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Australian Grape and Wine Authority

The Australian Wine Research Institute

Defining the nutritional drivers of yeast performance and matching yeast to must



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Project No.: AWR 1302
Author: Dr Simon Schmidt
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Table of Contents

1. ABSTRACT 4

2. EXECUTIVE SUMMARY 4

3. BACKGROUND 5

4. PROJECT AIMS AND PERFORMANCE TARGETS 6

5. METHOD 6

6. RESULTS AND DISCUSSION: 7

A. COMPARATIVE GENOMICS OF WINE YEAST 7

B. CONSTRUCTION OF WINE YEAST BARCODE COLLECTION 8

C. DEFINING OPTIMISED METHODS FOR THE CONDUCT OF POOLED INOCULUM FITNESS PROFILING EXPERIMENTS. 9

D. YEAST RESPONSE TO ENVIRONMENT - COMPARATIVE ANALYSIS OF MOLECULARLY BARCODED YEAST 10

E. GENETIC BASIS OF TOLERANCE TO DEFINED ENVIRONMENTAL CHALLENGES 15

7. OUTCOME/CONCLUSION: 16

A. PERFORMANCE AGAINST PLANNED OUTPUTS 16

B. COULD CHANGING THE METHODOLOGY/TECHNOLOGY HAVE IMPROVED THE OUTCOME? 16

C. PRACTICAL IMPLICATIONS 17

D. BENEFITS TO THE INDUSTRY 17

8. RECOMMENDATIONS: 17

9. APPENDIX 1: COMMUNICATION: 18

A. COMMUNICATION OF OUTCOMES 18

B. JOURNAL ARTICLES WRITTEN DURING PROJECT 18

C. PRESENTATIONS 19

10. APPENDIX 2: INTELLECTUAL PROPERTY: 19

11. APPENDIX 3: REFERENCES 20

12. APPENDIX 4: STAFF 20

13. APPENDIX 5: 20

14. APPENDIX 6: BUDGET RECONCILIATION 20

1. Abstract:

The complex interactions between yeasts and their environment are brought sharply into focus when wine fermentations fail to complete. Retrospective analyses of such failures are difficult or impossible. The relationship between yeast strain genetics and its interaction with the grape juice environment is the subject of this work. Two hundred commercially available wine yeasts, or yeasts isolated from wine, were sequenced to evaluate wine yeast genomic diversity. A representative subset of those sequenced yeasts was used to evaluate the fitness impacts of grape juice variables commonly associated with poor fermentation performance and the genetic determinants of some traits identified.

2. Executive summary:

The complex interactions between yeasts and their environment are brought sharply into focus when wine fermentations fail to complete. Retrospective analyses of such failures are difficult or impossible because of the many combinations of factors that may lead to this undesirable outcome. These factors include choice of yeast strain, of which there are many, grape juice composition and winemaking interventions.

Comparative genomics of yeast

The relationship between yeast strain genetics and a yeast's interaction with the grape juice environment is the subject of this work. Two hundred commercially available wine yeasts, or yeasts isolated from wine, were sequenced to evaluate wine yeast genomic diversity. Whole genome sequence data is now available for 200 wine yeast via the National Center for Biotechnology Information short read archive under the accession number SRP066835. This data archive contains genomic sequence information on wine, ale and cider yeasts of commercial and environmental origin, including species of *Saccharomyces cerevisiae*, *Saccharomyces uvarum* and hybrids of *S. cerevisiae* with non-*cerevisiae Saccharomyces* yeast including *S. eubayanus*, *S. paradoxus* and *S. kudriazevii*. The comparative analysis of this data has been published in the journal *Genes|Genomes|Genetics* (Borneman et al. 2016).

Yeast response to environment - comparative analysis of molecularly barcoded yeast

A fundamental property of any commercial yeast is the degree to which it can compete for resources in their working environment. Yeast competitiveness was assessed directly in this project using a representative collection of barcoded wine yeasts. Strain selection for this representative subset of 94 yeasts was informed by the results of comparative genomics. Each strain was tagged with a unique DNA barcode introduced into a phenotypically neutral location. The tags were required to identify strains in mixed cultures, thus enabling the parallel determination of fitness profiles in a range of industrially relevant media formulations. This was achieved through competition experiments in which differential fitness was assessed in response to environmental challenges.

Environmental variables commonly associated with poor fermentation performance, such as sugar concentration and temperature, were not discriminating factors of yeast strain fitness. Copper concentration and nitrogen availability were, however, powerful contributors to fitness variations between wine yeast strains. Fitness-based predictions of performance were validated using single inoculum fermentations. These experiments showed a high concordance between pooled culture fitness and individual strain performance profiles.

Following initial explorations of yeast and environment interactions that were assessed in defined medium, later stages of the project progressed to fitness profiling experiments using freshly prepared unfiltered juice. This enabled a comparison with defined medium results (fitness validation) using an environment complete with the rich microbial ecology that comprises a standard grape juice. Fitness of strains was evaluated in grape juice with different additives (modified

nitrogen, SO_2, increased sugar concentration etc.) and against a panel of juices. This work showed that juice preferences exist for some strains and lends weight to the idea that strain/must matching may offer tangible benefits when selecting strains for fermentation.

Uncovering the genetic basis of performance

To uncover the genetic basis of performance in specific environmental conditions, classical genetics (mating and screening of progeny) and modern genomics (whole genome sequencing of progeny) have been combined to identify regions of the genome that positively or negatively contribute to the performance of different strains. The primary focus has been on sulfite and copper tolerance, both of which are highly discriminatory for wine yeasts. The genetic basis of resistance to both of these stresses is well researched and much is already known about the evolutionary strategies that yeast use to tolerate these powerful selective agents. However, this work is uncovering an unlikely interdependence between these strategies, the exact nature of which is still being explored.

The project benefitted substantially from the involvement of several industry partners including Yalumba, Treasury Wine Estates through their Wolf Blass winery, and Pernod Ricard through their Orlando winery at Rowland Flat. The project also benefitted through access to sequencing resources at the Ramaciotti Centre for Genomics (UNSW Sydney, Australia).

3. Background:

There are many yeast strains available to the wine industry where there is limited detail available about how they perform in different contexts, both in terms of fermentation kinetics and flavour outcomes. The aim of this project was to build a knowledge base on how different yeast strains perform in different environments (i.e. grape juices of widely varying composition, representing a range of wine styles) and how winemakers can optimise fermentation efficiency, and best minimise the risk of suboptimal (stuck and sluggish) fermentations while delivering wine styles to winemaker specifications through collaborations with AWR 1301 ('Fit-for-purpose yeast').

To address the perennial problem of suboptimal (i.e. slow, sluggish and stuck) fermentations, a survey of Chardonnay grape juice composition was undertaken by the AWRI in 2009 to better understand juice parameters that contribute to fermentation performance. This survey built on previous of work at the AWRI and elsewhere that identified nitrogen and oxygen availability as being critical factors in fermentation efficiency and reliability. It led to the identification of potassium concentration and pH (and particularly their interaction) as key parameters influencing performance outcomes, and different yeast strains were found to differ in their sensitivity to these parameters (Schmidt 2011). However, serious limitations associated with characterising yeast strains one at a time became apparent during this work.

There have been numerous studies on the influences of specific environmental conditions on individual yeast strains. Some studies have simultaneously investigated two or three environmental conditions, for example lipids and oxygen, nitrogen and biotin, nitrogen and pantothenate, and pH and potassium. Due to the limited scope of work performed to date there is a lack of information about the broader context of nutrient interactions. There is also limited information on the wider range of nutrient interactions beyond pair-wise interactions. This is due to the number of experiments required to experimentally implement a multi-factorial design using conventional experimental approaches to fermentation where one of the design variables is yeast strain.

Technological developments in DNA sequencing and statistical approaches to analysis of sequence data have now enabled the simultaneous evaluation of large numbers of strains in many combinations of environments to assess how individual yeast strains behave in differing environmental conditions. The establishment of baseline performance maps allowed the evaluation of different winemaking practices on strain-specific performance profiles. This approach highlighted strain-specific limitations and essential requirements so that suboptimal fermentation conditions can be objectively minimised. Prior to this project, no such detailed information was available for a broad spectrum of strains.

This project contributed to objectives in Wine Australia's Programs 3.1, 3.2 and 3.3 by augmenting winemaking practices that will deliver improved fermentation efficiency with reduced risk of faults though informed use of available wine yeast (germplasm).

4. Project aims and performance targets:

a. Build a genomic database of wine yeast to facilitate strain selection and delineating available diversity
b. Define yeast strain-specific performance parameters for at least 100 commercially available wine strains, enabling the matching of strain to must
c. Evaluate the yeast strain specific efficacy of fermentation additives in modulating the performance of yeast in wine fermentations
d. Contribute to studies in AWRI Project 3.1.1, undertaking the identification of compounds that lead to undesirable (attenuated) sensory characters in wine arising from suboptimal fermentations
e. Identify robust wine yeast strains to be exploited in AWRI Project 3.2.2
f. Provide winemakers with knowledge that will enable them to deliver efficient and reliable fermentations and produce quality wine to market specification.

5. Method:

a. A genetically diverse set of commercially available wine yeast was selected through sequencing of genomic DNA extracted from 200 yeast strains. Sequencing was performed using Illumina HiSeq platforms running off Nextera prepared libraries. Relationships between strains were assessed through whole genome based phylogenetic cluster analysis (Borneman et al. 2016). All sequencing work was performed at the Ramaciotti Centre for Genomics (UNSW Sydney, Australia).

b. A barcoded commercial yeast strain collection was created and a pooled inoculum with equal representation of all barcoded strains generated. Choice of strains for this collection was informed by whole genome based phylogenetic analysis.

c. Pooled inocula were used to perform fermentations in a defined medium and in panels of different grape juices. Iterative rounds of experiments were performed with different combinations of key grape juice variables using factorial experimental designs. Grape juice variables chosen included those commonly measured in wineries and controllable by the winemaker.

d. Representative samples of all barcodes from individual, pooled inoculum fermentation replicates, treatments and time points were obtained by polymerase chain reaction (PCR). The PCR products were molecularly tagged using Illumina compatible indexing primers to allow concurrent analysis. Up to 96 samples could be analysed in a single sequencing run.

e. Relative frequencies of strains at the completion of the exponential growth phase in different environmental conditions were determined through enumeration of barcodes within molecularly tagged amplicon pools. Statistical evaluation of enumerated barcodes was performed via fitting of a Quasi-likelihood Generalised Linear Model (GLMs) using an empirical Bayes approach and then computing an Anova style F-test for each individual comparison that was made (Robinson et al. 2010). Data were filtered to remove strains with little or no informative data. After filtering, 87 strains generally remained in the data set. Prior to model fitting the data was normalised using the relative log expression (RLE) method described by Anders and Huber (2010). In addition,

the common and tagwise dispersions were also calculated. The different variances and the sources of variation were used in the modelling step to estimate significance.

f. Findings from pooled experiments were validated by evaluating individual strains in defined environmental conditions using 100 mL ferments fitted with non-return valves to limit exposure to atmospheric gas. The fermentation medium for validation experimental work was generally a minimal defined medium that models the composition of a Chardonnay juice as described in Schmidt et al. (2011). Fermentation vessels were stirred at 150 rpm and all fermentations were carried out at 17°C except where stated otherwise. Inoculation densities of 5 x10^6 were used for pooled inoculum work and 1 x 10^6 for single strain validation experiments.

6. Results and discussion:

a. Comparative genomics of wine yeast

Genome sequences are now available for 200 wine yeast, comprising *Saccharomyces cerevisiae*, *Saccharomyces uvarum* and hybrids of *S. cerevisiae* with non-*cerevisiae Saccharomyces* yeast including *S. eubayanus*, *S. paradoxus* and *S. kudriazevii*. The strains sequenced included commercial and non-commercial yeast. Commercial yeast strains were purchased as Active Dry Yeast from industry suppliers. Non-commercial strains were accessed from the AWRI wine microorganism culture collection and represent isolates collected from numerous wineries, both in Australia and overseas since the 1950s; many of these have not been previously characterised. Older wine yeast isolates provide some historical context to the relationships between the various wine yeasts in this study and offer a potential untapped source of diversity for strain phenotypic assessment.

Initial inter-genomic comparisons of the 200 sequenced wine yeast strains were made via alignment of each genome with that of the laboratory strain *S. cerevisiae* S288c. The shared differences of the various wine yeast, compared to S288c, form the primary comparative dataset used to construct a dendrogram (tree) of relationships between strains (Figure 1). Additional genetic elements not found within the genome of s288c as well as alignments to other *Saccharomyces* yeasts were made in parallel.

The overall structure of the dendrogram is flat; it has a shallow root structure. This indicates a high level of genetic similarity between strains, consistent with a recent point of divergence. The relationships between the different clades (higher order structures of the tree branches) are poorly defined, with all being equally related to each other. A more robust order begins to appear higher up in the branches of the tree within the small and tightly defined clades. Here the longer branches (and stronger statistical support for tree structure) speak to the influence of domestication through strain selection and breeding on the generation of increased levels of genetic divergence. Surprisingly, there are fewer directly identical strains than were expected prior to commencing this work.

Of the 200 strains sequenced, 54 (27%) form a clade (group) with a large degree of similarity to strain EC1118. EC1118 is a robust 'workhorse' yeast isolated many years ago in France from secondary fermentation of champagne. Subgroups within the larger EC1118 clade show particularly high levels of genetic divergence and evidence of breeding and selection. For example, seven strains are interspecific hybrids arising from crosses of non-*cerevisiae* strains with an EC1118-like parent (Borneman et al. 2016).

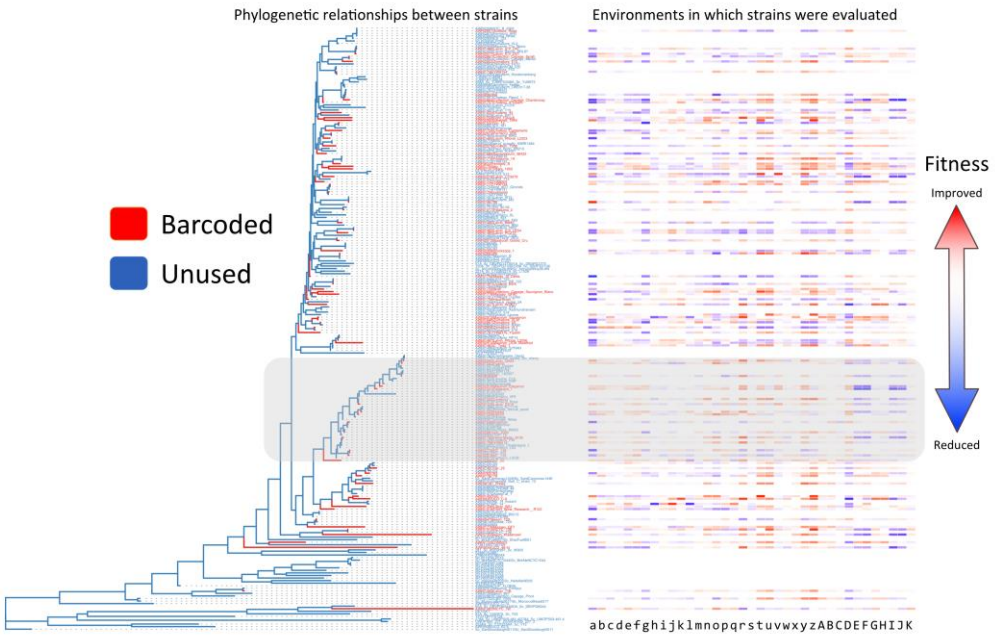


Figure 1. Heat map of yeast fitness profiles mapped against wine yeast phylogeny. In the heat map, red indicates improved fitness and blue indicates reduced fitness. On the dendrogram, strains marked in red indicate those that were barcoded. The subgroup shaded in grey indicates the predominant group of highly related yeasts closely associated with sparkling wine manufacture.

b. Construction of wine yeast barcode collection

The strategy for comparing relative fitness levels of many wine yeast strains in different environments required development of a methodology that enabled strains to be discriminated when harvested from mixed populations. This required the construction of a molecularly tagged (barcoded) wine yeast collection. The strains to be barcoded were chosen based on prior knowledge of their traits and genetic relatedness as determined from genome sequence data.

Barcodes were introduced into 94 strains. This was achieved in two stages. In the first a selectable marker (KanMX), flanked by LoxP sites and a pair barcodes (Caporaso et al. 2012) was integrated via homologous recombination into the HO locus of each selected strain. A second transformation with a non-integrative plasmid containing Cre recombinase stimulated the removal of the selectable marker between the LoxP sites (Carter and Delneri 2010), creating a 150 base pair barcode comprised of a LoxP scar flanked by a molecular tag at each end. Each strain was then cured of the non-integrative plasmid by growing overnight in a non-selective medium and screening for the loss of the antibiotic resistance encoded by the plasmid. The integrity of the barcode within each of these strains was verified by individually sequencing across the region in which the barcode was introduced. In the process of building the barcoded collection some strains were identified that had unanticipated inherent resistance to the selectable marker (G418). The barcoded strains are shown in red on the dendogram in Figure 1.

Inoculation ready pools were created by growing each barcoded strain individually in 50 mL liquid cultures to a defined optical density, mixing the cultures, changing the medium to

fresh YPD + Glycerol, rapidly freezing them in liquid nitrogen and storing at -80°C. This provided a ready stock for use directly in fitness profiling experiments. The creation of the barcoded wine yeast collection provides a resource for the investigation of yeast strain performance and genetic analysis of wine yeast for years to come. The direct effect of disruption of the HO locus in these wine yeasts is that spores dissected from strains in the collection can no longer mate with themselves. Therefore, strains from this collection can be used directly in genetic dissection experiments such a bulk segregant and quantitative trait analysis, an approach that will be discussed in greater detail later in this report.

c. *Defining optimised methods for the conduct of pooled inoculum fitness profiling experiments.*

At the outset of this project a demonstration was required that detecting a change in the abundance of a strain was possible within a mixed population. This was achieved by creating two pools of strains, one in which all strains were in equal proportions and one in which a subset of strains was mixed into a pool at a reduced concentration compared to other strains. These two pools were sequenced and analysed as described above. As shown in Figure 2, a subset of strains is clearly detected at a reduced frequency in the population, demonstrating the capability of the pooled sequencing approach for the detection of altered representation of individuals in mixed populations.

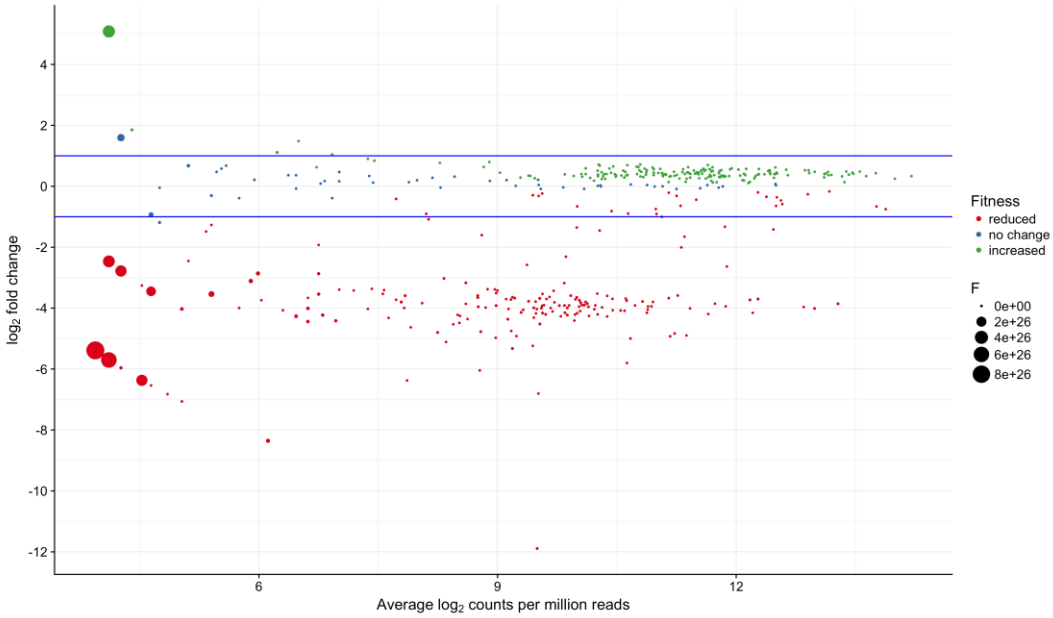


Figure 2. Detection of strain frequency changes in mixed populations by barcode sequencing. Each point represents an individual strain.

Two possible approaches to the conduct of fitness experiments is possible. One involves initiating a continuous culture where a treatment regime is applied to a culture in steady state and changes in population dynamics are compared before and after the change. The other approach is to use a serial batch regime where strains are used to initiate a standard fermentation. A sample from the first culture is then used to inoculate a second culture (serial batch). In this work, the relative merits of continuous culture and serial batch culture for the profiling of yeast strain fitness were determined. The comparison was made using a pooled inoculum comprised of 96 wine yeast gene deletion library

(WYGDL) mutants. Strains in the WYGDL collection have predictable phenotypes and therefore were the most suitable for such an evaluation. Ninety-six mutants were used to reflect the scope and scale of anticipated wine yeast barcode experiments.

Both continuous culture and serial batch culture successfully delivered expected fitness profiles for deletion mutants that had known phenotypes under the conditions tested. However, the resolution provided by serial batch cultures was clearly superior. Strains with a strong phenotype (e.g. a strain with deletion of the CUP2 gene indicating copper sensitivity) could be detected after only one passage through a treatment condition. Increased discrimination of strains with marginal fitness differences could be obtained through additional rounds of serial inoculation.

These results clearly identified serial batch inoculation as the method of choice for assessing the fitness of barcoded wine yeast strains. In addition, while chemostat culture is historically the method of choice for fitness profiling, the technical difficulties associated with its conduct make it difficult to implement in the context of experiments where many different conditions are examined simultaneously.

Finally, standard methods for the extraction of DNA from laboratory yeast had to be adapted to permit the uniform extraction from wine yeast with different morphologies. This was achieved through a modification to the Qiagen yeast genomic DNA extraction kit method involving a threefold increase in the concentration of lyticase used prior to cell lysis and a doubling of the lyticase incubation time.

d. Yeast response to environment - comparative analysis of molecularly barcoded yeast

A general approach adopted for comparing yeast fitness was devised in which eight environmental variables were tested in triplicate at four time points, each sample corresponding to the end of exponential growth phase in a serial inoculation regime. The overall approach was modelled on that taken by (Robinson et al. 2014). This configuration results in 96 samples, which is the limit imposed by the number of index sequences available in an Illumina dual indexed sequencing run on a HiSeq 2500 sequencer. Experiments were performed either in defined medium as described by (Schmidt et al. 2011) when testing the impacts of variations to specific compositional variables, or grape juice that had either been stored or freshly pressed. Grape juices were all derived from white grape varieties, either Chardonnay, Riesling, Pinot Gris or Sauvignon Blanc.

Some strains do not appear at all in the dataset or only occasionally. These strains are not even recoverable from the pooled inoculum. The missing strains are: 739, 793, 1430, 1501, 1787 and 2865. As a result, data for only 87 strains are reported. In addition, some strains deviate in their representation substantially from the inoculating pool once grown up in controlled defined medium. Some examples are 1427, 1716 and 1722, 1077 and 767. These strains are either detrimentally affected by the freeze-thaw process used to prepare the pools and inoculate the ferments or they may grow substantially less well in defined medium compared to YPD.

Table 1 summarises the compositional variables by experimental batch that were evaluated over the course of the project either using defined medium as a matrix for the exploration of effects on strain specific fitness or juice to explore gross matrix effects on the strain performance.

Table 1. Compositional variables assessed during this project

	Defined medium					Grape juice	
	Exp 1	**Exp 2**	**Exp 3**	**Exp 4**	**Exp 5**	**Exp 6**	**Exp 7**
1	High sugar	High potassium	DM_12°C	No vitamins	High copper	High YAN	YPD
2	High copper	High copper	High sugar_12°C	No biotin	Low biotin	High sugar	Defined medium
3	Low pH	High copper/ high potassium	DM_18°C	Biotin only	Low pH	High SO_2	Chardonnay RF
4	Low YAN	Low pH	High sugar_18°C	Biotin 0.1µg/L	High copper/ Low biotin	High sugar/ High YAN	Chardonnay BR
5	High potassium	Low pH/ High potassium	DM_28°C	No thiamine	Low pH/ Low biotin	High sugar/ High SO_2	Riesling EV
6	Low pH/ High potassium	Low pH/ High copper	High sugar_28°C	Thiamine 10 µg/L	Low pH/ High copper	High SO_2/ High YAN	Riesling WB
7	High sugar/Low YAN	Low pH/ High copper/ High potassium		No Pantothenate	Low pH/ High copper/ Low biotin	High sugar/ High SO_2/ High YAN	Sauvignon Blanc WB
8							Pinot Gris Y

The importance of matrix on the fitness response of strains is shown in Figure 3. In this experiment eight different medium types were compared: yeast extract peptone (YPD), defined medium and six different white grape juices. Most of the variation occurs with time as would be expected. With each re-inoculation there is more opportunity for strains to grow and therefore change the relative proportion of strains in the community. The changes in the make-up and relative representations of strains within the community are most obvious between time points 1 and 2. By time point 3 the structure of the community has stabilised and does not change between time points 3 and 4.

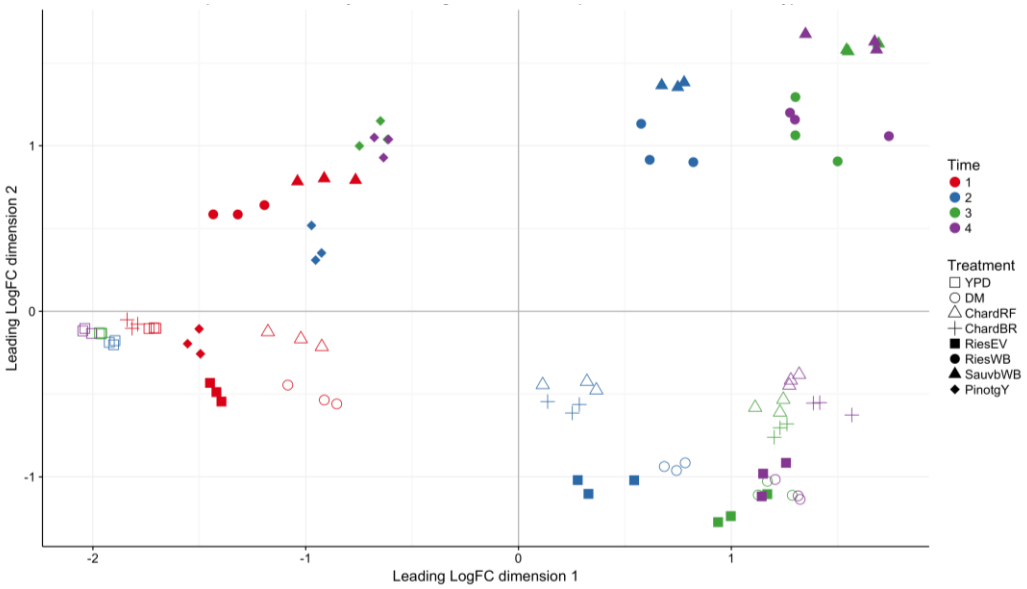


Figure 3. Multi-dimensional scaling plot showing general matrix effects on strain profiles over time. Different point shapes indicate different medium or juice matrices and the colour of the points indicates different time points.

There are some surprising features of this dataset. Firstly, the defined medium formulation is most similar in its effect on yeast strain representation to a Riesling from Eden Valley, despite having a substantially higher pH (3.5 vs 3.1). The other Riesling in the dataset has an impact similar to a Sauvignon Blanc. While the impact of the two Chardonnays is similar to each other despite substantial differences in the available nitrogen of the two juices (338 mg N/L and 170 mg N/L), the Pinot Gris does not have as great an impact over time as any of the other matrices, with the exception of YPD.

Figure 4 shows a more detailed picture of the impacts that the different juices have on specific strains. Compared to RF Chardonnay, there are there are only a few strains that show marginal juice preference for either the BR Chardonnay or EV Riesling. Specifically, strain 2078 does relatively better in the low YAN BR Chardonnay and strains 1502 and 2863 do marginally better in the low pH EV Riesling. Overall strains do not appear to perform differently in any of these juices. However, strain preferences are immediately evident when comparing RF Chardonnay with either WB Riesling or WB Sauvignon Blanc. In these cases, strains 1504, 228, 2280 and 934 all perform much worse in the Riesling and Sauvignon Blanc than they do in the RF Chardonnay. The compositional feature that these two juices have in common is their low pH; both are below 3.0.

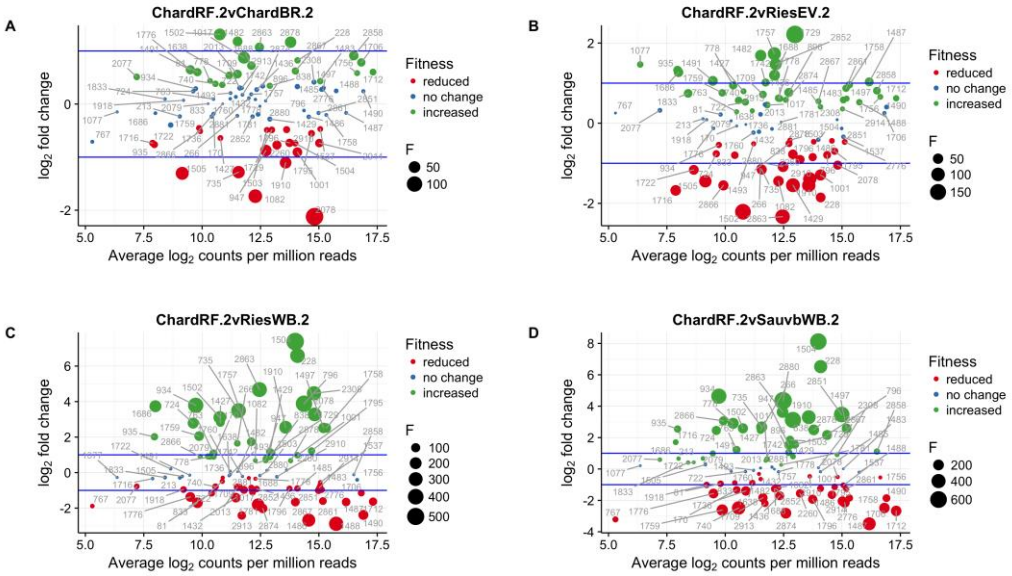


Figure 4 Strain specific performance profiles in RF Chardonnay compared to four other juices; A) RF Chardonnay – BR Chardonnay, B) RF Chardonnay – EV Riesling, C) RF Chardonnay – WB Riesling, D) RF Chardonnay – WB Sauvignon Blanc.

Of the many compositional variables assessed during this project only a handful were demonstrated to substantially alter the competitive fitness of yeasts in a strain specific manner. These variables were copper, sulfite, nitrogen and vitamin concentration. Low temperature, low YAN and high sugar had similar impacts across all strains and combinations of harsh conditions such as low pH, high copper and low biotin or high sugar, low YAN and high SO_2 put such a downward pressure on growth that no individual strain was able to thrive.

There are, however, some notable examples in particularly harsh conditions. In the complete absence of vitamins only one strain dominated. AWRI 1505, a hybrid of *S. cerevisiae* and *S. bayanus,* was able to grow and substantially dominate the culture where all other strains could not. This advantage was completely eroded by the addition of biotin alone, supporting previous work showing that biotin is one of the few vitamins critical for healthy fermentations. In subsequent experimental work it was shown that exceedingly small concentrations of this vitamin (0.1 µg/L) were sufficient to sustain normal growth in the presence of other vitamins, suggesting that in standard winemaking scenarios low concentrations of biotin would be unlikely to be growth limiting for wine yeast.

A second example of an individual yeast being particularly strong in a specific condition is the performance of another hybrid, this time between *S. cerevisiae* and *S. kudriavzevii* (AWRI 1503). This was one of a handful of strains whose competitive fitness was improved at 12°C compared to 28°C. Other strains were more competitive in the warmer temperature, specifically 796 and 1482. This type of temperature-based stratification in performance could be a basis for strain choice for red and white wine fermentations.

Two traits were studied in more detail: resistance to copper and sulfite. Both copper and sulfite can be present in high and unpredictable concentrations in juice because of intensive spaying in the vineyard or protective additions to the bins or crushers. Both copper and sulfite are anti-microbial agents and therefore their effects are inhibitory rather than stimulatory. They are notable because of their capability to discriminate amongst wine yeasts.

Panel A of Figure 5 shows the relative fitness of strains under elevated copper conditions. Although strongly inhibitory to growth for some strains, copper (at least at moderate concentrations) allows some strains to thrive, presumably because of a relief from nutrient constraints due to the suppressive effect on growth of competitor strains. Strains that not only survived but thrived under these conditions were 2913, 1432 and 796. On the other hand, there were a substantial number of strains on which elevated copper placed a particularly heavy burden. Strains 1487, 1537 and 2856 were representatives of that group. For these strains, copper limited their ability to form biomass; so, not only did they grow more slowly but the maximum number of cells they could form became limited. This can lead to ferments inoculated with them failing to complete in a timely manner when copper concentrations are substantially higher than normal.

Panel B in Figure 5 shows the relative fitness of strains under elevated sulfite conditions. In contrast the to the fitness profiles under high copper conditions, there were no major winners when concentrations of sulfite were high. There were many strains that survived, but did not necessarily thrive. However, the inhibitory effect was equally severe. While the impact on strains 228 and 1504 seemed to be particularly critical, there were many other strains for which inoculation into a high SO_2 must would result in extended lag times before obvious signs of fermentation became evident or possibly other strains would come to dominate the ferment before the inoculated strain was able to grow sufficiently. Another feature of the two conditions was that strains that performed well under high copper conditions tended not to not perform well under high sulfite conditions. Different sets of strains appeared to be better suited to either one condition or the other.

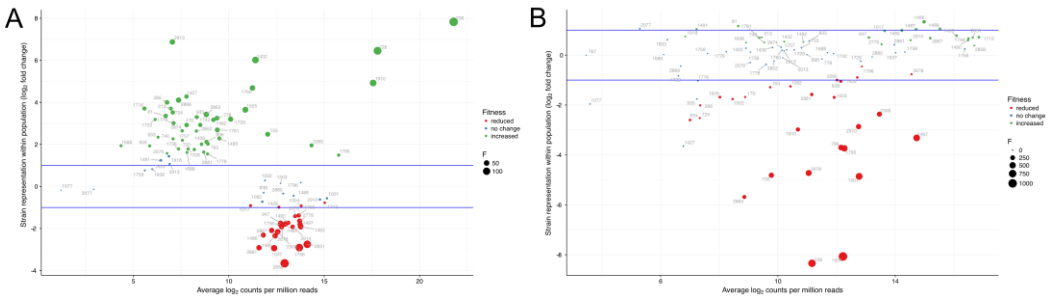


Figure 5. Strain specific performance profiles in media containing A) 10 mg/L copper, B) 18 mg/L free SO_2, compared to a medium with minimal or no copper or SO_2 respectively. Each point represents the fold change representation of a strain under the stated condition. Red indicates significant reduction in representation of a strain, green represents a significant improvement in the representation of a strain.

In summary, this work revealed many aspects of the yeast/environment interaction. A case can be made for the idea that at least one component of yeast strain choice for inoculated fermentations should be consideration of the conditions under which the ferment is to be carried out. A strain that performs well at low temperature may be at a competitive disadvantage at elevated temperature and strains that are competitive in high SO_2 conditions may be at a substantial disadvantage in high copper conditions. In this report a limited number of specific examples have been given but observations can be extended to a larger number of conditions including available nitrogen and pH. The relationship between copper and sulfite will be explored in more detail in the following section.

e. Genetic basis of tolerance to defined environmental challenges

The genetic aspects of copper and sulfite tolerance are rigorously studied fields with long histories. Yeast have evolved different approaches to dealing with these two inhibitory factors. Despite being inhibitory, both are required for the healthy growth of yeast. Copper is a cofactor required for protection against reactive oxygen species (Nevitt et al. 2012) and sulfite is a metabolic intermediate in the sulfate assimilation pathway required for the *de novo* synthesis of key amino acids (Yoshida et al. 2011). As a consequence, the cellular concentrations of both are tightly regulated in yeast.

The regulation of intracellular copper is complex. One major solution to the difficulty of managing free copper ions within the cell is to keep copper bound up. Yeast use a protein known as CUP1 as this sequestering agent (Fogel and Welch 1982). The number of copies of CUP1 in different yeasts has been related to a strain's ability to tolerate high concentrations of copper (Fogel et al. 1983). Copper concentrations in grape juice have been reported to vary from below detection thresholds to 7 mg/L (Schmidt et al. 2011). With wine strains varying greatly in their resistance to copper (Figure 5), a survey of CUP1 copy number was undertaken in the 87 strains for which copper tolerance information was available. These strains had between 2 and 60 copies of CUP1. However, the correlation between CUP1 copy number and copper tolerance was not strong. There were many examples of strains with a high CUP1 copy number but low fitness in a high copper medium. There are clearly other factors impinging on copper tolerance.

A classical genetics approach was undertaken to understand why a strain with high CUP1 copy number can be sensitive to copper. A copper resistant strain (796) was crossed with two copper sensitive strains (1537 and 1487) in two separate matings. The diploid progeny were sporulated and 100 spores of each assessed for their copper tolerance in 100 mL ferments. The spores were assigned to copper sensitive and tolerant pools and the genotypes of the pools assessed in a bulked segregant analysis (BSA), mapping single nucleotide polymorphism segregation in the bulks to identify alleles associated with copper tolerance or sensitivity (Magwene et al. 2011).

The cross between 796 and 1537 identified the CUP1 locus as the major contributor to differences in tolerance between those two strains. Quantitative PCR showed that 1537 has 18 fewer copies of CUP1 than 796. These findings are consistent with previous work that understands copper tolerance to be a function of CUP1 copy number. The cross between 796 and 1487 was between two strains that both have a large number of CUP1 copies. In this cross, BSA identified an allele tightly linked to SSU1. This gene encodes a sulfite efflux pump whose activity is associated with sulfite tolerance. The two parents used in this cross (796 and 1487) have low and high sulfite tolerance respectively which is contrary to their copper tolerance profiles. SSU1 has not previously been associated with copper sensitivity.

The relationship of SSU1 to copper sensitivity was established by deleting SSU1 in the haploid parents that were used to make the crosses used in the BSA analysis. It was shown that deletion of SSU1 in the 1487 haploid relieved it from copper sensitivity.

It is not yet clear why having improved sulfite tolerance may compromise a strain's copper tolerance. Answering this question is the basis of ongoing work. The finding suggests that yeast strains can be either copper tolerant or sulfite tolerant, but not both. This has significant implications for the isolation of broadly stress-tolerant yeasts that work in a range of commercial applications. In summary, CUP1 copy number and SSU1 activity status have been mapped for all 87 strains in the barcoded collection. This information can now be used to predict copper or sulfite tolerance in newly isolated strains, with potential for use in the wine industry or as markers in ongoing breeding efforts to generate strains with more robust performance characteristics.

7. Outcome/conclusion:

a. Performance against planned outputs

A summary of project performance against specific objectives is given below. Overall the project achieved the objectives set out in the original application.

i. A genomic database of wine yeasts was created. Information about the relationships between wine yeasts and the underlying sequence data relating to each strain are publicly available and can be accessed by other wine researchers.

ii. Yeast strain-specific performance parameters for 87 commercially available wine strains was generated, which is short of the 100 originally planned. While attempts were made to barcode 100 strains, only 94 were ultimately possible due to incompatibilities of some strains with the barcoding technique employed. As explained in the results, only 87 of these were routinely detected in sufficient quantity from a mixed pool to permit robust statistical evaluation of their fitness.

iii. Yeast strain-specific efficacy of fermentation additives in modulating the performance of yeast in wine fermentations was evaluated in both defined medium and Chardonnay juice. Yeasts that benefit from complex nitrogen addition were identified, as were strains whose performance was unchanged in response to more generous nutrient conditions.

iv. Identification of compounds contributing to attenuated characters was not pursued beyond working with project AWR 1301 to identify aromatic profiles of stuck ferments from a screen of wine yeast strains in red wine. In that work, clustering of strains based on aromatic profile did not identify a unique aromatic signature in ferments that failed to finish. As a result, this was not pursued further.

v. Six yeasts have been identified for use in future strain development work. These have undergone a rigorous screening process to evaluate their ability to complete fermentation of Chardonnay juice in a timely manner and to understand their baseline aromatic profiles.

vi. Knowledge derived from this project has been and continues to be delivered to stakeholders through trade journals and Wine Australia-funded extension mechanisms. Planned integration of data with the online ferment simulator will provide access to information in a usable form for all levy payers.

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

Some aspects did not progress to the extent that was originally envisaged, at least in part due to technical limitations resulting from evolution in sequencing technology. Specifically, the project was planned around use of a HiSeq 2000. Dual indexing, which was a requirement built into the design of the wine yeast barcode library, only became available on MiSeq and HiSeq 2500 sequencing platforms. The yield of sequence data from MiSeq platforms was insufficient to permit a rigorous statistical treatment of the experiments and sequencing on a HiSeq 2500 was more expensive than originally budgeted. Therefore, fewer experiments exploring yeast-environment interaction could be performed within the project budget than were originally envisaged. With sequencing being a continually evolving technology, it was important to adapt to the conditions and therefore all strain profiling work was performed on a HiSeq 2500. Had the design of the wine yeast barcode collection been approached differently, using a single barcode instead of two to define each yeast strain, there may have been more flexibility in the sequencing platforms that could be used in experimental work. This could have been

achieved through increasing the budget for the construction of the library and this may have saved subsequent costs in the analysis of experiments.

c. Practical implications

The work outlined in this report has three main practical implications

i. By understanding the relationships between a broad range of commercial yeasts and environmental isolates it is immediately possible to choose representative strain sets for research work. This capability will reduce redundancy in experimental designs and facilitate coordination across projects. With an increased focus on fewer strains, a more comprehensive picture can emerge about which strains to use and when.

ii. Through revealing strain-specific limitations in fermentation it will be possible to inform winemakers about potential problems before they arise and diagnose fermentation problems after the fact. Through understanding the nutrient requirements of individual yeast it is possible to advise which yeasts will benefit from nutrient additions and when an addition might have limited impact. Resources have also been developed that will continue to expedite research on fermentation performance issues and methodologies have been established that enable the efficient use of those resources.

iii. By uncovering the genetic basis of various phenotypic traits it becomes possible to categorise the performance attributes of strains without having to engage in expensive and time-consuming phenotyping trials for each new strain that is encountered. This will be of use in bioprospecting projects that aim to isolate many thousands of novel strains from natural and winery environments. These molecular markers can also be put to use in strain development programs where antibiotic-selectable markers cannot be used if those strains are to be deployed in the food and beverages industry.

d. Benefits to the industry

The AWRI's extensive experience in consulting and advising winemakers on rescue of suboptimal fermentations during vintage has made it abundantly clear that prediction of suboptimal fermentations is extremely poor beyond knowledge of sugar and nitrogen impacts. A detailed understanding of the performance interaction of yeast strain and environment has highlighted known, and identified previously unknown, predictive factors thereby improving the tools available to winemakers to better manage fermentation through objective matching of strain to must.

8. Recommendations:

Fitness profiling using a pooled inoculum was an effective and efficient tool to explore the impacts of environmental conditions on strain performance. Supported with appropriate statistical tools and assuming access to appropriate sequencing resources this is an approach that should find multiple applications in future research. Its main limitation is that it effectively assesses a strain's ability to grow, and fermentation performance issues are not always due to an impedance to growth, but may result from higher order nutritional deficiencies that have an impact on general resilience.

Future work will look to increase the complexity of the environment under investigation. Much of the work reported here was done in defined medium. While this is an important stepping stone, the limitations that stem from its basis in our limited understanding of the full complexity of the grape juice medium mean that this methodology must ultimately move to natural juice environments. This comes with its own cost to reproducibility; however, it promises to reveal much about both the phenotypic variability of yeast and also the as yet unseen components of juice that give rise to variable fermentation outcomes. One small window into this is evident in the work presented here

showing yeast strain fitness profiles between a Sauvignon Blanc and a Riesling from the same winery to be more similar than between two Rieslings. It may turn out to be the case that yeasts and their preferences can tell us as much about juice and its composition as experiments manipulating their environment can tell us about the yeasts themselves.

There is still substantial scope to investigate the genetic basis of physiological yeast traits. Gaining a better grasp of this aspect of yeast biology is a long-term thread that underpins much of the work reported here. The tools that are now available to address these questions have never been more powerful and accessible. The projects that have just been completed in the last four years have laid the foundations for the exploitation of this emergent and evolving technology. Staying on top of these developments and what they offer demands a continuous effort in their application because the lead times involved in re-establishing that knowledge is long.

Finally, it is anticipated the yeast/bacterial interactions is an area that requires closer attention. With the increase in popularity of simultaneous alcoholic/malolactic fermentations, warmer climates that bring new disease pressures and changes in the way winemakers approach the use of SO_2 both in the vineyard and the winery there is more opportunity for yeast and bacteria to interact in ways that the current generation of industry practitioners may not have experienced.

9. Appendix 1: Communication:

a. Communication of outcomes

Outcomes and knowledge generated during the progress of this project have been communicated to peers through peer-reviewed publications and conference presentations, and annual meetings with researchers at the University of Adelaide. Representatives of Wine Australia attend these meetings and therefore have had the opportunity to hear updates on project progress outside of the six- monthly reports that were provided for the duration of the project.

Information has been extended to industry stakeholders through articles in trade journals, presentations in the AWRI roadshow program and webinars.

In addition, results generated during this project will also be built into the AWRI Ferment Simulator. This application, which is available to levy payers through the AWRI-hosted WineCloud, enables users to specify compositional variables of their grape juice matrix. When yeast strains are selected that are not suitable in the conditions described the user can be given a warning that will alert them to the risk associated with the choice of that strain.

b. Journal articles written during project

Borneman, A. R., Forgan, A. H., Kolouchova, R., Fraser, J. A., Schmidt, S. A. 2016a. Whole genome comparison reveals high levels of inbreeding and strain redundancy across the spectrum of commercial wine strains of *Saccharomyces cerevisiae*. *G3: Genes|Genomes|Genetics 6*(4): 957–971.

Borneman, A. R., Schmidt, S. A., Pretorius, I. S. 2013. At the cutting-edge of grape and wine biotechnology. *Trends Genetics29*(4): 263–271.

Borneman, A., Chambers, P. J., Schmidt, S. A., Forgan, A., Kolouchova, R., Herderich, M., Johnson, D. (2016b). Wine yeast: where are they from and where are we taking them? *Wine Vitic. J.* 31(3): 47-49.

Schmidt, S.A., Costello, P.J. 2014. Nutrients to support primary and secondary fermentations: what are they and do they work? *AWRI Tech. Rev.* 211: 6-11.

Schmidt, S. A., Henschke, P. A. 2015. Production, reactivation and nutrient requirements of active dried yeast in winemaking: theory and practice. *Aust. J. Grape Wine Res. 21*(S1): 651–662.

Schmidt, S. A., Borneman, A. R., Kolouchova, R., McCarthy, J. M., Bellon, J., Herderich, M., Johnson, D. 2017. Spoilt for choice: Picking the right yeast in a vibrant market. *Wine Vitic. J.,* in press.

c. Presentations

Webinar - Simon Schmidt (2014) What you need to know about rehydration nutrients and nutrient additives in the management of alcoholic fermentation. Available from: https://www.youtube.com/watch?v=EQBfvw9CjNs&feature=youtu.be

Roadshow presentation - Managing stuck fermentations – Simon Schmidt (2015)

Schmidt, S. The fight for dominance in grape juice and the genetics underpinning yeast strain performance. 33rd International Specialised Symposium on Yeast, Cork, Ireland. 26-29 June 2017

Schmidt, S. Yeast competitive fitness in wine-like fermentation environments. 6th Yeast Products and Discovery, Adelaide, Australia. 2-4 December 2015.

10. Appendix 2: Intellectual Property:

This project has generated knowledge, know-how and data on the the nutritional drivers of yeast performance and matching yeast to must, which will be published and communicated as part of the project outcomes.

A barcoded strain collection of 87 strains has been created and CUP1 copy number and SSU1 activity status have been mapped for these strains. This information can be used to predict copper or sulfite tolerance in newly isolated strains with potential for use in the wine industry or as markers in ongoing breeding efforts to generate strains with more robust performance characteristics.

Six yeast strains have been identified for use in future strain development work, based on a rigorous screening process to evaluate their ability to complete fermentation of Chardonnay juice in a timely manner and their baseline aromatic profiles (AWRI 947, AWRI 1719, AWRI 1736, AWRI 1742, AWRI 1787 and AWRI 1939).

11. Appendix 3: References

Anders, S., Huber, W. 2010. Differential expression analysis for sequence count data. *Genome Biol.* 11(1): R106.

Borneman, A. R., Forgan, A. H., Kolouchova, R., Fraser, J. A., Schmidt, S. A. 2016. Whole genome comparison reveals high levels of inbreeding and strain redundancy across the spectrum of commercial wine strains of *Saccharomyces cerevisiae*. *G3. 6*(4): 957–971.

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Huntley, J., Fierer, N., Owens, S.M., Betley, J., Fraser, L., Bauer, M., Gormley, N., Gilbert, J.A., Smith, G., Knight, R. 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J.* 6 (8): 1621–1624.

Carter, Z., Delneri, D. 2010. New generation of loxP-mutated deletion cassettes for the genetic manipulation of yeast natural isolates. *Yeast* 27(9): 765–775.

Fogel, S., Welch, J.W. 1982. Tandem gene amplification mediates copper resistance in yeast. *Proceedings of the National Academy of Sciences of the United States of America* 79(17): 5342–5346.

Fogel, S., Welch, J.W., Cathala, G., Karin, M. 1983. Gene amplification in yeast: CUP1 copy number regulates copper resistance. *Curr. Genet*. 7(5): 347–355.

Magwene, P.M., Willis, J.H., Kelly, J.K. 2011. The statistics of bulk segregant analysis using next generation sequencing. *PLoS Comput. Biol.* 7(11): e1002255.

Nevitt, T., Oehrvik, H. Thiele, D.J. 2012. Charting the travels of copper in eukaryotes from yeast to mammals. *Biochim. Biophys. Acta* 1823(9): 1580–1593.

Robinson, D.G., Chen, W., Storey, J.D., Gresham, D. 2014. Design and analysis of Bar-seq experiments. *G3: Genes|Genomes|Genetics* 4(1): 11–18.

Robinson, M.D., McCarthy, D.J., Smyth, G.K. 2010. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 26(1): 139–140.

Schmidt, S.A., Dillon, S., Kolouchova, R., Henschke, P.A., Chambers, P.J. 2011. Impacts of variations in elemental nutrient concentration of Chardonnay musts on *Saccharomyces cerevisiae* fermentation kinetics and wine composition. *Appl. Microbiol. Biotechnol*. 91(2): 365–375.

Yoshida, S., Imoto, J., Minato, T., Oouchi, R., Kamada, Y., Tomita, M., Soga, T., Yoshimoto, H. 2011. A novel mechanism regulates H_2S and SO_2 production in *Saccharomyces cerevisiae*. *Yeast* 28(2): 109–121.

12. Appendix 4: Staff

Simon Schmidt, Anthony Borneman, Radka Kolouchova, Jane McCarthy, Angus Forgan, Paul Chambers

13. Appendix 5:

Nothing additional to report

14. Appendix 6: Budget reconciliation

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