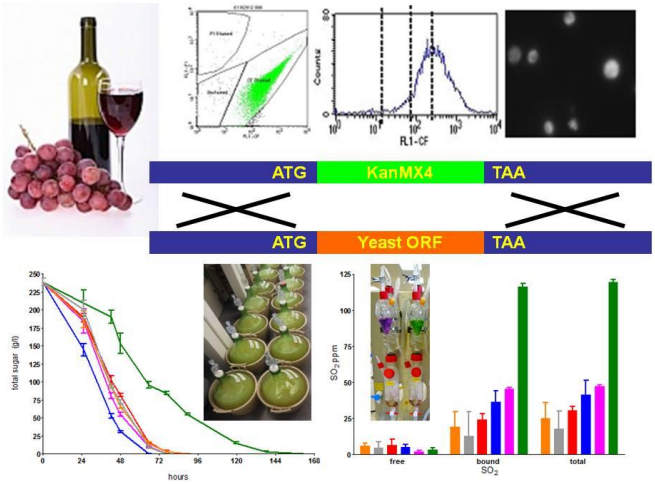
SCHOOL OF AGRICULTURE, FOOD AND WINE | THE UNIVERSITY of ADELAIDE

GWRDC GRAPE AND WINE RESEARCH AND DEVELOPMENT CORPORATION

INNOVATIVE MICROBIALS FOR WINEMAKING EXCELLENCE



FINAL REPORT to
GRAPE AND WINE RESEARCH & DEVELOPMENT CORPORATION

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Principal Investigator: **Professor Vladimir Jiranek**

Research Organisation: **The University of Adelaide**

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Table of contents

Abbreviations 4

Abstract 5

Executive Summary 6

Background 9

Project Aims and Performance Targets 11

Outputs

Outputs 1A & 2B: Hons/MSc students recruited 12

Output 1B: 'Fermentome' defined and short list of fermentation-crucial genes generated 14

Outputs 1C & 1D: Robust or novel yeast and/or bacterial strains generated and *Data on lab scale fermentation performance by superior strains* 27

Output 2A: Data on wines from lab scale fermentations 40

Output 2C: Pilot scale trials of selected candidate improved strains 53

Output 2D: Information on industry priorities in wine microbiology 57

Outputs 1E & 2E: Dissemination of data on strains and application strategies 58

Outcome/Conclusions 60

Recommendations 61

Appendix 1: Communication 62

Appendix 2: Intellectual Property 63

Appendix 3: References 64

Appendix 4: Staff 67

Appendix 5: Supplementary Data 68

Abbreviations

AE	Adaptive Evolution or Adaptively evolved (= Directed Evolution)
CDGJM	Chemically Defined Grape Juice Medium
cfu	Colony forming units
DAP	Di-ammonium phosphate
DE	Directed Evolution (= Adaptive Evolution)
FAN	Free Amino acid Nitrogen
FSO_2	Free sulfur dioxide (SO_2)
GC-MS	Gas Chromatography-Mass Spectrometry
HPLC	High Performance Liquid Chromatography
H_2S	Hydrogen sulfide
JSM_DAP	Juice starter medium with DAP
JSM_FAN	Juice starter medium with free amino acid nitrogen
MQ	MilliQ water
NaCl	Sodium chloride
OE	Over-expression
PBS	Phosphate buffered saline
ppm	parts per million (equates to mg L^{-1})
RO	Reverse osmosis
SD	Standard deviation
SNP	Single Nucleotide Polymorphism
SO_2	Sulfur dioxide
SSA	5-sulfosalicylic acid
TA	Titratable acidity
TSO_2	Total sulfur dioxide (SO_2)
YAN	Yeast Assimilable Nitrogen

Abstract

Making quality wine efficiently is critical for the sector's viability. This ability is inherently linked to the microbiology of the process. In response to winemaker surveys that highlighted the ongoing burden of fermentation difficulties, the Jiranek group undertook an 18-month project to capture earlier research outputs, with the aim of producing superior yeast and bacteria, application protocols and fundamental knowledge to improve both. This study enabled generation and selection of yeast or bacteria, variously with more robust performance and reduced H_2S and/or SO_2 production, along with considerable datasets of genes important for superior fermentation performance to guide future work.

Executive Summary

This project sought to generate superior microbial strains and applications of these and new knowledge on fermentation management to secure and improve wine quality. The key findings are listed below.

- The yeast 'Fermentome', i.e. genes and/or processes required by yeast to sense and respond to the multiple stresses encountered during growth in grape juice to allow complete and efficient fermentation, has been further defined in this project.
    - The High Sugar Fermentation (HSF) dataset first reported in project UA 05/01 was revisited in this study with additional genes confirmed as being necessary for fermentation completion in high sugar. This dataset was renamed the Fermentation Essential Genes (FEG) dataset and is made up of 93 genes.
    - Two Fermentation Relevant Yeast Gene (FRYG) databases were newly compiled in this study. The first, LIT_FRYG, is a collection of genes identified in previously published studies of single stress conditions that are of relevance to fermentation. The second, SGD_FRYG, is a collection of genes associated with fermentation relevant phenotype terms reported in the *Saccharomyces* Genome Database.
    - A comparison of the FEG dataset to the FRYG datasets identified eight genes not present in either the LIT_FRYG or the SGD_FRYG datasets. These genes represent unique targets for further investigation of their roles and associated biological pathways in relation to fermentation, since they have not previously been associated with any fermentation stresses.
    - Preliminary micro scale (1.8 mL) fermentation screening of a yeast gene overexpression library has identified genes putatively involved in decreasing fermentation duration (i.e. positive regulators of fermentation). Further laboratory scale (100 mL) fermentations of candidates from the preliminary screen will be conducted as part of our future project UA 1302.
    - Gene datasets redefined and newly developed in this study provide a valuable source of genetic information for future work in our subsequent project UA 1302. This information will be used to further understand the biological processes important for efficient and complete fermentation as well as providing targets for directed evolution of yeast strains for improved fermentation performance in oenological conditions.

- Vacuolar acidification was identified as a biological process required for efficient completion of high sugar fermentations in project UA 05/01. In this study, vacuolar acidification was monitored during fermentation of yeast strains with specific FEG dataset genes deleted. Vacuolar acidification was affected, as expected, in 20 of the yeast gene deletion strains as these genes had previously been associated with vacuolar acidification. However, a further 14 genes not previously associated with vacuolar acidification also exhibited vacuolar acidification defects. These results further support vacuolar acidification as an important biological process necessary for efficient fermentation in high sugar conditions. These findings are the subject of a publication to be submitted in July 2013 [1].

- Novel yeast strains were generated using non-recombinant methods including sporulation and mass mating, chemical mutagenesis and adaptive/directed evolution

approaches. These strain libraries were screened first in micro-scale fermentations and promising candidates subsequently screen in 100 mL and/or 10 L fermentations.

- Sporulation and subsequent mass mating of ~80 wine yeast strains, followed by ~190 generations of adaptive evolution were performed to generate novel yeast strains. Whilst time allowed for only a relatively small number of novel strains to be analysed (~120 strains in micro-scale and seven in 10 L fermentations), an extensive glycerol collection is available for further screening in a larger range of juice or chemically defined media for strains of interest.
- The adaptively evolved FM16 mixed population reported originally by McBryde *et al* [14] was used to identify new single isolates for screening in juice and chemically defined media. Promising strains were tested in 10 L white juice fermentations and showed fermentation performance equal to or better than the industry standard, EC1118. Yeast derived aroma compounds, key metabolites and SO_2 were analysed and whilst no significant differences were observed, differences were seen between wines made from strains EC1118, L2056 and FM16 evolved strains with higher levels of floral or fruit attributes in the FM16 derived strains.
- Yeast strains with low H_2S and SO_2 production have been produced through either chemical mutagenesis or by exploiting genomic heterozygosity observed in the wine yeast strains EC1118 and Distinction®. Three of these strains were tested in 10L fermentations with low H_2S and SO_2 production observed and comparable fermentation performance compared to our fastest evolved strain FM16-C7. This work was funded in part by a University of Adelaide Wine 2030 award to Dr Michelle Walker.
- White juice for 100 mL and 10 L laboratory fermentations was kindly provided by Treasury Wines (Wolf Blass, Barossa).
- Four malolactic fermentation efficient bacterial strains derived from SB3 have been generated by Directed Evolution (DE), these are still to be completely characterised and genome sequencing of these and the parent is underway.

- Our most promising evolved strain of interest FM16-C7 was provided to the Yalumba Wine Company (Barossa Valley) for testing as a rescue strain for sluggish/stuck fermentations. In the 2000 – 14000 L trials, FM16-C7 was not able to complete fermentation of these stuck fermentations, however it was able to reduce the amount of sugar by approximately half and showed no undesirable characters in the fermentations. These preliminary results suggest FM16-C7 may show promise as a rescue strain, however further trials to optimise the conditions needed for full rescue need to be determined. We hope to continue this collaboration with Yalumba during the 2014 vintage.

- During the course of this project, our high throughput screening methods have been re-evaluated and modified allowing for a greater number of sugar, FAN and malic acid determinations to be completed per unit of time than previously. This will increase productivity in our subsequent project UA 1302 where screening of new DE yeast and bacteria strains will be a high priority.

- Eight students have been trained and have contributed to this research during this project. These includes two summer scholarship students (both of whom have gone on to 2013 Honours degrees, one continuing in our group [Tek] and the other in a

University of Adelaide wine chemistry group [Li]), two visiting international students (one undergraduate [Houlès], one Masters [Mehrotra]), one Masters of Oenology [Lee] and three PhD students [Nguyen, Forrester, Zhang] who are all nearing completion.

- Our findings have been/are being disseminated through:
    - 3 annual lectures to undergraduate and postgraduate oenology/viticulture and biotechnology students (University of Adelaide)
    - 12 conference/seminar/workshop presentations (national and international)
    - 2 electronic magazine articles
    - 1 peer-reviewed publication (submitted July 2013)
    - 3 peer-reviewed publications (for submission in Aug/Sep 2013)
    - 1 provisional patent application

- Two further manuscripts for peer review journals are in preparation and will be submitted in the coming months.

- A provisional patent (McBryde, Gardner and Jiranek [No 207704] *Saccharomyces cerevisiae* strain and methods of use) was filed 28 June 2013 with support of Adelaide Research and Innovation.

Background

The quality of Australian wine is enhanced when winemakers can rely upon predictable fermentation outcomes in terms of consistent trouble-free completion and where yeast and/or bacterial supplement requirements are easily forecasted. However, problematic fermentations, which are either too slow or fail to complete and consequently require significant and costly intervention, are persistently reported by wine makers. It could actually be argued that problem fermentations are increasing in frequency for reasons such as a tendency towards grape musts with altered chemistry, such as higher sugars, due to stylistic or climate changes or the trend of non-interventionalist practices. In our 2010 survey of winemakers conducted as a prelude to this project, up to 8% of fermentations were identified as those with microbiological problems. Extrapolating to Australia's total production of ~1.1 billion litres (ABS 1329.0; 27-2-12), this equates to a total volume of ~96 million litres with a domestic wholesale value of ~$346 million. Whilst much of this would be salvaged, if a conservative 10% loss in value of this wine occurs due to it being unsaleable or even downgraded, this represents a loss of $34.6 million. Clearly research aimed at microbial process efficiency specifically within wine fermentation is of great value to the Australian wine industry.

In our previous projects UA05/06 ("Yeast and colour") and UA05/01 ("Better wine") we delivered improved yeast with robust fermentation properties under conditions of high stress (outperforming the industry gold standards EC1118 or PDM) as well as much related new knowledge. We also developed a multi-pronged approach to generate further yeast candidates, as well as having a collection of candidate improved strains awaiting allocation to specific wine making applications. Throughout these projects we also added to the development of in-house bioinformatics expertise to both manage and interrogate databases of yeast genes related to fermentation. This project therefore sought to funnel these collections, expertise and capabilities into industry specific outcomes outlined in the GWRDC's Program 2 – Winemaking Excellence. It was further aimed to develop superior microbial strains and applications of these and new knowledge on fermentation management to secure and improve Australian wine quality.

Given the multi-factorial basis for problem fermentations and the variety of fermentation outcomes sought by winemakers, it remains unlikely that a single strain of yeast or bacteria will be a panacea for all fermentations. Consequently a more comprehensive palate of application-specific strains is warranted. We sought to directly tackle the issue of problematic (protracted/stuck) primary (alcoholic) and secondary (malolactic) fermentation by producing superior strains of yeast and lactic acid bacteria (LAB) which exhibit fermentation robustness (i.e. fermentation reliability, vigour and reduced nutritional needs) and ideally novel metabolite profiles.

Having proven the concept in earlier projects, we aimed to use a holistic approach which harnesses the extensive expertise and capability of the Jiranek group in terms of yeast and bacterial genomics, strain optimisation and high-throughput robotic screening systems, and strain evaluation. Broadly, the research encompassed molecular and '-omic' technologies, microbiology, biochemistry and physiology in support of non-recombinant improvement by mutagenesis, directed evolution (DE) and classical breeding. We exploited our collections of candidate DE strains, datasets and expertise from completed projects UA05/05 ("Fructophilic yeast"), UA05/05 ("Yeast and colour") and UA05/01 ("Better wine") and GWR Ph0901 ("AE of LAB"). A bioinformatic interrogation of extensive datasets of genome sequences of

novel strains, mutant fermentation performance and relevant online datasets helped identify genes and hence targets or strategies for strain improvement. Genes associated with stress tolerance, nutrient efficiency and fermentation performance were of highest interest. Ultimately we sought to deliver superior yeast and LAB to enable more reliable and/or distinct winemaking outcomes.

Project Objectives

The project objectives 1-5 in the original proposal (UA1101) are stated below. Objectives 4 and 5 were not undertaken in this project but delayed and incorporated into the new GWRDC grant application UA1302 '*Fit-for-purpose yeast and Bacteria via Directed Evolution*' starting July 2013.

1. Undertake *in silico* analysis (data-mining) of databases specifically related to winemaking (wine yeast genomic, transcriptomic and metabolomic data). Yeast library screens conducted under winemaking conditions (in UA05/01 and UA05/06) will help define "winemaking essential" genes (i.e. for high sugar fermentation, flavour formation, growth at extreme temperatures) in order to direct optimisation of new strains with specific enhancements.

2. Characterise adaptively evolved (AE) strains of yeast (and LAB ex PhD project GWR Ph0901) on lab- and pilot-scale, for improved performance in specific problem fermentations as a lead-up to the commercialisation of these.

3. Analyse selected ferments and wines (from 1 & 2) using sensory approaches and metabolomics developed through UA05/01 with colleagues at Manchester, Cambridge and Bioplatforms Australia.

4. Define the genetic basis for desirable phenotypes of prodigious AE strains and use this to guide further evolution experiments for specific properties or else to generate or select non-recombinant variants of yeast and LAB with single and multiple enhancements.

5. Develop a bioinformatics capability to support multi-platform '-omics' approaches to strain development.

Project Aims and Performance Targets

The Outputs and Performance Targets outlined in the approved application 'Innovative Microbials for Winemaking Excellence' (UA1101) for an 18-month duration are shown below. The original target dates are shown as outlined in the application, although the original start date was brought forward from 1st January 2012 to 1st December 2011.

Outputs and Performance Targets 2011-2012

Year 1	Outputs	Target Date	Performance Targets
1A	Hons/MSc students recruited.	31/01/2012	Recruitment of 1 or 2 additional students to the project to support emerging areas.
1B	'Fermentome' defined and shortlist of fermentation-crucial genes generated.	30/06/2012	In-silico analysis (data-mining) of 'omics databases and yeast library screen data. Definition of wine essential genes making up the fermentome.
1C	Robust or novel yeast and/or bacterial strains generated.	30/06/2012	Improvement of strains through multiple rounds of mating, mutagenesis and AE approaches.
1D	Data on lab scale fermentation performance by superior strains.	30/06/2012	Micro-fermentation screens of above strains as well as candidates in existing collections from previous AE experiments undertaken.
1E	Manuscript prepared on data from bioinformatics work.	30/06/2012	Manuscript prepared for submission to peer-review and/or trade journal describing genes (and approach used to find them), which are critical to yeast fermentation performance in winemaking.

Outputs and Performance Targets 2012-2013

Year 2	Outputs	Target Date	Performance Targets
2A	Data on wines from lab scale fermentations.	31/12/2012	Complete secondary phase trials (100 mL to 10 L) with reduced set of candidate strains. Conduct analyses of wines by chemical and, where appropriate, sensory approaches.
2B	Hons/MSc students recruited.	31/01/2013	Recruitment of 1 or 2 additional students to the project to support emerging areas.
2C	Pilot scale trials of selected candidate improved strains.	30/04/2013	Conduct 100 - 1000L fermentations during 2013 vintage, to undertake sensory and wine compositional analysis.
2D	Information on industry priorities in wine microbiology.	30/06/2013	Initiate/conduct survey of industry in conjunction with GWRDC.
2E	Final report and dissemination of data on strains and application strategies.	30/06/2013	Preparation of final report and submission of findings for publication, seminars or workshops for researchers, yeast companies and winemakers.

Outputs 1A and 2B: Hons/MSc students recruited

- **Recruitment of 1 or 2 students to the project to support emerging areas for the duration of the project (January 2012 - July 2013).**

The Jiranek group has recruited a number of Masters students as well as undergraduate students to assist with the project over short periods of time (6 weeks to 4 months). Technical assistance was provided by Mrs Eva Tomczyk employed by the University (1 week; gratis). Two PhD students and two Masters students were employed on casual contract during vintage 2013 (1-2 months) to assist in the completion of the large workload.

These student recruitments and completions have seen the acheivement of **Output 1A** and **2A** and are summarised below. Additional staff and students have also contributed at various times. As these are above and beyond the performance targets for **Outputs 1A** and **2B** (which were limited to Hons/MSc students) they are not assigned to either Output (i.e. designated "NA" below).

PhD Students

Name	Thesis Title	Year Commenced	Output
Trung D. Nguyen	Generating robust yeast for successful fermentation in high sugar media	2010	NA
Jin Zhang	Assimilable nitrogen catabolism by wine yeast	2010	NA
Jade Forrester	Characterisation of the wine meta-metabolome: Linking sensory attributes to yeast genotype	2010	NA
Alice Betteridge	Evolution of superior lactic acid bacteria	2010	NA

Master of Oenology Student

Name	Thesis Title	Year Completed	Output
Andrea Lee	The formation of hydrogen sulfide and glutathione during wine fermentation of *sul1/sul2* sulfate transporter mutants	2012	1A

Honours Student

Name	Thesis Title	Year Commenced	Output
Ee Lin Tek	Evaluation of the oenological significance of genes contributing to a haploproficiency fermentation phenotype	2013	2B

Summer Scholars

Name	Project Area	Year	Output
Sijing Li	Robust or novel yeast	2012	NA
Ee Lin Tek	Yeast fermentome	2013	NA

Visiting Scholars/Staff

Name	Project Area	Year	Ouput
Anne Houlès	Robust or novel yeast	2012	NA
Shruti Mehrotra	Robust or novel yeast	2012	1A
Dr Michael Dow	Lab scale (10 L) ferments - secondary phase trials	2013	NA

Brenton Mui	Lab scale (10 L) ferments - secondary phase trials	2013	2B
Johannes Scharwies	Lab scale (10 L) ferments - secondary phase trials	2013	2B
Eva Tomczyk	Lab scale (10 L) ferments - secondary phase trials	2013	NA
Dr Monique Shearer	Lab scale (10 L) ferments - secondary phase trials	2013	NA

Output 1B: Fermentome defined and short list of fermentation crucial genes generated

- ***In-silico* analysis (data-mining) of 'omics databases and yeast library screen data.**
- **Definition of wine essential genes making up the fermentome.**

Contributors

Current PhD students: Jin Zhang, Trung Dung Nguyen, Jade (Forrester) Haggerty
Current Honours student: Ee Lin Tek
Postdoctoral fellow and/or student supervisors: Drs Michelle Walker, Jennie Gardner, Tommaso Liccioli, Joanna Sundstrom and Professor Vladimir Jiranek

Background

In project GWRDC UA 05/01 '*Better wine through novel and better informed application of microbiology*' we utilised a commercially available laboratory yeast deletion library (BY4743 *YFG::KanMX [2]*) to identify genes essential for the timely completion of high sugar fermentation referred to as HSF genes or the laboratory yeast Fermentome. Essentially these genes are required for yeast to sense and respond to the multiple stresses encountered during growth in grape juice to allow utilisation of all sugars. Importantly, these stresses also change over the course of fermentation with the dynamic changes that occur during oenological fermentation. 89 genes were identified, which when deleted, either gave rise to protracted fermentation (80 mutants) or fermentation arrest (9 mutants) under conditions of high sugar (200 g l^{-1}) and optimal nitrogen (450 mg l^{-1} FAN). Whilst fermentation under limited or altered assimilable nitrogen remains topical amongst winemakers, we were unable to study such conditions as the laboratory strain used was an auxotroph, requiring supplementation with specific amino acids in order to grow. Whilst at this time no prototrophic library was available for this project, we successfully used supplementation to allow fermentation performance to be assessed under optimal nitrogen conditions.

Recently, a prototrophic version of the library has become available [3] (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/) which together with a wine deletion library constructed by AWRI [4] will be utilised in the elaboration of the wine yeast Fermentome as part of the new GWRDC project UA1302 '*Fit-for-purpose Yeast and Bacteria*'.

In this project UA11/01 '*Innovative Microbials for Winemaking Excellence*' we have further defined the laboratory yeast Fermentome by in-depth analysis of key subsets of HSF genes via an *in silico* analysis as well as examination of other methods of gene manipulation (gene overexpression) that results in alteration of fermentation efficiency. We have also integrated previous data within this set, for example as part of generating nutritionally efficient yeast (UA05/01) we identified a number of genes that when deleted significantly improve fermentation efficiency under nitrogen limiting conditions (High Nitrogen Efficiency genes). These deletants were either haploid laboratory or wine yeast (△orf) or heterozygous (△orf/ORF) diploids (laboratory yeast). Together, these genes were also added as important members of the laboratory yeast Fermentome.

The aim of the current project UA11/01 was twofold: firstly, to define the Fermentome through *in silico* analysis (data mining) of published '-omics' datasets relevant to fermentation and secondly, to further interrogate highlighted biological pathways. In view of this, we also strove to enrich the fermentome dataset. To achieve this we analysed the

fermentation performance of a library of yeast gene over-expression mutants [5]. Genes that when over-expressed result in shortened fermentation duration are potentially positive regulators of fermentation and may be ideal targets for later strain development.

We have begun to investigate each highlighted individual gene and enriched processes linked to fermentation with a view to understanding their role in yeast adaptation to specific stresses during alcoholic fermentation. More specifically, we have found that biochemical processes such as maintenance of cellular ion homeostasis through the action of the V-ATPase are of vital importance for fermentation efficiency.

The 'Fermentome' dataset together with other complex datasets such as whole genome sequences are invaluable for yeast strain optimisation using techniques such as Directed Evolution. They allow informed targeting of specific selection pressures to determine potential phenotype outcome.

Methods

Confirmation of clonal identity of gene deletions in laboratory yeast strains

Genomic DNA was isolated from parent and deletant strains [6]. Gene deletions were confirmed by the following Polymerase Chain Reaction (PCR) using primers shown in **Supplementary Tables 1** and **2**.

1. gene-specific forward (A) and reverse (D) primers
2. gene-specific forward (A) and KanB primers or
3. gene-specific reverse (D) and Kan C primers

Polymerase chain reaction (PCR) was performed with 50 ηg genomic DNA of the appropriate strain using 2.5 units of Mango Taq™ DNA [7] or 0.5 units of Velocity proofreading DNA polymerase [8] per 25 µL reaction with a BIORAD C1000 thermal cycler [9]. PCR cycling conditions were as described on the *Saccharomyces* Genome Deletion Project website http://www-sequence.stanford.edu/group/yeast_deletion_project/deletions3.html. DNA sequencing was performed using 75 ηg of purified PCR product and 10 pmol of gene specific primers in a 12 µL volume using a AB3730*xl* capillary sequencer (AGRF, Brisbane)**.** DNA sequence alignment was performed using Geneious 5.6.1 software (http://www.geneious.com/) or LAST genome alignment software (http://last.cbrc.jp/) [10].

Classification and numeric enrichment of identified genes annotated to Gene Ontology (GO) terms using computational software tools

The Fermentation Essential Genes (or FEG) dataset replaces the HSF dataset reported in UA05/01, and includes 93 genes. The FEG dataset was analysed using GOToolBox; specifically GO-Stats (http://genome.crg.es/GOToolBox/) [11] which allowed the hierarchical clustering and over-representation (enrichment) or under-representation (depletion) of genes based on shared Gene Ontology (GO) terms. The clustering of genes based on their shared gene annotations was also determined using SGD Gene Ontology Slim Mapper (http://www.yeastgenome.org/cgi-bin/GO/goSlimMapper.pl). The interpretation of the GO terms identified was simplified using the web-based visualisation tool GO-*Module* (http://lussierlab.org/GO-module) [12].

Fermentation Relevant Yeast Genes (FRYG) database

Two databases denoted **F**ermentation **R**elevant **Y**east **G**enes (FRYG), were compiled from data available on the *Saccharomyces* Genome Database (http://www.yeastgenome.org/) and

from a literature survey. The databases were referred to as SGD_FRYG and LIT_FRYG, respectively, and were used in the *in silico* analysis. Statistical analysis of the results was performed using a hyper geometric algorithm as described by Martin et al. [11] to determine whether there was an enrichment (over representation) of genes identified in this study when compared to the FRYG datasets.

Evaluation of identified yeast deletants in laboratory scale (100 ml) fermentations
Triplicate 100 mL fermentations were performed in CDGJM_200 using 250 ml Erlenmeyer flasks fitted with airlocks [13]. N_2 sparging was omitted as previous studies demonstrated that anaerobic conditions were achieved within 4-6 hours of fermentation commencement (data not shown). Fermentation progress was monitored by periodic sampling (6-12 h) and enzymatic analysis of residual glucose and fructose [14] modified to suit a 96-well plate format. Fermentations were considered dry (completed) when the residual sugar was less than 2.5 g l^{-1}. Ammonia was measured enzymatically using glutamate dehydrogenase (Cat: 10197734001, Roche) whilst residual free amino acids were detected by the NOPA (*o*-phthaldialdehyde/N-acetyl-L-cysteine) method [15].

For the High Sugar Fermentation experimental work, the CDGJM_200 was enriched with polyphenols (200 g L^{-1} equimolar glucose and fructose; 450 mg L^{-1} FAN, 3 g L^{-1} polyphenol extract (Cat: Tppr, OenoProd, Sarl)). For the Haploproficiency (HP) experimental work fermentations were conducted according to Parker 2009 [16] with the nitrogen content in CDGJM_200 reduced to 75 mg L^{-1} FAN. For High Nitrogen Efficiency experimental work the nitrogen content of the CDGJM_200 was either 450 mg L^{-1} FAN or 55 mg L^{-1} FAN.

Screen of the over-expression library in the haploid wine yeast isoC9△leu2
Liquid handling of samples was performed using a CAS3800 robot (Corbett Robotics, Sydney). CDGJM-leu starter contained 450 mg L^{-1} FAN and lacked leucine. The nitrogen content in CDGM-leu_200 was the same as the starter medium, and the sugar content was 200 g L^{-1}, as equimolar glucose and fructose [17, 18]. The medium was spiked with 0.164 mg L^{-1} hexanol for determination of yeast derived aromatic compounds (Jade Haggerty, *pers. comm.*).

The over-expression library was kept at -80°C in 96 well plates and thawed prior to inoculation of 2 μL of each clone into 200 μL minimal drop-out medium (lacking leucine) [19] in 96 deep-well plates. Incubation was performed for 48 h at 28°C in a humidified box without shaking. After re-suspension by agitation on a mini vortexing shaker, 5.72 μL of culture was used to inoculate 200 μL CDGJM-leu starter medium in 300 μL 96-well plates and statically incubated for 24 h. The cells were resuspended and diluted 1 in 10 in fermentation medium (CDGJM-leu_200). Fermentation was performed in UV sterilised 96 deep-well plates with 1.8 mL CDGJM-leu_200 medium and 20 μL of 1/10 diluted starter culture. Each plate was sealed with a breathable seal (Cat: BF-400, Adelab Scientific) and incubated in a humidified container at 28°C for up to 19 days. Sacrifical plates were removed on days 1, 7, 10, 13, 15 and 19 for monitoring of fermentation progress by refractive index (°Brix). Individual plates were frozen at -20°C for later enzymatic analysis of residual glucose and fructose [14] Free amino nitrogen (FAN) [15], and GC-MS analysis [20].

Results and Discussion
Genes essential for high sugar fermentation
We reported in UA 05/01 on 89 genes which were required for High Sugar Fermentation (HSF); deletion of which resulted in fermentation protraction in homozygous diploid mutants

(or deletants). In this project (UA11/01) we have re-examined the genes involved in the assembly and structure of vacuolar ATPase, the major proton pump responsible for vacuolar acidification [21], a process identified as being essential and central to yeast adaptation during fermentation. In the original study we identified 10 structural genes of the vacuolar ATPase complex: 7 of the 8 subunits of the V_1 domain (*VMA2*, *VMA5*, *VMA7*, *VMA8*, *VMA10*, *VMA13*) and 3 of the 6 subunits of the V_o domain (*VMA1*, *VMA3*, *VMA16*) as shown in **Table 1**. *VPH2* and *PKR1* encoding V-ATPase assembly factors were also identified. However, four genes (*VMA4* (subunit E), *VMA6* (subunit d), *VMA11* (subunit c') and *VMA22* (assembly factor), contrary to expectation did not affect fermentation in the corresponding yeast deletants. The genes were sequenced within the corresponding commercial library mutants and not surprisingly were found to be incorrect. New clones were sourced from the supplier and their identity verified. Protracted fermentation was observed with all four mutants, which strongly corroborated our previous findings that the vacuolar ATPase was essential for vacuolar acidification. The enzyme's action as a proton pump to acidify the vacuole is intrinsically connected to cellular ion homeostasis (through the action of anti-porters utilising the generated membrane potential via a proton electrochemical gradient), as well as proteolytic activation (digestion of autophagasomes during autophagy or vacuolar proteolysis induced by starvation), endocytosis, and vacuole fusion [21, 22]. These processes were identified through Gene Ontology (GO) analysis of the 89 HSF gene dataset, which showed an enrichment of genes specifically annotated to the processes cellular ion homeostasis, (micro) autophagy and vacuole membrane fusion.

The HSF dataset was renamed as Fermentation Essential Genes (FEG) in this study and now includes 93 genes. GO analysis revealed the same GO terms as previously described; namely vacuolar acidification, cellular ion homeostasis, cellular pH regulation and autophagic processes. PCR amplification and DNA sequencing of selected clones within the dataset was undertaken (**Supplementary Table 1**) to have an indication of clonal error within the dataset. Furthermore, all except one (*RPA12*) of the 9 mutants that resulted in complete fermentation arrest (stuck fermentation) were authenticated by DNA sequencing of the PCR amplified deletion cassettes (*SIN3*, *ZAP1*, *NPT1*, *TPS1*, *TPS2*, *PLC1*, *DOA4*, and *SSQ1*). The clone corresponding to △rpa12 was shown to be incorrect, with DNA sequencing of the amplified product identifying the gene deletion as *ptk2::KANMX*. *PTK2* (*YJR059W*) encodes the protein kinase Ptk2p which is involved in ion homeostasis [23] and is responsible for the activation of plasma membrane proton pump Pma1p in response to glucose [24].

In silico analysis of fermentation essential genes with the use of the FRYG databases

FRYG (Fermentation Relevant Yeast Gene) databases were compiled from previously published genome wide studies that are performed under conditions relevant to industrial fermentation either manually from a compilation of selected literature or from the *Saccharomyces* Genome Database (phenotype database). The databases were complementary; the first (LIT_FRYG) was compiled from studies of conditions where usually only a single stress was imposed that also is known to occur during industrial fermentations. The second (SGD_FRYG), was a compilation of fermentation relevant phenotype terms specifically selected from the more general SGD phenotype terms database, whereby only null mutants (deletants) with a specific phenotypic response, i.e. decreased resistance or increased sensitivity were selected. The SGD_FRYG database was considered more inclusive, where 83 genes from the FEG dataset were also common, whilst mutants of 10 FEGs have not been previously reported to be affected under any fermentation related conditions (**Table 2**). Since fermentation outcome is reliant on yeast overcoming a multitude

Table 1: Genes required for assembly and function of vacuolar ATPase identified as being required for fermentation in UA05/01 and UA11/01 (adapted from Forgac [21])

Gene	Protein	Subunit Function
V-ATPase (V_1 domain*;* 8 subunit peripheral domain)		
VMA1 (*TFP1*)	A	ATP hydrolysis
VMA2	B	Non-catalytic ATP site, binds actin and aldolase, stator subunit
VMA5	C	Regulatory, stator subunit, binds actin
VMA8	D	Rotary subunit
VMA4	E	Stator subunit, binds RAVE and aldolase
VMA7	F	Rotary subunit
VMA10	G	Stator subunit
VMA13	H	Regulatory, stator subunit
V-ATPase (V_0 domain; 6 subunit integral domain*)		
VPH1	a (vph1p)	Proton pore, stator subunit, targeting (vacuole), binds aldolase
VMA3 (*CUP5*)	c	Proton pore, Rotary subunit, dicyclohexylcarodiimide binding)
VMA11 (TPF3)	c'	Proton pore, Rotary subunit, binds to assembly factor Vma21p
VMA16 (*PPA1*)	c"	Proton pore, Rotary subunit
VMA6	d	Rotary subunit, coupling
Assembly Factors		
VPH2 (VMA12)	Vph2p	Integral membrane protein; vacuolar H^+ ATPase (V-ATPase) assembly
PKR1	Pkr1p	V-ATPase assembly factor, functions with other V-ATPase assembly factors in ER to assemble V-ATPase membrane sector (V_o)
VMA22	Vma22p	Peripheral membrane protein; vacuolar H^+ ATPase assembly

* *VMA9* (*YCL005W-A*) and *SKP*1 (*YDR328C*) are not in the homozygous diploid deletion library. *SKP*1 is an essential gene for growth. *VMA9* encodes V_0 domain e subunit whilst Skp1p is part of RAVE comp

of stresses we would expect that genes associated with cross-tolerance to multiple stresses may be the most affected in our fermentation screen. Sensitivity to multiple or different stresses as identified in the FRYG databases gave an indication as to whether fermentation would arrest or become protracted; however this was certainly not a rule (**Table 3).**

The importance of appropriate vacuolar acidification for successful completion of fermentation is evident by the 20 genes annotated to this GO term within the 93 FEG dataset. We report on vacuolar acidification of all FEG mutants as demonstrated by flow cytometry of cells sampled 24 h post inoculation from 100 mL fermentations in CDGJM. Vacuolar acidification was monitored by the pH dependent hydrolysis of 6-CFDA in the vacuole. Increased fluorescence is observed in cells where vacuolar acidification malfunctioned (i.e. pH decreased). We examined both those genes annotated to this GO term as well as the remainder of the 93 FEG genes, which have not previously been associated with vacuolar acidification. Through this study we identified 33 genes, which when deleted, resulted in an increase in pH as demonstrated by 6-CFDA hydrolysis; 14 were already known to be associated with vacuolar acidification and this is the first report of the remaining 19 to have any involvement in vacuolar acidification. These findings are the subject of a manuscript being submitted in early July 2013 [1].

The FRYG datasets were also useful in elucidating which genes and processes were potential adaptation mechanisms that allow yeast to overcome fermentation stresses. For example, the disaccharide trehalose is associated with increased survival and cell protection against stresses such as heat [25] and ethanol [26]. Deletion of either *TPS1* or *TPS2* results in the cessation of fermentation probably because of the lack of trehalose in △tps1 and reduction in △tps2 mutants, respectively [27]. Over-expression of *TPS1* was recently shown to improve ethanol production in elevated temperatures (38°C), increasing yield by ~40% and the temperature threshold for growth to 42°C [28]. These traits have a potential application in the production of alcoholic beverages (wine) or biofuel if this genotype could be reproduced by non-recombinant methodologies. In this study we have shown that the stuck fermentation phenotype can be reversed, with the strain having a shorter fermentation duration than the parent strain BY4743 when *HXK2* (encoding hexokinase) is over-expressed in △tps1 (data not shown). We are now investigating whether other genes involved in hexose sensing and carbohydrate metabolism can be modified to positively affect fermentation. Those genes which result in increased fermentation efficiency due to increased tolerance towards fermentation stress (eg ethanol, elevated temperatures, etc) are potential candidates for the production of robust yeast by non-recombinant methodologies. A report of this work will be included in Trung D. Nguyen's PhD thesis.

Table 2: Fermentation Essential Genes not previously described as related to fermentation (via SGD_FRYG database analysis)

Systematic Name	Standard Name	Alias	Description
YBR131W	*CCZ1*	*CVT16*	Protein involved in vacuolar assembly, essential for autophagy and the cytoplasm-to-vacuole pathway
YDL076C	*RXT3*		Subunit of the RPD3L complex; involved in histone deacetylation; protein abundance increases in response to DNA replication stress
YDL117W	*CYK3*		SH3-domain protein located in the bud neck and cytokinetic actin ring; mutant phenotype and genetic interactions suggest a role in cytokinesis
YDR022C	*ATG31*	*CIS1*	Autophagy-specific protein required for autophagosome formation; may form a complex with Atg17p and Atg29p that localizes other proteins to the pre-autophagosomal structure
YER185W	*PUG1*		Plasma membrane protein with roles in the uptake of protoprophyrin IX and the efflux of heme; expression is induced under both low-heme and low-oxygen conditions; member of the fungal lipid-translocating exporter (LTE) family of proteins
YFL012W	*YFL012W*		Putative protein of unknown function; transcribed during sporulation; null mutant exhibits increased resistance to rapamycin
YFR053C	*HXK1*		Hexokinase isoenzyme 1; a cytosolic protein that catalyzes phosphorylation of glucose during glucose metabolism; expression is highest during growth on non-glucose carbon sources; glucose-induced repression involves hexokinase Hxk2p; HXK1 has a paralog, HXK2, that arose from the whole genome duplication
YHR171W	*ATG7*	*CVT2 APG11 APG7*	Autophagy-related protein and dual specificity member of the E1 family of ubiquitin-activating enzymes; mediates the conjugation of Atg12p with Atg5p and Atg8p with phosphatidylethanolamine, required steps in autophagosome formation
YJL036W	*SNX4*	*ATG24 CVT13*	Sorting nexin, involved in retrieval of late-Golgi SNAREs from post-Golgi endosomes to the trans-Golgi network and in cytoplasm to vacuole transport; contains a PX phosphoinositide-binding domain; forms complexes with Snx41p and with Atg20p
YOR267C	*HRK1*		Protein kinase; implicated in activation of the plasma membrane H(+)-ATPase Pma1p in response to glucose metabolism; plays a role in ion homeostasis; protein abundance increases in response to DNA replication stress

Table 3: Genes associated with decreased resistance or increased sensitivity towards stresses related to fermentation (SGD_FRYG) for the 9 deletants and vma mutants which give rise to stuck fermentation and protracted fermentation respectively

Acidic pH resistance	Hyperosmotic stress resistance	Oxidative stress resistance	Heat sensitivity	Starvation resistance	Ethanol resistance	Systematic Name	Standard Name
							Stuck mutants
↓	↓	↓	↓		↓	YDR074W	*TPS2*
↓	↓	↓	↓		↓	YDR069C	*DOA4*
	↓	↓	↓		↓	YLR369W	*SSQ1*
	↓	↓	↓		↓	YPL268W	*PLC1*
		↓		↓	↓	YJR059W	*PTK2*
↓					↓	YBR126C	*TPS1*
		↓	↓			YOR209C	*NPT1*
↓		↓				YJL056C	*ZAP1*
			↓		↓	YOL004W	*SIN3*
							V (H^+) ATPase
	↓	↓	↑	↓	↓	YEL051W	*VMA8*
↓		↓	↑	↓	↓	YKL080W	*VMA5*
↓	↓	↓	↑		↓	YPL234C	*VMA11*
↓	↓	↓	↑			YDL185W	*VMA1*
↓		↓	↑		↓	YGR020C	*VMA7*
	↓	↓	↑		↓	YHR026W	*VMA16*
↓		↓	↑		↓	YHR060W	*VMA22*
↓			↑	↓	↓	YOR270C	*VPH1*
		↓	↑		↓	YHR039C-A	*VMA10*
		↓	↑		↓	YEL027W	*VMA3*
		↓	↑		↓	YLR447C	*VMA6*
↓		↓			↓	YOR332W	*VMA4*
		↓			↓	YBR127C	*VMA2*
↓		↓	↑			YKL119C	*VPH2*
					↓	YJR033C	*RAV1*
					↓	YDR202C	*RAV2*
				↓		YMR123W	*PKR1*

Decrease in resistance, ↓; increase in sensitivity, ↑ *VMA13* (*YPR036W*) was not associated with any of the above fermentation related stresses.

Genes which confer haploproficiency and nitrogen efficiency during fermentation

In line with generating an expansive list of genes required for successful fermentation, i.e. the fermentome, we have also continued to work on genes which further ensure the probability of a desirable fermentation outcome. We reported in UA05/01 on fermentation haploproficiency in certain hemizygous diploid (*⊿orf/ORF*) deletants whereby fermentation duration was reduced in comparison to the parent (*ORF/ORF*) strain (Thomas Parker, past Honours student [16]). We have revisited 9 of the candidate genes (**Supplementary Table 2**). Five strains (*⊿whi2/WHI2*, *⊿pip2/PIP2*, *⊿pun1/PUN1*, *⊿ylr297w/YLR297W*, and *⊿fre7/FRE7*) were reconstructed in a protrophic laboratory yeast (△orf BY4741/S288c). These strains were evaluated in FAN-limited (75 mg L^{-1}) conditions. Whilst the fermentation performance of 3 strains was statistically different to the parent (one-way Anova), the minimally shortened duration (11.5 h) was not of sufficient magnitude in terms of an industrial fermentation (**Figure 1**). As genetic background can influence the phenotypic outcome of specific deletions these three genes and four others (*HSP150*, *IRC9*, *RMD6* and *RPL2B*) previously identified as being haploproficient for wine fermentation under FAN-limited conditions will be investigated in future. Briefly, hemizygous deletants in a wine yeast background (Lalvin L2056) are currently under construction and will be evaluated in 100 mL fermentations. The results will be reported through the new GWRDC project UA1302 '*Fit-for-purpose yeast and bacteria via directed evolution*'.

The additional experimental data obtained as part of Ee Lin Tek's Honours project will allow the publication of previous work on the subject (by Thomas Parker). We would like to recruit Ee Lin Tek as a PhD candidate, as she is a highly competent researcher, as part of the new project UA1302 to continue in related work. She is currently holding a GWRDC Honours Scholarship (GWR H1202).

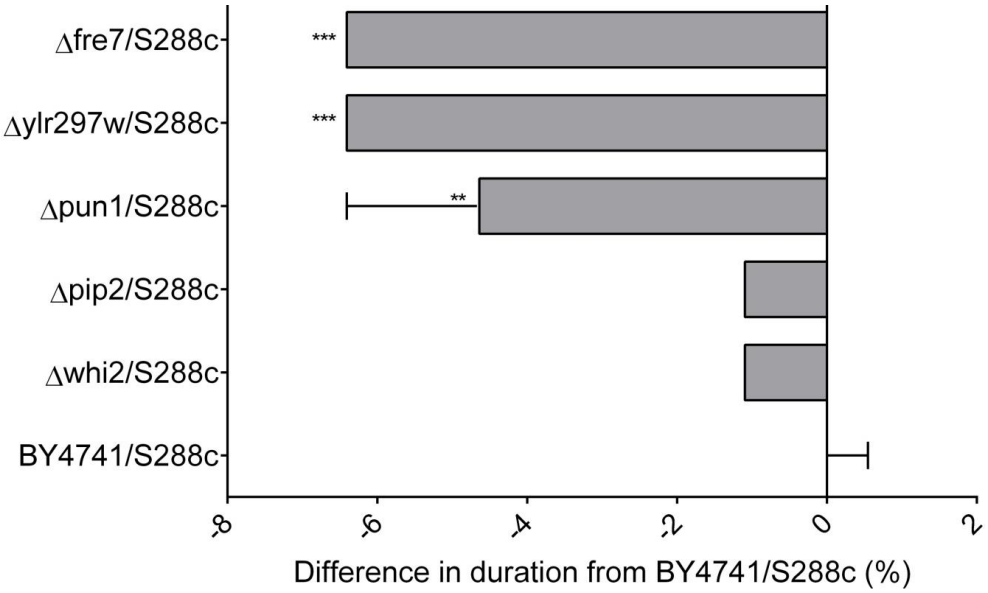


Figure 1: Percentage difference in fermentation duration relative to the parent strain BY4741/S288c for hemizygous deletants analysed in 100 mL fermentations. Fermentations were performed in CDGJM under FAN- limited (75 mg L^{-1}) conditions. Statistical differences are denoted by asterisks (*** $p<0.0001$, ** $p<0.001$).

Screening of over-expression library in haploid wine yeast

We reported in UA05/01 on the construction of a haploid wine strain (isoC9△leu2) as the genetic background for the transformation of an over-expression library in which the cloned genes are under the control of their own regulatory sequences [5]. We have successfully transformed the complete library (~1500 clones) into the haploid wine strain and confirmed the clonal identity and degree of plasmid retention for a representative number of clones (data not shown). Furthermore, screening conditions were developed to allow for both the identification of clones which result in enhanced or protracted fermentation in high sugar medium (CDGJM_200; 200 g L^{-1}, 450 mg L^{-1} FAN) and the impact on aroma profile, specifically 36 yeast derived volatiles.

We have successfully screened the library in micro-scale fermentation using the sacrificial plate method developed in this laboratory [29]. Fermentations were conducted in deep-well plates (1.8 mL) to allow residual sugar, nitrogen and GC-MS (yeast derived aromatic compound) analysis. The fermentation duration was unexpectedly long in comparison to the previous screens using a homozygous deletion library in the laboratory diploid strain BY4743. The parent strain isoC9△leu2(pGP564) which was transformed with the backbone plasmid pGP564 [5] and did not contain any other gene modifications, required 19 days to complete fermentation. The fermentation was terminated at 19 days because of concerns of plasmid instability, which was negating cell viability under these conditions. Nevertheless, analysis of residual sugar allowed the tentative identification of clones (and thus genes) that may enable shortened fermentation duration. Some clones were also inefficient at fermentation, with higher residual sugars at multiple time points throughout fermentation. These could be representative of genes which depress fermentation (negative fermentation regulators), however due to the observed occurrence of plasmid loss and a subsequent decrease in culture viability it must be noted that a number of these are probably unrelated to fermentation.

The extended fermentation duration (19 days) observed with the parent transformed with the plasmid pGP564 in what is considered an optimal nitrogen condition (450 mg L^{-1} FAN), makes redundant any attempts to undertake a fermentation screen under limiting nitrogen conditions (<100 mg L^{-1} FAN). We therefore will undertake such a screen using only the deletion library in the prototrophic laboratory yeast and wine strain background as part of the new project UA1302.

Collation of the various datasets obtained from this over-expression screen is in progress. It is envisaged that a list of potential clonal candidates will be available for evaluation by July 2013 in 100 mL fermentations. It must be noted that this library differs from other commercial over-expression libraries in that whilst expression is driven from the endogenous promoters of cloned genes, each construct has multiple genes rather than a single gene, not all of which are functional [5]. Using clonal identification data and GO analysis to examine which genes are annotated to particular GO terms for processes already determined as required for or affecting fermentation, we are in the process of determining which clones to evaluate in 100 mL fermentations. Perusal of the dataset specific for enhanced fermentation performance (170 genes; micro-screen results) has identified several clones which were previously shown to lead to protracted fermentation when deleted. These genes (*SLM4*, *TPS2*, *ATG33*, *VPS41*) together with others will be initially examined to determine the rigidity of the screen. Thirty five of the 170 genes were linked to abiotic stress and are therefore of interest with regards to fermentation.

Based on the findings of the laboratory scale evaluation, single genes will be chosen for re-cloning into pGP564 for further assessment. We envisage this approach will be appropriate, given the success we have had in the over-expression of *HXK2* in △tps1, leading to the amelioration of fermentation arrest (Nguyen, *pers. comm*.). **Figure 2** is representative of the fermentation profiles obtained in the micro-scale fermentation screen. Clones representing potential fermentation efficient strains and those with protracted fermentation are shown.

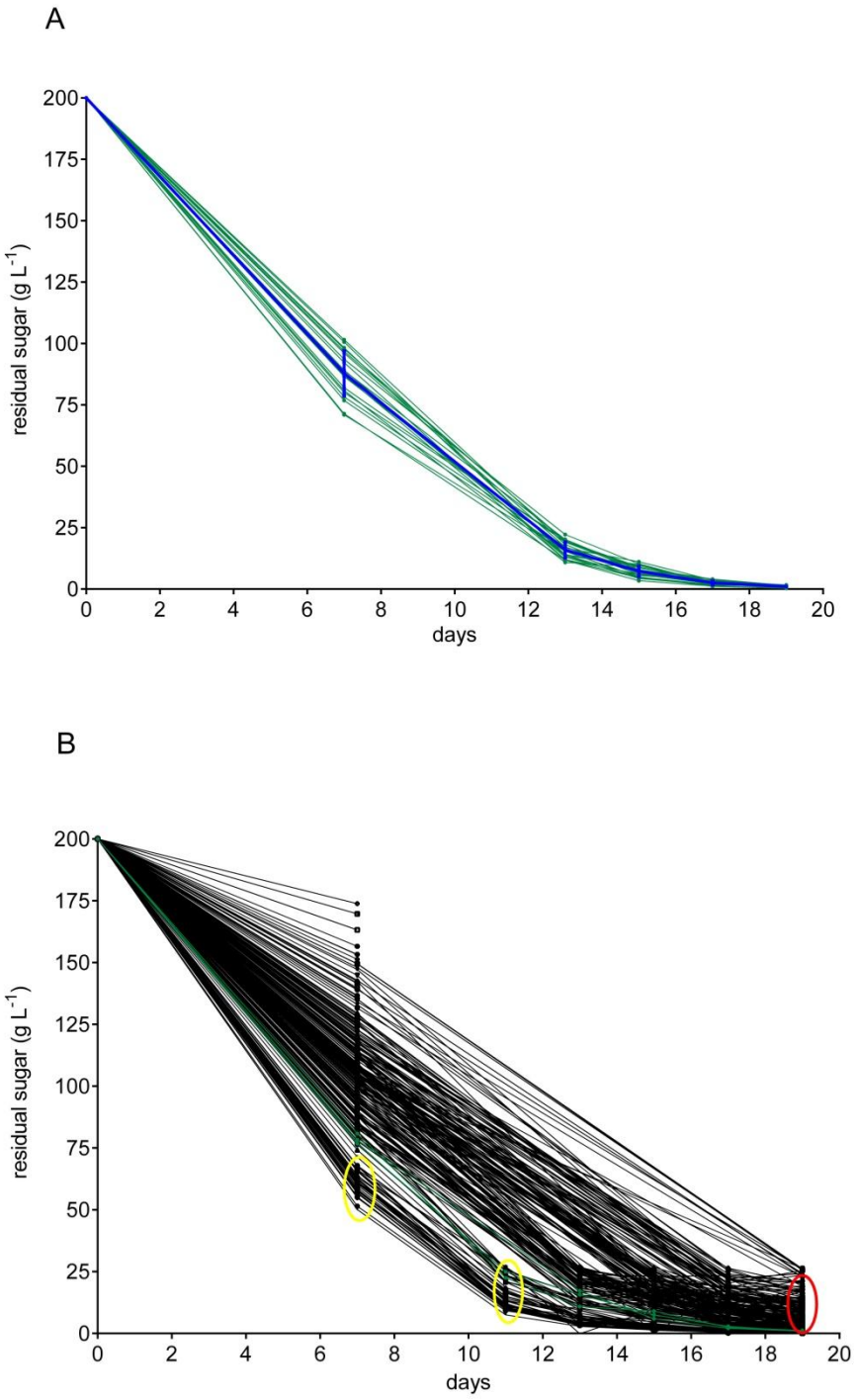


Figure 2: Fermentation profiles of 250 over-expression strains

Panel A represents the fermentation profiles of 21 replicates of the parent strain isoC9△leu2(pGP564) depicted in green. The average ± SD is depicted in blue. Panel B represents the fermentation profiles of 250 clones in the over-expression library. The fermentation curves of 3 replicates of the parent strain are shown in green. Those clones which utilise sugars more rapidly than the parent are highlighted in the orange circles. Residual sugars of two time-points were compared and those clones which had consistently lower sugars in both were selected for further analysis. The clones that fall within the red circle are representative of clones with genes that cause protracted fermentation.

Conclusions

We have made significant progress towards understanding the molecular mechanisms behind yeast adaptation to and fermentation activity in industrial fermentation, necessary for near or complete conversion of sugars to ethanol, as sought in wine and biofuel production. This has been achieved by identifying genes required for successful fermentation – the "Fermentome". Together with an *in silico* analysis of these genes through the compilation of the "Fermenation Relevant Yeast gene (FRYG) database" we have achieved a "top down" or Systems Biology view of the important mechanisms of fermentation efficiency, satisfying the performance target "*In silico* analysis of 'omics databases and library screen data and definition of wine essential genes making up the fermentome" for this **Output 1B**

Through understanding the genetic interaction of specific genes within biochemical pathways connected to fermentation outcome we hope to be able to foresee potential problems in 'negative' phenotypes in modified strains. For example the increased sulfur dioxide levels in low hydrogen sulfide producing strains which result from the mutation of sulfite reductase. This information together with sequencing data of evolved strains and the ongoing expansion of the fermentome dataset will be used in the new GWRDC project (UA1302) to construct strains as part of understanding directed approaches to strain enhancement. Four manuscripts are in preparation which will report on our research findings and thereby exceed the performance target (1 manuscript) for **Output 1E**.

Whilst recombinant technology (GM yeast and bacteria) is not looked upon favourably by the industry and consumers in Australia because of concerns (albeit unfounded) regarding safety and impact on the environment [30], there is still a role for this research and strain development paradigm. Genetic modification has already proven useful in demonstrating the potential in targeted improvement of industrial fermentation not only in kinetics but organoleptic properties of the product [31]. Recombinant strains are in use for the alcoholic beverage industry (eg sake), using technologies such as 'self cloning' [32] whereby gene modifications (whether deletion, or introduced mutations, etc) are introduced using gene sequences derived from the same species. Whilst non-recombinant paradigms, for instance adaptive evolution [17], are favoured, 'self cloning' may be the future in instances where specific fermentation traits (eg reduced alcohol and increased glycerol or desirable sensory attributes) are not obtainable by non-recombinant means to the extent observed with GM technologies [31].

Output 1C: Robust or novel yeast and/or bacterial strains generated

- **Improvement of strains through multiple rounds of mating, mutagenesis and AE approaches.**

Output 1D: Data on lab scale fermentation performance by superior strains

- **Micro-fermentation screens of above strains as well as candidates in existing collections from previous AE experiments undertaken.**

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Background
Within this Output improved strains were generated utilising multiple methods, each designed to capture the most desirable strain attributes. Generation of industrial microbes with the use of Directed Evolution (DE) offers a non-recombinant method of novel strain generation. DE is a form of Adaptive Evolution (AE) where experimental design, including selection criteria are intentionally set to enhance the chances of producing the most desirable isolate. We have used knowledge and materials from previous work UA05/01 and this study (**Output 1B**) to enable a more specific approach in the generation of such strains.

To maximise the chances of success, where possible, DE was performed with starter cultures with extensive genetic diversity. For example, to isolate yeast strains with improved fermentation efficiency many commercial yeast strains were collected, sporulated and then mated *en masse* to provide a starter culture with a large gene pool. This of course was not possible with bacterial cultures, where we aimed to isolate strains with robust and efficient malolactic fermentation capabilities and instead began with the current best commercially available strains. We also sought to improve current commercial 'low H_2S' yeast strains. Hydrogen sulfide production is still a problem in industry, being formed usually when nitrogen becomes limiting during fermentation. Other groups have released low H_2S strains previously [33], however industry have reported that these strains produce SO_2 in excess, which in many cases is undesirable. We examined the current commercial low H_2S strains as well as others for genetic diversity, in terms of genes of the sulfate reduction sequence. We also performed chemical mutagenesis followed by selection for mutants with reduced activity through the sulfate reduction sequence.

Methods
Sporulation and rare mating of commercial wine yeast
A collection of 86 commercial yeast strains (**Supplementary Table 3**) were inoculated from glycerol culture stocks (10 μL) in sporulation medium (**Appendix 5**) as 3 replicate 96 well plates, and incubated at 30 °C for 7 days in a humidified sealed container. Sporulation was confirmed by microscopy. The plates were incubated for a further 7 days at ambient temperature. One of these

plates was used for subsequent mass mating whilst two were kept for long term storage of the sporulating cultures as glycerol stocks.

Glycerol stocks of sporulating cultures

Yeast biomass was washed twice with 50 μL 1x phosphate buffered saline (PBS) (**Appendix 5**) and transferred to a new 96 well plate. 50 μL 80% glycerol was added to each well and cells mixed by gentle pipetting prior to freezing at -80°C.

Mass mating of wine yeast (sporulating cultures)

The agar containing the sporulating yeast culture was removed from each well with a sterile loop and transferred into a 50 mL sterile tube containing 25 mL 1x PBS. The cells were resuspended by gentle agitation, transferred to a sterile 50 mL tube and pelleted by centrifugation (5 min, 5000 rpm). Cells were twice washed in 1x PBS by suspension followed by centrifugation.

Vegetative cells were killed using diethyl ether. Cells were suspended in a 30 mL volume of equal parts diethyl ether and sterile MQ and incubated with agitation for 30 min (4 °C). Diethyl ether was removed following centrifugation and the cell pellet washed in sterile MQ, centrifuged and washed again. The ascus wall was removed by overnight digestion (30 °C, with agitation) using 15 μL β glucoronidase (Sigma) per 1 x 10^7 cells mL^{-1} of cells. The digested asci were washed twice in sterile MQ as described above, prior to being resuspended in YPD. After settling by centrifugation (1500 rpm, 5 min), the pellet was incubated overnight in YPD at ambient temperature to maximise the chance of mating.

Directed evolution: Sequential batch yeast fermentation

Batch fermentation was performed according to McBryde et al [17] in a 2 L maximum capacity Sartorius bioreactor under anaerobic conditions using 500 ml of highly clarified Chardonnay Juice (Adelaide Hills, 2010 vintage, sterile filtered (0.45 μm) and frozen). Juice analysis was conducted by AWRI Commercial Services (**Appendix 5**).

The initial fermentation was inoculated with the mixed mated culture (7.88 x 10^8 cells; viability not determined). Fermentation progress was monitored by enzymatic determination of residual glucose and fructose [14] using a commercially available kit (Cat: 139106, Boehringer Mannheim) adapted for 96-well plates. Fermentation was considered finished when the residual sugar was less than 2.5 g L^{-1}. The cells were harvested and used to inoculate fresh medium (sequential fermentation) at 5 x 10^6 cells mL^{-1}. Thirty four sequential batch fermentations were conducted representing ~190 generations, with sampling for candidate strain analysis every 25 generations. 1 mL samples were kept as glycerol stocks at -80 °C (**Appendix 5**).

Directed evolution: Continuous bacterial fermentations

The commercial *Oenococcus oeni* strain Lactoenos® SB3 (Laffort) was originally isolated from a commercial package and grown and maintained in Man Rogosa Sharpe (MRS) medium with 20% apple juice and stored in the same with 25% glycerol at -80 °C. Continuous culture was performed in a 2 L maximum capacity Sartorius bioreactor under anaerobic conditions using 1.1 L of MRS at 30 °C. Malolactic fermentation (MLF) performance was analysed in at least quadruplicate at 30 °C in 200 μL of MRS culture in 96 well plates, unless stated in the text. A sacrificial plate method was used and at least 4 time points were analysed over the course of fermentation. Residual malic

acid was quantified using a spectrophotometric method (Boehringer Mannheim) altered by us to suit high throughput analysis in small volumes (200 μL).

Generation of low H_2S and SO_2 producing yeast by chemical mutagenesis

The yeast strains Distinction® (Mauri Yeast Australia) and Lalvin EC1118 (Lallemand) were chemically mutagenized with ethyl-methane sulfonate (EMS) as outlined by Lawrence [34]. Yeast were grown in 50 mL YPD media overnight at 28°C prior to being washed twice in 15 mL of 0.1 M sodium phosphate buffer, pH 7.0, and finally resuspended in the same buffer at 2 x 10^8 cells mL^{-1}. A 1 mL sample was taken at time zero (T_0) prior to addition of 45 μL mL^{-1} EMS and incubation for 2 h with agitation. Samples (1 mL) were taken at 10 min intervals; the sample centrifuged and washed twice in 5% (w/v) filter-sterilized sodium thiosulfate. EMS-treated cells from each interval were resuspended in 1 mL YPD. An aliquot was kept for long-term storage as a glycerol stock at -80°C. The survival rate (%) was determined for each time point, with 50% survival rate chosen to minimise the number of mutations per cell. Aliquots of the glycerol stocks were diluted and grown on YPD to give 200 - 500 cfu per plate.

Selection of sulfate permease mutants

Sulfate permease mutants with impaired transport of sulfate ions (SO_4^{2-}) were selected using the toxic analogues potassium dichromate ($K_2Cr_2O_7$) and sodium selenate (Na_2SeO_4) [35, 36]. EMS treated colonies plated on YPD were then replica plated onto YPD (master plate) and YPD containing 5 mM $K_2Cr_2O_7$ and Na_2SeO_4 (selection plate) and grown for 2 days in the dark at 30°C. Colonies corresponding to the resistant colonies were taken from the master plate and restreaked onto the selection plates to confirm the growth phenotype. BiGGY agar (Oxoid, CM0589) was used to visually distinguish between low and high H_2S producers [37]. Respiratory deficient (petites) mutants were distinguished by the inability to grow on petite medium [38]. Potential sulfate permease mutants were kept as glycerol stocks for later evaluation. Methionine prototrophs were identified by growth on minimal drop out medium lacking methionine [38].

Molecular characterisation of sulfate permease mutants

Yeast genomic DNA was isolated from presumptive sulphate transport deficient strains and wine yeast EC1118, PDM (Mauri Yeast Australia) and Distinction® according to Adams et al. [6]. Two sulfate permease genes *SUL1* and *SUL2* and sulfite reductase genes *MET5* (β subunit) and *MET10* (α subunit) were amplified. Polymerase chain reaction (PCR) was performed using 0.5 units of Velocity proofreading DNA polymerase per 25 μL reaction [8] with a BIORAD C1000 thermal cycler [9]. The genes were amplified using terminal forward (A) and reverse (D) primer as per manufacturer's instructions. Primers used for PCR amplification and DNA sequencing are described in **Supplementary Table 4** (**Appendix 5**). DNA sequencing was performed by the Australian Genome Research Facility Ltd. (AGRF). Sequencing reactions were in a 96 well plate with each 12 μL reaction containing 75 ηg of gel-purified PCR product [39] and 10 pmol of gene specific primers. DNA sequence alignment was performed on Geneious 5.6.1 software and nucleotide substitution and amino acid change were noted between the parental strain and mutant strains [40]. Protein prediction SIFT software [41] was used to predict putative deleterious amino acid substitutions within mutant gene sequences.

Evaluation of candidate clones in micro-scale fermentation

Aliquots of the glycerol stocks from five time points in the DE experiment (**Output 1C**) were streaked out for single colonies onto YPD solid media. 187 individual colonies were picked and transferred into 600 μL YPD in 96 well plates and incubated overnight at 30 °C. Glycerol stocks were made of these, the mixed DE population, and commercial yeast EC1118 and L2056 in 300 μL flat bottomed 96 well plates. The cultures were used to inoculate the starter medium in the micro-scale fermentation described below.

Micro-scale fermentation

Liquid handling procedures were performed using a Corbett CAS3800 robot (Corbett Robotics, Sydney). Micro-scale fermentations using the sacrificial plate method [29] described in the GWRDC Report UA05/01 was used with the following modifications: 200 μL of starter media (comprised of equal volumes of Chardonnay juice and sterile MQ water) was inoculated with 2 μL of YPD culture in 96-deep-well plates with breathable sealing film (Cat: AXYBF-400, Axygen). The plates were statically incubated in a humidified container (30 °C, overnight). The cultures (0.2 mL) were diluted with 0.8 mL CDGJM starter medium and 10 μL (~1 $x10^6$ cells) used to inoculate 190 μL CDGJM used for the fermentation screen. 187 clones were evaluated in 11 plates; each plate was divided into four quadrants for replication as shown in **Figure 3**.

In later experiments, three media conditions were used in the evaluation of different subsets of candidate strains: Chardonnay juice (2010 Adelaide Hills), CDGJM (260 g L^{-1} sugar, 350 mg L^{-1} N), and CDGJM (230 g L^{-1} sugar, 125 mg L^{-1} N). The starter media used for each condition was CDGJM starter [17, 18]. Candidate strains which performed better than the parent in all 3 conditions were chosen for further evaluation in 100 mL to 10 L fermentations.

Glucose and fructose

Sugars were determined by a modified enzymatic analysis [14] in 96 well plates with a Infinite® 200 PRO microplate reader (Tecan Group Ltd) which has stacking capability allowing 10 plates to be analysed at one time. The detection limit of the assay was 0 – 3 g L^{-1}. Standards (200 μL) were added to the thawed 96 well plates (row 12) used for the fermentation screen (**Figure 3**). Standard concentrations were 1.0, 2.5, 7.5, 10.0, 25.0, 75.0, 150.0, 250.0 g L^{-1} (total as equimolar glucose and fructose). A dilution plate was made by pipetting samples and standards into a new 96 well plate to give a 1 in 66 dilution using MQ water (200 μL total volume). The enzyme assay [14] was performed in the microplate reader, using 6 μL of diluted sample and 200 μL reaction buffer (**Appendix 5**). The absorbance at 340 ηm (A0) was initially read prior to addition of 10 μL HK/G6P-DH solution. The plate was agitated for 15 min prior to reading the absorbance at 340 ηm(A1). 10 μL PGI solution was added and the plate was agitated for 15 min prior to reading the absorbance again (A2). A standard curve was generated using the absorbance values of the standards: A1-A0 (glucose) and A2-A1 (fructose) to determine the glucose and fructose concentration in the fermentation samples based on their absorbance values.

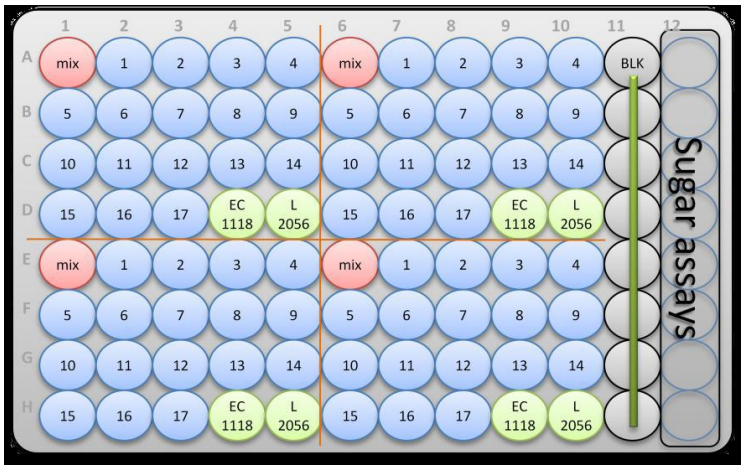


Figure 3: Set up of 96 well plates used in micro-scale fermentation screens
A 300 µl 96-well plate was divided into 4 quadrants; each representing one fermentation replicate. Wells A1, A6, E1, E6 were inoculated with the mixed culture, wells D4, D9, H4 and H9 were inoculated with EC1118 or PDM and wells D5, D10, H5, and H10 were inoculated with L2056. Row 11 represents the blank control (CDGJM_200) and row 12 was used for sugar assay standards.

NOPA determination of free amino nitrogen

Free amino nitrogen was determined by a modified enzymatic analysis [15] in 96 well plates with an Infinite® 200 PRO microplate reader (Tecan Group Ltd), with stacking capability allowing10 plates to be analysed at one time. Standards were made up with either isoleucine or mixed amino acids (as used in the chemically defined media [18]). Standards were made in MQ water at 5, 25, 50, 100, 150, 200, 300, and 450 mg L^{-1}. Use of isoleucine as a standard resulted in an underestimation of the absolute amount of nitrogen present in samples because amino acids such as arginine or glutamine have a single primary amino group, yet contain four or two nitrogen atoms. By using a mixed amino acid solution as a standard, an estimation of absolute amino acids was closer to the actual value. Standards (200 µL) were added to the thawed 96 well plates (row 12) used for the fermentation screen, starting with 5 mg L^{-1} (A12) and finishing at 350 mg L^{-1} (H12). 6 µL of sample were transferred into a new 96 well plate containing 100 µL of combined buffers i.e. 1:1 volume of NAC and NaOH/Boric acid solutions (**Appendix 5**) and mixed for 2 min. Absorbance was measured at 340 ηm (A0) and 10 µL of OPA solution (**Appendix 5**) was added and mixed for 15 min prior to reading of absorbance at 340 ηm (A1). A standard curve was generated using the absorbance values of the standards: A1-A0, in order to determine the amino acid nitrogen concentration in the ferment samples based on their absorbance values.

Results and Discussion

Mass mating and adaptive evolution to generate novel yeast

The fermentation performance of 80 wine yeast strains was evaluated prior to mass mating using the sacrificial plate method. Fermentation progress was determined as residual sugar and is shown in **Figure 4** and **Supplementary Table 5**. Whilst most appear to be able to ferment in this problematic media, 17 strains were observed to grow poorly in the juice as indicated by the inability to catabolise sugar **(Figure 4** and **Supplementary Table 6)**. This simple analysis highlights the great diversity in fermentation ability (and speed) within just this juice.

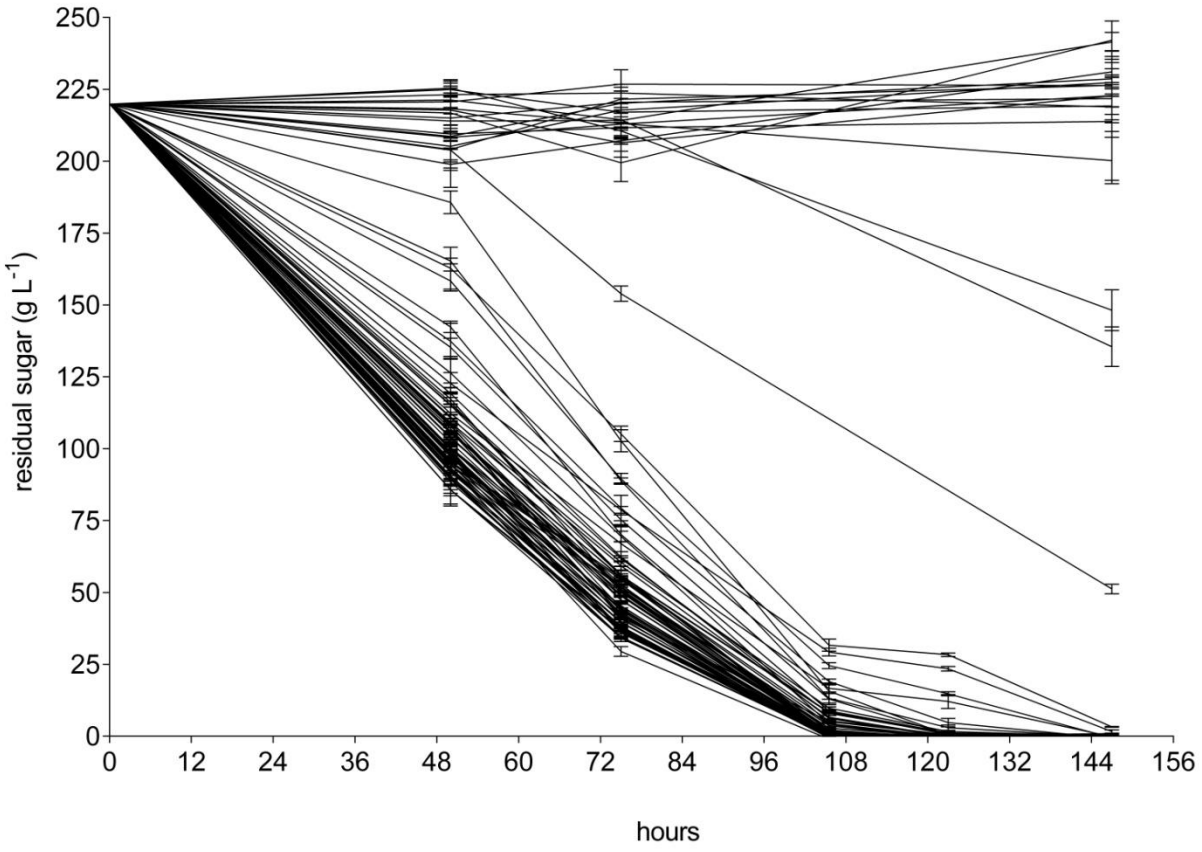


Figure 4: Fermentation performance of 80 commercial wine yeast in problematic highly clarified Chardonnay Juice (2010 Adelaide Hills) Yeast were grown in YPD in 96 well plates (28 °C) prior to inoculation into sterile Juice starter media (1:1 volume of Chardonnay juice and water) and incubated at 28 °C. Fermentation was performed at 28 °C in 96 well plates using the sacrificial plate method (4 replicates).

All of the strains (**Supplementary Table 3**) were used in the following DE experiment to generate new robust yeast suitable for fermentation in problematic juice/must. Each of the strains was sporulated and then mated *en masse* with the purpose of increasing the genetic variation within the population through meiotic segregation and random crossing of the progeny. Diethyl ether was used to kill off most of the vegetative parental population, which otherwise would have overcome the newly mated yeast that occur at a much low frequency. The selection medium in this DE strategy was a highly clarified Chardonnay juice (Adelaide Hills, 2010) with low YAN content, which had been repeatedly freeze-thawed. This juice was shown to be problematic in supporting fermentation (Liccioli, *pers. comm.*) and was therefore considered an ideal medium for adaption to harsh fermentation conditions rather than the readily fermentable CDGJM [17]. We hypothesised that this would tailor adaptation to other specifics of grape juice chemistry rather than a synthetic medium and improved fermentation performance should therefore be more easily transferable to other juices (Liccioli, *pers. comm.*) [42].

Sporulated cultures of ~80 wine strains were pooled and mated *en masse* prior to inoculation of 500 mL highly clarified Chardonnay juice (Adelaide Hills, 2010) in a chemostat. The DE experiment was conducted over ~190 generations in sequential batch fermentations, with samples removed every 25 generations (AE5: 25 gen.; AE10: 50 gen.; AE20: 100 gen.; AE31: 180 gen.). Clonal isolates were evaluated for fermentation performance against the mixed population and L2056 and EC1118 in 96 well plates initially using the same juice, and later a chemically defined grape juice (CDGJM) of varying sugar and nitrogen content.

Two time points were measured 98 h (data not shown) and 112 h, with strains chosen where residual sugar content was <20 g L^{-1} (98 h) and <5 g L^{-1} (112 h) as shown in **Supplementary Table 7**. Seven isolates were chosen for further investigation: AE5-5, AE5-135, AE10-100, AE20-91, AE20-178, AE31-58 and AE31-71 in 100 mL and 10 L fermentations (**Output 2A**).

<u>New clonal isolates of FM16 mixed population from previous DE experiment at 350 generations</u>
The mixed population (at 350 generations) from the original AE strategy described by McBryde et al [17] to generate evolved and improved strains from commercial yeast L2056 was also re-examined. This mixed population has previously yielded our most fermentation efficient strain so far, FM16-C7. New clonal isolates were obtained from the glycerol stocks, by plating on YPD.

187 single colonies were isolated from the FM16 mixed population derived at 350 generations in the AE experiment reported by McBryde et al [17]. These were evaluated in three conditions in micro-scale fermentations: Chardonnay juice (2010 Adelaide Hills), CDGJM (260 g L^{-1} sugar, 350 mg L^{-1} N), and CDGJM (230 g L^{-1} sugar, 125 mg L^{-1} N). **Supplementary Table 8** reports on the clones which have residual sugar <2.5 g L^{-1} (CDGJM) and <3.5 g L^{-1} (juice) at or close to end of fermentation. Five clones were chosen for 10 L evaluation: FM16-74, FM16-79, FM16-144, FM16-150, FM16-170 (**Ouput 2A**). These strains had improved fermentation efficiency in all conditions in the micro-fermentation screen.

The micro-scale fermentation screen allowed for high throughput evaluation of candidate strains in a time-efficient and economical manner. Whilst only several candidates were chosen for laboratory-scale trials (10 L juice fermentations) there is an extensive collection (in glycerol), which was able to be accessed as required. As part of the Output, we have modified the sugar analysis such that the enzymatic reactions only require one round of dilution (1 in 66) suitable for 0 – 350 g L^{-1} sugar (glucose and fructose). Together with the recently purchased Infinite® 200 PRO microplate reader (Tecan Group Ltd) we greatly improved our analysis throughput to 30 plates in 2 h (2640 samples). We have made similar modifications to enable high-throughput determination of FAN (NOPA) and malic acid.

<u>Directed evolution of *Oenococcus oeni*</u>
This research was undertaken by Ms Alice Betteridge as part of her PhD, also supported by the GWRDC (PH09/01). The commercial *Oenococcus oeni* strain Lactoenos® SB3 (Laffort) was chosen as a source strain since it has relatively robust secondary malolactic fermentation (MLF), i.e. is reported to tolerate up to 15 % alcohol, pH down to 3.3, up to 30 mg L^{-1} SO_2 and temperatures down to 16 °C. As this was the very first undertaking of Directed Evolution of bacteria, we wished to maintain as much control as possible, and this included using a medium of known composition. For this reason Directed Evolution was undertaken in a continuous culture of

defined medium, Man Rogosa Sharpe (MRS) broth. Initially MRS was supplied with 20% apple juice and 5% (v/v) ethanol, but over the course of a year (~ 400 generations) ethanol was gradually increased until it reached 15% (v/v). Over the course of the continuous culture the generation time changed from 13.5 hours to 31.8 hours. Weekly samples were taken throughout the course of the continuous culture and once the challenging ethanol concentration reached 13% the MLF performance of the mixed culture DE samples was evaluated. The MLF performance of a mixed culture from the final time point of the DE culture at 15% was extremely promising as it was able to complete MLF about 70 hours before its parent (**Figure 5**; 90 vs 160 h).

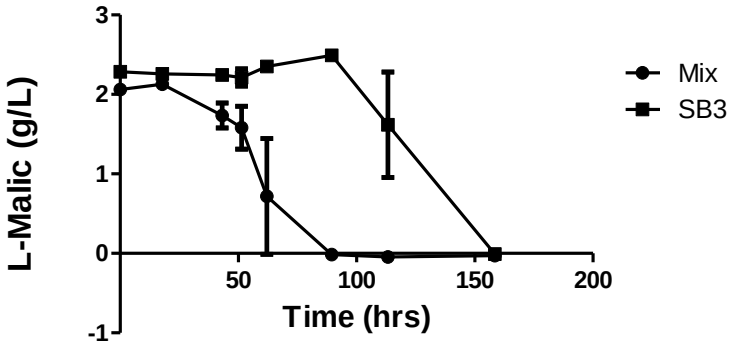


Figure 5: Malolactic fermentation (L-malic acid utilisation) by either a mixed evolved culture (Mix) from the DE SB3 culture at 15% ethanol or the original SB3 parent.

180 clones from the mixed DE culture (15% ethanol) were then isolated and tested individually for MLF performance in 200 µl cultures (MRS +15% ethanol). From this screen 26 of the best performing strains were chosen and reanalysed for MLF performance in 50 mL MRS cultures with 13 and 15% ethanol. The best performing strains, AB-2, AB-76, AB-89 and AB-90 were chosen for further evaluation. The MLF performance of these is currently being examined over a range of ethanol concentrations (13-19% [v/v]) and temperatures (12-30 °C). Future work will involve sequencing the genome of the best performing strain(s). We are currently undertaking genome sequencing of the parent strain SB3, including annotation in preparation for full analysis of evolved mutants. Importantly the capability to generate and analyse genomic data in house is of great benefit to future projects.

Production of low H_2S and SO_2 producing wine yeast strains

This project was funded in part by a University of Adelaide Wine2030 award to Dr Michelle Walker in 2012 (9 months) to produce second generation yeast which are of industry value. Here we focussed on producing yeast strains that were less likely to produce hydrogen sulfide (H_2S), especially under conditions of reduced nitrogen. Recently a number of wine yeast strains have become commercially available, which have a lower production of H_2S, for example Platinum ®, Distinction® and Advantage® (AB Mauri, Australia). These strains were reported to have alterations in the sulfate reduction sequence (SRS) [43], specifically within the sulfite reductase subunits encoded by *MET5* and *MET10*. Use of these strains can however lead to an overproduction of sulfur dioxide (often in excess of 100 ppm) that may result in wine that is out of the winemaker's specifications.

Sulfate is the most abundant inorganic sulfur compound in musts, ranging between 160-380 mg L^{-1} [43] to as high as 700 mg L^{-1} [44]. It is taken up and used within the SRS to provide sulfur for sulfur-containing amino acids methionine and cysteine. H_2S and SO_2 are intermediates of this pathway.

This study used two approaches. Firstly we made use of the existing genome heterozygosity around key genes involved in sulfite metabolism of currently available industrial wine yeast strains and secondly we continued research on low H_2S producing strains (constructed with the use of EMS) initiated by Ms Leah Adint (Masters student, 2011).

Examination of genome heterozygosity of genes of the SRS within commercial wine yeast

To alleviate the excess production of SO_2 within current commercial strains we sought to reduce the activity of the high affinity sulfate permeases encoded by *SUL1* and *SUL2*. This was intended to reduce the uptake of sulfate from the medium and thus reduce the flux through the SRS.

The *SUL1* and *SUL2* genes in the diploid wine yeast Distinction® were deleted using deletion cassettes (*⊿sul1::KanMX* and *⊿sul2::KanMX*) amplified from genomic DNA of laboratory yeast deletion mutants. Heterozygote transformants were sporulated and individual spore progeny containing the deleted genes as well as the wild-type (WT; unmodified) genes within the tetrad were kept. Only one or two of the four spores in each tetrad were viable, with no classical segregation of WT and deleted genes; all spore derived strains were diploid since Distinction® is a homothallic strain (i.e. able to switch mating types and rediploidise). Whilst these wine yeast deletants were to be used as 'proof of concept' strains to determine whether loss of sulfate transport via the sulfate permeases would decrease H_2S and SO_2 production, these strains have not yet been investigated due to time constraints. However, the WT spore progeny ('selfed' diploids) were subject to investigation because we wished to exploit observed genetic heterogeneity in wine yeast [45, 46]. Four strains were evaluated in 100 mL fermentations as reported in **Output 2A**: L1 (from the *SUL2* deletion experiment) and J1, F2, I1 (from the *SUL1* deletion experiment). The *SUL1* gene was sequenced in these mutants and compared to that from Distinction® and EC1118 [47]. **Table 5** shows the nucleotide changes in *SUL1* in the WT selfed diploids compared with EC1118. It was anticipated that the sequence of these mutants would be identical to Distinction®. The sequencing results were complicated since two distinct colony types were evident within two separate commercial packets (also from two separate batches) originally isolated by Ms Leah Adint and by Ms Andrea Lee (Lot #1382116). We refer to the two isolates as Dist 1 (large tan coloured colonies on BiGGY agar) and Dist 2 (small cream coloured colonies on BiGGY agar). Dist 2 was shown to behave according to Cordente et al [33] and displayed poor (reduced) fermentation activity in low FAN conditions, producing no measurable H_2S but large amounts of SO_2 (~100 mg L^{-1}, **Figure 7, Output 2A**). Distinction® and the other low H_2S producing strains (Advantage®, Platinium®) have been previously shown to exhibit a long lag phase and sluggish fermentation in the absence of DAP addition [48]. Sulfite accumulation due to defective sulfite reductase, is toxic to the cell, so it is exported as SO_2 by the *SSU1* sulfite exporter, giving rise to elevated levels in wine fermented with these strains. Here we report that Dist 1 was as vigorous in fermentation as EC1118 and had reduced H_2S and SO_2 compared to EC1118 [47]. Sequencing of the *MET5* and *MET10* genes in the two strains identified Dist 2 to be similar to strain 22.1; reported by Cordente et al [33], exhibiting mutation 2731G>A in *MET10*, which results in the amino acid substitution glycine to serine at residue 911 ($G^{911}S$). However the reported *MET10*

mutation in 22.1, 2523G>A, which introduces a stop codon at residue 841 ($W^{841}X$) was absent. These results suggest that this mutation may be detrimental to the yeast and therefore not stable and has been lost. None of the other mutations associated with the other low H_2S producing strains reported [33] could be found. Dist 1 was shown to have the same sequence as EC1118 for both *MET5* and *MET10*.

Table 5: Identification of SNPs in *MET 5* and *MET10* in PDM and Distinction® isolates Dist1 and Dist2 when compared to EC1118

PDM is the parent of Distinction® and is considered identical to EC1118. SNP highlighted in yellow was identified by Cordente et al (2009) in low H_2S wine strain Distinction®

Strains	***MET5***	***MET10***
PDM (sequence 1)	No change	514G>A ($A^{172}T$) 1372A>G ($T^{458}A$)
PDM (sequence 2)	1207T>G($Y^{403}D$)	514G>A ($A^{172}T$) 1372A>G($T^{458}A$) 1532C>T($T^{510}I$)
Dist 1	No change	514G>A ($A^{172}T$) 1278 R (A/G) ^{426}K 1372A>G ($T^{458}A$)
Dist 2	No change	514G>A ($A^{172}T$) 1278R (A/G) ^{426}K 1372A>G ($T^{458}A$) 2731G>A ($G^{911}S$)

The sequences of *MET5* and *MET10* were also compared between EC1118 and PDM to determine whether there were any sequence variations: PDM is commonly thought to be identical to EC1118. PDM is the parent of low H_2S producing strain Distinction® [33] and as mentioned earlier the isolates Dist 1 and Dist 2 were cultured from a commercial packet of Maurivin Distinction®. Both EC1118 and PDM had identical sequences at *MET5* (**Table 5**; sequence 1). However, a second experimental sequencing of PDM at MET5 (sequence 2) contained a T to G transversion at nucleotide 1207 ($Y^{403}D$) which requires further examination as to whether this is in fact a sequencing error. Three SNPs were identified in *MET10* derived from PDM when compared with EC1118 (**Table 5**). The mutations reported by Cordente et al [33] in Distinction® (2731G>A($G^{911}S$) and 2523G>A ($W^{841}X$) were not seen by us in the PDM *MET10* sequence. Both Dist 1 and Dist 2 have SNPs in *MET10* at positions 514; G>A($A^{172}T$) and 1372; A>G($T^{458}A$) when compared to EC1118. In addition, Dist2 contained a unique SNP 2731G>A($G^{911}S$) described by Cordente et al [33], which we believe to in fact be the mutation which confers the phenotypic difference of lowered H_2S production when compared with Dist 1. We are yet to confirm whether these two populations (Dist 1 and Dist 2) are an isolated occurrence or reflective of other packages of this wine yeast. Suffice to say that the EMS mutagenesis conducted by Ms Leah Adint was on the strain we now refer to as Dist 1. This strain was the principal clone in our culture stock. Whilst Dist 2 (small colonies) is present, it was not initially chosen as it does not appear to represent the majority of the population within a commercial package.

Sequencing of the *SUL1* and *SUL2* genes in Dist 1 and Dist 2 reported sequence differences when compared with EC1118 and the laboratory strain S288c. Mutations within strain 22.1 (which was commercialised as Distinction®) were not reported for *SUL1* and *SUL2* [33]. In this study we found that the *SUL1* gene in Dist 1 was identical to S288c whilst the *SUL1* gene in Dist2 displayed sequence heterozygosity, sequence similarities between EC1118 and S288c and novel mutations (**Table 6**). Whilst the sequence identity of key sequenced genes in F2, I1 and J1were identical, the fact that the fermentation performance of these strains was different [47] would suggest the presence of other as yet unidentified genome rearrangements, including a particular allele where polymorphism exists, as a result of meiosis during sporulation of the *SUL1/⊿sul1* or *SUL2/⊿sul2* transformants. Only one strain I1 would appear to be promising in terms of fermentation (**Output 2A**, 10 L fermentations), producing no H_2S and minimal SO_2. The ability to undertake whole genome sequencing would allow for the identification of mutations linked to this strain's fermentation phenotype.

Table 6: Comparison of nucleotide substitutions in *SUL1* in WT self-diploids *vs* EC1118

Nucleotide Position	EC1118	S288c	Dist 1	Dist 2	F2	I1	J1	L1
231	G, E^{77}	G, E^{77}	G, E^{77}	G/A, E^{77}	G>A, E^{77}	G>A, E^{77}	G>A, E^{77}	G, E^{77}
296	C, A^{99}	C, A^{99}	C, A^{99}	C/T, A^{99}/V^{99}	C>T, $A^{99}V$	C>T, $A^{99}V$	C>T, $A^{99}V$	C, A^{99}
1138	G, G^{380}	G, G^{380}	G, G^{380}	G>A, $G^{380}R$	G>A, $G^{380}R$	G>A, $G^{380}R$	G>A, $G^{380}R$	G, G^{380}
1764	G, K^{588}	G>T, $K^{588}N$	G>T, $K^{588}N$	G>T, $K^{588}N$	G, K^{588}	G, K^{588}	G, K^{588}	G>T, $K^{588}N$
2566	G, E^{856}	G>A $E^{586}K$	G>A $E^{586}K$	G, E^{856}	G, E^{856}	G, E^{856}	G, E^{856}	G>A $E^{586}K$

Nucleotide transitions (C to T, G to A) are denoted with symbol >. Heterogeneity at a nucleotide position and corresponding amino acid is denoted with symbol /. Amino acids are denoted by single letter code for example Lysine, K. Amino acid position is denoted with numeral (superscript)

Additional 'selfed' WT yeast have been isolated as part of this recombinant 'proof-of-concept' approach, in wine yeast PDM (low H_2S producer) and L2056 (moderate H_2S production). These strains still remain to be tested for fermentation performance and modulation of sulfur metabolism. Whilst previously we had only limited success in exploiting genotypic heterogeneity in generating new strains in terms of enhanced fermentation performance, low H_2S and SO_2 production appear to be feasible targets. The demand for reliable yeast which produce limited H_2S and SO_2 remains, as currently low H_2S producing strains have SO_2 levels inhibitory to MLF as well as close to exceeding some country's statutory limits.

<u>Low H_2S and SO_2 producing strains derived from ethyl methanesulfonate treated cultures</u>
As mentioned, part of this project's aim was to target the sulfate transport system, as a means of lowering H_2S liberation and controlling excessive SO_2 production by yeast cells without compromising growth and fermentation reliability. In this part of the study, two commercial strains were mutated using ethyl methanesulfonate and clonal isolates were screened on the toxic sulfate analogues sodium selenate and potassium dichromate. The strains were EC1118 (industry benchmark in fermentation performance) and Maurivin Distinction®, a second-generation yeast (low H_2S production <u>but</u> high SO_2 levels).

Selection of mutated cultures on YPD agar containing 5 mM sodium selenate and potassium dichromate yielded ~30 mutants in total (NB: 1-3 colonies were picked for some isolates). All of the mutants were methionine prototrophs indicating that whilst sulfate uptake was affected, there was still sufficient sulfur being taken up to make the amino acids methionine and cysteine essential for growth. These mutants were compared in single 100 mL fermentations (**Output 2A**) in CDGJM under high and low nitrogen conditions (data reported in Houlès, 2012 [49]). Five strains were chosen for further investigation, having good fermentation performance as well as low H_2S and SO_2 production: D13-1, D25-1 (derivatives of Distinction®) and E178-1, E211-1, E212-1 and E214-3 (derivatives of EC1118). The fermentation performance of these strains including sulfur metabolite production (H_2S, SO_2 and glutathione) was compared with their parents in 100 mL fermentations and 10 L fermentations (**Output 2A**). Selected gene sequences of these 5 mutants were also compared with the parent strains EC1118 and Distinction® as well as PDM (Mauri Yeast Australia) and laboratory strain S288c to identify which mutations were present within the key genes *SUL1*, *SUL2*, *MET5* and *MET10*. Detailed results are reported in Lee, 2012 [47] however **Table 7** shows the nucleotide changes in the *SUL1* and *SUL2* genes in the EMS derived mutants compared with EC1118 and S288c. The *SUL2* gene in all of the mutants except for E214 -3 was identical to the parent strains EC1118 and Distinction® (Dist 1); E214-3 had a G to A transition at position 1408 which resulted in the substitution of alanine to threonine ($A^{470}T$). All except for D25-1 displayed identical mutations, sharing similarity with EC1118 (nucleotides 1764, 2566) as well as Dist2 (nucleotide 1138). A silent mutation at nucleotide 231 (G>A) did not result in an amino acid substitution. Dist 2 displayed nucleotide dimorphism at base pair position 296 (C or T) resulting in either alanine (A) or valine (V) at amino acid 99. Both do not appear to be deleterious to sulfate transport function [41]. All of the mutants except D25-1 contained valine ($A^{99}V$) as a result of the C>T transition. D25-1 was identical to EC1118 at this position (C_{296}, A^{99}). D25-1 differed from the other mutants in having nucleotide dimorphism for nucleotide 1764 (G/T, K^{588}/ N^{588}). Both nucleotides would appear to be functional being present in S288c and EC1118. Sequencing analysis has demonstrated that in *SUL1* and *SUL2* genes in the various yeast strains there is genotypic heterogeneity [50], which has the potential to influence phenotypic outcome with regards to sulfur metabolism.

Conclusions

Within these **Outputs 1C** and **1D** we have successfully generated and isolated 12 promising new fermentation efficient yeast strains (from a mixture of random parents and new isolates from the mixed AE culture FM16), as well as ten new yeast isolates with reduced H_2S and SO_2 production (4 being the progeny of 'selfed' spores and 6 EMS mutant isolates of commercial strains. Through bacterial Directed Evolution we have also generated 4 new *O. oeni* strains that display efficient MLF in the presence of high alcohol. Each of these isolates have been examined in at least micro-fermentation scale, thus satisfying the performance targets of "Improvement of strains through multiple rounds of mating, mutagenesis and AE approaches" as well as testing of such in "micro-fermentation screens". We have also identified alleles found within genes of the sulfate reduction sequence between a set of commercial wine yeast strains including those marketed as being low H_2S producers. Together, with novel isolates already generated within this laboratory these new isolates amount to an excellent resource for development of new commercial strains if not direct commercialisation themselves.

Table 7: Comparison of nucleotide substitutions in *SUL1* and *SUL2* between Distinction® isolates Dist 1 and Dist 2, EMS mutants and EC1118

Nucleotide Position (*SUL1*)	**EC1118**	**S288c**	**Dist 1**	**Dist 2**	**D25-1**	**D13-1**	**E178-1**	**E211-1**	**E212-1**	**E214-3**
231	G, E^{77}	G, E^{77}	G, E^{77}	G/A, E^{77}	G>A, E^{77}	G>A, E^{77}	G>A, E^{77}	G>A, E^{77}	G>A, E^{77}	G>A, E^{77}
296	C, A^{99}	C, A^{99}	C, A^{99}	C/T, A^{99}/V^{99}	C, A^{99}	C>T, $A^{99}V$	C>T, $A^{99}V$	C>T, $A^{99}V$	C>T, $A^{99}V$	C>T, $A^{99}V$
1138	G, G^{380}	G, G^{380}	G, G^{380}	G>A, $G^{380}R$	G>A, $G^{380}R$	G>A, $G^{380}R$	G>A, $G^{380}R$	G>A, $G^{380}R$	G>A, $G^{380}R$	G>A, $G^{380}R$
1764	G, K^{588}	G>T, $K^{588}N$	G>T, $K^{588}N$	G>T, $K^{588}N$	G/T, K^{588}/N^{588}	G, K^{588}	G, K^{588}	G, K^{588}	G, K^{588}	G, K^{588}
2566	G, E^{856}	G>A $E^{586}K$	G>A $E^{586}K$	G, E^{856}	G, E^{856}	G, E^{856}	G, E^{856}	G, E^{856}	G, E^{856}	G, E^{856}

Nucleotide Position (*SUL2*)	**EC1118**	**S288c**	**Dist 1**	**Dist 2**	**D25-1**	**D13-1**	**E178-1**	**E211-1**	**E212-1**	**E214-3**
1408	G, A^{470}	G>A, $E^{856}K$	G, A^{470}	G, A^{470}	G, A^{470}	G, A^{470}	G, A^{470}	G, A^{470}	G, A^{470}	G>A, $A^{470}T$

Mutants derived from EMS mutagenesis of EC1118 are denoted with the prefix E, whilst those derived from the Distinction® are denoted with the prefix D.
Nucleotide transitions (C to T, G to A) are denoted with the symbol >. Heterogeneity at a nucleotide position and corresponding amino acid is denoted with the symbol /.
Amino acids are denoted by single letter code for example Lysine, K. Amino acid position is denoted with numeral (superscript)

Output 2A: Data on wines from lab scale fermentations

- **Complete secondary phase trials (100 mL to 10 L) with reduced set of candidate strains. Conduct analyses of wines by chemical and, where appropriate, sensory approaches.**

Contributors
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Casual Staff: Dr Michael Dow, Brenton Mui, Johannes Scharwies, Eva Tomczyk, Dr Monique Shearer

Background
Novel strains generated in **Output 1C** and previous projects were tested here in larger scale fermentations (0.1-10 L) to analyse their performance under conditions that are more similar to those encountered in industry. This scale of experiment also allows for a more thorough analysis (including chemical and sensory) and an ability to more tightly control experimental conditions. Where possible we also analysed fermentation attributes in a number of different grape juices, varying vintage, variety and region so as to model the variable challenges these microbes may face in industry.

Methods
Laboratory-scale (100 mL) fermentations (AE experiment)
Triplicate 100 mL fermentations were performed in white juice in 250 mL Erylenmeyer flasks fitted with airlocks [17, 51] using manual shaking on a Ratek shaker (120 rpm) or the automated fermentation facility (www.agwine.adelaide.edu.au/wine/facilities/msff/) or N_2 sparging was omitted as the fermentations become anaerobic within 4-6 hrs of commencing (data not shown). Fermentation progress was monitored as accumulative weight loss due to CO_2 evolution, and enzymatic analysis of residual glucose and fructose [14].

Laboratory-scale (100 mL and 10 L) fermentations – low H_2S producing strains
For candidates for low H_2S production, 100 mL fermentations were performed in white juice or CDGJM (100 mg L^{-1} nitrogen, 200 g L^{-1}sugars) with H_2S gas detection tubes (1-150 ppm; Cat: 120 SB and 25-2000 ppm; Cat: 120SF, Air Met Scientific) fitted into dry airlocks. Hydrogen sulphide production was detected through the chemical conversion of either silver nitrate to silver sulfide (1) or lead acetate to lead sulfide (2) and measured as arbitrary units using the scale on the detection tubes.

(1) $H_2S + Ag \rightarrow 2AgNO_3 + Ag_2S$ [white to black colouration] (120SF; Kitagawa)
(2) $H_2S + Pb \rightarrow PbS + 2CH_3CO_2H$ [white to brown colouration] (120SB; Kitagawa)

Selected candidate strains were evaluated for fermentation performance and H_2S and SO_2 production in 10 L fermentations using a variety of white juices as described below.

10 L grape juice fermentations
Fermentations (10 L) were conducted in triplicate using hot water washed demijohns. Juice parameters were analysed using an OenoFoss Analyser (Foss), sugar by enzymatic analysis [14] using a Infinite® 200 PRO microplate reader (Tecan Group Ltd), and SO_2 by the aspiration method [52].

Juice was sourced from Treasury Wine Estates (Wolf Blass, Barossa) in 220 L drums and stored 1-4 days at 4 °C before use. The contents were mixed using N_2 gas before being decanted into demijohns (9 L) prior to inoculation of the fermentation. Tartaric acid and DAP additions were not made to juice unless stated.

Yeast were grown on YPD agar and used to inoculate 100 ml YPD medium. The strains were grown overnight (30 °C) before inoculation at 5 x 10^6 cells mL^{-1} into 1 L of JSM_DAP or JSM_FAA starter medium (**Appendix 5**). Following 24 h growth, the starter cultures were used to inoculate 9 L juice at 5 x 10^6 cells mL^{-1}. Fermentations were conducted in triplicate at ambient temperature (22°C). Samples were collected regularly and analysed for residual glucose and fructose content as described above. Terminal samples (1 mL and 50 mL) were collected, clarified by centrifugation and frozen for subsequent metabolite analysis. Hydrogen sulfide (H_2S) was monitored daily using a Kitagawa AP20 aspirating pump and 1-60 ppm gas detection tubes (Cat: AP20 and 120SD, Air Met Scientific).

Metabolite analysis

Organic acids, glycerol, acetaldehyde and ethanol concentrations were determined by high performance liquid chromatography (HPLC) [13] and total SO_2 and volatile acidity by determined by aspiration [51].

Yeast aroma compounds

Relevant compounds were analysed by GC-MS according to Siebert et al [20] using 20 ml wine samples (clarified by centrifugation, sterile filtered (0.22 μm) and kept at 4°C for up to one week). GC-MS samples were prepared on the day of analysis in a sealed GC vial containing a 2 mL sample + 7.9 mL sterile MQ + 0.1 mL octanol standard + 2 g NaCl. Samples were shaken prior to loading onto the GC-MS carousel. The octanol standard was freshly prepared in a volumetric flask (1 drop (~0.009 g) dissolved in 100 mL sterile MQ). GC-MS analysis was conducted by Mrs Jade Haggerty, University of Adelaide.

Glutathione determination

A glutathione assay kit (Sigma, Cat: CSO260) was used in 96-well plates [47, 52]. Working solutions were prepared according to the manufacturer's instructions. A calibration curve was constructed by serial dilution with 5% (v/v) 5-sulfosalicylic acid (SSA) of a 125 μM glutathione standard (1.95 μM to 125 μM or 0.6 mg L^{-1} to 38.4 mg L^{-1}). Wine samples were diluted 1:2 and juice samples diluted 1:10 with 5% SSA and 10 μL of the diluted sample was used in the reaction scheme. The assay mixture was prepared fresh for each 96 well plate reaction set-up. Reagents required 5 minutes of incubation at room temperature before the enzymatic REDOX reaction was activated with the addition of 0.16 mg mL^{-1} NADPH in each well. The 5% SSA was used as the reagent blank and a base absorbance reading at 412 ηm (time point zero) was recorded prior to the addition of NADPH. A spectrophotometer with in-built Kineticalc version 3.03 rev 4 software (μQuant, BioTek Instrument Inc.) [53] was used to read absorbance at 412 ηm in one minute intervals up to 5 minutes and a final reading was recorded at 10 minutes post NADPH activation. The detection limit was ~40 mg L^{-1} GSH, with reliable determination of total glutathione at 2 minutes post NADPH addition if SO_2 was present (>25 mg L^{-1}) in the sample as there is minimal REDOX effect from SO_2.

Comparative evaluation of FM16-C7 and Uvaferm 43 or PDM in restarting fermentation

A series of fermentations were conducted to evaluate the performance of FM16-C7 in restarting yeast alcoholic fermentation. Red and white wines were sourced from Yalumba

Wine Company (Barossa Valley). The residual sugar content varied from 5 to 90 g L^{-1}. In wines with <20 g L^{-1} residual sugar, sugar content was standardised at 20 g L^{-1} using fructose. The protocol for restarting fermentations described by Lallemand was followed. Yeast strains were grown on YPD agar before inoculation into YPD medium. After 24 h growth at 30 °C, 25% of the culture was used in the production of the starter A (**Appendix 5**). The starter A culture was grown for 24 h before being used in Starter B (**Appendix 5**). The 100 mL starter B culture was grown again for 24 h. Triplicate 100 mL fermentations were prepared using 25% starter B culture and 75% stuck wine in an Erylenmeyer flask fitted with a water-filled airlock. Fermentations were incubated at 30 °C, with regular monitoring of residual sugars [14].

Results and Discussion

The 100 mL and 10 L fermentation data on strains obtained from this project (sporulation, mass mating and AE in Chardonnay juice) and new clones derived from previous AE strategies outlined in UA05/01 are reported together for this Output. Fermentations with low H_2S producing strains are reported separately (100 mL and 10 L fermentations).

Robust and novel yeast generated by mass mating and adaptive evolution

100 mL fermentations were conducted in the high throughput fermentation facility using Chardonnay juice (2010 Adelaide Hills) on the five FM16 clones and 7 strains (mating/DE isolated as part of this project) chosen from the micro-fermentation screen (**Output 1D**) and commercial yeast EC1118, L2056 and/or PDM. In the case of the FM16 clones, fermentation was also compared against the mixed FM16 population from which the clones were isolated. Fermentation progress was monitored as cumulative CO_2 loss (g 100 g^{-1}) [54] as shown in **Figure 6**. Strains AE10-100, AE20-91 and AE20-178 were chosen for further evaluation in 10 L white juice fermentations. FM16 clones FM16-74, FM16-79, FM16-144, FM16-150 and FM16-170 were chosen for initial evaluation (10 L Chardonnay, 2012, Riverland), of which the latter three were further investigated in a variety of white juices. The fermentation performance of 6 other strains isolated in previous AE strategies were also examined in 10 L fermentations as shown in **Table 8**.

Trials were conducted in four white juices sourced from 3 vintages. Juice from 2010 and 2011 (frozen at -20°C) and thawed 2-3 days, 4 °C followed by 24 h at ambient temperature prior to inoculation. Juice from 2012 and 2013 was sourced from Treasury Wine Estates and cold settled (1-3 days, 4 °C) before inoculation. As mentioned in the interim report the strains derived from mass mating were found to be inferior because of negative attributes (excessive foaming, reductive odours) and poor fermentation performance. The fructophilic strain's performance in juice did not reflect the performance in chemically defined grape juice (CDGJM), where it was observed to have increased fructose uptake and catabolism [42]. See **Supplementary Figures 1 to 3** for fermentation profiles of the individual strains.

Aroma compounds, key metabolites and SO_2 were also analysed from fermentation samples. Thirty six yeast-derived aroma compounds were compared qualitatively by GC-MS analysis from the fermentation samples from EC1118, L2056 and FM16 derivatives. The relative amount of each compound from each fermentation sample was analysed by 2-way ANOVA. Whilst there were generally no significant differences noted for most of the compounds, several differences were observed between the wine strains EC1118 and L2056 and evolved strains (FM16 derivatives) as shown in **Supplementary Table 9**. These tended to be compounds associated with floral or fruit attributes (**Supplementary Table 10**). The overall sensorial effect on the aroma profile of the wines however was not determined. However,

basic sensory tests as reported in the interim report (Feb 2013) showed the same or greater acceptance of the wine from FM16-C7 versus L2056 and EC1118. The other FM16 derivatives were not examined because of time constraints and will be examined in greater detail as part of the new project UA1302.

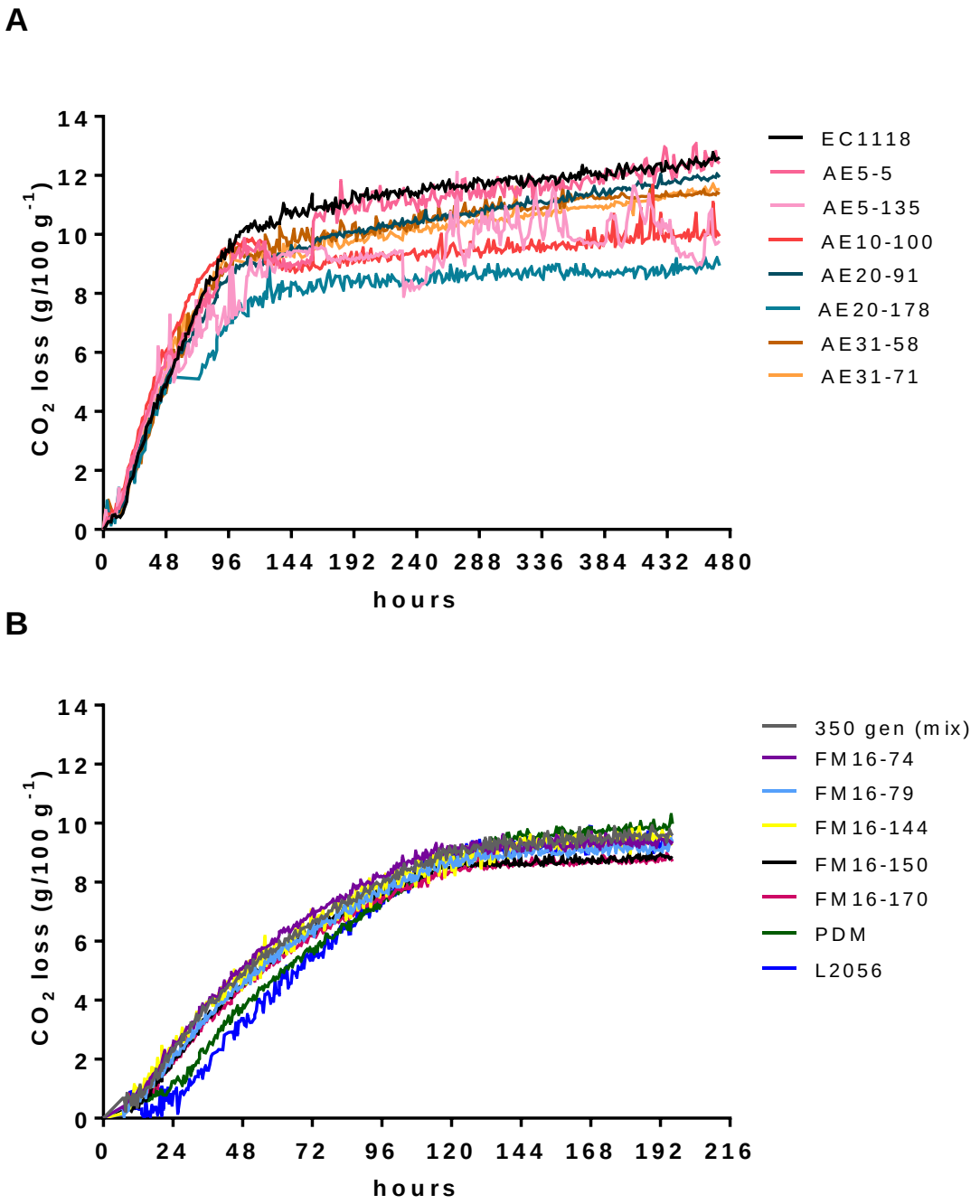


Figure 6: Triplicate 100 mL fermentations conducted in Chardonnay juice

Fermentation A: Yeast were grown in YPD (28 °C) prior to inoculation into sterile juice starter media with amino acids (JSM_AA) and incubated at 28 °C. Yeast were inoculated at 5 x 10^6 cells mL^{-1} into sterile Chardonnay (2012, Riverland). Fermentation was performed at 22 °C in the fermentation facility. Juice parameters were 254 mg L^{-1} YAN, 20.8 °Brix, pH 3.25, 6.6 g L^{-1} TA.

Fermentation B: Yeast were grown in YPD (28 °C) prior to inoculation in sterile juice starter media (1:1 volume of chardonnay juice and water) and incubated at 28 °C. Yeast were inoculated at 5 x 10^6 cells mL^{-1} into sterile Chardonnay (2010, Adelaide Hills). Fermentation was performed at 22 °C in the fermentation facility. Juice parameters were 152 mg L^{-1} YAN, 19.4 °Brix, pH 3.1, 4.2 g L^{-1} TA

Table 8: Evaluations of fermentation performance conducted on candidate yeast strains (100 mL and 10 L laboratory trials)

Strain	AE selection pressure/comments	100 mL	10 L
EC1118		✓	✓
PDM		✓	✓
AWRI 796			✓
Distinction®		✓	✓
L2056		✓	✓
AE5-5	Juice (this study)	✓	✓
AE5-135	Juice (this study)	✓	✓
AE10-100	Juice (this study)	✓	✓
AE20-91	Juice (this study)	✓	✓
AE20-135	Juice (this study)	✓	✓
AE31-58	Juice (this study)	✓	✓
AE31-71	Juice (this study)	✓	✓
FM16 (350 gen; mixed culture)	Oxidative stress, low inoculum [17]	✓	
FM16-C7	As above [17]		✓
FM16-74	As above, isolated this study [17]	✓	✓
FM16-79	As above, isolated this study [17]	✓	✓
FM16-144	As above, isolated this study [17]	✓	✓
FM16-150	As above, isolated this study [17]	✓	✓
FM16-170	As above, isolated this study [17]	✓	✓
A12*	Cold stress (McBryde unpublished)		✓
B11*	Cold stress (McBryde unpublished)		✓
TEE9	Fructose-only growth [42]		✓
SORB E1*	1M sorbitol (McBryde unpublished)	✓	✓
SORB C1*	1M sorbitol (McBryde unpublished)	✓	✓
SORB H1*	1M sorbitol (McBryde unpublished)	✓	✓
D13-1	EMS, selenate/dichromate	✓	✓
D25-1	EMS, selenate/dichromate	✓	✓
E178-1	EMS, selenate/dichromate	✓	
E211-1	EMS, selenate/dichromate	✓	✓
E212-1	EMS, selenate/dichromate	✓	
E214-3	EMS, selenate/dichromate	✓	
I1	Spore progeny	✓	✓
F2	Spore progeny	✓	
J1	Spore progeny	✓	
L1	Spore progeny	✓	

*ARC project: *Use of innovative, Adaptive Evolution strategies to develop superior yeast for ready application in the wine and related industries* McBryde, Grbin and Jiranek (2008).

All fermentation experiments were conducted in triplicate. 100 mL fermentations were performed in the Fermentation Facility (University of Adelaide). 10 L fermentations were undertaken in four white juice varieties over 3 vintages (Chardonnay vintages (2010, 2012-2013); Riesling (2010-2012), Semillon (2013), Sauvignon Blanc 2013). Not all the strains were examined in each juice.

Low H_2S and SO_2 producing yeast by chemical mutagenesis and spore heterozygosity

As reported in **Output 1C**, we generated yeast strains which exhibit low H_2S without over-production of SO_2 during fermentation. This was achieved through the modification of the sulfate transporters Sul1p and Sul2p, through random chemical mutagenesis and the selection of mutants defective in sulfate transport. We also exploited genetic heterozygosity within the strain to identify spore progeny that did not produce H_2S and had reduced SO_2 compared to Distinction®.

100 mL fermentations

Ten candidate strains (**Table 8**) were evaluated in 100 mL fermentations using defined media of varying nitrogen composition (refer to [47, 49]). Six EMS-derived strains were evaluated in two white juice fermentations, since these were the focus of DNA sequencing (**Output 1C**). Key metabolites, H_2S, SO_2 and glutathione were determined at the end of fermentation. The fermentation performance of isolates Dist1 and Dist2 (described in **Output 1C**) was also compared. Fermentation in both 2013 Chardonnay and 2013 Verdelho grape juices were examined (**Figures 7 and 8**). In the Chardonnay juice, where nitrogen content was not limiting, the new strains fermented as well as the parent strains. The only poor performer was Dist2. In the Verdelho juice, the EMS-derived strains had a longer lag phase, but were able to complete fermentation in a similar time to the parents.

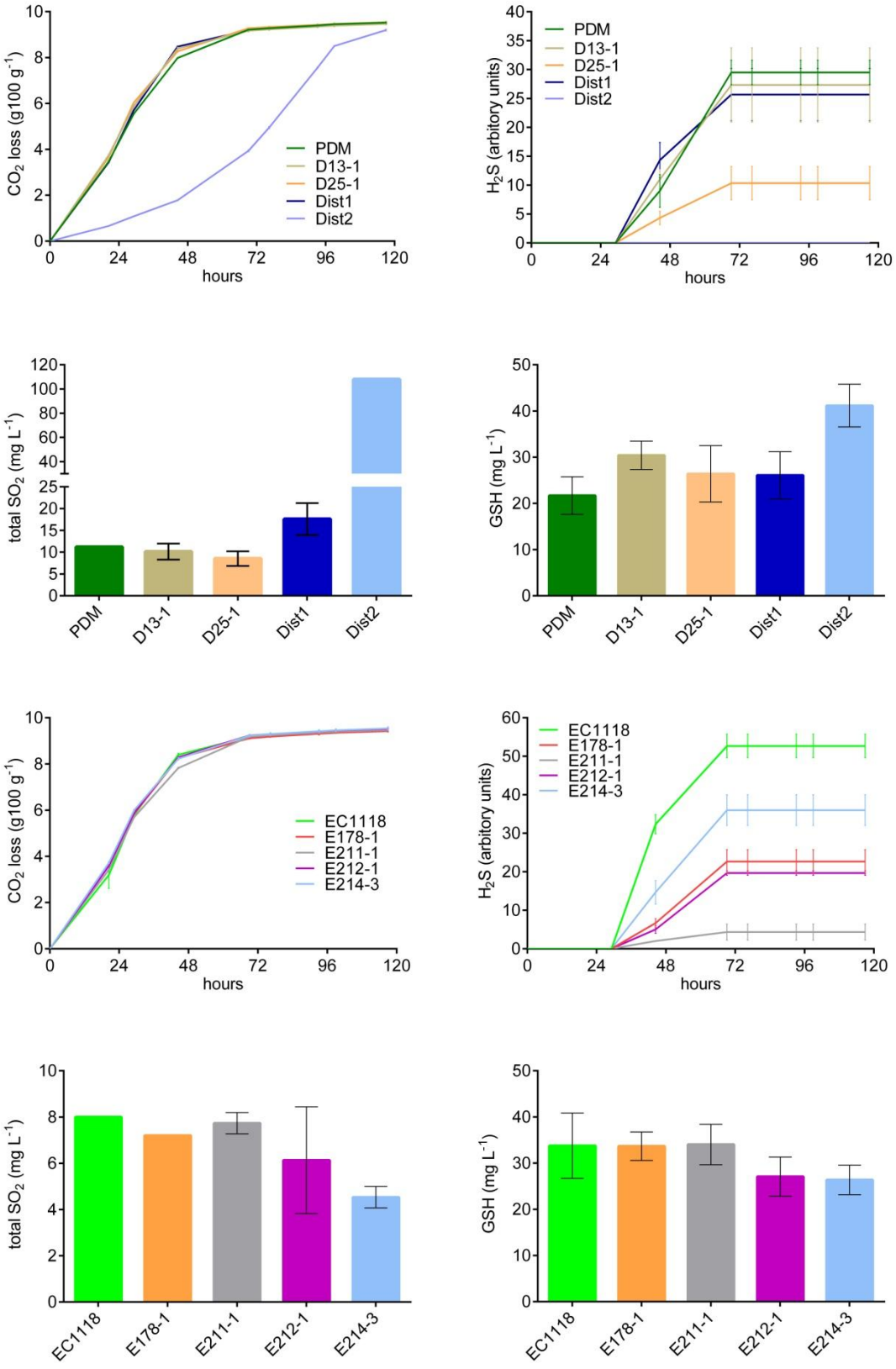


Figure 7: Evaluation of low H_2S producing wine strains in 100 mL Chardonnay fermentations. Triplicate 100 mL fermentations were performed at 28 °C in Chardonnay juice (sourced from the Waite Campus). H_2S, SO_2 and glutathione (GSH) content are shown. Juice parameters: 377 mg L^{-1} YAN, 20.6 °Brix, pH 3.41, 4.9 g L^{-1} Titratable acidity.

No discernible differences were noted in the key metabolite profile (sugars, glycerol, organic acids and ethanol) for the EMS mutants and Dist1 in both juice fermentations. However, as mentioned, Dist2 displayed poor fermentation kinetics and did not complete fermentation within the timeframe (58 g L^{-1} residual sugar). Acetic acid was variable with the highest amounts being seen in E178-1 (1 g L^{-1}), E212-1 (2.4 g L^{-1}) and E214-3 (3.1 g L^{-1}) as compared to the other EMS derived strains (data not shown). These values are much higher than acceptable in terms of legal limits and odour threshold, but are not uncommon in small laboratory fermentations. Wines produced by Dist2 had much higher total SO_2 levels (110 mg L^{-1}) than any of the other strains. The laboratory's isolate of Distinction® (Lab-Distinction®) would appear to be the same as Dist1, as both produce 30% less H_2S than EC1118, whilst there was zero H_2S detected from Dist2. Dist2 demonstrated the properties (zero H_2S and high SO_2) of Distinction® as reported by Mauri Yeast Australia. Whilst there was no significant difference in SO_2 production between the EMS derived strains, all produced significantly less than EC1118 with E211-1 observed to produce the least amount of hydrogen sulfide (**Figure 7**).

As glutathione is an important antioxidant in juice and wine, we measured glutathione content in the thawed terminal samples. We were interested in determining whether reduced flux of sulfur in these strains with altered sulfate uptake, also was detrimental to glutathione biosynthesis. Significant differences were noted between Lab_Distinction® and E178-1, E212-1 and E214 (Tukey, one-way ANOVA, $p<0.0001$ test; CI 0.95). E212-1 and E214-3 however were no different when compared to their own parental stain EC1118, whilst E178-1 produced the least amount of total glutathione (**Figure 8**). E211-1 produced similar amounts of glutathione to the parent strain EC1118.

Whilst the *SUL1* and *SUL2* genes were sequenced in the above EMS mutants, it is difficult to make a direct link with the observed SNPs within these gene ORFs and the strain properties, especially since E178-1, E211-1 and E212-1 all had the same sequences at *SUL1* and *SUL2*, yet variable H_2S production: clearly other gene sequences are conferring these changes. The only exception would be that of E214-3, a *sul2* mutant exhibiting a single nucleotide change at base pair position 1408 with an amino acid substitution ($A^{470}T$) which has the potential to result in a non-functional Sul2p. This may attribute to the observed reduction in H_2S and SO_2 levels in this mutant. Sequence analysis of the *SUL1* and *SUL2* genes in the remainder of the strains (**Output 1C**, **Table 7**) did not correspond to the fermentation results, suggesting that other genes involved in sulfur metabolism may be affected. Two such genes may be *SSU1* [55] encoding the sulfite exporter Ssu1p and its upstream transcriptional activator (*FZF1* gene); both are involved in sulfite detoxification and are implicated in sulfite tolerance [56]. Future work will include investigation of these genes.

The spore progeny F2, I1, J1 and L1, derived from Distinction® were also examined in triplicate 100 mL fermentations under low nitrogen conditions in CDGJM (**Figure 9**). As expected these strains had variable fermentation attributes. I1 was the fastest strain overall, completing fermentation in 135.5 h, with F2 finishing in 162.3 h and the remainder (including EC1118 and Distinction®) at 180 h. Whilst J1 and L1 produced no detectable SO_2, the H_2S levels were greater than EC1118 and the laboratory isolate of Distinction® (Lab-_Distinction®). I1, however, which had no observable growth defect and excellent fermentation efficiency produced limited H_2S and SO_2 (**Figure 9**) and uncompromised glutathione levels (data not shown), thus was considered suitable for further evaluation.

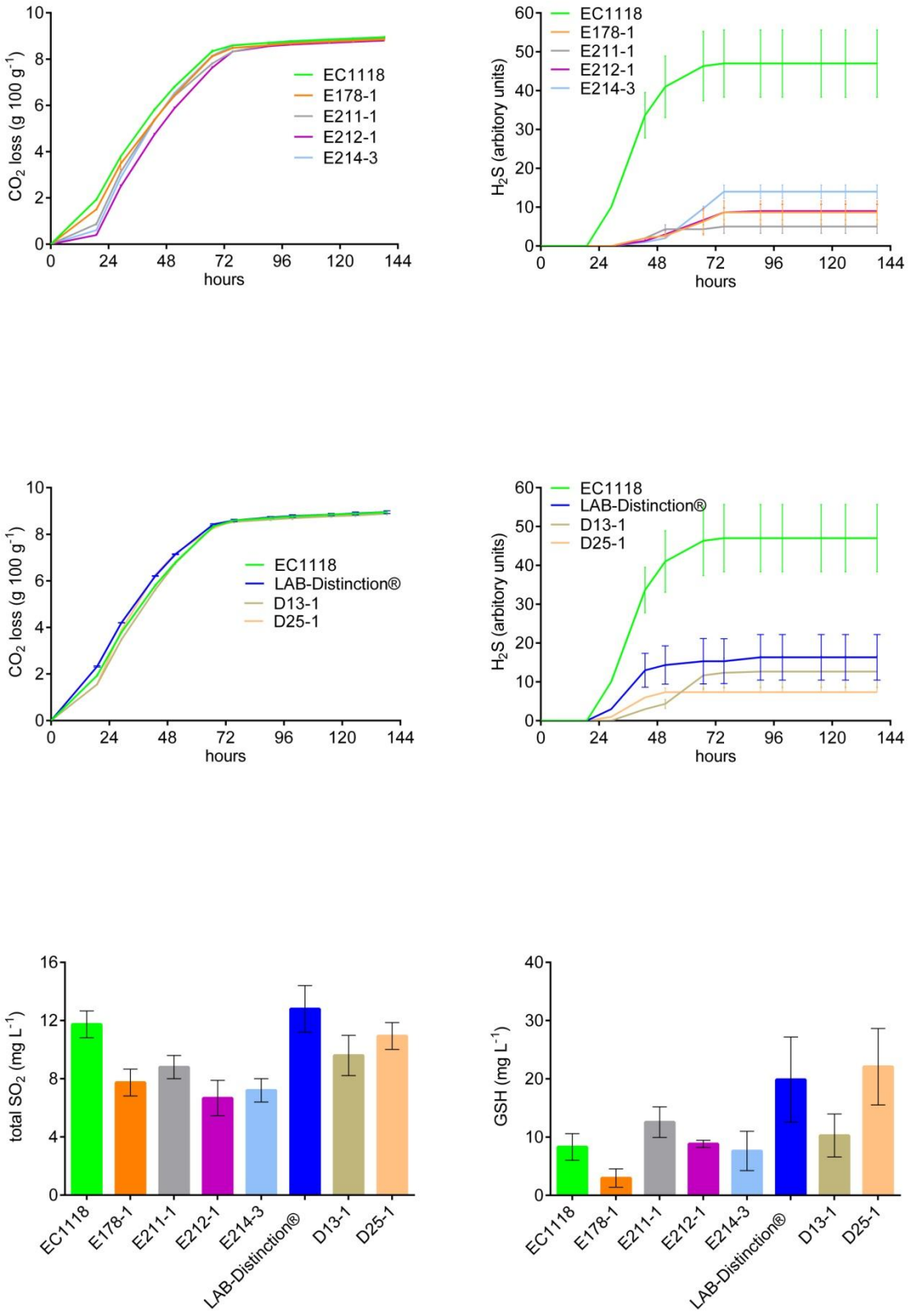


Figure 8: Evaluation of low H_2S producing wine strains in 100 mL Verdelho fermentations. Triplicate 100 mL fermentations were performed at 28 °C in Verdelho juice. H_2S, SO_2 and glutathione (GSH) content are shown. Juice parameters: 248 mg L^{-1} YAN, 18.6 °Brix, pH 3.1, 4.9 g L^{-1} TA, 11 mg L^{-1} FSO_2, 26 mg $L^{-1}TSO_2$

10 L fermentations

The four most promising low H_2S strains (E211-1, D13-1, D25-1 and I1) were compared in a series of 10 L white juice fermentations against PDM and Distinction® and FM16-C7 (our most fermentation efficient evolved wine yeast strain) for fermentation performance and H_2S and SO_2 production. Whilst H_2S was difficult to measure in the 10 L fermentations, being in nearly all cases undetectable, SO_2 production was consistently less than PDM whilst Distinction® produced the highest levels (refer to **Supplementary Figures 4** and **5**). The exceptions were where fermentation was problematic, for example Riesling 2011 and Chardonnay 2010, where the differences were reduced. The comparable fermentation performance of I1 (and to a lesser extent D25-1) to our fastest evolved strain FM16-C7, together with the decreased liberation of SO_2 make these candidates worthy of further investigation. In addition, the other strains from the H_2S project require further evaluation as 10 L juice fermentations. We envisage this work and fermentations of increased volume could be undertaken either next vintage or the coming vintage in California (at UC Davis).

Comparative evaluation of FM16-C7 and Uvaferm 43® in restarting fermentation

Since our previously reported strain FM16-C7 appears to have a very efficient fermentation phenotype, we investigated whether this strain could be used as a rescue culture for arrested fermentations. A series of fermentation trials were conducted to compare FM16-C7 to Uvaferm 43® and other strains commonly used in industry for this purpose. Three initial trials were conducted using a partially fermented Semillon juice (2012, Adelaide Hills, inoculated with L2056), which was arrested before completion by cold settling and sequential filtration through a 2μm and 0.2 μm membrane. Six 'arrested musts' were produced from this with varying residual sugar for use in these initial trials. Three experiments were conducted as shown in **Supplementary Figure 6** whereby several wine strains including some produced as part of this project (FM16-C7 and Tee9) were evaluated for restoration of fermentation. The effect of increasing the ethanol content and addition of unsaturated fatty acids was also investigated.

In unadulterated juice, all the strains were able to restore fermentation (**Supplementary Figure 6 A)** although the timing of completion varied between the strains. Addition of 5% (v/v) ethanol to increase the ethanol content resulted in a severe protraction of fermentation compared to the control treatment (Control; EC1118) as shown in **Supplementary Figure 6 B**. Three strains were unable to restore fermentation: fructophilic strain Tee9, FM16-C7 and EC1118. Fermichamp® and Uvaferm® were able to restore fermentation, but still showed protraction (243.5 h and 386 h duration as compared to the control, 73.5 h). When unsaturated fatty acids were included (**Supplementary Figure 6 B**) fermentation was also protracted, with Tee9 being unable to restore fermentation. In this case FM16-C7 performed similarly to Uvaferm®. Of interest was the observation that fatty acid supplementation lead to an initial increase in fermentation rate in EC1118 and Fermichamp®, however, the final outcome of fermentation was not improved as compared to the control (EC1118). Octanoic acid (C8-unsaturated fatty acid) and decanoic acid (C10- unsaturated fatty acid) are products of alcoholic fermentation, and are important in the organoleptic properties of wine. These lipophilic fatty acids have been previously reported to inhibit growth and biomass development during fermentation, with toxicity increasing under acidic conditions (≤ pH 3) [57]. The mode of action of these acids is thought to be through the disruption of the membrane potential, as the molecules are able to cross the lipid membrane in an undissociated form (cited in [58]). Toxicity is known to increase with decrease in molecule size, for example decanoic acid > octanoic acid >> ethanol [58]. It is not surprising therefore that the addition of ethanol led to the severe protraction of fermentation, following re-

inoculation with Fermichamp® and Uvaferm®, and the continued arrest of EC1118, FM16-C7 and Tee9, most likely due to strain sensitivity to the ethanol.

In three subsequent experiments FM16-C7 was compared against Uvaferm 43® in its ability to restart a number of red and white wines which were arrested at different sugar concentrations. In cases where the sugar content was less than 20 g L^{-1}**,** fructose was added, since it is most commonly the last monosaccharide to be consumed during fermentation. A total of 12 wines were investigated across 3 experiments as shown in **Supplementary Figure 7**. Neither yeast were unable to 'rescue' any of the Shiraz or Shiraz/Mataro wines (3 experiments) and one Chardonnay wine (Experiment C, Wine 1), although the starting sugar content was comparable. This suggests that there is something within the chemistry of these wines which is inhibitory to fermentation. In the case of 2 Chardonnay wines (Experiment B, Wines 1 and 2) FM16-C7 did not restore fermentation, whilst with the Riesling wines (Experiment A, Wines 3 and 4) both FM16-C7 and Uvaferm 43® were comparable in and were able to complete fermentation, even when these wines had high residual sugar.

A commercial preparation of yeast hulls (BIOCELL, Laffort) was used in Starter media A and B (**Appendix 5**). Yeast hulls have been shown to ameliorate the inhibitory action of unsaturated fatty acids, through their adsorption [58]. Whilst such preparations are useful, it would appear that in this case, there were additional inhibitory factors not necessarily fatty acids (or esters) which were the cause of the fermentation arrest. Common factors are very high initial sugar contents, vitamin or nitrogen deficiencies, anti-microbial compounds, high clarification of the juice and extreme fermentation temperatures (cited in [58]). The latter is usually not a problem given most wineries have adequate temperature control of their fermentations. It would be of interest to determine whether in these particular juices, there was a specific deficiency such as potassium, which is known to influence fermentation outcome [59].

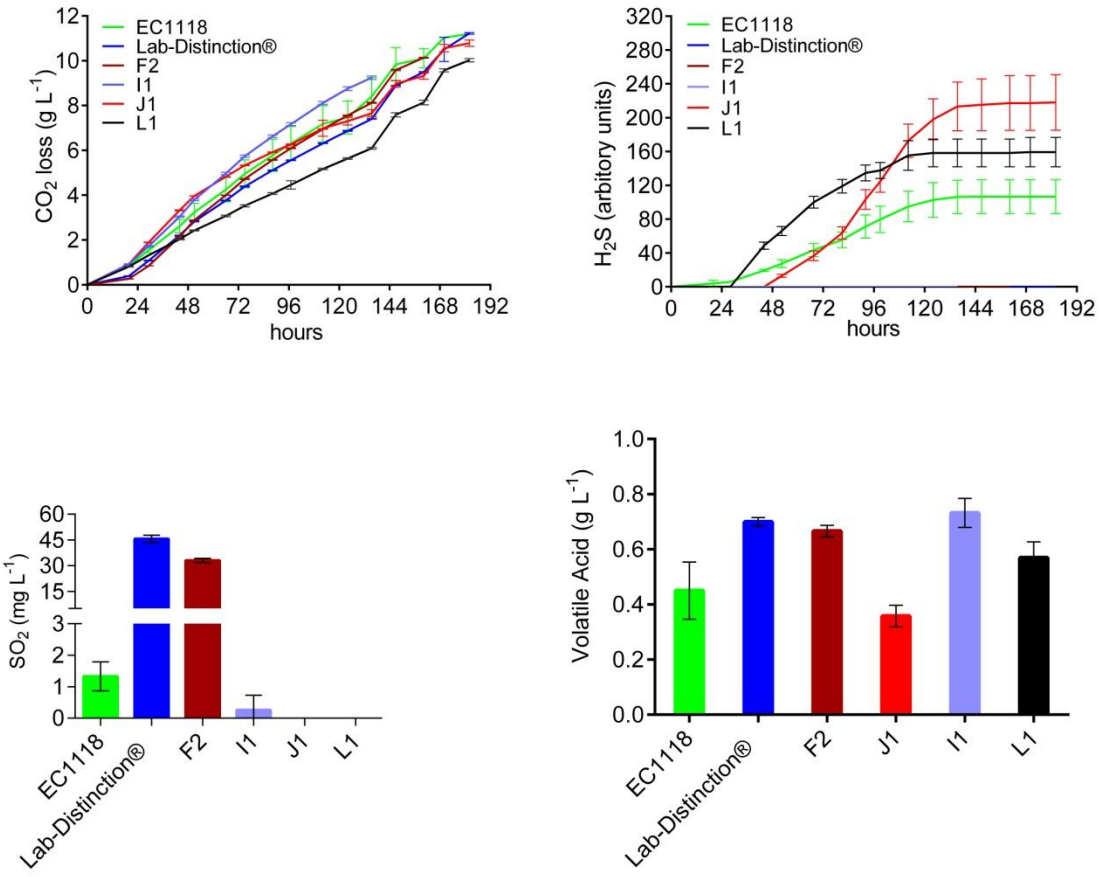


Figure 9: Evaluation of low H_2S producing wine strains in 100 mL CDGJM (100 mg L^{-1} N as ammonium sulfate) Triplicate 100 mL fermentations were performed at 28°C in CDGJM (100 mg L^{-1} N as $NH_4(SO_2)_2$ and 200 gL^{-1} sugar). H_2S production and SO_2 and volatile acidity are shown.

Conclusions

In this **Output 2A** we have achieved our performance target "Complete secondary phase trials (100 mL to 10L) with a reduced set of candidate strains and conduct chemical and sensory analysis where appropriate". In brief, the 20 best DE strains were evaluated for fermentations in 0.1 – 10 L juice fermentations, with the majority being evaluated in 10 L. Fermentation in a number of different juices was examined so as to evaluate any differences in medium dependent fermentation profile. Overall, the most efficient strain under almost every condition was FM16-C7 with a number of other evolved strains having slightly improved fermentation efficiency. A suite of volatile aroma compounds was also examined where a number of differences relating to fruity aromas were noted. Interestingly informal sensory performed in-house revealed that wines made with FM16 derived strains commonly had pleasant fruity and floral aromas. These observations are however yet to be confirmed with a full sensory analysis.

Further examination of low H_2S mutants generated in **Output 1C** within larger scale fermentation revealed that a number of strains generated here (in particular I1 and E211-1) indeed only produced low or no H_2S and had a significant reduction in SO_2 production (often in the order of 5-10 fold) and were able to ferment a range of juices as efficiently as their parents.

Finally we examined whether the fermentation efficient FM16-C7 and /or the fructophilic strain TEE9 generated by this laboratory (UA05/05) could be used as a rescue culture for arrested primary fermentations. Whilst we found that under some conditions, FM16-C7 was able to complete arrested fermentation, it did not have an improved performance when compared to industry standards Uvaferm 43® or Fermichamp®. Informal analysis appeared to suggest benefits linked to the sensory properties (absence of undesirable aromas) through the use of FM16-C7.

Output 2C: Pilot scale trials of selected candidate improved strains.

- **Conduct 100 - 1000L fermentations during 2013 vintage, to undertake sensory and wine compositional analysis**

Contributors
Postdoctoral fellow and/or student supervisors: Drs Tommaso Liccioli and Professor Vladimir Jiranek
Industry collaborators: Yalumba Wine Company (Angaston, Barossa Valley)

Background
The evolved strain FM16-C7 [17] has consistently demonstrated to be a robust wine yeast, often out-performing the industry standard strains Lalvin EC1118 (Lallemand) and PDM (Mauri Yeast Australia) in 10 L white juice fermentations (**Output 2A**). The strong fermentation performance of FM-16 and pleasant aroma of the finished wine, suggests it as a potential strain for restarting alcoholic fermentation in instances where sugar catabolism is arrested at 5 g L^{-1} or greater in problematic juices. Uvaferm 43® (Lallemand) is routinely used for restarting stuck fermentations with high fructose to glucose ratios because of its ability to consume fructose more readily than other wine yeast, high alcohol tolerance, strong fermentation and neutral affect on the wines. Whilst trialling of FM16-C7 was limited to 100 mL to 10 L fermentations in the laboratory (white and red fermentations) as reported in **Output 2A**, negotiations with the Yalumba Wine Company have allowed preliminary industrial trials of the strain as an alternative to Uvaferm 43® during the latter part of the 2013 vintage. These trials, particularly those involving restarting arrested fermentation often occurred in parallel with those performed in the laboratory.

Methods
Yeast were inoculated into 50 mL sterile juice (diluted to ~140 g L^{-1} total sugar) in 250 mL conical flasks and incubated with shaking (28 °C) to a density of 1 x 10^8 cells mL^{-1}. 50 mL of this culture was inoculated into 2 L sterile juice (diluted to ~140 g L^{-1} sugar) in a 5 L flask and grown until a density of 1 x 10^8 cells mL^{-1}. 150 L of juice was pasteurised, diluted down to ~140 g L^{-1} sugar and inoculated with the 2 L culture in a tank (25 °C, air flow 5 litres/min/10 hL).

Once cell density reached 1 x 10^8 cells mL^{-1}, the culture was used as the start up culture in a sequential (step-wise) adaptation to the 'stuck' wine. Firstly, the start up culture was inoculated at 1-2 x 10^6 cells mL^{-1} into 150 L diluted juice and grown aerobically (air flow 5 litres/min/10 hL) at 25°C. After a decrease in sugar (0.3 - 0.5 g L^{-1}), the above culture (150 L) was added to 50 L diluted juice and 50 L stuck base wine (total volume 250 L) and cultured with aeration and agitation at 25 °C. When fermentation was observed to start (decrease in residual sugar), the 250 L culture was added to 250 L of stuck wine (total volume 500 L) and grown with agitation and under anaerobic (CO_2 addition) conditions at 25 °C. Again with a Baumé drop (approx. 18 g L^{-1} of sugar), the 500 L culture was added to 500 L of stuck base wine (1000 L) and incubated under the same conditions as for the 500 L culture. This step wise procedure was repeated twice for 2000 L and 4000 L volumes using an equal volume of culture and stuck base wine. Informal sensory analysis was performed by the winemaker at each stage of the restoration procedure. The value (decline in sugar content) used to determine active fermentation was dependent on the individual wine.

Results and Discussion

FM16-C7 as a yeast suitable for rescue of arrested fermentation

FM16-C7 was provided to the Yalumba Wine Company after signing of a Materials Transfer Agreement (for IP protection) for trials of its use on sluggish/stuck fermentations during the 2013 vintage. Three examples of such trials are shown below, whereby the cultures ranged from 430 L to 4000 L and wine volumes from 2000 L to 14000 L. In two out of the three examples, the yeast did not completely consume the sugar and the cultures had to be re-rescued with the commercially available fructophilic yeast Uvaferm 43®. The chemical parameters of 3 stuck wines prior to inoculation with FM16-C7 are described in **Table 9**. In the cases where the yeast was not able to complete fermentation, FM16-C7 did reduce the amount of sugar by approximately half. The yeast culture itself produced no undesirable characters in any of the fermentations. In these cases it is worth noting that no yeast nutrients were utilised.

Uvaferm 43® is a reliable strain as suggested by the manufacturer Lallemand for the rescue of arrested primary fermentation. These preliminary results suggest that FM16-C7 may provide an alternative in stuck fermentations with residual sugar content less than 10 g L^{-1}. Whilst the evolved strain is not as reliable as Uvaferm 43®, it produced no undesirable characters. Given that the trials were conducted towards the end of vintage, when it was vital to process any outstanding wines in tank, Uvaferm 43® was used in most cases because of its known reliability. In the 2014 vintage, with agreement from Yalumba, we aim to trial FM16 against Uvaferm 43® using the tanks currently used to conduct 200 L scale red and white ferments as part of 2 GWRDC funded PhD projects (Aaron Hayes, GWR Ph1010; Simon Dillon, GWR Ph 1108). The aim of the trial would be to determine the optimum conditions for use of FM-16 in treating stuck fermentations including the use of nutrient additives such as BIOCELL (Laffort) or NUTRIENT VIT END™ (Lallemand).

Pilot scale trials of selected candidate improved strains

We detailed in the interim report Feb 2013 that we were in correspondence with the Yalumba Wine Company regarding pilot fermentation trials (500-1000 L) during the 2013 vintage. Yalumba could not consent to the trials because of lack of space, thus we will pursue further negotiations for 2014 vintage to use their research facility (200 L fermentors) to trial both the selected strains and FM16-C7 (see above). Other wineries will be approach as well. We have previously undertaken vintage trials at UC Davis (UA05/01) to take advantage of an 'out of season' vintage. This provides a good option for further evaluating the strains identified in **Output 2A**. In each case, IP protection would be assured through the completion of a Materials Transfer Agreements. Our aim is to conduct triplicate fermentations in white juice and red must for 2 strains: the benchmark strain EC1118 (or PDM) and I1 (a spore derivative of Distinction®; **Output 1C**). This data would make a valuable addition, being more relevant to industrial use rather than smaller scale laboratory fermentations, for the purpose of IP protection and commercialisation opportunities.

Conclusion

The performance target of this **Output 2C** was to "conduct 100-1000 L fermentations during the 2013 vintage and undertake sensory and wine compositional analysis". The Yalumba Wine Company (Barossa Valley) became involved as an industry partner and conducted some trials with the fermentation efficient strain FM16-C7. We were hoping to extend the number of strains analysed, however, this was not possible due to limited tank space at Yalumba. We instead expanded our in-house analysis at the 10 L scale, with many juices, as reported in **Output 2A**. Nonetheless, the ability of FM16-C7 to rescue arrested industrial

fermentations was trialled at Yalumba. Whilst it seems this strain does not necessarily offer an improvement on currently available commercial rescue yeasts (in terms of fermentation kinetics) these experiments have continued to enhance an ongoing dialogue and a positive research relationship with one of our important Industry partners.

Table 9: Chemical parameters of 3 stuck Shiraz wines prior to inoculation with evolved strain FM16-C7

Wine codes	Yeast culture (L)	Wine (L)	sugar ($g\ L^{-1}$)	TA ($g\ L^{-1}$)	pH	Alcohol (%)	Malic acid ($g\ L^{-1}$)	VA ($g\ L^{-1}$)	Fermentations considered dry (<2.5 $g\ L^{-1}$) as of 22 May 2013
Barossa Shiraz 1	4000	6600	2	6.6	3.8	14.9	0	0.9	Yes - 18 d post addition
Barossa Shiraz 2	430	2000	18.3	7.2	3.56	14.4	0.9	0.8	No - 12g L^{-1} sugar. Re-rescued with Uvaferm 43®
Barossa Shiraz 3	3800	14000	20	7.9	3.8	13.9	3.3	0.5	No - Re-rescued with Uvaferm 43®

Output 2D: Information on industry priorities in wine microbiology.

- **Initiate/conduct survey of industry in conjunction with GWRDC.**

As part of the GWRDC strategic RD & E Plan 2012-17, the GWRDC commissioned Mark Gishen (Gishen Consulting) to review the current knowledge on yeast and bacterial germplasm, and identify the industry needs for this area. We contributed to this review process, as well as having already conducted our own survey in Oct 2010 of 29 winemakers responsible for ~25% of the volume of Australia's industry. All scales of production were represented (21% <1000t; 33% =1000 t0 5000t; 46%>5000t) in our survey. Both the review and our survey were in strong agreement. Winemakers desire an increased diversity of yeast and bacterial strains to increase efficiency; better management of alcoholic and malolactic fermentation; affording greater options to differentiate their wines both locally and overseas. The findings of the recently conducted Gishen review do not reveal any significant changes in winemaker's opinions, other than a greater desire for cooperation between researchers and industry in terms of a desire for industry relevant information and trialling of strains, without commercialisation encumbrances. As such, we considered that another survey (as proposed in **Output D**) would provide little new information and conducted no further survey.

Outputs 1E and 2E: Disseminated Findings

- Manuscript prepared for submission to peer-review and/or trade journal describing genes (and approach used to find them), which are critical to yeast fermentation performance in winemaking.
- Preparation of final report and submission of findings for publication, seminars or workshops for researchers, yeast companies and winemakers.

The Jiranek group has disseminated project findings through written media, electronic magazines, invited seminar presentations and poster presentations and talks at a number of national and international conferences. In addition outlines of the project has been communicated when Vladimir Jiranek (Project Supervisor) visited wineries and yeast suppliers as potential industry collaborators.

Output 1E has been delayed up until now, with three manuscripts describing the lab yeast 'Fermentome' and related gene(s) now in the final stages of preparation or submission in July 2013. Additional experimental work and data analysis was required to ensure the reports were suitable for publication.

Referred journal article (available upon request)
Walker, M.E., T.D., Nguyen, T. Liccioli, F. Schmid, N. Kalatzis, J.F. Sundstrom, J.M. Gardner and V. Jiranek. (2013) Genome-wide identification of the Fermentome; genes required for successful and timely completion of fermentation by *Saccharomyces cerevisiae.* BMC Genomics. (submitted).

Electronic magazine articles
Jiranek, V. (2013) Darwin's Drop. The Evolution of Wine. E-Science **4**:38 (http://sciences.adelaide.edu.au/schools-resources/e-science/).

Jiranek, V. (2012) Researchers developing new yeast strains to reduce winemaking costs. *R&D At Work – GWRDC Newsletter. Feb 2012.* (http://www.gwrdc.com.au/wp-content/uploads/2012/04/RD_Work_FEB12.pdf).

Conference, workshop and seminar presentations
Jiranek, V. (2013) Data from characterisation of evolved yeast and screening of yeast libraries to identify genes important for fermentation. *Seminar presentation as part of workshop W28, 15th Aust. Wine Ind. Tech. Conf., Sydney (13-18 July 2013).*

Walker, M.E., Lee, A., Houlés, A., Adint, L., Li, S. and Jiranek, V. (2013) Second generation yeast with reduced hydrogen sulfide and sulfur dioxide production. *Poster presentation, 15th Aust. Wine Ind. Tech. Conf., Sydney (13-18 July 2013).*

Walker, M.E., Liccioli, T., Schmid, F., Gardner, J. M., Kalatzis, N., Grbin, P. R., and Jiranek, V. (2013) Genome screening as an approach to understand the cellular mechanisms behind yeast adaptation during fermentation to allow for successful completion. *Poster presentation, 15th Aust. Wine Ind. Tech. Conf., Sydney (13-18 July 2013).*

Gardner, J.M., Walker, M.E., Bilsland, E., Liccioli, T., Sundstrom, J. F., Oliver, S., and Jiranek, V. (2013) Unravelling the efficient fermentation phenotype of an evolved wine yeast. *Poster presentation, 15th Aust. Wine Ind. Tech. Conf., Sydney (13-18 July 2013).*

Zhang, J., Gardner, J.M., Walker, M.E., Atsorga, M., Sundstrom, J.F., Grbin, P.R. and Jiranek, V. (2013) Investigation of the genetic basis of high nitrogen efficiency (HNE) in wine yeast. *Poster presentation, 15th Aust. Wine Ind. Tech. Conf., Sydney (13-18 July 2013).*

Trung D. N., Walker, M. E., Gardner, J. M., and Jiranek, V. (2013) Vacuolar acidification may play a key role in the ability of yeast to successfully complete industrial fermentation. *Poster presentation, 15th Aust. Wine Ind. Tech. Conf., Sydney (13-18 July 2013).*

Liccioli, T., Gardner, J., Walker, M., Sundstrom, J., Shearer, M., and Jiranek, V. (2013) Re-starting stuck wine fermentations using an evolved wine yeast. *Poster presentation, 15th Aust. Wine Ind. Tech. Conf., Sydney (13-18 July 2013).*

Jin, G., Hayes, A.M., Jiranek, V., and Grbin, P.R. (2013) Identification of bacteria associated with 'wild' or uninoculated malolactic fermentations in red wine. *Poster presentation, 15th Aust. Wine Ind. Tech. Conf., Sydney (13-18 July 2013).*

Jiranek, V. (2013) Evolutionary engineering and genome wide screening to improve wine microorganisms. *Presentation, 61st German Winegrowers´ Cong., Stuttgart (23-27 April 2013).*

Jiranek, V. (2012) "From Vine to Wine". Research Tuesdays Seminar Program, University of Adelaide. http://blogs.adelaide.edu.au/researchtuesdays/2012/11/19/vladimir-jiranek/

Betteridge, A., Grbin, P. and Jiranek, V. (2012) Enhanced winemaking efficiency: evolution of a superior lactic acid bacteria. *Oral presentation, Crush 2012 – the Grape and Wine Sci. Symp., Adelaide, 15-16 November 2012.*

Zhang, J., Gardner, J., Walker, M., Sundstrom, J., Grbin, P. and Jiranek, V. (2012) Investigation of the genetic basis of High Nitrogen Efficiency (HNE) in wine yeast. *Oral presentation, Crush 2012 – the Grape and Wine Sci. Symp., Adelaide, 15-16 November 2012.*

<u>Lectures to undergraduate and postgraduate viticulture, oenology and biotechnology students</u>
Jiranek, V. (2011-2013) Wine yeast optimisation. *Microbiology for Viticulture and Oenology (1 lecture),* 2nd year course, University of Adelaide.

Jiranek, V. (2011-2013) Enhanced use of yeast and other microorganisms in fermentative processes used in food & beverage production. Biotechnology Practice III (2 lectures), 3rd year course, University of Adelaide.

Outcome/Conclusions

The project has succeeded in achieving and typically exceeding the project outputs and performance targets that remained relevant through the life of the project. We also contributed to the remaining Output as part of the GWRDC-initiated review of research priorities in wine microbiology under the 'Yeast and bacterial germplasm' investment priority for 2013–14.

This study has reconfirmed the utility of directed evolution as well as the established techniques of mutagenesis and classical genetics in securing microbial strains of superior performance. What has been particularly helpful in accelerating outputs from these techniques, which often require large number of candidates to be evaluated, have been our sequential, high throughput screening approaches. From this work it is also clear that our micro- and laboratory-scale fermentation protocol can generally indicate performance on a larger scale in a range of juices.

This study also extended an earlier analysis (UA 05/01) of fermentation performance of disruptants from the laboratory yeast deletion library to include strain checking of shortlisted disruptants in 100 mL fermentations. Greater certainty of relevance of highlighted genes was thereby achieved.

Through the above, the project has produced: 1) a fuller definition of the Fermentome (as well as the FRYG databases), 2) significant data supporting the importance of correct function of the vacuole in yeast tolerance of fermentation conditions, 3) a collection of yeast strains with robust fermentation performance and potential for use as 'rescue' yeast, 4) yeast strains that improve on existing 'low-H_2S' strains, 5) an apparent world first application of directed evolution to improvement of wine lactic acid bacteria, 6) extensive characterisation of several candidate strains, in some cases at an industrial scale, 7) greater expertise in the Jiranek group relevant to microbial characterisation and optimisation, which can be applied to future projects to benefit the wine sector, 8) numerous disseminations of the project findings, and 9) several research students with expertise relevant to the field of wine microbiology.

The use of non-recombinant methods (directed evolution, classical genetics, etc) to generate many of the improved strains detailed herein, ensures that they are able to be adopted by industry as soon as they can be made commercially available – no special release approval is required for these strains. To that end and as a prelude to commercialisation, at least one strain is being protected by way of a patent application. Wider availability of this and other strains is being actively pursued with the hope that benefits in terms of more efficient production and/or high quality wine are realised by the sector as soon as possible.

Recommendations

The project has yielded a large number of tangible and intangible outputs of potential benefit for the sector. Foremost are optimised microbial strains as well as a greater understanding of the genetic basis for desired properties of these and other wine microbes.

The Jiranek group therefore proposes to extend these studies along with the process of bringing key developments to the industry as soon as possible. The new GWRDC project UA1302 (*Fit-for-purpose yeast and bacteria via directed evolution*) will be heavily informed by the findings of the current project and thereby will capture and help bring to realisation many of the potential benefits detailed herein.

Appendix 1: Communication

Communication and dissemination of findings from this research has been achieved through publications, conference, workshop and seminar presentations detailed in **Outputs 1E** and **2E**. Outlines of the work have also been provided to wine industry groups and international academics visiting the Waite Campus. In addition the work has been detailed as cutting–edge technology in wine microbiology through lectures to undergraduate and postgraduate oenology/viticulture students as well as BSc (Biotechnology) students at the University of Adelaide.

Appendix 2: Intellectual Property

The fermentation efficient phenotype of FM16-C7 under multiple conditions makes it an ideal candidate for use in industry. As such we have submitted a provisional patent application for this strain:

> McBryde, C., J. Gardner and V. Jiranek. (2013) *Saccharomyces cerevisiae* strain and methods of use. Provisional patent (Australia). pp 1-26. No: 207704.

As part of the above process, the strain (designated FM16-7) has also been deposited at The National Measurement Institute as part of the Budapest Treaty: Accession number V13/006528.

Strains derived by EMS mutagenesis, or sporulation and rediploidisation, with the intention of yielding strains with reduces H_2S and SO_2 liberation may also be of potential interest. Given some of these were derived from commercially available strains (eg Distinction®).

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Appendix 4: Staff

The following staff contributed to this project:

The University of Adelaide
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Undergraduate students:
Sijing Li (Summer School Scholarship – 6 weeks)
Shruti Mehrotra (International Internship – 6 weeks)
Ee Lin Tek (Summer School Scholarship – 6 weeks)

Masters students:
Andrea Lee (Masters of Oenology)
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Collaborators:
Yalumba Wine Company (Angaston, Barossa Valley)
Wolf Blass - Treasury Wine Estates (Nuriootpa, Barossa Valley)

Appendix 5: Supplementary Data

Sporulation medium (per litre)

Potassium acetate (1%)	10 g
Yeast extract (0.1%)	1 g
Glucose (0.05%)	0.5 g
Bacto-agar (2%)	20 g

Autoclave 20 min, 121°C

YPD medium (per litre)

Yeast extract (1%)	10 g
Bacto-peptone (2%)	20 g
Glucose (2%)	20 g
Bacto-agar (2%) – for plates	20 g

Autoclave 20 min, 121°C

10x Phosphate buffered saline (per litre)

Na_2HPO_4 (anhydrous)	10.9 g
NaH_2PO_4 (anhydrous)	3.2 g
NaCl	90 g

Adjust pH to 7.2.

Filter sterilize (0.22 μm)

Glycerol stocks of yeast cultures

Liquid yeast culture	1.0 mL
80% sterile glycerol	0.5 mL

In 2 mL screw cap eppendorf tube, mix and freeze -80°C

Juice Starter Media with amino acids (JSM_AA)

Nutrient supplements were made according to McBryde et al. [17] and Jiranek and Henschke [18]. Juice nitrogen content was determined and adjusted to 350 - 450 mg L^{-1} FAN using 25 x amino acid stock

white juice	10 L (same as juice used for fermentation)
100 x salts	100 mL
500 x trace minerals	40 mL
80 x vitamins	185 mL
500 x Tween 80/ergosterol	40 mL
25 x amino acids	variable mL

Make up to 20 L with RO water, filter sterilize (0.45 μm)

Juice Starter Media with DAP (JSM_DAP)
Nutrient supplements were made according to McBryde et al. [17] and Jiranek and Henschke [18]. Juice nitrogen content determined and adjusted to 350 - 450 mg L^{-1} FAN using DAP

white juice	10 L (same as juice used for fermentation)
100 x salts	100 mL
500 x trace minerals	40 mL
80 x vitamins	185 mL
500 x Tween 80/ergosterol	40 mL
DAP	variable g

Make up to 20 L with RO water, filter sterilize (0.45 μm)

Juice Parameters: Chardonnay (2010) sourced from Adelaide Hills

°Brix		19.4
pH		3.1
Titratable acidity	(g L^{-1})	4.2
Ammonia	(mg L^{-1})	35.0
FAA	(mg L^{-1})	123.0
Total YAN	(mg L^{-1})	152.0
Free SO_2	(mg L^{-1})	27.0
Total SO_2	(mg L^{-1})	77.0

Starter A culture (per 50 mL)

YPD culture	12.5 mL	25%
Stuck wine	12.5 mL	25%
RO water	25.0 mL	50%
BIOCELL (Laffort) - yeast cell walls	0.75 g	1.5%

Starter B culture (per 100 mL)

Starter A culture	10 mL	10%
Stuck wine	85 mL	85%
RO water	5 mL	5%
BIOCELL (Laffort) - yeast cell walls	1.5 g	1.5%

50x Tris-Acetate-EDTA (TAE) buffer (per litre)

		Final concentration
Trizma Base	242 g	40 mM
Glacial acetic acid	57.2 mL	20 mM
EDTA pH 8.0	100 mL	1 mM

1x TAE buffer (per litre)

		Final concentration
50x TAE buffer	20 mL	2% (v/v)
MQ Water	980 mL	98% (v/v)

1% Agarose gel in TAE buffer (per litre)

		Final Concentration
Agarose (Astral Scientific)	10 g	1% (w/v)
1x TAE buffer	1000 mL	~ 99% (v/v)
GelRed™	40 μL	~ 0.004% (v/v)

Enzymatic determination of glucose and fructose (buffers and solutions)

NADP/ATP reaction buffer (pH 7.6):
0.75 M triethanolamine
10 mM Magnesium sulfate heptaydrate
1.1 mM $NADP^+$ (nicotinamide adenine dinucleotide phosphate)
8 mM ATP (adenosine 5'-triphosphate)

To make up 50 mL:
Dissolve 7 g of triethanolamine and 0.125 g of $MgSO_4.7H_2O$ in 40 mL of MQ
Adjust to pH 7.6 (using 5M NaOH)
Add 50 mg $NADP^+$ and 250 mg ATP
Bring the volume up to 50 mL
Store at -20°C, will keep for several months. Once thawed, keep at 4°C up to ~4 weeks.

Hexokinase/Glucose-6-phosphate dehydrogenase (HK/G6P-DH)
Dilute 500 µL of HK (420 U/mL) + G6P-DH (210 U/mL) in 9.5 mL of MQ
Refrigerate at 4°C up to ~4 weeks.

Phosphoglucose Isomerase (PGI)
Dilute 20 µL of PGI (1000 U/mL) in 980 µL of MQ
Refrigerate at 4°C up to ~4 weeks.

Glucose and fructose Standards
The individual standards are made in sterile MQ and kept at -20°C as 1 mL aliquots. Once thawed, keep at 4°C up to ~4 weeks.
Glucose or fructose standards: 0.5, 1.25, 3.75, 5.0, 12.5, 37.5, 75.0, 125.0 g L^{-1}. The individual standards for glucose and fructose are mixed together in one solution, bringing the final sugar concentration of the standards to 1.0, 2.5, 7.5, 10.0, 25.0, 75.0, 150.0, 250.0 g L^{-1}.

NOPA determination of primary amino acids (Buffers and solutions)
OPA (o-phthaldialdehdye) solution (per litre)
95 % (v/v) ethanol
6.71 g OPA
Stable for 2 years at 4°C (in dark)
10 mL is sufficient for 500 samples.

NAC (N-acetyl-L-cysteine) solution (per litre)
1.83 g NAC in 1 L MQ water
Stable for 2 years at 4°C (in dark)
150 mL is sufficient for 500 samples.

Sodium hydroxide /o- boric acid solution (per litre)
25.58 g NaOH
56.45 g ortho-boric acid
Stable for 2 years at 4°C (in dark)
150 mL is sufficient for 500 samples.

Supplementary Table 1: Gene specific primers for PCR amplification and DNA sequencing of 44 FEG genes

Primer Name	Primer Sequence (5'-3')	Primer Name	Primer Sequence (5'-3')
Kan B	CTGCAGCGAGGAGCCGTAAT	Kan C	TGATTTTGATGACGAGCGTAA T
VMA13-AF	CGCTGTGTTGTATATTGCTCTAAAA	VMA13-DR	GAGGAGGCGTCATGAAAAAG
CCR4-1-AF	TCAAAGGTAAAGTAGCCGTTTAGGCCT	CCR4-1-DR	AAAGAAACAGGACGAAAAAGATACGC
CCR4-2-AF	TCGTGCTCTAGCACTGATAAGTAAC	CCR4-2-DR	GGTTTGGTTAAATAATTTTGGCTCT
VMA16-AF	CGTTAACGGTTATTATGCTGAGTCT	VMA16-DR	TTAAAGAAGAACCCTGACTATCGTG
VAM3-AF	GACACAGGAAGAGCAAAACTAAGAT	VAM3-DR	AAGTAGAAAAAGGAAAAAGGGCATA
PAF1-AF	ATATCGAAGTTGACCATACGAAAAG	PAF1-DR	TATTTGTGTCAGTGTAACTGCTGGT
YDR433W-AF	TGGAAGTTGTTTACTCTAAATTGCC	YDR433W-DR	AAGGATGTTAAATGTTATCATGGGA
DBF2-AF	TCATGGTTAGGGCTCTTCTACATAC	DBF2-DR	CCCAACATGAATGCTTAAGTTTAGT
BUD27-AF	AGTAACCTCTCACAGGTATTGCTTG	BUD27-DR	AAATCGGTAACTCTGGTATGTTCAG
VMA2-AF	TATTGGACTTTTGGGATGTTGTACT	VMA2-DR	CAAGTGTATTGCAATGTTCTTTGTC
TFP1-AF	CGATTAGACTCCACTTTGCTTCTAT	TFP1-DR	GGGTGTAAACCATTGCCTACTATTT
PEX1-AF	ATGAGTCCATAATACTGTACTGGCG	PEX1-DR	TATATATGCAACTTCGGATTTCGTT
VMA3-AF	GAGGAACTGAATTCTTTATCAGCAA	VMA3-DR	ATAGTGATCTCATCGTACCCATTGT
DIA2-AF	CTGCGTTCGTAGTAAAATCAGAAAT	DIA2-DR	TGGAACAATTTCCTCTTATGATGTT
IES6-AF	TGCATAGTTTATTAGTCTGTGCCTG	IES6-DR	GTGGCTATTTTGTCGATAGACACTT
DCC1-AF	GGCAGCGTATAGTATAACCAGAAAA	DCC1-DR	TTGATTTCAACATCTTCCAGTTGTA
VMA5-AF	AAAACAATTTTCCGTAAAGAGAAGC	VMA5-DR	TAGTGCTAAATCCTGTAGATTTCCG
VMA10-AF	TTGTATTTGAACTCTTTTATTCCGC	VMA10-DR	CGGCGTTTAGTATTTTTCGTTAATA
XRS2-AF	GTATTGAAGCAATTTGTAAGCTGGT	XRS2-DR	TCCAATTTTAAGATTTTCACTCTGC
CYK3-AF	AACAAGAGAAAAGGCCAAAATTAAC	CYK3-DR	CGTTCTGTTTGATAAGTAGGGGATA
CNM67-AF	TGCCAAAATTAAAAAGAATAGATCG	CNM67-DR	AAAAGCTCATAGTAGCAGGTCTTCA
VMA7-AF	TCACCCAGATACCTATTTCTTACCA	VMA7-DR	TCTTTATTGAAGCATTAGCCAAATC
YCL007C-AF	TGTTTTGTGCAGTGTATTACCAACT	YCL007C	ACAGGAATACTGTGAGGAGATGAAC
VPH2-AF	GGGGAACTTTTAGGATATTCTGTGT	VPH2-DR	TAGGCCATAATTAATTCCCATAACA

Supplementary Table 1: (cont.)

Primer Name	Primer Sequence (5'-3')	Primer Name	Primer Sequence (5'-3')
ERG6-AF	CTGTTGCCGATAACTTCTTCATTGC	ERG6-DR	GGCCTGCTAGCAATGAACGTGCTA
SPC72-AF	TGCTATCAACATAACAAGCAAGAAG	SPC72-DR	GATGAGAAATCTCTTGGGATACTGA
VPH1-AF	CTCAGATAAGTCGATATGGAAAACC	VPH1-DR	AGGATTTGTCAACTGTTTAGACCTG
HOG1-AF	GAGATTATTCGGCATTTTGACATAC	HOG1-DR	CTCTTCGGTAAAAGCACAGAATTAG
VMA4-AF	GCATTCAGATCTAACCATCAATTTC	VMA4-DR	TGGATGTGTCAGCATACTTTCTAAG
VMA6-AF	GTACCCTTCTTATCTTACAGACGCA	VMA6-DR	ACGCTTGTAACAAACTAAGGATGTC
VMA11-AF	TGCTTTTATATGTTTTCCCTCTACG	VMA11-DR	AATATCCATTGCTAAATCAGCAGAC
VMA22-AF	TACTGCTAAATGGAAAGCTGAAAAC	VMA22-DR	GTGGATGACGTGCTAAAATTCTACT
OPI1-AF	AAGTTGAGTACTTGAAGGTCGAAGA	OPI1-DR	GAAGAGTTACCCCTGATATTTGGAT
VPS3-AF	ATTTTTGAAGGATTTGAGAGGAGTT	VPS3-DR	CCAGAAGATTTTCATAGGCAAGTAA
STV1-AF	CCTCCCCTAAATATCATTCTCAGAT	STV1-DR	TTCCCCTGTTAATTTACTACGACAG
RPA12-AF	CATTGAAATGCTTAAAATGTGTGAA	RPA12-DR	GAGTTTGTAGCTCTTTATCACGCAC
SIN3-AF	AGTACGGGTAATGATATCGGATTTT	SIN3-DR	GTGAATCACTAGACGATTTTGGAGT
ZAP1-AF	CTGCGATTTACTCTAGGGATCTTC	ZAP1-DR	CTTCGGTTACCTAGTTGTCACTCAT
TPS1-AF	AGAACAAGGAACAAAGTCCAAGC	TPS1-DR	GGGAGAGAAAGAAAGAGAGAGAAAA
TPS2-AF	ACAATCTCGATTCTCATTTTCTTTG	TPS2-DR	GTAGTACCCTCTTTTACCTACCGCT
SSQ1-AF	AACCATTATTTATTTGCTTTCCACA	SSQ1-DR	CTCAAGAGGGATTGAAAGATGTTAG
NPT1-AF	TCGGAGTGCTGATAGTAGCTTATTT	NPT1-DR	GTGAATGAATTAAGAAAGCAAAGGA
PTK2-AF	TCCTATAGCCATTCAAGCCTACATA	PTK2-DR	TAATTGTTGTTTGCCCAATACTTTT
PLC1-AF	TTTTTATATATCCTCCCTCTCACCC	PLC1-DR	AAAGCACAAAACTTCAGAAAGAAGA

Forward primers are denoted with the letter A and reverse primers with the letter D. Gene specific A and D primer sets were used for PCR amplification.

Supplementary Table 2: Primers used to amplify and sequence gene deletion (*⊿orf::KanMX*) cassettes in 9 yeast strains exhibiting haploproficiency (HP) during fermentation

Gene Name	PrimerName	Primer Sequence (5'-3')	Primer Name	Primer Sequence (5'-3')
WHI2	YOR043W_A	GCATAGGCATAGTGATAGAGTGTGA	ΔYOR043W_D	CTATTGTTTTATACCGGATCAATGC
PIP2	YOR363C_A	AAAAGCCGCAGATCAATAAGATT	YOR363C_D	TATTGTAAATGGAATAAACTGGCGT
PUN1	YLR414C_A	TATTTCCAAATCGGGCGTACTAT	ΔYLR414C_D	ATTATTTCTGGCTCTTCTCCATTTT
YLR297W	YLR297W_A	AAGAGTAAAGAGATGAGTTTTGCGA	YLR297W_D	TAGATATACTGCAAACCACGATCAA
FRE7	YOL152W_A	AAAAGTGTCAAATTATAGTGCTCGC	YOL152W_D	TTCTCACTGAGGTTTTCATCCTTAC
RMD6	YEL072W_A	GCTTGTACATTCATTCTGGCTAGTT	YEL072W_D	TGCTATAAGTAGTGCAATTTGAATGTT
HSP150	YJL159W_A	GTCTGTAGTGATAAGTCGCCAACTC	YJL159W_D	ACACATTAGGACTGGCTTATTTTTG
IRC9	YJL142C_A	TCTACACAAAAGCATCGTATGAAAA	YJL142C_D	GCAAGCTTAGGCATGTAGACATTAT
RPL2B	YIL018W_A	GAGAAAAAGAAGGAATTCTCAAAGG	YIL018W_D	GGAGGTAGGATTATACGATCTGGTT
	Kan C	TGATTTTGATGACGAGCGTAAT		

Forward primers are denoted with the letter A and reverse primers with the letter D.

Supplementary Table 3: Commercial wine yeast used in generation of new wine yeast by intra-specific hybridisation (sporulation and mating)

Strain name	Yeast Manufacturer	Strain name	Yeast Manufacturer
Maurivin B	Mauri Yeast Australia	AWRI 350	Mauri Yeast Australia
Uvaferm VRB®	Lallemand	Oenoferm® Bouquet	Erbsloeh
Uvaferm SVG™	Lallemand	Montrachet	Red Star
Blastosel Grand Cru	Perdomini IOC	VIN13	Anchor Wine Yeast
Oenoferm® Tipico	Erbsloeh	IOC B2000	IOC
LW 679	?	Premier Cuvée	Red Star
Fermivin 7013	DSM	10VB	?
Fermicru VR5	DSM	46 EDV	Lallemand
O16	Lallemand	Safale S-04	Fermentis
Enoferm AMH™	Lallemand	Lalvin C	Lallemand
Levuline Als	Oeno France	Lalvin BM45	Lallemand
Karoovin NT7	DSM	Ba11™	Lallemand
Lalvin HK8	Lallemand	(Siha)-4 activ	SIHA (Begerow)
Lalvin EC1118™	Lallemand	Lalvin D254 ICV®	Lallemand
IOC BR9002	IOC	Lalvin 71B	Lallemand
Uvaferm BDX™	Lallemand	Sauvignon	?
Lalvin S6U	Lallemand	(Siha)-3 activ	SIHA (Begerow)
Enoferm SYRAH™	Lallemand	Fermichamp®	Oenobrands
Lalvin ICV D47™	Lallemand	IOC 18-2007	IOC
Rhone 2056	Lallemand	Côtes des Blanc	Red Star
Primeur	Mauri Yeast Australia	Pasteur RED	Red Star
Zymaflore F15	Laffort	Flor sherry	Red Star
Vitileuvre LX4	Lallemand	Lalvin RHST®	Lallemand
Oenoferm® InterDry	Erbsloeh	UVAFERM PM	SIHA (Begerow)
SYR D80	Lallemand	Levure sèche active	LeSaffre
SYR	Lallemand	PDM	Mauri Yeast Australia
Fermirouge® 7303	DSM	Zymaflore VL3®	Laffort
Lalvin QA23™	Lallemand	Lalvin DV10™	Lallemand
IOC BR8000	IOC	AWRI R2	Mauri Yeast Australia
Lalvin M	Lallemand	Maurivin™ Cru-Blanc	Mauri Yeast Australia
Lalvin Rhone L2056®	Lallemand	Lalvin T73™	Lallemand
Enoferm R2	Lallemand	Actiflore B0213	Laffort
Enoferm Burgundy (BGY)™	Lallemand	Zymaflore RX60®	Laffort
Uvaferm CEG®	Lallemand	Lalvin Rhone	Lallemand
Lalvin Bourgoblanc (CY3079)®	Lallemand	BP725	Mauri Yeast Australia
Maurivin A	Mauri Yeast Australia	Maurivin AWRI Fusion	Mauri Yeast Australia
Lalvin M69™	Lallemand	AWRI 1503	Mauri Yeast Australia
AWRI 796	Mauri Yeast Australia	UCD 713	UC Davis
Enoferm AVISE	Lallemand	Anchor NT202	Oenobrands
Blastosel CULTIVAR	Perdomini IOC	UCD 522	UC Davis
Lalvin V1116 (K1)™	Lallemand	Uvaferm 43®	Lallemand
Fermiblanc® Arom	DSM	ICV D254	Lallemand
Enoferm T306	Lallemand	W-27	Lallemand

Supplementary Table 4: Gene specific primers for PCR amplification and DNA sequencing of four sulfur reduction pathway genes

Primer Name	Primer Sequence (5'-3')	Primer Name	Primer Sequence (5'-3')
MET5 A	TTCATCACGTGCGTATTATCTCTTA	MET5 D	TTTATTCTTCACCTCGTTTTCATTC
MET5 A1	CAATTACCACCACACAGAGG	MET5 D1	GACAGGTTAACAATGACAGTGG
MET5 A2	GGAGATTGCTAACGAAGAGAC	MET5 D2	GGCCTCTTGTAATTGAGCTTC
MET5 A3	GATCAGCTTAGCAAAAACCTCTC	MET5 D3	CTTGCTGCTAGTCTCTTTGC
MET5 A4	CCCTATTAACCAGAAAAGATCC	MET5 D4	GGTACCATTGCCTGAAGTATC
MET5 A5	CAAGACGATCGTGACATTAGAG	MET5 D5	GTGGTTTAACCCAGTTTCATC
MET5 A6	CTGTAGATGATATGGGTGTCG	MET5 D6	CCGAACTAGCCGAAGGATAAC
MET10 A	AAAGAAAACACTATCAACATTCCCA	MET10 D	ATCTCAGTTCATTGTAGTCTCGCTT
MET10 A1	GGAAACCCATAGTCATCTTCG	MET10 D1	GGATTCTAAAGACCCATAAGTG
MET10 A2	CCAAAGTTATTGCACCAGATG	MET10 D2	CATTCTGCGACATCTAGACC
MET10 A3	GAAGCATTTAGACGTAGCTGC	MET10 D3	CTTCGTTAGAGGCATATGGAAT
MET10 A4	GAATCCGATGTTGTCTTAGTCC	MET10 D4	GCAAAGCTTGAGTAATATCTGG
MET10 A5	CTTTCTCAAGAGACCAACCTC	MET10 D5	CGCAGGGTTTAGAGGTTAAAG
SUL1 A	TCGAACACTGTCATTTGAAATTATG	SUL1 D	GGACATTTGTAGAAAATAGGCTCAA
SUL1 A1	CATTAACGTCTCAAAACCAGGTC	SUL1 D1	GTAGTGACATTACAGCGACCG
SUL1 A2	GCCCCAATCTATGTCATACGC	SUL1 D2	CCAGAAAACGGAGTGCGCACG
SUL1 A3	CCTGACCAAGAACTTATTGCG	SUL1 D3	GTCATAATGCCTTGAGCAGTTC
SUL1 A4	CGTTCGATCATGGATACTCAAG	SUL1 D4	CTTTCGCCATTCAACATACTC
SUL2 A	AGTTGATTAGTTGTTGTTGGCTGTT	SUL2 D	GCTTTCATGTTTTTCTATCTGTGGT
SUL2 A1	CTCGTACTAAAGCCTGGAAG	SUL2 D1	GAGGAGTACAACCCATACTGC
SUL2 A2	GGTTTACTGCGGATCTTATTGC	SUL2 D2	CCTAAAAGGTTCGAGACACCA
SUL2 A3	GTCCAAATTTGCCTGCATCC	SUL2 D3	GATCATTCCATGGACGGTCAG
SUL2 A4	GGCTATCAGAGAGCTACACCT	SUL2 D4	CGGTTGTATTTCTTTTGTTCCTGAG

Forward primers are denoted with letter A and reverse primers with letter D. Gene specific A and D primer sets were used for PCR amplification. Nested forward and reverse primers with a numerical designation eg SUL1 A1 and D1 were used to sequence the entire gene in both directions, to generate overlapping contigs spanning the entire gene.

Supplementary Table 5: Fermentation performance of commercial wine yeast in problematic Chardonnay juice (2010 Adelaide Hills, clarified)

Strain	Mean sugar g L^{-1}	± SD	N	Strain	Mean sugar g L^{-1}	± SD	N
(Siha)-4 activ	-1.06	0.07	4	IOC 18-2007	0.83	0.32	4
D254 ICV	-0.81	0.14	4	NT202	0.84	0.70	4
Premier Cuvée	-0.78	0.12	4	Uvaferm PM	0.85	0.21	4
DV10	-0.71	0.32	4	AWRI 1503	0.94	1.24	4
Actiflore B0213	-0.68	0.19	4	V1116	0.34	0.28	4
UCD 522	-0.63	0.28	4	Lalvin C	0.38	0.52	4
(Siha)-3 activ	-0.59	0.17	4	Lalvin M	0.46	0.36	4
S6U	-0.27	0.29	4	CY3079	0.49	0.62	4
RHST	-0.24	0.17	4	PDM	0.52	0.52	4
Zymaflore VL3	-0.19	0.60	4	IOC B2000	0.56	0.41	4
Fermiblanc AROM	-0.14	0.88	4	Uvaferm 43	0.58	0.36	4
Montracht	-0.13	0.09	4	SVG	0.62	0.54	4
AWRI R2	-0.05	0.33	4	Vitileuvre LX4	0.71	0.55	4
L2056	0.03	0.06	4	IOC 18-2007	0.83	0.32	4
BM45	0.05	0.19	4	NT202	0.84	0.70	4
L2056	0.09	0.29	4	Uvaferm PM	0.85	0.21	4
Zymaflore RX60	0.12	0.42	4	AWRI 1503	0.94	1.24	4
Oenoferm Bouquet	0.26	0.34	4	F15	1.06	0.23	4
SYRAH	0.29	0.63	4	Bal1	1.12	0.57	4
VRB	0.30	0.10	4	QA23	1.30	0.56	4
V1116	0.34	0.28	4	ICV D47	1.32	1.09	4
Lalvin C	0.38	0.52	4	PDM	1.41	1.17	4
Lalvin M	0.46	0.36	4	Enoferm Burgundy	1.87	0.91	4
CY3079	0.49	0.62	4	Cru-Blanc	2.05	1.68	4
PDM	0.52	0.52	4	VR5	2.15	1.26	4
IOC B2000	0.56	0.41	4	VIN13	2.85	0.66	4
Oenoferm Bouquet	0.26	0.34	4	Levaline Als	3.69	2.26	4
SYRAH	0.29	0.63	4	Fermivin 7013	3.71	2.39	4
VRB	0.30	0.10	4	T73	4.16	3.29	4
V1116	0.34	0.28	4	EC1118	4.40	1.84	4
Lalvin C	0.38	0.52	4	T306	5.10	0.94	4
Lalvin M	0.46	0.36	4	Sauvignon	5.37	0.77	4
CY3079	0.49	0.62	4	Levure sèche active	6.18	3.65	4
PDM	0.52	0.52	4	M69	6.62	1.12	4
IOC B2000	0.56	0.41	4	Flor sherry	7.94	0.41	4
Oenoferm Bouquet	0.26	0.34	4	(Siha) activ hefe 3	8.14	0.69	4
SYRAH	0.29	0.63	4	IOC BR8000	8.39	0.94	4
VRB	0.30	0.10	4	BGY	8.84	1.10	4
V1116	0.34	0.28	4	Blastosel CULTIVAR	9.76	0.90	4
Lalvin C	0.38	0.52	4	Maurivin A	12.93	3.84	4
Lalvin M	0.46	0.36	4	IOC BR9002	13.25	3.34	4
CY3079	0.49	0.62	4	O16	15.67	5.57	4
PDM	0.52	0.52	4	Blastosel Grand Cru	16.65	2.43	4
IOC B2000	0.56	0.41	4	AWRI 350	19.04	1.69	4
Uvaferm 43	0.58	0.36	4	Oenoferm InterDry	24.58	2.05	4
SVG	0.62	0.54	4	Cotes des blanc	29.29	2.71	4
Vitileuvre LX4	0.71	0.55	4	Karoovin NT7	31.67	4.22	4

Fermentations were conducted in micro-scale in 96 well plates in problematic Chardonnay juice (2010 Adelaide Hills, clarified) using the sacrificial plate method. Residual sugar was measured enzymatically and values are the mean of 4 replicates (N) ± standard deviation of the mean at 105.5 h.

Supplementary Table 6: Commercial wine yeast which displayed poor sugar catabolism and growth in problematic Chardonnay juice when evaluated in micro-scale fermentations

Wine yeast strains
Mauri B, Oenoferm TIPICO, LW 679, Enoferm AMH, HK8, Primeur, Fermirouge 7303, AWRI 796, Enoferm AVISE, Bordeaux Red, 71B, Fermichamp, Pasteur RED, BP725, AWRI Fusion, SYRAH D80, W-27

Supplementary Table 7: Mass mating and adaptive evolution to generate new yeast. Micro-scale fermentation evaluation of selected yeast isolates

Residual sugar (g L^{-1}) was measured at 98 h (data not shown) and 112 h. The average of four replicates (per strain) ± standard deviation (SD) are reported. The corrected average values (corr ave) were calculated based on average data from 10 standard curves (10 plates) per sugar analysis experiment. Residual sugar (<5 g L^{-1}) is highlighted in light green (112 h). Strains highlighted in yellow were chosen for 100 mL fermentations. AE mixed population; Mix.

AE 05						
Strain	ave	±	SD	corr ave	±	SD corr
EC1118	21.48	±	7.60	20.44	±	8.11
Mix	12.65	±	3.66	12.22	±	4.75
5	3.00	±	1.34	3.43	±	1.00
18	4.25	±	6.24	5.16	±	5.28
65	4.31	±	1.68	4.69	±	1.64
135	6.63	±	1.35	8.31	±	1.72

AE 10						
Strain	ave	±	SD	corr ave	±	SD corr
EC1118	16.60	±	5.34	16.71	±	7.01
Mix	10.46	±	3.15	11.94	±	3.86
83	3.41	±	1.40	4.17	±	1.44
100	-0.76	±	0.85	0.16	±	0.90
116	4.05	±	2.90	5.65	±	2.95
137	2.37	±	2.50	2.80	±	2.24
152	4.67	±	1.64	6.49	±	1.44
168	0.26	±	2.01	-0.03	±	1.36

Supplementary Table 7 (cont'd)

AE 20

Strain	ave	±	SD	corr ave	±	SD corr
EC1118	10.21	±	3.11	10.28	±	3.41
Mix	2.71	±	3.55	2.99	±	3.21
1	2.82	±	1.72	2.19	±	1.58
4	5.44	±	3.17	4.71	±	2.63
5	4.44	±	3.00	3.80	±	2.56
8	3.30	±	3.14	2.87	±	2.59
9	0.27	±	0.53	-0.27	±	1.47
11	0.00	±	0.00	0.00	±	0.00
12	4.85	±	3.88	5.25	±	2.16
13	5.08	±	4.42	4.06	±	4.19
14	0.59	±	1.18	0.24	±	2.36
15	2.12	±	2.89	2.02	±	3.17
18	4.03	±	4.95	6.83	±	2.43
20	0.92	±	0.24	0.85	±	1.23
25	4.41	±	1.36	5.41	±	1.56
26	2.94	±	1.08	1.84	±	2.25
30	2.00	±	0.56	1.95	±	1.28
33	2.22	±	2.72	2.23	±	3.26
34	2.45	±	1.30	3.12	±	1.44
37	4.55	±	1.26	5.18	±	1.34
38	1.32	±	1.03	0.83	±	1.75
43	2.00	±	2.40	2.22	±	2.86
44	1.94	±	1.59	3.15	±	1.54
48	0.88	±	1.32	1.27	±	1.15
51	2.48	±	2.04	2.37	±	2.43

AE 31

Strain	ave	±	SD	corr ave	±	SD corr
EC1118	11.62	±	5.04	10.58	±	4.05
Mix	4.23	±	3.11	4.27	±	2.66
1	2.00	±	2.54	2.25	±	1.75
8	-0.62	±	1.98	0.71	±	1.65
9	3.50	±	2.83	3.30	±	1.87
10	-2.77	±	0.46	-1.01	±	0.29
11	-0.88	±	0.94	0.39	±	0.46
12	5.41	±	5.33	4.84	±	4.10
13	4.08	±	3.34	3.87	±	2.36
16	-0.95	±	1.11	0.46	±	0.75
17	3.97	±	5.48	3.69	±	3.85
18	-1.73	±	0.92	-0.27	±	0.57
23	3.31	±	5.08	4.02	±	4.99
24	-0.04	±	1.10	0.74	±	1.08
32	0.60	±	1.34	1.36	±	1.31
34	1.17	±	3.39	1.91	±	3.31
37	3.40	±	2.42	3.88	±	2.29
42	0.42	±	2.96	1.03	±	2.79
45	2.84	±	1.54	3.30	±	1.45
48	0.97	±	2.41	1.52	±	2.27
58	4.47	±	0.88	3.71	±	0.76
71	1.48	±	2.39	1.26	±	2.02
75	3.57	±	5.55	2.98	±	3.97
83	0.97	±	1.79	1.01	±	1.24
88	4.00	±	3.49	3.22	±	2.57

Supplementary Table 7 (cont'd)

AE 20						
Strain	ave	±	SD	corr ave	±	SD corr
52	2.32	±	1.55	2.85	±	1.74
55	3.58	±	2.53	3.45	±	2.44
57	4.67	±	2.17	4.51	±	1.84
58	1.31	±	0.89	1.20	±	1.77
59	3.04	±	5.67	2.16	±	4.40
60	5.14	±	4.53	4.93	±	3.83
61	0.80	±	1.05	0.79	±	1.24
63	0.97	±	1.98	0.84	±	1.58
64	2.14	±	0.00	1.55	±	1.60
65	5.02	±	3.08	4.82	±	2.56
66	3.17	±	3.51	3.27	±	2.98
67	0.37	±	2.40	0.55	±	1.81
68	-1.00	±	1.18	-0.24	±	0.93
70	-0.73	±	0.59	0.04	±	0.51
71	-2.79	±	0.59	-1.51	±	0.21
72	-0.90	±	2.01	-0.15	±	1.70
73	1.35	±	1.46	1.44	±	1.59
76	1.73	±	1.35	1.96	±	1.45
78	-0.61	±	1.48	-0.25	±	1.65
81	1.12	±	1.47	1.22	±	1.61
85	5.84	±	0.79	6.31	±	0.90
86	2.50	±	2.13	2.85	±	2.19
89	0.41	±	1.76	0.42	±	1.95

AE 31						
Strain	ave	±	SD	corr ave	±	SD corr
97	1.71	±	1.08	2.86	±	0.99
102	1.84	±	3.53	2.94	±	3.49
106	3.72	±	3.81	4.85	±	3.78
107	-0.35	±	0.83	0.82	±	0.82
108	1.52	±	2.24	2.66	±	2.18
109	-0.28	±	1.90	0.24	±	1.34
117	4.78	±	3.04	3.95	±	2.18
119	4.81	±	5.25	3.88	±	3.69
121	2.90	±	0.66	2.58	±	0.41
127	3.01	±	2.70	2.71	±	2.31
130	2.31	±	0.79	2.12	±	0.72
135	4.02	±	2.20	3.58	±	1.92
136	3.87	±	1.37	3.46	±	1.20
141	3.00	±	2.49	2.69	±	2.12
163	0.92	±	3.04	2.70	±	2.88
166	4.81	±	3.52	6.30	±	3.29
167	1.39	±	2.09	3.20	±	2.00
168	0.29	±	2.29	2.10	±	2.12
170	1.59	±	1.80	3.36	±	1.65
171	-0.13	±	1.73	1.70	±	1.61
173	0.48	±	2.25	2.29	±	2.07
174	2.63	±	3.29	4.32	±	3.09
177	3.61	±	3.55	5.25	±	3.45

Supplementary Table 7 (cont'd)

AE 20						
Strain	ave	±	SD	corr ave	±	SD corr
91	1.54	±	2.82	2.19	±	2.32
109	4.81	±	3.06	4.84	±	2.91
118	4.38	±	3.55	4.43	±	3.36
128	2.63	±	0.81	2.43	±	0.74
129	-0.66	±	1.23	-0.49	±	0.90
130	5.88	±	2.01	5.23	±	1.72
131	0.03	±	1.22	0.12	±	1.08
132	4.76	±	0.86	4.31	±	0.82
136	-1.63	±	1.13	-1.00	±	0.80
138	-0.09	±	0.79	0.01	±	0.61
145	-0.25	±	2.71	1.29	±	2.22
147	2.00	±	1.45	2.44	±	1.42
150	3.10	±	2.30	3.75	±	2.20
152	3.54	±	2.88	4.28	±	3.08
153	4.51	±	3.83	5.20	±	3.83
154	4.36	±	2.01	4.81	±	1.94
157	3.82	±	1.63	4.53	±	1.56
159	3.80	±	1.65	4.26	±	1.58
169	4.34	±	3.19	3.78	±	3.85
173	3.99	±	3.22	3.37	±	3.83
178	-0.05	±	1.04	-0.47	±	0.96

Supplementary Table 8: Micro-scale fermentation evaluation of new clonal isolates from FM16 mixed population (350 generations):

Residual sugar (g L^{-1}) was measured and the average of four replicates (per strain) ± standard deviation (SD) are reported. The values reported refer to sugar determinations at 118 h (Chardonnay juice; end of ferment = 129 h) and 336 h (end of ferment: CDGJM 260 g L^{-1} and 230 g L^{-1} sugar). Residual sugar <2.5 g L^{-1} (CDGJM) and <3.5 g L^{-1} (Chardonnay juice) is highlighted in light green. Strains highlighted in yellow were chosen for 100 mL and 10 L fermentations.

CDGJM 260 g L^{-1} sugar			
Colony	Average	±	SD
5	1.67	±	1.57
8	2.14	±	0.17
10	0.54	±	0.67
11	1.50	±	1.02
16	1.46	±	1.30
17	18.30	±	2.36
18	2.08	±	1.97
19	9.89	±	14.43
24	6.13	±	3.31
26	1.36	±	0.60
28	1.79	±	0.88
29	11.09	±	1.53
30	2.08	±	1.15
32	0.93	±	0.54
33	2.54	±	1.29
34	28.26	±	2.01
35	10.36	±	3.71
41	6.86	±	2.21
43	3.68	±	1.34
47	10.11	±	0.52
48	3.12	±	0.77
49	13.78	±	2.17

CDGJM 230 g L^{-1} sugar			
Colony	Average	±	SD
5	1.19	±	0.83
8	0.31	±	0.56
10	-0.50	±	0.06
11	-0.06	±	0.25
16	0.83	±	0.52
17	0.83	±	0.52
18	0.72	±	0.75
19	9.84	±	14.55
24	4.75	±	0.79
26	-0.26	±	0.17
28	0.61	±	0.64
29	10.77	±	2.49
30	1.31	±	0.55
32	18.56	±	0.83
33	1.49	±	0.85
34	1.49	±	0.85
35	2.23	±	3.11
41	1.10	±	0.75
43	0.59	±	0.67
47	2.28	±	1.36
48	-1.18	±	0.18
49	-1.18	±	0.18

Chardonnay Juice			
Colony	Average	±	SD
5	3.65	±	1.21
8	5.34	±	0.77
10	3.77	±	0.81
11	5.27	±	0.86
16	5.72	±	2.31
17	9.50	±	0.90
18	2.58	±	2.22
19	4.68	±	0.29
24	4.02	±	2.02
26	2.51	±	1.60
28	2.65	±	0.55
29	5.32	±	0.56
30	3.42	±	0.73
32	2.37	±	0.90
33	2.99	±	0.95
34	16.16	±	2.72
35	7.09	±	1.56
41	8.06	±	0.93
43	5.69	±	1.72
47	7.93	±	1.23
48	6.27	±	1.75
49	9.03	±	1.60

CDGJM 260 g L^{-1} sugar			
Colony	Average	±	SD
50	2.88	±	1.26
51	4.64	±	2.15
53	9.17	±	4.46
55	2.99	±	2.41
56	9.90	±	2.69
58	23.31	±	1.59
63	17.80	±	1.67
64	1.74	±	1.11
66	5.67	±	2.84
67	26.20	±	4.73
68	1.20	±	1.48
74	0.91	±	1.44
77	7.70	±	1.96
79	1.83	±	1.95
80	3.59	±	3.07
81	14.50	±	0.78
82	3.45	±	1.35
83	19.84	±	3.65
86	4.49	±	1.07
91	3.86	±	1.45
93	5.08	±	1.07
95	4.13	±	1.73
97	3.93	±	1.32
98	1.77	±	1.54
99	1.70	±	1.02
100	7.40	±	1.33

CDGJM 230 g L^{-1} sugar			
Colony	Average	±	SD
50	-1.09	±	0.22
51	-1.09	±	0.22
53	1.40	±	2.30
55	-0.78	±	0.28
56	3.34	±	3.82
58	9.42	±	3.45
63	13.45	±	2.61
64	-0.87	±	0.29
66	22.00	±	3.66
67	10.91	±	2.49
68	10.91	±	2.49
74	-1.22	±	0.16
77	0.33	±	0.33
79	-0.66	±	0.33
80	-1.13	±	0.24
81	5.78	±	1.17
82	-1.10	±	0.07
83	-1.10	±	0.07
86	-0.16	±	0.33
91	-0.02	±	0.85
93	0.91	±	1.25
95	-0.53	±	0.32
97	1.55	±	0.71
98	-0.51	±	0.43
99	-0.97	±	0.45
100	-0.97	±	0.45

Chardonnay Juice			
Colony	Average	±	SD
50	6.28	±	1.69
51	7.04	±	1.74
53	6.39	±	1.06
55	3.56	±	0.65
56	5.85	±	2.01
58	9.00	±	0.73
63	10.26	±	2.19
64	3.37	±	1.00
66	4.38	±	0.35
67	9.37	±	1.77
68	2.99	±	1.25
74	2.46	±	2.10
77	5.14	±	3.36
79	2.18	±	1.41
80	1.86	±	1.71
81	4.01	±	1.44
82	4.30	±	1.27
83	9.46	±	0.81
86	2.94	±	1.55
91	17.62	±	25.20
93	4.83	±	0.92
95	3.57	±	1.34
97	4.31	±	1.25
98	3.82	±	0.75
99	3.73	±	0.75
100	55.32	±	0.46

CDGJM 260 g L^{-1} sugar			
Colony	Average	±	SD
101	2.90	±	1.61
102	3.78	±	1.23
104	8.81	±	7.09
110	3.88	±	1.87
115	6.25	±	2.42
120	4.56	±	2.51
121	6.85	±	1.90
122	6.76	±	4.18
123	6.90	±	1.17
125	2.88	±	2.20
126	6.85	±	4.97
128	11.36	±	2.91
132	1.54	±	0.72
133	1.82	±	1.03
134	30.29	±	5.54
138	3.04	±	1.36
139	4.68	±	2.59
141	2.71	±	2.06
144	2.34	±	1.61
149	3.89	±	1.13
150	1.08	±	0.64
151	46.63	±	6.14
152	3.28	±	1.70
153	7.79	±	6.49
154	11.50	±	5.55
155	10.57	±	3.45

CDGJM 230 g L^{-1} sugar			
Colony	Average	±	SD
101	-0.06	±	0.59
102	-0.06	±	0.59
104	1.46	±	2.24
110	1.04	±	0.76
115	1.02	±	0.48
120	-0.61	±	0.28
121	-0.32	±	1.28
122	0.34	±	0.51
123	0.46	±	1.27
125	-0.97	±	0.33
126	-0.11	±	1.47
128	1.74	±	0.72
132	-0.95	±	0.13
133	-1.05	±	0.21
134	-1.05	±	0.21
138	-0.33	±	0.22
139	0.92	±	0.82
141	-0.61	±	0.37
144	-0.55	±	0.26
149	0.14	±	0.44
150	-0.71	±	0.07
151	-0.71	±	0.07
152	-0.34	±	0.25
153	-0.34	±	0.25
154	0.13	±	0.77
155	1.25	±	0.69

Chardonnay Juice			
Colony	Average	±	SD
101	3.85	±	0.32
102	3.77	±	0.67
104	2.95	±	0.24
110	3.70	±	0.84
115	4.67	±	1.50
120	3.88	±	0.60
121	4.40	±	0.67
122	3.95	±	0.67
123	4.64	±	0.87
125	3.64	±	0.46
126	5.05	±	0.74
128	6.29	±	1.42
132	3.77	±	0.35
133	3.20	±	0.54
134	14.11	±	0.91
138	2.98	±	1.09
139	2.99	±	1.67
141	3.34	±	0.94
144	4.66	±	0.54
149	3.20	±	0.47
150	3.26	±	0.41
151	14.53	±	1.18
152	2.47	±	0.25
153	3.81	±	0.64
154	3.74	±	2.64
155	4.38	±	1.04

CDGJM 260 g L^{-1} sugar			
Colony	Average	±	SD
158	7.49	±	2.13
161	14.05	±	3.16
164	5.04	±	2.85
165	13.02	±	2.29
166	5.77	±	1.71
167	4.38	±	1.49
168	43.77	±	2.15
169	5.52	±	3.30
170	1.49	±	1.42
171	5.23	±	1.08
172	0.75	±	1.43
173	2.11	±	2.01
175	5.67	±	4.37
179	2.37	±	2.13
180	3.28	±	2.68
L2056 P1	-0.11	±	0.26
L2056 P2	-0.47	±	0.04
L2056 P3	0.02	±	0.11
L2056 P4	-0.89	±	0.02
L2056 P5	-0.96	±	0.06
L2056 P6	-0.70	±	0.04
L2056 P7	-0.69	±	0.30
L2056 P8	-0.37	±	0.13
L2056 P9	-0.62	±	0.31
L2056 P10	-0.55	±	0.19
L2056 P11	-1.55	±	0.16

CDGJM 230 g L^{-1} sugar			
Colony	Average	±	SD
158	-0.26	±	0.38
161	11.40	±	1.25
164	-0.13	±	0.29
165	2.28	±	0.88
166	-0.09	±	0.17
167	-0.12	±	0.32
168	-0.12	±	0.32
169	0.22	±	0.35
170	0.22	±	0.35
171	1.56	±	0.81
172	-0.05	±	0.47
173	1.44	±	0.36
175	1.09	±	0.97
179	0.59	±	0.77
180	1.48	±	0.51
L2056 P1	0.83	±	0.52
L2056 P10	0.22	±	0.35
L2056 P11	17.03	±	5.38
L2056 P2	1.49	±	0.85
L2056 P3	-1.09	±	0.22
L2056 P4	10.91	±	2.49
L2056 P5	3.77	±	0.78
L2056 P6	-0.06	±	0.59
L2056 P7	23.62	±	3.64
L2056 P8	14.80	±	1.06
L2056 P9	-0.34	±	0.25

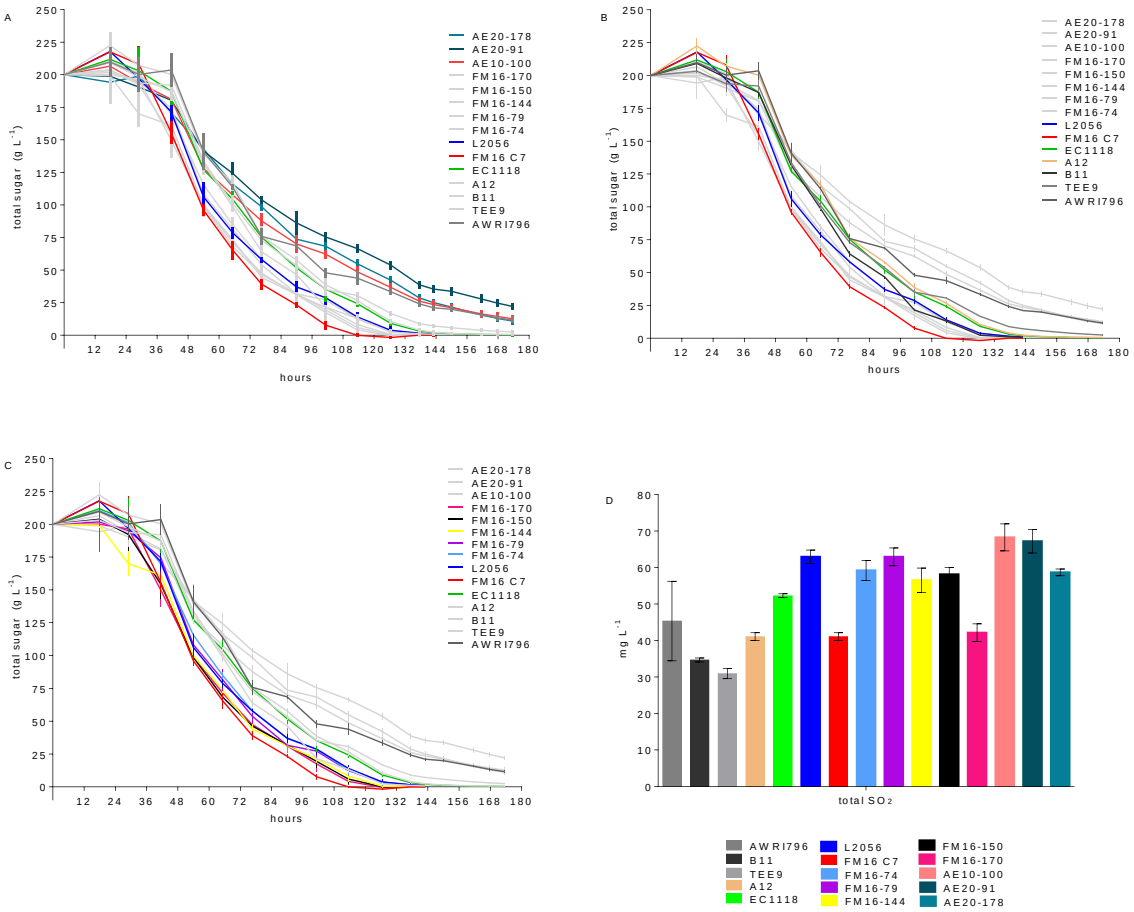
Chardonnay Juice			
Colony	Average	±	SD
158	5.17	±	0.92
161	3.14	±	1.91
164	2.50	±	0.86
165	5.12	±	1.62
166	3.09	±	1.03
167	2.46	±	0.50
168	12.93	±	0.92
169	3.16	±	1.47
170	2.03	±	0.26
171	6.46	±	1.02
172	4.45	±	1.03
173	5.01	±	2.43
175	7.08	±	0.53
179	5.04	±	0.58
180	4.23	±	0.39
L2056 P1	7.13	±	1.03
L2056 P10	13.19	±	1.14
L2056 P11	11.83	±	3.78
L2056 P2	10.39	±	0.54
L2056 P3	10.12	±	4.37
L2056 P4	9.58	±	1.88
L2056 P5	11.03	±	1.31
L2056 P6	9.90	±	1.72
L2056 P7	12.66	±	4.63
L2056 P8	13.19	±	1.98
L2056 P9	12.61	±	2.12

CDGJM 260 g L^{-1} sugar			
Colony	Average	±	SD
Mix P1	8.07	±	2.18
Mix P2	6.75	±	4.20
Mix P3	14.54	±	3.25
Mix P4	8.21	±	1.96
Mix P5	12.78	±	3.43
Mix P6	10.47	±	4.54
Mix P7	13.11	±	4.22
Mix P8	14.26	±	4.28
Mix P9	7.62	±	7.86
Mix P10	16.64	±	4.74
Mix P11	16.77	±	3.70
PDM P1	0.13	±	0.35
PDM P2	-0.07	±	0.13
PDM P3	1.19	±	0.62
PDM P4	-0.13	±	0.42
PDM P5	0.53	±	0.35
PDM P6	0.46	±	0.28
PDM P7	1.52	±	0.58
PDM P8	0.87	±	0.15
PDM P9	0.59	±	0.79
PDM P10	1.95	±	0.85
PDM P11	-0.89	±	0.55

CDGJM 230 g L^{-1} sugar			
Colony	Average	±	SD
Mix P1	0.83	±	0.52
Mix P10	0.22	±	0.35
Mix P11	17.03	±	5.38
Mix P2	1.49	±	0.85
Mix P3	-1.09	±	0.22
Mix P4	10.91	±	2.49
Mix P5	3.77	±	0.78
Mix P6	-0.06	±	0.59
Mix P7	23.62	±	3.64
Mix P8	14.80	±	1.06
Mix P9	-0.34	±	0.25
PDM P1	4.67	±	1.03
PDM P10	-0.31	±	0.21
PDM P11	0.67	±	0.81
PDM P2	1.08	±	0.70
PDM P3	0.03	±	0.30
PDM P4	-0.43	±	0.57
PDM P5	-0.10	±	0.75
PDM P6	-0.32	±	0.77
PDM P7	0.15	±	0.54
PDM P8	-0.63	±	0.24
PDM P9	-0.19	±	0.23

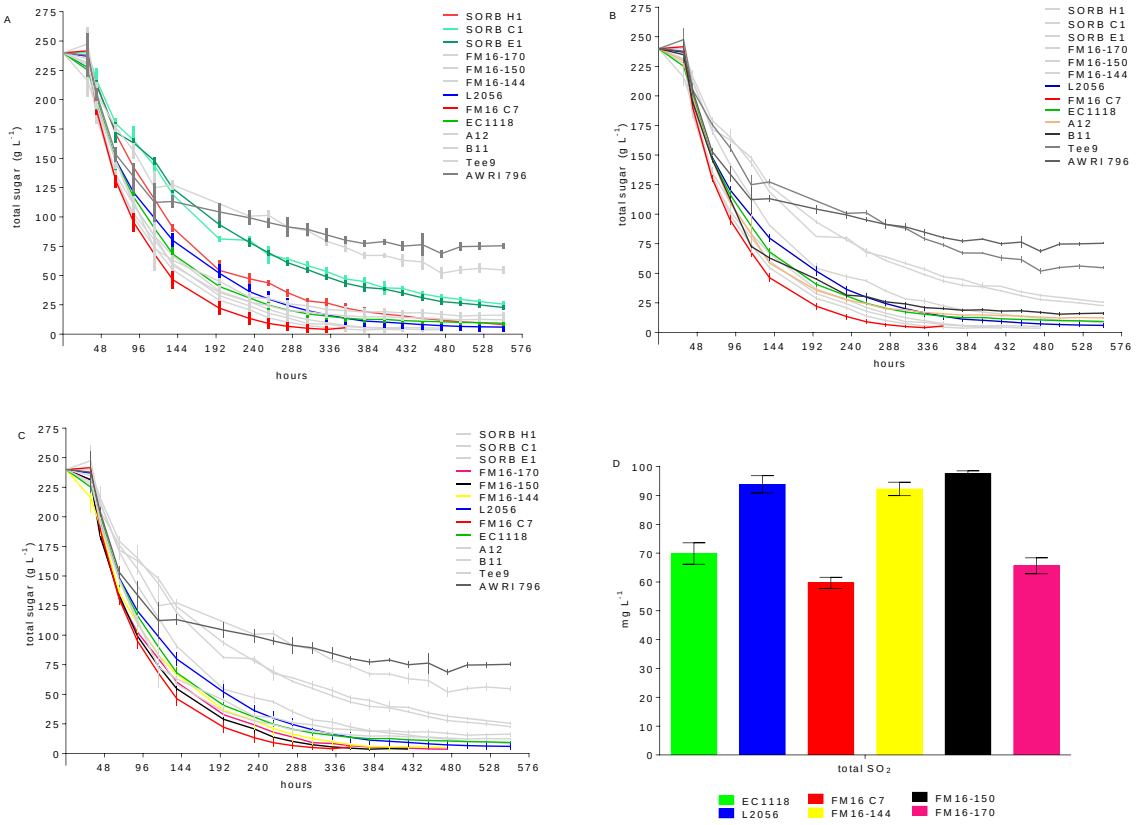
Chardonnay Juice			
Colony	Average	±	SD
Mix P1	4.74	±	3.58
Mix P10	3.49	±	1.63
Mix P11	9.74	±	1.02
Mix P2	5.76	±	2.39
Mix P3	10.59	±	1.74
Mix P4	7.88	±	1.51
Mix P5	5.97	±	2.50
Mix P6	7.35	±	1.65
Mix P7	6.40	±	1.67
Mix P8	7.61	±	1.55
Mix P9	5.54	±	0.87
PDM P1	6.22	±	0.51
PDM P10	5.95	±	3.20
PDM P11	6.76	±	1.72
PDM P2	5.43	±	0.90
PDM P3	8.18	±	0.89
PDM P4	6.47	±	0.50
PDM P5	5.57	±	1.08
PDM P6	6.67	±	1.62
PDM P7	5.09	±	0.65
PDM P8	5.86	±	1.22
PDM P9	5.79	±	0.91

Supplementary Figure 1: Evaluation of new wine strains in 10 L fermentations in Chardonnay juice (Riverland, 2012 vintage)



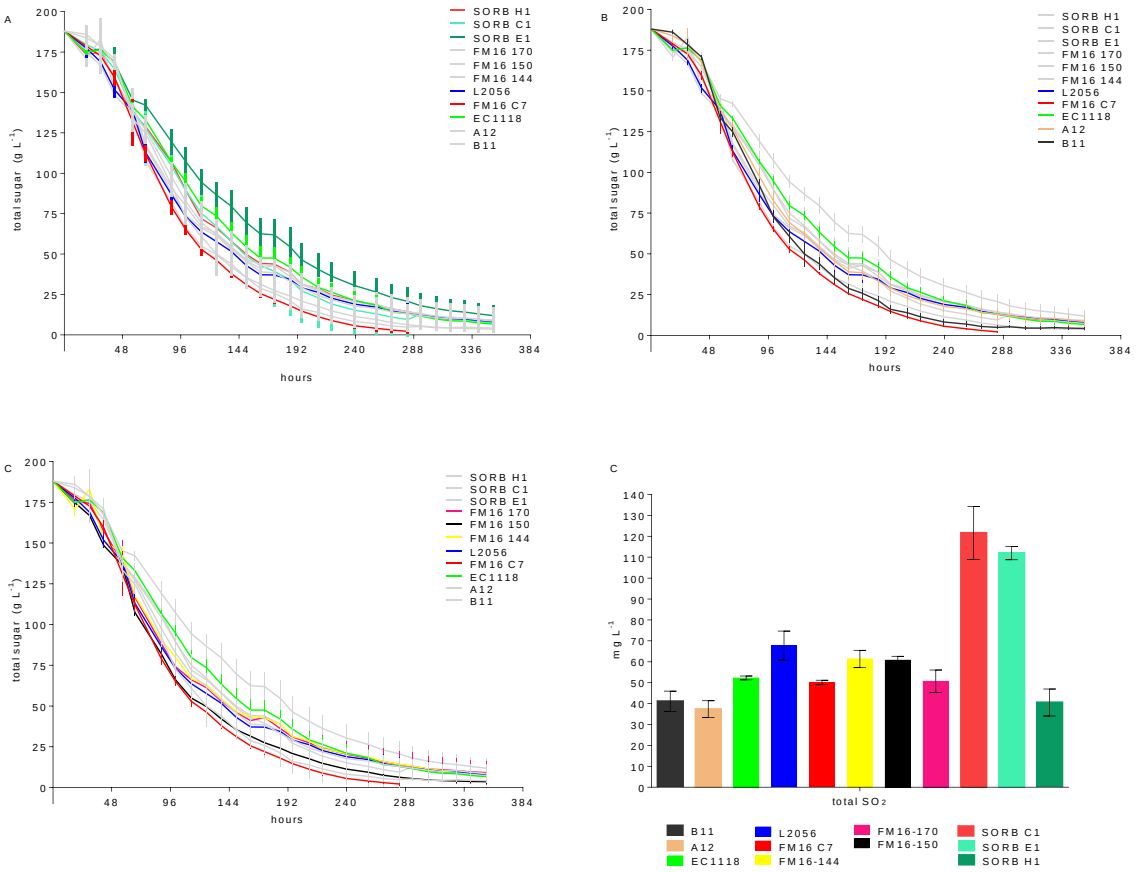
Eleven new wine strains were evaluated in 10 L fermentations (triplicate, 22 °C) – Ferment 1. Strains were compared to EC1118 as the industry standard, AWRI 796, L2056 and the evolved strain FM16-C7. The following strains are highlighted in panels (A) mating/AE strategy, (B) A12, B11 and TEE9 and (C) FM16 derivatives. Total SO_2 (mg L^{-1}) is shown in Panel D. Juice parameters: 254 mg L^{-1} YAN, 20.7 °Brix, pH 3.25, 6.6 g L^{-1} TA, 18 mg L^{-1} FSO_2, 49 mg L^{-1} TSO_2.

Supplementary Figure 2: Evaluation of new wine strains in 10L fermentations in Chardonnay juice (Padthaway, 2012 vintage)



Nine new wine strains were evaluated in 10 L fermentations (triplicate, 22 °C) – Ferment 2. Strains were compared to EC1118, AWRI 796 and L2056 commercial yeast and the evolved strain FM16-C7. The following strains are highlighted panels (A) Sorbitol AE, (B) A12, B11 and TEE9 and (C) FM16 derivatives. Total SO_2 (mg L^{-1}) was determined for only the FM16 derivatives as shown in Panel D. Juice parameters: 287 mg L^{-1} YAN, 11.8 Bé, pH 3.85, 4.5 g L^{-1} TA, 7 mg L^{-1} FSO_2, 49 mg L^{-1} TSO_2. Tartaric acid was added to juice (1 g L^{-1}) to give final pH of 3.54 and 6.5 g L^{-1} TA.

Supplementary Figure 3: Evaluation of new wine strains in 10 L fermentations in Semillon juice (Adelaide Hills, 2012 vintage)



Eight new wine strains were evaluated in 10 L fermentations (triplicate, 22 °C) – Ferment 3. Strains were compared to EC111, the industry standard, and L2056 the parent of the evolved strain FM16-C7. The following strains are highlighted in panels (A) Sorbitol AE, (B) A12, B11 and TEE9 and (C) FM16 derivatives. Total SO_2 (mg L^{-1}) is shown in Panel D. Juice parameters: 209 mg L^{-1} YAN, 10.9 Bé, pH 3.33, 7.17 g L^{-1} TA, 10 mg L^{-1} FSO_2, 46 mg L^{-1} TSO_2.

Supplementary Table 9: Comparison between strains for yeast derived aroma compounds produced during fermentation.

No differences are denoted by grey shading. Statistical differences determined by 2-way Anova: **** p<0.0001, ***p<0.001, **p<0.01, *p<0.05, ns not significant.

Ferment 1	propanoic acid	acetaldehyde	acetic acid	ethyl dodecanoate	2-phenyl ethyl acetate	decanoic acid	2-methyl butyl acetate	ethyl acetate	ethyl octanoate	octanoic acid
EC1118 vs FM16 C7				****		**			****	****
EC1118 vs L2056		*		****		*			****	****
EC1118 vs FM16 144	**			***		*				****
EC1118 vs FM16 150				****		***		*		****
EC1118 vs FM16 170				****	*	*	*	**	***	****
FM16 C7 vs L2056					****		***			
FM16 C7 vs FM16 144	**						*		****	
FM16 C7 vs FM16 150					****		***	*	**	
FM16 C7 vs. FM16 170					****		****	**		
L2056 vs FM16 144	**	**	*		*				****	
L2056 vs FM16 150									**	
L2056 vs FM16 170		***	*							
FM16 144 vs FM16 150	**				*					
FM16 144 vs FM16 170	**				**				*	

Ferment 2	ethyl octanoate	octanoic acid	2-phenyl ethyl acetate	2-phenyl ethanol	decanoic acid	acetic acid	ethyl butanoate	2,3-butandione	acetalde-hyde	2-methyl butanol / 3-methyl butanol
EC1118 vs FM16 C7	*	*	****					*	**	
EC1118 vs L2056									**	*
EC1118 vs FM16 144			***	*		***			****	**
EC1118 vs FM16 150			****	****		****			****	***
EC1118 vs FM16 170		*	*	**		****		***	*	
FM16 C7 vs L2056			**			**			****	**
FM16 C7 vs FM16 144	**					****	*		****	**
FM16 C7 vs FM16 150				**	*	****			****	****
FM16 C7 vs FM16 170						****			****	
L2056 vs FM16 144									***	

L2056 vs FM16 150			**	*					**	
L2056 vs FM16 170						**				
FM16 144 vs FM16 170									****	
FM16 150 vs FM16 170									***	*
Ferment 2	ethyl 2-methyl propanoate	benzyl alcohol	ethyl butanoate	2,3-butandione	2-methyl propanol	propanol	hexanol	ethyl hexanoate	ethyl propanoate	acetoin
EC1118 vs FM16 C7				*				****	*	*
EC1118 vs L2056		*	ns	ns			****	**		**
EC1118 vs FM16 144					****		****			
EC1118 vs FM16 150	*				**		****	**		
EC1118 vs FM16 170	*			***		**	**	****		
FM16 C7 vs L2056		***					****	***		
FM16 C7 vs FM16 144		**	*	ns	*	*	****	****		
FM16 C7 vs FM16 150	*	***					****	**		
FM16 C7 vs FM16 170	**						****			
L2056 vs FM16 150	*									
L2056 vs FM16 170	*					**				*
FM16 144 vs FM16 170						***		**		
FM16 150 vs FM16 170							*			
Ferment 3	propanoic acid	acetoin	ethyl propanoate	butanol	2/3-methyl butanoic acid	2,3-butandione	acetic acid	2-phenyl ethyl acetate	2/3-methyl butyl acetate	2-phenyl ethanol
EC1118 vs FM16 C7				****		*		****	****	*
EC1118 vs L2056		**			*	****	*			
EC1118 vs FM16 144		**				****	****			
EC1118 vs FM16 150			*	ns		****				
FM16 C7 vs L2056		*		ns		****	****			
FM16 C7 vs FM16 144		*		****				****	****	
FM16 C7 vs FM16 150		*		****		*	***	****	****	*
FM16 C7 vs FM16 170	*			****		*		***	***	*
L2056 vs FM16 144				****		**	**	****	***	*
L2056 vs FM16 170	**									

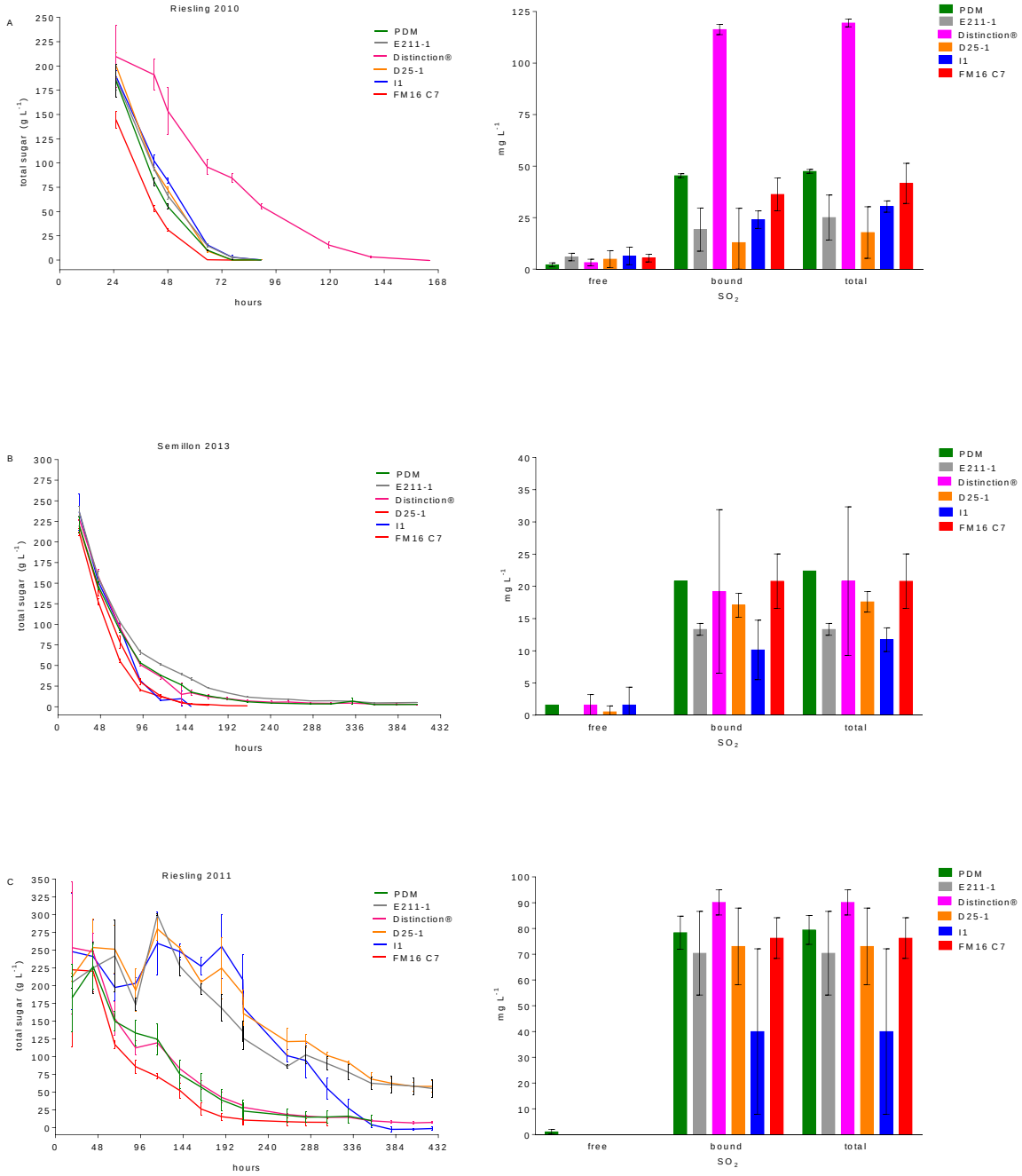
FM16 144 vs FM16 150	*									
FM16 144 vs FM16 170	****	*					**			
FM16 150 vs FM16 170	***									
Ferment 3	ethyl acetate	acetic acid	ethyl propanoate	2-phenyl ethanol	ethyl dodecanoate + hexanoic acid	hexyl acetate	butanol	2/3-methyl butanoic acid	2/3-methyl butyl acetate	2,3-butandione
EC1118 vs FM16 C7				*	****	***	****		****	*
EC1118 vs L2056		*			****			*		****
EC1118 vs FM16 144		****			****					****
EC1118 vs FM16 150			*		****					****
FM16 C7 vs L2056		****			****					****
FM16 C7 vs FM16 144	**					****	****		****	
FM16 C7 vs FM16 150	*	***		*		****	****		****	*
FM16 C7 vs FM16 170	**			*		****	****		***	*
L2056 vs FM16 144	*	**		*		****	****		***	**
FM16 144 vs FM16 170		**								
FM16 144 vs FM16 150		*								
Ferment 3	acetoin	propanoic acid								
EC1118 vs FM16 C7										
EC1118 vs L2056	**									
EC1118 vs FM16 144	**									
FM16 C7 vs L2056	*									
FM16 C7 vs FM16 144	*									
FM16 C7 vs FM16 150	*									
FM16 C7 vs FM16 170		*								
L2056 vs FM16 170		**								
FM16 144 vs FM16 150		*								
FM16 144 vs FM16 170	*	****								
FM16 150 vs FM16 170		***								

Supplementary Table 10: Aroma compounds formed by *Saccharomyces cerevisiae* shown to differ between EC1118, L2056 and evolved FM16 strains

Compound	aroma description*
Ethyl esters	
Ethyl isobutyrate (ethyl 2-methyl propanoate)	sweet, rubber
Ethyl butanoate (ethyl butyrate)	apple, fruity, orange, pineapple
Ethyl octanoate	fruit, fat
Ethyl decanoate	grape
Ethyl propanoate	fruity
acetates	
2-methylbutyl acetate (2-methyl butanoate)	fruit
3-methylbuytl acetate (3-methyl butanoate)	banana
2-phenylethyl acetate	rose, honey, tobacco
Ethyl acetate	pineapple, fruity solvent, balsamic
Hexyl acetate	fruit, herb
Acids	
Ccetic acid	sour
2-methylbutanoic acid	cheese, sweaty
3-methylbutanoic acid (iso-valeric acid)	cheese, sweaty
Propanoic acid	pungent, rancid, soy
Hexanoic acid	sweat
Octanoic acid	sweat, cheese
Decanoic acid	rancid, fat
Alcohols	
Benzyl alcohol	sweet , flower
Isobutanol	wine, solvent, bitter
2-methyl butanol	wine, onion
2-phenyl ethanol	honey, spice, rose, lilac
n-propanol	alcohol, pungent
2,3-butandiol	fruity
C6 alcohols	
1-hexanol	resin, flower, green
Miscellaneous	
2,3-butanedione	butter
Acetoin	butter, cream
Acetaldehyde	pungent, ether

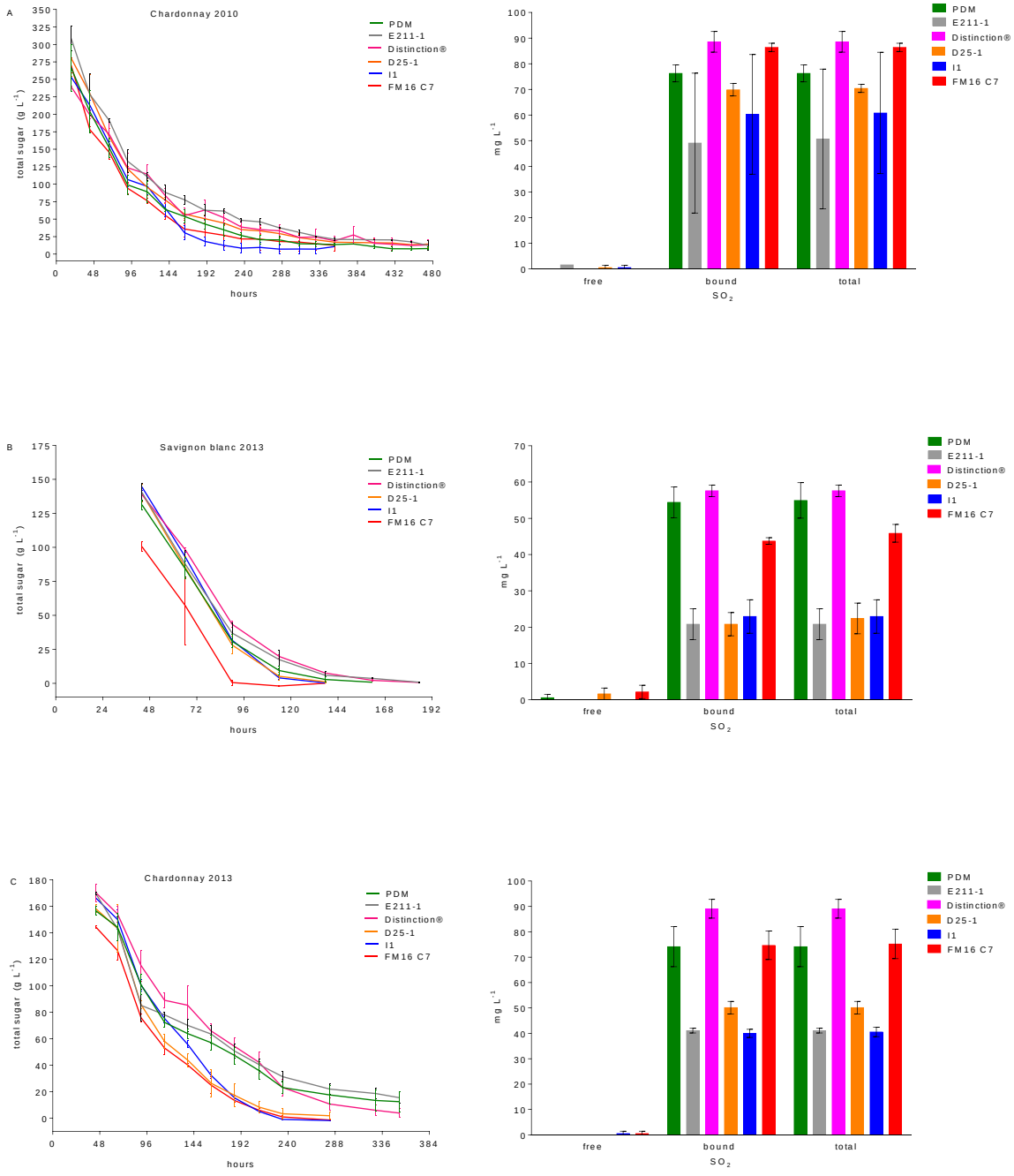
* Aroma descriptors are from Flavornet [60]

Supplementary Figure 4: Evaluation of low H_2S producing wine yeast in 10 L white juice fermentations



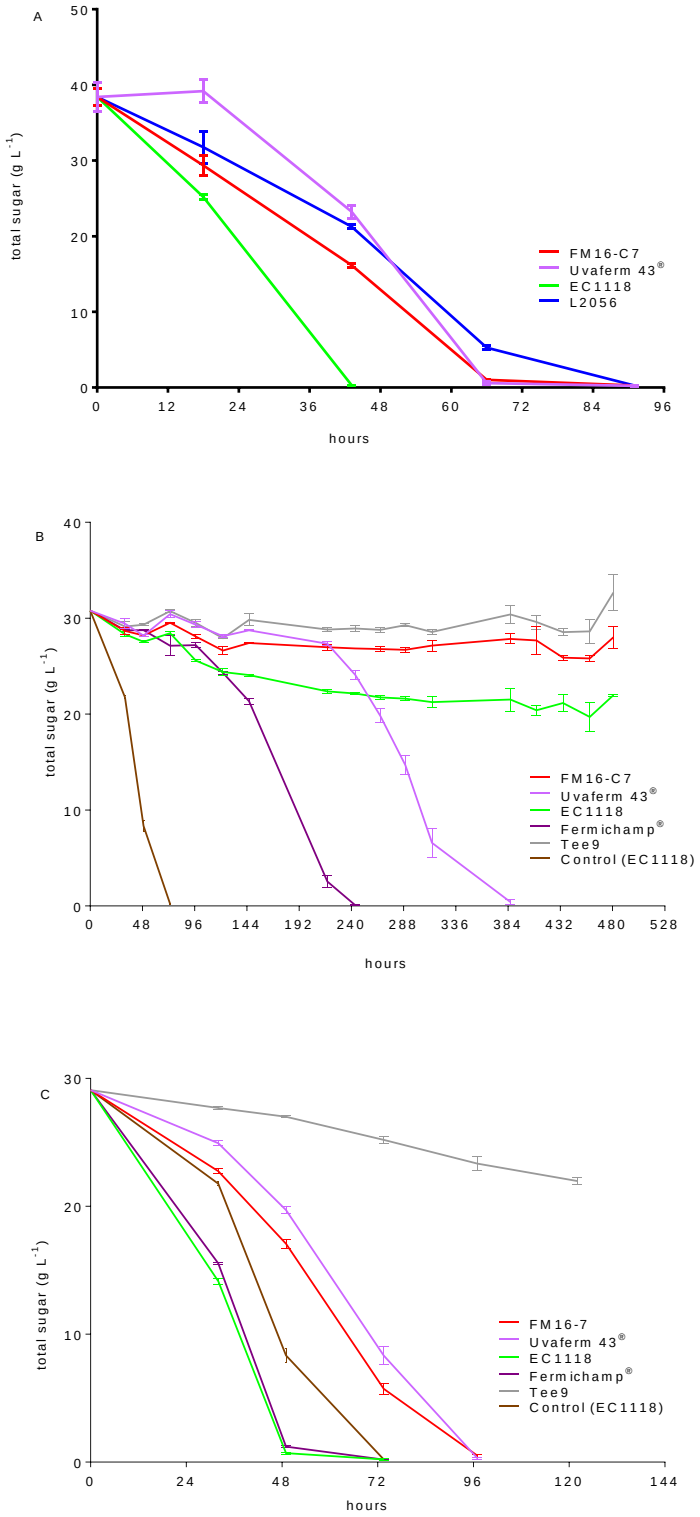
Three low H_2S producing strains were evaluated in 10 L white juice fermentations (triplicate, 22 °C). Strains were compared to PDM, Distinction® and the evolved strain FM16-C7. Strains were initially propagated in YPD, followed by Juice Starter Media with DAP (JSM_DAP). Fermentations were inoculated at 5 x 10^6 cells mL^{-1}. No adjustments were made to TA or nitrogen content unless stated.
The sugar utilization profiles (total sugar g L^{-1}) and SO_2 (mg L^{-1}) are shown for each fermentation. Values are the average of 3 replicates ± SD. The following juice fermentations are shown in panels:
A: Riesling, 2010 (112.83 mg L^{-1} YAN, 18.6 °Brix, pH 3.18, 5.63 g L^{-1} TA, 11.2 mg L^{-1} FSO_2, 54.4 mg L^{-1} TSO_2). YAN adjusted with 100 mg L^{-1} DAP & 400 mg L^{-1} BIOCELL addition.
B: Semillon 2013 (220 mg L^{-1} YAN, 21.2 °Brix, pH 3.12, 18 mg L^{-1} FSO_2, 32 mg L^{-1} TSO_2). This was a blend of 2 batches with corresponding TA of 6.46 and 7.19 g L^{-1}respectively. TA of the blended juice was not determined.
C: Riesling, 2011 (72.4 mg L^{-1} YAN, 17.5 °Brix, pH 3.09, 8.7 g L^{-1} TA, 8 mg L^{-1} FSO_2, 83.2 mg L^{-1} TSO_2). 250 mg L^{-1} DAP was added to juice.

Supplementary Figure 5: Evaluation of low H_2S producing wine yeast in 10 L white juice fermentations



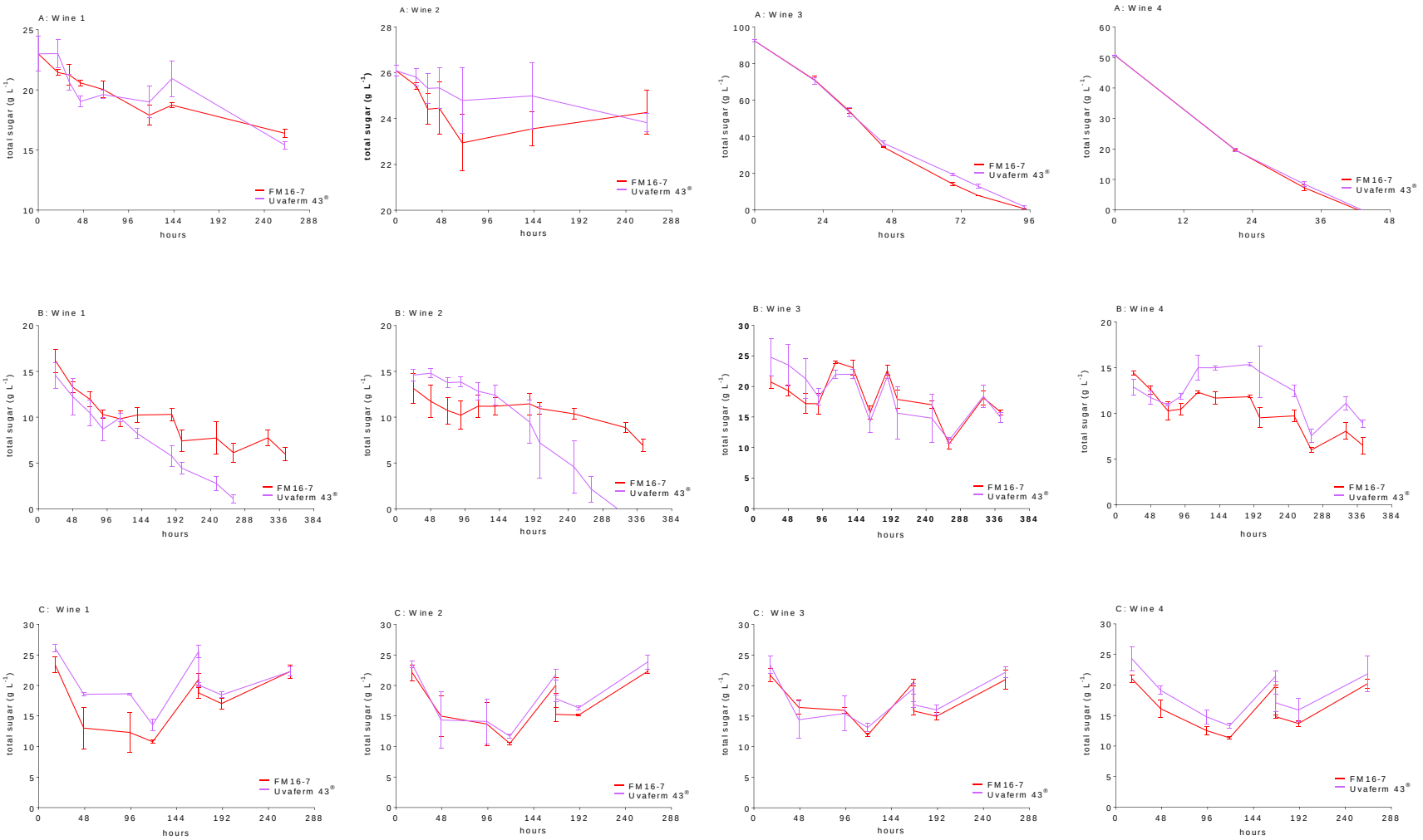
Three low H_2S producing strains were evaluated in 10 L white juice fermentations (triplicate, 22 °C). Strains were compared to PDM, Distinction® and the evolved strain FM16-C7. Strains were initially propagated in YPD, followed by Juice Starter Media with DAP (JSM_DAP). Fermentations were inoculated at 5 x 10^6 cells mL^{-1}. No adjustments were made to TA or nitrogen content unless stated.
The sugar utilization profiles (total sugar g L^{-1}) and SO_2 (mg L^{-1}) is shown for each fermentation. Values are the average of 3 replicates ± SD. The following juice fermentations are shown in panels:
A: Chardonnay, 2010 (180.4 mg L^{-1} YAN, 18.4 °Brix, pH 3.47, 5.4 g L^{-1} TA, 8 mg L^{-1} FSO_2, 44.8 mg L^{-1} TSO_2). YAN adjusted to 250 mg L^{-1} with DAP.
B: Sauvignon Blanc 2013 (243 mg L^{-1} YAN, 18.3 °Brix, pH 3.65, 5.5 g L^{-1} TA, 3.2 mg L^{-1} FSO_2, 35.2 mg L^{-1} TSO_2).
C: Chardonnay 2013 (316.3 mg L^{-1} YAN, 19.4 °Brix, pH 3.84, 5.2 g L^{-1} TA, 6.4 mg L^{-1} FSO_2, 43.2 mg L^{-1} TSO_2).

Supplementary Figure 6: Effect of ethanol and unsaturated fatty acids on wine yeast in restarting primary (alcoholic) fermentation



A Semillon juice was fermented with L2056 and arrested at 37.5 g L^{-1} total sugar by cold settling and filtration to remove the yeast. Comparative fermentations (triplicate, 22 °C) were performed in the partially fermented juice using FM16-C7 and other wine strains. The juice was treated as follows: (A) No addition, (B) Addition of 5% (v/v) ethanol, (C) addition of 3 mg L^{-1} octanoic acid and decanoic acid. In the case of fermentations A and B, one set of fermentations, i.e. Control (EC1118) had no additions.

Supplementary Figure 7: Evaluation of FM16-C7 and Uvaferm43® in restarting fermentation



Experiment A: Wine 1: Shiraz 1 (20 g L^{-1} sugar), Wine 2: Shiraz 2 (3 g L^{-1} sugar, adjusted to 20 g L^{-1} with fructose), Wine 3: Riesling 1 (127.9 g L^{-1}), Wine 4: Riesling 2 (86.4 g L^{-1}). **Experiment B**: Wine 1: Chardonnay 1 (7.5 g L^{-1} sugar), Wine 2: Chardonnay 2 (5.2 g L^{-1} sugar), Wine 3: Shiraz/Mataro (7.95. g L^{-1}), Wine 4: Shiraz (1.6 g L^{-1}). **Experiment C**: Wine 1: Chardonnay (2 g L^{-1} sugar), Wine 2: Shiraz/Mataro (3.7 g L^{-1} sugar), Wine 3: Shiraz 1 (3 g L^{-1}), Wine 4: Shiraz 2 (2.3 g L^{-1}) For B and C all wines adjusted to 20 g L^{-1} with fructose