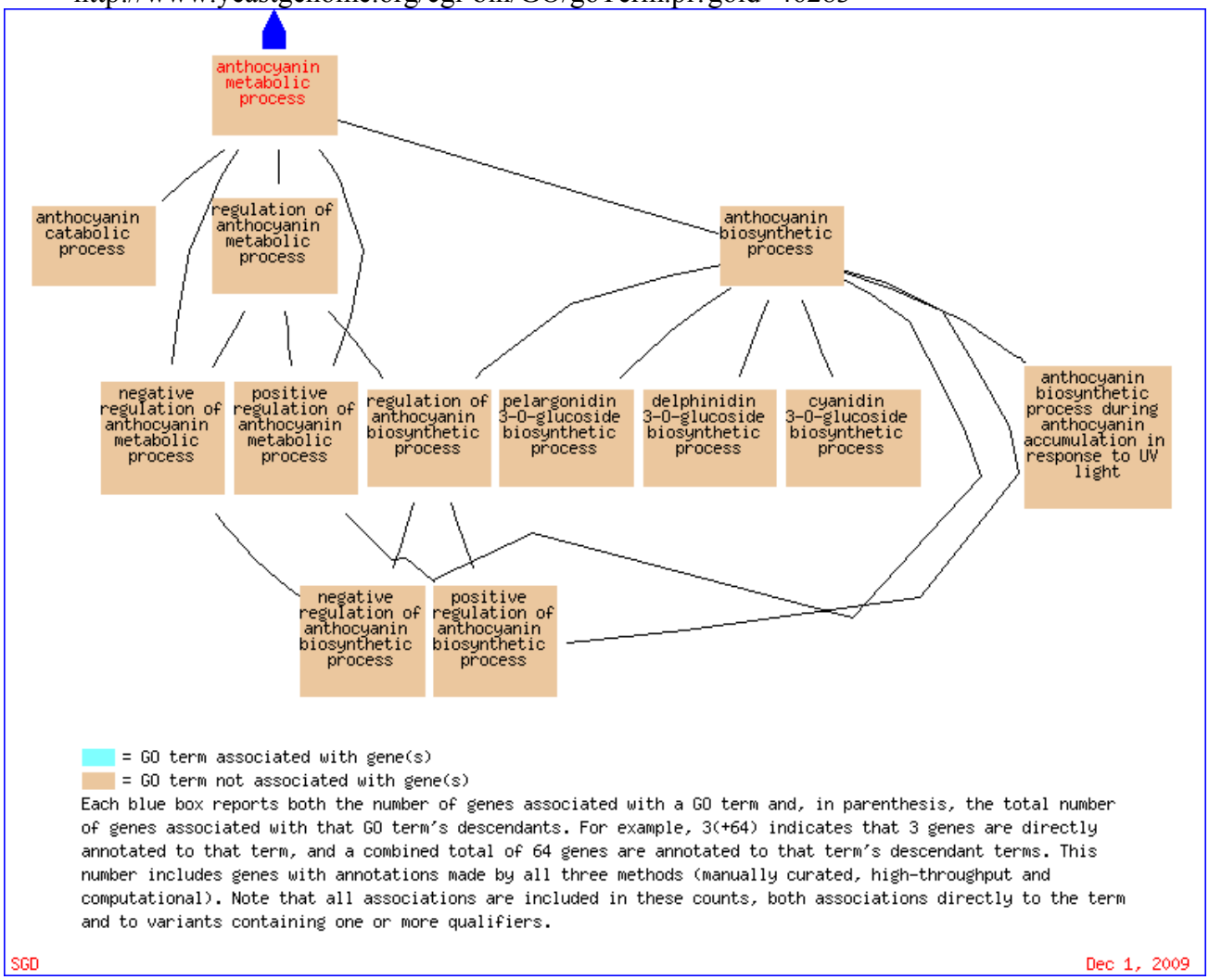
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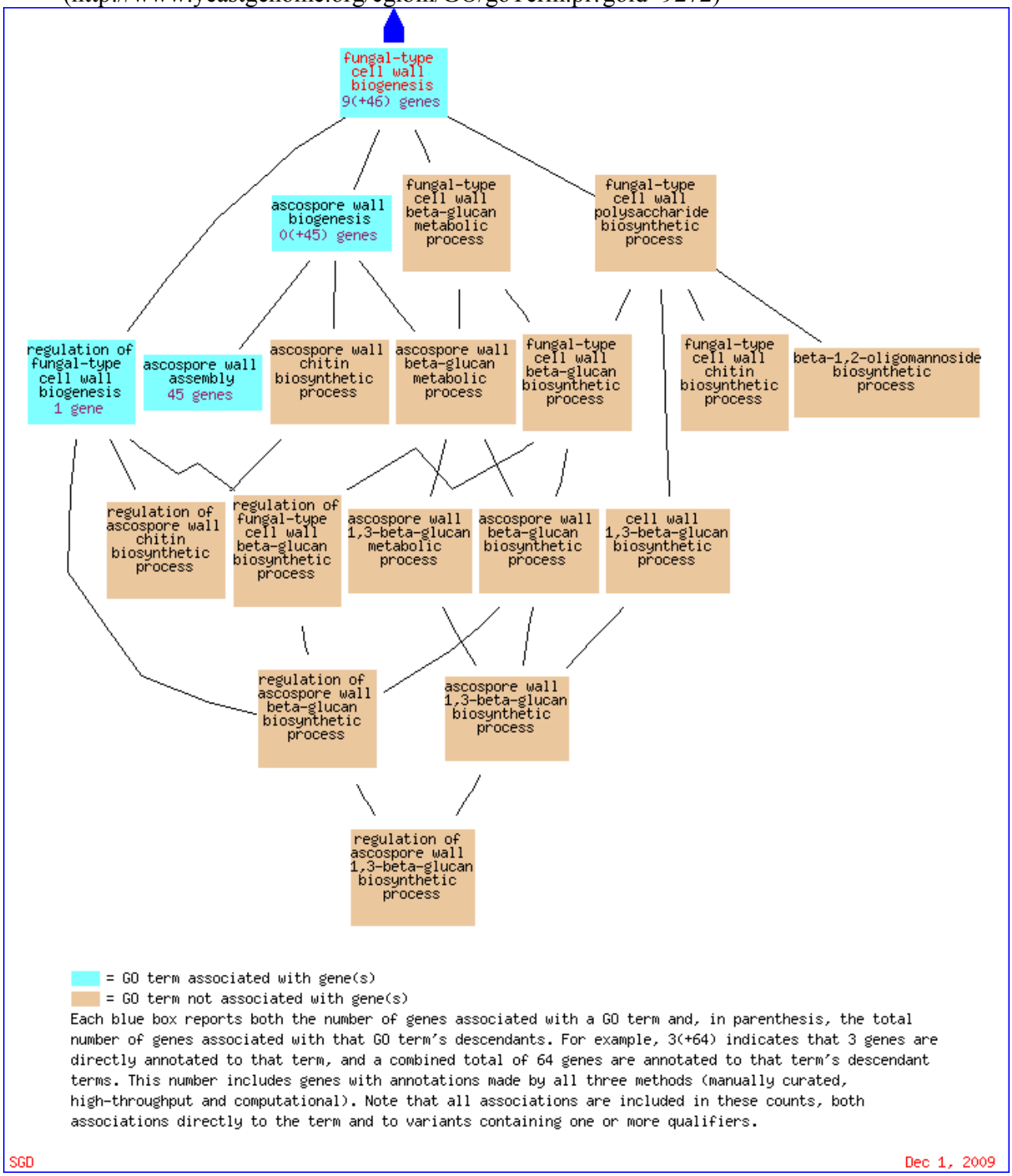
Appendix 4.3. GO term: anthocyanin catabolic process
(http://www.yeastgenome.org/cgi-bin/GO/goTerm.pl?goid=46284)



Appendix 4.4. GO term: anthocyanin metabolic process
http://www.yeastgenome.org/cgi-bin/GO/goTerm.pl?goid=46283



Appendix 4.5. GO term: Cell wall biosynthesis
(http://www.yeastgenome.org/cgibin/GO/goTerm.pl?goid=9272)



Appendix 5: Staff
The following staff contributed to this project:

The University of Adelaide
Assoc Prof Vladimir Jiranek (Project Supervisor)
Dr Paul Grbin (Project Supervisor)
Dr Michelle Walker
Dr Frank Schmid
Tommaso Liccioli
Alana Capaldo
Victoria Harris
Tristan Warren (PhD student)

The Australian Wine Research Institute
Dr Markus Herderich (Project Supervisor)

Appendix 6: Budget reconciliation
To be supplied with the hard-copy report.