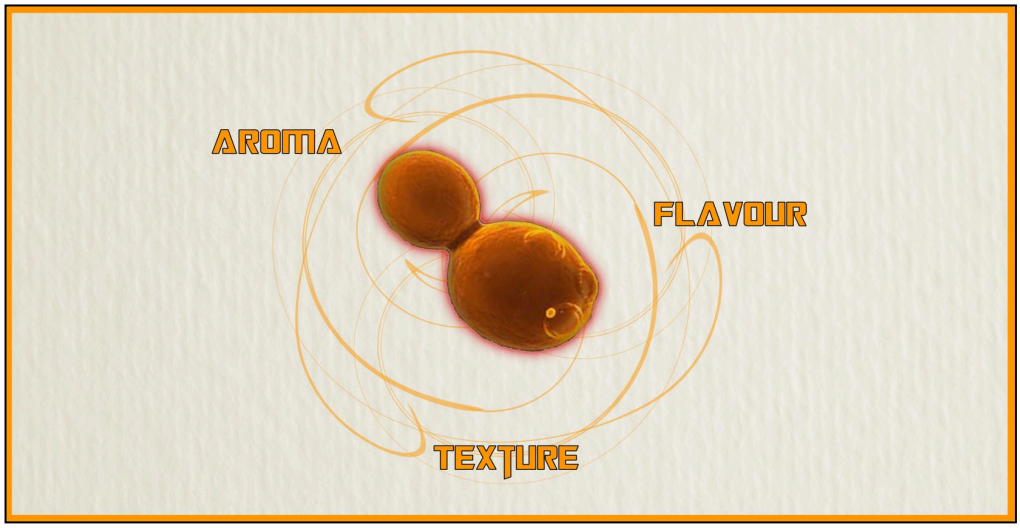
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AWRI Australian Wine Research Institute

Putting microbial diversity to work in shaping wine style



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1. Abstract

There is substantial genetic diversity both within and between different yeast species, with significant potential to provide benefits to wine producers. This project has put that genetic diversity to work by characterising existing strains to help build the foundations of future strain development, introducing new traits through targeted selection, and expanding the range of characteristics available through the introgression of genetic elements not previously accessible in a winemaking context. In achieving these tasks the project team has also developed new methods which will help to build future generations of wine yeast.

2. Executive summary

A general constraint in microbial strain development programs can be access to germplasm free of third-party IP constraints. To this end, the project team sought to identify and gain access to *Saccharomyces cerevisiae* isolates that could be used for our ongoing strain development program. Three robust *S. cerevisiae* strains were identified (AWRI 1742, 1939 and 1796), which exhibited strong fermentation kinetics, an absence of negative quality attributes and produced a variety of compounds associated with positive aromas in wine.

Physiological characterisation of yeasts isolated by Project 4.4.3 was undertaken to identify novel characteristics and opportunities for strain refinement. In total, 250 genetically distinct yeast isolates were screened on solid media for winemaking-relevant traits. Just over 1% were classified as low-H_2S producers. Seven of the 250 wild isolates exhibited potential for varietal thiol release during fermentation. One isolate exhibited robust fermentation kinetics in trial ferments and had a high-thiol-releasing phenotype.

Recent changes to the definitions of genetically modified organisms by the Office of the Gene Technology Regulator prompted an exploration of the use of CRISPR methodology to modify diploid yeast genotype. The approach typically makes use of a molecular repair feature of many organisms called non-homologous end joining (NHEJ). NHEJ is suppressed in diploid yeast, leading to low frequency of multi-allelic modification. The project team explored several approaches to overcome this issue and identified an approach that stimulates NHEJ in diploid wine yeast which substantially improved the efficiency of the CRISPR method. This development has laid the foundation for the use of this approach in the future development of robust yeast for industrial fermentations.

The non-*Saccharomyces* yeast genera *Hanseniaspora* can have a very active metabolism of the amino acid phenylalanine, which enhances the formation of benzaldehyde and benzyl alcohol, precursors to the 'flinty' compound phenylmethanethiol. The potential for strain-specific production of different benzenoid-derived compounds by a subset of *H. vineae* and *H. osmophila* strains was investigated. Even though some of the isolates from this genera produced higher concentrations of both benzaldehyde and benzyl alcohol than selected *S. cerevisiae* yeasts, this was not translated into increased formation of phenylmethanethiol.

The aromatic higher alcohols 2-phenylethanol (2-PE), tryptophol, and tyrosol are yeast-derived compounds that modulate the aroma and palate of fermented beverages such as beer, wine, and sake. A group of five variants of the widely used industrial wine strain AWRI 796, previously isolated due to their elevated production of the desirable 'rose-like aroma' compound 2-PE, were characterised in pilot-scale fermentation of a Chardonnay must. Results showed that these variants increased both the concentration of 2-PE and modulated the formation of the higher alcohols tryptophol, tyrosol, and methionol, in addition to volatile sulfur compounds derived from the amino acid methionine. This work highlighted the connections between nitrogen and sulfur metabolism in yeast during fermentation.

Such increased production of specific compounds may not suit all wine styles. Extensive trialling of three of the 'rose' yeast strains (AWRI 2940, AWRI 2965 and AWRI 4390) at pilot scale over several vintages has allowed an assessment of which wine styles may be more compatible with enhanced

'rose' aromas. It was demonstrated that the 'rose' yeast benefitted varieties with neutral profiles such as Chardonnay and Pinot Gris, sparkling Chardonnay and Grenache rosé.

The foundation yeast used for the production of the original 'rose' yeast is not an ideal commercial foundation for the production of sparkling wines. Therefore, a second generation of 2-PE overproducing yeasts was generated to improve compatibility with this winemaking style. A pilot-scale winemaking trial assessed the suitability of second generation 2-PE overproducing yeast for primary and secondary fermentation during sparkling wine production using the traditional method. The most prominent effect was achieved when the 'rose' yeasts were used during primary fermentation, with 2-PE being produced at between five to seven times higher concentration than the control. While the yeast were capable of conducting secondary fermentation, there was no compositional or sensorial benefit to their use at this winemaking stage. Both 'rose' yeasts had distinct effects on sparkling wine style. AWRI 4390 resulted in wines with more intense 'rose' and 'fruity' aromas than the control, while AWRI 2965 was characterised by an intense 'brioche' aroma.

Interspecific hybridisation is the term used when two different species are mated together. The project team has used this process to introduce genetic diversity into traditional wine yeasts and to harness traits from more distantly related yeasts that can then be used in winemaking. While the blending of genomes often results in additive combinations of characteristics, occasionally, a hybrid may exhibit characteristics different from either parent.

Two new hybrids containing partial genomes of *S. eubayanus* and *S. arboricola* were created during the project. This achievement completed the collection of possible hybrids between *S. cerevisiae* and other *Saccharomyces* yeasts with complementary mating type features from the *sensu stricto* group.

The seven hybrids in this completed set were characterised in pilot-scale fermentations of red and white grapes. The red wines differed across nine descriptive attributes. Key among the more positive attributes were fruity aromas of cherry, red berry, baking spice and vanilla. However, some strains produced wines with more prominent 'boiled egg' and 'canned vegetable' aromas. This work identified hybrid strains that appear well suited to the production of red wine.

White wines made using hybrids differed in yellow colour intensity, tropical fruit aroma, passionfruit aroma, banana aroma, honey aroma, stone fruit flavour and banana flavour. Overall, the *S. mikatae* hybrid strain AWRI 1504 appeared most favourable to white wine production, imparting stone fruit aroma and flavour.

One of the hybrids in the set, a cross between *S. cerevisiae* and *S. uvarum*, displays robust fermentation properties in high-sugar juices while producing wines with lower concentrations of acetic acid than either parent. The team set about discovering the genetic basis of the 'low acetic acid' trait in the *uvarum* hybrid. The genome of the hybrid was picked apart by sporulating it to generate hundreds of progeny. This work pointed toward the loss, or retention, of a specific *uvarum* chromosome being a defining feature in the hybrid, responsible for the strain's ability to produce a very low concentration of acetic acid.

3. Acknowledgements

This work was supported by Australia's grapegrowers and winemakers through their investment body Wine Australia with matching funds from the Australian Government. The AWRI is a member of the Wine Innovation Cluster in Adelaide, SA.

The project team collaborated with the Biochemistry and Biotechnology group of the School of Oenology in Universitat Rovira i Virgili (URV) in Tarragona (Spain) to characterise some of the novel

strains developed in this project, which has resulted in two 'Final Degree projects in Biotechnology' and the publication of a mini-review.

The team would also like to acknowledge:

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- Dimitra Capone (University of Adelaide, formerly AWRI) for her help with polyfunctional thiol analysis
- Tracey Siebert for her help with the analysis of benzenoid-derived metabolites
- Alex Schulkin for his help with the analysis of non-volatile compounds derived from aromatic amino acid metabolism.
- Joe Rossi for his help in developing an improved CRISPR-Cas9 method.

The project team collaborated with staff in Projects 3.1.3 and 3.1.4 on aspects of the work, and would like to acknowledge Keren Bindon, Stella Kassara and Luke Qi for their help with analysis and interpretation of wine phenolics and polysaccharides.

The entire sensory team of Desiree Likos, Eleanor Bilogrevic, Damian Espinase Nandorfy, Wes Pearson, Leigh Francis and panellists from the external AWRI-trained descriptive analysis panel are acknowledged for their help with the conduct and interpretation of sensory trials.

Finally, the project team would like to acknowledge Yalumba Wines, in particular Louisa Rose and Greg Nattrass, for their assistance in the conduct of commercially relevant winemaking trials.

4. Background

While many wine yeasts are currently available for winemaking, extensive genetic analysis has shown the genetic diversity among these yeasts to be extremely shallow. This limited depth means there is scope to expand the genetic diversity of wine yeasts through breeding and selection. This project builds on previous Wine Australia-funded work in which *Saccharomyces cerevisiae* was mated with non-*cerevisiae* members of the *Saccharomyces* genus to produce genetically complex hybrids and work where non-GM methods of selection were employed to develop low H_2S and low acetate producing yeasts. Together these breeding and selection strategies deliver non-genetically modified germplasm for winemakers seeking a point of differentiation in their wines.

A review published on flavour-active wine yeasts (Cordente et al. 2012) discussed the yeast 'flavour phenotypes' available to winemakers and a more recent review detailed the nuances of secondary metabolite production via aromatic amino acid metabolism (Cordente et al. 2019). These reviews highlighted the range of aromatic products that can enhance fermented beverage flavour and revealed the limited level of understanding about the environmental and genetic factors that determine their production. The potential of non-*Saccharomyces* yeast has also been detailed in a review by Jolly et al (2014). These reviews suggest that the bioconversion potential of non-*cerevisiae* and non-*Saccharomyces* yeast can be harnessed to the benefit of commercial fermentation industries.

The AWRI is well placed, with a reservoir of Australian yeast germplasm to draw upon (Project 3.2.3). Previous projects have generated yeast flavour volatile profile datasets in defined medium and Chardonnay juice (AWR 1301) providing a baseline understanding of strain-dependent characteristics that can be employed. It is still the case that inoculation with selected active dry wine yeast represents one of the lowest cost inputs in wine production, yet with one of the most significant impacts on winemaking outcomes. In putting the available diversity to work, the current project has contributed a set of tools that winemakers can use to help achieve their stylistic ambitions.

5. Project aims

This project, together with others underway in the Australian grape and wine community, contributed to the following broad aims:

- An improved toolbox to create a target wine style.
- New or enhanced yeast and bacterial germplasm to enhance winemaking processes and realise performance and economic gains, through improved fermentation efficiency and differentiation of wine products/styles.

The project objectives were to:

1. Review existing yeast strain stock to identify opportunities for strain refinement
2. Review microorganisms identified by the bioprospecting project (4.4.3) to identify opportunities for strain refinement
3. Develop new interspecies hybrids to complete the hybrid portfolio
4. Develop methods to produce non-GM yeasts through marker-assisted breeding
5. Develop non-GM wine yeast with modified aromatic profiles from alterations to amino acid biosynthetic pathways
6. Produce non-GM yeasts through marker-assisted breeding
7. Conduct pilot- and industry-scale trials evaluating new yeast strains
8. Deliver practical advice and extension materials for winemakers.

In addressing these objectives, the intention of the project was to identify yeasts with desirable characteristics to serve as a foundation for subsequent development, make use of those yeasts to create second-generation yeasts with specific use cases front of mind, and to expand the available genetic diversity of wine yeast through targeted introduction of novel genetic material.

6. Methods

6.1 Laboratory-scale fermentations

Laboratory-scale fermentations were performed in triplicate in different fermentation media, including an unfiltered Pinot Noir must, a filtered Chardonnay must and a chemically defined must (CDM). Fermentations were conducted at 17°C in 100 or 250 mL Schott bottles fitted with stir bars. The lids of the Schott bottles were fitted with gas detector tubes to measure the release of H_2S during fermentation. Fermentation progress was followed by CO_2 weight loss, measured every 24 hours. All yeast strains were obtained from the AWRI wine microorganism culture collection.

6.2 Pilot-scale winemaking

6.2.1 Pilot-scale winemaking of Grenache

Small-lot wines were produced by WIC Winemaking Services, according to a standardised red winemaking protocol. Grenache grapes (2017 vintage) from a high-value McLaren Vale vineyard were hand-picked. Crushed and destemmed grapes were apportioned into 50 kg lots and inoculated with different yeast strains in triplicate and fermented on skins at 20°C. Wines were pressed and seeded for malolactic fermentation. Wines were bottled in screw-cap-sealed bottles (375 mL). All yeast strains were obtained from the AWRI wine microorganism culture collection (AWRI 778, 1776, 1833, 2260, 2878 and 2914).

6.2.2 Pilot-scale winemaking of Chardonnay

Small-lot wines were produced by WIC Winemaking Services, according to a standardised white winemaking protocol. Chardonnay grapes from high-value vineyards from the Adelaide Hills (2015 vintage) and McLaren Vale (2018 vintage) regions were hand-picked. Nineteen-litre ferments were conducted in triplicate at 15°C. Once the alcoholic fermentation had finished, the wines were sulfured, cold-stabilised and bottled in screw-cap sealed bottles for sensory and chemical analysis. In the 2015 vintage, the control AWRI 796 and the high-2-PE producing strain AWRI 2940 were evaluated. In the 2018 vintage, the control AWRI 796 and the five 'rose' strains AWRI 2936, 2940, 2965, 4124 and 2969 were evaluated.

6.2.3 Pilot-scale winemaking of sparkling Chardonnay

Small-lot wines were produced by WIC Winemaking Services, according to a standardised sparkling winemaking protocol. The wines were produced using the traditional method (bottle fermented), with hand-picked Chardonnay grapes from the Adelaide Hills (2019 vintage). Different strain combinations were used for the primary and secondary fermentations. Chardonnay must was partitioned into 30-litre vessels and inoculated with either the control strain (PDM) or one of the two 'rose' yeasts (AWRI 2965 or 4390). After completion of both alcoholic and malolactic fermentations, base wines were cross-flow filtered. Sugar was added to each of the base wines at a level that would result in ~6 Bar pressure following secondary fermentation. The base wines were then split and inoculated with either the control or one of the 'rose' yeasts to initiate secondary fermentation, which was completed in-bottle. The wines were matured for eight months on yeast lees and disgorged, with sugar added to achieve a style similar to a Brut wine.

6.2.4 Pilot-scale winemaking Riesling – hybrid trial

A vintage trial was conducted (in triplicate fermentations) at Yalumba using Riesling grapes sourced from the Barossa Valley. Grapes were crushed and chilled to 14°C before being settled. Clarified juice was transferred to 200 L stainless steel barrels and inoculated with AWRI 838, AWRI 1501 *S. paradoxus*, AWRI 1502 *S. cariocanus*, AWRI 1503 *S. kudriavzevii*, AWRI 1504 *S. mikatae*, AWRI 3060 *S. eubayanus* and AWRI 4122 *S. arboricola*. Wines received a sulfur dioxide addition (60 mg/L) at completion of fermentation and were then bentonite fined and cross-flow filtered to bottle.

6.2.5 Pilot-scale winemaking of Shiraz - hybrid trial

The Shiraz vintage trial was undertaken in triplicate 50 L fermentations by WIC Winemaking Services using grapes sourced from the Barossa Valley. Crushed grapes received pectinase (AB Biotek Rohavin L) and SO_2 (50 mg/L) additions prior to yeast inoculation and Mauriferm Activator (250 µg/L) addition. Wines were inoculated with the following yeast; AWRI 838, AWRI 1501, AWRI 1502, AWRI 1503, AWRI

1504, AWRI 1505, AWRI 3060 and AWRI 4122. MLF bacteria (Pinnacle Malosafe) were inoculated on the third day of fermentation. At fermentation completion wines received another SO_2 addition (80 mg/L) after which they were cold settled, filtered and bottled.

6.3 Analysis of benzenoid-derived compounds

A GC-MS analytical method for benzaldehyde, a potential precursor of phenylmethanethiol (PMT), as well as the related compounds benzyl alcohol, benzyl acetate and benzoic acid was developed. The methodology developed is described in Espinase Nandorfy et al. (2022, in preparation).

6.4 Analysis of volatile sulfur compounds

Volatile sulfur compounds were analysed according to Siebert et al. (2010) and Cordente et al. (2022) using static HS-GC-SCD on an Agilent 7890B GC, coupled to an Agilent 8355 SCD and equipped with a Gerstel MPS2 XL autosampler.

Polyfunctional thiols (including PMT and varietal thiols 3-MH, 3-MHA and 4-MMP) were initially quantified by LC-MS/MS as per Capone et al. (2015). After modifications to improve sample throughput, polyfunctional thiols were quantified as described in Cordente et al. (2022).

6.5 Analysis of volatile fermentation-derived compounds

Targeted analyses of fermentation-derived compounds (higher alcohols, acids and esters) were performed by Metabolomics SA (Adelaide) by GC-MS using a stable isotope dilution assay (Siebert et al. 2005).

6.6 Analysis of non-volatile compounds

The concentrations of sugar, ethanol, glycerol and organic acids were measured by HPLC using a Bio-Rad HPX-87H column (Nissen et al. 1997). The higher alcohols tyrosol (TyrOH), tryptophol (TOL) and its sulfonated adduct tryptophol-2-sulfonate (TOL-SO_3H) were analysed on an Agilent 1200SL HPLC using a Phenomenex Kinetex PFP column, as described in Cordente et al. (2021).

6.7 Analysis of haze-forming proteins

The concentrations of haze-forming proteins, including chitinases and thaumatin-like proteins, were measured using ultrahigh-performance liquid chromatography (UHPLC) (Culbert et al. 2017).

6.8 Protein heat stability test

Protein stability of wines was assessed by heating the wines to 80°C followed by measuring wine turbidity using nephelometry (Pocock & Waters 2006).

6.9 Sensory analysis

The detailed sensory studies conducted for the different pilot-scale winemaking trials used a quantitative sensory descriptive analysis method as described in (Heymann et al. 2014). All panellists were part of the external AWRI-trained descriptive analysis panel and had extensive experience in wine sensory descriptive analysis.

6.10 Polysaccharide analysis

Polysaccharide and individual monosaccharide moieties were analysed using dialysis size exclusion/hydrolysis preparation and subsequent HPLC detection and quantification (Bindon et al. 2016).

6.11 Analysis of tannin concentration

Total tannin concentration was determined using a methylcellulose precipitable tannin (MCPT) assay with relative UV absorbance at 280 nm (Mercurio et al. 2007).

6.12 Wine colour and phenolics analysis

Total phenolics and wine colour measures were calculated using the modified Somers colour assay (McRae et al. 2012).

6.13 *S. cerevisiae* x *S. uvarum* hybrid (AWRI 1572) daughter strain generation

Mitotically growing cells were transferred to acetate medium to induce sporulation. Clonal isolates of daughter strains were formed by the transfer of individual spores to nutrient medium using a micromanipulator.

6.14 Characterisation of acetic acid production phenotype

Fermentations (10 mL) were conducted in Chardonnay juice. At completion of fermentation, wines were analysed for acetic acid concentration (Boehringer Mannheim enzymatic bioanalysis kit).

6.15 Genetic mapping of acetic acid phenotype

Hybrid strains were categorised into two pools based on acetic acid production: high and low acetate producers. DNA from cohort pools was sequenced using an Illumina NovaSeq by the Ramaciotti Centre for Genomics. Raw sequence reads were mapped to a reference genome. The reference genome was a digital concatenation of the reference genomes of *S. cerevisiae* and *S. uvarum*. The average read depth in overlapping 10 kb windows was divided by the overall average read depth of the strain (mean centred) and $\log_2$ transformed.

6.16 Generation of hybrid yeast with loss of *S. uvarum* chromosome 4

A construct was designed that programmed a large protein to attach to the centromere region of *S. uvarum* chromosome 4 (Reid et al. 2008). Integration of the construct into the hybrid genome inactivated the centromere, resulting in loss of *S. uvarum* chromosome 4 in some mitotic progeny. A chromosomal marker system (Bellon et al. 2018) was used to confirm the loss and/or retention of each of the 16 chromosomes of *S. cerevisiae* and *S. uvarum* in the resultant progeny.

6.17 *S. uvarum* chromosome 4 segmental and gene deletions

Chromosomal regions were deleted in segments with a selectable marker construct (Geneticin antibiotic resistance) designed to loop out regions along the length of *S. uvarum* chromosome 4. Gene deletions were obtained by inserting the selectable marker construct into genes of interest, thus inactivating the gene.

7. Results and discussion

7.1 Review of existing yeast strain stock to identify opportunities for strain refinement

7.1.1 Characterisation of winery isolates strains for our breeding program

A general constraint in strain development programs can be access to germplasm free of third-party IP constraints. To this end, the project team sought to identify and gain access to *S. cerevisiae* isolates which could be used for our ongoing strain development program. Three robust *S. cerevisiae* strains were identified within the AWRI wine microorganism culture collection. These strains (AWRI 1742, 1939 and 1796) had been isolated from natural fermentations in a commercial winery in South Australia. Whole genome sequencing had previously demonstrated that two of the strains (AWRI 1939 and 1796) were part of the PdM clade which contains almost a third of the strains used by the wine industry (Borneman et al. 2016), while AWRI 1742 was a member of a more genetically diverse group.

The contribution of these strains to the chemical composition of wine was assessed in laboratory-scale fermentations of Chardonnay. All three strains produced low concentrations of a series of compounds associated with negative aromas in wine (volatile sulfur compounds and acetic acid) while producing varied concentrations of compounds associated with positive aromas. In particular, AWRI 1742 stood out as producing high concentrations of esters associated with 'fruity' aromas. The three strains also exhibited strong fermentation kinetics in Chardonnay. These positive fermentation characteristics, together with the ability to sporulate and a high percentage of viable spores, confirmed these strains to be an excellent set of resources for future strain development programs.

7.1.2 High-throughput screening of *Saccharomyces cerevisiae* in solid media for relevant winemaking traits

While hundreds of wine yeasts are commercially available for winemaking, extensive genetic analysis has shown the genetic diversity among these yeasts to be extremely shallow. An important alternative source of genetic diversity is the environment of winemaking itself. A separate project running in parallel, Project 4.4.3 Bioprospecting Australian microbial genetic diversity, tapped into that reservoir and isolated a large number of previously uncharacterised wine yeast strains. The isolated yeasts were genetically characterised as part of that project.

Project 3.2.1 embarked on a physiological characterisation of the yeasts isolated by the Project 4.4.3 team to identify novel characteristics and opportunities for strain refinement. A group of genetically distinct wild *S. cerevisiae* isolates, numbering 250 in total, was screened on solid media for winemaking-relevant traits (H_2S production, 'tropical' thiol release and ability to grow in low nitrogen concentrations). These strains were compared to a set of 92 *S. cerevisiae* strains from the AWRI culture collection, which contained a significant percentage (50%) of commercial wine yeasts with known winemaking traits.

Most wild isolates had a moderate to high-H_2S production potential on solid media. Just over 1% of the strains (three out of 250) could be classified as low-H_2S producers. A significantly larger percentage of low-H_2S strains were present within the cohort of commercial wine yeasts (8.7%), which indicates that, to some extent, a low-H_2S phenotype has been selected by commercial manufacturers.

The ability of a strain to grow on a solid media with the amino acid cysteine as the sole nitrogen source was used as a high throughput method to screen yeast's potential to release 'tropical' thiols during fermentation. Seven of the 250 wild isolates (2.8%) displayed strong growth on this selective media, while 4% of strains showed moderate growth. Similar percentages were observed within the reference group, with 3.2% and 5% of strains showing strong and moderate growth on cysteine, respectively.

The seven wild isolates that grew well on cysteine were later evaluated in laboratory-scale fermentations of Chardonnay for their ability to release the 'tropical' thiols 3-MH and 4-MMP. The wild isolates were compared with two commercial wine yeast controls (a low- and a high-thiol-releasing strain). Two of the seven wild strains (wild-6 and 7) produced significantly higher concentrations of 3-MH than those released by the low-thiol control strain AWRI 796 (Figure 1). Of particular interest was wild-7, which exhibited robust fermentation kinetics together with a high-thiol-releasing phenotype.

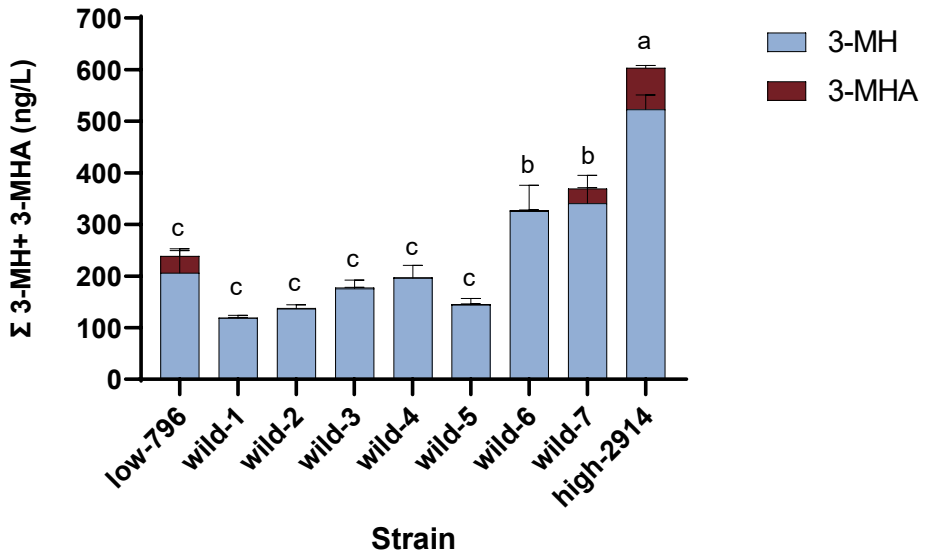


Figure 1. Concentration of thiols 3-MH (blue) and 3-MHA (red) after fermentation of a Chardonnay must (100 mL). Seven *S. cerevisiae* wild isolates were assessed, alongside a low- (AWRI 796) and a high-thiol-releasing (AWRI 2914) control.

7.1.3 Screening of non-*Saccharomyces* yeast for the production of benzenoid-derived compounds associated with 'flinty' characters

'Flint' is a highly sought-after character in some white wine styles, particularly in segments of the premium Australian Chardonnay market. Phenylmethanethiol (PMT), also known as benzylmercaptan, has been associated with this 'flint' character in wine. The benzenoid-derived compound benzaldehyde is believed to be one of the two precursors of PMT, the other being the sulfur donor H_2S.

The non-*Saccharomyces* yeast *Hanseniaspora vineae* has a very active metabolism of the amino acid phenylalanine, which results in the formation of benzaldehyde and benzyl alcohol at concentrations higher than those produced by *S. cerevisiae*. Therefore, the project team investigated the potential for strain-specific production of different benzenoid-derived compounds by a subset of *H. vineae* strains from the AWRI culture collection, as well as by the related species *Hanseniaspora osmophila*.

Seven of the nine strains (four *H. vineae* and three *H. osmophila*) were able to finish alcoholic fermentation in CDM, although at a lower rate than the *S. cerevisiae* controls. While the *H. vineae* strains produced significantly higher concentrations of benzaldehyde and benzyl alcohol than their *S. cerevisiae* counterparts, this did not translate into increased formation of PMT (Figure 2). The low overall production of H_2S by *H. vineae* during fermentation is one possible explanation for the poor conversion of benzaldehyde to PMT. On the other hand, the three *H. osmophila* strains produced levels of PMT similar to that of the *S. cerevisiae* strains (Figure 2). This aspect of the project complements objectives from Project 3.4.2 (Sulfur compounds).

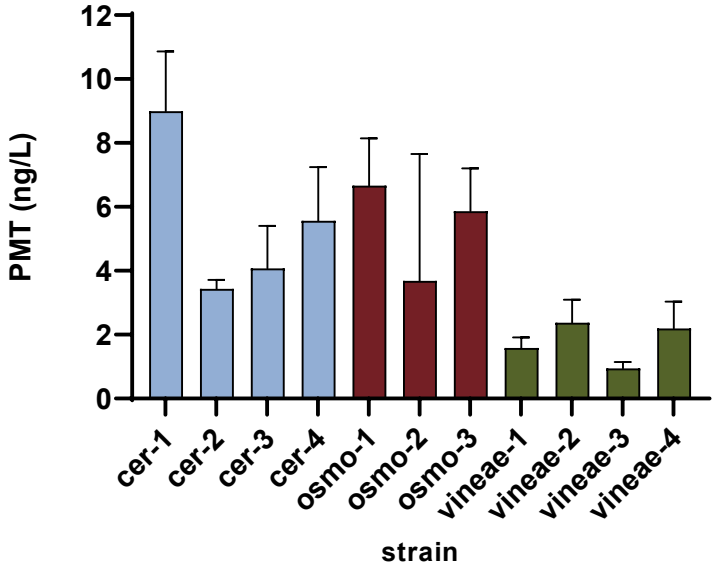


Figure 2. Formation of the 'flinty' compound PMT after fermentation of 100 mL of a chemically defined medium. Seven non-*Saccharomyces* strains were assessed, including three *H. osmophila* (red) and four *H. vineae* (green). Four *S. cerevisiae* strains were used as reference (blue).

H. vineae is also characterised by its ability to produce high concentrations of the 'rose' aroma compound 2-phenylethyl acetate (2-PEA). The four *H. vineae* strains produced extremely high concentrations of 2-PEA (~ 30 times more than the *S. cerevisiae* strains) in CDM, as well as high concentrations of less desirable compounds such as acetic acid and ethyl acetate. Interestingly, osmo-3 produced exceptionally high concentrations of the 'fruity' ethyl esters, ethyl 2-methylpropanoate, ethyl 2-methylbutanoate and ethyl propanoate. These results identified one of the *H. osmophila* strains (osmo-3) as a promising candidate worthy of further characterisation. Its potential for production of the 'flinty' compound PMT and 'fruity' ethyl esters while displaying similar fermentation kinetics to commercial *S. cerevisiae* wine strains is a desirable combination of traits.

7.2 Yeast modulating red wine aroma: formation of 'tropical' thiols

Yeast can produce a wide range of flavour and aroma compounds during winemaking. This variation in the potential for flavour compound production means that wine yeast choice can be a simple and effective way to optimise and differentiate wine style while conducting risk-free fermentations. One of the difficulties with choosing an appropriate strain is that our understanding of the influence on wine style imparted by different yeast strains is incomplete. In particular, surprisingly little information is available about the effect of yeast strains on the final flavour and aroma of red wines.

To fill this gap, the project team undertook a series of experiments to evaluate the effect of yeast strain on Grenache. Six strains from the AWRI wine microorganism culture collection, whose diverse volatile aroma profiles in laboratory-scale fermentation of Shiraz had previously been demonstrated, were chosen for the work. Pilot-scale (50 kg) fermentations were conducted and an analysis of volatile aroma compounds in the resulting wines confirmed significant strain-dependent variations in wine composition. The wines varied in the concentration of the 'tropical' thiols 3-mercaptohexan-1-ol (3-MH) and 3-mercaptohexyl acetate (3-MHA), and also a range of acetate esters associated with 'fruity' characters.

Sensory descriptive analysis of the wines confirmed that the differences in wine composition resulted in important differences in aroma and flavour. Two of the strains (AWRI 2914 and 1833) produced wines that were rated highly in some desirable aroma attributes such as 'red fruit' and 'cherry/confectionery', and rated lowest in reductive ('drain') aromas (Figure 3). These results showed that the choice of yeast strain for primary fermentation can greatly affect the final aromas of

Grenache wines. This study shed light on how yeast strain choice can be used to positively influence red wine style while avoiding the formation of undesirable compounds. The results were published in an industry article in *AWRI Technical Review* (Cordente et al. 2018) and a peer-reviewed article (Cordente et al. 2022).

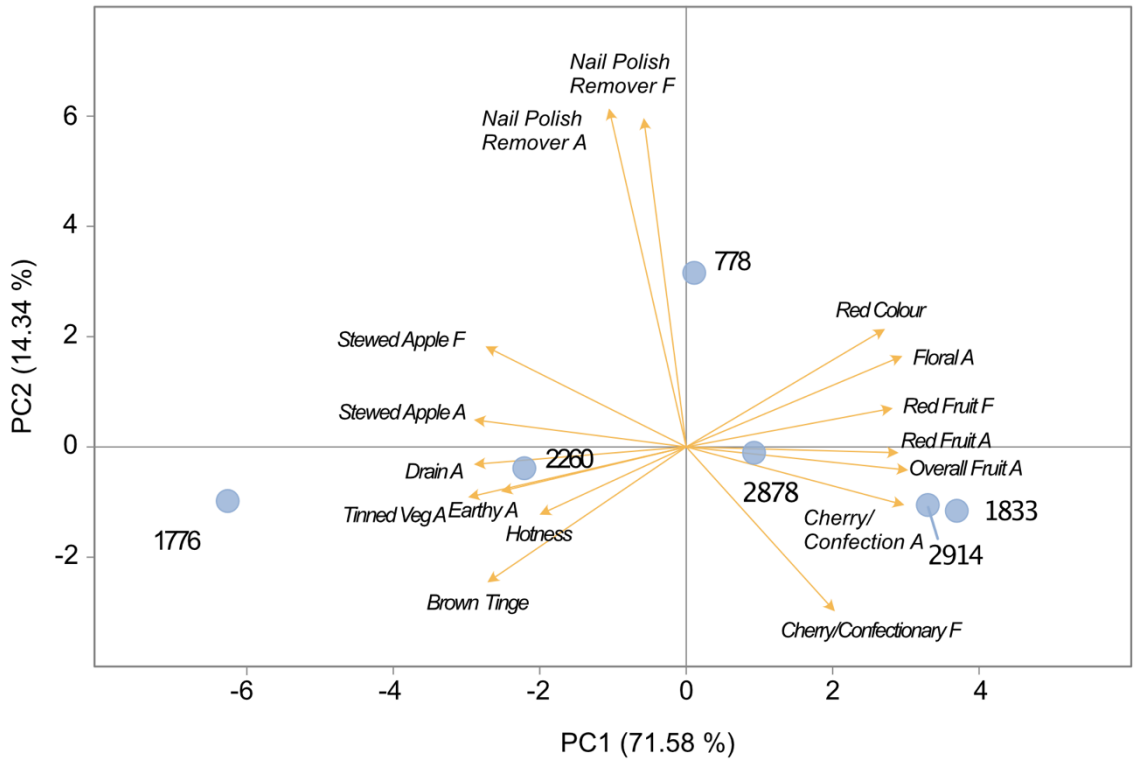


Figure 3. Principal component (PC) biplot of significant (P<0.05) and close to significant sensory attributes (P<0.10) on PC1 and PC2 for the six yeast strains in the Grenache wines. A: Aroma, F: Flavour.

The results of the Grenache study prompted an in-depth exploration of 'tropical' thiol release by yeast in other red cultivars. Although the formation of 'tropical' thiols has been extensively studied in white wines, little is known about their formation and sensory impact in reds. This aspect complemented other research objectives from Projects 3.4.2 (Formation and fate of sulfur compounds associated with negative attributes in wines) and 3.1.1 (Identification and control of volatile compounds responsible for important sensory attributes).

Previously, the yeast *IRC7* gene, encoding an enzyme with β-lyase activity, was identified as the primary genetic determinant affecting the release of 'tropical' thiols during wine fermentation. A molecular tool based on *IRC7* genotype was developed to predict yeast potential for thiol release during fermentation. The molecular assessment permitted the classification of 179 wine yeast strains as low-, moderate- or high-thiol releasers. The classification was validated for a subset of 80 strains in laboratory-scale fermentation of a chemically defined must (CDM). Overall, the characterisation of wine yeast according to *IRC7* status showed that most commercial yeast strains are not very efficient at releasing thiols from their precursors. Further information can be found in Cordente et al. (2019).

As a further demonstration of the utility of the β-lyase *IRC7* as a molecular marker, the project team evaluated 11 *S. cerevisiae* strains, with varying *IRC7* genotype status, in laboratory-scale fermentations of a Pinot Noir must. These ferments exhibited marked differences in 3-MH concentration, which varied by almost 18-fold in the resulting wines (Figure 4). The variation in 3-MHA concentration was even larger, nearly 43-fold. A strong positive correlation between yeast β-lyase activity and thiol release was observed in the Pinot Noir wines. These results were published in Cordente et al. (2022).

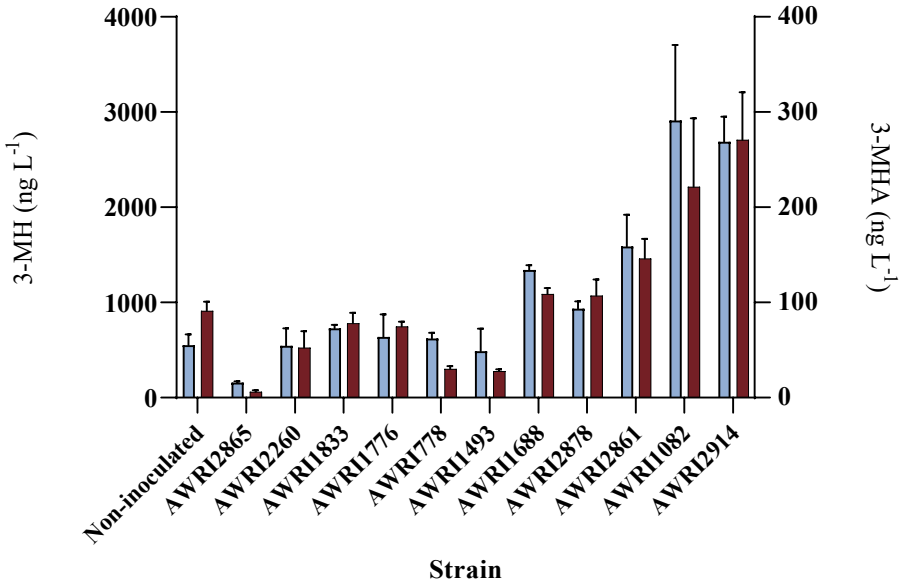


Figure 4. Concentration of thiols 3-MH (blue) and 3-MHA (red) in Pinot Noir wines (200 mL) made using eleven different yeast strains. All bars show the mean of three treatments with error bars showing standard deviation. The yeast strains are shown in ascending order according to their specific β-lyase activity.

7.3 Methods available for producing non-GM yeasts through marker-assisted breeding

The yeast *ARO9* gene encodes an enzyme with aminotransferase activity, which is involved in aromatic amino acid metabolism in yeast. A higher expression of this gene has been linked with increased production of aromatic amino acids and the higher alcohols, 2-phenylethanol (2-PE) and tryptophol (TOL). A reporter gene system was developed using the promoter region of *ARO9* (Aro9pr) to drive expression of an enzyme that exhibits bright blue fluorescence (BFP). Simple spectrophotometric methods could then be used to assess a strain's 2-PE production potential. The activation of Aro9pr::BFP and consequent yeast strain fluorescence correlated well with the ability of a group of strains to produce 2-PE. This reporter system could be used as a sensor to characterise environmental factors contributing to 2-PE production by yeast. Further information about this technology can be found in Cordente et al. (2021).

Whole genome sequencing of a subset of 16 strains that overproduce the 'rose' aroma compound 2-PE showed that these strains accumulated mutations in two genes (*TYR1* and *ARO4*) involved in the biosynthesis of aromatic amino acids. Specific mutations in these two genes were proven to be responsible for the overproduction of 2-PE by these strains. These two genes can be used as molecular makers for the breeding of strains with enhanced 'rose' aromas. Further information can be found in Cordente et al. (2018).

As described in section 7.2 the *IRC7* gene can be used as a molecular marker for the breeding of strains with enhanced 'tropical' thiol production. Further information can be found in Cordente et al. (2019).

7.4 Non-GM wine yeast with modified volatile or non-volatile profiles from alterations to amino acid biosynthetic pathways

Across the wide range of commercial yeast strains available to winemakers there is substantial variation in their production of aroma compounds, but some desirable compounds are not typically produced at sufficiently high concentrations to affect wine aroma and flavour. Examples include the yeast-derived compounds 2-PE and 2-PEA, which are associated with 'rose/floral' aromas in wine.

Generally, the concentrations of these compounds in wines are below their aroma sensory threshold, particularly in white wines, so their potential contribution to wine aroma is considered to be minimal.

7.4.1 Stop and smell the roses: novel yeast that impart 'floral' aromas in wine

By using a classical yeast strain development technique, which involved the use of toxic amino acid analogues as a selective agent to drive alterations in amino acid metabolism, more than 60 non-genetically modified (non-GM) yeast that produce high concentrations of the 'rose' aroma compounds 2-PE and 2-PEA were generated.

The first generation of 'rose' yeasts derived from the commercial wine strain AWRI 796 was produced in a previous Wine Australia funded project. Pilot-scale Chardonnay wines (2015 vintage) produced with one of these strains, the highest 2-PE producer AWRI 2940, were rated notably higher for 'floral' aroma than those made with a control strain. Interestingly, AWRI 2940 also produced wines with a more intense 'yellow colour intensity' and were more bitter than the control wine. Compositional analysis showed that AWRI 2940 not only produced higher concentrations of 2-PE, but also of the related higher alcohols tyrosol (TyrOH) and TOL.

7.4.2 Effects of yeast-derived higher alcohols on aroma and palate attributes during Chardonnay ageing

In this project, a second group of four 'rose' strains derived from AWRI 796 was characterised. These strains exhibited a spectrum of 2-PE, TyrOH and TOL production potential. To determine how variations in these Ehrlich pathway-derived higher alcohols modulate sensory properties (aroma, palate and appearance) of white wines, Chardonnay wine was produced (2018 vintage) using these 'rose' strains. The wines were assessed by a trained sensory panel providing a quantitative descriptive analysis of the wines after cellaring in bottle for three and 15 months.

After three months, the only wine perceived as being more 'floral' was the one with the highest 2-PE concentration (AWRI 2940). The other strains in the trial were not perceived differently from the control, despite the wines containing between 2- and 12-fold higher 2-PE concentrations. Interestingly, 'floral' aromas were more prominent after 15 months in bottle, highlighting the stability of higher alcohol-based aromas such as 2-PE, as opposed to some 'fruity' or 'estery' aromas that tend to decrease with time in bottle. At 15 months, the intensity of the 'floral' aromas in wine showed a strong positive correlation with the concentrations of both 2-PE and 2-PEA produced by the different wine yeast.

This trial also shed light on the effect of the higher alcohol TOL on the palate of white wines. Unlike the related higher alcohols 2-PE and TyrOH, which were found to be quite stable with time in bottle, a substantial percentage of the initial TOL produced by yeast during fermentation was converted into its sulfonated adduct, tryptophol-2-sulfonate (TOL-SO_3H), during storage. The equilibrium between TOL and its adduct was achieved after six months in bottle, and longer storage time (up to 18 months) did not lead to further increases in the concentration of TOL-SO_3H. Wines made with the highest TOL producer AWRI 2940 were scored notably lower in 'sweetness' and slightly higher in 'bitterness'. A strong negative association was observed between the attribute 'sweetness' and the compounds TOL and TOL-SO_3H, and these two compounds were also positively associated with 'bitterness'. In relation to TyrOH, the results showed a limited effect of this compound on white wine's palate attributes.

Of the five 'rose' strains assessed, AWRI 2965 showed the highest potential to be used as a white winemaking yeast. This strain imparts accentuated and lasting 'rose/floral' aromas to wines, with a good palate and no off-flavours. These results have been published in an industry article (Cordente et al. 2021), and in a peer-reviewed paper (Cordente et al. 2021).

7.4.3 Second generation of 'rose' strains: bringing floral aromas to different yeast backgrounds for sparkling wine production

Another factor influencing the performance of these 2-PE enhancing isolates is the parent strain from which they were originally derived. With poor pH and SO_2 tolerance, the original parent is not optimal for sparkling wine production. A second generation of amino acid analogue resistant strains (24 in total) were produced in two alternative genetic backgrounds, AWRI 1742 and AWRI 1939, to overcome this issue. In particular, AWRI 1939 was initially considered to be a good candidate for sparkling wine production as strains with a similar genetic background (PDM and EC1118) are widely used in the wine industry for this purpose. The overproduction of 2-PE and 2-PEA by the second generation of 'rose' strains was confirmed in laboratory-scale fermentations in both a CDM and a Chardonnay must. Similar to the first generation of yeast derived from AWRI 796, these second-generation strains exhibited a spectrum of 2-PE and TOL production potential.

In collaboration with the University Rovira i Virgili (URV) in Spain, three 'rose' strains derived from AWRI 1742, the moderate 2-PE producer AWRI 4235 and the high 2-PE producers AWRI 4234 and 4236, were fully characterised in CDM. Strain AWRI 4235 showed the shortest lag time and reached the highest population density of all the strains tested. The high population densities achieved contributed to this strain achieving fermentation times faster than those of the parent from which it was derived.

Strains AWRI 4234 and 4236 were characterised by slower fermentations kinetics, and very high production of the higher alcohols 2-PE and TOL. Genome sequencing confirmed that all three 'rose' strains shared a similar, previously uncharacterised, mutation in the same amino acid (Cys108) of Tyr1p, an enzyme involved in the biosynthesis of tyrosine. A 3-D model of Tyr1p was constructed, and Cys108 was found to be located adjacent to the catalytic amino acid Ser107. It was hypothesised that mutations in the vicinity of the critical Ser107 were likely to negatively affect the activity of Tyr1p, which might explain the overproduction of 2-PE by these strains.

One of the second-generation isolates, AWRI 4390 (derived from AWRI 1939), was selected for a pilot-scale winemaking trial to assess its suitability for sparkling wine production, both during primary and secondary fermentation. The moderate 2-PE producer AWRI 2965 (derived from AWRI 796), and the widely used commercial strain PDM, were also included in the trial. The sparkling wines were produced using the traditional method (bottle fermented) using Chardonnay grapes.

The most prominent effect was achieved when either of the two 'rose' yeast were used during primary fermentation, with concentrations of 2-PE produced between five and seven times higher than the control PDM. The amount of 2-PE produced during secondary fermentation was limited compared to that produced during primary fermentation. Formal sensory analysis of the wines showed that yeast strain choice during primary fermentation had the greatest sensory impact (Figure 5). Even though both 'rose' yeast produced similar amounts of 2-PE, their impact on sensory attributes was markedly different: AWRI 4390 resulted in wines with more intense 'rose' and 'fruity' aromas than the control, while AWRI 2965 was characterised by an intense 'brioche' aroma.

Despite the moderate levels of TOL (below 10 mg/L) produced, no significant conversion into the sulfonated adduct was observed after approximately 10 months in bottle. This suggests that this reaction might depend on the physico-chemical characteristics of the wine such as pH, temperature, SO_2 and O_2 concentrations. In summary, the results from this sparkling winemaking trial indicated that AWRI 4390 may offer winemakers an alternative yeast with which to produce a distinctive sparkling base wine with desirable 'floral' aromas, while also exhibiting robust fermentation kinetics. These results have been published in Cordente et al. (2021).

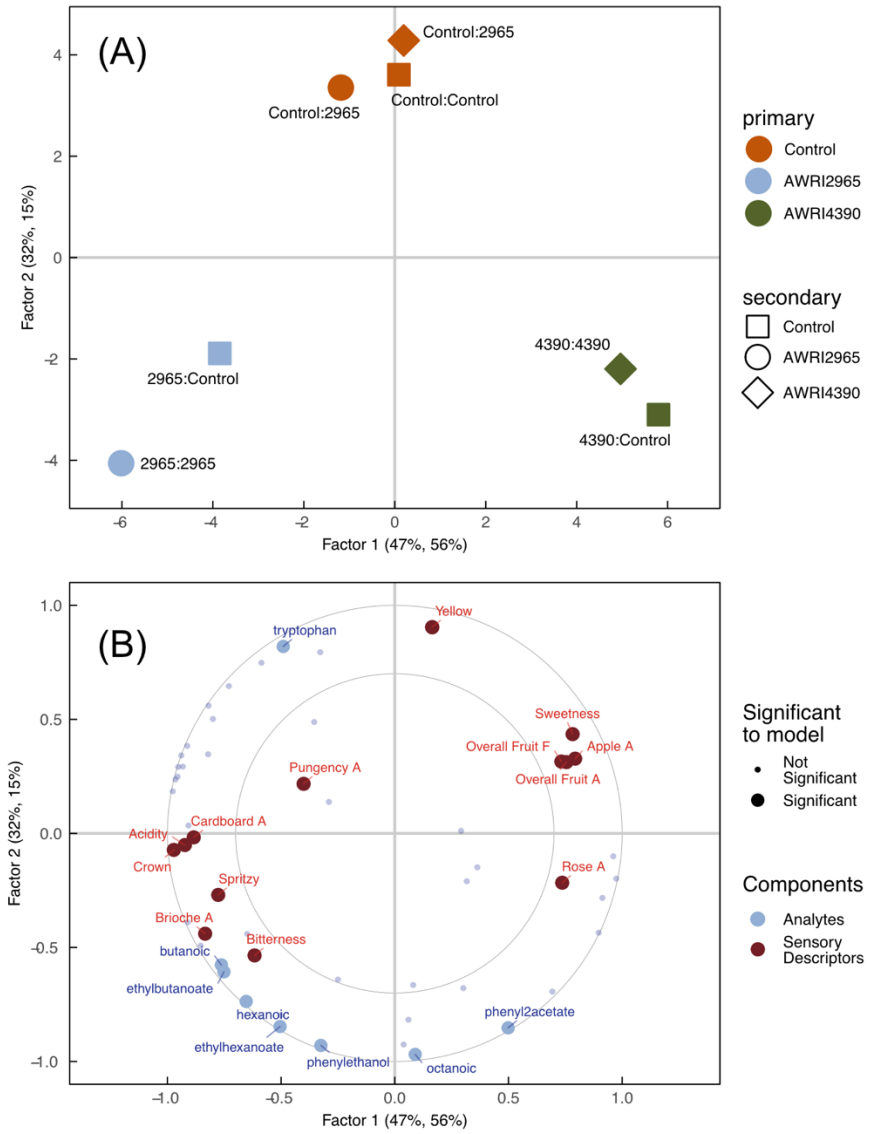


Figure 5. The effect of 2-PE overproducing yeast strains on composition and sensory characteristics of sparkling wine. Factors 1 and 2 for the scores (A) and loadings (B) from a partial least squares regression analysis. The control strain PDM, as well as the 'rose' yeasts 2965 and 4390 were used in both primary and secondary fermentations.

7.4.4 Which wine styles are more compatible with intense 'floral' aromas?

Extensive trialling of three of the 'rose' yeast (AWRI 2940, AWRI 2965 and AWRI 4390) at pilot scale over several vintages has allowed the project team to assess which wine styles may be more compatible with enhanced 'rose' aromas, including white (Semillon, Chardonnay, Riesling and Pinot Gris), rosé (Grenache), red (Shiraz and Tempranillo) and sparkling (Chardonnay). Regardless of the style of wine, the different 'rose' yeasts produced significantly higher concentrations of 2-PE than different commercially available strains widely used in the Australian wine industry. Depending on the variety assessed, the 'rose' yeasts produced between two and 12 times more 2-PE than the reference strain, well above its sensory threshold. Larger increases, up to 25-fold, were observed for 2-PEA, which has an even lower detection threshold.

These pilot-scale wines were subjected to either informal sensory evaluation or a more formal quantitative sensory descriptive analysis using the highly trained AWRI sensory panel. The sensory data demonstrated that the 'rose' yeast had the largest sensorial effect in white varieties, particularly those with neutral or less aromatic profiles such as Chardonnay and Pinot Gris. Positive results were also obtained in sparkling Chardonnay wines, as well as in a Grenache rosé. On the other hand, this

was not the case for red wines, as Shiraz wines produced with AWRI 2940 were perceived as being out of balance and did not meet expectations for the flavour profile of a Shiraz wine. These results are summarised in Cordente et al. (2021).

7.4.5 Investigation of the role of 'rose' strains in yellow colour formation in white wine

Sensory analysis of Chardonnay pilot-scale wines (vintages 2015 and 2018) produced by different 'rose' strains showed that these yeasts not only affected wine aroma but also resulted in wines with an increased 'yellow colour intensity'. Firstly, it was validated whether the 'yellow colour intensity' scores from the sensory data (based on human eye perception) correlated with a more objective measure of colour using a UV-Vis method (CieLAB). A high correlation (R^2 = 0.78) was obtained between these two estimates of yellow colour in wine.

To investigate whether some of the metabolites overproduced by the 'rose' yeast during fermentation were responsible for this increase in yellow colour intensity, base Chardonnay wines were spiked with increasing concentrations of either TOL, its sulfonate adduct TOL-SO_3H, TyrOH or 2-PE. No increase in 'yellowness' was observed when any of these compounds were spiked up to wine-like concentrations. While the identity of the compound(s) responsible for this enhanced yellow colour attribute remains elusive, the 'rose' strains isolated in this project could perhaps be used to improve the overall appearance of light-coloured white wines.

7.4.6 *Saccharomyces cerevisiae* strains that are resistant to toxic tyrosine and tryptophan analogues

The 'rose' strains described previously were isolated using a toxic analogue of the aromatic amino acid phenylalanine. In yeast, the biosynthesis of the three aromatic amino acids (phenylalanine, tryptophan and tyrosine) proceeds via a common pathway, sharing some of the precursors and enzymatic activities for their biosynthesis.

A range of toxic analogues of the amino acids tyrosine and tryptophan were used to select for strains with an altered aromatic amino acid catabolism. The isolation of variants with an altered tryptophan metabolism was of particular interest to understand the role of yeast in the formation of the off-flavour indole ('plastic/chemical-like' aroma). In addition, tryptophan is also the precursor of some bioactive compounds such as serotonin and melatonin, which exert positive effects on human health.

Isolation of strains resistant to toxic analogues of tyrosine proved to be difficult, and only 11 resistant variants were isolated. These strains showed a similar volatile profile to that of the parent strain in a laboratory-scale fermentation in Chardonnay.

A total of 350 strains resistant to a toxic analogue of tryptophan were isolated in two different genetic backgrounds. Nineteen strains with an altered tryptophan biosynthetic pathway were selected and further characterised in laboratory-scale fermentations in both a synthetic juice and Chardonnay. Volatile and non-volatile chemical analysis of wines produced by these strains showed a similar profile to that of the 2-PE overproducing strains previously isolated using toxic analogues of phenylalanine. None of the tryptophan-resistant strains produced quantifiable amounts of the off-flavour indole.

7.5 Production of an HO knockout yeast strain in a diploid background to generate non-GM yeasts that exhibit genetic stability during sporulation

The CRISPR/Cas9 system is a powerful genetic modification technology that can be used to edit parts of the genome of living organisms. It has been recently established by the Office of the Gene Technology Regulator (OGTR) of Australia that an organism modified through the CRISPR-Cas9 system that does not use a nucleic acid template to guide genome repair is classified as a non-genetically modified organism (non-GMO) from a non-food regulatory standpoint.

7.5.1 Improvement of existing CRISPR-Cas9 technologies in yeast

The existing Cas9-based methodology has been modified to produce knockout yeast strains more efficiently in a diploid background without the need to use any guide repair DNA. This can be achieved by activating the natural non-homologous end joining (NHEJ) DNA repair pathway in yeast. NHEJ is less

accurate than the ubiquitous homology recombination pathway at repairing DNA double-strand breaks induced by the endonuclease Cas9, which ultimately might cause the inactivation or knockout of a gene of interest.

While the primary target was the *HO* gene, involved in mating type switching in yeast, the *ADE1* gene reporter system was targeted initially to validate the modified Cas9-based method. This method allowed for a quick visual identification (red *vs* white colonies) of yeast cells in which a genetic modification (knockout) has occurred.

Initial experiments were successfully conducted in the industrial diploid wine yeast AWRI 796. When the endonuclease Cas9 was overexpressed in yeast, only 0.4% of the colonies (1 in 250) had a knockout in *ADE1* (red colour). However, when the classical Cas9-based method was combined with the activation of the NHEJ pathway in yeast, the frequency of *ADE1* knockouts was much larger, between 3% and 20%, depending on the specific method used to activate NHEJ. This indicated a more efficient pathway for the non-GM modification of diploid yeast, which will be used to produce gene inactivations in future work.

7.6 Development of new interspecies hybrids to complement hybrid portfolio

The *Saccharomyces* species are a clade of yeast that evolved from a common ancestor, and all can grow in high sugar environments while tolerating high ethanol concentrations. While the yeasts within this clade are considered closely related in a physiological sense, they are highly divergent from a DNA sequence perspective: the level of divergence within this clade is similar to that between a human and a mouse. Strains of *S. cerevisiae* have the greatest ethanol tolerance of the clade and have become the mainstay of not only winemaking, but also many other fermentation industries.

Yeasts within the *sensu stricto* subgroup of *Saccharomyces* are interfertile, meaning they can breed with one another. In earlier work, interspecific hybridisation was used to harness the metabolic differences of other species of the *sensu stricto* clade for winemaking potential. A new breed of wine yeast was generated by mating a robust *S. cerevisiae* wine yeast strain with other members of the *sensu stricto* clade to deliver yeast with novel and wide-ranging flavour and aroma attributes. The work in this project built on that framework by completing a stable of yeast hybrids with six different *Saccharomyces* species.

Interspecific hybridisations between two newly classified *Saccharomyces* species (*S. arboricola* and *S. eubayanus*) were successfully achieved with different *S. cerevisiae* strains (AWRI 838, AWRI 947, NT 116, N96). This accomplishment contributed to the completion of a portfolio of six different *Saccharomyces* interspecific hybrid strains, each having the same neutral wine yeast parent strain, AWRI 838, but with different non-*Saccharomyces* parents (AWRI 1501 *S. paradoxus*, AWRI 1502 *S. cariocanus*, AWRI 1503 *S. kudriavzevii*, AWRI 1504 *S. mikatae*, AWRI 3060 *S. eubayanus* and AWRI 4122 *S. arboricola*).

The completion of this portfolio has enabled a comparison of these hybrids to be made in a series of industry trials (described below). These trials, conducted in Riesling and Shiraz, assessed what the genomes of these different *Saccharomyces* species could bring to winemaking.

7.6.1 Contribution of hybrid yeasts to Riesling wine

7.6.1.1 Sensory properties of Riesling wine made using hybrid yeast

A primary question was whether hybrid yeast could be used to make wines that have quantitatively different sensory properties. To this end, a quantitative sensory descriptive analysis was performed to assess the contribution of different hybrid yeast to Riesling wine style. A total of 34 sensory descriptors were defined that described the appearance, aroma and palate of the wines. There was strong evidence ($P < 0.05$) that there were strain-specific differences between the wines in seven attributes rated by the panel. These attributes included: yellow colour intensity, tropical fruit aroma, passionfruit aroma, banana aroma, honey aroma, stone fruit flavour and banana flavour. In addition, there was evidence ($P < 0.15$) for strain-specific differences between the wines in a further eleven

attributes: apple aroma, stone fruit aroma, floral aroma, cooked veg aroma, pungency, sweetness, acidity, tropical fruit flavour, passionfruit flavour and cut grass flavour, which were close to being significant.

PCA plots (Figure 6) were created using the 18 attributes for which there was evidence of a strain-specific effect on a sensory attribute. Principal components 1, 2 and 3 explained 36.4%, 26.0% and 15.2% of the variation in the data, respectively. Hybrid yeast strains AWRI 1504, 1502 and 1503 plotted to the right of the figure, were rated highly in those attributes highly positively loaded on PC1, notably tropical fruit aroma, passionfruit aroma, banana confection aroma, passionfruit flavour and stone fruit flavour. Wines from hybrid AWRI 4122 were also rated moderately highly in some of these attributes. Conversely, wines from parent *S. cerevisiae* strain AWRI 838 and hybrid AWRI 1501 were plotted to the left of the figure, being rated lower in intensity of these attributes, and highly in the close to significant (P<0.15) attribute cut grass flavour, which was negatively loaded on PC1. Hybrid AWRI 1505 was plotted higher on the vertical axis, being rated highly in yellow colour intensity and honey aroma, while hybrids AWRI 3060 and 1503 were plotted lower, being highly rated in cooked veg aroma.

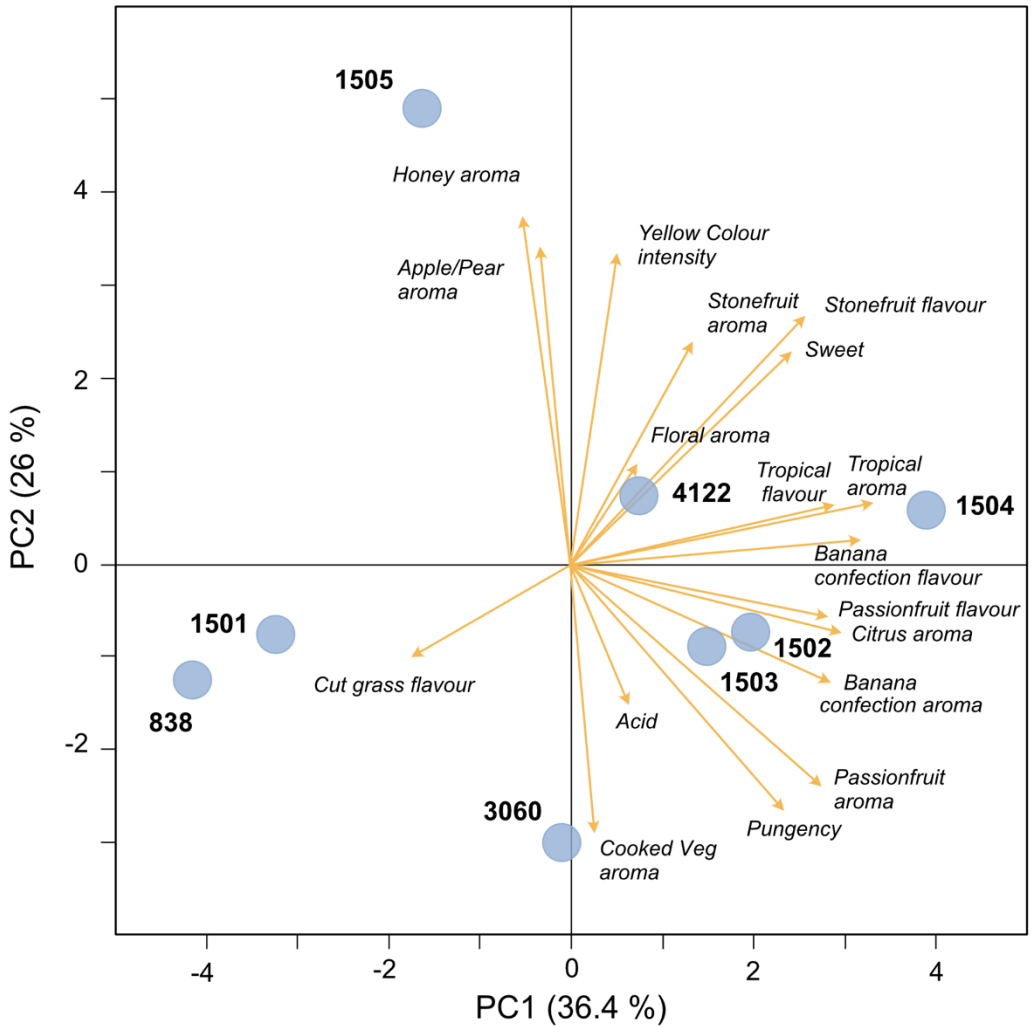


Figure 6. Principal component biplot of significant (P<0.05) and close to significant sensory attributes (P<0.15) on principal components 1 and 2 for Riesling wines made using seven hybrid yeasts and an *S. cerevisiae* wine strain, with the different yeasts signified with blue dots.

The wines made by the hybrid strains were generally rated higher in flavour attributes relating to tropical-style fruit (tropical fruit, passionfruit, banana), stone fruit and floral aroma than wines made by the parent *S. cerevisiae* strain AWRI 838. Overall, the *S. mikatae* hybrid strain AWRI 1504 received the highest scores for aroma and flavour attributes, including stone fruit aroma and flavour (150%

relative to parent strain AWRI 838), which may be relevant to recent AWRI research into this sensory attribute.

7.6.1.2 Contribution of hybrid yeasts to the composition of Riesling wine

Quantitative analysis of volatile flavour compounds corroborated the sensory reports. Hybrid-made wines contained higher concentrations of fruit and floral-associated compounds relative to their *S. cerevisiae* parent; AWRI 838: 2-phenylethyl acetate (floral) 315% AWRI 1503 (*S. kudriavzevii*), 400% AWRI 1504 (*S. mikatae)*, 335% AWRI 3060 (*S. eubayanus*) and 390% AWRI 4122 (*S. arboricola*) hybrids; 2-methylbutyl acetate (banana) 170% AWRI 1503, 185% AWRI 1504 and 200% AWRI 4122; ethyl butanoate (fruit) 135% AWRI 1502 *S. cariocanus* hybrid; ethyl hexanoate (green apple) 180% AWRI 1502 and 150% AWRI 1504 (Table 1).

Table 1. Effect of hybrid yeast on the composition of Riesling wine. Table shows the mean concentration as µg/L (n= 3) with the percentage relative to AWRI 838 shown in brackets.

Strain	2-Phenylethyl acetate	2-Methylbutyl acetate	Ethyl butanoate	Ethyl hexanoate
AWRI 838	80 (100)	1773 (100)	254 (100)	342 (100)
AWRI 1501	131 (163)	1404 (79)	202 (80)	348 (102)
AWRI 1502	98 (122)	1730 (98)	342 (135)	613 (179)
AWRI 1503	252 (315)	3058 (170)	250 (98)	431 (126)
AWRI 1504	320 (400)	3322 (187)	346 (136)	507 (148)
AWRI 1505	58 (72)	1028 (58)	96 (38)	178 (52)
AWRI 3060	269 (335)	1969 (111)	208 (82)	294 (86)
AWRI 4122	312 (390)	3687 (208)	174 (68)	309 (91)

Polysaccharides are known to influence mouthfeel attributes such as viscosity, astringency and hotness in wines. While the actual physiological mechanism for these changes in mouthfeel is not known, polysaccharides have been shown to disrupt the interaction between salivary proteins and phenolic compounds, perhaps due to the formation of ternary complexes involving protein, polyphenol and polysaccharide (Gawel et al. 2018).

A quantitative analysis of the total polysaccharide and individual monosaccharide moieties (Table 2) in the Riesling wines was undertaken. Total polysaccharide concentrations varied considerably between the wines, with the *S. uvarum* hybrid (AWRI 1505) producing the highest concentration (530 mg/L) and the *S. cariocanus* hybrid (AWRI 1502) the lowest (275 mg/L). These values represent 150% and 75% of the concentration produced by *S. cerevisiae* parent AWRI 838 respectively.

Table 2. Total polysaccharide and monosaccharide concentrations of Riesling wines produced by hybrid yeasts. Concentrations (mg/L) are expressed as the mean with different letters indicating significant difference ($p < 0.05$) using one-way ANOVA of means with Tukey comparisons.

Strain	Total polysaccharide	Mannose	Galacturonic acid	Galactose	Arabinose
AWRI 838	369 b,c	81 b,c	96 a	77 a	68 a
AWRI 1501	315 c,d	90 b,c	84 a,b	70 a,b,c	43 b,c
AWRI 1502	275 d	65 c	85 a,b	46 c	38 c
AWRI 1503	350 b,c,d	93 b,c	81 a,b	72 a,b,c	58 b,c
AWRI 1504	314 c,d	76 c	79 a,b	63 b,c	55 a,b,c
AWRI 1505	530 a	230 a	65 b	91 a	72 a
AWRI 3060	411 b	119 b	63 b	60 b,c	65 a
AWRI 4122	288 c,d	65 c	80 a,b	59 b,c	46 b,c

The differences in yeast-specific total polysaccharide concentration were underpinned by differences in the concentration of four monosaccharide moieties: mannose, galacturonic acid, galactose and arabinose. Mannose residues arise from mannoproteins released from yeast cell walls during fermentation and from autolysis at the maturation stage. Galactose, arabinose and galacturonic acid residues originate from the grape and are extracted during grape crushing and fermentation; arabinose and galactose are derived from arabinogalactan proteins (AGPs), which exist in the grape vacuole; while galacturonic acid is released from pectins embedded within the cell. Mannose contributed most to the high total polysaccharide level of AWRI 1505, with a concentration of 230 mg/L. This was roughly three times the concentration of the AWRI 838 wine, at 81 mg/L.

Studies have shown that the perception of viscosity can be enhanced and the bitterness of grape seed tannin reduced by the addition of mannoproteins and AGPs in model white wine (Vidal et al. 2004). In addition, it has also been noted that the presence of polysaccharides can increase the overall flavour of wines (Jones et al. 2008). It is possible that some of the differences in the sensory qualities of wines made using AWRI 1505 relative to other wines in the set may be attributed to greater enhancement of some flavours and aromas due to the increased concentration of polysaccharides.

As polysaccharides are known to affect astringency by disrupting the interaction between polyphenols and salivary proteins (Carvalho et al. 2006), the wines were analysed spectrophotometrically by the Folin-Ciocalteau Micro Method for phenolic content as gallic acid equivalents (GAE). However, no major differences in total phenolic content were observed, with all wines containing concentrations between 74.4 and 78.9 mg/L gallic acid equivalents.

Protein content and stability of the wines were analysed at ferment completion (Table 3). Some differences in haze-forming protein concentrations of wines were evident at fermentation completion (Table 3). The *S. eubayanus* (AWRI 3060) and *S. arboricola* (AWRI 4122) hybrids produced wines with the highest total protein concentrations at 163 and 159 mg/L respectively. The *S. mikatae* hybrid (AWRI 1504) produced wines with the lowest concentration (124 mg/L), while the *S. cerevisiae* parent (AWRI 838) produced an intermediate amount of 133 mg/L.

Table 3. Mean concentrations (mg/L) and nephelometric turbidity units (NTU) of post fermentation Riesling wines (n=2) made with seven different yeast. Letters indicate that treatments belong to different groups (at alpha = 0.05).

Strain	Total haze-forming protein (mg/L)	Heat test (NTU)
AWRI 838	133 b,c	21.5 a
AWRI 1501	126 b,c	14.8 c
AWRI 1502	130 b,c	16.1 c
AWRI 1503	131 b,c	16.6 b,c
AWRI 1504	124 c	21.4 a,b
AWRI 1505	136 b	8.7 d
AWRI 3060	163 a	21.7 a
AWRI 4122	159 a	24.8 a

The wine made using the *S. uvarum* hybrid (AWRI 1505) stood out as being much more protein-stable than the other wines in the set. The heat test results for this wine were far lower (8.7 NTU) than other wines with similar total protein concentrations (16-21 NTU). There was no correlation between total protein concentration and the results of the standard heat test (Pearson r = 0.471). This is consistent with previous work showing a poor correlation between wine protein concentration and haze formation potential (Sluyter et al. 2015). There was evidence (Pearson r = -0.723, P = 0.043) for a relationship between wine haze formation potential and total mannose concentration. As mentioned above, mannose is derived from yeast mannoproteins. Yeast mannoproteins have long been known to suppress haze formation in wine (Schmidt et al. 2009).

TDN (1,1,6-trimethyl-1,2-dihydronapthalene) is an important bottle age odorant in Riesling wine, imparting kerosene-like characters. The basis for TDN formation is known to be vineyard derived but an initial yeast-mediated step appears to be important for its formation in wine (Grebneva et al. 2019). For this reason the concentrations of acetal, TDN and related C_{13}-norisoprenoids from the proposed TDN formation pathways were quantified in the hybrid Riesling trial wines to determine if non-*S. cerevisiae* species could play a role in modulating TDN evolution. Each wine was assessed using optimised hydrolysis conditions (pH 1.7, 75°C and 60 minutes incubation) to quantify yeast effects on future TDN evolution.

Analysis of the wines following hydrolysis revealed that only the *S. cariocanus* hybrid (AWRI 1502) was able to decrease potential TDN concentrations. *S. cariocanus* hybrid wines contained TDN at a concentration of 99 µg/L, 16 µg/L less than the wines produced by the *S. cerevisiae* parent (AWRI 838) (115 µg/L). Other hybrid strains tended to produce wines with less total TDN, but the magnitude of the decreases was small (Table 4). There was no evidence that the wines differed in the concentration of the other norisoprenoid compounds analysed. Future analyses of the *S. cariocanus* hybrid-made wines will reveal if the difference in TDN concentrations as young wine have a sensory effect following bottle ageing.

Table 4. C13-norisoprenoid concentrations from hydrolysis of Riesling wines produced by hybrid yeasts. Concentrations (µg/L) are expressed as the mean of fermentation triplicate, with different letters within each column indicating significant difference ($p < 0.05$).

Yeast strain	TDN	vitispirane	Riesling acetal	actinidol	TPB
AWRI 838	115 a	37.4 a	37.5 a	42.7 a	16.3 a
AWRI 1501	110 a,b	36.5 a	36.5 a	41.0 a	16.3 a
AWRI 1502	99 b	36.0 a	36.0 a	41.0 a	16.2 a
AWRI 1503	108 a,b	37.2 a	37.2 a	42.1 a	16.4 a
AWRI 1504	110 a,b	36.9 a	36.9 a	41.5 a	16.3 a
AWRI 1505	104 a,b	36.0 a	36.0 a	40.6 a	16.1 a
AWRI 3060	116 a	38.1 a	38.1 a	44.1 a	16.3 a
AWRI 4122	112 a,b	36.9 a	36.9 a	42.9 a	16.4 a

In summary, the Riesling vintage trial established that wine yeast generated through interspecific hybridisation of a robust commercial *S. cerevisiae* wine yeast with other *Saccharomyces* species from the *sensu stricto* clade can deliver valuable outcomes in terms of flavour and aroma diversity. In addition, the hybrid yeast produced wines with different macromolecule matrices that have the potential to bring benefits to wine in areas of palate structure such as viscosity/fullness perception and bitterness.

7.6.2 Contribution of hybrid yeasts to Shiraz wine

In contrast to the white wine study described above, the predominant characteristics of red wine are related to red fruit, with a different set of compositional factors underpinning those characters. Furthermore, grape berry phenolic material plays a significant role in the overall perception of red wine. These features of red wine provided the opportunity to explore how non-*cerevisiae* based hybrids influence not only the aromatic qualities of wine, but also the textural elements. Do they bring with them enzymes that can alter the extraction of phenolic material and do they change yeast's physical properties sufficiently to alter their retention in red wine?

A quantitative sensory descriptive analysis after five months in bottle identified 22 attributes that described the appearance, aroma and palate of the wines. Nine attributes differed amongst the treatments. Six of these were related to aroma. The attributes cherry' and red berry were rated highest in wines made by hybrids AWRI 1505 (*S. uvarum*) while baking spice and sweetness were most highly rated in wines made using AWRI 1502 (*S. cariocanus*) (Figure 7). Hybrid strains AWRI 1501 (*S.*

paradoxus) and AWRI 4122(*S. arboricola*) produced wines with elevated canned vegetable and boiled egg aromas. AWRI 1502 and AWRI 838 rated lowest for these attributes. Wines made using AWRI 4122 were rated as the most astringent and as having the highest opacity.

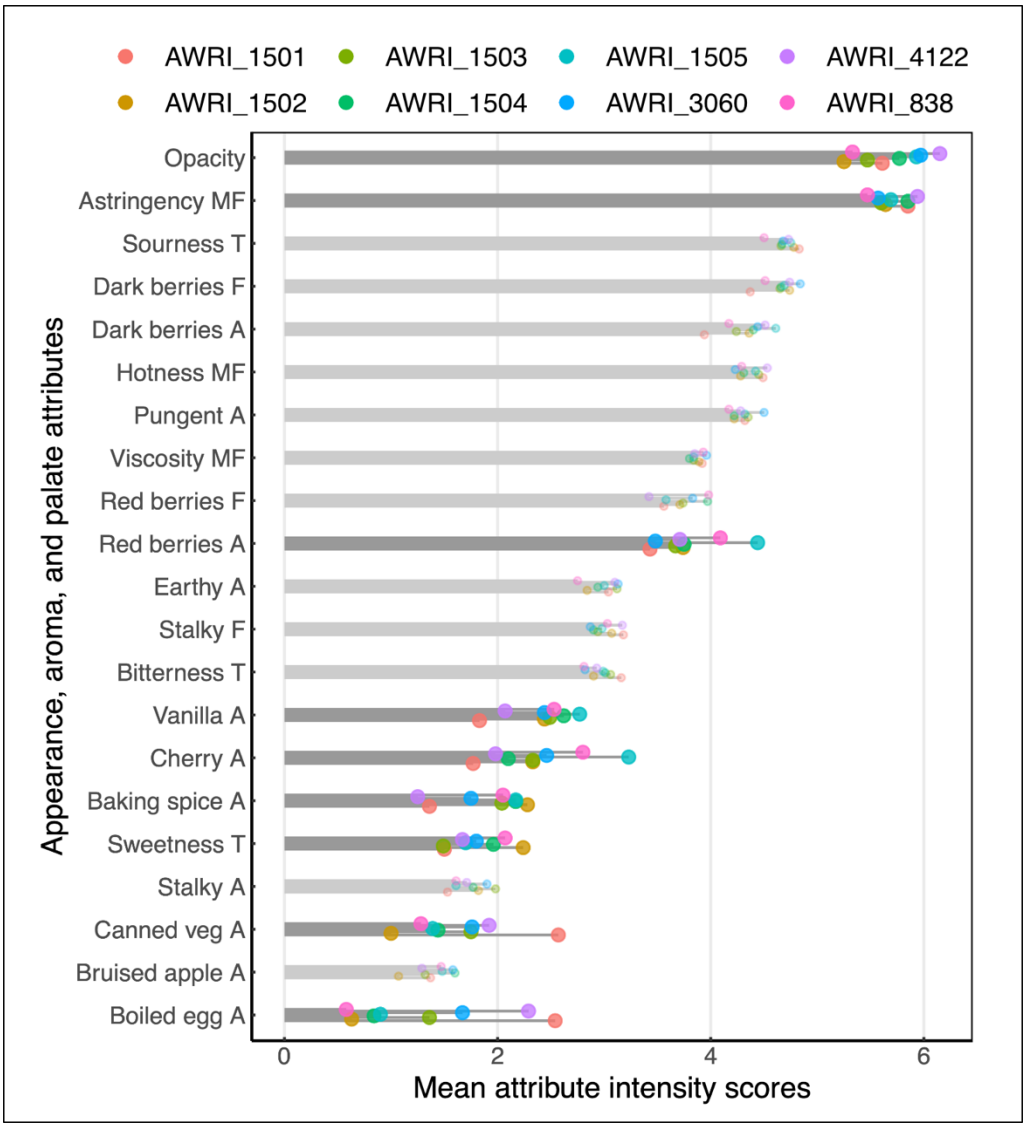


Figure 7. Mean attribute scores from quantitative sensory descriptive analysis of Shiraz wines made with seven hybrid yeast and a *S. cerevisiae* wine yeast. Sensory attributes for which there was evidence of an effect of yeast strain ($P < 0.05$) are shown with larger and more strongly coloured dots and lines.

Compositional analyses identified varied concentrations in a range of yeast-derived volatile compounds. AWRI 1505 and AWRI 3060 produced almost double the concentration of ethyl-2 and ethyl-3 methylbutanoate (Table 5), a compound previously associated with red fruit attributes in red wine (Day et al. 2021). Concentrations of compounds associated with floral attributes such as 2-phenylethanol were also 1.7-fold higher in AWRI 1505 wines. This was consistent with AWRI 1505 wines being highly rated for red berry aroma. Across the hybrid yeast, ethyl hexanoate and 2-methylbutyl acetate concentrations were similar to those found in the control wine (AWRI 838).

Table 5. Concentration of volatile fermentation compounds in hybrid Shiraz wines relative to *S. cerevisiae* parent AWRI 838 wines expressed as percentage

Yeast strain ID	Ethyl-2-methyl butanoate	Ethyl-3-methyl butanoate	Ethyl butanoate	Ethyl propanoate	Ethyl-2-methyl propanoate	Ethyl hexanoate	2-Methylbutyl acetate	2-Phenylethyl acetate	2-Phenyl ethanol
AWRI 838	100	100	100	100	100	100	100	100	100
AWRI 1501	131	130	151	260	156	128	113	123	150
AWRI 1502	149	150	156	185	146	139	65	85	136
AWRI 1503	131	140	123	136	157	106	100	156	143
AWRI 1504	126	128	149	152	161	114	101	142	169
AWRI 1505	181	185	124	162	257	88	123	203	177
AWRI 3060	200	184	129	156	310	95	91	170	172
AWRI 4122	139	143	113	134	273	79	122	167	146

There was strong evidence ($P < 0.05$) that all wines made using hybrid yeast had higher tannin concentrations than wines made using the *S. cerevisiae* parent. The magnitude of the increase ranged from 220 mg/L in AWRI 1503 wines to 566 mg/L in AWRI 4122 wines (Figure 8A). Consistent with other work (Day et al. 2021) there was a good correlation (Pearson $r = 0.86$, $P = 0.013$) between tannin concentration and astringency if AWRI 3060 wines were excluded from the analysis. AWRI 3060 wines were one of the lowest rated for astringency and there may have been other features of this wine that suppressed the perception of that attribute.

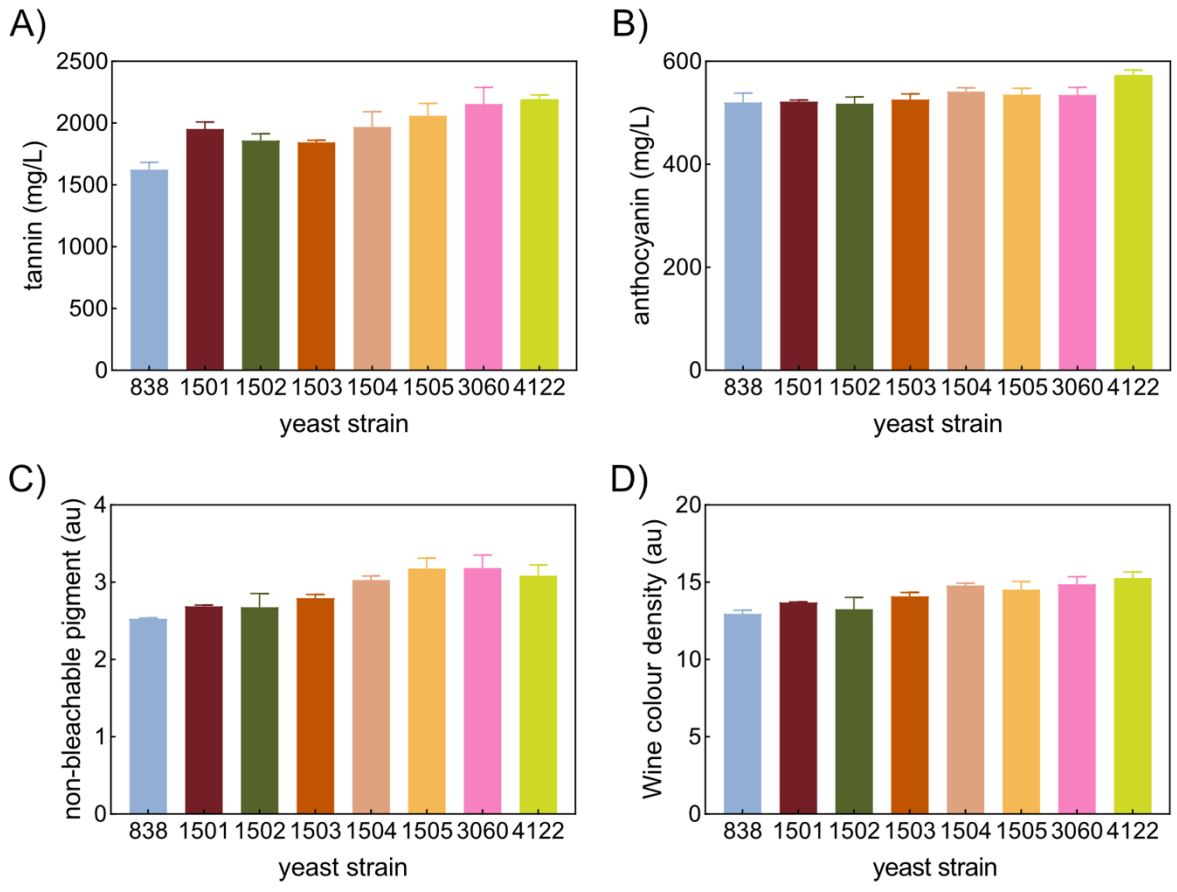


Figure 8. Effects of hybrid yeast on phenolic indices of Shiraz wine

AWRI 4122 wines contained the highest total anthocyanin concentrations (Figure 8B) and were the only wines for which there was evidence (P = 0.0004) that anthocyanin concentration differed from the control (AWRI 838). Wine colour density was also highest in the AWRI 4122 wines (Figure 8D). Both of these analytical measures were consistent with AWRI 4122 receiving the highest score for opacity in quantitative sensory assessments (Figure 7).

Wines made by *S. eubayanus* hybrid AWRI 3060 contained the highest concentrations of total polysaccharide (Table 6) with a mean increase in concentration relative to AWRI 838 wines of 163 mg/L (95% CI, 30, 296). Both yeast-derived (mannose) and grape-derived (galacturonic acid) polysaccharide sources contributed to the higher polysaccharide concentration. It is possible that this higher concentration of total polysaccharides was a contributing factor to the low perceived astringency of AWRI 3060 wines. Such observations have been made elsewhere (Vidal et al. 2004).

Table 6. Total polysaccharide and monosaccharide concentrations (mg/L) of Shiraz wines produced by hybrid yeasts

Yeast strain ID	Total polysaccharide	Mannose	Rhamnose	Glucuronic acid	Galacturonic acid
AWRI 838	627 b	128 b,c	63 b	10.6 b,c	236 c,d
AWRI 1501	775 a,b	162 a	77 a,b	9.6 b,c	315 a,b
AWRI 1502	673 a,b	148 a,b	71 a,b	9.4 b,c	252 a,b,c,d
AWRI 1503	625 a,b	148 a,b	64 a,b	8.5 c	216 d
AWRI 1504	697 a,b	145 a,b	73 a,b	10.9 b,c	266 a,b,c,d
AWRI 1505	649 a,b	125 b,c	70 a,b	12.4 a,b	249 b,c,d
AWRI 3060	790 a	160 a	78 a	15.2 a	328 a
AWRI 4122	720 a,b	109 c	74 a,b	15.7 a	306 a,b,c

There was no evidence for strain-associated variation in the concentration of low molecular weight sulfur compounds in wines made by the different hybrids ($P > 0.05$) except for methyl thioacetate (MeSAc) ($P < 0.0001$). All of the wines made with hybrid yeasts had higher concentration of this analyte (Figure 9), ranging from 6.4 µg/L (AWRI 1503) to 10.7 µg/L (AWRI 4122) compared to 2.1 µg/L produced by AWRI 838. MeSAc has been shown to contribute to the formation of methanethiol (MeSH) in wines post-bottling. The highest MeSAc concentrations were observed in wines made by AWRI 1501 and AWRI 4122 and this result was consistent with the sensory analyses that rated both of these wines as having higher canned vegetable and boiled egg attributes.

The Shiraz trial highlighted strain AWRI 1505, a hybrid with *S. uvarum*, as being the most promising for red wine production among the hybrid yeasts tested. This strain was rated low in 'reductive' off-aromas and highest in cherry and red berry aromas.

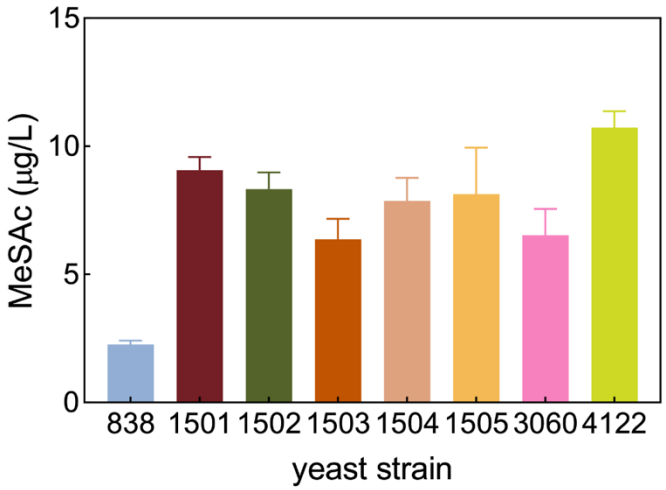


Figure 9. Methyl thioacetate (MeSAc) concentrations in Shiraz wines made using hybrid yeast

7.7 Identification of genetic elements contributing to low acetate production fermentation in an *S. cerevisiae* x *S. uvarum* hybrid

An output from a previous Wine Australia project was the generation of an S*. cerevisiae* x *S. uvarum* hybrid exhibiting low acetate production in high-sugar conditions (Bellon et al. 2015). The concentration of acetate produced by this hybrid was not intermediate between the two parents, but significantly less than both, suggesting an unusual genetic interaction.

To identify the genetic determinants of the low acetate trait, the project team applied a genetic tool known as bulk segregant analysis. Put simply, the strain under investigation is sporulated, producing a large number (hundreds) of daughter strains that vary in the low acetate trait. Each of these is characterised, the amount of acetic acid they produce in a defined condition is quantified, and they are separated into groups based on how much acetic acid they produce. The genomes of these two groups are then sequenced separately and compared to a reference genome. The analysis highlights parts of the genome that segregate with the trait of interest.

A chromosomal marker system was used to confirm presence/absence of each species' chromosomes in all isolates. Low-resolution genotyping identified some weak correlations with gross chromosomal structural rearrangements. High-resolution genetic mapping of bulk segregants indicated loss of *S. uvarum* chromosome 4 in all high acetic acid-producing hybrid progeny (Figure 10).

That chromosomal loss in this hybrid is a causative factor in the modulation of acetic acid concentration in wine was demonstrated by deleting the entirety of chromosome 4 derived from *S. uvarum*. This was achieved by applying the method of chromosome ablation described by Reid et al (2008).

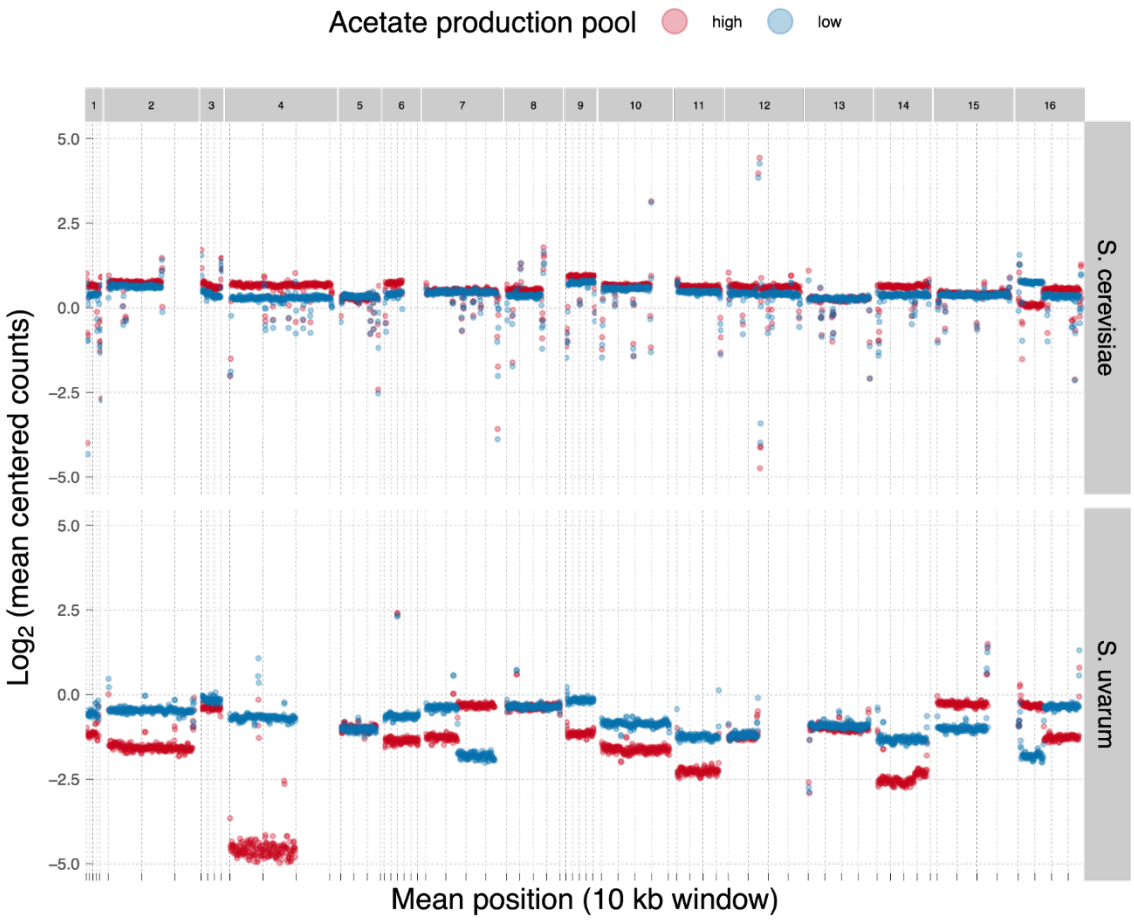


Figure 10. Genome sequencing depth of high-acetate and low-acetate hybrid yeast separated into pools of high (red) and low (blue) acetic acid production potential. The yeast used to make the pools were created from the sporulation of AWRI 1571. Raw sequence reads were mapped to a reference genome. The reference genome was a digital concatenation of the reference genomes of *S. cerevisiae* and *S. uvarum*. The average read depth in

overlapping 10 kb windows was divided by the overall average read depth of the strain (mean centred) and $\log_2$ transformed.

Hybrid transformants that had lost *S. uvarum* chromosome 4 were confirmed by a chromosomal marker system. The same marker system was used to confirm that all other *S. cerevisiae* and *S. uvarum* chromosomes were retained in the *S. uvarum* chromosome 4-deleted strains. Chromosome 4 deletion mutants (CEN4-1 and CEN4-2) produced four-fold higher concentrations of acetic acid in Chardonnay juice fermentation than the parent strain AWRI 1572, demonstrating the causal relationship between chromosomal retention and the low-acetic acid trait in this hybrid strain (Figure 11).

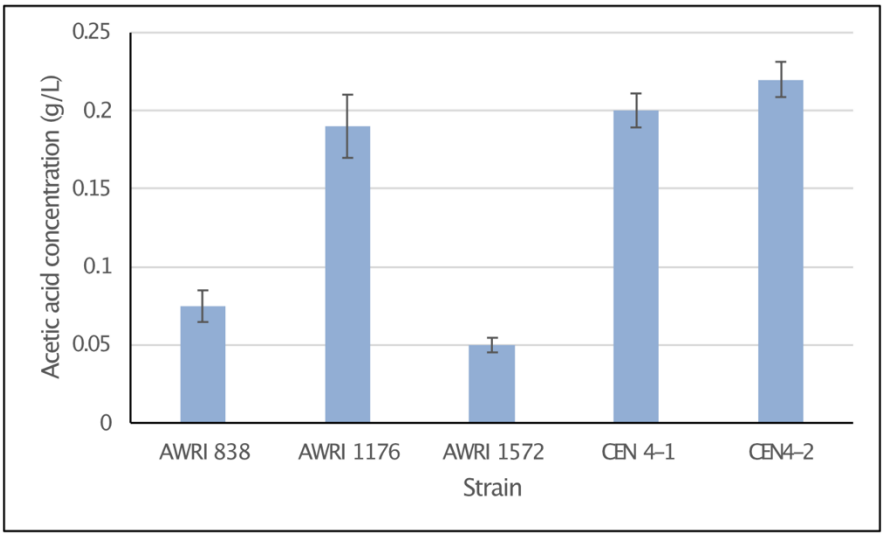


Figure 11. The effect of chromosome 4 loss from AWRI 1572 on acetic acid concentrations in Chardonnay wine

To locate the region of chromosome 4 responsible for the low-acetate trait, segmental deletions were induced in the *S. uvarum* chromosome 4 of AWRI 1572. Strains with missing different parts of the chromosome were again evaluated for acetic acid production capacity. Of the 130 mutant strains screened, 12 strains producing high acetic acid concentrations were molecularly typed using inverse PCR and sequencing to pinpoint the chromosomal region deleted. Four areas of interest were identified and individual gene deletions within those regions were constructed.

Deletion of a single gene, *NBP2*, recovered 50% of the acetic acid concentration that could be ascribed to whole chromosome loss (Figure 12). *NBP2* is a negative regulator of the high osmolarity glycerol pathway (HOG) in yeast, a pathway that typically regulates glycerol production in response to environmental stress. However, the contribution of *NBP2* to the low-acetate trait is not the complete picture: *NBP2* alone is not responsible for the low acetic acid phenotype of the hybrid parent strain AWRI 1572. Bioinformatic analysis identified another four genes important in the HOG pathway on chromosome 4. It is possible that the low acetic acid production phenotype displayed by AWRI 1572 is polygenic and that these other genes involved in the HOG pathway on chromosome 4 also contribute to the production of reduced acetic acid in the *S. cerevisiae* x *S. uvarum* hybrid.

High levels of acetic acid due to osmoadaption during fermentation in *S. cerevisiae* have been reported previously (Heit et al. 2018). The high sugar levels in grape juice generate osmotic stress in yeast cells early in fermentation. It is likely that the interplay between *S. uvarum* chromosome 4 and chromosomes of *S. cerevisiae* in the hybrid genome contribute to better osmotic adaption during fermentation leading to lower acetic acid production in wine.

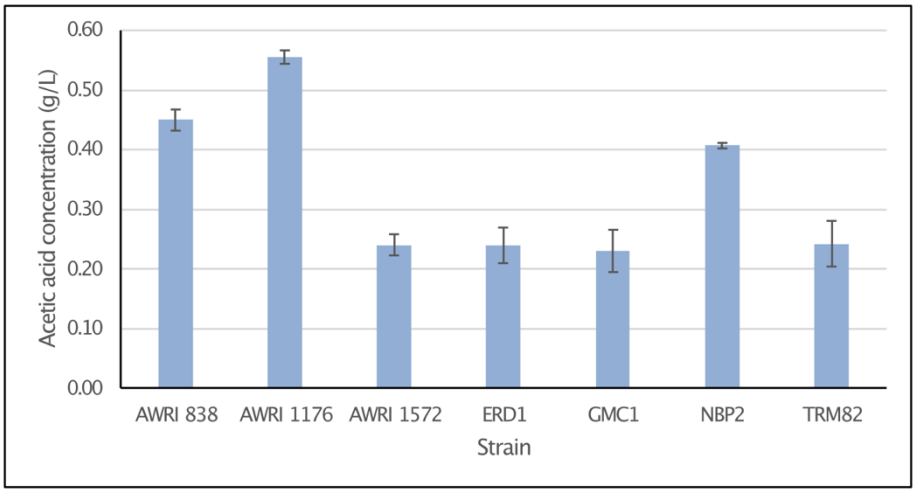


Figure 12. The effect of single gene deletions within *S. uvarum* chromosome 4 of AWRI 1572 on acetic acid concentrations in Chardonnay wine

8. Outcome and conclusion

8.1 Performance against planned outputs

8.1.1 Review of existing yeast strain stock to identify opportunities for strain refinement.

Review of existing yeast strain stock in the extensive AWRI culture collection has allowed us to identify several strains with interesting winemaking properties. In particular, one of the non-*Saccharomyces* strains evaluated, belonging to the *H. osmophila* species, produced laboratory-scale wines with high concentrations of the 'flinty' compound PMT and some 'fruity' esters, while displaying similar fermentation kinetics to commercial *S. cerevisiae* wine strains. In addition, three robust *S. cerevisiae* strains were identified (AWRI 1742, 1939 and 1796) that exhibited strong fermentation kinetics, an absence of negative quality attributes and produced a variety of compounds associated with positive aromas in wine. The rights to the future use of these three yeasts have been retained by AWRI.

8.1.2 Review of microorganisms identified by the bioprospecting project (4.4.3) to identify opportunities for strain refinement.

In total, 250 genetically distinct yeast isolates were screened on solid media for winemaking relevant traits. Just over 1% were classified as low-H_2S producers. Seven of the 250 wild isolates exhibited potential for varietal thiol release during fermentation. One isolate exhibited robust fermentation kinetics in trial ferments and had a high thiol releasing phenotype.

8.1.3 Development of new interspecies hybrids to complete hybrid portfolio.

Two new hybrids containing partial genomes of *S. eubayanus* and *S. arboricola* were created during the project. This achievement now completes the collection of possible hybrids between *S. cerevisiae* and other *Saccharomyces* yeasts with complementary mating type features from the *sensu stricto* group. For a full list of strains generated and characterised during the conduct of the project, refer to Table 7 in Section 11.

8.1.4 Methods available for producing non-GM yeasts through marker assisted breeding.

Initial research on an improved CRISPR-Cas9 methodology showed the potential of this technology to efficiently generate non-GM alterations in industrial diploid wine yeast.
Furthermore, identification of causative mutations in *ARO4* and *TYR1* that give rise to alterations in aromatic amino acid metabolism, and identification of *NBP2* which contributes to low acetic acid production in wine yeast will facilitate future selection of yeasts with these traits.

8.1.5 Non-GM wine yeast with modified aromatic profiles from alterations to amino acid biosynthetic pathways

As a result of extensive research on the different 'rose' strains isolated in this project, we now have a better understanding of the formation of different compounds derived from aromatic amino acids by yeast, as well as their potential effect on different wine sensory properties (aroma, palate and appearance). As an example, strain AWRI 2940 has been used as a tool for Project 3.1.3 (Texture) to study the role of the higher alcohol TOL and its adduct TOL-SO_3H in the mouthfeel properties of both white and red wines.

Extensive trialling of three of the 'rose' yeast (AWRI 2940, AWRI 2965 and AWRI 4390) at pilot scale over several vintages has allowed assessment of which wine styles may be more compatible with enhanced 'rose' aromas. Two of these 'rose' yeast strains have been commercialised by AB Biotek, with the names of AWRI Rosa (AWRI 2965) and AWRI Rosa Intense (AWRI 2940). These strains are recommended to be used with white varieties such as Pinot Gris, Riesling, Chardonnay, Colombard and Semillon.

8.1.6 Pilot- and industry-scale trials evaluating new yeast strains.

The seven hybrids comprising the completed hybrid set were characterised in pilot-scale fermentations of red and white grapes. The red wines differed across nine descriptive attributes. Key among the more positive attributes were fruity aromas of cherry, red berry, baking spice and vanilla. However, some strains produced wines with more prominent 'boiled egg' and 'canned vegetable' aromas. This work has identified hybrid strains that appear well suited to the production of red wine.

In white wine there was strong evidence ($P < 0.05$) that there were strain-specific differences between the wines in seven attributes. These attributes included: yellow colour intensity, tropical fruit aroma, passionfruit aroma, banana aroma, honey aroma, stone fruit flavour and banana flavour. Overall, the *S. mikatae* hybrid strain AWRI 1504 received the highest scores for aroma and flavour attributes, including stone fruit aroma and flavour (150% relative to parent strain AWRI 838), which may be relevant to other recent AWRI research into this sensory attribute.

Extensive trialling of three of the 'rose' yeast (AWRI 2940, AWRI 2965 and AWRI 4390) at pilot-scale over several vintages allowed an assessment of which wine styles may be more compatible with enhanced 'rose' aromas, including white (Semillon, Chardonnay, Riesling and Pinot Gris), rosé (Grenache), red (Shiraz and Tempranillo) and sparkling (Chardonnay). The sensory data demonstrated that the 'rose' yeast had the largest sensorial effect in white varieties, particularly those with neutral or less aromatic profiles such as Chardonnay and Pinot Gris. Positive results were also obtained in sparkling Chardonnay wines, as well as in a Grenache rosé.

8.1.7 Practical advice and extension materials for winemakers

One of the fundamental ways that the outputs from this project are extended to industry is through the commercial release of yeast strains. The project team has been actively involved in supporting efforts to license and release strains through third party suppliers. Strains that have been released during the project term include 'AWRI Rosa', 'AWRI Rosa Intense', 'AWRI Paragon', 'AWRI Uvamax', 'AWRI Celebrate' and 'Exotics Novello'.

In addition to commercial release of yeast strains, the project team communicates with industry about yeast strain developments through industry articles, seminars and workshops. Workshops presenting results from this project were delivered at both the 17th and 18th AWITC events.

8.2 Could changing the methodology/technology have improved the outcome?

In hindsight there are certain lines of enquiry that were not as fruitful as initially hoped. It is not considered that the methodology or technology could have been improved to deliver better or more timely outcomes. In fact, in many cases throughout this work, new technologies or methodologies had to be developed to achieve project outcomes.

However, some methods, while powerful, are very resource intensive. Quantitative-trait-loci (QTL) methodology is a case in point. This is an approach that can assign genetic contributions from genomic elements to traits evident in a progeny population. With an easy method for screening the population

for a trait of interest, the process may be relatively fast. However, few such easy methods exist for fermentation-related traits, for which hundreds of replicated fermentations may be required. If the trait is the production of a specific metabolite, hundreds of quantitative analyses need to be undertaken before approaching the question of the genetic underpinning of a trait. This can involve years of work. Improved rapid and low-cost analytical methods for a variety of analytes can facilitate such work, by reducing the cost and duration of such an enquiry.

8.3 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 reinforced previous observations that yeast strain choice can be used to modulate red wine flavour and aroma attributes to great effect.

Foundational developments in the technology used to direct modifications in the genome of diploid wine yeast will facilitate future developments in yeast breeding, serving as an enabling technology in strain development.

8.4 Benefits to the industry

The Australian wine industry benefitted from this project through:

a) The commercialisation of yeast strains developed within the project, which are a resource that can be used directly to enhance or modulate wines. Through partnerships with yeast suppliers, outputs from the project have a direct route to market.
   - Two strains that overproduce the rose aroma compound 2-PE were commercialised by the yeast manufacturer AB Biotek during the project period. Another strain, the moderate 2-PE producer AWRI 4390, has the potential to be commercialised for sparkling wine production. Further research will have to be conducted with this strain to assess its viability to be used for sparkling winemaking.
   - Interspecific hybridisation within the *Saccharomyces* species resulted in four yeast being commercialised during this current project period. AB Biotek has released three active dry yeast (ADY) products: AWRI Paragon (AWRI 1501 *S. cerevisiae* x *S. paradoxus*), AWRI Uvamax (AWRI 1505 *S. cerevisiae* x *S. uvarum*), AWRI Celebrate (AWRI 2526 *S. cerevisiae* x *S. mikatae*), while Anchor has released one ADY product, Exotics Novello (AWRI 2795 *S. cerevisiae* x *S. cariocanus*).
   - Ongoing development and refinement of these strains means ongoing support and product improvement over time. As a result, continuing wine industry investment in this area provides opportunities for process improvement.

b) Ongoing characterisation of strains that have been developed help to focus the use-case for some of these new strain developments and provides support to suppliers of licensed project outputs. Through industry trials and large pilot-scale trials of strains in reds and whites, the project has helped to identify the types of wine to which newly developed strains might be most suited, helping winemakers to understand when and where new strains might benefit their product.

c) Foundational work such as the identification and characterisation of microbial germplasm unencumbered by third-party licensing or ownership restrictions will expedite future germplasm development, reducing the financial and temporal cost associated with acquiring the rights to genetic material.

9. Recommendations

This project has seen the culmination of many years of work on hybrids between *S. cerevisiae* and non-*cerevisiae Saccharomyces* yeasts. Two large head-to-head trials in red and white wine together with a number of smaller trials demonstrated the versatility and utility of these yeasts. While this project effectively wraps up hybrid generation work at AWRI, it should not be forgotten that the process used

to generate the hybrids, as described in this report, also generated a large amount of hybrid germplasm material with different rearrangements of the genetic material from the non-*cerevisiae* yeast that were used to make them. This germplasm, while not selected as optimal for winemaking in the first instance, is a large resource of untapped genetic diversity whose value has yet to be realised.

An earlier project, AWR 1302, suggested QTL analyses could be performed with a relatively small number of pairwise crosses to identify molecular features that are responsible for physiological traits in yeast. This suggestion was realised in this project to identify the molecular basis of a low acetate trait in one of the hybrid yeasts. The execution of such a program is labour intensive, however the outcome is progressive discovery of previously uncharacterised genetic features with relevance to commercial winemaking. Once identified, these molecular targets need to be introduced into commercial yeast lineages for their utility to be realised. This is the case for the *S. uvarum* gene set identified in this report. Future work should seek to demonstrate that it can be used to modulate acetic acid production in a wider host range than the hybrid in which it was identified.

The identification and characterisation of microbial germplasm unencumbered by third-party licensing or ownership restrictions should be used in subsequent projects as foundational material in which to breed future generations of Australian wine yeast. These strains have robust genetic features and physiological traits that make them ideal for that task. As an example, the moderate 2-PE producer (AWRI 4390) was generated from one of these unencumbered yeasts, and has the potential to be commercialised for sparkling wine production.

Further research is needed with some of the *H. osmophila* strains evaluated in this project to prove their suitability for wine production. This should occur as part of a concerted effort to broaden the scope of microbial germplasm that is currently used by food and beverage industries generally, and the Australian wine industry in particular.

10. Appendix 1: Communication

Outcomes and knowledge generated during the progress of this project were communicated through peer-reviewed publications and conference presentations. In addition, annual meetings were held with researchers at the University of Adelaide and with 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 was extended to industry stakeholders through articles in trade journals, presentations in the AWRI roadshow program, webinars, and workshops at both the 17th and 18th Australian Wine Industry Technical Conferences.

10.1 Publications (non-peer-reviewed)

Cordente, A.G., Schmidt, S., Nandorfy, D.E., Francis, L., Bilogrevic, E., Solomon, M., Pisaniello, L., Siebert, T.E. 2018. Yeast strain selection - an easy and effective way to drive wine style in Grenache. *AWRI Tech. Review* 326: 7–12.

Cordente, T., Schmidt, S., Nandorfy, D.E., Francis, L., Solomon, M., Pearson, W. 2021 Stop and smell the roses: novel yeast that impart 'floral' aromas in wine. *AWRI Tech. Rev.* 250: 5-11.

10.2 Publications (peer-reviewed)

Bellon J.R., Ford C.M, Borneman A.R., Chambers P.J. 2018. A novel approach to isolating improved industrial interspecific wine yeasts using chromosomal mutations as potential markers for increased fitness. *Front. Microbiol.* 9: 1442.

Grebneva Y., Bellon J.R., Herderich M.J., Rauhut D., Stoll M., Hixson J.L. 2019 Understanding yeast impact on 1,1,6-Trimethyl-1,2-dihydronaphthalene formation in Riesling wine through formulation-pathway-informed hydrolytic assay. *J. Agric. Food Chem.* 67: 13487–13495.

Cordente, A.G., Solomon, M., Schulkin, A., Francis, I.L., Barker, A., Borneman, A.R., Curtin, C.D. 2018. Novel wine yeast with *ARO4* and *TYR1* mutations that overproduce 'floral' aroma compounds 2-phenylethanol and 2-phenylethyl acetate. *Appl. Microbiol. Biotechnol.* 102: 5977-5988.

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10.3 Presentations

Bellon, J.R. 2019. Breeding new life into the ancient art of winemaking., 17th AWITC workshop, Adelaide.

Bellon, J.R 2022. Introducing a new breed of wine yeast., 18th AWITC workshop Adelaide.

Cordente, A.G. Generación de levaduras que intensifican aromas florales en vino (Spanish) Enoforum (Zaragoza-Spain, April 2022)

Cordente, A.G. 2021. Modulation of 'fruity' thiol production by yeast in red wines Crush Grape and Wine Symposium, Adelaide.

Cordente, A.G. 2019. Stop and smell the roses: non-GM yeast that impart 'floral' aromas in wine. 17th AWITC workshop, Adelaide.

Cordente, A.G. 2019. Yeast modulation of wine flavour and aroma. National Science Week event, Adelaide.

Cordente, A.G. 2019. Yeast modulation of wine flavour and aroma. AB Biotek, Sydney.

Cordente, A.G. 2018. Overview of strain development at AWRI. Visit from MP Nicolle Flint and Senator the Hon. Zed Seselja (Assistant Minister for Science, Jobs and Innovation), AWRI.

Cordente, A.G. 2017. Generación de levaduras vínicas para mejorar la calidad del vino (Spanish). Universitat Rovira i Virgili, Tarragona, Spain.

Cordente, A.G. 2017. Generation of non-GM yeast that impart "rose" aromas in wine. ComBio, Adelaide.

Schmidt.S.A. 2019. A tale of two alleles? If only it were that simple: Genetic contributions to volatile thiol production by wine yeast. Joint Symposium: Yeast and fermented beverage flavour. Sonoma County, California, USA, 24-26 April 2019.

Schmidt, S.A. and Prior, J. 2022. A palette of yeast for your wine palate (workshop) 18th AWITC. Adelaide, 26 June 2022.

10.4 Posters

Bellon et al. 2018. Wine can increase fitness? Just ask yeast! Yeast Genetics Meeting, Stanford University, California.

Bellon et al. 2019. When less is more: Streamlining the interspecific hybrid yeast genome for improved fermentation. 17th AWITC, Adelaide

Cordente et al. 2019. A rose by any other name: novel wine yeast that impart floral aromas. 17th AWITC, Adelaide

Cordente et al. 2022. Modulation of 'fruity' thiol release during fermentation of red musts by wine yeast. 18th AWITC, Adelaide.

11. Appendix 2: Intellectual property

Program IP has been communicated through presentations and publications and resides in the public domain. Copyright in any reports, posters or presentations is held by the AWRI. Copyright in publications remains with the publisher. No inventions or patentable discoveries have been identified from the work. No Program IP has been commercialised at the time of writing this report.

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 Table 7.

Table 7. List of yeasts generated during the conduct of the project

Category	**Characterised strains**	**Parental strains**
'Rose' aroma variants (11)	AWRI 4234, 4235, 4236	AWRI 1742
'Rose' aroma variants (13)	AWRI4390	AWRI 1939
Variants resistant to toxic analogues of tyrosine (11)		AWRI 2940
Variants resistant to toxic analogues of tryptophan (350)		AWRI 1742, AWRI 1939
S. eubayanus hybrids (2)	AWRI3060 AWRI3061	AWRI 838 AWRI 2759 (*S. eubayanus*) [2]
S. arboricola hybrids (2)	AWRI4122 AWRI4123	AWRI 838 AWRI 2280 (*S. arboricola*) [2]
S. arboricola hybrids (6)		AWRI 947 AWRI 2280 (*S. arboricola*) [2]
S. arboricola hybrids (2)		AWRI 1544 (N96) [3] AWRI 2280 (*S. arboricola*)[4]
S. mikatae hybrids (20)	AWRI5009	AWRI 947 AWRI 1529 (*S. mikatae*) [4]
S. mikatae hybrids (20)		AWRI 1540 (NT116) [1] AWRI 1529 (S. *mikatae*) [4]
S. cariocanus hybrids (27)	AWRI5010	AWRI 947 AWRI 1528 (*S. cariocanus)* [4]
S. uvarum hybrids for acetic acid phenotype (300)		AWRI 1572 (AWRI838 x AWRI1176)

[1]NT116 is a commercial strain developed by Infruitec-Nietvoorbij (South Africa) and licensed by Anchor Yeast
[2]These strains are publicly available from the CBS-KNAW (Netherlands) yeast collection and were obtained under their standard MTA
[3]AWRI1544 is a strain received from Stellenbosch University (South Africa) under their standard MTA
[4]These strains are publicly available from the NCYC (UK) yeast collection and were obtained under their standard MTA

12. Appendix 3: References

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13. Appendix 4: Staff

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14. Appendix 5: Other supporting material (including raw data, if appropriate)

Not applicable