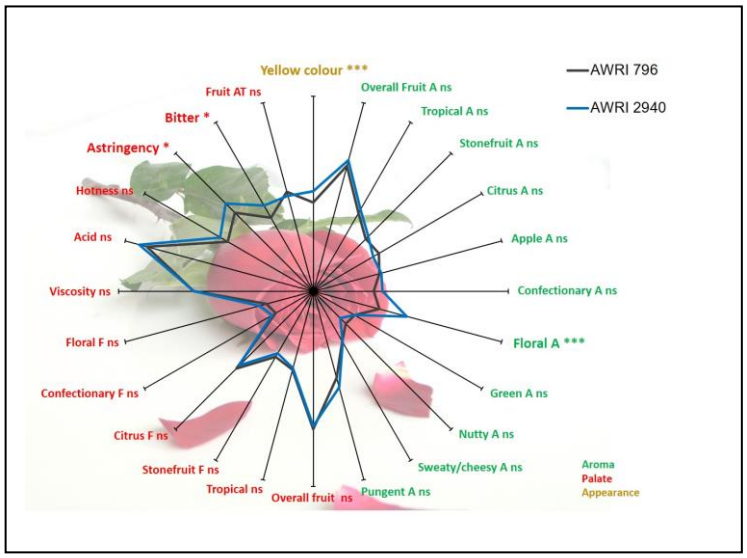
Australian Government

Australian Grape and Wine Authority

The Australian Wine Research Institute

Enhanced winemaking outcomes and wine style diversification through provision of fit for purpose yeast starter cultures



FINAL REPORT to

AUSTRALIAN GRAPE AND WINE AUTHORITY

Project Number: **AWR 1301**

Principal Investigator: **Christopher Curtin (to August 2016), Simon Schmidt (from August 2016)**

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Author: Dr Chris Curtin and Dr Simon Schmidt

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1. Abstract:

While it is common practice for winemakers to choose yeast starter cultures based on their perceived impact on wine style, as much as on their capacity to reliably complete fermentation, this choice has generally been guided by anecdotal evidence rather than objective data. In this project, replicated small-scale fermentations and pilot-scale winemaking revealed the extent to which existing yeast strains can influence composition and sensory properties of red wine. Through correlation to genome sequences, genetic markers were identified for key flavour compounds. Finally, novel *Saccharomyces* interspecies hybrids were generated along with variants of AWRI796 that impart 'rose' aromas in wine.

2. Executive summary:

Once grapes have been harvested and crushed, Australian winemakers are confronted by the need to choose from more than one hundred commercially available yeast strains to start their fermentations. For some winemakers the choice is straightforward, they base their decision solely upon reliability of fermentation completion and may in fact use the same yeast strain for all wine styles. Others make use of yeast germplasm resources to accentuate desired flavours and minimise the production of off-flavours, choosing strains that are 'fit-for-purpose' considering the wine style they are seeking to achieve. To date there has been a lack of objective data winemakers could draw upon to make this choice, typically limited to technical information for a single supplier's strain portfolio, or scientific studies involving a relatively small number of strains. This project was designed to generate knowledge concerning the potential flavour impacts various commercially-available strains may have on wine style, in parallel with work to better understand their fermentation performance and reliability (Project AWR1302). In other words, the aim was to better define what purpose available strains are fit for. The data sets generated were then used to guide development of novel yeast strains that offer Australian winemakers unique opportunities to craft new wine styles and achieve their stylistic goals.

Outcome 1: Knowledge of potential value that can be derived from available yeast genetic diversity to shape wine flavour profile and texture.

In previous work at the AWRI, the potential for yeast strain to shape white wine style had been demonstrated for a small number of strains chosen based upon anecdotal evidence. To provide a more systematic dataset informing winemakers on choice of yeast, for this study strains were selected based on genome sequencing performed in Project AWR1302. This ensured that available genetic diversity was represented across an even split of commercially available and Australian winery isolates.

Small-scale replicated laboratory fermentations were performed using 94 yeast strains in a standardised model grape juice and a Shiraz juice. The latter was modified first-run juice to which commercially available grape phenolic extracts were added to mimic the phenolic composition of a complete Shiraz must. This enabled comparison of flavour compound production in real juice to that observed in a model system, and also exploration of the impact of yeast strain on phenolic composition of red wine.

The results show that model grape juice is suitable for evaluating production of many key yeast-derived volatile aroma compounds, though for a subset of analytes it was evident that yeast metabolism was substantially altered in real grape juice relative to defined medium. This is consistent with other research that has examined the influence of grape composition on yeast-derived flavour compound formation. Nevertheless, when considered together, the experiments in model grape juice and real grape juice provide insight into the extent of variation between existing yeast strains with regards to their potential flavour impacts. Strains could be grouped according to their overall aromatic profiles by applying clustering algorithms. Winemakers interested in harnessing yeast that boost production of desired aroma compounds can use these datasets to make an informed choice from

amongst several strains, taking into consideration their performance-related properties and any preference for a particular supplier.

A major focus in this study was to determine the extent of yeast influence on red wine style, extending prior research that suggested Shiraz quality parameters were modulated according to yeast strain. In addition to laboratory-scale fermentations exploring yeast-phenolic interactions in the absence of grape solids, winemaking trials were performed in collaboration with AWRI Project 3.1.4 (texture). A follow-up winemaking trial was performed incorporating yeast strains studied throughout this project, shown at laboratory scale to yield the greatest differences in wine composition. Data on wine volatiles confirm that profiles observed at laboratory scale were replicated in pilot-scale fermentations. Together, these datasets emphasise that choice of yeast has a marked influence on red-wine style, and amongst available yeast strains there are several groups that have distinct properties that can be harnessed by winemakers.

Outcome 2: Knowledge of the potential to control yeast flocculation late in fermentation to improve processing efficiency

Cell-to-cell binding can result in the formation of large clusters of cells that settle to the bottom of the fermentation vessel, a process known as flocculation. This process is particularly helpful at the end of fermentation, making wine clarification and filtration easier. As a proof-of-concept study, gene technologies were used to construct a wine strain where the onset of flocculation could be triggered at any point during fermentation by modifying an external signal.

To gain insight into natural variation amongst wine yeast regarding the timing and strength of flocculation, two complementary approaches were taken. First, a novel high-throughput sedimentation-rate assay was devised and applied to end of fermentation samples of yeast from model grape juice fermentations described under outcome 1. The samples were transferred into model wine solutions where known triggers of flocculation were varied, namely pH and ethanol concentration, and some samples were transferred to lower temperatures. Second, a collection of 94 barcoded wine yeast strains developed in project AWR1302 were pooled and used to perform fermentations under varied conditions (pH, ethanol, temperature). The barcodes were used to detect which strains remained in suspension throughout fermentation and which strains flocculated at different stages of fermentation. Together these datasets revealed some strains that flocculate throughout fermentation and are unaffected by fermentation conditions, and other strains that flocculate strongly at the end of fermentation.

Fermentation and processing difficulties can also be associated with strains that are overly flocculent or flocculate inappropriately. The methods developed as components of this project were ultimately used not only to characterise flocculation behaviour but to alter it. Work on low alcohol yeasts in AWRI Project 3.3.1 identified strains capable of producing wine with reduced alcohol that were excessively flocculent and had only limited application in winemaking. Non-flocculating versions of those yeast have been isolated by applying the methods originally designed to characterise yeast flocculation behaviour.

Outcome 3: Knowledge of the impact that genome composition of wine yeast interspecies hybrids has on their potential to modulate wine style

Genome composition of hybrids generated in this project was assessed using custom-designed molecular assays that detect the presence or absence of representative chromosomal regions from each parent species, and by whole-genome sequencing. Genome stability was assessed by comparison of genome structure shortly after hybrid generation with structure following fermentation completion or in some cases after passaging cultures for hundreds of generations. Due to their method of creation *cerevisiae*/non-*cerevisiae* hybrids are generally triploid as determined by flow cytometry. The hybrids generated at the AWRI have demonstrated a surprising degree of genetic stability during mitotic growth. Examples of partial chromosome loss were encountered but these were often the

consequence of the hybridisation event itself rather than propagation. Once a lineage was established its genetic makeup and phenotypic profile remained unchanged and thus represent suitable source material for commercial-scale propagation.

Outcome 4: Development of novel wine yeast strains

While results generated in this project demonstrate the extent of natural variation that exists amongst wine yeasts, and their potential to impact wine style, there is no single 'perfect' yeast. Each exhibits a combination of desirable and undesirable attributes. Furthermore, among commonly used wine yeast strains some properties are not available. Genome sequences (AWR1302) and wine compositional data from fermentations performed in this project were drawn upon to explore the potential use of genome-wide-association (GWAS) to identify genetic markers for strain development. Several potential markers were identified for production of volatile aroma compounds, such as those responsible for 'rose' aroma in wine, and work was initiated to confirm their relevance.

In parallel to the generation of these foundational datasets and platforms for future strain development, in this project classical strain development approaches were used to generate new interspecies hybrids and variants of an industry 'workhorse' wine strain (AWRI796) that improved one of its known defects and introduced a novel property.

The portfolio of interspecies hybrids generated by the AWRI was expanded to include hybrids with recently discovered *Saccharomyces* species: *S. arboricolus* and *S. eubayanus*. The winemaking potential of these two species was unknown prior to initiation of this work, though the industrial relevance of *S. eubayanus* is well established through its role as one parent of lager yeast. The capacity of lager yeast to ferment wort at low temperature prompted investigations of the low-temperature fermentation potential of wine strain x *S. eubayanus* hybrids alongside hybrids with another known cold-tolerant species, *S. kudriavzevii*. Screening of wine yeast interspecific hybrids generated between these species identified a number of hybrid yeast strains that complete fermentation at 12°C, but with a protracted timeline (15-18 days). However, two hybrid strains showed robust fermentation properties at 12°C and completed fermentation in a timely manner (9-12 days).

Newly generated hybrids, along with others generated in earlier projects, were also evaluated for use in sparkling wine production. Some of this work was performed in collaboration with Plumpton College in the UK and this has led to considerable interest in using interspecific wine yeasts for sparkling wine production.

AWRI796 was chosen as a starting point for strain improvement activities due to its relatively simple genomic organisation (its chromosomal copies are almost identical to one another) and widespread use in the wine industry. One of the known flaws of this strain is its tendency to produce high levels of succinic acid, which can lead to an imbalance in acidity and 'salty' flavour in wine (Coulter et al. 2004). Mutants of AWRI796 were successfully isolated that produced 40% less succinic acid than AWRI796 in white wines in laboratory trials.

Drawing upon knowledge of genes involved in production of volatile compounds responsible for 'rose' aroma in wine, 2-phenylethanol and 2-phenylethyl acetate, a selection strategy was implemented to isolate variants of AWRI796 with enhanced production of these compounds. This strategy succeeded without extensive accumulation of other mutations, meaning that isolated variants were essentially identical to AWRI796 aside from a very small number of changes to their genomes (< 10 nucleotides). These changes translated to production of between 5- and 50-fold increases in 'rose' aroma compounds relative to AWRI796, dependent upon grape variety fermented. Pilot- and industry-scale trials of these novel strains demonstrated a profound impact of these strains on wine sensory properties. Generally, favourable profiles were seen in white varieties; however, descriptors associated with these strains in red varieties were more varied and not always positive.

3. Background:

Previous AWRI projects (AWRI 1.3.1 and AWRI 1.3.3) were successful in developing new yeast starter cultures for Australian wine producers that minimise hydrogen sulfide formation, enhance 'fruity' volatile thiol formation, and create opportunities for wine style diversification. With the exception of low H_2S strains, which drew upon detailed understanding of genes involved in the sulfate reduction pathway, these products were developed empirically. Despite the important 'imprint' yeast can leave on a wine's flavour profile, there is an incredibly shallow understanding of the genetics of yeast-derived flavour. Even for the Ehrlich pathway (which produces higher alcohols and volatile fatty acids), many genes involved are known, but it is not clear why one yeast has a tendency to produce more of a particular higher alcohol than another. The AWRI is well placed, with a reservoir of Australian yeast germplasm diversity to draw upon (AWRI Project 3.2.5), to develop understanding of flavour profile diversity and underlying genetics.

The review published on flavour-active wine yeasts (Cordente et al. 2012) discussed the yeast 'flavour phenotypes' currently available to winemakers, while highlighting a lack of progress towards genomics-assisted yeast breeding. A lack of comprehensive yeast flavour profiling datasets has limited systematic studies examining the genetic basis of flavour compound formation to a few targets – specifically, production of acetic acid (Marullo et al. 2007) and the volatile thiol 4MMP (Roncoroni et al. 2011). Attempts have been made to correlate production of esters and higher alcohols to transcriptomic data (Rossouw et al. 2008). Genome wide association studies are much more likely to facilitate targeted yeast breeding, particularly where performing interspecies hybridisation. The concept that particular alleles (copies) of flavour genes may determine overall flavour profile has received relatively little attention to date (Bisson and Karpel 2010).

Inoculation with selected active dry wine yeast represents one of the lowest cost inputs in wine production, yet has a significant impact on winemaking outcomes. Even though some strains are favoured over others for particular applications, often due to their perceived flavour effects, there is a lack of systematic data on wine yeast flavour profile diversity. Improved understanding of diversity already available amongst commercial starter cultures will ensure winemakers can choose fermentation yeast most congruent with their stylistic intentions, and will underpin development of fit for purpose starter cultures tailored for Australian winemaking requirements.

4. Project aims and performance targets

The objectives of this project were to:

1. Understand the potential value that can be derived from yeast genetic diversity to shape wine flavour profile and mouth-feel, predominantly for *Saccharomyces* species (complementary objectives in AWRI Projects 3.2.3 and 3.5.3) but also selected non-*Saccharomyces* yeasts.
2. Understand the potential for more efficient processing of wine post-alcoholic fermentation through controlled timing and strength of yeast flocculation.
3. Develop knowledge of relative contributions of divergent genomes in interspecies hybrids to wine flavour profiles and winemaking outcomes, to support effective breeding and hybridisation methodology.
4. Use genomics-assisted breeding and interspecies hybridisation to develop novel yeast strains targeting traits identified as important for Australian winemakers by Wine Australia and through industry consultation, with initial priority targets of enhanced red wine flavour, production of 'rose' aromas, avoidance of faults (H_2S, VA, succinic acid), optimal flocculation behaviour, and stability in performance. Yeast strains identified in AWRI Project 3.2.3 as having desirable traits will be incorporated in strain development.

5. Methods:

5.1. Conduct and analysis of small-scale fermentations for aromatic profiling

A large number of commercial, indigenous, and genetically diverse yeasts were used to separately ferment chemically defined and real grape juices. The choice of yeasts was guided by genomic data generated in AWR1302. Metabolomic analyses of fermentation volatiles were performed, enabling the relative proportions of >50 compounds to be determined, and multivariate yeast strain 'flavour phenotypes' were defined. Fermentations were performed in triplicate. Hydrogen sulfide production during fermentation was monitored using mercuric chloride detector tubes as described by Ugliano and Henschke (2010). Volatile metabolites were measured as previously described (Siebert et al. 2005, 2010). Concentrations of glycerol, acetate, ethanol and organic acids were determined as described by Schmidt et al. (2011).

5.2. Flocculation characteristics of these yeasts were assessed using complementary approaches:

5.2.1.High-throughput sedimentation rate assay of individual strains

Multifactorial screening experiments exploring yeast strain x environmental interactions on flocculation behaviour were conducted by taking end of fermentation yeast samples from 5.1 and resuspending them in media with varied composition (pH, ethanol), and incubating them for 24 hours at different temperatures. A high-throughput sedimentation rate assay, developed in this project, was then used to determine how quickly the yeast samples dropped out of suspension. Briefly, in deep 96-well plates the samples were agitated, and subsampled into a standard 96-well plate for measurement of optical density. After a defined period, the deep well plates were resampled for measurement of optical density. Difference between the two measures was indicative of yeast in the deep-well plates falling out of suspension due to enhanced flocculation behaviour under the experiment conditions.

5.2.2. Competitive sedimentation rate assay

A sedimentation rate assay using the barcoded yeast collection developed in AWR1302 was developed to independently compare the flocculation behaviour of yeast. In this assay a mixed population of yeast were grown under varied conditions (sugar concentration, pH, temperature) in fermentation vessels, then subsampled into a tall cylindrical flask with a sample port. After a defined period, subsamples were taken from the top and bottom of the cylinder, for comparison with the original sample. Custom-amplicon sequencing as described in AWR1302 was used to generate quantitative data on the strain composition of the samples, enabling detection of strains that were over-represented in 'bottom samples' due to their flocculation behaviour. The 'cylinder-sedimentation' approach was used to inform design of a selection strategy for generation of non-GM wine yeasts with altered flocculation properties.

5.3. Determining genomic factors contributing to variations in aromatic profiles

Building on the knowledge of variation in strain aromatic profiles and genomic datasets (AWR1302), bioinformatic approaches were used to correlate genomic features with desirable strain traits. Genomic correlations were used to develop genetic markers for strain development, with the goal of optimising more than one trait relevant to red winemaking. The intention was to perform quantitative-trait-loci (QTL) analysis to validate these and generate additional markers, and the foundations were laid for this work through selection of stable haploid variants (through sporulation) of wine yeast displaying traits of interest. During this project, new methods for yeast modification were developed and published (Ryan et al. 2016), based upon the bacterial CRISPR-cas9 system. These methods enable precise and efficient manipulation of industrial yeast strain genomes, with one common application being the verification of genetic markers. In this project, CRISPR protocols were implemented and used to verify genetic markers for production of 'rose' aroma compounds, by 'swapping' gene alleles (copies) between strains.

5.4. Methods for the development of new strains

5.4.1.Mutagenesis and selection

Two traits were targeted for introduction to the industry workhorse yeast strain AWRI796 – enhanced production of 'rose' aroma compounds, and reduction of this strain's tendency to overproduce succinic acid, which can lead to perceived mouth-feel and taste faults. AWRI 796 was mutagenised with 6% ethyl methane sulfonate for a duration that resulted in a survival rate of ~ 60%. Cultures of AWRI 796 that had either been mutagenised or not were plated onto YPD agar containing toxic analogues of phenylalanine (p-Fluoro-DL-phenylalanine [PFP] or o-Fluoro-DL-phenylalanine). Isolates able to grow on the toxic analogues were subsequently screened for alterations to their aroma profiles.

5.4.2. Rare mating for interspecies hybridisation

Saccharomyces cerevisiae wine strains previously shown to hybridise efficiently with other *Saccharomyces* species were used to evaluate the potential for new hybrids to be constructed with recently described species *S. eubayanus* (one of the parental species of lager yeast) and *S. arboricolus*. The methods for their creation are described in Bellon et al (2011). Winemaking potential of novel hybrids generated by rare-mating was investigated through laboratory-scale fermentation experiments.

5.5. Conduct of pilot-scale winemaking experiments

In parallel to small-scale laboratory fermentations, pilot-scale benchmarking trials were performed across three vintages, with commercial or newly generated yeast strains in each trial, to evaluate sensory impact on Shiraz, Cabernet Sauvignon and/or Grenache. Wines produced in year 1 of the project were evaluated over two years to determine the effects of storage on red wines with different yeast-generated flavour profiles.

6. Results/discussion:

6.1. Aroma profiling of representative wine yeast

6.1.1.Establishing baseline profiles in defined medium

A small-scale fermentation protocol was established and validated using three commercial yeast strains known for their divergent aromatic profiles (AWRI796, AWRI838, AWRI1688 – a monosporic isolate of wine strain Zymaflore VL3). These strains were used to ferment a defined medium representative of Chardonnay juice (formulation described in Schmidt et al. 2011) in 100 mL fermentation vessels fitted with non-return valves to minimise any impact from access to oxygen. An early issue was batch variation in reported concentrations of aroma compounds due to the technical approach employed for aroma compound quantification. Various strategies to address this were explored including C^{13} labelled internal standards and various data normalisation approaches. Geometric-mean normalisation (across three control strains) proved to be an effective method of standardising the data so that comparisons across multiple batches of fermentations could be made. This necessitated the inclusion of all three control strains in every fermentation batch, reducing the number of strains included in the study.

Aromatic profiling of 94 strains (listed in Appendix 5, Table 1) was undertaken. Volatile metabolite production profiles and VSC formation datasets were compiled for fermentations conducted in chemically defined grape juice (CDGJ) in year 1 of the project, and heat maps depicting the relative concentration of each analyte for all strains were constructed (see Figure 1 for example). Two-way clustering groups yeasts together based upon their overall volatile aroma compound profiles, and shows which analytes are correlated. Some examples are evident where genetically related strains are also grouped by their aroma compound profiles, for example AWRI838 and others from the 'PDM' clade described in Project AWR1302 – in this

case AWRI2260, AWRI1001, AWRI2910 (Qa23, Petaluma 1 and Elegance). Interestingly, interspecies hybrids that have AWRI838 as a parent also displayed a similar profile for key compounds, namely volatile fatty acids and their ethyl esters. Also evident was a cluster of strains that could release significantly higher concentrations of tropical volatile thiols (3MH, 3MHA, 4MMP), including AWRI1487, AWRI1537, AWRI2858 and AWRI2861 (L2056, Vin13, VL1 and X5 respectively).

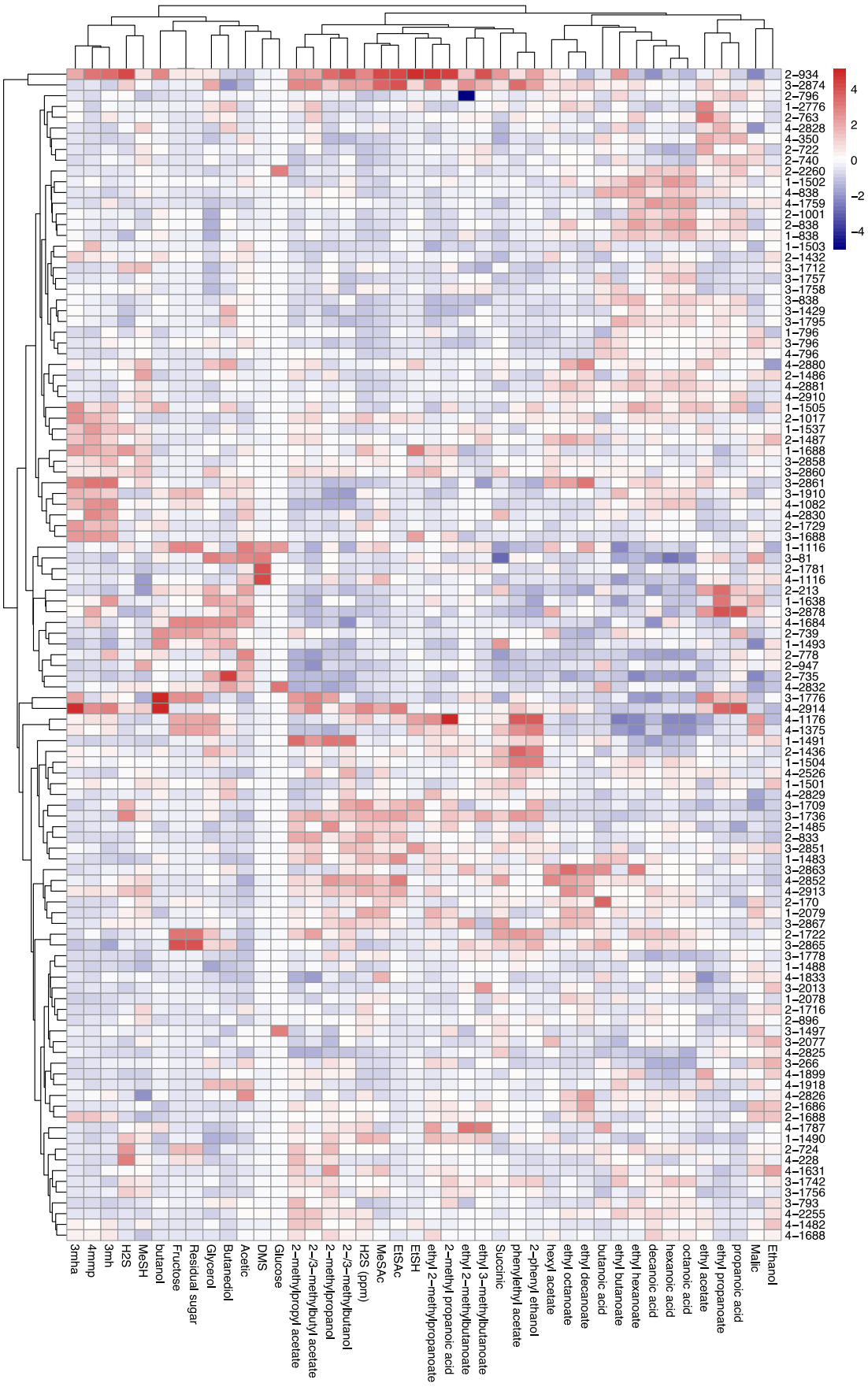


Figure 1. Clustered heat map showing how yeast-derived fermentation products and wine yeast strains vary in their aromatic profiles. Yeast strains are shown by their AWRI wine microorganism culture collection numbers and are preceded by a number indicating the batch in which the ferments occurred. Only chemical analytes with significant differences between strains within each batch of fermentations are included.

6.1.2.Evaluating variation in wine yeast aroma compound production during laboratory-scale fermentation of Shiraz grape juice

An independent set of experiments was conducted to evaluate flavour profile variation using a red juice as fermentation medium. Shiraz juice was prepared from freshly harvested grapes during the 2015 vintage. Initial chemical and microbial analyses were performed with the aim of evaluating juice sterilisation methods. In this regard DMDC (Velcorin) proved to be a useful stabilising agent for grape juice, allowing storage at least six months. Pilot fermentations were conducted with two strains and different rates of supplementation with a commercial grape phenolic extract, allowing final juice composition to be optimised prior to initiation of strain evaluations. Analyses of esters, alcohols and acids were completed but vinyl phenols analysis was not undertaken as fermentations were conducted in a red grape juice - these compounds form anthocyanin adducts and are reported at trace levels in red wine.

Of the 94 strains tested, 11 (~12%) did not complete fermentation (residual sugar > 4 g/L). Of these, two were commercial strains, two were environmental isolates, two were strains of *Saccharomyces uvarum* and three were winery isolates. A summary graph showing variation in volatile concentrations in wines fermented with the 94 yeasts is shown in Figure 2. Notable in the degree of variation between strains are some of the acetate esters such as 2-methyl butyl acetate, 2-methyl propyl acetate and hexyl acetate, for which 14, 20 and 31-fold variances were observed respectively between lowest producer and highest producer. Even greater variation was observed for acids and their ethyl esters, specifically butanoate and ethyl-2-methyl butanoate, which varied more than 60-fold. H_2S concentration in the finished wine ranged from undetectable to as much as 2 µg/L for some strains.

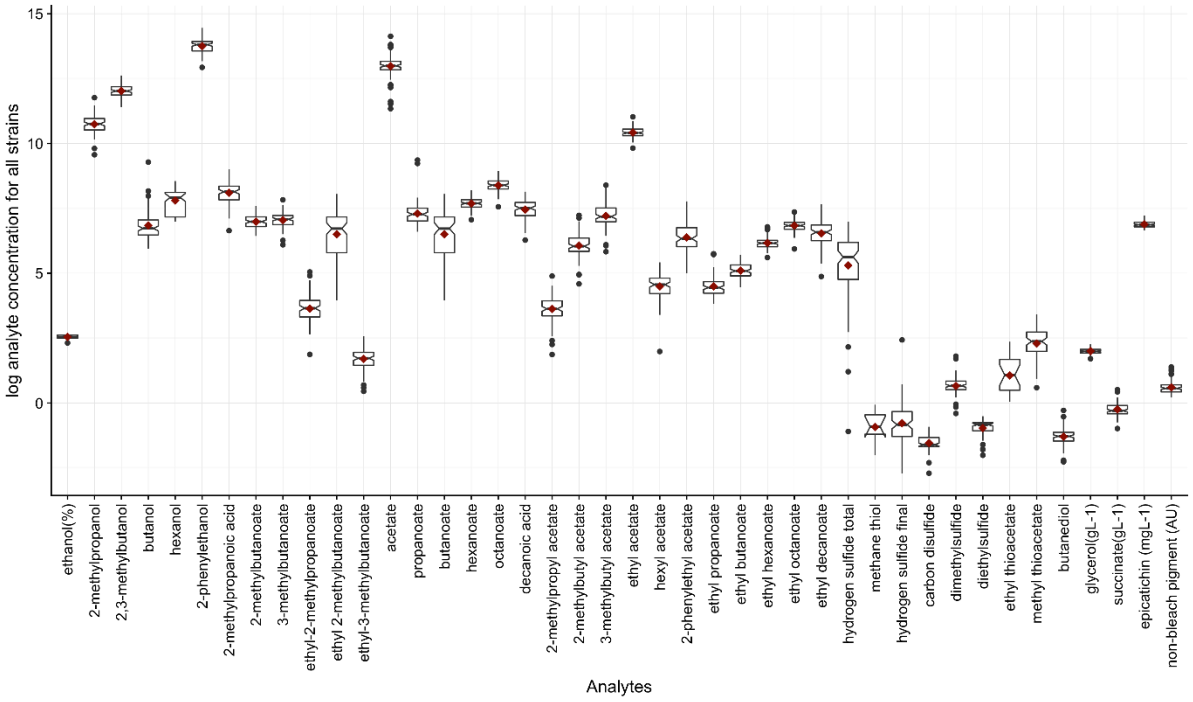


Figure 2. Boxplot showing variation in headspace volatile concentrations of wines fermented with 94 wine yeast. Box shows 25th and 75th percentiles with centre line showing the median. The whiskers show largest and smallest values with points plotting outliers. Red diamond shows the mean. Values are log (µg/L) except where noted.

6.1.3.Correlations of aroma profiles obtained in defined medium and grape juice

For metabolites measured in common for defined medium and grape juice fermentations, approximately 70% displayed statistically significant positive correlations (Figure 3). Some metabolites that were not correlated have previously been shown to be affected by juice precursor content, a key example being hexyl acetate (Dennis et al. 2012). It was also evident that several strains behaved quite differently in CDGJ and real juice.

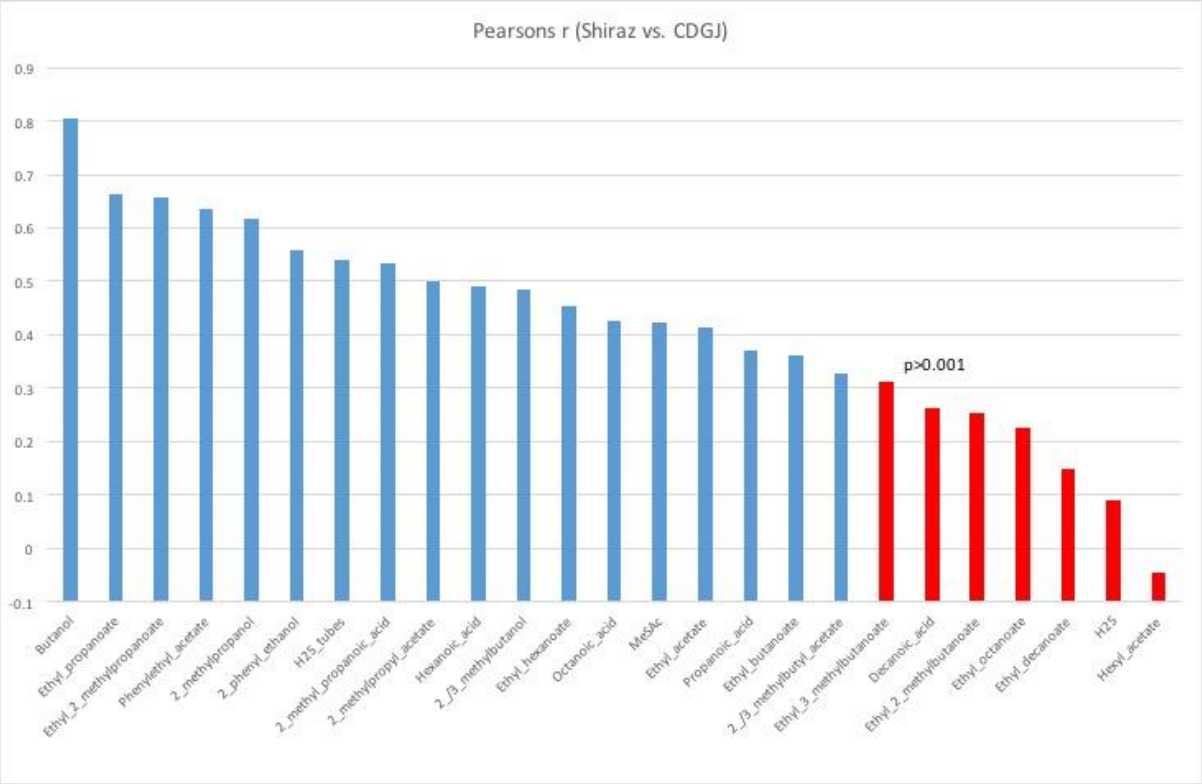


Figure 3. Pearson r correlation statistic for yeast-derived fermentation products measured following fermentation of defined medium and Shiraz juice.

6.1.4.Aromatic profiles of selected yeast determined at pilot scale

To better understand and contextualise the variation in wine observed in 100 mL laboratory-scale fermentations, pilot-scale experiments were performed over two vintages. In the 2014 vintage, a subset of strains was chosen that broadly represented the laboratory variation observed (RX60, F15, AWRI1503, AWRI 796, NT50, BDX, L2323, CLOS, EC1118 and UVAferm HPS). These strains were used to ferment 40 kg of Shiraz in triplicate.

All strains fermented to dryness, and produced wines with similar basic composition within commercially acceptable ranges. As observed at the laboratory scale, significant differences in tannin concentration were evident in wines sampled after alcoholic fermentation. Pre-filtration tannin concentrations varied by up to 30% depending on the strains used. Differences in total polysaccharide content were also seen, mainly due to differing levels of extraction of grape-derived polysaccharides. Strain L2323 is known for its pectinolytic activity (van Wyk and Divol 2010) and yielded the highest concentration of polysaccharides post-fermentation. Somewhat surprisingly, wines made with UVAferm HPS did not contain higher levels of yeast-derived polysaccharides.

Differences in the concentration of key aroma compounds from 1.5 to 2-fold were observed. Aroma compounds associated with 'dark berry' descriptors such as ethyl propanoate, ethyl 2-methyl propanoate and ethyl 2-methyl butanoate varied significantly, along with similar magnitudes of difference in 'red berry' compounds ethyl butanoate, ethyl hexanoate and ethyl octanoate. Wines made with BDX and 1503 contained higher concentrations of methanethiol, while those made with BDX also contained elevated concentrations of dimethylsulfide.

Sensory descriptive analysis of wines showed differences in 'dark fruit' and colour intensity as opposed to 'vegetal' and 'earthy', with 1503 and BDX, and to a lesser extent NT50 and UVAFERM, being low in 'dark fruit'/colour and higher in 'earthy' and 'vegetal'. BDX, 2323, RX60, EC118 and F15 were also lower in 'red fruit' flavour. Differences in astringency were not evident due to an apparent normalising effect of small-scale pad filtration. These observations are summarised in the Principal Component Analysis (PCA) presented in Figure 4.

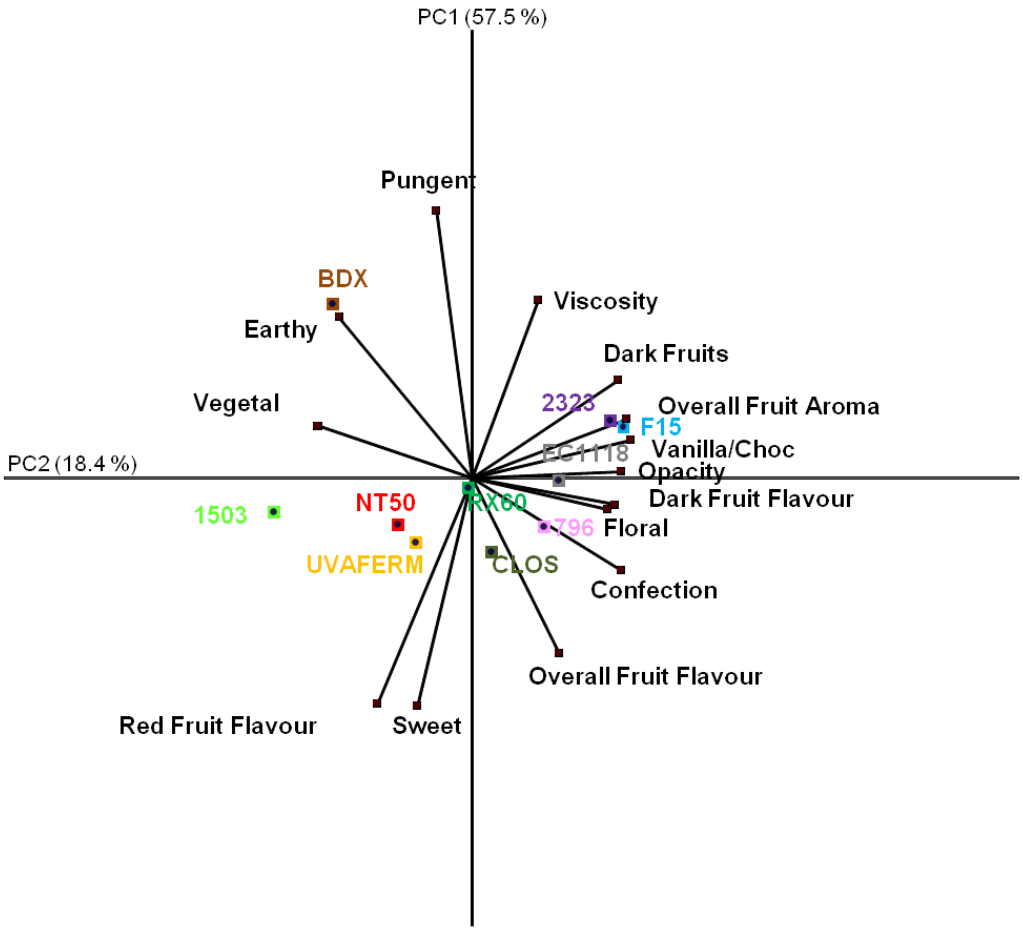


Figure 4. Scores and loadings bi-plot for PCA of attributes and mean values of treatments, showing PC1 and PC2

A second sensory assessment was performed on the 2014 vintage trial wines, providing a 24-month time-point to follow the initial ~9-month assessment. Wines retained some of the key aroma differences observed previously, predominantly separated by attributes such as 'floral', 'vegetal', 'dark fruit', 'herbal' and 'vanilla/chocolate', although the extent of aroma differences

between wines was less than observed at 9 months. Interestingly, the wines had developed significant differences between strains for mouth-feel attributes such as 'viscosity', 'sweet', 'salt', 'astringency', 'bitter' and 'hotness', not observed at the 9-month assessment.

Aromatic profiling of a broad range of yeast in defined medium (6.1.1) and red juice (6.1.2) identified cohorts of strains that produced wines with similar chemical profiles. Replicated pilot-scale winemaking trials were undertaken to determine whether the profiles observed in laboratory-scale screening work were representative of that which might be observed at a larger scale. Six strains were chosen that were representative of the cohorts previously identified. These strains were used to ferment 50 kg of McLaren Vale Grenache.

While these wines have not yet been bottled, post-fermentation analysis of the wines shows that all 6 wines display very different chemical profiles (both volatile and non-volatile) and the results are consistent with the trends observed in the red and synthetic grape juice (SGJ) small-scale ferments (Figure 5). For example, strains AWRI 778 and 1776 produced high and low amounts of acetic acid, respectively, in all three fermentation experiments. Strain AWRI 2260 produced high concentrations of different ethyl esters derived from medium chain fatty acids, while strain AWRI 2914 produced high concentrations of acetate esters and butanol. Finally, strain AWRI 1833 is characterised by a low flavour phenotype (low concentrations of 'fruity' esters). These results confirmed that model ferments using SGJ were good predictors of a strain's capacity to produce different chemical compounds important for the flavour and aroma of wine. Once sensory analysis of these wines has been performed it will, therefore, be possible to interpolate the sensory profiles of other strains used in laboratory-scale experiments based upon their volatile aroma compound profiles.

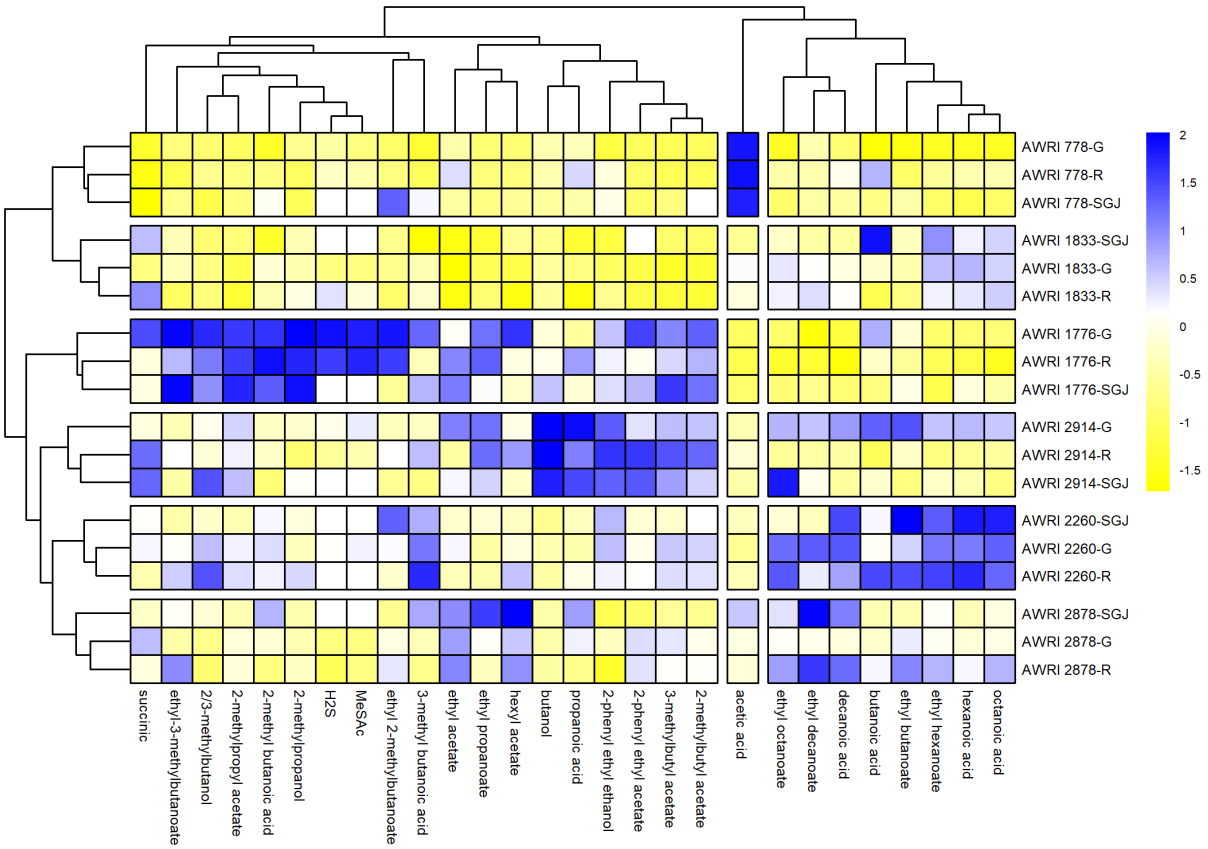


Figure 5. A comparison of aromatic profiles in wines made using six different yeasts used to ferment synthetic grape juice (SGJ), Shiraz juice without solids (R) or Grenache grapes (crushed and destemmed) in 100 mL (SGJ and R) or 50 kg (G) volumes.

6.2. Controlling the timing and strength of yeast flocculation

6.2.1.Measurement of flocculation behaviour

Cell to cell binding resulting in the formation of large clusters of cells that settle to the bottom of the fermentation vessel is known as flocculation. This behaviour involves a nonsexual, homotypic and reversible aggregation of yeast cells to form multicellular masses containing thousands of yeast cells. Yeast flocculation is highly complex in terms of phenotypes, adhesion mechanisms, signalling pathways, responsible genes and regulatory networks. In most biotechnological applications, yeast biomass is separated from the culture media after fermentation or production has finished. Specifically, in brewing and winemaking, yeasts are removed after fermentation by either filtration, flotation or racking, which add costs to the overall process. In theory, efficient yeast flocculation could make clarification and filtration easier and thus lower costs related to biomass separation benefitting these industries substantially.

At the initiation of this project a key challenge was to be able to measure the flocculation behaviour of many yeast strains. Two separate approaches were taken. In the first, a high-throughput assay based on monitoring kinetics of optical density change detected directly at the bottom of micro plate wells was developed to address this need. The assay involved monitoring the optical density of a culture in deep well plates by transferring a sample taken at a defined depth after a specific duration of sedimentation, to a fresh multi-well plate. The optical density of the new plate could then be assessed and combined with data from other samples to estimate sedimentation rates.

The second approach to measurement of flocculation behaviour involved using a barcoded wine yeast collection (produced as a component of Wine Australia project AWR1302) to compare the sedimentation rates of 87 strains in vertically extended fermentation tubes. Measurements for enrichment of strains in samples taken from the tops and the bottoms of the tubes over time was achieved through extraction of DNA and barcode sequencing as described by Robinson et al. (2014).

6.2.2.Profiling of yeast strain flocculation

Using the high throughput sedimentation assay described in 6.2.1, 99 yeast strains were screened, identifying several strains that flocculate heavily during fermentation in chemically defined wine, and a separate group that exhibit poor sedimentation behaviour. Additionally, employing a collection of barcoded wine yeast strains, the flocculation behaviour of 87 strains in response to several environmental conditions was determined. These environmental conditions included chemically defined grape juice (CDGJ) with decreased pH, increased sugar concentration and decreased fermentation temperature. Six of these strains were able to strongly sediment at the end of fermentation under the above-mentioned conditions and also in Chardonnay juice (Figure 6Figure 2). Two of these wine yeasts, AWRI1688 and AWRI1759, were selected for pilot-scale trials in vintage 2017 in order to evaluate filterability, lees density and sensory profile.

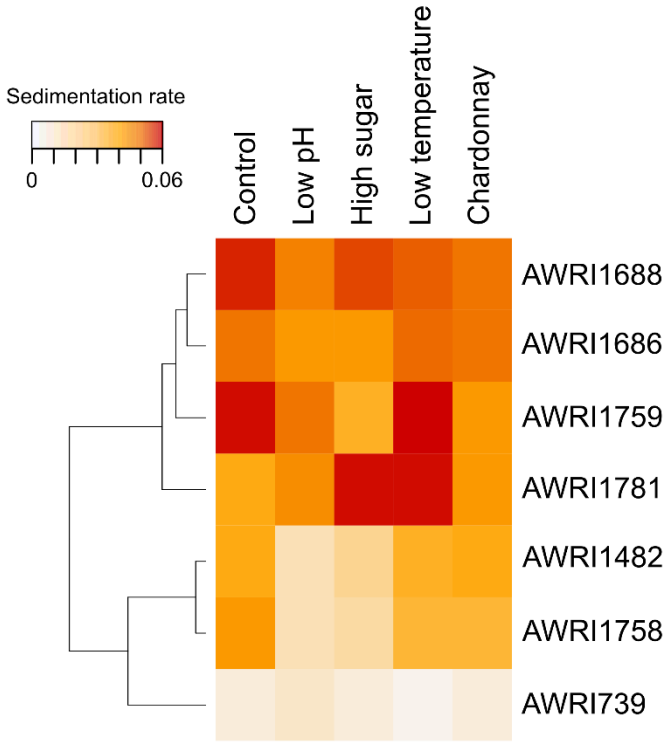


Figure 6. Flocculation behaviour of seven *S. cerevisiae* strains in different environmental conditions. Conditions include: CDGJ (control), CDGJ with lower pH, CDGJ with increased sugar concentration, CDGJ fermented at lower temperature, Chardonnay must. Colour indicates sedimentation rate calculated as ΔOD600/min.

6.2.3.Modification of flocculation behaviour through the application of adaptive evolution

As a proof-of-concept study, several gene modification approaches were evaluated to enable controlling flocculation onset. Successful strategies would enable induction of flocculation in wine yeast at any point during fermentation by modifying an external signal. The approach taken was to stimulate expression of a gene known to affect flocculation, *FLO1*. Ten promoters that had previously been shown to be responsive to environmental stimuli were evaluated but most were found to lack the tight control required of this system; that is, they stimulated flocculation in the absence of a stimulus. One was identified that had minimal background activity. The promoter of MF(alpha)1 linked to *FLO1* could stimulate flocculation on demand by addition of the commercially available yeast peptide hormone a-factor. This work demonstrated that the complexities of the flocculation process can be circumvented by a rational synthetic gene design.

A selection strategy capable of isolating non-flocculent variants of overly flocculent wine yeast was developed based on a variation of the screening strategy described in 6.2.1. This strategy made use of differential sedimentation rates in vertically extended fermentation tubes to capture genetically variant strains with altered flocculation traits. Fermentation in elongated tubes, initiated by inoculation with samples taken from the upper part of a previous fermentation (sequential batch fermentations), permitted the isolation of strains with modified flocculation behaviour.

The differential sedimentation strategy was employed to alter the flocculation behaviour of two highly flocculent strains, *S. cerevisiae* AWRI350 and *M. pulcherrima* AWRI1149. The strong flocculation characteristics that they exhibited negatively affected the kinetics of fermentation. Non-flocculent variants of both strains were obtained. That the strains ultimately derived from

the experiments were the same as those used to initiate the experiment was demonstrated using whole genome sequencing of parental and derivative strains. The non-flocculent isolates showed faster fermentation kinetics and similar production of primary metabolites, highlighting potential winemaking applications for these evolved isolates.

6.3. Interspecies yeast hybrids

6.3.1. Genomic structure and stability of interspecific hybrids

Mating in *Saccharomyces* species is typically between haploid cells of the opposite mating type. However, generation of interspecific hybrids between *cerevisiae* and non-*cerevisiae* species of *Saccharomyces* was performed using a diploid *cerevisiae* parent that had undergone a mating type switch at the mating type locus. These strains are homozygous (either a/a or α/α) at that locus allowing the diploid *cerevisiae* strain to mate with haploids of the opposite mating type. One consequence of this approach is that new hybrids will be initially polyploid, meaning that they will have a larger number of copies than the standard two homologous sets of chromosomes. This imbalance in gene copy number can potentially lead to genome instability to varying degrees with parts of chromosomes, whole chromosomes or large parts of one of the genomes being jettisoned because of incompatibilities that can occur during the mitotic and meiotic reproduction (Antunovics et al. 2005, Marsit et al. 2017). Large-scale genomic instability would have consequences for the stability of traits as they relate to winemaking and ultimately for the usefulness of the strain. It was therefore important to assess the genomic stability of strains generated as part of this work.

Hybrid stability was evaluated using PCR-RFLP with markers specific to the ends of each arm of all 16 chromosomes with specific restriction enzymes capable of distinguishing between chromosomes derived from *cerevisiae* and non-*cerevisiae* parents (Bellon et al. 2013). In total 150 end-of-ferment isolates from two independently generated *cerevisiae*/*mikatae* hybrid strains (CxM1 and CxM4) were evaluated in this way. In two out of 150 isolates of CxM1 and four out of 150 isolates of CxM4, chromosomal abnormalities were detected indicating loss or partial loss of chromosomes derived from *S. mikatae*. No isolate showed loss of DNA on more than one chromosome. Flow cytometry indicated no loss of ploidy for these hybrids.

A similar approach was used to assess the genomic stability of hybrids between *S. cerevisiae* and *S. bayanus*. In this work, hybrid stability was assessed after 200 mitotic generations to evaluate suitability of hybrids for commercial propagation. Again no loss of chromosomes was detected with PCR-PFLP and flow cytometry indicated a triploid genome complement (Bellon et al. 2015).

The genomic content of hybrids between *S. cariocanus* and *S. cerevisiae* strain AWRI947 (a *S. cerevisiae* wine yeast isolate outside the EC1118/PDM clade) was confirmed through molecular typing (ribosomal DNA, targeted PCR for all 16 yeast chromosomes) demonstrating the presence of the genome of both parent species. Flow cytometry analysis established that the hybrids had DNA content consistent with them being triploid. Targeted PCR analysis of both arms of all 16 yeast chromosomes (32 molecular markers) revealed some instability in the AWRI947 x *S. cariocanus* hybrids, whereby a small section on the left arm of chromosome 6 from the *S. cerevisiae* parent was lost in some individual clones during the initial hybridisation event. Subsequent analysis of their fermentation performance did not reveal changes to fermentation characteristics that could be associated with loss of that segment of chromosome 6.

In summary, interspecific hybrids generated between diploid *S. cerevisiae* and various non-*cerevisiae Saccharomyces* species are mitotically stable. Any genomic changes observed appear to occur during their initial generation. Poor performing hybrids are selected against in early rounds of screening. Hybrid strains that can grow and ferment efficiently have a low probability of phenotypic loss resulting from subsequent propagation.

6.3.2.Aroma profiling of interspecific hybrids

The aroma profiles of interspecific hybrids generated from *S. cariocanus*, *S. arboricola* and *S. eubayanus* species were evaluated in laboratory-scale Chardonnay juice fermentations. Differential production of flavour active compounds was observed to occur in hybrids between the different species. For instance, ethyl decanoate production showed an additive effect in *S. cerevisiae* x *S. eubayanus* hybrids, whereas a repressive effect was evident in *S. cerevisiae* x *S. arboricola* hybrids as these hybrid strains produced lower concentrations than either of the parent strains (less than half the concentration of the lower parent).

Industry trials in Tempranillo and Grenache (rosé) were completed using newly generated hybrids between wine yeast NT116 and several different *Saccharomyces* species: *S. kudriavzevii* (AWRI 2790); *S. cariocanus* (AWRI 2795); *S. arboricola* (AWRI 3054); *S. eubayanus* (AWRI 3058). Wines produced from these hybrids were showcased in a workshop at the 16th AWITC in July. While these trials were not conducted in a replicated format that permitted formal sensory descriptive analysis, informal commentary on the wines was sought from workshop participants. In Tempranillo wine, the *cerevisiae* parent was described as 'spicy' with a 'rich mid-palate'. To this background, the *cariocanus* hybrid was noted as being more complex with a softening of the palate and the *eubayanus* hybrid added 'confectionary' and 'chalky' attributes. These trials showed that hybrids of *S. arboricola* and *S. eubayanus* can perform in a winery context to an acceptable standard and that they generate wines with altered stylistic attributes.

6.3.3.Fermentation performance of interspecific hybrids in extremes of temperature

A small number of interspecific hybrid strains (*S. cerevisiae* x *S. kudriavzevii*, *S. cerevisiae* x *S. uvarum*, and *S. cerevisiae* x *S. eubayanus*) were shown to be able to grow robustly in laboratory assays on YPD agar plate at both high (37°C) and low (12°C) temperatures. In fact, together with ethanol tolerance, temperature-based selection is a key criterion enabling the selection of many of the hybrids generated in this and previous projects. The question was posed whether ability to grow at high (37°C) or low (12°C) temperature on plates was an indicator of fermentation capacity at those temperatures.

Despite being able to grow on plates at 37°C none of the hybrids could ferment Chardonnay juice to completion at that temperature. This was also the case for the *S. cerevisiae* parent. At 12°C some of the hybrids exhibited accelerated sugar consumption profiles compared to the *S. cerevisiae* parent. This was the case for both *kudriavzevii* and *eubayanus* hybrids although only data for the *kudriavzevii* hybrids are shown in (Figure 7). Not all hybrids exhibited the same fermentation capacity. Two of the three hybrids in the experiment shown in Figure 7 performed no better than the *S. cerevisiae* parental strain despite demonstrating robust growth on plates at than temperature.

These experiments demonstrated that hybrids provide a pathway for the development of enhanced fermentation attributes that can exceed the capability of performance driven *S. cerevisiae* yeasts in specific conditions. They also highlighted the need for condition-specific performance evaluations to ensure that sought-after traits are ultimately captured in the strains that make their way to production environments.

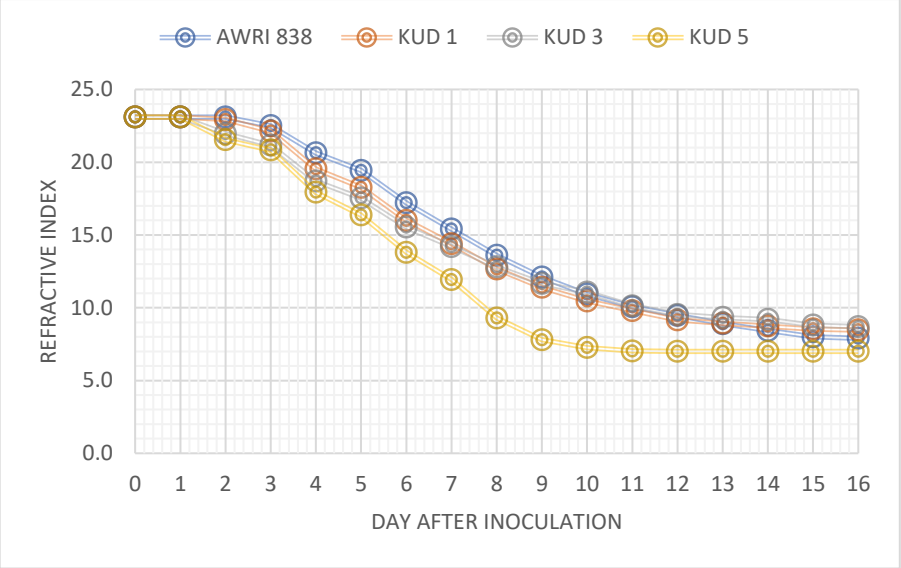


Figure 7- Fermentation kinetics of *S. cerevisiae* x *S. kudriavzevii* hybrid yeast at 12°C

6.3.4.Hybrids to reduce the volatile acidity of dessert wines

Sweet dessert wines are made from grapes with extremely high sugar content. When *S. cerevisiae* is in a high sugar environment it produces glycerol as a compatible solute. To balance the production of glycerol, acetic acid is also produced. The more glycerol that is produced, the more acetic acid is also produced. Previous work has shown that some strains of *S. bayanus* contribute less acetic acid to wines than *S. cerevisiae* (Castellari et al. 1994). However, *S. bayanus* generally lacks robust fermentation characteristics that would enable its direct industrial application. The combined traits of *S. bayanus* and *S. cerevisiae* suggested that progeny of a cross might have the potential to efficiently ferment high sugar juice while producing desirable sensory attributes.

Interspecific hybrids yeasts were generated (Bellon et al. 2011) and their ability to ferment high sugar juices was assessed. Trials were conducted in Chardonnay supplemented with glucose and fructose in equal amounts. At the highest sugar concentration assessed (355 g/L) the two hybrid strains produced wines with less residual sugar than either the *S. cerevisiae* or *S. bayanus* parent. Likewise, acetic acid concentrations in the finished wines were also lower (0.65 g/L), being almost half that of the *S. cerevisiae* parent and less than half that of the *S. bayanus* parent (Figure 8). Surprisingly, glycerol concentrations of the hybrids were higher than both parental strains (Bellon et al. 2015).

The experiments in chaptalised Chardonnay juice were replicated in botrytised Riesling juice with similar results. Acetic acid concentrations were again lower; however, glycerol concentrations were not different between any of the strains. Ethyl acetate concentration, the other key contributor to volatile acidity, was significantly less only for hybrid 1572. These hybrids provide an opportunity for winemakers to minimise acetic acid concentrations in wine styles that are traditionally fraught with volatile acidity issues.

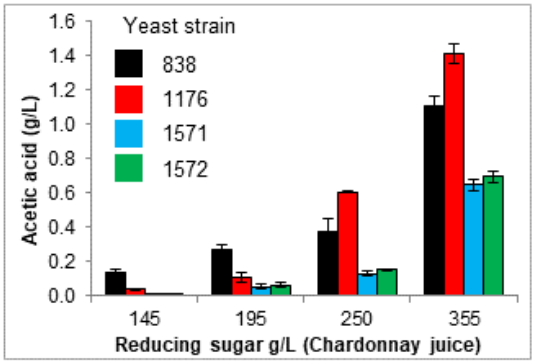


Figure 8. Acetic acid production of hybrids (1571 and 1572) in comparison to parental strains (838 and 1176) in juices with increasing sugar concentration.

6.4. Development of novel yeast strains using classical mutagenesis breeding

6.4.1. Minimising the production of succinic acid in high-succinic acid yeast

Succinic acid production during fermentation can lead to undesirable increases in total acidity (TA), and has the potential to increase perceived 'saltiness' of wine. The commercial strain Maurivin AWRI796 is known for its capacity to produce greater quantities of succinic acid, and this can be seen as a risk for some winemakers.

To isolate low-succinic-producing yeast derived from AWRI796, a classical mutagenesis technique was used based on the incubation of yeast cells with the chemical agent ethyl methanesulfonate. After mutagenesis, a large number of cells ($3x10^8$) were spread on glycerol solid plates containing the antifungal agent cerulenin, as selection pressure. Fifty-nine mutants that appeared after 5-6 days of incubation at 30°C were isolated.

Initial screening was performed in 96-well microplates (600 µL) in two synthetic grape juices and a Chardonnay juice. The fermentation performance of approximately 50% of the mutagenised strains was equivalent to the parent strain AWRI796. Of those, many produced succinic acid levels 20-40% lower than the parent. To confirm these results, laboratory-scale fermentations (200 mL) were performed in two Chardonnay juices with the selected strains (30 in total), and four of them displayed a consistent low succinic acid phenotype (on average 50% less than the parent) and similar fermentation performance to the parent.

One of these strains (AWRI 2955) was selected for a pilot-scale fermentation trial (50 kg) in Shiraz grapes (2016 vintage). In these conditions, strain AWRI 2955 produced 10% less succinic acid than the parent strain AWRI796 (2.34 vs 2.57 g/L). It should be noted that in this particular trial AWRI796 didn't display a high succinic phenotype in comparison to the other strains. Titratable acidity of the wine produced by AWRI2955 was also slightly less than the wine produced by the parent strain (5.5 vs 5.9 g/L, pH =7). These results show that AWRI2955 can be used to reduce succinic acid production relative to AWRI796, and under (yet to be elucidated) conditions where AWRI796 does overproduce this acid the reduction is expected to be more substantial.

6.4.2. Producing yeasts with enhanced floral attributes

While varietal 'floral' aromas in wine are typically the product of grape-derived terpenoid flavour compounds, products of yeast metabolism, 2-phenylethanol (2-PE) and 2-phenylethyl acetate (2-PEA), are considered important contributors to 'rose' aroma (Vilanova et al. 2013). The compounds can enhance the 'floral' sensory properties of wine and other fermented foods and beverages (Fukuda et al. 1990, Duenas-Sanchez et al. 2014). Generally, the concentrations of 2-PE and 2-PEA in finished wines are below their aroma perception thresholds (Vilanova et al. 2013), meaning their contribution to wine style is minimal.

To generate a novel yeast that imparts 'floral' aromas, a well-known and widely used wine yeast strain, AWRI796, was exposed to a chemical selection process previously applied to saké and baking yeast (Fukuda et al. 1990, Duenas-Sanchez et al. 2014). Chemically mutagenised and non-mutagenised populations of AWRI 796 were spread onto plates containing a toxic analogue of the amino acid phenylalanine. Only cells that carry mutations in key phenylalanine biosynthetic pathway genes (that enable the cell to make more of its own supply of this essential amino acid) can grow in the presence of this toxic analogue. A by-product of cells making large amounts of phenylalanine is that more is available for biosynthesis of the flavour-active compounds 2-PE and 2-PEA. All yeast derived using this and related protocols are not genetically-modified (GM) and are suitable for use by the Australian wine industry.

One-hundred and fifty-three AWRI796 mutants were screened for 'floral' aromas, and seventeen of those were used to conduct fermentations in the laboratory. The resultant wines were analysed for a suite of volatile aroma compounds. Ten mutants that produced significantly higher concentrations of 2-PE and 2-PEA had their whole genomes sequenced; importantly six of these were 'spontaneous mutants' that had been derived without use of the mutagenic agent, which means they carried a small number of changes (<10 nucleotides out of 12 million) in their genomes. This enabled identification of two genes, Tyr1 and Aro4, in the aromatic amino acid biosynthetic pathway that harboured possible causative mutations. Further evaluation of these genes and the mutations identified in them is discussed in 6.5.2.

6.4.3.Pilot-scale and industry trials of 2-phenylethanol overproducing strains

The 'rose' strain AWRI 2940 has been trialled in several pilot-scale ferments ranging from 20 L (whites and rosé) to 50 kg (red), for the last three vintages. Three different white wines (Chardonnay'15, Semillon'17 and Riesling'17), two rosé wines (Grenache'16 and Pinot Meunier'17), and three red wines (Tempranillo' 16, Shiraz'15 and Pinot Noir'17) have been produced. In all the trials, strain 2940 produced consistently higher levels of 2-PE and 2-PEA than the control strain AWRI796.

When compared to the control, the increase in the production of the 'rose' aroma compounds by strain AWRI 2940 was higher in the rosé wines, followed by the red wines, and lowest in the white wines. Formal sensory analysis of the Chardonnay'15 wines found that the wine made with the strain AWRI 2940 displayed pleasant and desirable 'floral' and 'rose' aromas (Figure 9). These wines contained 7- and 37-fold higher concentrations of 2-PE and 2-PEA respectively. This was not the case for the Shiraz'15 wine though, in this particular case, the high levels of the 'rose' aroma compounds seemed to mask the overall fruit ('red'/'dark') aromas of the wines.

An industry trial of two different 2-phenylethanol (2-PE) overproducing strains (AWRI 2940 and AWRI2965) was also recently completed in Riesling and Semillon. AWRI2965 wines had a slightly different aromatic profile to AWRI2940. Chemical analysis of the wines confirmed the 2-PE overproduction phenotype of both strains in white wines. In addition, an informal sensory of the wines confirmed the pleasant 'rose'/'floral' and 'honey' aromas imparted by strain AWRI2940 in both Riesling and Semillon, as observed previously for Chardonnay.

6.4.4.Additional traits of 2-phenylethanol overproducing strains

In addition to enhanced 'floral' characters, wines made with AWRI2940 were observed to be more bitter (Figure 9). Additional chemical analysis of the pilot-scale wines produced in the last three vintages showed that both strains AWRI2940 and AWRI2965 not only overproduce 2-PE but also a range of phenolic compounds derived from the metabolism of aromatic amino acids in yeast. Two of these compounds (tyrosol and tryptophol) are particularly interesting because they have been shown to contribute to the perception of bitterness. Informal assessment of different red and white wines indicated that the perceived bitterness of white wines produced with AWRI2940 is less than that of red wines.

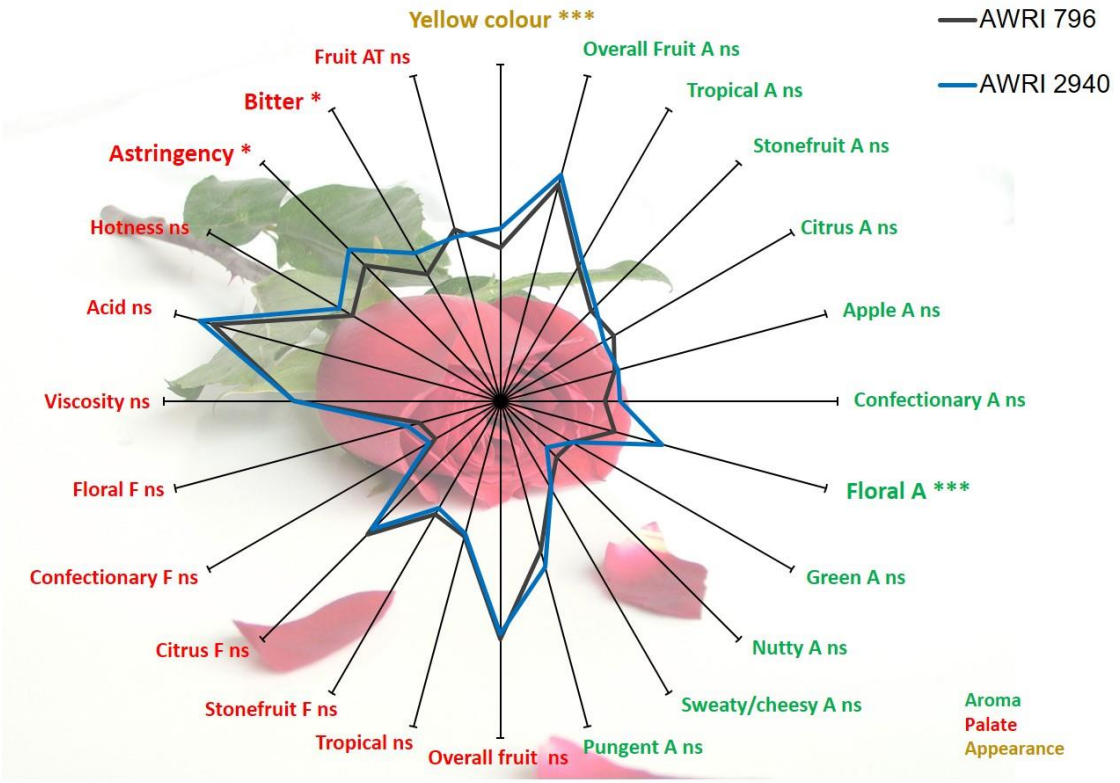


Figure 9. Mean ratings for aroma and flavour attributes for Chardonnay wines (20 L scale) produced using AWRI 796 and the 'rose' strain AWRI 2940 (n = three fermentation replicates × 10 judges). Aroma and flavour attributes are shown in green and red, respectively. ns, not significant; *p < 0.05; ***p < 0.001.

6.5. Genetic determinants of flavour profiles

6.5.1. Discovering potential genetic determinants of aroma compound production by genome wide association studies and quantitative trait locus analysis

Several candidate strains for QTL analysis exhibiting differences in VSC production, 2PE/2PEA production, and production of volatile fatty acids were tested for sporulation rate and spore viability. Due to the tendency of *S. cerevisiae* wine strains to switch mating types after sporulation and re-diploidise (mother-daughter mating), derivatives of candidate strains that have had this capacity disabled (by genetic modification) have been used. Some candidates displayed poor sporulation frequency and/or viability, or their spores were difficult to separate. The latter was resolved by troubleshooting the spore germination and separation protocol. Twelve strains with promising sporulation characteristics were re-sporulated and their stable haploid derivatives retained for further characterisation. Two batches of haploid derivatives (51 progeny from seven candidate strains for QTL) were evaluated in fermentation experiments in chemically defined grape juice, and individuals with appropriate production of volatiles (as observed for original diploid parent) selected.

Among the different volatile compounds analysed in the small-scale ferments, the production of 2-PE and 2-PEA displayed a huge variability between strains (30-fold difference). Therefore, a genome wide association study (GWAS) approach was considered appropriate to find the genetic determinant(s) involved in the formation of these 'rose' aroma compounds. For the GWAS study, a total of 20 wine strains were selected, half of them with a high 2-PE production phenotype, and the other half with the opposite phenotype (low 2-PE producers).

Three ‘gene’ candidates with high scores were identified on the basis that different versions of these genes are conserved between the high- and the low- 2-PE producers. In other words, for each of these three genes, one version is conserved amongst the high 2-PE producers, while a different version is conserved amongst the low 2-PE producers. Interestingly, only one of the three genes appeared to be associated with the pathway that leads to the formation of these aromatic compounds.

6.5.2.Demonstrating genetic determinants of phenylethanol production using CRISPR

Mutations in two genes, Tyr1 and Aro4, were identified following whole genome sequencing of PE overproducing isolates. A new technique for genomic editing (CRISPR-CAS9) was implemented to enable alteration of both gene copies in diploid industrial wine yeast strains to identify causative genetic factors in PE overproduction. Using this technique, a total of six allele swaps in the Tyr1 and Aro4 genes were successfully completed in two different yeast strain backgrounds (AWRI 1631 and AWRI 796). Both Tyr1 and Aro4 are involved in yeast amino acid metabolism, and previous findings indicated that mutations in these genes might be responsible for the enhanced ‘rose’ aroma (2-PE overproduction) of the non-GM wine yeast strains generated in this output. These allele swaps allowed evaluation of the relative contribution of different gene variants to the high 2-phenylethanol trait.

2-phenylethanol production by strains carrying mutations in either Tyr1 or Aro4 was assessed in small-scale fermentations. Chemical analysis of the wines produced confirmed that mutations in both genes contribute to the overproduction of 2-PE and 2-PEA. However, mutations in Tyr1 had a bigger impact than those in Aro4 with regards to the overproduction of both 2-PE and 2-PEA by yeast.

7. Outcome/conclusion:

7.1. Performance against planned outputs

7.1.1.Understand the potential value that can be derived from yeast genetic diversity to shape wine flavour profile and mouth-feel.

The approach of combining integrated laboratory and pilot-scale winemaking guided by genomic sequence data (project AWR1302) achieved the planned output.

Work performed towards this output has provided comprehensive volatile and non-volatile chemical datasets for laboratory-scale wines made with a significant cross-section of commercially available yeast strains. These datasets can be used to inform winemakers’ choice of yeast, and also as a reference against which to evaluate the potential novelty of newly developed strains.

Pilot-scale winemaking trials highlighted the extent of yeast impact on red wine aroma and mouth-feel, with some differences evident up to 24-months post-bottling. Reference strains shown at laboratory scale to impart divergent aroma compound profiles were incorporated into a vintage trial in 2017. Pending results of sensory analysis for these wines, it is anticipated that the data can be used to assign sensory profiles to a large number of strains currently used by the Australian wine industry.

7.1.2.*Understand the potential for more efficient processing of wine post-alcoholic fermentation through controlled timing and strength of yeast flocculation.*

This output was achieved through complementary high-throughput screening approaches to determine how many strains amongst the 94 studied in this project flocculate, and more importantly, how many flocculate late in fermentation in response to known wine-relevant parameters (low pH, high ethanol). The potential for some strains to flocculate strongly in response to a shift in temperature was also investigated.

7.1.3. *Develop knowledge of relative contributions of divergent genomes in interspecies hybrids to wine flavour profiles and winemaking outcomes, to support effective breeding and hybridisation methodology.*

Several new hybrids were generated in this project with the goal of building a collection where wine yeast parents are combined with each non-*cerevisiae Saccharomyces* parent (*S. arboricola, S. eubayanus, S. uvarum, S. kudriavzevii, S. paradoxus*). An additional focus was to incorporate strains that were unencumbered with third-party intellectual property rights.

While the AWRI was the first laboratory in the world to successfully generate hybrids between 'PDM'-clade wine yeasts and *S. arboricola* and *S. eubayanus*, it proved a significant challenge to develop hybrids with other *S. cerevisiae* parents. This slowed progress on planned investigations comparing relative contributions of *cerevisiae* and non-*cerevisiae* genomes across the intended multi-dimensional collection. Nevertheless, various hybrids were generated and their properties investigated through laboratory-scale, pilot-scale and industry fermentation trials.

7.1.4. Use genomics assisted breeding and interspecies hybridisation to develop novel yeast strains targeting traits identified as important for Australian winemakers by Wine Australia and through industry consultation, with initial priority targets of enhanced red wine flavour, production of 'rose' aromas, avoidance of faults (H_2S, VA, succinic acid), optimal flocculation behaviour, and stability in performance. Yeast strains identified in AWRI Project 3.2.3 as having desirable traits will be incorporated in strain development.

Plans to draw upon validated genetic markers by cross-breeding of IP-unencumbered strains were not fully realised due to deficiencies in mating fitness for many of the studied strains. Several potential markers were identified for production of H_2S and 'rose' aroma compounds, and potential breeding strains exhibiting high production levels for volatile compounds associated with 'red' and 'dark' fruit aromas in red wine were characterised.

A strength of this project was the implementation of multiple strain development approaches in parallel. Thus, while marker-guided breeding was not implemented, classical mutagenesis, breeding, adaptive evolution, and interspecies hybridisation were all effectively applied to yield novel strains.

Adaptive evolution was used to generate non-flocculent variants of a known heavy-flocculating wine strain, AWRI350, and a promising low-alcohol non-*Saccharomyces* strain *Metschnikowia pulcherrima* AWRI1149.

Interspecies hybrids generated in this project show promise in applications for low-temperature and high-sugar fermentations, along with sparkling wine production.

Classical mutagenesis and selection were successfully applied to isolate variants of popular wine yeast AWRI796 that either reduced production of succinic acid or increased production of volatile aroma compounds responsible for 'rose' aroma in wine.

7.2. Could changing the methodology/technology have improved the outcome?

Methodologies were adapted during the project to ensure robustness in large-scale screening of flavour compound production profiles, casting into doubt some recently published studies where insufficient replication and cross-batch validation were performed to enable comparison of large numbers of yeast strains.

Implementation of quantitative-trait-loci (QTL) methodology for optimisation of wine yeast traits was hampered by the relatively poor sporulation efficiency and spore viability of many industrial strains. This component of the project may have been more successful if all sequenced strains had been pre-screened for mating proficiency prior to choosing those to

use in fermentation screenings. To progress genetic marker validation, in parallel CRISPR-Cas9 based genome editing was implemented in years 3 and 4 of the project.

Similarly, generation of novel interspecies hybrids using different *S. cerevisiae* wine yeast parent strains proved challenging. This was partly due to apparent incompatibilities between some strains when used in rare-mating, in combination with incomplete data on parent strain traits that could be leveraged to select for hybrids. Choice of *S. cerevisiae* parental strains was guided by genetic differences (largely within or outside of 'PDM'-clade) and observed differences in flavour compound production. Screening of all strains for an expanded range of traits, including resistance to various inhibitors not relevant to winemaking, may have allowed design of more efficient hybrid-selection strategies.

7.3. Practical implications

While the impact of yeast strain on white wine style has been well established, in red varieties it has been assumed that choice of yeast strain is less important due to the time these wines spend in maturation prior to release. Results generated in this project demonstrated that young red wines made with different yeast strains differed in their aroma profiles 12 months post-fermentation and that some of these differences were evident 27-months post-fermentation. Furthermore, texture-related sensory descriptors became significantly different between yeast strains as the wines matured.

7.4. Benefits to the industry

Drawing upon datasets generated in this project and AWR1302, winemakers can make a more informed choice of yeast strain to match their winemaking goals. The observed redundancy in strain impact, where multiple strains displayed similar profiles, means that this choice can be made across yeast suppliers. This may enhance the ability of wine producers to negotiate with yeast suppliers and could thereby reduce costs of production.

Novel strains that are commercialised can be rapidly adopted by Australian winemakers, with the knowledge that support is readily available at the AWRI. In this project commercialisable strains were generated that can be used to shape wine style in unique ways, along with improved versions of existing strains (e.g. low-succinic AWRI796, non-flocculant AWRI350). Techniques developed in this project were also used to improve a low-alcohol non-*Saccharomyces* yeast strain, AWRI1149, by correcting its tendency to flocculate when inoculated into red grape must. Use of strains that impart unique flavour profiles (e.g. 'rose' aroma) can be used by winemakers to establish a point of difference in style, enhancing their ability to market their wine at a premium. The low succinic acid producing variant of AWRI796 can be used to reduce the risk of quality loss in some vintages due to acid-imbalance, thereby avoiding quality loss and downgrading.

Finally, the foundations have been laid for genetic marker guided breeding, which will enable the wine industry to rapidly draw upon available yeast germplasm to generate new strains for new purposes as they are defined.

7.5. Recommendations

The data generated in this project, and project AWR1302, provide an unprecedented foundation that can be further expanded. When suppliers introduce new strains into the Australian market, or new strains are developed in Wine Australia-funded projects, they can be benchmarked by sequencing their genomes and performing laboratory-scale fermentations alongside reference strains.

Various QTL analyses can be performed with a relatively small number of pairwise crosses, and now that CRISPR-Cas9 tools are available, downstream validation is achievable. Strains unencumbered by third party intellectual property constraints have been identified and will form the foundation of future strain development efforts. These strains, together with those

that will be identified in bioprospecting projects that are already underway, will provide the next generation of wine yeasts featuring much broader genetic and phenotypic diversity compared to wine yeasts available today.

In addition to harnessing untapped sources of genetic diversity, mutagenesis and selection have demonstrated their capacity to introduce phenotypic diversity into existing strains. This should be an ongoing approach to diversifying wine yeast phenotypes in the absence of acceptance of GM technology. The extent to which the Ehrlich pathway can be manipulated to alter or enhance wine yeast flavour profiles has not yet been fully explored and opportunities remain to produce yeast with aromatic attributes not present in the market today.

8. Appendix 1: Communication:

8.1. Communication of the outcomes

Outcomes and knowledge generated during the progress of this project have been communicated to peers through peer-reviewed publications and conference presentations. In addition, annual meetings were held with researchers at the University of Adelaide and representatives of Wine Australia to discuss updates on project progress outside of the regular six-monthly reports that were provided for the duration of the project.

Information has been extended to industry stakeholders through articles in trade journals, presentations in the AWRI roadshow program, webinars, and workshops at both the 15th and 16th Australian Wine Industry Technical Conferences.

8.2. Journal articles written during project

Solomon, M., Francis, L., Pearson, W., Barker, A., Kassara, S., Bindon, K., Smith, P. 2015. The influence of yeast strain on Shiraz wine composition and sensory properties. *AWRI Technical Review* (216): 11-14.

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9. Appendix 2: Intellectual Property

Various new yeast strains were generated during the course of this project, and those that have been used in pilot- and industry-scale winemaking trials are sufficiently characterised for evaluation of commercial application. A list of developed yeast strains is presented in the table below.

Category	Characterised strains	Parental strains
'Rose' aroma variants	AWRI2940, AWRI2965	AWRI796[1]
Low succinic acid producers	AWRI2955-2963	AWRI796[1]
S. eubayanus hybrids	AWRI3058	AWRI1540 (NT116)[2] AWRI2759 (*S. eubayanus*)[3]
S. arboricola hybrids	AWRI3051	AWRI1540 (NT116)[2] AWRI2280 (*S. arboricola*)[3]
S. cariocanus hybrids	AWRI2794	AWRI947 AWRI1528 (*S. cariocanus*)[3]
Reduced flocculation strains	AWRI3050 AWRI3878	AWRI1149[3] AWRI350[4]

[1]AWRI796 is a commercial strain licensed to AB Mauri from AWRI

[2]NT116 is a commercial strain developed by Infruitec-Nietvoorbij (South Africa) and licensed by Anchor Yeast

[3]These strains are publicly available from the CBS-KNAW (Netherlands) yeast collection and were obtained under their standard MTA

[4]AWRI350 is a commercial strain licensed to AB Mauri from AWRI

10. Appendix 3: References

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11. Appendix 4: Staff

Chris Curtin, Toni Cordente, Cristian Varela, Jennifer Bellon, Caroline Bartel, Markus Herderich, Paul Chambers, Simon Schmidt

12. Appendix 5: Supplementary Data

Table 1. AWRI numbers of yeast strains screened

	Batch 1	**Batch 2**	**Batch 3**	**Batch 4**
1	**796**	170	81	228
2	**838**	213	266	350
3	1116	722	793	**796**
4	1483	724	**796**	**838**
5	1488	735	**838**	1082
6	1490	739	1429	**1116**
7	1491	740	1497	1176
8	1493	763	**1688**	1375
9	1501	778	1709	1482
10	1502	**796**	1712	1631
11	1503	833	1736	1684
12	1504	**838**	1742	**1688**
13	1505	896	1756	1759
14	1537	934	1757	1833
15	1638	947	1758	1899
16	**1688**	1001	1776	1918
17	2078	1017	1778	2255
18	2079	1432	1795	2526
19	2776	1436	1910	2825
20		1485	2013	2826
21		1486	2077	2828
22		1487	2851	2829
23		1686	2858	2830
24		**1688**	2860	1787
25		1716	2861	2832
26		1722	2863	2852
27		1729	2865	2880
28		1781	2867	2881
29		2260	2874	2910
30			2878	2913
31				2914

13. Appendix 6: Budget reconciliation

The project's budget reconciliation statement will be submitted separately.