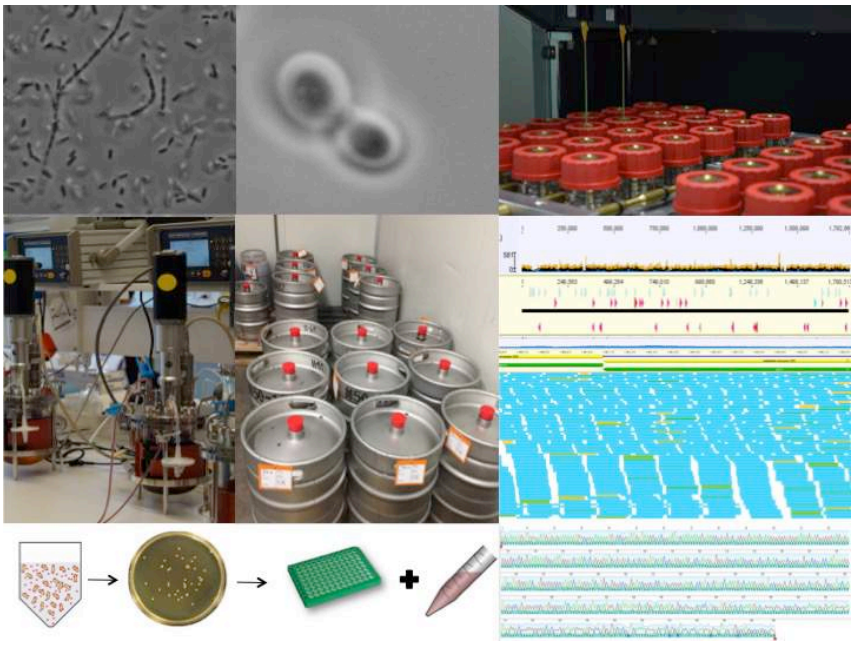
Australian Government

Australian Grape and Wine Authority

SCHOOL OF AGRICULTURE, FOOD AND WINE

THE UNIVERSITY of ADELAIDE

Fit-for-Purpose Yeast and Bacteria via Directed Evolution



FINAL REPORT to

AUSTRALIAN GRAPE AND WINE AUTHORITY

Project Number: **UA1302**

Principal Investigator: **Professor Vladimir Jiranek**

Research Organisation: **University of Adelaide**

Date: **30th December 2017**

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Abbreviations

CDGJM	Chemically Defined Grape Juice Medium
DAP	Diammonium Phosphate (Ammonium Dibasic Phosphate)
DE	Directed Evolution
FAN	Free Amino acid Nitrogen
FCDGJM	Fermented Chemically Defined Grape Juice Medium
FEG	Fermentation Essential Genes
FSO_2	Free sulfur dioxide
TSO_2	Total sulfur dioxide
LYDL	Lab Yeast Deletion Library
LAB	Lactic acid bacteria
MFCA	Medium Chain Fatty Acids
MLF	Malolactic fermentation
MRS	De Man, Rogosa and Sharpe medium
MRSAJ	MRS with Apple Juice
NGS	Next Generation Sequencing
ORF	Open Reading Frame
PMS	Potassium Metabisulfite
RCDGJM	Red CDGJM
RFCDGJM	Red Fermented CDGJM
SD	Standard deviation
SNP	Single Nucleotide Polymorphism
WYDL	Wine Yeast Deletion Library
WGS	Whole Genome Sequencing

Abstract

This project sought to generate 'fit-for-purpose' yeast and lactic acid bacterial (LAB) strains better suited to problematic wine and juice conditions via Directed Evolution (DE). DE involves growing an organism in a stressful environment where it can mutate and adapt over time. Screening of DE populations and LAB isolates from un-inoculated fermentations has generated 5 yeast and 8 LAB improved strains. The molecular basis behind the observed improved performance during fermentation was also investigated. Our findings demonstrate the feasibility of this approach to generate more efficient yeast and LAB strains tailored for juice and wines with multiple stressors.

Executive Summary

The use of specifically selected yeast and bacteria to complete both primary and secondary fermentation still remains the most commonly used and arguably most reliable method to ensure desirable fermentation outcomes in new-world industrial winemaking. Many commercial strains are currently available, however feedback from industry reports that even with these, problem fermentations still exist. We expect this is also exacerbated by the potential for increased variability in the composition of grape musts, impacted by factors such as climate change as well as viticultural practices targeting particular wine styles. This project sought to generate 'fit-for-purpose' yeast and lactic acid bacterial strains better suited to problematic juice conditions, as reported by industry (high sugar, low nitrogen, low pH) and the presence of microbial inhibitors such as sulfur dioxide and medium chain fatty acids. Reliable fermentation outcomes will translate into further improvements to sustainable and economical wine production with enhanced or consistent wine quality. The objectives of this project are defined in three broad areas; (i) Novel and improved lactic acid bacteria isolates, (ii) Novel and improved wine yeast (*Saccharomyces cerevisiae*) isolates and (iii) Definition of the wine yeast fermentome.

Directed evolution of yeast and lactic acid bacteria, along with evaluation of bacterial isolates from un-inoculated fermentations has generated 5 improved yeast (*Saccharomyces cerevisiae*) strains and 9 bacteria (8 *Oenococcus oeni* and 1 *Lactobacillus plantarum* strain). These improved strains were evaluated first in chemically defined media and then in a range of juices or wines at volumes up to 20 L, as appropriate. Evaluation in a range of juices/musts is important to ensure reliability in the many varied conditions as occurs in industry. Results were compared to widely used industry standard robust microbes; for instance Uvaferm 43 (yeast) and VP41 (bacteria). The large number of cultures generated and isolated in this project represents a diverse array of novel strains and is an excellent resource for future microbial improvement projects. Further details on outcomes from our 3 broad objectives follow:

(i) Over 800 bacterial strains were isolated from directly evolved cultures and other suitable sources and tested for desirable attributes. In particular, ability to grow and undertake malolactic fermentation in wines with combinations of low pH, high alcohol and the presence of SO_2 and medium chain fatty acids was assessed. Improved lactic acid bacteria selected in this project had a range of desirable attributes that matched those sought. Typically they have increased tolerance to SO_2 and lower pH, and in some cases, can still undertake malolactic fermentation in the presence of 18% (v/v) ethanol. Depending on the conditions, these tolerances resulted in a reduction of malolactic fermentation by up to 50%, or completion of MLF whilst the parent strains did not (i.e. became stuck). Given that leaving wine unprotected by SO_2 whilst waiting for malolactic fermentation to complete increases the risk of microbial spoilage a reduced malolactic fermentation time is highly desirable. Such strains show strong potential for commercialisation and distribution to the wine industry.

(ii) Twelve wine yeast were chosen as source strains for improvement and these were subjected to two conditions of directed evolution (i) exposure to the combination of high sugar and ethanol and low pH and (ii) high fructose and ethanol content (i.e. as per a late-stage fermentation).. Later saturated fatty acids (sub-products of ethanolic fermentation) were also added to increase stress. A range of methods were utilized in order to increase genetic diversity of evolving cultures, for instance chemical mutagenesis and *en masse* sporulation and subsequent rare-mating. From these, 1,500 yeast strains were selected and tested for fermentation capabilities from a bank of 25

separately evolved cultures. The fermentation performance of our in-house yeast hybrid collection (250 cultures) was also evaluated. Five evolved yeast cultures were found to have superior fermentation performance, as measured by total fermentation duration i.e. the time taken to utilize all sugars. For instance strain RM7-71 fermented all available sugars 23 hours before the commercial yeast Uvaferm 43 (well known to be capable of rapid fermentation) in two separate experiments of different media and scales. Again, these strains have excellent potential for wider availability through commercialisation.

Whole genome sequencing using Illumina technology was used to determine the genetic basis of the oenological improvement in the yeast and bacterial strains of interest. This information will highlight which biochemical processes have been modified and thus which are likely to confer the desirable attribute. Parent strains were also sequenced to allow a direct comparison. Overall, 18 lactic acid bacteria including 12 *O. oeni* and 5 *Lb. plantarum*, and 6 wine yeast were sequenced, to investigate performance variation. All genome sequences have been received and identification of genomic changes is well underway and is expected to be completed in future studies.

In the first group of *O. oeni* strains sequenced (generated from SB3 and subsequently A90; Betteridge et al 2015 and 2017) a total of 19 single nucleotide polymorphisms (SNPs) were found in strains 2-49 and it's further evolved isolate 3-83 (Jiang et al 2017) in comparison to A90. Ten SNPs were found in regions that code genes (coding regions) and previous reports suggest that modifications of these genes could impact the cell envelope, fatty acids biosynthesis, DNA translation and homeostasis of internal pH. We expect it is the modification of these pathways that leads to the improved performance of these evolved bacterial strains. Interestingly six SNPs are common to both strains with only two of these in coding regions. These genes encode acyltransferase and a membrane protein. Further studies will investigate how modification of these particular genes could confer robust and efficient malolactic fermentation.

Evaluation of the SNPs of three evolved wine yeast strains (C7H_B4, RM5_15 and RM3_25) and their parent strains (FM16_C7H, 71B and Q7) is also underway. The SNPs from a previously evolved wine yeast isolate (FM16-7, also investigated in project UA 11/01) have been defined. We found 207 in regions that are predicted to affect protein function. Of these no specific processes are highlighted as being central to this genotype, thus we hypothesise that the fermentation efficiency of this particular strain is conferred by multiple genes fine-tuning multiple processes.

(iii) Definition of 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. Multiple stresses were applied during fermentation screening with a focus on one of the most common stresses encountered by yeast in a wine fermentation, nitrogen deficiency.

Ninety-three deletion mutants (in a laboratory yeast strain), exhibit protracted fermentation when grown in a chemically defined grape juice (Walker et al., 2014). We termed these genes **F**ermentation **E**ssential **G**enes (UA 11/01) and reported them as the first data set of the Fermentome. Twenty of these mutants present with vacuolar dysfunction during the early stages of fermentation (Nguyen et al 2017). Except for

Δhog1, Δpbs2 and Δvph1 mutants, where dysfunction was directly related to osmolality, the remainder exhibit increased vacuolar dysfunction independent of sugar concentration. This highlights the biological importance of vacuolar function to cell adaption to the environment of a grape juice fermentation.

We expanded upon the fermentome by screening gene deletion mutants in the more appropriate background of a wine strain (AWRI 1631). Since the current wine yeast deletion library is only partially complete, another laboratory yeast deletion library was also screened. This particular library has the advantage of not carrying any auxotrophies (mutations that result in requirement of amino acid supplementation) thus allowing studies in limiting nitrogen conditions. Overall, growth, fermentation duration and nitrogen utilization of 8,220 gene deletants were screened when supplied with limiting or optimal nitrogen. 671 genes from the laboratory and 201 from the wine yeast libraries were found to be required for efficient fermentation (Peter et al 2018). We compiled these together with other relevant datasets from published literature to build a database of genes affecting many facets of fermentation. Cross comparison of this rich dataset is enabling insights into this complex biological process. For example, we compared genes that harbor SNPs in the evolved wine yeast (FM16-7), finding seven genes that were common to two or more of the fermentation screening datasets generated in this project. This method focuses further studies toward the genes most likely to confer the yeasts capacity for efficient fermentation.

Mutations that resulted in a shortening of fermentation duration were also of great interest. Through a tiered screening approach 15 wine yeast deletion mutants were found to be capable of faster fermentation under nitrogen limitation (*c.a.* 15-59% time reduction). These genes are associated with the biological processes of protein modification, transport, metabolism, ubiquitination (*UBC13, MMS2, UBP7, UBI4, BRO1, TPK2, EAR1, MRP17, MFA2 and MVB12*), signalling (*MFA2*) and amino acid metabolism (*AAT2*), alluding to the importance of these processes to yeast performance in fermentation conditions. Screening of an overexpression library was also undertaken, here 60 genes are candidates for further analysis since their over-expression results in shortened fermentation duration.

The Δmfa2 deletant was further investigated to better understand how *MFA2*, when deleted, confers fermentation efficiency in limited nitrogen conditions. This is the first report of *MFA2* having a role in fermentation, as it has previously only been associated with mating type cell regulation (Peter, 2018). The construction and phenotype evaluation of recombinant strains (harbouring targeted genetic modifications) allow for proof of a direct gene and phenotype relationship. Targeted deletion of *MFA2* across multiple strains appear to show that the effect of this gene on fermentation efficiency is strain specific.

Also, in this project, genes related to hydrogen sulfide production from cysteine treatment during alcoholic fermentation have been identified. Overexpression of *TUM1* was shown to increase thiols 3MH and 3MA in chemically defined grape juice (Huang et al., 2016, 2017).

Across these activities, several candidate strains have been highlighted for commercialisation because of their superior performance or potential sensory contribution, knowledge about key gene knock-outs or modifications can be applied to strain construction. Accordingly, we have identified several gene modifications that lead to improved fermentation outcomes, which we intend to reconstruct in different

wine yeast strains using CRISPR Cas9 gene editing technology as part of a PhD project and the new bilateral agreement between Wine Australia and the University of Adelaide (Wine Microbiology).

During this project 8 PhD students [Betteridge, Long, Zhang, Nguyen, Haggerty, Peter, Jiang, Huang], and 4 Masters of Viticulture and Oenology students [Mi, Wang, Lui, Li] have been trained. Eight have been awarded their degrees, and 5 have continued careers in research [Betteridge, Zhang, Nguyen, Long] or education in Agricultural Science [Haggerty]. All have contributed to this project during their research. In addition, training of undergraduates has been undertaken in wine microbiology [Ricciotti, 3rd Yr; Lim, 2nd Yr) through 13 week research placements.

Our findings have been/are being disseminated through:

- Lectures to undergraduate and postgraduate Oenology/Viticulture [Grbin, Jiranek], Microbiology [Peter 2016] and Chemical Engineering and Biotechnology students [Watson 2013-16, Sumby 2017]
- 22 conference/seminar/workshop presentations (national and international)
- 4 industry articles
- 9 peer-reviewed publications
- 3 peer-reviewed publications (under review; late 2017)
- 2 further manuscripts for peer review journals are in preparation and will be submitted shortly.

Background

A most fundamental requirement of winemaking is reliable and predictable fermentations: reliable in terms of trouble-free completion and predictable in terms of fermentation duration, yeast/bacterial supplement needs and appropriate production of desirable and undesirable metabolites. To this day winemakers report fermentations that are too slow or fail to complete, often requiring amelioration with nutrients or chemicals, heating, modification of acidity or re-inoculation, amongst others. The need to monitor these fermentations appropriately and undertake the necessary interventions is both timely and costly. Fermentations failing to proceed as expected face a significant risk of oxidative and microbial spoilage and marked loss of wine value.

If anything, the challenge of achieving reliable and predictable fermentations is increasing. On one hand winemakers are seeking to reduce additives (incl. nutrients) or are pursuing low temperature fermentation for stylistic reasons and thereby expecting more from yeast. On the other hand more 'difficult' juices (e.g. higher in sugar, lower in nutrients, more disease affected/carry higher loads of spoilage microbes/requiring more SO_2), are being thrust upon winemakers by climate extremes.

Production of 'fit-for-purpose' yeast and bacteria by the proven strategy of Directed Evolution and underpinned by knowledge from definition of genes essential for specific fermentation scenarios offers a highly achievable solution to these challenges faced by today's wine industry. Decreasing the risk of problem fermentations will result in improved profit margins, not only via preservation of a portion of ~$35 million (a conservative estimation (10%) of the annual loss in potential value or product), but also in significant production costs associated with management of the 10% of fermentations considered problematic. Benefits also extend to the remaining ~90% of fermentations not necessarily reported as problematic, thereby giving further cost reductions. Predicted more reliable (shorter) fermentations will require less inputs (energy, additives, labour, etc) and result in less wastage with the salvage of poorer quality fruit, thereby reducing overall environmental impact. Greater profitability and sustainability will assist the wine industry to maintain its critical employment role, particularly in regional areas. This will be achieved by enhancing profitability and expanding markets domestically (through new products), as well as helping provide sought after, ideally high value wine to emergent overseas markets such as India and China.

Project Objectives

Using Directed Evolution of wine microbes (pioneered by us in 2002 via UA01/04 and GWR-Ph0901), we will produce for the Australian wine industry a bank of 1) robust and tailored lactic acid bacteria (LAB) and 2) robust and tailored yeast produced by a process informed by 3) characterisation of the *wine* yeast genes and processes essential to fermentation. We will exploit leads on metabolic pathways contributing to successful wine-like (multi-stressor) fermentations, identified as part of the 'Fermentome' (UA 1101) and comparison of the *wine* and *laboratory* yeast fermentomes, to reveal those genes specifically important to wine yeast. Information derived from this work will enable alternate or more logical targeting of selective pressures used in yeast Directed Evolution approaches to drive emergence of the desired optimised strains.

The specific objectives were to:

1. Generate (via directed evolution; DE) and characterise new isolates of LAB for efficient MLF when faced with typical wine stresses e.g. low nutrient availability; cold; high ethanol, sulphur dioxide or tannin content; presence of toxic fatty acids

or viticultural chemical residues.

2. Generate (via DE) and characterise new *Saccharomyces cerevisiae* isolates for efficient alcoholic fermentation with acid, SO_2, high sugar (or other features of Botrytis-affected juice) or cold.

3. Generate (via DE) and characterise new yeast isolates with enhanced or novel attributes such as malic acid metabolism, increased or stage-dependent flocculation/sedimentation, and reduced H_2S and SO_2.

4. Define the *wine* yeast Fermentome (ie a database of fermentation-essential genes) by using AWRI's partial wine yeast deletion library. Then compare this with the *laboratory* yeast Fermentome (from UA1101) to identify wine yeast specific fermentation-essential genes/processes. We also used the AWRI deletion library and newly completed prototrophic laboratory yeast library to identify genes and understand nutrient use and its impact on fermentation reliability.

5. Guide design of DE experiments via outcomes from 4. Use characterisation from 1-3 (especially genome sequencing) to support IP protection and further evolution experiments.

Project Aims and Performance Targets

The Outputs and Performance Targets outlined in the approved application 'Fit-for-Purpose Yeast and Bacteria via Directed Evolution' (UA1302) for a 4 year duration are shown below. The original target dates are shown.

Outputs and Performance Targets 2013-2014

Year 1	Outputs	Target Date	Performance Targets
1A	Data on strains and levels of key stressors for up to 3 improved attributes established for subsequent Directed Evolution (DE) studies	31/12/2013	Preliminary screening trials to make initial selection of strains and preliminary evaluation of the levels of the key stressors to achieve the sought-after improvements.
1B	Wine yeast Fermentome (database of fermentation essential genes): Stage 1 - preliminary screening (up to 300 genes).	31/03/2014	Source and screen wine yeast and prototrophic lab yeast deletion libraries (~7,500 deletants) under standard and/or low nutrient availability at the micro-titre scale in order to broadly identify key genes/processes/potential stresses for further investigation and exploitation.
1C	Documented outcomes from Annual Project Meeting.	31/05/2014	Hold Annual Project Meeting to review and discuss results and suggest future project plans (with relevant staff from AWRI and GWRDC).
1D	At least 1 candidate 'fit-for-purpose' yeast or LAB strain.	30/06/2014	Conduct DE experiments targeting up to 3 fermentation attributes/strains, development of screening conditions, intermittent screening of evolving populations, identification of candidate improved strains.
1E	Summary of data on fermentation performance of promising candidate yeast (and available LAB) strains in laboratory scale fermentations.	30/06/2014	High throughput screening of large number of isolates according to attribute in question. To conduct in conjunction with Output FY1C using micro- and lab-scale fermentations.
1F	Wine yeast Fermentome (database of fermentation essential genes): Stage 2 secondary screening (to up to 100 genes).	30/06/2014	Evaluation of candidate yeast deletants in lab-scale fermentations under variable conditions to confirm the roles of the deleted genes in fermentation performance and process.

Outputs and Performance Targets 2014 -2015

Year 2	Outputs	Target Date	Performance Targets
2A	Wine yeast Fermentome (database of fermentation essential	31/12/2014	Bioinformatic analysis of wine yeast Fermentome and comparison to laboratory yeast Fermentome (from UA1101) in order to identify up to 20

	genes): Stage 3 - final selection (to up to 20 genes).		wine yeast specific fermentation - essential genes/processes.
2B	Data demonstrating relative success of candidate strains from the DE experiments.	31/12/2014	Assess success of initial DE experiments to yield promising yeast and LAB strains on the basis of relative performance. Decide whether to abandon, continue or refine conditions of ongoing DE experiments. Initiate 2nd round of DE experiments for added attributes or strains.
2C	Documented outcomes from Annual Project Meeting.	31/05/2015	Hold Annual Project Meeting to review and discuss results and suggest future project plans (with relevant staff from AWRI and GWRDC).
2D	Summary of data on fermentation performance of promising candidate yeast (and available LAB) strains in laboratory -scale fermentations.	30/06/2015	High throughput screening of large number of isolates according to attribute in question. To be conducted concomitantly with Output FY2B and as candidates become available.
2E	Lead strains generated to date evaluated (kinetic and sensory) for practical suitability.	30/06/2015	WIC Winemaking Services to conduct large scale trials (-100 L) to obtain fermentation kinetic data. Preliminary sensory analysis to be conducted 'in house' and with industry representatives.
2F	Data from Years 1 and 2 (DE experiments and characterisation of 'Fermentome' database) disseminated through at least two extension mechanisms.	30/06/2015	Subject to any IP restrictions, preparation of papers for submission to peer-reviewed journals, and poster presentations at wine industry relevant conferences (e.g. CRUSH), describing properties of the initial DE strains generated/isolated and the wine yeast Fermentome.

Outputs and Performance Targets 2015 -2016

Year 3	Outputs	Target Date	Performance Targets
3A	Genome sequence of 1 - 3 promising isolates (yeast or bacteria).	31/03/2016	Genome sequencing and assembly of up to 2 LAB and 1 yeast isolate initiated as a route to determining the basis of their phenotype and to support IP protection. Final numbers will be determined by state of progress of DE and selection experiments.
3B	Summary of data on fermentation performance of promising	30/06/2016	Ongoing high throughput screening of large numbers of isolates of yeast and LAB according to attributes in question

	candidate yeast (and available LAB) strains in laboratory scale fermentations.		and subsequent assessment in lab scale fermentations.
3C	Summary of comparison of Fermentome data with genome sequences of evolved strains.	30/06/2016	Performance of a comparison of Fermentome vs genome sequence data to identify genes/processes etc relevant to fermentation and as the basis for possible strategies for better fermentation management or as additional opportunities for DE experiments. Prepare a manuscript for submission for publication in a peer-reviewed journal if appropriate.
3D	Genome sequences for additional yeast and bacterial isolates (up to 8).	30/06/2016	Genome sequencing continued from initiation in FY3a and used to characterise isolates (up to 4 LAB and 4 yeast across the project) as strains become available and data deemed useful.
3E	Data on practical suitability (kinetic and sensory) of strains generated to date.	30/06/2016	WIC Winemaking Services to conduct large scale trials (-100 L) to obtain fermentation kinetic data for additional strain(s) generated to date. Preliminary sensory analysis to be conducted 'in house' and with industry representatives.
3F	Documented outcomes from Annual Project Meeting and stop/go review point with GWRDC.	30/06/2016	Hold Annual Project Meeting to review and discuss results and suggest future project plans (with relevant staff from AWRI and GWRDC). Review progress with GWRDC and stop/ continuation /initiation decision made on specific strain attribute targets or newly arisen target priorities.

Outputs and Performance Targets 2016 -2017

Year 4	Outputs	Target Date	Performance Targets
4A	Recombinant 'proof-of-concept' strains.	31/12/2016	Construction of recombinant strains to confirm importance of any observed gene modifications or highlighted deletion mutants. Findings prepared for submission for publication in a peer-reviewed journal (subject to any IP restrictions).
4B	Documented outcomes from discussions with yeast and bacteria manufacturers for purposes of strain	31/12/2016	Discussions with various yeast and bacteria manufacturers (e.g. Lallemand, Mauri Australia, Christian Hansen and Laffort) with a view to commercialisation and to

	commercialization.		facilitate industry-wide trials from vintage 2017.
4C	Data on the characterisation of lead evolved strains.	30/06/2017	Most promising yeast and/or LAB strains (ideally at least one candidate for each target attribute) to have been evaluated as appropriate e.g. in fermentations up to 100 L (via WIC Winemaking) or 1OOO+L (via collaborating wineries [at no cost]), chemical and sensory analysis, genome sequencing.
4D	Documented outcomes from Annual Project Meeting.	30/05/2017	Hold Annual Project Meeting to review and discuss results and suggest future project plans (with relevant staff from AWRI and GWRDC).
4E	Project findings disseminated to Australian Wine Industry and researchers.	30/06/2017	Preparation and submission of at least 2-3 manuscripts in academic peer-reviewed journals, and at least 2 manuscripts in industry trade magazines. Where available, and subject to acceptance, at least 2 posters, at least 1 oral presentations, and at least 1 workshop (tastings) at suitable industry conferences.

Two consecutive 3 month extensions to the project were approved in order to complete work on UA1302 target/outcomes, as well as complete work from previous projects, as well as supervision of HDR students in the final stages of their Honours and PhD candidatures. The 3 project outputs 8 to 10 in the application are detailed below:

Output and related WA Project number	Performance Target	Target Date	Progress
7. UA1302	Upgrade of the T-Bot to enable analysis of 384 parallel fermentations(instead of 96)	30/12/2017	Yet test platform as 4 (96 x 30 mL) separate units
8. UA1101	Identification of yeast genes that affect the production of hydrogen sulfide	30/12/2017	Yet to resume ● Finalise sequence data for select genes in SAP pathway for parent and EMS mutants ● Write up as academic and/or industry articles for publication
9. UA1405	Identification of yeast genes that affect the colour of wine	30/12/2017	In progress ● Analyse fermentation and colour data ● Write up as academic and/or industry articles for publication
10. UA1101	Identification of genes that reduce fermentation duration when overexpressed	30/12/2017	Yet to resume ● Evaluate 60 strains in 100 mL fermentations ● Cloning of individual genes and repeat evaluation (as part of bilateral agreement)

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1.0 Generation of microbes with improved fermentation characteristics by Directed Evolution or classical methods

Output 1A: Data on strains and levels of key stressors for up to 3 improved attributes established for subsequent Directed Evolution (DE) studies.

- Preliminary screening trials to make initial selection of strains and preliminary evaluation of the levels of the key stressors to achieve the sought-after improvements.

Output 1D: At least 1 candidate 'fit-for purpose' yeast or LAB strain.

- Conduct DE experiments targeting up to 3 fermentation attributes/strains, development of screening conditions, intermittent screening of evolving populations, identification of candidate improved strains.

Output 2B: Data demonstrating relative success of candidate strains from the DE experiments.

- Assess success of initial DE experiments to yield promising yeast and LAB strains on the basis of relative performance. Decide whether to abandon, continue or refine conditions of ongoing DE experiments. Initiate 2nd round of DE experiments for added attributes or strains.

Contributors (Yeast DE)

Postdoctoral fellows: *Drs Michelle Walker, Tommaso Watson, Jennie Gardner, Joanna Sundstrom and Professor Vladimir Jiranek*

Contributors (LAB DE)

Current PhD students: Jiao Jiang
Postdoctoral fellows: *Dr Krista Sumby, Joanna Sundstrom, Associate Professor Paul Grbin and Professor Vladimir Jiranek*

Background

This study aimed to optimise *Saccharomyces cerevisiae* and lactic acid bacteria (LAB) including *Oenococcus oeni* and *Lactobacillus plantarum* for more efficient and reliable alcoholic and malolactic fermentation in juice and wine. This was done using a multipronged approach.

Directed Evolution of yeast was undertaken with a wide variety of starter cultures, and stressors, as well as employing both continuous and sequential batch culture. A panel of yeast with desirable attributes was initially evaluated for suitability and these were then chosen as

starter cultures for Directed Evolution in various stress media by sequential batch fermentation. We also utilised the classical techniques of mutagenesis and breeding (through hybridisation) to generate starter cultures with a greater genetic diversity. These techniques are used to increase the chances of generation of improved strains through DE. We also took advantage of previously generated evolved strains (FM16_C7H and Tee9; projects UA0501, UA1101) as genetic resources as well as starter cultures for further improvement. In the case of FM16_C7H DE was also undertaken in bioreactors where a combination of both batch and continuous culture with larger cultures was possible.

Improvement of lactic acid bacteria (LAB) was also tackled in a number of ways, using both classical microbial isolation techniques and Directed Evolution of strains from both our lab collection and industry. LAB improvement strategies comprised of 4 main approaches. Firstly Directed Evolution of a previously evolved ethanol tolerant strain, A90 (derived from SB3) was undertaken. In the initial stages of this project A90 was characterised for resistance to combined pH and ethanol stress in both MRSAJ and Red Fermented Chemically Defined Grape Juice Medium (RFCDGJM). A90 showed a similar viability in RFCDGJM compared to its parent, SB3, indicating the need for further improvement. DE was carried out to determine 1) if DE can be applied to further improve A90 in a wine-like environment using combinations of stressors to generate more superior strains with better general stress resistance; 2) how much further A90 could be developed. A continuous culture of A90 was established in a bioreactor and grown in a wine-like environment for approximately 350 generations with increasing ethanol and sulfur dioxide (SO_2), and decreasing pH over time.

The second approach used DE of *Lb. plantarum* strain K45 (Lallemand), which whilst able to conduct MLF when co-inoculated with yeast rapidly lost viability after approximately 3 days, and often failed to complete MLF. This was confirmed by testing for minimum inhibitory concentration (MIC's) of wine stressors, and subsequent DE in FCDGJM and RFCDGJM where supplementation with MRS was necessary to prevent cell death. *Lb. plantarum* is the LAB most typically used as an alternative to *O. oeni* in winemaking to carry out MLF. If a homofermentative strain is chosen *Lb. plantarum* will not increase volatile acidity and has a large potential to contribute positively to wine aroma. An important requirement of MLF is that the process is reliably completed in a timely manner. When an LAB starter strain is added to wine, it encounters multiple stressors, including low pH and high ethanol concentrations. Directed Evolution (DE) was used to improve *Lb. plantarum* for more efficient and reliable MLF. Many strategies were used during the project to obtain an improved phenotype of K45 including; UV mutagenesis, EMS mutagenesis, batch culture and continuous culture. Screening of isolates from these experiments was performed in Red FCDGJM at both the 0.2 mL and 50 mL scales to select for evolved strains.

The third approach used DE via continuous culture of Lalvin 4X (VL92) and Lallemand nuovi Ceppi Oo4 (KS21), chosen for previously characterised esterase (Matthews et al. 2006) and beta-glucosidase activities (Grimaldi et al. 2005). DE was performed as per A90, but hasher conditions (wine) were introduced earlier to see if it was possible to increase recovery of improved isolates.

The fourth approach involved strains were isolated from un-inoculated fermentations. Three *O. oeni* isolates, G20, G55 and G71 isolated from high ethanol Grenache (17%) and one *O. oeni* isolate recently isolated from Clare Valley Shiraz. These were screened in RFCDJM to test MIC's and in wine for their suitability as new starter cultures.

Methods

M 1.1 Microbial Strains

Yeast strains and media

Yeast strains were routinely kept at 4 °C on YPD agar plates (1% yeast extract, 2% bactopeptone, 2% dextrose, 2% agar) for short term storage. DE cultures (50 mL) from each batch fermentation were centrifuged at 5000 rpm, 5 min and the cell pellet resuspended in 5 mL YPD containing 26% glycerol) before storage at -80 °C. Yeast were sporulated using PRE5 (0.8% yeast extract, 0.3% Bacto Peptone and 10% glucose) and SPOR2 (0.5% potassium acetate) media (Codón et al., 1995). Yeast strains used in this study are described in **Table 1.1**.

Table 1.1: Yeast strains used in this study

Yeast Strain	Strain description/ oenological attribute	Source
F2 hybrid set (EC1118 x FM16 C7H)		
166 F2 hybrids	F2 spore progeny from F1 hybrid 3-13. 34 tetrads (136 spores) and 30 random spores	UA0501, Jiranek lab
Stage 1 DE		
Maurivin B	Malic acid utiliser	Maurivin Australia
Lalvin 71B *#	Malic acid utiliser, fruity aroma character	Lallemand
Uvaferm 43 #	Robustness, restart yeast for stuck fermentations	YSEO, Lallemand
Fermichamp® #	Fructophilicity, restart yeast	Oenobrand
Rhone Lalvin 2056 (L2056)	Varietal character, low SO_2	YSEO, Lallemand
FM16 C7H *# (C7H)	Evolved strain of L2056, improved robustness	McBryde et al 2006, Jiranek lab
Enoferm Simi White TM	Aroma	Enoferm, Lallemand
Tee 9 *#	Evolved strain of AWRI 796, improved fructophilicity	Liccioli, 2010; Jiranek lab
Lalvin QA23TM	β-glucosidase activity, increased thiols	YSEO, Lallmand
Q7 *#	Evolved strain of QA23, improved proline utilisation	Long, 2014; Jiranek lab
Q2 *#	Evolved strain of D254, improved proline utilisation	Long, 2014; Jiranek lab
C7H BIO **	FM16 C7H, mixed population, evolved in bioreactor for 90 generations (batch culture)	This study
DE Stage 2		
E3**	Hybrid (FM16 C7H x Q7)	UA1101
Q1**	EMS mutant of QA23, salt tolerant	UA1101

E44	EMS mutant of 71B, increased malic acid utilisation	This study
M16	EMS mutant of 71B, increased sugar utilisation	This study
P2-1	EMS mutant of 71B, increased malic acid utilisation	This study
RM1**	Random sporulated and mated population; FM16 C7H x Q7	This study
RM2	Random sporulated and mated population; FM16 C7H x Tee 9	This study
RM3**	Random sporulated and mated population; Q7 x Tee 9	This study
RM4**	Random sporulated and mated population; Q7 x 71B	This study
RM5**	Random sporulated and mated population; FM16 C7H x 71B	This study
RM6	Random sporulated and mated population; Tee 9 x 71B	This study
RM7**	Random sporulated and mated population; Uvaferm 43 x Q2	This study
RM8**	Random sporulated and mated population; Fermichamp x Q2	This study
RM9	Random sporulated and mated population; Uvaferm 43 x Maurivin B	This study
RM10**	Random sporulated and mated population; Fermichamp x Maurivin B	This study
RM11**	Random sporulated and mated population; FM16 C7H, Q7, Tee 9, 71B, Uvaferm 43, Q2, Fermichamp, Maurvin B	This study
Mixed culture**	FM16 C7H_A, C7H_B, C7H BIO_A, and C7H BIO_B, Q1, E3, E44	This study
C7H_BIO_CC**	C7H BIO mixed culture further evolved in continuous culture for 160 generations. C7H BIO_CC 50G and C7H BIO_CC 100G are the 50 and 100 generation mixed cultures	This study

* strains used in Stage 1 DE experiments
# strains used in *en masse* sporulation and mating
** DE populations evaluated either at 0.2 mL or 100 mL scale for fermentation performance

Bacterial strains and media

The bacteria used in this study are described in **Table 1.2**. Initial growth of strains was in MRS (Amyl Media) supplemented with 20% Apple Juice (Golden Circle or Coles brand) (MRSAJ). Bacterial strains were cultured from glycerol stocks kept at -80 °C in MRSAJ with 30% glycerol. Low pH modified MRSAJ agar (MRSAJ_pH3.5) and CDGJM:MRSAJ agar (50:50 at pH 3.5) were used for screening isolates after UV and EMS mutagenesis. MRSAJ_pH3.5 was made by washing (3x) and autoclaving 2x bacteriological agar separately to 2x MRS containing 10 g L^{-1} L-malic acid. After autoclaving 20% apple juice was added to the combined agar and MRS along with 1 ml filter sterilised bromophenol blue solution (10 g L^{-1} stock) as a pH indicator. CDGJM:MRSAJ agar was made by washing (3x) and

autoclaving 2x bacteriological agar and then adding filter sterilised 50:50 mix of CDGJM and MRS (pH 3.5). For screening and DE experiments, Red Fermented Chemically Defined Grape Juice Media (RFCDGJM) was made by fermenting Chemically Defined Grape Juice Medium supplemented with 5% (v/v) grape tannin extract (GrapeEX, Tarac Technologies) with *Saccharomyces cerevisiae* (PDM, Lallemand) until dry (total sugar < 2.0 g L^{-1}). MRSAJ was supplemented with 5 g L^{-1} DL-malic acid (Sigma-Aldrich, 6915-15-7). MRSAJ and RFCDGJM were then modified by adding analytical grade 100% ethanol (Chem-Supply, 64-17-5). The pH of the media was adjusted to pH 3.5 with trace amounts of 10 M NaOH or 37% (v/v) HCl. Final ethanol concentration and pH of the media were checked by a Wine ME/DMA 4500M Alcolyser (Anton Paar) and a CyberScan 1100 pH meter (Eutech). Modified MRSAJ media and FCDGJM were filter sterilised (0.22 μm) before use.

M 1.2 Yeast strain identification using Delta PCR

Genomic DNA was extracted according to Adams et al. (1998). Strains were characterised by interdelta typing according to Legras and Karst (2003) using primers Delta 2 (GTGGATTTTTATTCCAACA) and Delta 12 (TCAACAATGGAATCCCAAC). PCR amplification was as follows: 95°C, 5 min (initial denaturation), 2 cycles of 95°C, 30 sec; 42 °C, 30 sec; 95 °C, 30 sec; 42 °C, 30 sec; 72 °C, 2 min, followed by 29 cycles of 95 °C, 30 sec; 45 °C, 30 sec; 72 °C, 2 min and final extension of 72 °C, 10 min. PCR reactions were held at 12 °C.

M 1.3 Generation of F2 hybrid progeny from a genetic cross between EC1118 and FM16 C7H

Intra-specific hybridisation of EC1118 and FM16 C7H (evolved from L2056) was reported in UA0501. One hybrid, H3-13, was plated on YPD agar for single colonies; two of which were sporulated and tetrads were separated using a dissecting microscope. The F2 spore progeny grown on YPD agar for 2 days before being grown in overnight in 96-well plates in YPD at 28 °C prior to storage as glycerol stocks.

M 1.4 *En masse* sporulation and mating of yeast

Yeast strains were sporulated in liquid culture according to Codón et al. (1995) with modifications. Briefly, stationary-phase culture grown in YPD was diluted 1 in 100 into 10 mL PRE5 medium and grown 4 h at 30 °C with shaking. The cells were then centrifuged, washed twice with deionised water, and resuspended into 10 mL SPOR2 medium and incubated at 22 °C with shaking for 7 days. Sporulated cultures were stored at 4 °C up to 2 months prior to use. One mL cultures were washed in sterile deionised water and incubated with 15 μL β-glucuronidase from *Helix pomatia* (Sigma) overnight at 30 °C and stored at 4 °C up to 1 week prior to use. Rare (spore-spore) mating was undertaken using 0.25 mL of each strain which were mixed. The cells were centrifuged (3000 rpm), resuspended in 1 mL YPD and incubated 2 days at 22 °C. The mated cultures (1 mL; RM 1-11, Table 1) were added to individual flasks containing 100 mL Stress Medium C (Stage 2 DE, batch 18).

Table 1.2: Bacterial strains used in this study

Bacterial Strain	Strain description/ oenological attribute	Source
Preliminary screening trials & DE round 1		
A89	*O. oeni* DE strain derived from SB3	Alice Betteridge (GWR PH 1308)
A90	*O. oeni* DE strain derived from SB3	Alice Betteridge (GWR PH 1308)
K45	*Lb. plantarum* not lasting for entire MLF duration (if more than 3 days)	Lallemand
KS72	Clonal isolate of K45	This study
KS78	UV treated K45 isolate	This study
DE Round 2		
KS2 (Lalvin 4X (VL92))	*O. oeni* strain, chosen for previously characterised esterase and beta-glucosidase activities	Laffort
KS21 (Lallemand nuovi Ceppi Oo4)	*O. oeni* strain, chosen for previously characterised esterase and beta-glucosidase activities	Lallemand
Isolates from un-inoculated ferments		
O. oeni strains G1, G5, G8, G20, G26, G28, G39, G46, G47, G48, G41, G55, G63, G66, G71, G72, G83, G84, G92, G99, G101, G106, G107 and G108	Isolates from an un-inoculated ferment vintage 2012 (high ethanol Grenache)	Barossa Valley
Lb. hilgardii strains G76, G102 and G103 (unpublished data)	Isolates from an un-inoculated ferment, vintage 2012 (high ethanol Grenache)	Barossa Valley
Oe16	Isolate from un-inoculated ferment, vintage 2016 (Shiraz)	Clare Valley, Adelina wines

M 1.5 Micro-fermentation screens for tolerance to single stress conditions: Yeast

Nine wine yeast (2 clonal isolates of each strain) were evaluated in CDGJM with 4 stress conditions: **1**: 200 g L^{-1} sugar and 450 mg FAN L^{-1}, pH 3.5; **2**: 200 g L^{-1} sugar and 450 mg FAN L^{-1}, pH 2.8; **3**: 200 g L^{-1} sugar and 450 mg FAN L^{-1}, pH 3.5 + 5% ethanol and **4**: 320 g L^{-1} sugar and 450 mg FAN L^{-1} pH 3.5. Strains were grown in 10 mL YPD overnight, and inoculated at 2.5 x 10^6 cells mL^{-1} into 10 mL CDGJM starter medium. 0.2 mL of the starter culture was diluted with 0.8 mL fresh starter medium and used to inoculate 4 microtiter plates

for each stress condition (10 µL culture into 190 µL medium). The plates were sealed with breathable clear film and incubated at 30 °C in a CO_2 incubator at 20% CO_2 and 70-85% humidity. At specific intervals during fermentation, one plate was removed, re-sealed with an impermeable film and stored for sugar analysis.

M 1.6 Micro-fermentation evaluation of F2 hybrids originating from H3-13 (EC118 x FM16 C7H)

Fermentation performance was evaluated in micro-fermentation scale in CDGJM with (i) 200 g L^{-1} sugar, 468 mg N L^{-1}, (ii) 320 g L^{-1} sugar, 450 mg N L^{-1} and (iii) 260 g L^{-1} sugar, 90 mg N L^{-1} as described above.

M 1.7 10 mL screens for tolerance to single and multiple stress conditions: Bacteria

Pre-cultures from glycerol stocks were incubated at 30 ˚C, for 5 days for *O. oeni* and 2 days for *Lb. plantarum*, in 8 mL of MRSAJ medium. The liquid cultures were then diluted with fresh MRSAJ to an optical density 600 nm (OD_{600}) of 1.0 (Helios Cuvette spectrophotometer, Thermo Scientific) before 500 µL of *O. oeni* or 250 µL of *Lb. plantarum*, was added to 8 mL fresh MRSAJ. After a 24-hour incubation at 30 ˚C, cells were in the middle of exponential phase (OD_{600} = 0.8–1.0) and harvested. The cultures were then re-diluted with MRSAJ to adjust their OD_{600} to 0.5 before being inoculated into experimental media (250 µL into 8 mL) as described in **Table 1.3**. All cultures were incubated at 22 ˚C for 10 days. Each culture was prepared in triplicate.

After inoculating *O. oeni* into experimental media, samples were taken at time points between 0 and 240 hours. A ten-fold dilution series of each sample (10^{-1}–10^{-5}) was made and 5 µL of culture spotted onto the surface of MRSAJ agar plates, which were then incubated at 30 ˚C with 20% (v/v) CO_2 for 2-7 days (depending on species). Colony forming units (CFUs) were counted.

Table 1.3: Media used in bacterial stress tolerance assays

Media	pH range or fixed value	Ethanol range or fixed value % (v/v)	SO_2 ppm	Sugar g L^{-1} (Glu:Fru 50:50)	Malic acid g L^{-1}
MRSAJ-1	5.5	12–18 (2% (v/v) increments)		< 5	5
MRSAJ-2	2.8–3.6 (0.2 increments)	0		< 5	5
MRSAJ-3	2.8–3.6 (0.2 increments)	12		< 5	5
MRSAJ-4	3.5	12–18 (2% (v/v) increments)		< 5	5
MRSAJ-5	3.5	12.5	0-50 (10 ppm increments)	< 5	5
FCDGJM-1	2.8, 3.1, 3.3, 3.5	12		< 5	3.5
FCDGJM-2	3.5	14–18 (2% (v/v) increments)		< 5	3.5
RFCDGJM-1	2.8–3.6 (0.2 increments)	12		< 5	3.5
RFCDGJM-2	3.5	12–18 (2% (v/v) increments)		< 5	3.5
RFCDGJM-3	3.5	12.3%	0-40 (10 ppm increments)	< 5	3.5
RCDGJM	3.5	2		260, 290, 320	3.5

M 1.8 Micro-fermentation screen for malic acid utilisation

Malic acid utilisation in yeast

Micro-fermentations were similarly conducted in malic acid containing media under aerobic (in a humidified plastic box) and reduced O_2 conditions (in CO_2 incubator). Synthetic starter medium was used in place of CDGJM starter medium. Four conditions were evaluated: Condition E (2% malate medium pH 3.5; aerobic growth); Condition F (2% malate medium pH 6.0; anaerobic growth); Condition G (2% malate medium pH 3.5; anaerobic growth); Condition H (2% malate medium pH 6.0; aerobic growth). Growth was measured as optical density at 600 nm (OD_{600}) on an Infinite® 200 PRO microplate reader (Tecan Group Ltd) at the start of the experiment and daily. Media formulation is described in **Appendix 5**. L-malic acid was measured enzymatically using thawed samples (Vintessentials L-Malic Acid Enzymatic Analysis Kit; 4A165); with volumes reduced to suit microtiter plates.

Malic acid utilisation in bacteria

Characterisation of K45, SB3 and A90 was conducted in MRSAJ and RFCDGJM with 15%

(v/v) ethanol. Preliminary characterisation of DE isolates was performed in micro-scale fermentations in micro-titre plates when LAB were evolved for approximately 150, 250 and 350 generations using the method described by Betteridge et al. (2017). Randomly picked colonies were inoculated into MRSAJ in deep-well plates, grown for 3 days at 30 ˚C before a 1 in 4 dilution in fresh RFCDGJM and introduction of 10 µL of each culture into 190 µL of the appropriate medium in a microtitre plate. Replicate plates (10 copies) were used to allow sacrificial sampling at the required intervals. Plates sealed with titre tops were incubated at 22 ˚C for 12-20 days with 20% (v/v) CO_2. L-malic acid was measured using an enzymatic test kit (4A165, Vintessential laboratories, Australia) with modifications so that a Tecan spectrophotometer (Infinite 200 PRO, Tecan, Männedorf, Switzerland) could be used to read absorbance. Specifically, each well of a 96 well micro-titre plate was dosed with 70 µL buffer, 14 µL nicotinamide adenine dinucleotide, 70 µL distilled water, 0.7 µL glutamate oxaloacetate transaminase and 5 µL sample or one of the standards (ranging from 0–3.0 g L^{-1}). The plate was incubated at 22 ˚C for 3 minutes and the first absorbance was read at 340 nm; 7 µL of the 1:10 diluted L-malate dehydrogenase was added and mixed into each well; the plate was incubated at 22 ˚C for 15 minutes before the second absorbance was measured at 340 nm. L-malic acid in each sample was calculated from standard curves prepared with known concentrations. Strains that fermented more efficiently were then evaluated by laboratory-scale fermentations in 15 or 50 mL tubes in various media. The best performing strains were further characterised in red wines. To monitor laboratory-scale fermentations, 200 µL samples were collected aseptically every 24 to 48 hours. Half of the sample was immediately frozen at -20 ˚C for subsequent analysis of L-malic acid, while the remainder was used for microbial enumeration. All fermentations were conducted at 22 ˚C. The micro-scale screening experiments were performed in quadruplicate while all larger laboratory-scale fermentations were performed in triplicate.

M 1.9 Evaluation of fermentation performance in 100 mL scale using automated robotic platform (T-bot)

A robotic fermentation platform based on the Tecan Evo 200 was developed for automated sampling of 96 fermentation bottles (refer to UA1101). Fermentations were performed individually or in triplicate in 100 mL CDGJM or 0.45 µM filtered white juice. Starter cultures in YPD were grown 48 h prior to inoculation at 1%. Fermentation temperature was dependent upon individual experiments.

M 1.10 Sequential batch DE of microbes

Sequential batch DE of yeast: flasks

Batch DE fermentations were conducted using 100 mL CDGJM in Erlenmeyer flasks fitted with glass airlocks. The composition of the medium was varied throughout the Stage 1 and Stage 2 DE experiments, as shown in the **Appendix 5**. Yeast strains were inoculated into CDGJM starter medium using ~0.5 mL glycerol cultures and grown for 48 h. Cell numbers (total and viable) were measured on the FACSCalibur instrument using FACS analysis after staining with propidium iodide. Fermentations were inoculated to 2.5 x 10^5 viable cells mL^{-1}, and cell numbers recalculated. Cell numbers were measured throughout fermentation in order to determine the number of generations obtained per batch fermentation.

Sequential batch DE of yeast strain FM16 C7H: bioreactor

FM16 C7H was used for sequential batch fermentation of 500 mL of CDGJM (30 g L^{-1} fructose, 10 g L^{-1} glucose, 150 mg N L^{-1}), with increasing ethanol concentration (from 8 to 12.5%; see Supplementary data) in a 1L bioreactor (*BIOSTAT® A plus*, controlled using the *MFCS/DA A plus 2.1* software - Sartorius BBI System GmbH). After exhaustion of all sugars

(< 2.5 g L^{-1}), an aliquot of culture was retained to inoculate fresh medium at 5 x 10^6 cells mL^{-1}. This process was repeated 25 times with an average cell growth of 3.7 generations in each batch, totalling ~90 generations of growth under increasing selective pressure.

Sequential batch DE of lactic acid bacteria

Lb. plantarum K45 was subjected to both continuous and batch culture throughout the duration of the project. Initial batch DE fermentations were conducted using 500 mL MRS, pH 3.5 (5 g L^{-1} L-malic acid and tartaric acid) with increasing ethanol. Cell biomass was from a mixed population directly from a freeze dried sample (Lallemand). Each batch lasted 10 days. This resulted in cell death by batch #3 (pH 3.5, 12% ethanol), thus it was decided to trial batch ferments in FCDGJM (1.6 g L^{-1} total SO_2) and Grenache wine (8 g L^{-1} total SO_2) using the following conditions **(Figure 1.1)**.

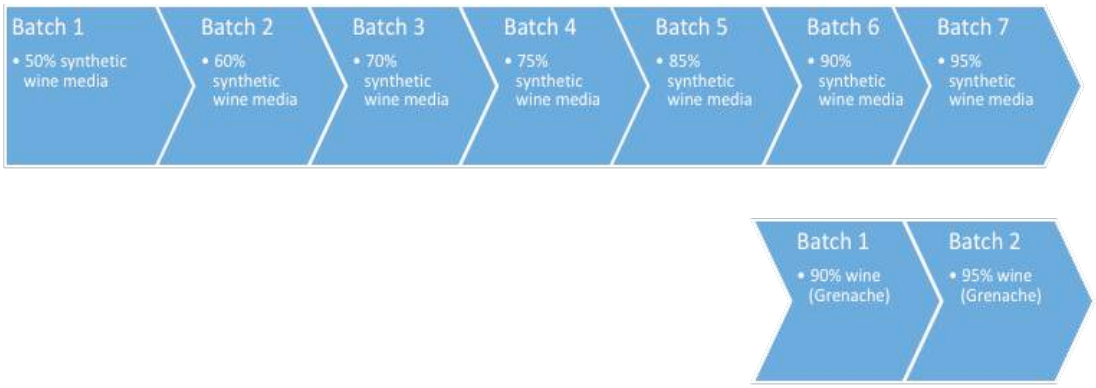


Figure 1.1: Initial batch DE cultures for *Lb. plantarum* K45.

Batch cultures of *Lb. pantarum* K45 were reinitiated in either FCDGJM or Grenache, starting at 50:50 FCDGJM or Grenache:MRS and finally dropping to 5% MRSAJ in Schott bottles (Batch 7) with increasing ethanol concentration. From Batch 7 ethanol was increased and pH dropped to 3.3 over the DE experiment until Batch 12, with an average cell growth of 5 generations each batch, totalling ~60 generations of growth under increasing selective pressure. Additionally batch culture was performed following UV and EMS mutagenesis (see below), as described in method section M 1.12.

M 1.11 Continuous DE of microbes in a bioreactor

Continuous DE of FM16 C7H in a bioreactor

The previously evolved C7H (90 generations) population was grown in continuous culture for 6 months in a medium consisting of white juice:H_2O:YPD (45:45:10). After inoculation of 500 mL of sterile medium in the 1L bioreactor, a steady-state equilibrium was initially created with a dilution rate of 0.18 h^{-1} (i.e. 90 mL h^{-1} were continuously fed into the 500 mL volume in the bioreactor). The cell concentration at this equilibrium was measured with a turbidity probe, registering a value of ~1.5 units (corresponding to ~1.18 cells mL^{-1}). To control the level of stress for the DE, a "feedback loop" system was used, similar to that described by Brown and Oliver (1982). Briefly, a stressor was added every time the value of the turbidity probe was above 0.9 (corresponding to a cell density of ~10^7 cells mL^{-1}) and stopped once the cell density dropped below such set point. In this way, the population was allowed to maintain a minimum level of reproductive fitness in order to recover sufficient cell

density, before being completely washed out of the bioreactor. This approach guaranteed control of the concentration of the stressor to drive adaptation, while maintaining viability for an extended time. The stressor consisted of a solution of 100 g L^{-1} tartaric acid and 50% (v/v) ethanol. As the rate at which the stressor was added matched that of the feed medium, while the stressor was added, the dilution rate doubled (0.36 h^{-1}) and the speed of the exhaust pump was adjusted accordingly to maintain the operative volume of 500 mL constant.

The description of the algorithm used for this DE experiment was:

feed pump = always on (10%). if the TURB value > 0.9, the stressor pump switch on (10%) and the rate of the waste pump jumps from 10% to 20%.
Algorithm for the Sartorius software (where SUBSA was the feed pump, SUBSB the stressor pump and TURB the value of the turbidity probe): [SUBSA.Setpoint = (((TURB.Value < 0.9) * 0) + (TURB.Value >= 0.9) * 10))]. [SUBSB.Setpoint = (((TURB.Value < 0.9) * 10) + (TURB.Value >= 0.9) * 20))].

The culture was sampled every ~50 generations and kept at -80 °C with glycerol (15% v/v) for subsequent measurement of fermentation kinetics compared with the parental strain (see Output 1D).

Continuous DE of LAB in a bioreactor

The establishment of a continuous DE system using a sterilised Biostat A plus bioreactor (Sartorius BBI system GmbH, Germany) was based on the method previously used in this laboratory (Betteridge et al. 2017) with a few modifications. DE began in 1100 mL of either FCDGJM (*Lb. plantarum*) or RFCDGJM (*O. oeni* A90, *O. oeni* KS2, *O. oeni* KS21) at pH 3.5 with 10.93% (v/v) ethanol at 22 °C. When the bacteria were in late-exponential phase, fresh RFCDGJM supplemented with extra ethanol was fed into the bioreactor continuously at a rate of 12 mL/h to maintain turbidity of 0.07-0.11, which was equivalent to an OD_{600} of 0.3-0.6. Ethanol concentration was increased gradually to 13.5% (v/v) and pH was decreased to 3.3, over 350 days. For all *O. oeni* strains DE was then moved to a second stage by gradually changing the medium to wine: 2015 Shiraz and RFCDGJM were supplied together to the culture, with the Shiraz feed gradually increasing over 70 days (MFCS/Win v3.0 config 2355 software; Sartorius BBI system GmbH, Germany) and according to a set culture turbidity as described. DE was terminated when the ethanol concentration reached 16.5% (v/v). Samples from the bioreactor were collected weekly for L-malic acid analysis and enumeration of bacteria. The culture was sampled every ~25 generations and kept at -80 °C in glycerol (15% v/v) for subsequent measurement of fermentation kinetics compared with the parental strain (see Output 2D).

M 1.12 Mutagenesis of *Lb. plantarum*

An overnight culture of *Lb. plantarum* was grown in MRSAJ from a freeze-dried packet (Lallemand). The liquid cultures were then diluted with fresh MRSAJ to an optical density 600 nm (OD_{600}) of 1.0, which previous cell growth experiments had shown to be ~1.6 x 10^8 CFU/mL for this strain. 2 mL was inoculated into 100 mL of MRSAJ (Schott bottle). After a 12 h incubation at 30˚C, OD_{600} was measured and cells were diluted to OD_{600} = 1 in MRSAJ and 250 µL was aliquoted into 6 x 10 mL tubes containing CDGJM (pH 3.5, 12.25% ethanol) plus the treatments as displayed in the following matrix (**Table 1.4**).

Table 1.4: Matrix of ethanol shock treatments pre-mutagenesis (16 treatments total)

pH	Ethanol (%)			
3.5	12.25	14	16	18
3.3	12.25	14	16	18
3.1	12.25	14	16	18
2.8	12.25	14	16	18

Cells were incubated for 1 h at 21 °C then centrifuged for 10 min at 5000 rpm. Cells were washed 2x in 100 mM potassium phosphate buffer (pH 7.5) and resuspended in 10 mL of MRSAJ. Spot plates were used as described in previous methods to calculate % lethality. Lethality was calculated using the following formula:

$$\text{Lethality (\%)} = (1-N_A/N_C) \times 100\%$$

Where N_A is CFU of sample treated with decreasing pH and increased ethanol and N_C is CFU of the control sample (CDGJM no additions). The optimal shock condition is calculated on the basis of the sub-lethal dosage (~ 50% lethality). Once the optimal condition for shock was established, UV mutation was performed under acidic stress conditions.

An overnight culture of *Lb. plantarum* was grown in 10 mL MRSAJ, from a mixed population stored at -80°C in glycerol. The liquid cultures were diluted with fresh MRSAJ to OD_{600} of 1.0, and 2 mL was inoculated into 100 mL of MRSAJ (Schott bottle). After a 12 h incubation at 30˚C, OD_{600} was measured and cells were diluted in potassium phosphate buffer (pH 7.0) in two separate bottles to either OD_{600} = 1 or either OD_{600} = 0.5 and 250 µL was aliquoted into 3 x 10 ml tubes containing CDGJM (pH 3.1, 12.25% ethanol). UV mutagenesis was carried out using a UV cross-linker (UVItec CL-508) in a petri-dish for 0, 5, 10, 15, 20, 25, 30, 45, 60, & 75 sec. Samples were covered in foil and serial dilutions were plated onto MRSAJ agar and incubated in the dark (to prevent reactivation) at 30˚C with 20% (v/v) CO_2 for 2 days before CFU's were counted. Lethality was calculated using the following formula:

$$\text{Lethality (\%)} = (1-N_T/N_U) \times 100\%$$

Where N_T is CFU of sample exposed to UV and N_U is CFU of the control sample (T=0). The optimal UV exposure time was calculated on the basis of the sub-lethal dosage (~ 50% lethality). The UV mutagensised cells were screened using micro-fermentation and one UV strain was selected to be included in EMS mutagenesis.

The same acid shock method was used with EMS (ethyl methane sulfonate) mutagenesis. Initial EMS experiments used *Lb. plantarum* K45 cells diluted to 1 x 10^8 CFU/mL in MRSAJ with the addition of either 0 µL/mL (control), 12 µL/mL or 45 µL/mL EMS for 2 h. Cells were incubated at 21 ˚C in a rolling shaker and 750 µL samples were taken every 15 min. An equal volume of 5% sodium thiosulfate was added to the sample, prior to mixing and centrifugation. The cell pellet was washed twice in wash buffer (100 mM potassium phosphate, pH 7.5) and resuspended in MRSAJ. Serial dilutions were plated onto MRSAJ agar to plot a 'kill' curve. The rest of the sample was stored in glycerol (30% final concentration) at -80 ˚C.

EMS treatment failed to kill the *Lb. plantarum* K45 cells even after 2 hours and the protocol was modified. Briefly for the next round of EMS mutagenesis *Lb. plantarum* K45 cells or *Lb. plantarum* K45_UV mutagenised cells (KS78) were diluted to 1 x 10^8 CFU/mL in MRSAJ with the addition of either 0 µL/mL (control) or 120 µL/mL EMS for 4h. Cells were incubated at 28 ˚C in a rolling shaker and sampled every 30 min. Sodium thiosulfate was added and cells were washed, plated and stored as before. Batch fermentation was undertaken in Schott bottles after both UV and EMS mutagenesis in fermented chemically defined grape juice media (FCDGJM) and MRS for ~150 generations.

M 1.13 Screening LAB for improved MLF performance.

The generation of new 'fit-for-purpose' bacteria within the timeframe of the project was reliant on DE experiments being conducted on a number of bacteria using multiple stressors as well as generating increased genetic diversity through UV and EMS mutagenesis (*Lb. plantarum*). Two strains were initially chosen - an *O. oeni* (A90) and a *Lb. plantarum* (K45). With the success of the previous proof-of-concept directed evolution (DE) in *O. oeni*, a new DE was carried out to determine if DE could be applied to further improve A90 in a wine-like environment using combinations of stressors to generate more strains with better general stress resistance. A continuous culture of A90 was established in a bioreactor (PhD student Jiao Jiang, GWR PH 1308). Samples of the population in the bioreactor were collected at three significant times during the DE to screen for improved isolates based on L-malic acid consumption and growth.

Isolates from both continuous and batch cultures were tested for fermentation performance using micro-fermentation screening techniques (**Figure 1.2**). When screening L-malic acid consumption using sacrificial plating (96 well plates) of mixed isolates from various stages of the DE experiment was used as an indicator of MLF performance capability of each isolate. Included in screens were the parent strain and mixed population samples from current bioreactor and batch experiments. Clonal isolates were evaluated before a decision was made to modify strains/conditions or to continue with the DE strategies.

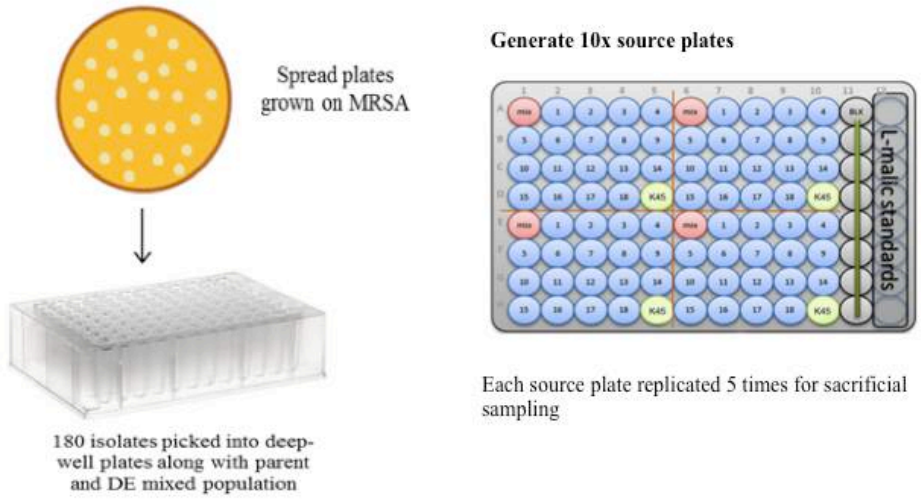


Figure 1.2: Procedure for high throughput screening of LAB for improved MLF performance.

M 1.14 Evaluation of un-inoculated ferment sample for MLF performance

With a view towards increasing our toolbox of LAB isolates, *O. oeni* and *Lb. hilgardii* clonal isolates from a high ethanol Grenache fermentation were also investigated for MLF efficiency. MLF performance in RFCDGJM was evaluated alongside previous DE strains (A90 and A89) and their parent strain SB3. AFLP data (unpublished) was used to construct a phylogenetic tree depicting evolutionary relationships between different *O. oeni* strains. Twenty four unrelated *O. oeni* strains together with 3 *Lb. hilgardii* clones isolated from the Grenache, were tested. Superior L-malic acid consumption performance was screened for as described in M 1.13 using FCDGJM pH 3.4, 15% (v/v) ethanol.

Summary of Outcomes

Improvement of yeast and lactic acid bacteria was undertaken on a wide variety of starter cultures, selected from a panel of strains with desirable attributes. The magnitude of these desirable attributes was quantified in this project. It was important to do this since fermentation outcomes can be widely variable depending on experimental set up.

Specific stressors were chosen e.g. matching those that Australian winemakers wish to see overcome (as communicated by Wine Australia). We examined the effect on bacterial growth and malolactic fermentation of the addition of ethanol, sulfur dioxide and medium chain fatty acids as well as the reduction of pH. The effect of the combined stress of high sugar and low pH on yeast growth, alcoholic fermentation and malic acid utilization (by yeast) was measured as well as the effect on growth of additions of ethanol, high/low temperature, free sulfite and concanamycin A on solid media. Comparative performance for each yeast and bacterial strain gave a clear indication of strain capabilities and also provided an initial benchmark on which attributes could then be improved upon. This information is of great value to industry as it provides an independent evaluation of comparative strain performance.

Both classical techniques of mutagenesis and breeding as well as the modern approach of directed evolution were used. Single isolates from each treatment were analysed to detect improvements in fermentation dynamics.

Twelve wine yeast were chosen as source strains for improvement and these were subjected to two conditions of directed evolution (i) tolerance to the combination of high sugar and ethanol and low pH and (ii) improved fructose utilization and ethanol tolerance. Later saturated fatty acids (sub-products of ethanolic fermentation) were also added to further increase stress. 25 culture/medium combinations were evolved for up to 158 generations and the fermentation performance of approximately 90 isolates of 14 of these pools were analysed for improvement. Analysis was performed in micro-fermentations in different media/juice types as per executive summary, so as to capture the isolates capable of improved performance under varied conditions. This culture collection of over 1500 strains represents a diverse array of evolved strains and is an excellent resource available for future screening.

The fermentation performance of our in-house collection of yeast hybrids (250 strains generated by breeding) was also analysed. Three strains with superior performance (Q1, E3 and M16) were evaluated in 8 different juices and fermentation performance was found to be similar to the well known robust and efficient wine yeast strain Uvaferm 43. These strains represent excellent alternatives for rapid fermentation where winemakers wish to diversify their microbial toolkit.

The effort to improve lactic acid bacteria focused on multiple strain sources; for instance, A89 and A90 (previously evolved in-house from the commercially available Laffort strain SB3), Laffort sourced strain Lalvin 4X (VL92), Lallemand strains, K45 (*Lb. plantarum*) and nuovi Ceppi Oo4 (KS21) as well as 28 strains (*O. oeni* and *Lb hilgardii*) isolated in-house from un-inoculated wine fermentations.

The previously evolved, ethanol-tolerant lactic acid bacteria (*O. oeni*) A90, derived from the commercial strain SB3 was evolved for a further 350 generations with increasing ethanol and sulfur dioxide and decreasing pH. SB3, developed and distributed by Laffort is marketed as being "adapted to the most difficult conditions, including stuck malolactic fermentation". In commercial circumstances it is reported to be able to withstand up to 15% alcohol, a pH of 3.1 and up to 40 mg/L of total sulfur dioxide. In this study, further evolved isolates were capable of up to a 50% reduction (9 vs 18 days) in fermentation duration in a number of wines. Thus we expect use of these strains in industry would result in decreased duration and increased reliability of malolactic fermentation, particularly in harsh conditions. These strains are also now being evaluated for commercialization by Laffort to the wine industry.

Lb. plantarum strain K45 was provided by Lallemand as a strain of interest, since it has excellent potential for rapid malolactic fermentation and positive contributions to mouthfeel. However this strain rapidly loses viability when co-innoculated with yeast and consequently MLF often fails to be completed. Directed evolution was undertaken on this strain for 300 generations in increasing ethanol and decreasing pH. Isolates tested had improved growth but unfortunately malolactic fermentation was not improved concomitantly. This raises the interesting possibility that these two biological processes can be uncoupled.

Directed evolution was also undertaken on *O. oeni* strains KS2 and KS21 in conditions of increasing ethanol, sulfur dioxide and decreasing pH. KS21 was evolved for 320 generations and 15 isolates were chosen for further evaluation with a view to the best isolates being commercialised. KS2 could not be resurrected after 320 generations, future studies will test earlier generational isolates.

Results and Discussion

Output 1A 1.0 Preliminary screening trials for selection of yeast strains and key stressors to achieve sought-after outcomes

Twelve wine yeast were evaluated for their ability to complete fermentation in 100 mL of modified CDGJM (320 g L^{-1} sugar, 300 mg L^{-1} FAN, 5 g L^{-1} L-malic pH 2.8, adjusted with L-tartaric acid) in Erlenmeyer flasks (**Figure 1.3**). No strains were able to complete fermentation, becoming stuck after 540 hours. Malic acid consumption was minimal for all strains (ranging from 4% for Q2; to 17-18% for 71B and Maurivin B, respectively). These strains are reported to metabolise up to 20-40% and 56% of malic acid, respectively, which is useful for acid reduction of highly acidic juices. 71B was of particular interest, being associated with a characteristic fruity aroma due to the production of stable esters.

The effect of the following stress factors on fermentation outcome was evaluated: high sugar (from 200 g L^{-1} to 320 g L^{-1}), low pH (from pH 3.5 to pH 2.8) and addition of ethanol (5%). Thus the following three fermentation attributes were analysed; capability to ferment high concentrations of sugar at increased acidity and in the presence of elevated ethanol. Nine strains were fermented in CDGJM incorporating single stress factors in micro-fermentations. Growth was affected in the high sugar medium or when ethanol was added, resulting in increased fermentation duration. Surprisingly, a decrease in pH to 2.8 did not adversely affect

growth or fermentation (**Supplementary Figure 1.1**). Malic acid consumption was evaluated separately; the 9 strains being grown in malic acid containing medium at pH 6 and pH 3.5 with and without 20% CO_2 to determine the conditions for optimum malic acid consumption (**Table 1.5**). Malic acid consumption by all strains was minimal throughout the duration of the fermentation (258 h). Malic acid consumption was best at pH 3.5 and in aerobic conditions, followed by anaerobic conditions (akin to wine fermentation conditions). Lalvin 71B was the best performing strain (12.6% utilisation at pH3.5, anaerobic conditions and 9.1% at pH 3.5, aerobic conditions). Later screening was performed in CDGJM (with 3 g L^{-1} malic acid) rather than this medium (see **Supplementary Figures 1.2 and 1.3**). Nevertheless, these findings are consistent with the optimal pH for de-acidification at 33.5, confirming that non-dissociated organic acids enter *S. cerevisiae* cells by simple diffusion (Delcourt et al 1995). The ability to degrade extracellular malic acid is dependent upon the facilitated diffusion of the dicarboxylic acid, as well as the efficacy of the mitochondrial NAD(P)-dependent malic enzyme, Mae1p, converting malic acid to pyruvate under anaerobic conditions (Redzepovic et al 2003). The authors allude to a differential expression of *MAE1* in 71B (malic acid utiliser) compared to EC1118 (a non-utiliser), which is dependent upon malic acid and glucose content. However, the late expression did not lead to increased enzyme activity (Redzepovic et al 2003). Whilst *Saccharomyces cerevisiae* is a poor utiliser of L-malic acid, our results indicate that malic acid and sugar consumption are 'coupled', with strains considered good utilisers (71B and Maurivin B) being slower to ferment than poor ones (**Supplementary Figure 1.3**). 71B was selected as a suitable candidate for further improvement via DE and EMS mutagenesis, because of its aroma attributes (Output 1D 1.1 and 1.4).

Other conditions examined in growth assays on solid media (YPD pH 3.5) with added single stressors were used to determine the tolerance of twelve strains to different concentrations of the following: ethanol (8%, 10%, 12%); concanamycin A (200 nM, 300 nM and 500 nM) – an inhibitor of vacuolar acidification: a process essential for pH homeostasis; temperature (8°C, 17°C, 30°C, 40°C), and free sulfite (12.5 mg, 25 mg per 50 mL YPD, pH 3.5). Growth on malic acid indicator plates (with and without glucose) was also tested. The results are reported in **Supplementary Table 1.1.** The toxicity of concanamycin A precluded its use in routine DE experiments, although it inhibited fermentation at 300 nM (Nguyen et al. 2018). Whilst growth sensitivity to this chemical on solid media was strain dependent, it is still useful as a selection for mutants following mutagenesis.

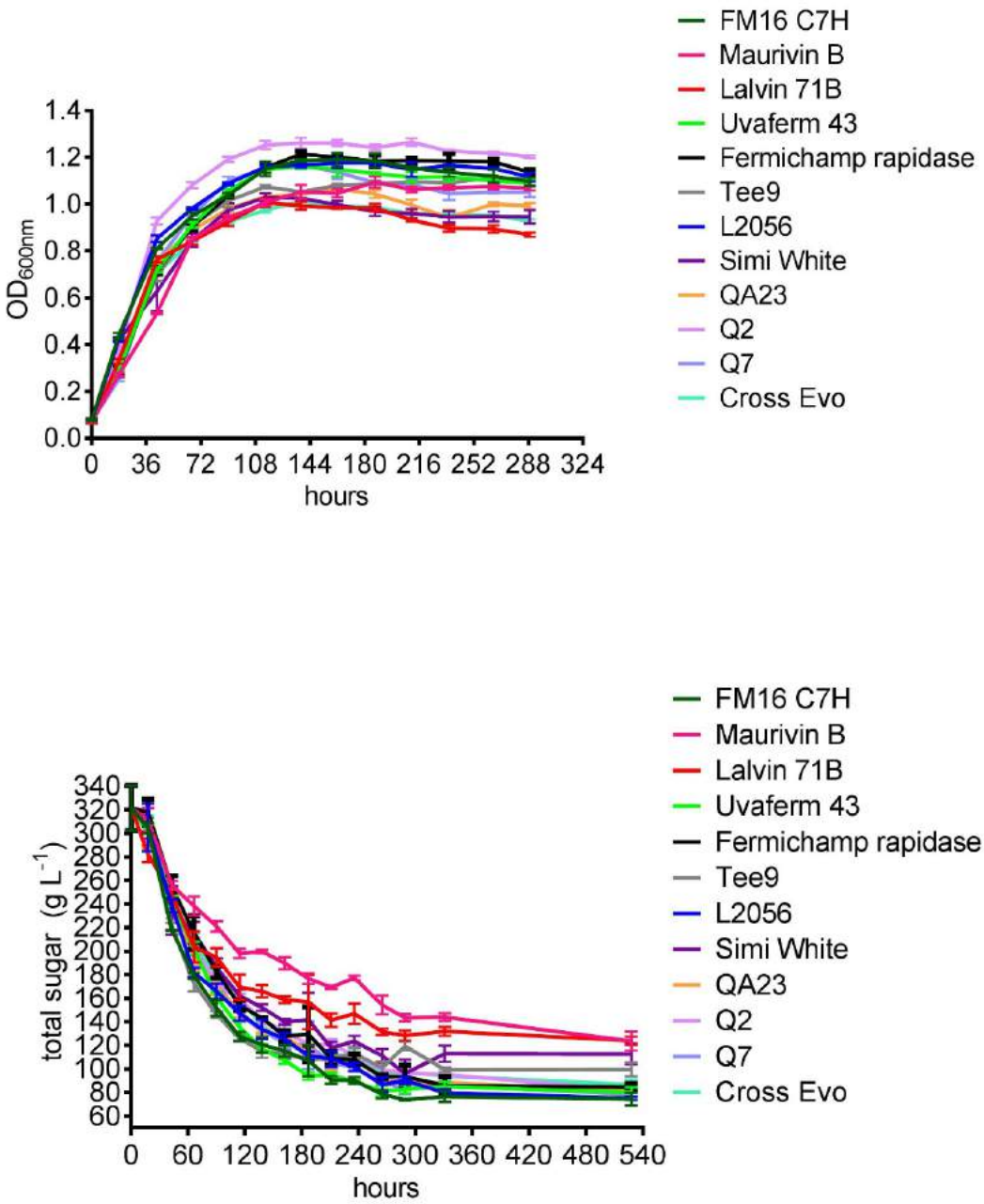


Figure 1.3: Evaluation of the fermentation performance of 12 wine yeast in modified CDGJM.
Fermentations were performed at 28 °C in 100 mL CDGJM (320 g L^{-1} sugar, 300 mg L^{-1} FAN, 5 g L^{-1} L-malic, pH 2.8) at 28 °C. Strains were initially propagated in YPD, followed by CDGJM starter medium. Fermentations were inoculated at 5 x 10^6 cells mL^{-1}. The sugar utilization profiles (total sugar g L^{-1}) and growth (OD_{600}) are shown. Values are the average of 3 replicates ± SD.

Table 1.5: Micro-fermentation evaluation for malic acid utilisation by commercial wine yeast

		% utilisation									
MEDIUM COMPOSITION		**CDGJM**	**Fermichamp**	**FM16 C7H**	**L2056**	**Lalvin 71B**	**Maurivin B**	**QA23**	**Simi White**	**Tee 9**	**Uvaferm 43**
2% malate medium pH 3.5 AEROBIC	L malic acid g L^{-1}	9.75 ± 0.40	9.12 ± 0.48	9.11 ± 0.38	8.88 ± 0.43	8.45 ± 0.34	8.92 ± 0.41	9.09 ± 0.47	9.02 ± 0.52	8.94 ± 0.47	9.05 ± 0.51
	% utilisation		6.5	6.6	9.0	13.4	8.5	6.8	7.5	8.4	7.2
2% malate medium pH 6.0 AEROBIC	L malic acid g L^{-1}	9.75 ± 0.40	9.57 ± 0.46	9.46 ± 0.37	9.56 ± 0.38	9.52 ± 0.38	9.52 ± 0.36	9.46 ± 0.38	9.49 ± 0.42	9.61 ± 0.37	9.39 ± 0.31
	% utilisation		1.9	3.0	2.0	2.4	2.4	3.0	2.7	1.5	3.8
2% malate medium pH 3.5 ANAEROBIC	L malic acid g L^{-1}	9.75 ± 0.40	9.45 ± 0.66	9.33 ± 0.73	9.33 ± 0.74	8.99 ± 0.65	9.33 ± 0.74	9.47 ± 0.69	9.33 ± 0.70	9.41 ± 0.67	9.30 ± 0.56
	% utilisation		3.1	4.3	4.4	7.8	4.3	2.9	4.4	3.5	4.7
2% malate medium pH 6.0 ANAEROBIC	L malic acid g L^{-1}	9.75 ± 0.40	9.26 ± 0.35	9.33 ± 0.29	9.27 ± 0.48	9.20 ± 0.39	9.29 ± 0.34	9.26 ± 0.37	9.36 ± 0.27	9.38 ± 0.30	9.27 ± 0.37
	% utilisation		5.1	4.3	5.0	5.7	4.7	5.1	4.0	3.8	5.0

Micro-fermentations were performed in minimal medium at pH 3.5 and 6.0 in the presence (anaerobic) and absence of 20% carbon dioxide (aerobic). 20 g L^{-1} DL malic acid (~ 10 g L^{-1} L malic acid) was added to the medium. L-malic acid utilisation was calculated from the residual L-malic acid content at the end of fermentation as a percentage of the initial. Residual L-malic acid was measured spectrophotometrically via L-malate dehydrogenase and glutamate-oxaloacetate transaminase catalysed reactions. The average values represent for residual malic acid over the duration of the ferment (258 h) ± SD.

Output 1A 2.0 Preliminary screening trials for selection of bacterial strains and key stressors to achieve sought-after outcomes

The initial LAB strains chosen were *Lactobacillus plantarum* K45 (isolated from a commercial freeze dried sachet of *Lb. plantarum*) and two previously isolated *O. oeni* clones A89 and A90 (**Table 1.2**). Initial characterisation on MRS medium (Cat. # AM103, Amyl Media, Victoria, Australia), supplemented with 20% apple juice (MRSAJ) agar plates with increasing ethanol, and decreasing pH established that *Lb. plantarum* was more sensitive to decreasing pH (in MRSAJ, no ethanol, **Figure 1.4**) than *O. oeni* isolates A90 and A89 **(Figure 1.5).**

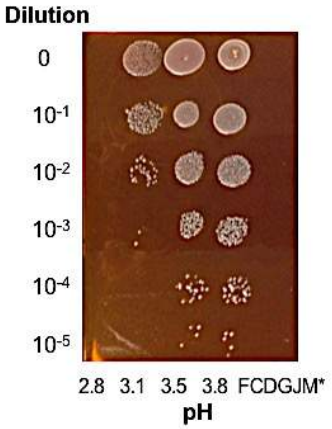


Figure 1.4: Survival of *Lb. plantarum* at different pH when grown in FCDGJM or MRS media. Cultures were incubated at 22 °C for 7 days prior to evaluation.

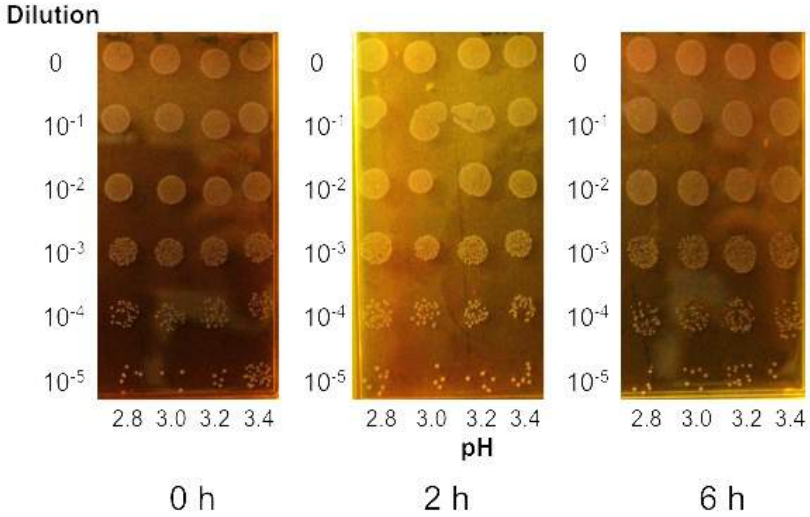


Figure 1.5: Tolerance of *O. oeni* A90 to different pH when grown on MRSAJ (no ethanol).

Initial characterisation in MRSAJ with increasing ethanol, established that *Lb. plantarum* K45 is tolerant of ethanol up to 18% in MRS at pH 5.5 (**Figure 1.6**), and it was subsequently hypothesised that the ethanol tolerance may be pH dependent. *O. oeni* was also tolerant of ethanol at pH 5.5, but showed reduced viability in MRSAJ after 6 h in ethanol 16% (v/v) (**Figure 1.7**).

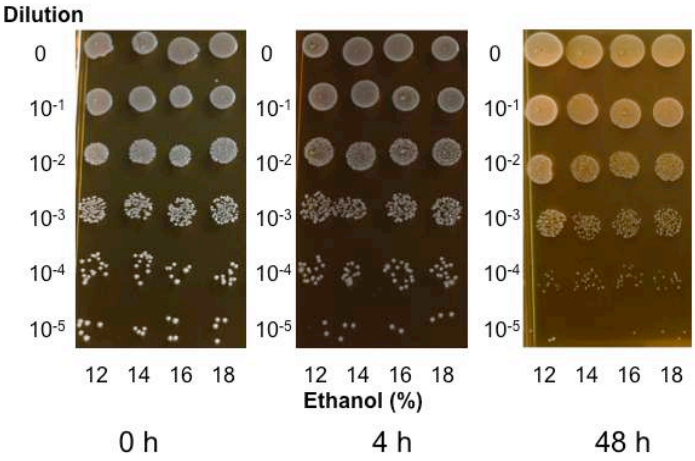


Figure 1.6: Minimum Inhibitory Concentration (MIC) of ethanol when *Lb. plantarum* was grown on MRSAJ (pH 5.5).

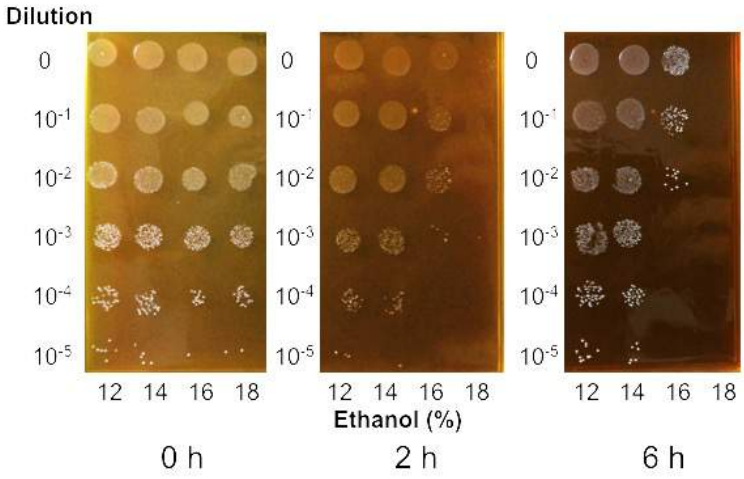


Figure 1.7: Ethanol tolerance of *O. oeni* A90 in MRSAJ (pH 3.5).

All three LAB strains were subsequently tested for ethanol tolerance at reduced "wine-like" pH of 3.5 in FCDGJM and ethanol tolerance was greatly reduced at this lower pH (**Figures 1.8 and 1.9**) with *Lb. plantarum* K45 showing no CFU on spot plates after 24 h at pH 2.8 and pH 3.1 when grown on CDGJM (18% ethanol). All three LAB were tolerant of pH as low as 3.1 in the presence of 10-12% ethanol, but did not survive at pH 2.8 **(Figures 1.8 and 1.9)**. The tolerance of *Lb. plantarum* K45 was also examined with increasing amounts of SO_2, at pH 3.5 with 12% ethanol (example **Figure 1.10**). After 2 h there were no viable cells with SO_2 levels above 10 ppm. Other conditions considered in the further characterisation of the

LAB strains included temperature and medium chain fatty acids (MCFA). Initial experiments focused on increasing tolerance of *O. oeni* A90 and *Lb. plantarum* to low pH and ethanol in Chemically Defined Grape Juice Media (CDGJM). For further results on MIC's of *O. oeni* A89 and A90 please refer to Jiao Jiang's PhD thesis (2017).

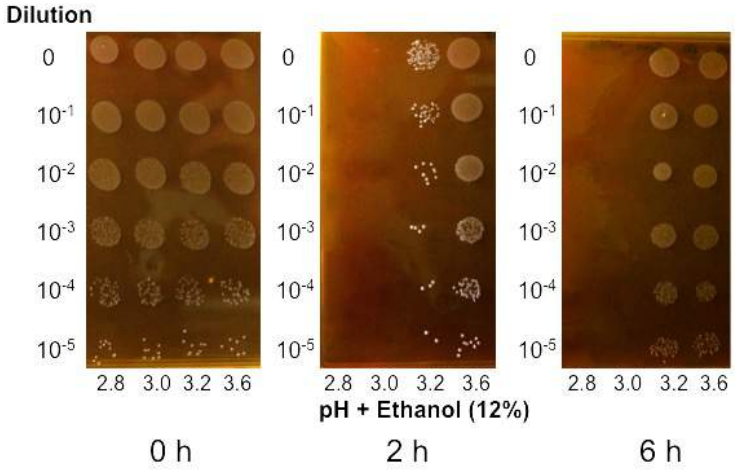


Figure 1.8: Tolerance of *O. oeni* A90 to combined pH and Ethanol stress in MRSAJ.

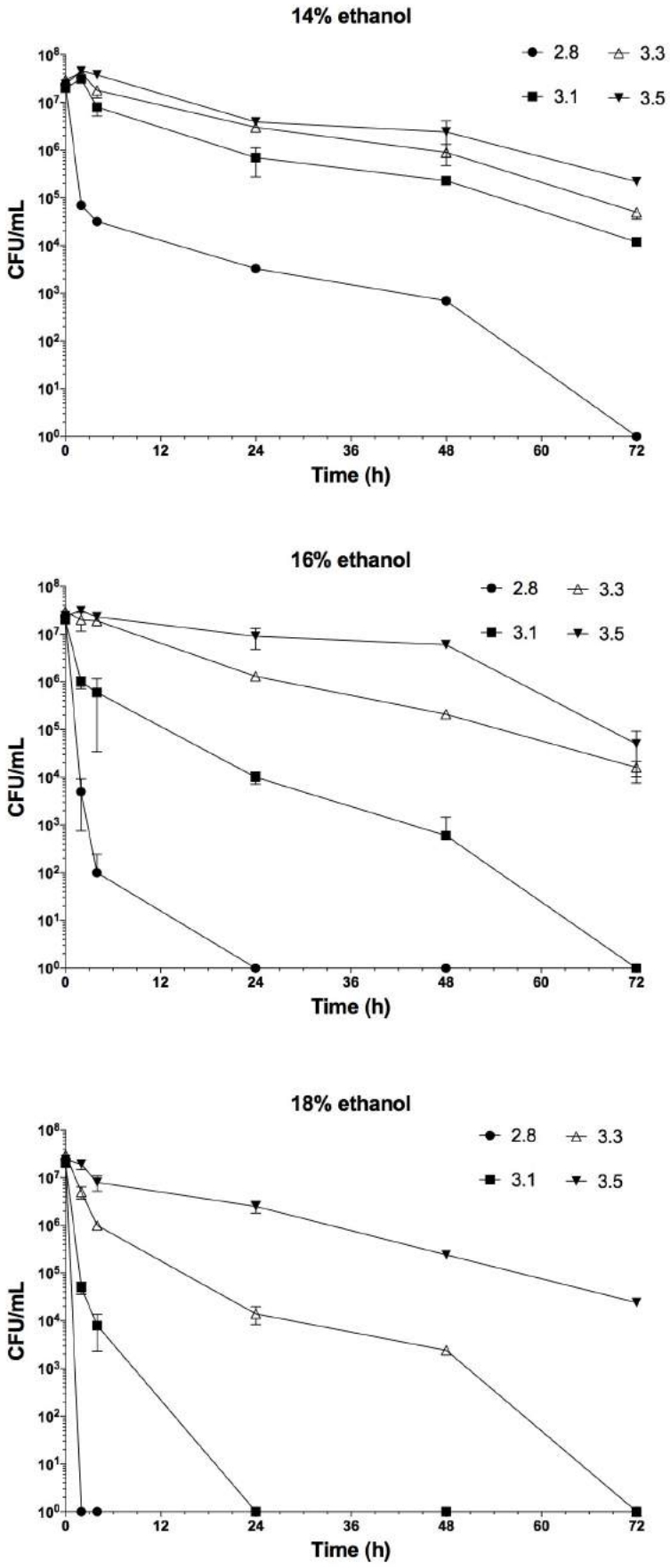


Figure 1.9: Determination of the minimum inhibitory concentrations (MICs) for ethanol in combination with pH for *Lb. plantarum* grown in FCDGJM

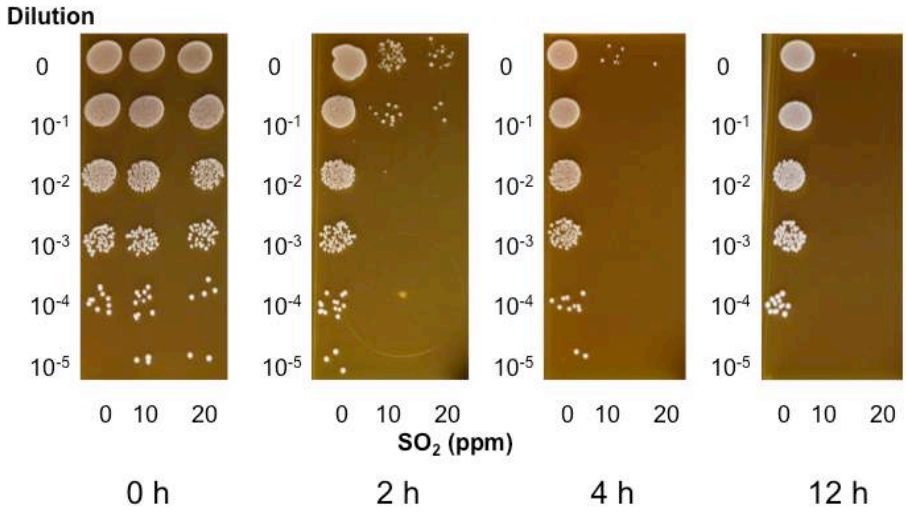


Figure 1.10: Tolerance of *Lb. plantarum* to sulphite (SO_2) when grown on MRSAJ (pH 3.5)

The effect of MCFA's on a clonal isolate of *Lb. plantarum* K45 (KS72) was measured along with L-malic acid consumption (**Figure 1.11**). The addition of 520 mg L^{-1} hexanoic acid (C6) and octanoic acid (C8) did not show any inhibitory effect on viability in RFCDGJM (**Figure 1.11 A and B**). By comparison, decanoic acid (C10) did prove inhibitory at higher concentrations. Little effect was seen at 5 mg L^{-1} (**Figure 1.11 C**), while an intermediate degree of MLF inhibition was observed at 10 mg L^{-1}. No consumption of L-malic acid was observed for any strains in the presence of 10 and 15 mg L^{-1} C10 in RFCDGJM (**Figure 1.11 C**). Dodecanoic acid (C12) proved to be highly inhibitory at concentrations between 5 and 15 mg L^{-1}, with a rapid decline in viability and absence of MLF (**Figure 1.11 D**). Given the likelihood that mixtures of MCFA occur in wine, a combination of the four used in this study was also examined. A total amount of 1, 2.5, or 5 mg L^{-1} of mixed MCFA did not inhibit bacterial fermentation or growth when compared to the control (**Figure 1.11 E**). However, when the combined C8, C10 and C12 was increased to 7.5 mg L^{-1}, an obvious inhibition of viability and MLF occurred (**Figure 1.11 E**). Overall, *Lb. plantarum* K45 consumed L-malic acid at a steady rate for the first 2 days after which there was minimal consumption despite there being enough viable cells to carry out MLF. This is a known problem with this species and DE was undertaken to improve viability and MLF performance.

O. oeni A90 was chosen as the parent strain for PhD student Jiao Jiang to carry out DE experiments and the data on MIC for SB3, A90, and A89 can be found in her PhD thesis titled "Use of directed evolution to generate multiple-stress tolerant *Oenococcus oeni* for enhance malolactic fermentation". Interestingly *O. oeni* species showed much greater tolerance for MCFA's than *Lb. plantarum* when tested under similar conditions. In the case of *O. oeni,* addition of 520 mg L^{-1} C8 did not show any inhibitory effect on either MLF progress or growth for all *O. oeni* strains assessed in RFCDGJM. Decanoic acid did prove inhibitory to *O. oeni*, but at higher concentrations than with *Lb. plantarum*. Minimal effect was observed at 5 and 10 mg L^{-1} while an intermediate level of MLF inhibition was observed at 15 mg L^{-1} and no consumption of L-malic acid was observed for any strains in the presence of 20 mg L^{-1} C10 in RFCDGJM. Cell viability was reduced with C8 but to ~10^3 CFU/mL over 10 days (Jiang, 2017) and did not decline as rapidly as *Lb. plantarum*. Dodecanoic acid proved to be

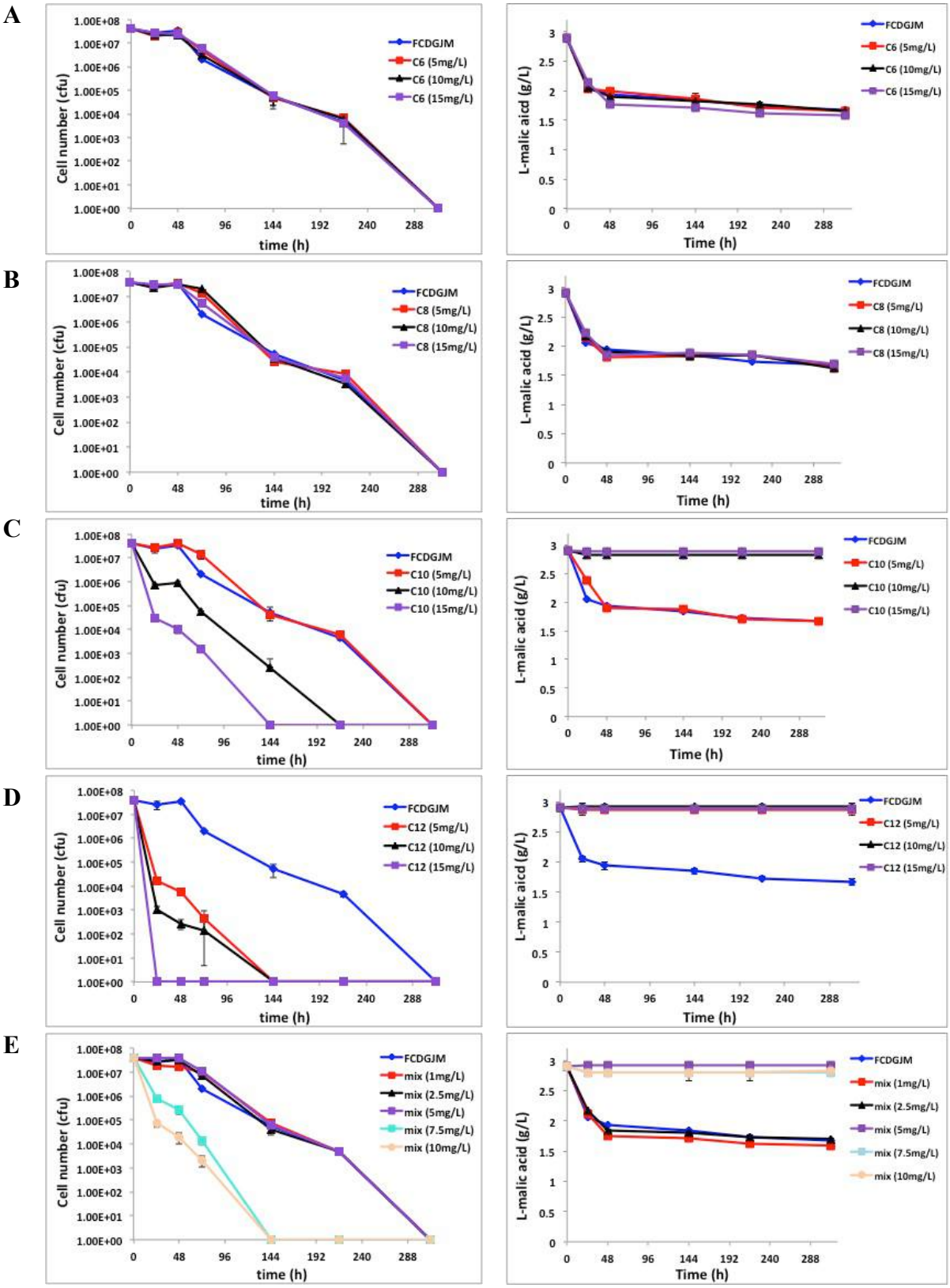


Figure 1.11: Growth of *Lb. plantarum* KS_72* in red FCDGJM with selected MCFA's.
Panels A: hexanoic acid (C6), B: octanoic acid (C8), C: decanoic acid (C10), D: dodecanoic acid (C12), E: mix of equal amounts of C6, C8, C10 and C12 acids.
*(KS72 is originally from K45 freeze dried packet)

highly inhibitory at concentrations between 10 and 20 mg L^{-1}, whereby viability dropped to between 10^3 and 10^4 CFU/mL and no MLF was detectable. At the lowest concentration examined (5 mg L^{-1}) MLF was delayed with SB3, and the DE strains finished MLF before A90. Equal amounts of C8, C10 and C12 acids to give a total of 4.5 or 9 mg L^{-1} MCFA did not inhibit bacterial fermentation or growth. However, when the combined C8, C10 and C12 was increased to 13.5 mg L^{-1}, an obvious inhibition of MLF occurred (Jiang, 2017). Both malolactic activity and viability of all strains were strongly affected with the addition of 18 mg L^{-1} mixed MCFA; a much higher concentration than needed to effect *Lb. plantarum*.

DE experimental conditions were established based on the MIC data related to wine stress (i.e. pH, ethanol and SO_2) – see Output 1.A 2.0. The initial experiments focused on increasing tolerance of *O. oeni* A90 and *Lb. plantarum* to low pH and ethanol in fermented chemically defined grape juice media (FCDGJM). The first challenge was to reduce the stress levels such that they were below the MIC, such that cell viability was not greatly affected during both batch and continuous culture. Initial attempts in CDGJM resulted in cells dying in batch cultures and it was decided to supplement the media with a small amount of MRS to extend cell survival. Initial conditions were FCDGJM with 20% MRS (which was reduced over time), low pH (3.5), with ethanol content gradually increased over time.

Output 1D 1.0 Stage 1 DE experiments and intermittent evaluation of candidate strains with the aim of generating improved yeast strains

The generation of new 'fit-for-purpose' yeast within the timeframe of the project was reliant on DE experiments being conducted on a number of yeast using several stressors as well as generating increased genetic diversity through mutagenesis and intra-specific hybridisation.

Five strains were initially chosen, targeting four oenological traits in Stage the 1 DE strategy: Rescue 'stuck' fermentation (FM16 C7H; evolved from L2056 (UA1101), Proline utilisation (Q2 (EMS mutant of D254), Q7 (EMS mutant of QA23; Long 2014), Malic acid utilisation (Lalvin 71B) and Fermentation reliability (L2056).

Two batch DE strategies were used: (i) improved tolerance to high sugar, ethanol, and low pH and (ii) improved fructose utilisation and ethanol tolerance. These strategies are presented diagrammatically in **Figure 1.12**. The yeast populations and different conditions used in the DE program for strain improvement, together with the number of batch fermentations undertaken and total (cumulative) generations is shown in **Supplementary Table 1.2**.

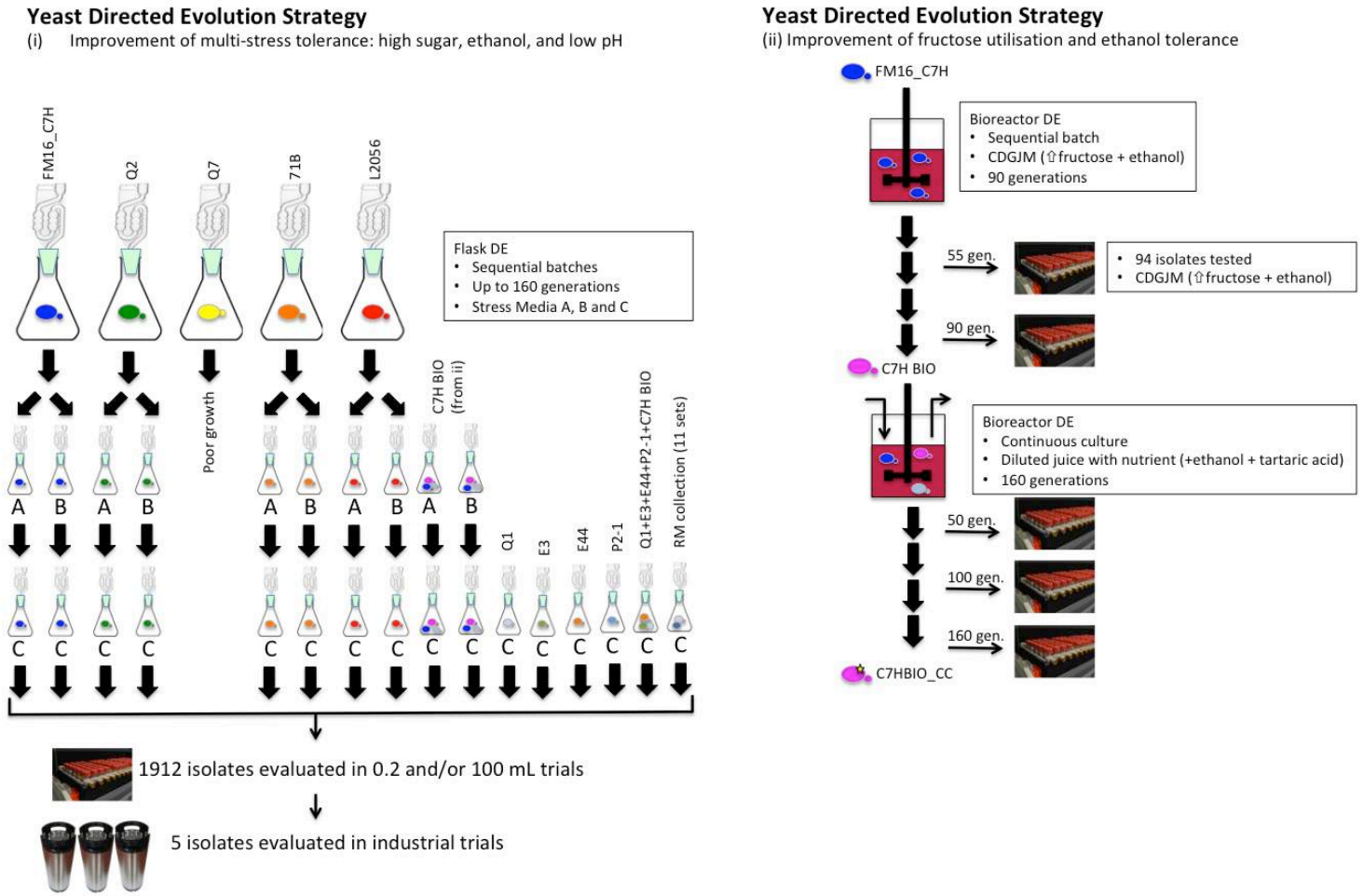


Figure 1.12: Overview of the yeast Directed Evolution strategies (i) and (ii).
Yeast DE was undertaken for improvement in 2 broad conditions (i, ii), using both batch and continuous culture. These approaches were undertaken with 5 wine yeast strains (FM16 C7H, Q2, Q7, 71B and L2056). Multiple rounds of DE were undertaken (indicated by the arrows) with a range of stress media (A,B,C). Key differences in experimental approaches are highlighted. The most promising clones were then evaluated in lab (0.2 and 100 mL) and industrial (20 L) trials.

Output 1D 1.1 Sequential batch DE (in flasks) targeting DE strategy (i) tolerance to multiple stresses

The five strains were initially evolved for up to 18 generations (as 3 sequential batch fermentations), with yeast grown in Starter Medium 1 and inoculated at 5 x 10^6 cells mL^{-1} in Stress Medium A* (**Appendix 5**). However, these stringent conditions resulted in fermentation arrest, with cultures being harvested between 350 and 400 h. Q7 was eliminated from the experiment because of poor growth. The remainder were further evolved for 60 generations (7 batch fermentations) under 2 stress conditions: Stress Medium A (**Appendix 5: multiple stressors**) and Stress Medium B (**Appendix 5: ethanol tolerance**). Yeasts were cultured in Starter Medium 2 (Supplementary data), and the inoculation rate was decreased to 2.5 x 10^5 viable cells mL^{-1} to increase the generation number per batch. The total generations (6776) was dependent upon the strain, and whether fermentations were re-inoculated from earlier experiments if growth failed. The evolved population derived from Cross Evolution was not viable after the 10^{th} fermentation.

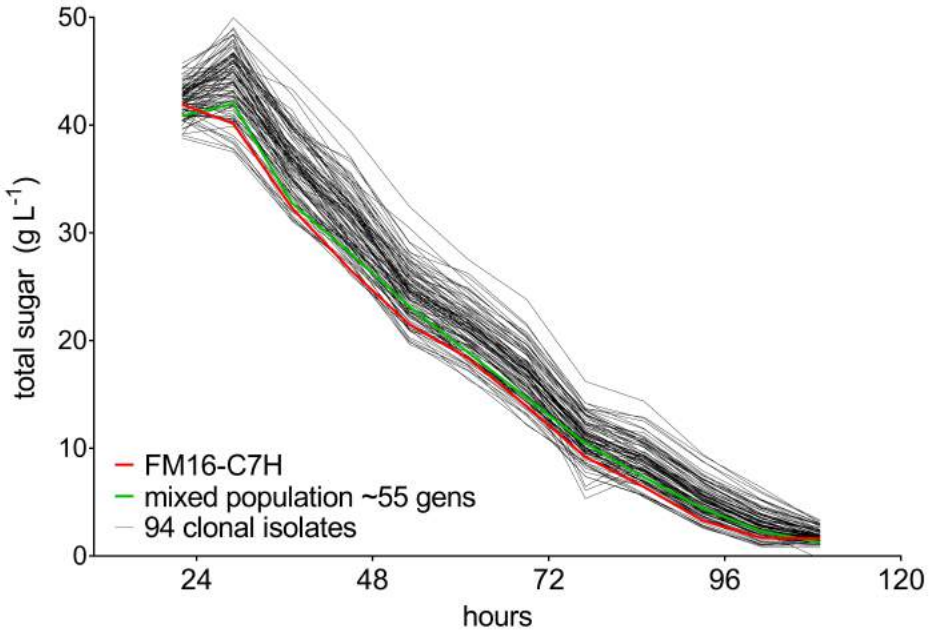
L2056, FM16 C7H, 71B and Q2 were evolved for 3 sequential batches ($11^{th}13^{th}$ fermentations). The total generations varied from ~40 generations for Q2 to 9096 generations for C7H, L2056 and 71B in Stress Media A and B. The DE populations were monitored for cross-contamination using delta PCR for molecular typing of the parent strains and evolving populations. Earlier DE cultures were used to re-inoculate the DE experiment when contamination was found e.g. 71B Stress medium B and Q2 Stress Media A and B.

Output 1D 1.2 Sequential batch DE (in bioreactor) targeting DE strategy (ii) improved fructose utilisation and ethanol tolerance

FM16 C7H was fermented in 25 sequential batches representing ~90 generations in a 1L bioreactor containing 500 mL of CDGJM (30 g L^{-1} fructose, 10 g L^{-1} glucose, 150 mg N L^{-1}), with increasing ethanol concentration (812.5%). At the end of each fermentation (i.e. total sugar concentration < 2.5 g L^{-1}), an aliquot of culture was retained to inoculate fresh medium at 5 x 10^6 cells mL^{-1}.

The fermentation performance of ~90 randomly selected isolates from the culture sampled at ~55 and 90 generations, the mixed population and the parent strain FM16 C7H, was evaluated using conditions similar to that used in the bioreactor, except for ethanol and N content (**Figure 1.13**). These tests aimed to identify if fermentation kinetics of any of the isolates outperformed that of the parent. Isolates from the 90 generation evolved population were generally more robust then the parent. Greater heterogeneity was evident from clones isolated from the 90-generation time point (**Figure 1.13**) when compared to 55-generation time point. Whilst improvement was observed, it was decided that further selective pressure would be necessary to generate genotypes that further reduced fermentation duration. Thus, the mixed culture from the 90-generation population (C7H BIO) was used as the starting culture for the continuous culture strategy (Strategy i, Output 1D 1.3) and batch DE in flasks (Strategy ii, Output 1D 1.8).

A



B

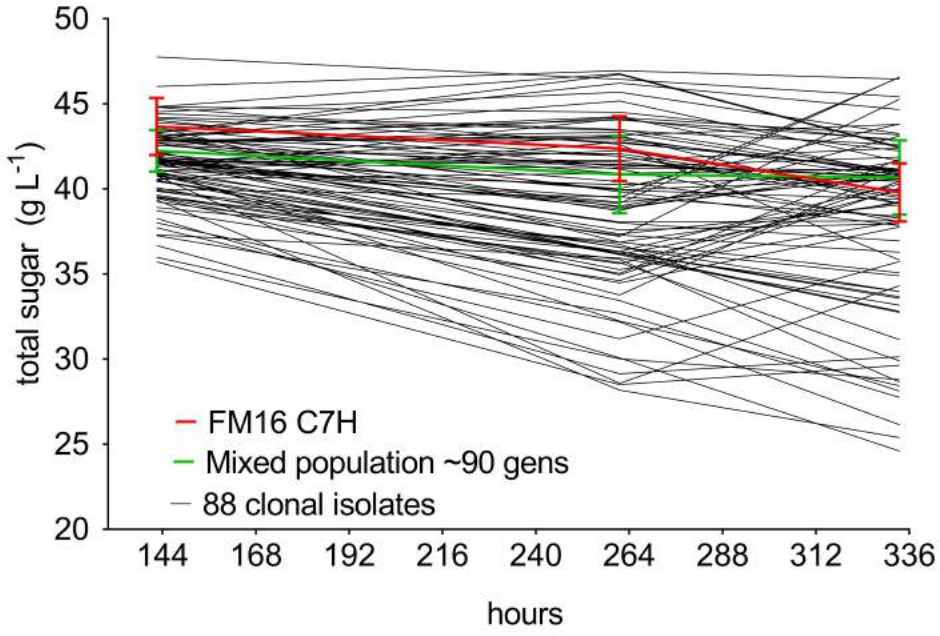


Figure 1.13: Evaluation of the fermentation performance of ~90 clonal isolates from batch fermentation of FM16C7H in bioreactor, at 55 and 90 generations.

Panel A: 55 generations. Fermentations were performed at 28 °C in 100 mL CDGJM (10 g L^{-1} glucose, 40 g L^{-1} fructose, 75 mg L^{-1} FAN, 12% (v/v) ethanol). Panel B: 90 generations. Fermentations were performed in CDGJM (10 g L^{-1} glucose, 40 g L^{-1} fructose, 55 mg L^{-1} FAN, 12% ($^{v}/_{v}$) ethanol. Strains were initially propagated in Starter Medium 2 (see Supplementary Data). Fermentations were inoculated at 1% with the starter culture. The sugar utilization profiles (total sugar g L^{-1}) are shown. Values (with error bars) are the average of 3 replicates ± SD.

Output 1D 1.3 Directed Evolution of the mixed C7H BIO population by continuous culture

Continuous culture is reliant on the maintenance of a set culture density (turbidity) in the bioreactor via an automated control loop based on a turbidity probe, inlet and outlet pumps and associated software. The installation of 3 new bioreactors in 2015, funded through a grant variation, enabled continuous culture for yeast (1 reactor) and LAB (2 reactors) rather than only batch culture. Both batch and continuous culture were then undertaken to maximise the likelihood of generating 12 evolved yeast strains with desirable fermentation characteristics. However, because of the long timeframe (~300 h) per batch fermentation, continuous culture, whilst limited to a single culture condition, would in theory reduce the time required to achieve the planned target of ~200 generations required to stably incorporate beneficial mutations within the genome (McBryde et al 2006).

The previously evolved C7H BIO population (Output 1D 1.2) was grown in continuous culture for 6 months in a medium consisting of white juice:H_2O:YPD (45:45:10). The grape juice was changed (source and variety) periodically to prevent the population becoming conditioned to a particular juice. The population was evolved for ~160 generations over 6 months prior to evaluation, with samples collected every 50 generations (C7H BIO_CC) and kept at -80°C for subsequent evaluation (Output 2D).

Output 1D 1.4 Increased genetic heterogeneity through chemical mutagenesis

Wine yeast 71B was treated with ethyl methane sulfonate (EMS) and mutants screened on selection plates for increased tolerance to ethanol or malic acid. 71B was observed to be resistant to EMS, with 70% survival after using the Herskowitz Lab Protocol (http://biochemistry.ucsf.edu/labs/herskowitz/ems.html). Treated cells from 120 and 135 min samples were plated for single colonies on YPD. ~1700 colonies were replica plated in duplicate onto selective media (12% ethanol (YPD, pH 3.5 or Malic Acid Indicator Agar or MAI; **Appendix 5**). Thirty isolates were selected as potential malic acid users (dark green colonies on pH indicator, bromocresol green) and 14 isolates with increased ethanol tolerance on 12% ethanol). The isolates were evaluated as part of Output 1E as 100 mL fermentations (**Supplementary Figures 1.2 and 1.3**). Strains M16 (efficient sugar utilisation) and E44 and P2-1 (efficient malic acid consumers) were then further evolved by DE (Output 1D 1.8). Isolates P11-1 and P11-2 (from MAI screen) were heavily flocculant during fermentation in CDGJM (260 g L^{-1} sugar, 350 mg L^{-1} N, 3 g L^{-1} L-malic acid, pH 3.5). The remainder are part of our 'in-house' collection and require further evaluation.

Output 1D 1.5 Increased genetic heterogeneity through rare spore mating

Commercial wine yeast, chosen for their distinct properties, are often heterozygous (Bradbury et al 2006) and therefore, are potential sources of genetic diversity from either the re-diploidised 'selfed' progeny or when mated (F1 hybrid progeny) and re-sporulated (F2 hybrid progeny) in terms of fermentation attributes. However, as wine yeast do not readily mate, the procedure, termed rare or spore-spore mating, relies on sporulation and mating of the haploid spore progeny prior to their switching mating type and re-diploidising. Previous work on new strains generated from intra-specific hybridisation (F1 hybrids) and 'selfed' monosporic clones is reported in UA 0501 and UA0104, respectively.

In this study, 2 clonal isolates of the F1 hybrid H3-13 (EC1118 x FM16 C7H) were sporulated and a total of 34 tetrads (each with 4 viable spores; 136 spores) and 30 random spores (not from tetrads) were dissected. Strains were grown in CDGJM (200 g L^{-1} sugar, 468 mg N L^{-1}) where 136 strains performed similarly to the parent and 29 were protracted. 94 of the 136 strains which performed similarly to the parent were further evaluated in CDGJM (260 g L^{-1} sugar, 90 mg N L^{-1}) and CDGJM (320 g L^{-1} sugar, 450 mg N L^{-1}). All strains (with the exception of 5, which were protracted), behaved similarly to parents H3-13, EC1118 and FM16 C7H in both conditions.

It was considered that the phenotypic diversity was insufficient to be of value in the strain improvement program, so was abandoned at this point. Nonetheless, this strain collection may be of value in the future as this method is routinely used in genetic mapping of oenological traits, where two strains, which are genetically different with distinct phenotypes are mated and subsequently sporulated (Brice et al 2014, Huang et al 2014).

Output 1D 1.6 Increased genetic heterogeneity through *en masse* sporulation and rare mating (RM)

A selection of commercial and in-house yeast strains with desirable attributes (FM16 C7H, Q2, Q7, Tee9, 71B, Uvaferm 43, Maurivin B, Fermichamp) were mated in pairs with the view of combining desirable attributes (RM1 to RM11, **Table 1.1**). *En masse* sporulation and mating was undertaken and mated cultures were used as inocula for sequential batch DE in flasks (Output 1D 1.8, Stress Medium C). Each population was evolved separately. RM11 was evolved as a 'competitive fitness' experiment whereby we expect the fittest strain(s) to dominate the population. RM2, 6 and 9 were eliminated from further experimentation as they did not grow in the initial batch fermentation. RM8 (Fermichamp x Q2) flocculated when grown in Stress Medium C. The total generations for each population are reported in **Table 1.6**.

Output 1D 1.7 Evaluation of existing collection of monosporic and hybrid strains for fermentation performance

Our 'in-house' collection consisting of ~250 monosporic clones and hybrid strains from the current project and others (UA1101, UA0501 and UA0401) were evaluated for fermentation performance (no replication) in CDGJM (300 g L^{-1} sugar, 350 mg N L^{-1}, 5 g L^{-1} L malic acid, pH 2.8) at 22°C. Two strains were selected, based on their efficient fermentation performance (Q1 and E3) and strain M16 (generated in this study). Q1, an EMS mutant of QA23 was selected for growth on 1.9 M NaCl; E3 is a hybrid between C7H and Q7 and M16 is an EMS mutant of 71B having increased sugar utilisation. The strains were evaluated as part of Output 1E, in 8 different juices in triplicate 100 mL fermentations (**Figure 1.14**). Q1 and E3 behaved similarly to Uvaferm 43, except for one juice fermentation where all 3 strains except for Uvaferm 43 became arrested. M16 in general was slower than the remainder, however it was able to complete most fermentations. Strains Q1 and E3 were further evolved in sequential batch (flask) DE (Output 1D 1.8) propagated in CDGJM starter:YPD (1:1) and inoculated at 5 x 10^6 cells mL^{-1}. Progress was monitored as sugar consumption. Values are average of 3 replicates ± SD.

Output 1D 1.8 Stage 2 DE strategy: batch flask fermentations targeting (i) tolerance to multiple stresses and ethanol tolerance

A second stage was introduced into the sequential batch DE program after the 15th batch of fermentation (~100 generations). The medium used was modified to prevent yeast fitness being evolved specifically to one condition. Several new strains were also included: C7H BIO (Output 1D 1.2), Q1 and E3 (Output 1D 1.7), E44 and P2-1 (Output 1D 1.4), and a mixed culture (evolved populations from Stage 1 DE: FM16 C7H_A, C7H_B, C7H BIO_A, and C7H BIO_B, Q1, E3 and E44, were pooled). A further 8 batches were performed resulting in cultures evolved for up to 158 generations (**Table 1.6**). P2-1 and E44 were eliminated after 13 batches (1517; 14 gen) because of poor growth performance. As described above, rare-mated populations (Output 1D 1.6) were also included. We expect the original parent strain, monosporic isolates and hybrids (zygotes) to be present within the mated population, and it was anticipated that only the fittest would survive and become enriched during the short timeframe of adaption (up to 46 gen).

Stress Media A and B were modified to include ethanol and C8 and C10 fatty acids and low pH as the stressors (Stress Medium C; **Appendix 5**). Preliminary investigations were undertaken to determine the tolerance of different wine yeast including a number of evolved strains (this study) to different concentrations of the lipophilic octanoic (caprylic) and decanoic acid (data not shown). These saturated fatty acids are sub-products of ethanol fermentation, ranging from 0.7 to 23 mg L^{-1} (Viegas et al., 1989). Viegas and coworkers demonstrated octanoic and decanoic acids, at concentrations up to 16 and 8 mg L^{-1} respectively, decreased the maximum specific growth rate and the biomass yield at 30°C as an exponential function of the fatty acid concentration and increased the duration of growth latency. Toxicity increased with a decrease in pH in the range of 5.4 to 3.0, indicating that the undissociated form was toxic to the cell. In our study, octanoic acid was more effective in protracting fermentation contrary to Viegas' results. Fermentation protraction was related to the concentration of octanoic acid, regardless of the concentration of decanoic acid (**Figure 1.15**). Protraction was only noted mid-fermentation, with the cells recovering to complete fermentation as observed with the control. The increased malic acid utilisation in the treated cells alludes to membrane disruption, which allows diffusion of malic acid into the cell. Both decanoic acid and octanoic acid were added as part of the DE strategy initially at 6 mg L^{-1} each to Stress Media A and B; the concentration later reduced to 3 mg L^{-1} in Stress Medium C. For details on the evaluation of the resultant cultures refer to **Outputs 1E, 2D** and **3B** (laboratory scale) and **Output 3E** (20 L scale). Shortlisted individual isolates were identified by Delta PCR. An example of which is shown in **Supplementary Figure 1.4**, corresponding to the strains **from Table 2.5**.

Table 1.6: Total number of cell generations of sequential batch DE (flasks)

Yeast Strain	Total generations evolved	Fermentation Evaluation (100 mL)
L2056_ A (sequential batch DE in flasks, Stress Medium A and then C)	96.2	
C7H_ A (sequential batch DE in flasks, Stress Medium A and then C)	138.2	*
71B_ A (sequential batch DE in flasks, Stress Medium A and then C)	90.6	
Q2_ A (sequential batch DE in flasks, Stress Medium A and then C)	42.2	
BIO C7H_A (90 generations from seq batch chemostat) + 10.2 generations in Stress Medium A and then C	100.2	
L2056_ B (sequential batch DE in flasks, Stress Medium B and then C)	94.5	
C7H_ B (sequential batch DE in flasks, Stress Medium B and then C)	158.3	*
Lalvin 71B_B (sequential batch DE in flasks, Stress Medium B and then C)	63.6	
Lalvin 71B_B (sequential batch DE in flasks, Stress Medium A and then B and then C)	84.7	
C7H BIO_B (90 generations from batch chemostat) + 59.2 generations in Stress Medium B and then C	149.2	*
Q1 (EMS isolate QA23 1.9M NaCl, sequential batch DE in flasks, Stress Medium C)	64.5	*
E3 (C7H x Q7 hybrid, sequential batch DE in flasks, Stress Medium C)	55.3	*
Mixed culture (C7H_A, C7H_B, C7H BIO_A, and C7H BIO_B, Q1, E3, E44 mix, sequential batch DE in flasks, Stress Medium C)	46.1	*
E44_B (sequential batch DE in flasks, Stress Medium B and then C)	14.6	
RM1_C (sequential batch DE in flasks, Stress Medium C)	39.1	*
RM2_C (sequential batch DE in flasks, Stress Medium C)	3.5	
RM3_C (sequential batch DE in flasks, Stress Medium C)	38.6	*
RM4_C (sequential batch DE in flasks, Stress Medium C)	41.2	*
RM5_C (sequential batch DE in flasks, Stress Medium C)	37	*
RM6_C (sequential batch DE in flasks, Stress Medium C)	2.8	
RM7_C (sequential batch DE in flasks, Stress Medium C)	39.1	*
RM8_C (sequential batch DE in flasks, Stress Medium C)	29.8	
RM9_C (sequential batch DE in flasks, Stress Medium C)	0.1	
RM10_C (sequential batch DE in flasks, Stress Medium C)	43.3	*
RM11_C (sequential batch DE in flasks, Stress Medium C)	29.2	*

Total number of generations are shown. Strains were evolved in different Stress medium during the course of up to 23 batch fermentations. The following are denoted by a suffix which represents the original medium the strain was evolved from: Stress medium A, _A; Stress medium B, _B; and Stress medium C, _C. *Cultures further evaluated for fermentation performance at 100 mL (**Outputs 1E, 2D, 3B)**

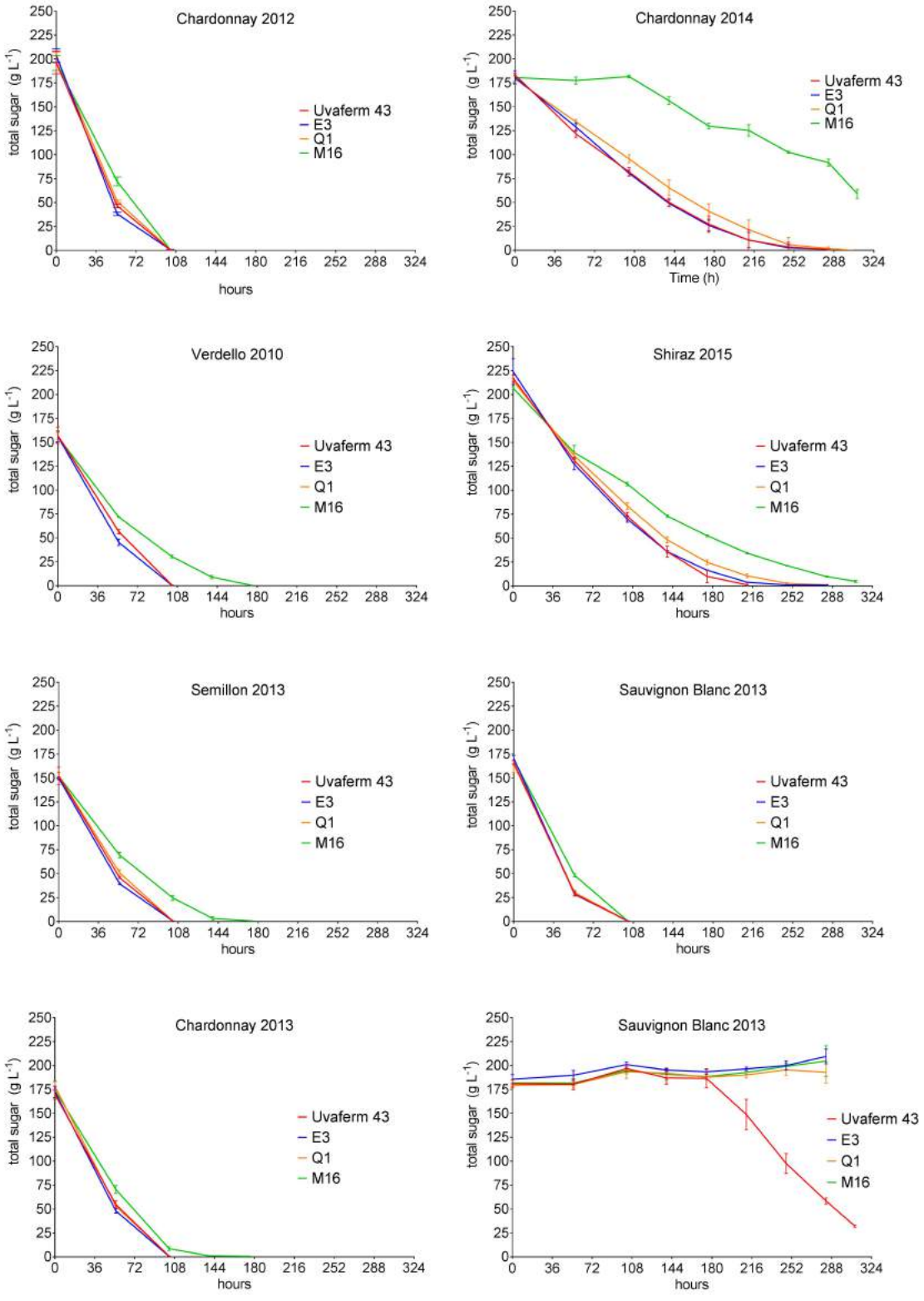


Figure 1.14: Fermentation performance of Q1, E3 and M16 in comparison to Uvaferm 43 in 8 different white juices. Q1, E3 and M16 were fermented in 100 mL thawed, filtered juice at 22°C. Strains were propagated in CDGJM starter:YPD (1:1) and inoculated at 5 x 10^6 cells mL^{-1}. Progress was monitored as sugar consumption. Values are average of 3 replicates ± SD.

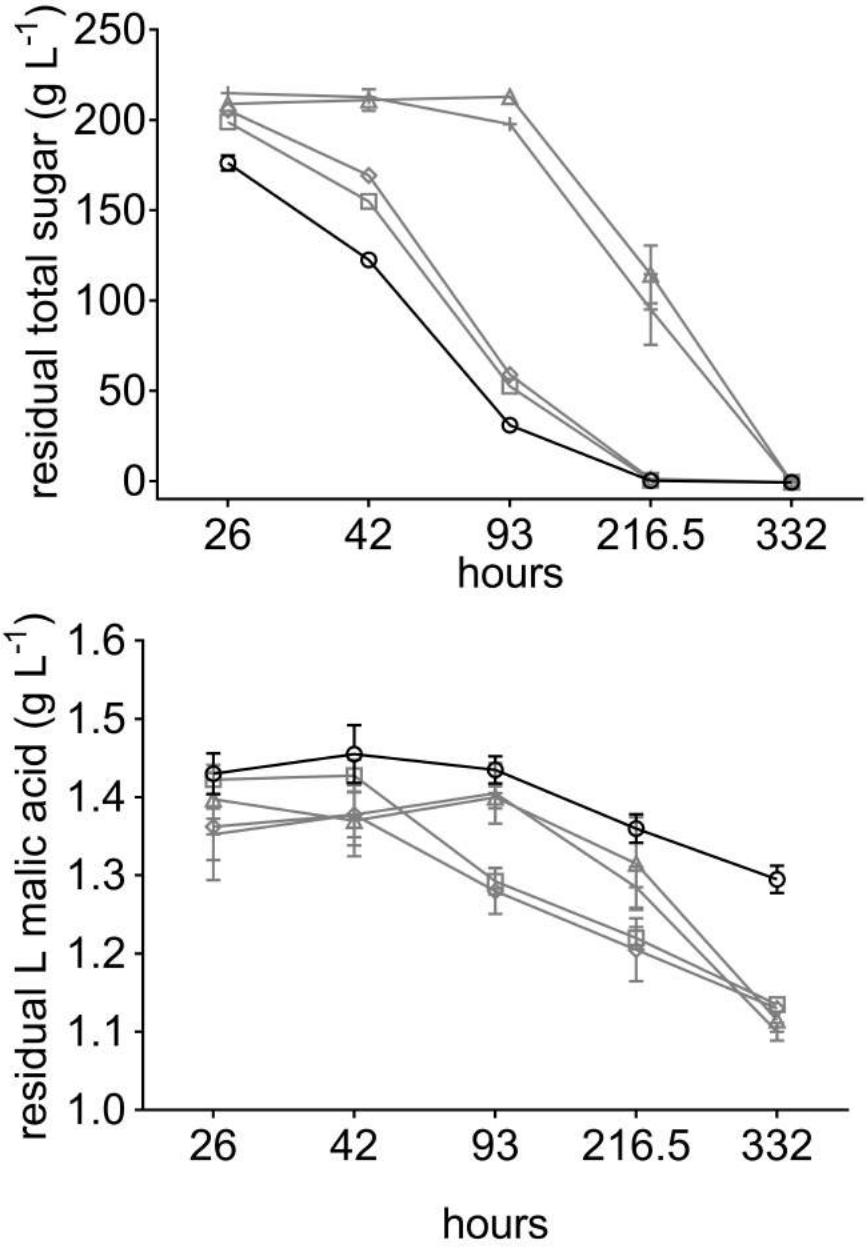


Figure 1.15: Effect of differing concentrations of octanoic and decanoic acid on fermentation performance. 71B was fermented in CDGJM (200 g L^{-1} sugar, 350 mg N L^{-1}, 3 g L^{-1} DL malic acid (1.5 g L^{-1} L malic acid) pH 3.5. Medium chain fatty acids were added to the medium as follows: Control; 0 mg L^{-1} octanoic and decanoic acid (○); 6 mg L^{-1} each of octanoic and decanoic acid (□); 12 mg L^{-1} of octanoic and 6 mg L^{-1} decanoic acid (△); 6 mg L^{-1} octanoic and 12 mg L^{-1} decanoic acid (◇); and 12 mg L^{-1} octanoic and 12 mg L^{-1} decanoic acid (+). Sugar and malic acid values are the average of 3 replicate fermentations.

Output 1D 2.0 Lactic acid bacteria, Stage 1 DE and evaluation of candidate strains with the aim of generating candidate improved strains

The generation of new 'fit-for-purpose' lactic acid bacteria within the timeframe of the project was reliant on the DE experiments to be undertaken on several bacteria using multiple stressors, as well as generating increased genetic diversity in *Lb. plantarum* strains through mutagenesis (UV and EMS). The overarching strategy is presented diagrammatically in Figure 1.16. Two strains were initially chosen, 1 *O. oeni* (A90) and 1 *Lb. plantarum* (K45). With the success of the previous proof-of-concept directed evolution (DE) in *O. oeni*, a new DE was carried out to determine if DE could be applied to further improve A90 in a wine-like environment using combinations of stressors to generate more strains with better general stress resistance.

Output 1D 2.1 Sequential batch DE of *Lb. plantarum* in Schott bottles, targeting tolerance to multiple stresses.

Initial DE experiments were performed in batch culture, whilst the bioreactors were upgraded in the first years of the project, to allow for continuous culture. *Lb. plantarum* was chosen with the view of broadening stress tolerance during fermentation. Strain K45 was subjected to multiple batch culture under increasingly stressful conditions in Fermented Chemically Defined Grape Juice Media (FCDGJM, 12.5% ethanol, pH 3.3).

The DE of *Lb. plantarum* used a number of approaches to increase genetic diversity. As *Lb. plantarum* has a functional DNA mismatch repair system, fewer genetic changes would be expected over 300 generations as compared to *O. oeni* which lacks *mutS* and *mutL*, encoding two key enzymes in the Methylated Mismatch Repair (MMR) pathway. The correction of mismatches by MutS and MutL decreases the spontaneous mutation rate of a species, therefore a defect or absence of the MMR system leads to an increase in mutation frequency. This is discussed in more detail in Betteridge et al. (2017).

A sequential batch fermentation approach has been used with some success for dairy or probiotic LAB (Teusink et al. 2009; Bachmann et al. 2012; Douillard et al. 2016) and by our group to improve wine yeast (McBryde et al 2006). The sequential batch approach has the potential advantage of exposing the culture to all of the compositionally different phases of the batch culture, thereby allowing evolution in response to more diverse conditions.

Additionally, UV and chemical mutagenesis, to increase genetic diversity and therefore the likelihood of obtaining the desired project outputs, preceded DE batch experiments. Batch fermentations of UV mutagenised *Lb. plantarum* K45 were undertaken due to a limited number of bioreactors being available for continuous culture. This was conducted in FCDGJM (12.5% ethanol pH 3.2) with the addition of 5% MRS.

An important factor during the search for improved phenotypes by DE is the time span for

the selection experiment. Regardless of the experimental approach applied in DE, improved phenotypes are reported to appear across a wide range of generations (100 and 10,000 generations) (Dragosits and Mattanovich 2013). A typical DE experiment is performed for somewhere between 100 and 2000 generations and usually takes a few weeks to a few months. During this time several phenotypes will occur at first and compete for 'dominance' in the total population. In this study, the evolving population was regularly monitored to determine when clones with improved properties were apparent and the magnitude of any such improvements relative to the parent. In order to validate a screening method, L-malic acid consumption using sacrificial sampling (96 well plates) of mixed isolates from various stages of the DE experiment was undertaken. Included in this screen were the parent strain and mixed population samples from bioreactor and batch experiments.

Output 1D 2.2 Batch fermentations of of *Lb. plantarum* UV and EMS mutants.

The initial selection of suitable conditions for batch DE of *Lb. plantarum* as described in the methods required considerable trouble-shooting. Once it was established that less MRS could be used to ensure cell growth (from previous batch experiments), a second round of batch DE experiments was initiated. UV and chemical mutagenesis were used to increase genetic diversity and the likelihood of obtaining the desired project outputs. The UV-treated cells were tested in FCDGJM supplemented with apple juice instead of MRS (**Figure 1.17**), as part of identifying the initial conditions for batch DE. Cell growth was poor when apple juice was used instead of MRS and it was therefore decided to use 5% MRS supplemented into the FCDGJM to enable enough growth for DE to occur.

Batch fermentations of EMS and EMS+UV treated *Lb. plantarum* K45 in FCDGJM (12.5% ethanol, pH 3.2 with the addition of 5% MRS

Batch fermentations of UV mutagenized *Lb. plantarum* K45 were undertaken due to a limited number of bioreactors being available for continuous culture. This was conducted in FCDGJM (12.5% ethanol pH 3.2) with the addition of 5% MRS. The initial attempt at EMS mutagenesis of K45 was unsuccessful as the strain was highly resistant. The method was subsequently modified to increase the treatment duration and concentration of EMS which resulted in 50% lethality after 3 hours. Batch fermentations were then initiated as for the UV treated cells (**Figure 1.16**).

Output 1D 2.3 Directed Evolution of LAB by continuous culture

The DE experiments were delayed due to the need for existing bioreactors to be upgraded. Continuous culture experiments are reliant on the maintenance of a set culture density (turbidity) in the bioreactor via an automated control loop based on a turbidity probe, inlet and outlet pumps and associated software. During this period waiting for the necessary equipment (probe) purchases, repairs and upgrades the AWRI agreed to the temporary use (for up to 3 months) of two of their bioreactors. These bioreactors were used for initial test experiments to establish the culture conditions for bacteria. Once the University of Adelaide bioreactors were operational, we initiated the cultures and DE experiments in our own bioreactors.

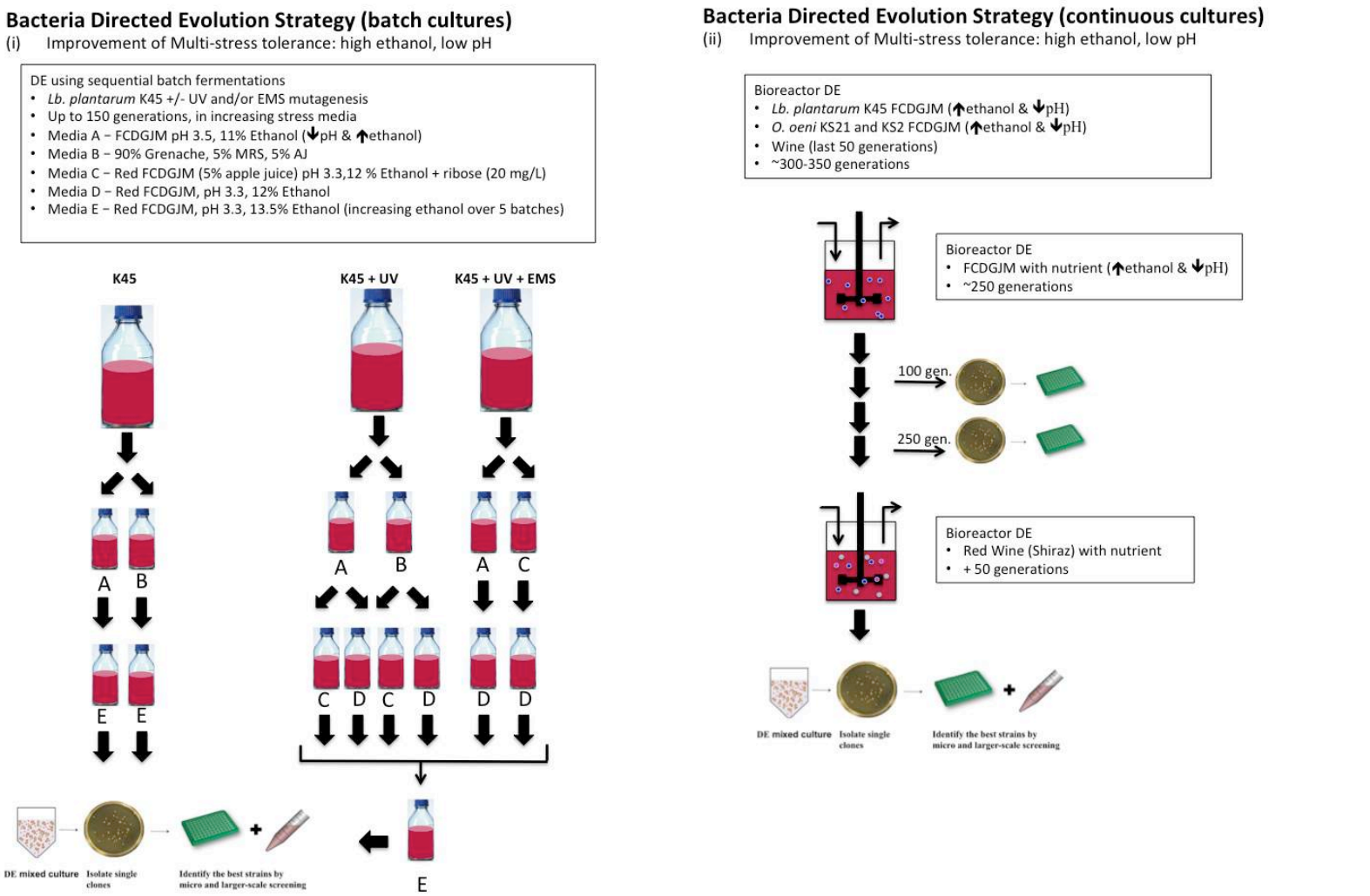


Figure 1.16: Overview of the bacterial DE Strategy

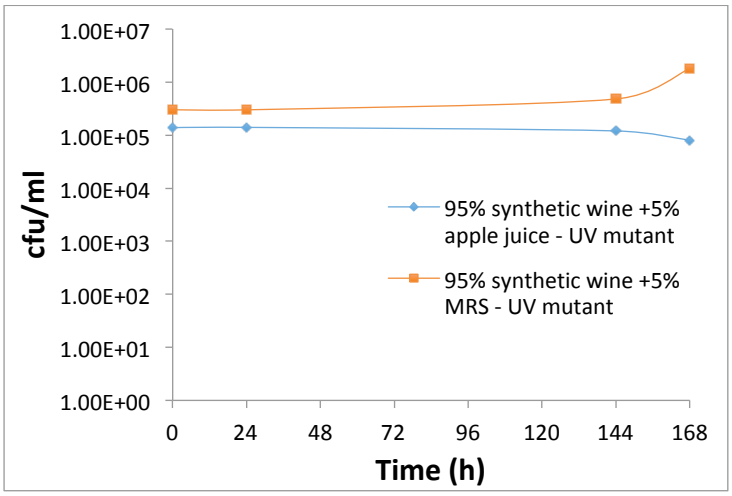


Figure 1.17: Growth of *Lb. plantarum* K45 UV treated mixed culture (T = 75 s) in CDGJM 95% w apple juice addition (AJ) or 5% MRS addition.

Directed Evolution of *Lb. plantarum* K45 by continuous culture

To broaden the stress tolerance of *Lb. plantarum* K45, continuous cultures w FCDGJM (increased ethanol/low pH) for DE. Separate batch DE cultures of strains grown under different stressors were also trialled. Initial validation of th turbidity probe was carried out to confirm the correlation between growth an turbidity as measured by the probe (**Figure 1.18**).

As reported (Output 1A 2.0), the minimum inhibitory concentrations (MIC) o and SO_2 in combination and as single 'wine-related' stresses were establi *plantarum*, which were subsequently used to set up the initial DE conditions. *L* K45 underwent continuous fermentation in a bioreactor fitted with a turbi monitor growth. The strain was cultured in Fermented Chemically Defined medium (FCDGJM, 12.5% ethanol, pH 3.3) with the inclusion of some MR growth. During the course of DE the ethanol content increased toward 13.5% pH decreased to 3.3. The total number of elapsed generations for each strain v based on doubling times calculated at different points during the DE experiment time-frame of the experiment. Accordingly, approximately 300 generations were *Lb. plantarum* K45.

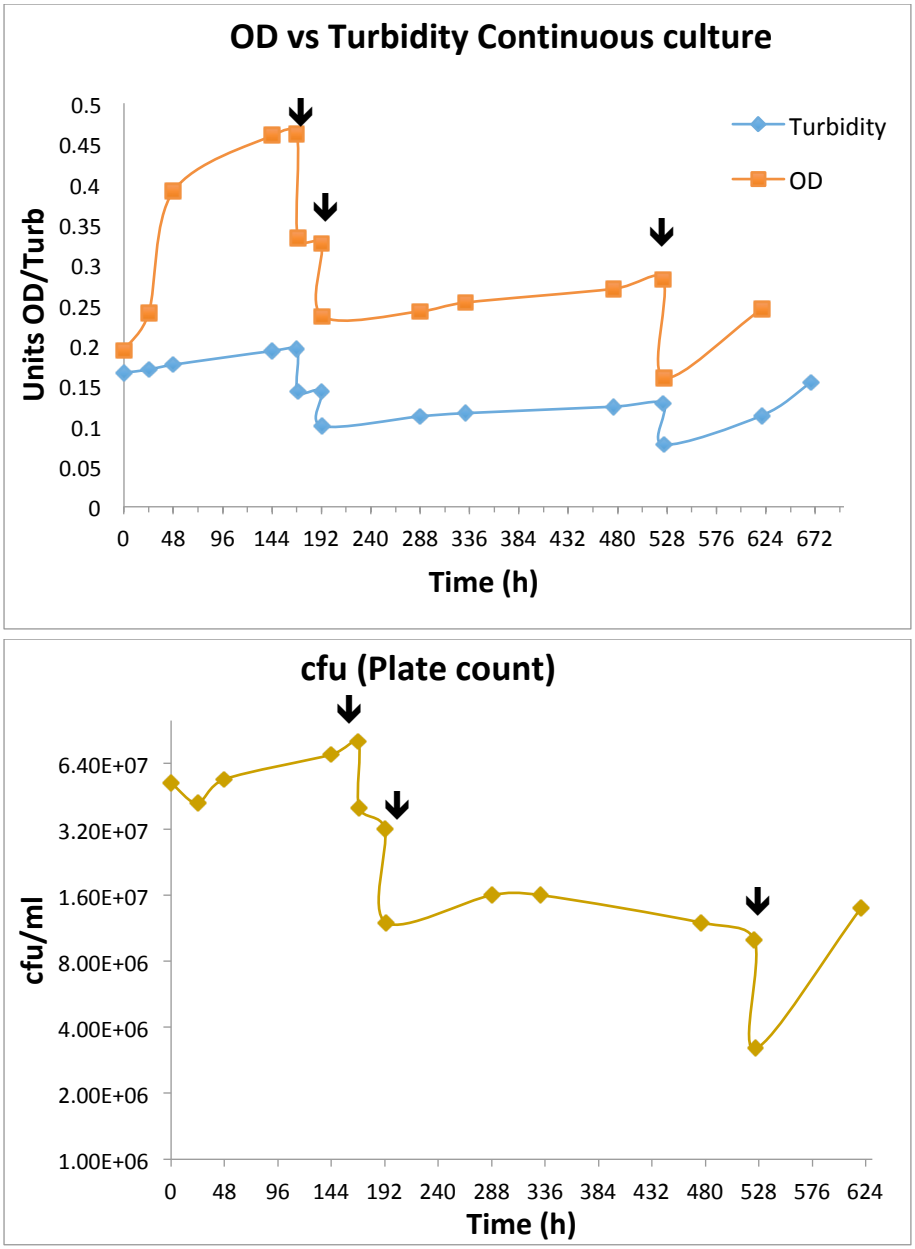


Figure 1.18: Validation of turbidity probe for use as indicator of cell growth during continuous culture. Turbidity as measured by the bioreactor was compared to both OD_{600} and CFU via plate count. ↓, media feed.

Output 1D 2.4 Directed Evolution of *O. oeni* A90 by continuous culture

A continuous culture of A90 was established in a bioreactor and grown in a wine-like environment for approximately 350 generations with increasing ethanol and sulfur dioxide (SO_2), and decreasing pH over time (PhD student Jiao Jiang). The evolving population was sampled at three significant times during the DE and screened for improved isolates based on L-malic acid consumption and growth. The DE of *O. oeni* A90 has been very successful. A third micro-fermentation of *O. oeni* DE isolates (350 generations) identified 34 isolates with shortened fermentation duration. These were evaluated together with the superior isolates from previous screens at a 50 mL scale in red FCDGJM (Fermented Chemically Defined Grape Juice Medium with polyphenol extract). The best 3 were tested for their minimum inhibitory concentration (MIC) in RFCDGJM for tolerance to: ethanol (at pH 3.5), pH (at

12% ethanol), or SO_2 and medium chain fatty acids (at 12% ethanol, pH 3.5). Two strains showed improved performance. *O. oeni* A90 DE work was carried out by Jiao Jiang and is reported in her PhD thesis (2017).

Output 2B 2.5 Directed Evolution of additional LAB

DE of KS2 and KS21 by continuous culture

Directed evolution of LAB has proven very successful, and was extended to include 2 new strains, KS2 and KS21, as part of Output 2B to increase the range of new DE strains. Continuous culture was undertaken, whereby ethanol and sulfur dioxide (SO_2) were gradually increased whilst pH was decreased. *O. oeni* KS21 was grown for approximately 320 generations, whilst *O. oeni* KS2 did not survive the final DE conditions, and will need to be revived from an earlier glycerol sample at a later date.

The establishment of DE with the second round of strains was less complicated than the first round as a large amount of trouble-shooting had already been completed. Fifteen isolates from KS21 DE were tested with the parent in 15 mL fermentations in both RFCDGJM; and wine (Section 2.0, Methods M 2.6, Results 2.5).

Output 2B 2.6 Screening for improved MLF performance during DE.

In order to validate a screening method, L-malic acid consumption was measured in 96 well microtitre plates, whereby the fermentations were replicated on several plates and a single plate was 'sacrificed' or sampled. Mixed isolates from various stages of the DE experiment including the parent strain, and mixed population samples from the bioreactor and batch experiments were evaluated by this sacrificial plating method.

The initial DE experiment with *Lactobacillus plantarum* K45 was in continuous culture with increasing ethanol in FCDGJM + 10% MRS, pH 3.5, 13.0% ethanol with the addition of 2.5 g L^{-1} L-malic acid. The original K45 strain (which was a mixed culture) was grown in continuous culture, in FCDGJM + 10% MRS (12.5% (v/v) ethanol) for ~70 generations. This DE population displayed improved growth as compared to the parent strain.

DE batch cultures of UV mutagenized cells were also grown in FCDGJM + 10% MRS (12.5% (v/v) ethanol) for ~50 generations, whilst the pH was slowly decreased. Again, the DE culture out-performed the parent when tested for growth. The mixed cultures (bioreactor continuous culture (BR) and UV treated cells) were compared against the parent in 15 mL fermentations, firstly in MRSAJ, pH 3.5, 12.5% (v/v) ethanol, 2 g L^{-1} L malic acid and secondly, in RFCDGJM, pH 3.5, 12.5% (v/v) ethanol 2 g L^{-1} L-malic acid. Growth (based on cell counts) was either equal to or increased relative to the parent, in MRSAJ, pH 3.5, 12.5% (v/v) ethanol. However, L-malic acid consumption after 192 h was incomplete for all strains but the parent; with the UV mutagenized cells being the next best. In RFCDGJM, pH 3.5, 12.5% (v/v) ethanol 2 g L^{-1} L-malic acid, consumption was also slower than the parent for both UV mutagenized and BR cells (300 h vs 192 h for the parent). This experiment was

repeated with increased ethanol (15% (v/v)) and none of the strains (including the parent) finished.

It was observed (after screening) that although the population was growing well under increasing ethanol content, L-malic acid consumption was reduced, with some clones not completing fermentation (**Figure 1.19**).

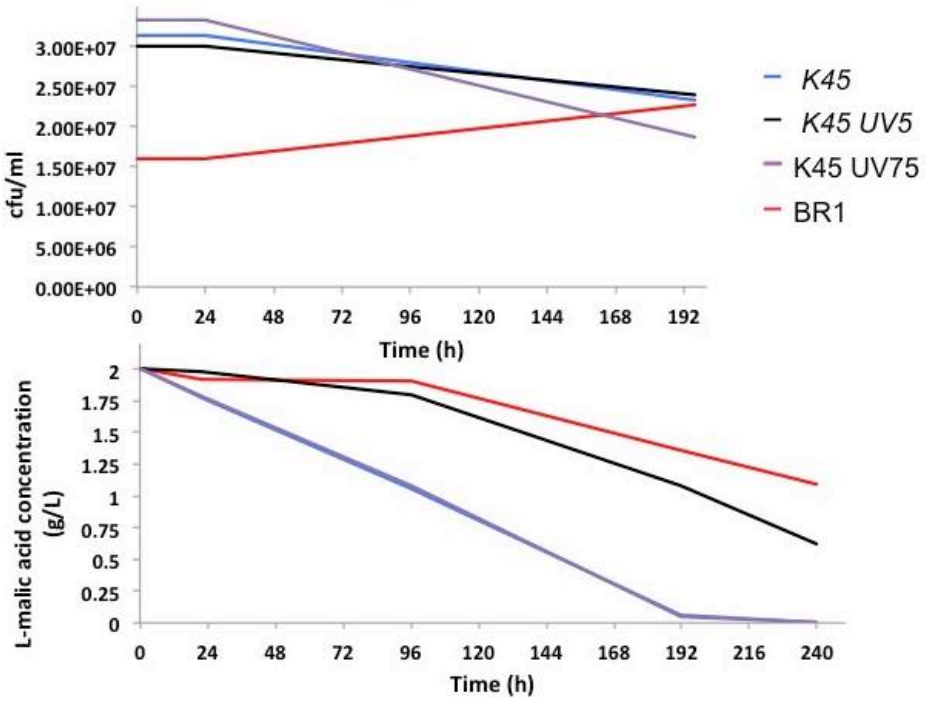


Figure 1.19: Screening of mixed culture from earlier Directed Evolution experiments for growth and L-malic acid utilization. Screening was in 15 mL cultures of MRSAJ, 12.5% (v/v) ethanol, pH 3.5, 2 g L^{-1} L-malic acid. K45 (parental culture), K45 UV5 (UV treated cells; 5 seconds), K45 UV75 (UV treated cells; 75 seconds), BR1 (mixed culture from bioreactor continuous culture).

Clonal isolates from both continuous culture and UV treated cultures (156 of each) were screened as laboratory-scale (200 µL and 50 mL) fermentations. From this initial screen none of the clonal isolates were significantly improved, with ~40% of the bioreactor isolates being comparable to the parent. Furthermore, the results suggested that the DE strategy was inadvertently selecting for faster growth and in some cases reduced L-malic acid utilization (**Figure 1.20**). It was decided to continue the DE to ensure that the number of generations achieved was sufficient to stabilise any beneficial mutations resulting in improved fermentation performance. From this first screening L-malic acid consumption appears to be uncoupled from growth for *Lb. plantarum* K45, i.e. increased growth under stress in the bioreactor did not equate to better L-malic acid consumption. This could be because of competition between sugar and L-malic acid transport. It is possible that sugar can be transported by the same route as L-malic acid and by selecting for growth over time (as happens in a long DE experiment) we have inadvertently selected for improved sugar transport rather than L-malic acid transport. During DE, an improved phenotype or property

is often associated with increased fitness. During direct competition of an ancestral microbial strain and an adapted strain, the increased fitness of the adapted variant is assumed to result in its increased frequency in the total population. However, selection for improved fitness in a specialised environment often leads to significant trade-offs in other stressful or selective conditions (Bono et al. 2017, Kassen et al. 2002). In the context of this study, the best phenotype is not necessarily the one with the highest fitness in a single (CDGJM) condition, but the one that shows increased performance and the least trade-offs in highly variable conditions, such as that found in wine.

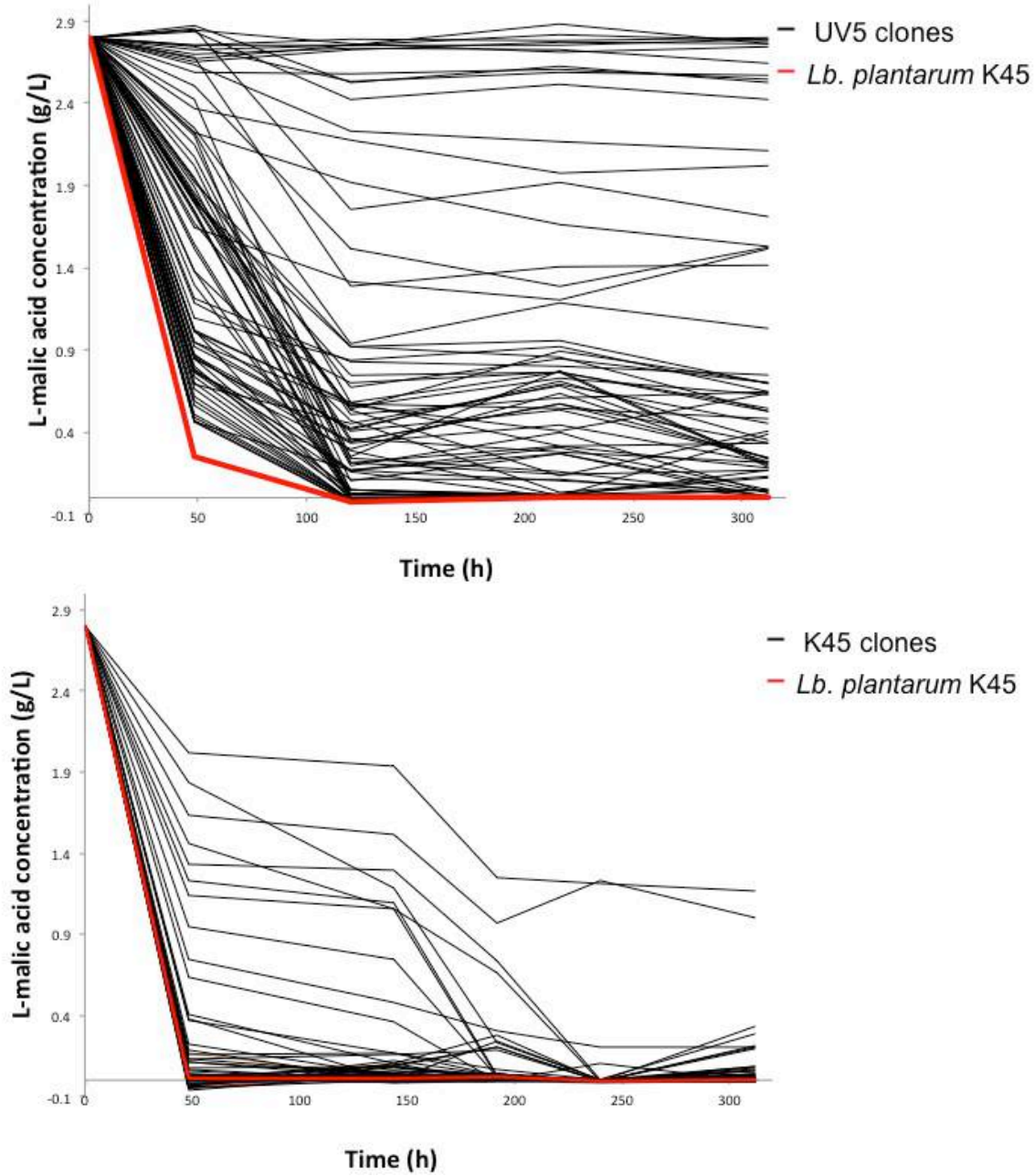


Figure 1.20: Screening of clonal isolates from DE experiments (50 generations) for L-malic acid utilization. Micro-fermentations were conducted in red FCDGJM, 15% (v/v) ethanol, pH 3.4, 2.8 g L^{-1} L-malic acid. *Lb. plantarum* K45 (original mixed culture as parent), K45 clones (clonal isolates from DE experiment), UV5 clones (clonal isolates from UV treated cells; batch DE experiment).

The best performing K45 isolate from the screen (**Figure 1.20**) was renamed as KS72 and chosen as the isolate to use for further DE experiments. It was decided after the initial screens at ~50 generations, to increase the content of L-malic acid of the medium up to 7.5g L^{-1}. *Lb. plantarum* KS72 was again grown in continuous culture with increasing ethanol to ~300 generations in FCDGJM + 10% MRS with the addition of 5 g L^{-1} L-malic acid. The final conditions were pH 3.3 and 15.0% ethanol. Additional DE batch cultures of UV mutagenised and EMS mutagenised K45 were also conducted concurrently (FCDGJM + 10% MRS pH 3.5, 14% ethanol, 2.5 g L^{-1} L-malic acid). The use of multiple DE conditions, mutagenesis and screening of large numbers of candidates was aimed at increasing the likelihood of successful isolation of improved strains.

Output 1D 2.7 Evaluation of un-inoculated ferment samples for MLF performance

O. oeni and *Lb. hilgardii* clonal isolates from a high ethanol content Grenache fermentation were investigated in parallel. MLF performance in wine was evaluated alongside the DE strains (**Figure 1.21**). Whilst the 3 *Lb. hilgardii* clones performed similarly, differences were noted between some of the other strains. The two best performing *O. oeni* (natural isolates) were evaluated in 5 L ferments along with one *Lb. hilgardii* and two *O. oeni* DE strains (see section 3.0).

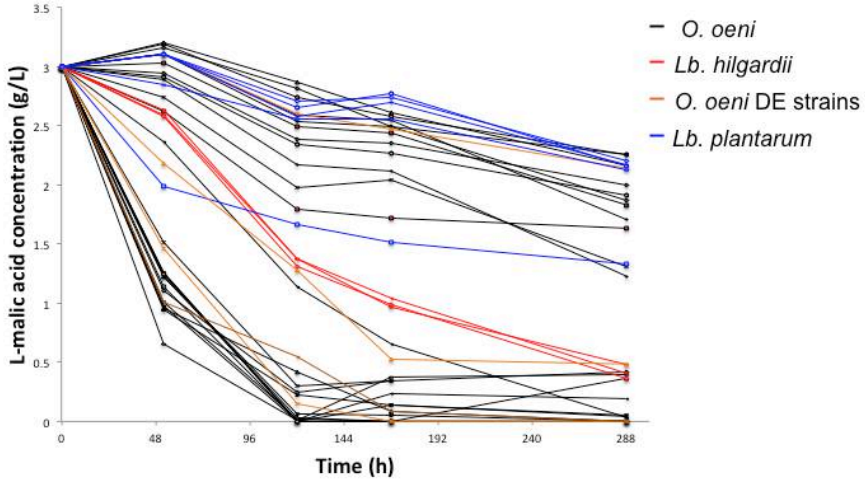


Figure 1.21: Micro-plate screen of un-inoculated ferment samples from a high ethanol (17%) Grenache.

The strains were evaluated as part of Output 2E, in Section 3.0. For details on the evaluation of the resultant cultures refer to Outputs 1E, 2D and 3B (laboratory scale) and Output 3E (20 L scale).

2.0 Fermentation performance of promising candidate microbes with improved fermentation attributes in laboratory scale fermentations

Output 1E: Summary of data on fermentation performance of promising candidate yeast (and available LAB) strains in laboratory scale fermentations.

- High throughput screening of large number of isolates according to attribute in question. To conduct in conjunction with Output 1C using micro- and lab-scale fermentations.

Output 2D: Summary of data on fermentation performance of promising candidate yeast (and available LAB) strains in laboratory-scale fermentations.

- High throughput screening of large numbers of isolates according to attribute in question. To be conducted concomitantly with Output 2B and as candidates become available.

Output 3B: Summary of data on fermentation performance of promising candidate yeast (and available LAB) strains in laboratory-scale fermentations.

- Ongoing high throughput screening of large numbers of isolates of yeast and LAB according to attributes in question and subsequent assessment in lab scale fermentations.

Contributors (Yeast DE)

Postdoctoral fellows: *Drs Michelle Walker, Tommaso Watson, Jennie Gardner, Joanna Sundstrom and Professor Vladimir Jiranek*

Contributors (LAB DE)

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Background

A summary of the data on the fermentation performance of the most promising candidate yeast and LAB strains in laboratory-scale fermentations for the combined years 1 to 3 (Outputs 1E, 2D and 3B) are reported below.

Methods

M 2.1 Evaluation of yeast fermentation performance in microscale (0.2 mL)

The DE populations (Output 1D 1.8) were evaluated in micro-fermentations and the best performing strains were selected for further evaluation. Single colony isolates and the mixed population were compared against the parent strains and reference Uvaferm 43 in three media; filtered white juice, or filtered white juice with 5% ethanol or with additional sugar (50 g L^{-1}). Strains were inoculated into 0.6 mL YPD in a 96 deep-well plate, sealed with a cloth seal and grown 2 days at 28°C without agitation. 0.2 mL of juice starter medium was inoculated with 2 µL of the YPD culture and grown for 1 day (without agitation). The culture was diluted with 0.8 mL fresh starter medium and used to inoculate 4 microtiter plates for each condition (10 µL culture into 190 µL medium). The plates were sealed with breathable clear film and incubated at 30°C in a CO_2 incubator at 20% CO_2 and 70-85% humidity. At specific intervals during fermentation, one plate was removed, re-sealed with an impermeable film and stored for sugar analysis.

M 2.2 Evaluation of yeast fermentation performance in 100 mL cultures

Fermentations were performed on a robotic fermentation platform (T-bot). The strains were evaluated at 22°C as single or triplicate fermentations in 100 mL Stress Medium A or 0.45 µM filtered white juice with added ethanol (5%). Starter cultures were grown 2448 h in 10 mL Starter Medium 2 or juice starter medium (45% juice: 45% YPD: 10% H_20) prior to inoculation of fermentations at 1%. Samples were collected at programmed intervals and stored for sugar analysis.

M 2.3 Evaluation of LAB MLF performance in microscale (0.2 mL)

The DE populations (Output 1D 1.8) were evaluated in micro-fermentations and the best performing strains were selected for further evaluation. Single colony isolates and the mixed population were compared against the parent strains RFCDGJM as described previously (Section 1).

M 2.4 Micro-plate screen of LAB bioreactor clones

180 clones isolated from continuous DE culture were screened in RFCDGJM (pH 3.3, 13% ethanol) for increased L-malic acid consumption. Plates were set up for sacrificial sampling as described earlier. The parent strain used in the bioreactor, KS72 originated from a screen of L-malic acid consumption of parental isolates from the original freeze-dried packet.

M 2.5 Micro-plate screen of *Lb. plantarum* EMS mutants

90 isolates from each EMS mutagenesis experiment with strain KS72 and KS78 were screened in RFCDGJM for increased L-malic acid consumption. Strain KS78 is an isolate from the original UV mutagenesis and subsequent batch culture screening that was subjected

to EMS treatment as with KS72. Plates were set up for sacrificial sampling as described in M1.13.

M 2.6 Evaluation of MLF performance in 15 mL and 50 mL cultures

All the strains were cultured in MRSAJ before inoculation into the experimental medium. Except for the micro-scale screening experiments, for which a simplified inoculation regime was used (see M 1.13), growth of bacterial starter cultures was monitored at 600 nm (OD_{600}) using a Helios Cuvette spectrophotometer (Thermo Scientific). At an OD_{600} of 1.2, the liquid cultures were diluted with MRSAJ to OD_{600} = 1.0. A final volume of 2% (v/v) of starter culture was inoculated into each experimental medium (15 mL or 50 mL). In the larger scale ferments, viable cell numbers were determined by serially diluting the sample with ultra-pure deionised water followed by spot-plating 5 µL droplets on MRSAJ agar plates. These were incubated at 30 ˚C with 20% (v/v) CO_2 for 27 days (*Lb. plantarum* 2d, *O. oeni* 7d) before the colonies were counted.

MLF performance of *Lb. plantarum* in 15 mL and 50 mL cultures

Thirty isolates were selected based on performance in micro-fermentation screens, and evaluated in 15 mL and 50 mL fermentations in RFCDJGM 15% (v/v) ethanol, pH 3.5 as per M2.6. Ten isolates were chosen and screened together with the parents in a 50:50 mix of RFCDGJM and Grenache (as used in original batch cultures) with the final conditions being 16% (v/v) ethanol, pH3.2. The isolates tested were; KS72, KS72_15, KS72_87, KS72_26, KS72_36, KS78, KS78_E93, KS78_E35, KS78_E15, and KS78_E70. Cells were inoculated at a higher rate in this assay as recommended by Lallemand. However these conditions were too harsh and none of the isolates finished, so the ferments were repeated again but with the medium diluted with water to pH 3.5 and 14.12% (v/v) ethanol.

The best clonal isolates from both continuous culture and UV mutagenised cultures were evaluated in 15 mL RFCDGJM, together with the 6 most promising *Lb. plantarum* clones from all experiments (selected from 200 µL and 50 mL fermentations) and the parent strain. The isolates were tested against the previously established MIC's of the parent strain for pH, ethanol, and osmotic stress (**Table 2.1**) to determine whether the new strains were robust under varying conditions. Osmotic stress was chosen as an additional stressor, given the use of *Lb. plantarum* as a co-inoculum.

Unfortunately, the DE strains did not show improved performance relative to the parent strain in any of the tested conditions. It was then decided to screen additional strains (K45, KS72, KS72_4, KS72_15, KS72_16, KS72_26, KS72_36, KS72_37, KS72_87, KS72_107, KS72_114, KS72_135, KS78, KS78_E3, KS78_E15, KS78_E70, and KS78_E106) from the previous 50 mL ferments under fewer conditions (**Table 2.2**).

Table 2.1: Levels of stressors in CDGJM and RFCDGJM for minimum inhibitory concentration (MIC) assays.

Base medium	MIC's tested		
	Ethanol (%)	pH	Glucose:Fructose (50:50) g L^{-1}
RFCDGJM	12.5, 13.5, 15.5, 17.5	3.5	0
RFCDGJM	12.5	2.8, 3.0, 3.2, 3.4	0
CDGJM +17% RFCDGJM	2	3.4	260, 290, 320

Table 2.2: Levels of stressors in RFCDGJM and red wine used to evaluate malic acid utilisation in *Lb. plantarum* DE isolates

Base medium	Ethanol (%)	pH
RFCDGJM	13, 15	3.4
Mataro	13	3.4
Shiraz	15.6	3.6

MLF performance of KS21 and KS2 in 15 mL cultures

KS2 and KS21 were grown under highly stressful conditions for 380 days, with the final conditions being in wine (pH 3.1, 16% ethanol, 25 mg L^{-1} SO_2). Attempts were made to isolate cells from this final stage, but only 11 colonies grew on MRSAJ plates from KS21 and none for KS2. It was decided to check glycerol stocks of earlier time-points, to determine when death had occurred, and rescue the isolates from a viable glycerol culture. Samples were also plated onto three different media: MRSAJ agar, FCDGJM agar, FCDGJM (70% FCDGJM:30% AJ) agar. Two cells grew on MRSAJ agar for strain KS21 DE (Dec 2016) and earlier glycerol (KS21 October 2016) had numerous colonies but was not used. KS2 failed to grow on plates from the same time-points. It remains to be determined when KS2 lost viability during the DE experiment

.

Fifteen isolates were screened in RFCDGJM (pH 3.2, 13.8% ethanol). Cultures were grown to 1.3 OD_{600} and inoculated 1:50 into 14 mL RFCDGJM. Fermentations were performed in triplicate at 21˚C. The best isolate from this experiment were then tested in four wines (**Table 2.3**).

Table 2.3: Stress parameters in wine used to test performance of *O. oeni* KS21 DE isolates

Base medium	Ethanol (%)	pH
Merlot (2016)	14.68	3.4
Merlot (2017; frozen must from 2015 vintage)	15.1	3.4
Shiraz	15.6	3.45
Mataro	13.0	3.4

MLF performance of isolates from un-inoculated ferments in 15 mL cultures

The ability of *O. oeni* strain Oe16 to conduct MLF was examined together with additional LAB isolates by Master of Viticulture & Oenology student Xingwei Mi (Drs Sumby and Grbin supervisors). Oe16 was evaluated under different pH, ethanol and SO_2 treatments in RFCDGJM. Cold stabilised RFCDGJM was adjusted based on experimental design and sterile filtered (0.2 μm) before use. pH was adjusted using HCl (37%) or NaOH (10 M). Alcohol concentration was adjusted by the addition of 100% ethanol and potassium metabisulfite (PMS) was used for SO_2 adjustment. pH, ethanol and SO_2 were assessed in single parameter experiments. The standard RFCDGJM condition was 2.5 g L^{-1} L-malic acid, pH 3.6 and 12% (v/v) ethanol. Adjustment for each assay is shown in **Table 2.4**. The fermentation experiments were conducted in duplicate at 23 °C and L-malic acid was measured every 24-48 hours. Isolates were cultured in 10 mL MRSAJ at 30 °C for 48 hours, and then sub-cultured in 50 mL of medium (50% MRS-AJ and 50% FCDGJM (without red grape skin extract)) at 30°C for 48 hours. Before inoculation into the final FCDGJM, the cultures were adjusted to $OD_{600} = 1.0$ and inoculated at 1:50 for a 45 mL culture.

Table 2.4: The chosen levels of pH, ethanol and SO_2 for tolerance assay

Stress factors	Stress levels			
pH	3.2	3.4	3.6	3.8
Ethanol (% (v/v))	12	14	16	18
SO_2 (mg L^{-1})	10	20	30	40

Summary of Outcomes

1,912 potentially improved yeast and 800 bacterial isolates were screened at a laboratory scale for desirable fermentation attributes. A multi-staged screening approach was used where the most promising isolates from each stage were progressed to the next stage. Later stages had increased replication, larger volumes and performance in a number of different media was also evaluated. Results were compared to widely used industry standard robust microbes, Uvaferm 43 (yeast) and VP41 (bacteria).

Twelve evolved wine yeast had significantly improved fermentation efficiency in media,

similar to which they were evolved, in comparison to their parents and Uvaferm 43. The most promising 8 of these were evaluated in 2 juices and defined medium, each modified to resemble a challenging must. Variable performance was seen in these media with strain RM7-71 performing well in all three conditions, similar to Uvaferm 43.

Further testing of evolved bacterial strains derived from A90 resulted in selection of the three most promising, these being 1-161, 2-49 and 3-83. Performance was consistently superior to parents SB3 and A90 across four wines. 15 improved strains were selected from KS21 and one (G55) from un-inoculated fermentations. Interestingly G55 was capable of undergoing malolactic fermentation in the presence of 18% ethanol.

Such strains will be further shortlisted and the best performing sent to starter culture suppliers for evaluation ahead of release to winemakers.

Results and Discussion

2.0 Periodic evaluation of evolved yeast populations from sequential batch DE

The yeast DE populations were evaluated at intervals to determine whether genetic changes within the population resulting from temporal exposure to multiple stresses, were noticeable as fermentation variability within the population. Typically ~90 single isolates were compared to the parents and the mixed population, in the presence of stressors with a view to identifying isolates with efficient fermentation performance. Over the entirety of this study a total of 1912 isolates were examined in either 0.2 mL or 100 mL scale in CDGJM or white juice with added sugar (50 g L^{-1}) or 5% ethanol.

From Stage 1 sequential batch (flask and bioreactor) DE cultures, three populations evolved for between 50 and 90 generations were evaluated (88 single colony isolates from each). These being; 1. C7H BIO (56 generations), 2. C7H BIO (94 generations) and 3. C7H_A (67 generations), each evaluated in 100 mL fermentations. The medium (CDGJM) was modified to include stressors similar to the source DE experiment. For C7H BIO this was 30 g L^{-1} fructose, 10 g L^{-1} glucose with either 75 mg N L^{-1} and 12% ethanol (1) or 55 mg N L^{-1} and 13% ethanol (2). For C7H_A, this was Stress Medium A. No significant improvements in fermentation performance were observed (data not shown). However, population variability between the individual colony isolates suggested that the populations were becoming heterogeneous, and that DE should be continued.

Selected populations were evaluated when cultures reached between 100 and 150 generations as juice fermentations with added sugar or ethanol. At 100 generations, the populations C7H_B (29 isolates), C7H BIO_A (27 isolates) and C7H BIO_ B (39 isolates) performed similarly to FM16 C7H and corresponding mixed population, or worse (data not shown). These findings may be due to the small number of isolates tested as well as the strains not being adapted to grape juice (~240 g L^{-1}). The mixed culture DE culture pooled from several DE experiments (**Output 1D** 1.8) was evaluated at 130 and 150 generations. At 130 generations, 84 clonal isolates were analysed and the population was found to be diverse, with many strains having extended lag phases prior to normal exponential growth which was reflected in protracted fermentation (**Supplementary Figure 2.1**). After 150 generations, the mixed culture DE population (80 clonal isolates examined) appeared to be less heterogeneous with 3 population clusters with various lag phases in growth (data not shown). It is expected

that this population would become more homogenous with time.

The 13 evolved populations (**Output 1D** 1.8, **Table 1.6**) and the C7H continuous culture (C7H_BIO_CC) were evaluated as micro-fermentations. The fermentation performance of 92 isolates from each population (1288 clonal isolates in total) were evaluated in juice (Marsanne, Semillon or Viognier) with or without additional sugar (50 g L^{-1}) or 5% ethanol (see Methods). The DE populations RM8 (Fermichamp x Q2) and RM10 (Fermichamp x Maurivin B) were not further evaluated as 100 mL fermentations because of time constraints.

The 90 most promising clonal DE strains (in all 3 conditions) were first evaluated as non-replicated 100 mL fermentations in similar stress conditions used for the DE (except for the continuous culture) ie. CDGJM (210 g L^{-1} sugar, 350 mg N L^{-1}, 5% (v/v) ethanol, 2 mg L^{-1} each of octanoic and decanoic acid, at pH 3.2). Twelve strains were observed to have significantly improved fermentation efficiency when compared to the parents and Uvaferm 43 (**Figure 2.1**). The commercial yeast strain Uvaferm 43 was used as a benchmark since it is routinely used in industry to restart stuck fermentations.

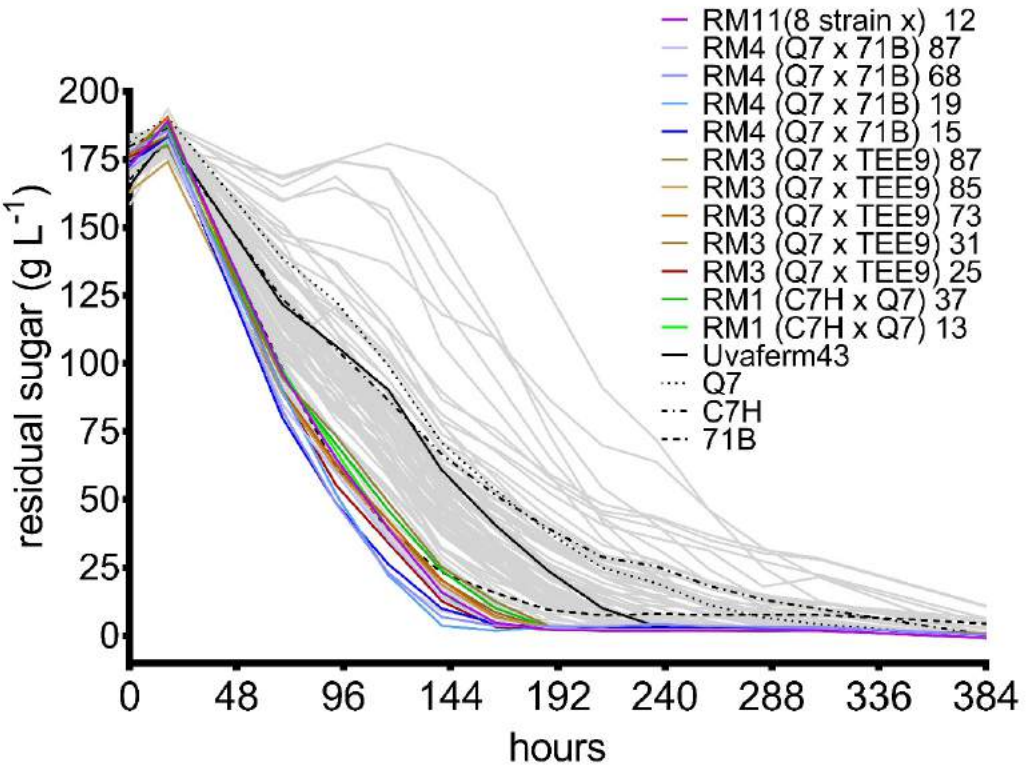


Figure 2.1: Fermentation performance of the best 90 candidate evolved strains in modified CDGJM as non-replicated 100 mL fermentations. Q1, E3 and M16 were fermented in 100 mL thawed, filtered juice at 22 °C. Strains were propagated in CDGJM starter:YPD (1:1) and inoculated at 1% (~1 x 10^6 cells mL^{-1}). Progress was monitored as sugar consumption. RM represents random sporulation and mating of strains with 'x' meaning genetic cross; numeral denotes single colony isolate.

These 12 strains, together with others representative of the best of each of the 14 DE populations were tested in two separate fermentation experiments to examine whether the fermentation improvement was media-specific. The ability to successfully complete

fermentation in a range of juices and winemaking conditions (without impacting on wine composition and quality) is a measure of a strain's suitability for the wide variety of challenges found in industrial fermentations.

Triplicate 100 mL fermentations were undertaken with selected strains in modified CDGJM (210 g L^{-1} sugar, 350 mg N L^{-1}, 5% ethanol, with 2 mg L^{-1} of octanoic acid and decanoic acid) at pH 3.2. Tee 9 (evolved from AWRI 796) and RM7_71 (Uvaferm x Q2) outperformed Uvaferm 43, whilst the others were comparable to one of the parents, which suggested that the isolates were likely to be monosporic clones. Our best evolved strain, C7H (Project UA1101) was originally evolved from L2056 over 350 generations (McBryde et al., 2006). In the current project it was further evolved under different stress conditions, with the goal of improving fermentation efficiency. Evaluation at 100 mL scale suggested that the strain had been further adapted as shown by the improved sugar consumption (**Table 2.5**). However, upon strain identification using Delta PCR (**Supplementary Figure 1.4**), it would appear that the strain is closely related to Q7 than C7H as is C7H B27. The isolates C7H_A86 and C7H_Bio B34 look similar to C7H. For the C7H_B DE experiment it will be necessary to screen earlier stages to determine when the contamination by Q7 occurred.
Interestingly, the RM3 isolates except for RM3_73, appear to be either closely related to Q7 and perhaps represent monosporic clones. RM3_73, together with RM4 isolates and RM5_15 differ from the parents and may be hybrids. Genome sequencing is required to determine the evolutionary relationship between the strains. The 3 clonal isolates of RM11 are all identical suggesting that this strain in the mixture, has dominated the population over time.

A second experiment was undertaken in which the 8 best performing strains were evaluated as triplicate 100 mL fermentations in 3 different media: 1. Semillon juice (2016, Waite vineyard), 2. Viognier juice (2016, McLaren Vale) and 3. CDGJM (210 g L^{-1} sugar, 350 mg N L^{-1}, 5% ethanol (v/v), pH 3.2). The juices were pH adjusted to 3.2 with tartaric acid. Increased stress was applied by the inclusion of 30 g L^{-1} glucose, 30 g L^{-1} fructose, 5% ethanol (v/v), and 3 mg L^{-1} MFCA. Fermentation performance of individual strains varied with medium, with all strains unable to utilise all sugars in CDGJM within 594 h when the experiment was terminated (**Figure 2.2**). In all three media RM7-71 (Uvaferm 43 x Q2) was capable of performing similarly to Uvaferm 43, and both outperformed all other strains.

The 5 best performing strains, together with their parents, were further evaluated in larger scale (20 L white and 50 kg red) fermentations (see Outputs 1E, 2D and 3B).

Table 2.5: Fermentation performance of yeast isolates from DE populations

Strain	Residual sugar g L^{-1}	Duration (h)
FM16 C7H	22.53 ± 12.52	474*
C7H_B4	5.79 ± 2.56	474*
C7H_B27	5.13 ± 6.89	474*
C7H_BIO B34	15.99 ± 15.01	474*
C7H_A86	8.57 ± 5.75	474
Q7	0.77 ± 4.56	474
RM1 (Q7 x C7H) 13	0.21 ± 2.65	474
RM1 (Q7 x C7H) 37	0.33 ± 3.85	474
Tee9	-1.73 ± 0.18	332
RM3 (Q7 x Tee) 25	-1.84 ± 0.10	401
RM3 (Q7 x Tee) 31	3.24 ± 8.75	474*
RM3 (Q7 x Tee) 73	-1.95 ± 0.06	425
RM3 (Q7 x Tee) 85	9.84 ± 5.15	474*
RM3 (Q7 x Tee) 87	1.78 ± 5.51	474
71B	16.88 ± 0.76	474*
RM4 (Q7 x 71B) 15	1.54 ± 0.79	474
RM4 (Q7 x 71B) 19	-1.74 ± 0.16	332
RM4 (Q7 x 71B) 68	6.05 ± 0.49	474*
RM4 (Q7 x 71B) 71	-1.72 ± 0.06	332
RM4 (Q7 x 71B) 87	11.00 ± 1.64	474*
RM5 (C7H x 71B) 15	5.13 ± 4.08	474*
Uvaferm 43	-1.7 ± 0.12	355
Fermichamp	-1.79 ± 0.11	425
Q2	35.29 ± 11.83	474*
RM7 (Uvaferm x Q2) 71	-1.76 ± 0.01	332
Mixed culture - 65	1.12 ± 3.162	474
Mixed culture - 39	5.16 ± 5.19	474*
RM11-12	2.39 ± 6.25	474*
RM11-26	0.42 ± 2.20	474
RM11 - 66	1.2 ± 2.99	474

Strains were compared with the parent strains and Uvaferm 43 (as industry benchmark strain) in triplicate 100 mL fermentations in modified CDGJM. Values represent total sugar (g L^{-1}) for 3 replicate fermentation samples ± SD at 474 h. The fermentation duration represents the hours after inoculation for the fermentations to become dry. * denotes fermentations were not complete when experiment was terminated.

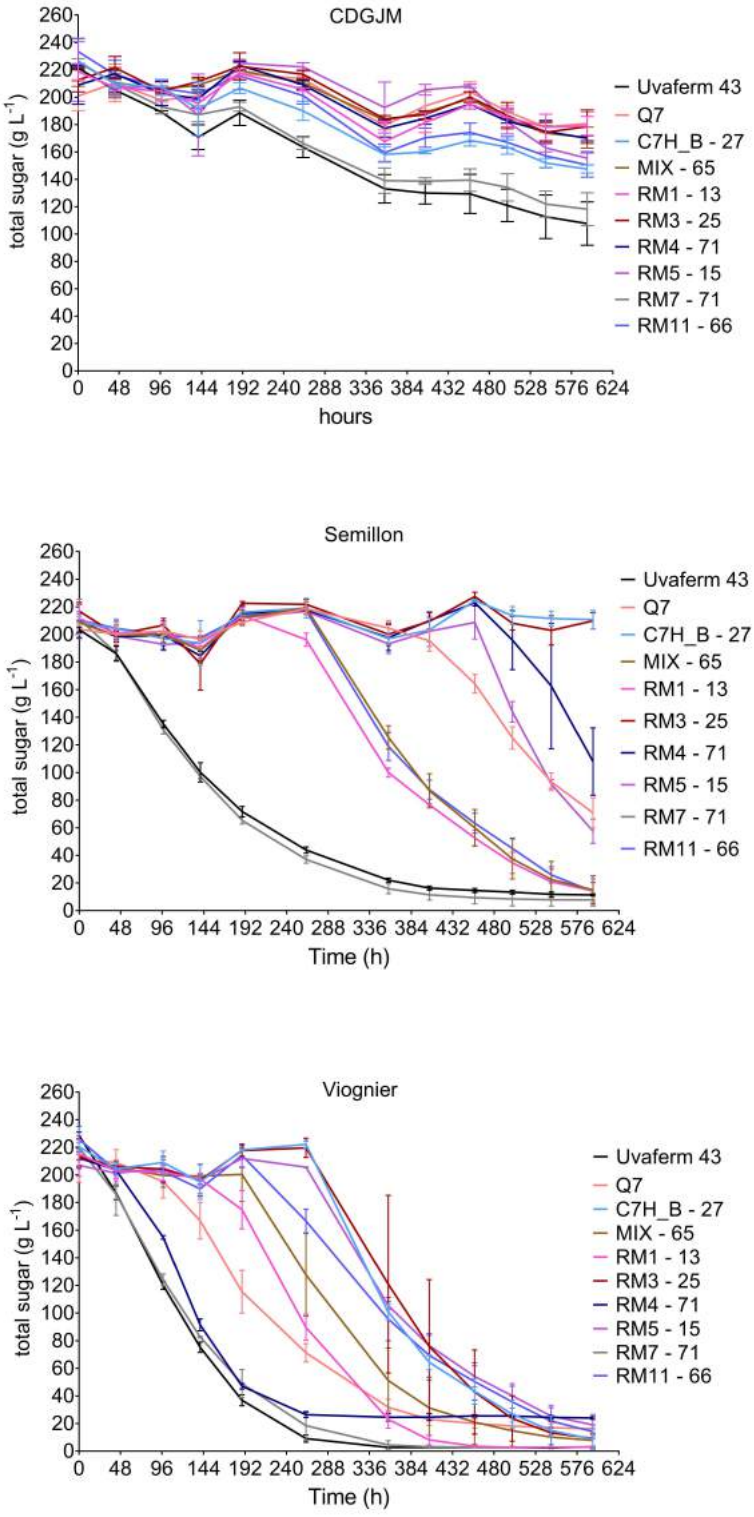


Figure 2.2: Fermentation performance of evolved strains in 3 media.
100 mL (non- replicated) fermentations of Modified CDGJM, Semillon or Viognier juice at 25 °C. Ethanol and MCFA were added to media and then pH was adjusted to 3.2. Strains were propagated in CDGJM starter:YPD (1:1) and inoculated at 1% (~1 x 10^8 cells mL^{-1}). Progress was monitored as sugar consumption.

2.1 Periodic evaluation of evolved LAB populations from sequential batch DE and continuous culture

The LAB DE populations were evaluated at intervals to determine whether genetic changes within the population resulting from temporal exposure to multiple stresses are noticeable as fermentation variability within the population. Typically ~160 single isolates were compared to the parents and the mixed population in the presence of stressors (RCDGJM), with a view to identifying isolates with efficient MLF performance. Over the entirety of this study a total of ~800 isolates were examined in either 0.2 mL, 15mL or 50 mL fermentations in RCDGJM, RFCDGJM or wine.

The bioreactor conditions were changed for *Lb. plantarum*, after noticing a decoupling of L-malic acid consumption from growth, to include an extra 5g L^{-1} L-malic acid in the feed medium (FCDGJM + MRS, pH 3.4, 14% ethanol) to "select" for clones that were efficient in L-malic acid consumption. The bioreactor isolates were rescreened at approximately 250 generations (8 months).

A large number (180) of clonal isolates was screened in RFCDGJM (pH 3.3, 13% ethanol) (**Figure 2.3**). Decoupling of L-malic acid consumption was not observed and several isolates appeared to consume L-malic acid faster than the parent. These strains require evaluation on a larger scale.

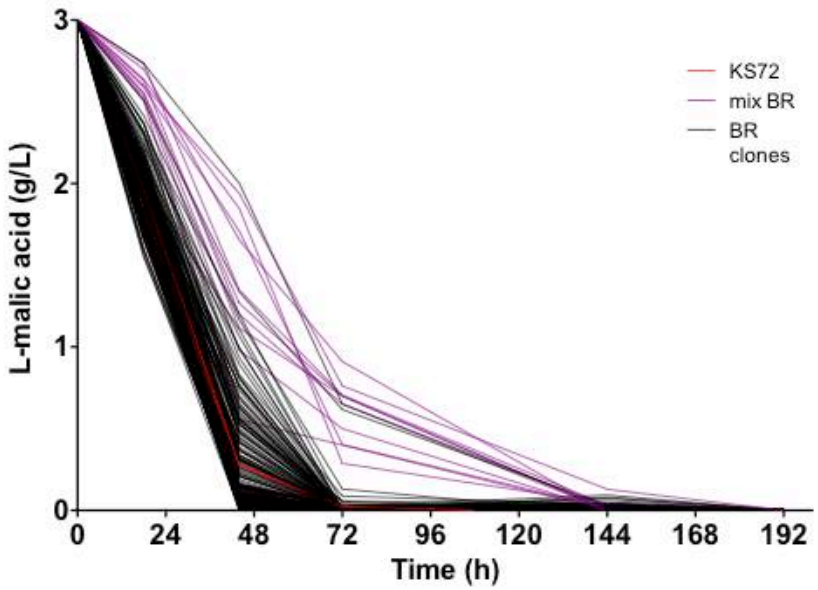


Figure 2.3: Malic acid utilisation by 180 continuous culture isolates in 0.2 mL fermentations conducted in RFCDGJM (pH 3.3, 13% ethanol).

2.2 Micro-plate screen for MLF performance of *Lb. plantarum* EMS mutants

108 clones isolated from each of the EMS mutagenesis experiments with *O. oeni* KS72 and KS78 were screened in RFCDGJM for increased L-malic acid consumption (**Figures 2.4** and **2.5**). From this screen it appears that these isolates from two rounds of mutagenesis are the most likely candidates for improved L-malic acid consumption and isolates that were faster than the parent were then selected for testing at a larger scale.

Various pre-selection methods were trialled prior to screening to increase the chance of selecting an improved strain from the EMS experiments. Micro-fermentations proved most reliable. Screening on agar plates containing L-malic acid and pH indicator (consumption leading to a change in pH and indicator colour) was unsuccessful (data not shown).

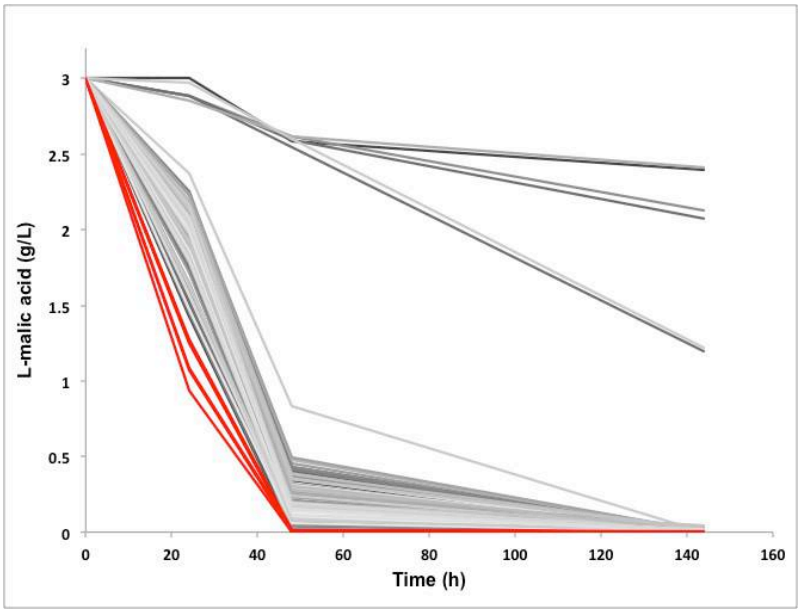


Figure 2.4: 108 EMS mutants were screened in RFCDGJM for improved MLF.
All isolates were screened for increased L-malic acid consumption alongside the parent (KS72 – red lines).

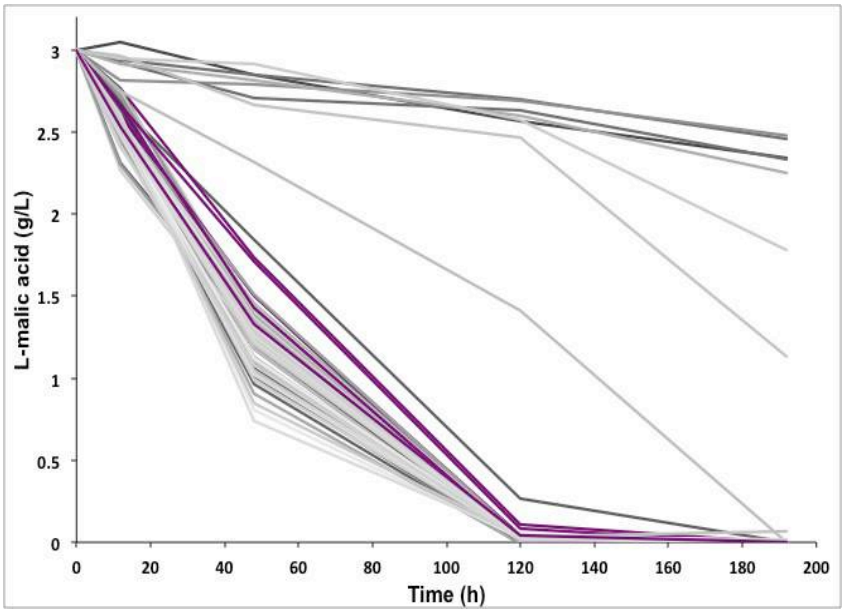


Figure 2.5: 108 clones isolated from EMS experiments were screened for MLF in RFCDGJM. All isolates were screened for increased L-malic acid consumption alongside the parent (KS78 – purple lines).

2.3 Evaluation of MLF by *Lb. plantarum* isolates grown in 15 mL and 50 mL cultures

Thirty promising *Lb. plantarum* clones from continuous culture and EMS experiments were selected from micro-fermentation results (396 clones) and were evaluated in 50 mL RFCDGJM fermentations alongside the parental strains (K45, KS72, KS78) (**Figure 2.6**). Seventeen isolates were then screened in RFCDGJM (15% (v/v) ethanol, pH 3.5; **Figure 2.7**).

Eight promising *Lb. plantarum* clones from continuous culture and EMS experiments were selected based on improved malic acid consumption when grown as 50 mL cultures. They were further evaluated in 50 mL 50% RFCDGJM, 50% Grenache medium, alongside parental strains (KS72 and KS78). The small difference in acid consumption between KS72 and KS72_87 under these conditions (**Figure 2.8**), suggests that the conditions were not harsh enough to differentiate them.

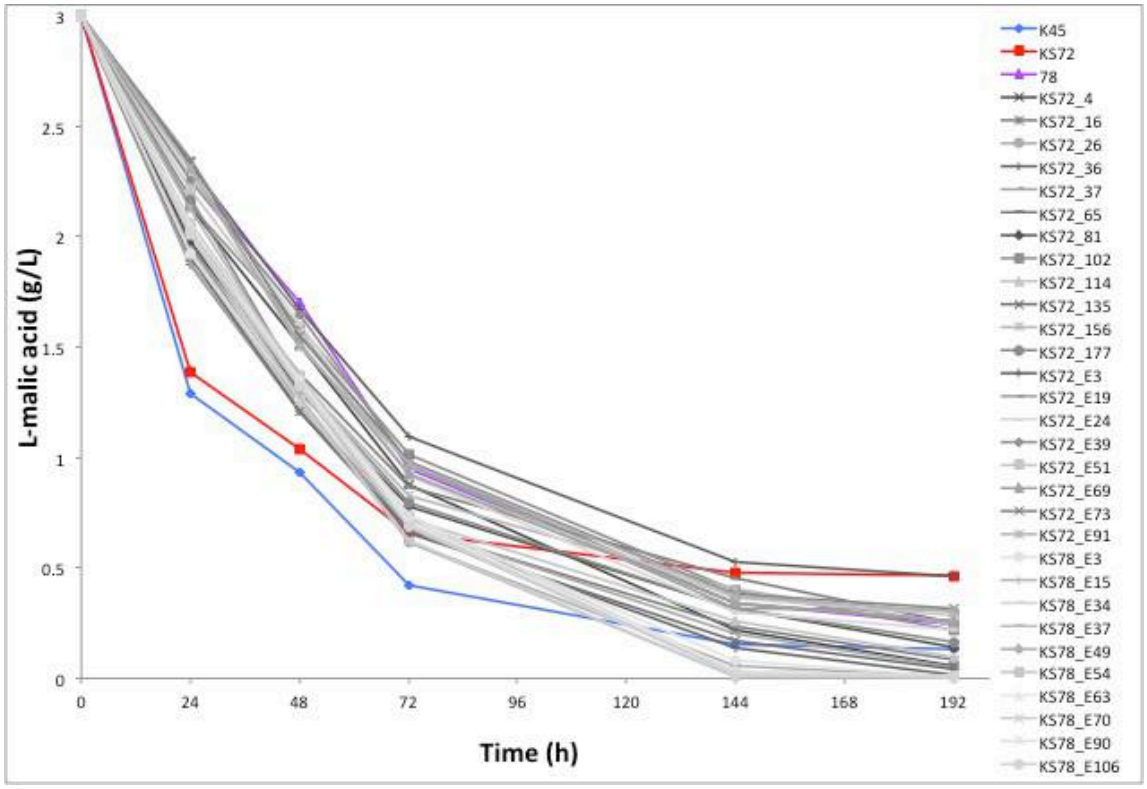
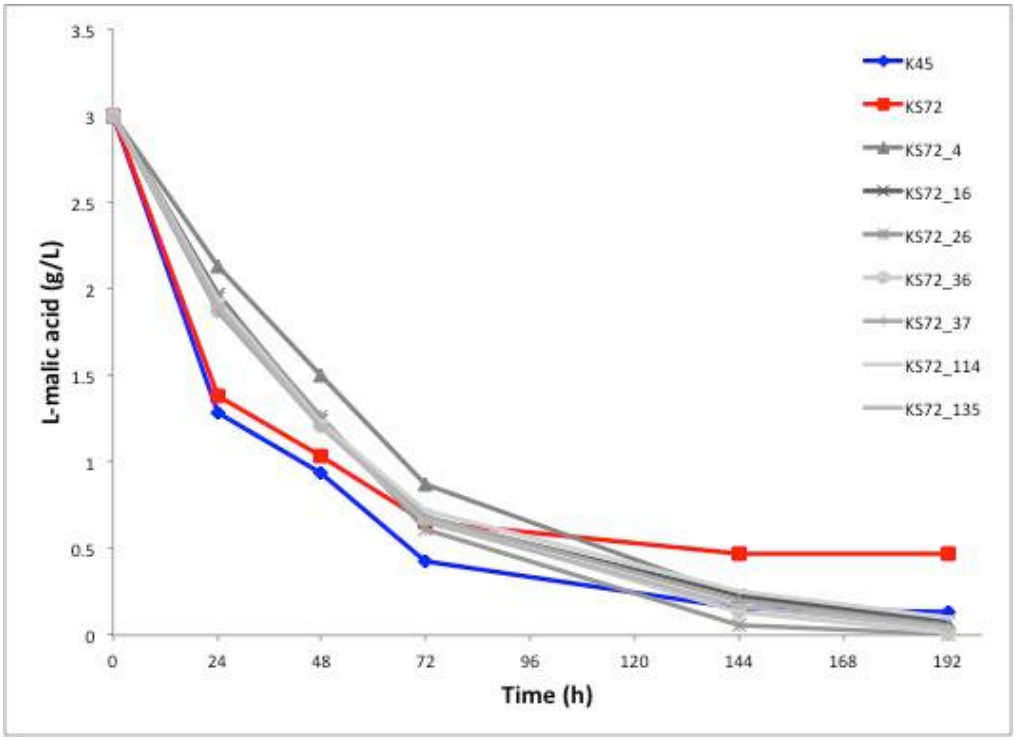


Figure 2.6: 30 isolates from continuous cultures in a bioreactor and EMS experiments were screened for improved MLF in Red FCDGJM. Conditions were RFCDGJM (15 % (v/v) ethanol, pH 3.5) tested for increased L-malic acid consumption alongside the parents (K45, KS72 and KS78).

Further screening of clonal isolates from both continuous culture and UV-treated cultures was performed in 15 mL RFCDGJM. The 6 most promising *Lb. plantarum* clones from all experiments (0.2 mL and 50 mL fermentation data) and the parent strain were tested against the previously established MIC's of the parent strain for pH, ethanol, and osmotic stress (**Table 2.1**) to determine if the new strains are robust under varying conditions. Unfortunately the DE strains did not show improved performance relative to the parent strain in any of the tested conditions. It was then decided to screen 15 additional strains from previous 50 mL ferments under fewer conditions (**Table 2.2)**.

Thirteen of the tested strains had improved malic acid consumption in RFCDGJM (13% ethanol, pH 3.4) but not in RFCDGJM (15% ethanol, pH 3.4). All of the strains, including the parent, failed to finish MLF. Furthermore, none of the DE strains were able to perform MLF in either of the Mataro or Shiraz wines (**Table 2.2**), whereas the parent strain finished after 5 days. Initial inoculation rates were similar across the experiments (2 x 10^7 CFU/mL), and all but one isolate maintained viability in all tested media over the 7 days. The DE isolates appear to have adapted to a narrow range of conditions and the reason for this needs to be investigated.

A



B

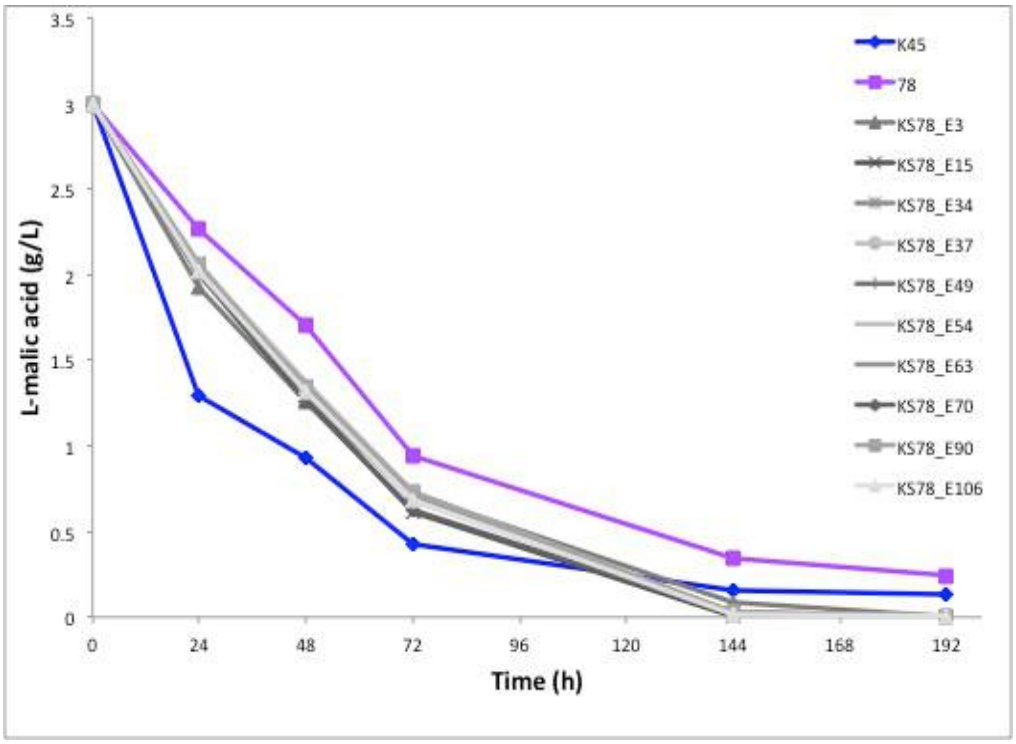


Figure 2.7: MLF screen of the best 17 *Lb. plantarum* isolates from bioreactor and EMS experiments. Isolates were screened in Red FCDGJM (15 % (v/v) ethanol, pH 3.5). A; Bioreactor isolates, B; EMS isolates. K45 is the original strain used in this study, whilst KS78 and KS72 are the parent strains of the evolved isolates.

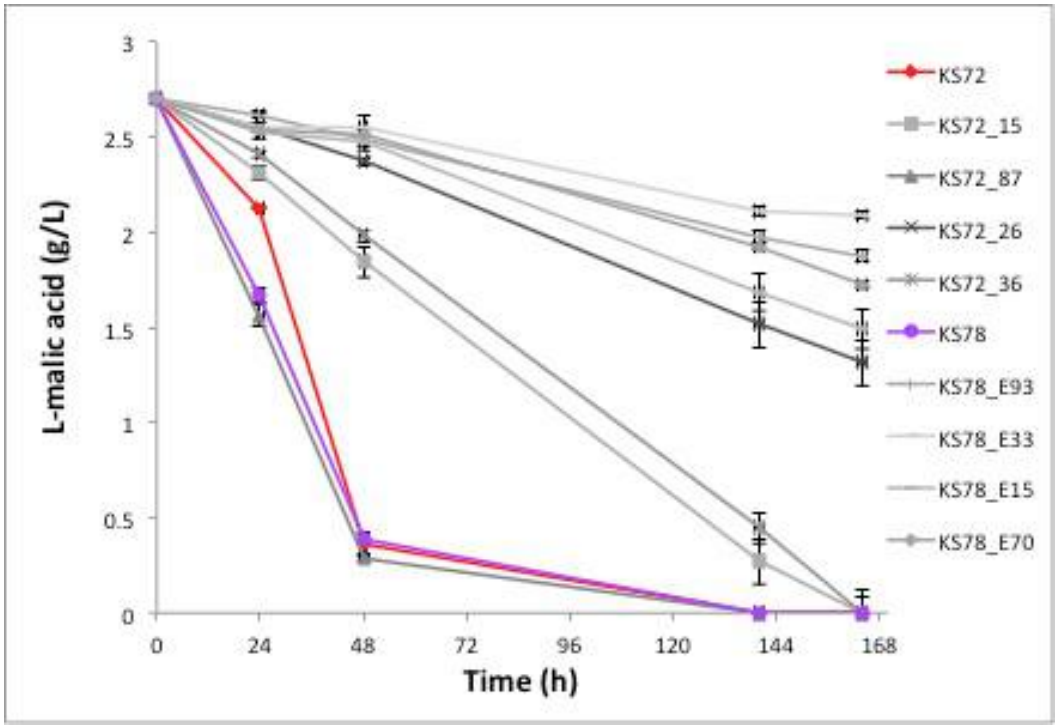


Figure 2.8: MLF performance by the best 8 *Lb. plantarum* isolates from bioreactor and EMS screen in 50:50 RFCDGJM:Grenache (14.12 % (v/v) ethanol, pH 3.5) medium. KS72 and KS78 are the parent strains.

The data suggests that under the DE conditions, improved biomass has been selected at the expense of L-malic acid consumption and it appears that DE with *Lactobacillus* sp. is more difficult to predict. It has been reported that in DE, mutations that cause deleterious effects on fitness arise due to genome erosion more often than advantageous mutants (Perfeito et al. 2007). When this occurs a population remains viable within its specific environment, but its fitness is reduced for growth in any other environment and is therefore highly specialised. DE has been successfully used with LAB. For example, *Lactobacillus plantarum* growth on glycerol under anaerobic conditions is too slow to be accurately measured; however, after approximately 500 generations under continuous selection using glycerol as a limiting factor, growth rate was improved by over 10-fold (Teusink et al. 2009). Another example is the replication of the natural adaptation of *Lactococcus lactis* from a plant niche to growth in milk in 1,000 generations under laboratory conditions (Bachmann et al. 2012).

More recently, Douillard et al. (2016) conducted a DE experiment with *Lactobacillus rhamnosus* using 4 stress conditions and reported that after 1000 generations the population was still heterogenic. The authors reported that whilst there was no adaptation pattern to *in vitro* conditions under all stress conditions, larger chromosomal deletions were associated with specific stressors. Unlike *O. oeni*, *Lactobacillus* sp. possess the mismatch repair (MMR) pathway, which may reduce the number of SNP's 'fixed' in the genome over time, as compared to *O. oeni*.

Whilst *Lb. plantarum*, DE showed modest improvement, whole genome sequencing was still initiated for the candidate strains, with a view toward better understanding the genes needed

for successful adaptation of *Lb. plantarum* to wine and in order to investigate the cause of the reduced MLF phenotype. Parent strain K45 was sequenced together with 5 DE isolates (of varying performance) and they will be checked for any genetic rearrangements/loss and SNP's to help determine why the DE strains have reduced fitness with regard to MLF. This will also allow us to better direct any new DE experiments with this organism. Future work should also include rescreening EMS isolates (216 total) for co-inoculation performance and the ability to finish MLF under different conditions.

2.4 Periodic evaluation of *O. oeni* A90 DE

Jiao Jiang, (GWRPh1308) carried out DE of *O. oeni* A90 in parallel with this project and under the supervision of Dr Krista Sumby, Dr Joanna Sundstrom, Dr Paul Grbin and Professor Vladimir Jiranek. The results of the final screen during DE are presented graphically (**Figure 2.9**). However for more detailed information please refer to Jiao Jiang's PhD thesis (2017) titled "Use of directed evolution to generate multiple-stress tolerant *Oenococcus oeni* for enhance malolactic fermentation".

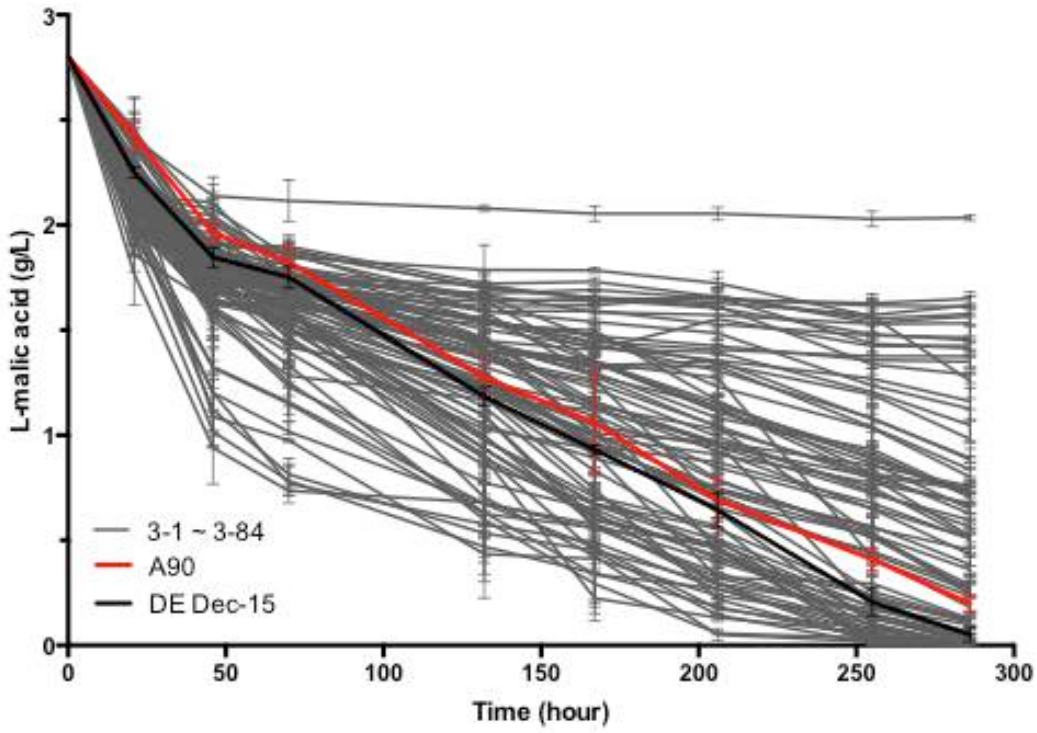


Figure 2.9: Evaluation of malic acid consumption by clonal isolates from *O. oeni* A90 DE (300+ generations). Isolates were screened in 75% stuck Shiraz and 25% RFCDJGM (14.5% (v/v) ethanol, pH 3.4, 25.8 mg L^{-1} SO_2)

Our findings demonstrated the feasibility of using laboratory directed evolution to generate novel *O. oeni* strains that are more suited to winemaking conditions. Screens of individual isolates taken throughout the DE process identified strains with improved MLF performance. Subsequent trials confirmed the superior performance of these strains in an array of wines, indicating a broad applicability in wine fermentation. More in-depth characterisation of the

evolved strains is on-going. To our knowledge, this study is the first successful application of DE to *O. oeni* to produce isolates better suited to an environment with multiple wine related stressors.

2.5 Evaluation of *O. oeni* KS21 DE

Fifteen KS21 DE isolates were screened in RFCDGJM (pH 3.2, 13.8% ethanol) (**Figure 2.10**). All isolates outperformed the parent strain in L-malic acid consumption, despite the parent having increased growth in the tested media. A second 15 mL screen of KS21 DE isolates was undertaken in RFCDGJM (pH 3.2, 13.8% ethanol) and in Shiraz (pH 3.45, 15.6% (v/v) ethanol). Prior to this screen the top 6 isolates were regrown on MRSAJ (from glycerol stocks) and single colonies picked and named according to their origin. Multiple isolates were retested in the second screen. None of the isolates finished MLF in the Shiraz, but all finished in the RFCDGJM and the best two strains from both screens were evaluated in wine (**Table 2.3**).

Tolerance to common inhibitors in wines is of great importance to the wine bacterium *O. oeni*. Therefore viability and L-malic acid consumption of *O. oeni* strains in different wines were taken as an indication of the effect of combined stressors of ethanol, pH and SO_2. These stressors were introduced in the DE experiment, and levels of these stressors were increased over time.

Evolved strains from KS21 were able to survive in all the tested wines (**Table 2.3**) and consumed more L-malic acid in the Merlot (2017), where unlike the parent, the DE strains finished MLF, and also finished faster than the parent in the Shiraz wine. MLF duration was equal to the parent in the other two tested wines. Future work will include testing these DE isolates further in additional wines. These results, along with the results of Jiao Jiang (2017) indicate that DE has the potential to generate strains with intended improvements. Now that the evolved strains have shown improvement they may need to be subjected to further DE as was the case for A90, with the addition of SO_2 and MCFA. This was not done during the first round due to time constraints and the need to test the DE candidates in wine.

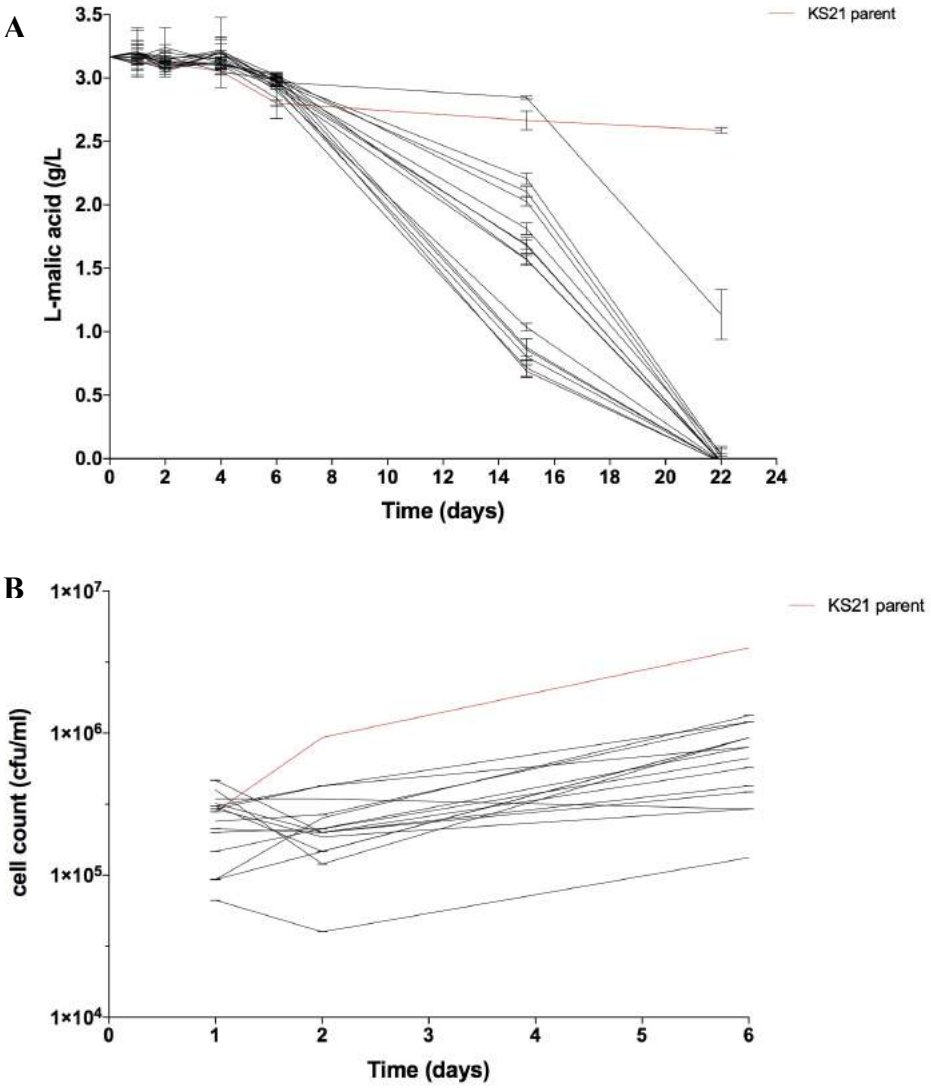


Figure 2.10: 15mL screen of KS21 DE isolates in RFCDGJM (pH 3.2, 13.8% ethanol).
A; L-malic acid consumption, **B;** growth.

2.6 Evaluation of *O. oeni* isolates from an un-inoculated fermentation (15 mL cultures)

Grenache isolates

When the effect of pH (14% ethanol) was examined the results were similar (**Figure 2.11**), but isolate G55 had the fastest L-malic acid consumption at low pH (2.8, 14% (v/v) ethanol). VP41 showed no L-malic acid consumption for this assay due to an inoculation error. The effect of pH was also tested at 12% (v/v) ethanol, but there was no significant difference between the isolates (data not shown). When the effect of ethanol was assessed (pH 3.5), VP41 consumed L-malic acid the fastest at 14% (v/v) and 16% (v/v) ethanol, but all isolates finished (**Figure 2.12**). However, when the ethanol was increased to 18% (v/v) G55 was the fastest and consumed the most L-malic acid, but none of the isolates finished MLF. There was no L-malic acid consumption for all isolates at 20% (v/v) ethanol. For the SO_2 trial,

VP41 was the fastest again, but all finished and in this case G71 was the slowest (**Figure 2.13**).

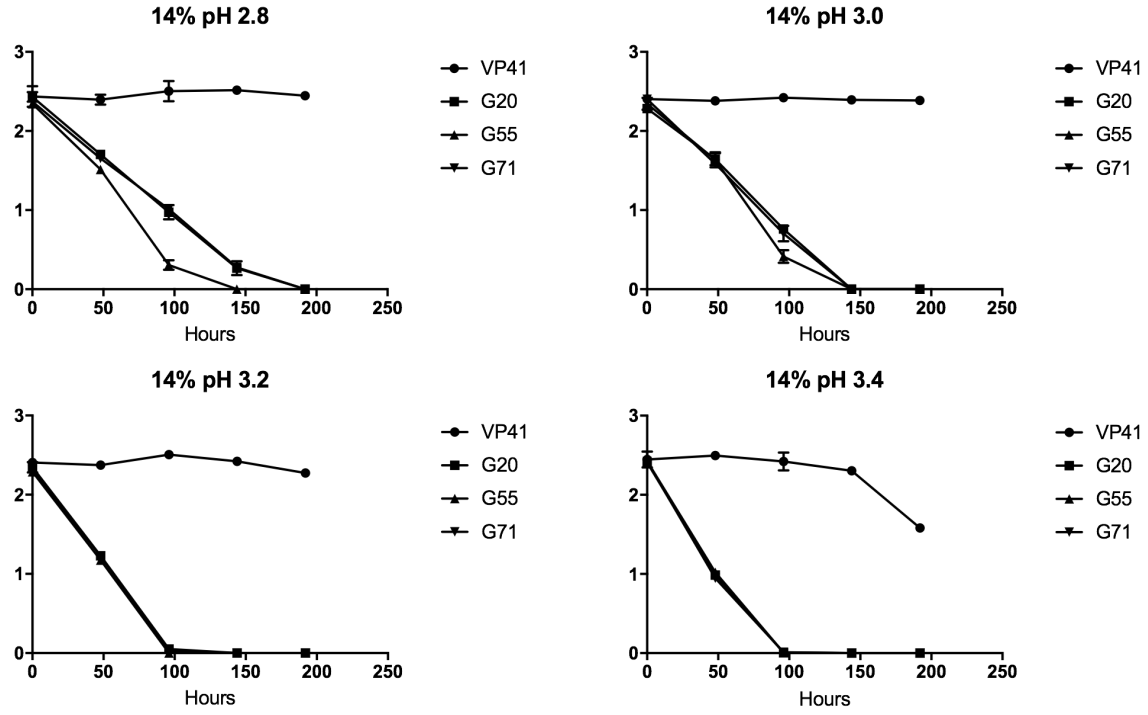


Figure 2.11: Evaluation of isolates, from un-inoculated Grenache ferments, for performance in RFCDGJM (14% (v/v) ethanol) with different pH levels.

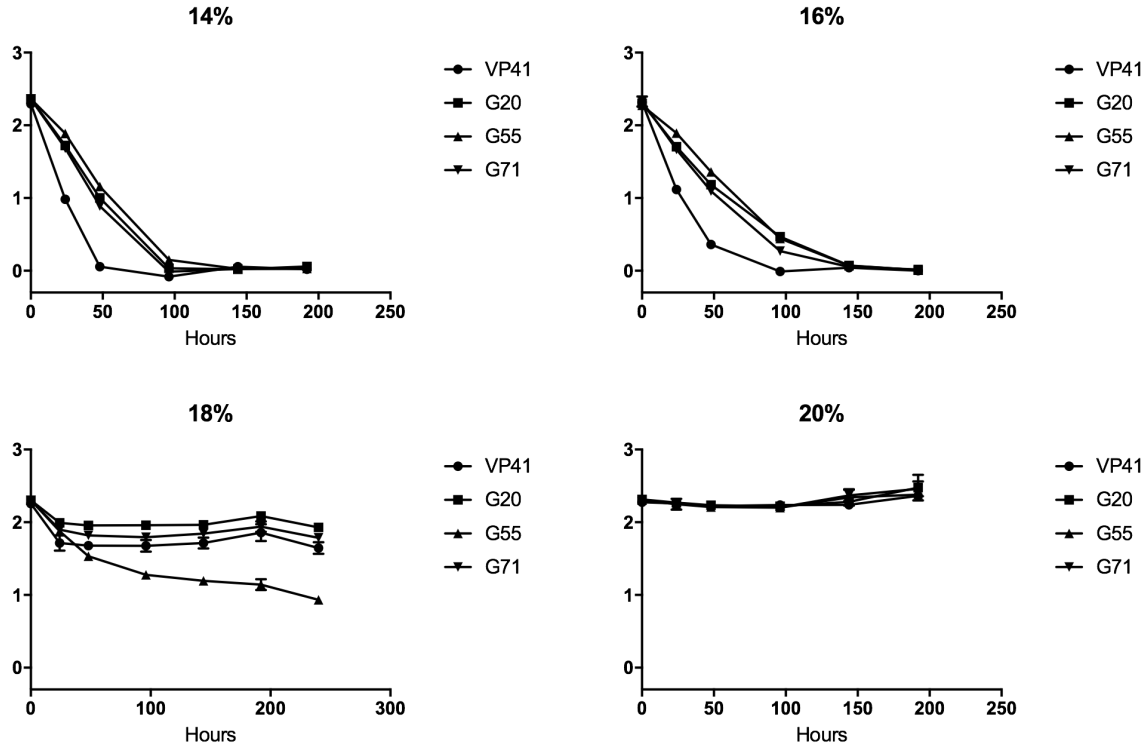


Figure 2.12: Evaluation of isolates from un-inoculated Grenache ferments for performance in high ethanol conditions in RFCDGJM (pH 3.5) with increasing ethanol concentration.

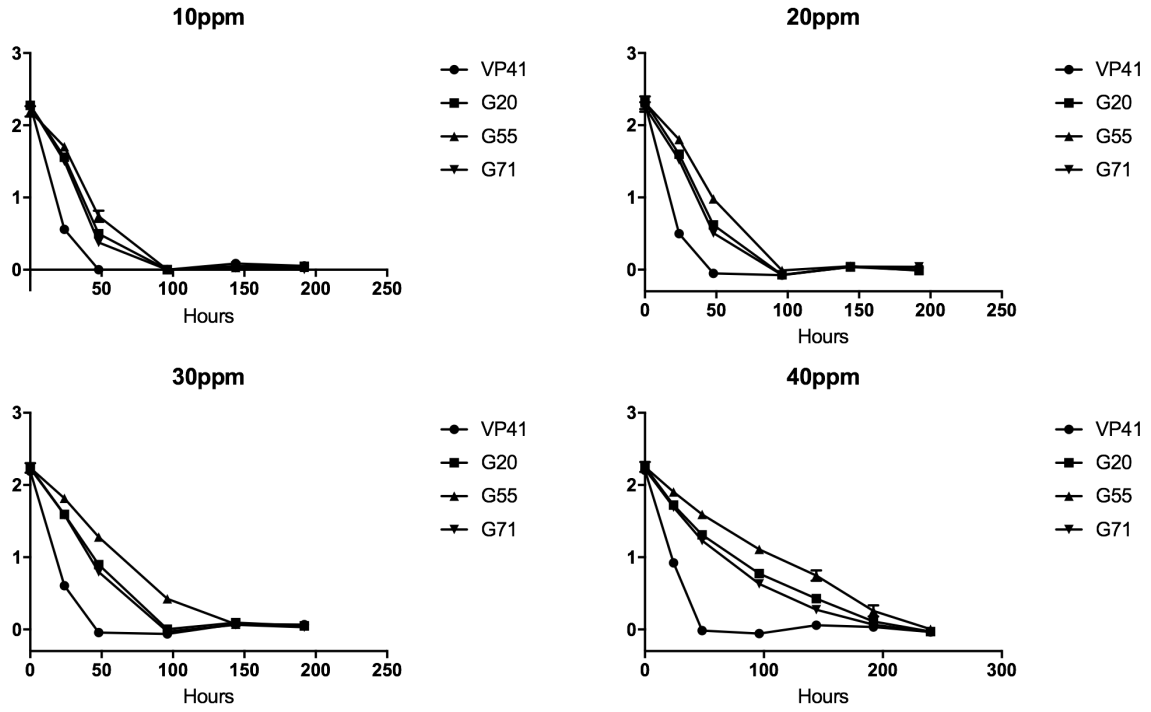


Figure 2.13: Evaluation of isolates from un-inoculated Grenache ferments for performance with SO_2 additions in RFCDGJM (12.3% ethanol, pH 3.5) with increasing SO_2 concentration.

Shiraz isolate Oe16

MIC's and performance in wine for un-inoculated ferment isolate *O. oeni* Oe16 were tested by Master of Viticulture and Oenology students Xingwei Mi and Yin Liu under the supervision of Dr Sumby and Assoc. Prof. Grbin. The results from this can be found in their thesis (Xingwei Mi, 2016 and Yin Liu, 2017). In summary, *O. oeni* Oe16 showed strong ethanol tolerance and rapid fermentation kinetics. In the pH tolerance assay *O. oeni* Oe16 was able to finish MLF at all the pH levels assessed. In the ethanol tolerance assay, Oe16 also showed strong ethanol tolerance, but about 150 hours lag was observed at 16% ethanol. For the SO_2 trial, Oe16 exhibited lower malolactic activity in the presence of increased SO_2.

3.0 Industrial scale winemaking trials of evolved microbes

Output 2E: Lead strains generated to date evaluated (kinetic and sensory) for practical suitability.

- WIC Winemaking Services to conduct large scale trials (~100 L) to obtain fermentation kinetic data. Preliminary sensory analysis to be conducted 'in house' and with industry representatives.

Output 3E: Data on practical suitability (kinetic and sensory) of strains generated to date.

- WIC Winemaking Services to conduct large scale trials (~100 L) to obtain fermentation kinetic data for additional strain(s) generated to date. Preliminary sensory analysis to be conducted 'in house' and with industry representatives.

Output 4C: Data on characterisation of lead evolved strains.

- Most promising yeast and/or LAB strains (ideally at least one candidate for each target attribute) to have been evaluated as appropriate e.g. in fermentations up to 100 L (via WIC Winemaking) or 1000+L (via collaborating wineries (at no cost), chemical and sensory analysis, genome sequencing.

Contributors (Yeast DE)
Postdoctoral fellows: *Drs Michelle Walker, Tommaso Watson, Jennie Gardner, Joanna Sundstrom and Professor Vladimir Jiranek*

Contributors (LAB DE)
Current PhD students: Jiao Jiang
Staff: Dr Alice Betteridge, Dr Jun Nimi
Postdoctoral fellows: *Dr Krista Sumby, Associate Professor Paul Grbin and Professor Vladimir Jiranek*

Background

A most fundamental requirement of winemaking is reliable and predictable fermentations: reliable in terms of trouble-free completion and predictable in terms of fermentation duration, yeast/bacterial supplement needs and appropriate production of desirable and undesirable metabolites. To this day winemakers report fermentations that are too slow or fail to complete, often requiring amelioration with nutrients or chemicals, heating, modification of acidity or reinoculation, which is both time consuming and costly. Reliable and predictable primary and secondary fermentations remain a challenge, with winemakers seeking to reduce additives (incl. nutrients) or are pursuing low temperature fermentation for stylistic reasons, as well as having to handle more 'difficult' juices (e.g. higher in sugar, lower in nutrients, more disease affected/carry higher loads of spoilage microbes/requiring more SO_2). Whilst

there are a large number of commercial yeast and bacteria available, not all have the ability to conduct fermentation reliably under these conditions. To date, *S. cerevisiae bayanus* strain, Uvaferm 43 (Lallemand) is the industry standard for tackling high sugar fermentations, or restarting 'stuck fermentations'. Whilst extremely reliable and generally undergoing a 'clean fermentation', there have been anecdotal aroma shortcomings reported such as 'onion notes' from high levels of benzene compounds (Zhao et al., 2012). Malolactic fermentation still remains problematic and unreliable throughout industry. This is thought to be mainly due to culture sensitivity to high concentrations of ethanol and SO_2, as well as acidic conditions.

In this study we have produced new microbial strains evolved under wine related stress conditions with the aim of providing an alternative yeast to Uvaferm 43, with more desirable aromatic properties, as well as more reliable LAB monocultures. Importantly confirmation of these strains capabilities to perform reliably in industrial conditions and when in the presence of common stressors has been undertaken. Evaluation of strains at these larger scales allows for a more comprehensive analysis, allowing confidence in predictability of performance when available commercially. The fermentation performance of cultures in a range of juices as well as metabolite compositions and effects on sensory profiles of finished wines have been analysed.

Methods

M3.1 Yeast strains

Yeast used for this Output were the most promising candidates generated from **Outputs 1E**, **2D** and **3B** (**Table 3.1**). For each experiment strains were originally cultured on YPD plates inoculated from glycerol stocks.

Table 3.1: Yeast used in Winery-scale fermentations

Strain	Description	Source
RM3_25	Colony isolate from random sporulated and mated population (Q7 x Tee 9) evolved for 38.6 gens	This study
RM4_71	Colony isolate from random sporulated and mated population (Q7 x 71B) evolved for 41.2 gens	This study
RM5_15	Colony isolate from random sporulated and mated population (C7H x 71B) evolved for 37 gens	This study
RM7_71	Colony isolate from random sporulated and mated population (Uvaferm 43 x Q2) evolved for 39.1 gens	This study
C7H_ B4	Colony isolate from FM 16 C7H evolved for 158.3 gens total	This study
Tee 9	Evolved strain of AWRI 796, improved fructophilicity	Liccioli, 2010; Jiranek lab
Q7	Evolved strain of QA23, improved proline utilisation	Long, 2014; Jiranek lab
FM16 C7H	Evolved strain of L2056, efficient sugar catabolism	McBryde et al 2006; Jiranek lab

Lalvin 71B	Malic acid utiliser, fruity aroma character	Lallemand
Uvaferm 43	Robustness, restart yeast for stuck fermentations	YSEO, Lallemand

M 3.2 Winery scale yeast fermentations - Mataro

Fermentations were conducted using 80 kg of 2016 Mataro grapes from the University of Adelaide (UA) Waite Campus vineyard in 100 L water-jacketed fermenters (WIC Winemaking Service). QA23 and Q7 cultures were grown overnight at 28 °C in 200 mL YPD on a shaker platform (140 rpm) before being transferred to ~10 L sterile-filtered starter medium (5 L white grape juice, 5 L water and 20 mL vitamin stock; Henschke and Jiranek, 1993). Starter cultures were grown overnight to ~2 x 10^8 cells mL^{-1} at 28°C with ~10 L/min sterile-filtered compressed air diffused using a metal scinter. Each 80 kg fermentation vessel was then inoculated at 5 x 10^6 cells mL^{-1} using 2 L of the starter cultures. A total of 8 fermentations was conducted at 28 °C, comprising of QA23 and Q7 and two different treatments (with or without aeration during fermentation at ~2 L/min). Each fermentation was supplemented with 1 g L^{-1} of proline, totalling 50 g per vessel (this anticipating ~50 L of liquid extracted from 80 kg of grapes). Fermentation management included two manual mixing operations per day followed by monitoring of fermentation progress by refractive index (°Brix). At each time point, clarified samples of the medium (centrifugation, 1 min at 20,000 rcf) were collected for subsequent measurement of residual sugar and nitrogen.

M 3.3 Winery scale yeast fermentations - Chardonnay

Chardonnay juice was sourced from Wolf Blass, Barossa. The juice was racked and settled at 0°C for 3 days. The juice parameters were: 12.1 Bé, 219 mg L^{-1} YAN, 4.42 g L^{-1} L malic acid, 16 mg L^{-1} FSO2, 49, mg L^{-1} TSO_2, 6.79 g L^{-1} TA at pH 3.3. Strains were inoculated into 50 mL YPD and grown for 2 days before being transferred directly into 600 mL starter medium (filter sterilised 45% Chardonnay juice, 45% water and 10% YEPD). The cultures were grown at 30°C with shaking for 2 days.

30 L beer-style fermenters were filled with 19 L of juice. A small amount of dry ice was also added to reduce juice oxidation upon filling and fermenters were fitted with an airlock. 200 mL of starter culture was inoculated to each and transferred into a 22°C room. Fermentations were performed in triplicate. After 2 days, 200 ppm DAP was added and fermentations catabolised all sugars within 10 days. The wines were racked and since triplicate fermentations were virtually indistinguishable, 1.8 L of each triplicate was blended to yield 5.4 L of wine per strain tested. 50 ppm SO_2 was added, airlocks fitted and the bottles placed at 2°C to settle for 1 month. The wines were then bottled into 650 mL brown bottles, fitted with crown seals and stored at 2°C (6 bottles per wine/strain).

M 3.4 Winery scale yeast fermentations - Cabernet Sauvignon

Strains were inoculated into 10 mL YPD and grown overnight before being transferred directly into 600 mL starter medium (45% Semillon Waite 2013 juice, 45% water and 10% YEPD, filter sterilised). The cultures were grown at 30°C with shaking for 1 day.

Cabernet Sauvignon grapes were hand-picked from the Alverstoke Orchard at the Waite Campus, and crushed in a small crusher-destemmer at CSIRO facilities, Waite Campus. SO_2 (50 ppm) was added to the fruit before crush. 20 kg of must was transferred into 30 L beer-

style fermenters. The sugar content was 22.4 °Brix.

Fermentations were conducted in triplicate, each inoculated with 200 mL starter culture and placed at 22 °C. After 24 h, food grade sugar (sucrose, 50g/kg must) was added. After 48 h, *Oenococcous oeni* (Lallemand O-MEGA™) was added at 1.5 g/100 L (as per the manufacturer's instructions). MLF completed in 14 days and then wines were racked and blended as above. Airlocks were fitted and the bottles placed at 22°C to settle. Wines were bottled after 1 month into 650 mL brown glass bottles with crown seals (6 bottles per wine) and stored at 2°C.

M 3.5 Winery scale malolactic fermentation

Bacteria used for this Output were the most promising candidates generated from Section 2.0 (**Table 3.2**). For each experiment, strains were originally cultured on MRSAJ plates inoculated from glycerol stocks.

Table 3.2: Bacteria used in large-scale fermentations

Strain	Description	Source
SB3	Commercially available starter	Laffort
A90	Evolved strain of SB3, improved ethanol tolerance in MRSAJ	Betteridge, 2014; Jiranek lab
A89	Evolved strain of SB3, improved ethanol tolerance in MRSAJ	Betteridge, 2014; Jiranek lab
G55	*O. oeni* isolate from high ethanol Grenache	Gang 2012; Jiranek lab
G71	*O. oeni* isolate from high ethanol Grenache	Gang 2012; Jiranek lab
G103	*Lb. hilgardii* isolate from high ethanol Grenache	Gang 2012; Jiranek lab
VP41	Commercial fast fermenting *O. oeni* strain	Lallemand
2-49	*O. oeni* evolved strain of A90, improved multi-stress tolerance in RFCDGJM	Jiang 2017; Jiranek lab
3-83	*O. oeni* evolved strain of A90, improved multi-stress tolerance in RFCDGJM	Jiang 2017; Jiranek lab

M 3.6 Bottle (5L) scale MLF – Shiraz and Shiraz-Grenache blend

Screening of clonal isolates of both *O. oeni* and *Lactobacillus* identified 3 clonal isolates that have a promising MLF phenotype. These have been tested as 5 L triplicate fermentations (no headspace) in two wines (Shiraz and a Shiraz-Grenache blend). Five LAB strains were trialed along with two commercial strains (SB3 and VP41). The fermentation performance of strains VP41, A89 and A90 and their parent (SB3), G55, G71 and G103 was evaluated to confirm improved performance was not restricted to defined media.

Both wines were made in a similar style. One tonne of Shiraz grapes from Mount Jagged (SA) or one tonne of a blend of Grenache (40%) and Shiraz (60%) grapes from the Waite Vineyard at the University of Adelaide (SA) were crushed at the Hickinbotham Roseworthy Wine Science Laboratory into a stainless steel one-tonne open fermenter. The must was

inoculated with the yeast PDM at a rate of 250 mg L^{-1}. The fermentation was then plunged twice daily until 2 Bé, before the musts were pressed and the wine fermented to dryness. The strains were then used to inoculate both the Shiraz and Shiraz-Grenache blend wines, which were supplemented with ethanol to give a concentration of 14.18% (v/v) and 16.05% (v/v) respectively. The Shiraz had an initial pH of 3.44 and an initial concentration of 2.6 g L^{-1} L-malic acid and was supplemented with 0.8 g L^{-1} DL-malic acid to achieve a final pH of 3.40 and final concentration of approximately 3 g L^{-1} L-malic acid. The Shiraz Grenache blend had an initial pH of 3.71 and a final concentration of 1.82 g L^{-1} L-malic acid and was subsequently supplemented with 2 g L^{-1} tartaric acid and 1.3 g L^{-1} DL-malic acid to achieve a final pH of 3.34 and a final concentration of approximately 2.5 g L^{-1} L-malic acid.

5 L of each wine was transferred to glass vessels leaving minimal headspace and fitted with airlocks. All LAB strains were grown to an OD_{600} >1.0 and washed in a 50:50 mixture of wine and water. They were then inoculated into 5 L selected wine in triplicate. Samples (1 mL) were taken regularly and analysed for L-malic acid consumption. At the completion of MLF (< 0.1 g L^{-1} L-malic acid) SO_2 was added (60 mg L^{-1}) and fermentations were cold-settled for 1 week prior to bottling. The finished wine was filtered through a 0.8 µm Bevassure cartridge in stainless steel housing (Cuno Pacific Pty Ltd), followed in-line by a 0.45 µm Waterra FHT-45 disposable filter (Air-met Scientific, Australia) and bottled into 330 ml crown sealed bottles. Bottled wines were stored at 15°C prior to sensory and HPLC analyses.



M 3.7 Winery scale bacteria MLF - Mataro

Fermentations were conducted using Mataro wine from M 3.1 (this study). The resultant Mataro wine was blended with Merlot wine (14.8% ethanol, pH 3.5) and MLF was conducted in duplicate in 200 L drums with parent strain *O. oeni* SB3 and DE strain A90. Sufficient inoculum for 200 L fermentations was prepared through a 3-step method. Briefly, 5 mL of pre-cultured bacteria of each strain was transferred into 45 mL MRSAJ to make a 50 mL culture. This 50 mL culture was inoculated into 450 mL MRSAJ in Schott bottles, and

incubated for 5 days by which time the cultures became turbid. Cells were pitched into 5L sterile wine-juice mix and incubated at 22˚C for 5 days. The final inoculation into duplicate (200 L) fermentations was performed at a rate of 1.25% (v/v) for each strain.

M 3.8 Winery scale bacteria MLF - Shiraz

In order to assess the impact of a wine matrix and potentially other organisms (e.g. yeast), a lab-scale MLF (250 mL) in Shiraz was performed with all bacterial strains pre-cultured from glycerol stocks. Strain 2-49 finished MLF by Day 32 whilst 3-83, A90 and SB3 suffered from stuck fermentation from Day 24. Trials were also conducted in 50 L kegs to more closely mimic industrial fermentation conditions. Strains SB3, A90 and 3-83 were inoculated into the Shiraz at ~5×10^6 CFU/mL to commence MLF. Due to poor growth of the starter, strain 2-49 was inoculated at 2–4×10^6 CFU/mL.

Three stages were involved to prepare inoculum for the 50 L fermentations. For each strain, 5 mL pre-cultured bacteria was transferred into 45 mL MRSAJ to make a 50 mL culture and incubated for 4 days. The 50 mL culture was sub-cultured into 450 mL MRSAJ in Schott bottles, and incubated for 5 days. Cells were harvested by centrifugation, washed and re-suspended in 100 mL sterile wine-juice mix and incubated at 22 ˚C for 4 days. The OD_{600} reached 0.39 ± 0.05 before inoculation. The final inoculation of triplicate 50 L aliquots of non-sterile Shiraz was performed at a rate of 3% (v/v) for each strain.

M 3.9 Sensory analysis

M 3.9.1 Sensory analysis DE yeast

The study ‘Fit for Purpose Yeast and Bacteria via Directed Evolution’ was approved by the Human Research Ethics Committee at the University of Adelaide (approval number H-2015-213) to undertake sensory analysis of wines made using evolved yeast and lactic acid bacteria.

Sensory analysis was conducted on wines after 4 months storage in bottle at 2 °C. One bottle of each wine was opened for a preliminary sensory analysis (descriptive notes by Dr Gardner; researcher and winemaker) and preference testing by 5 experienced wine researchers. Wines displaying the largest sensory variations were selected for more rigorous sensory evaluation. Further sensory analysis was conducted by Dr Yaelle Saltman at the University of Adelaide Sensory labs, Southern Barnes, Waite Campus. Wines produced by RM5_15 and C7H_B4 were compared against that from Uvaferm 43 in 2 tests: Preference test (44 participants) and triangle test (25 participants).

M 3.9.2 Sensory analysis LAB 5 L MLF

Informal tasting of samples by staff members suggested that sensory differences in the wine were subtle except for VP41 Shiraz-Grenache blend, which had an oxidized character. To

determine the sensory differences among the samples, free choice profiling (FCP) was used. These experiments are described in a paper due to be re-submitted and will be available upon publication.

M 3.10 HPLC and GCMS analysis of key metabolites

Ethanol, glycerol, organic acids and sugars were measured by HPLC (Li et al., 2017) and GCMS analysis of yeast derived aroma compounds was undertaken by Metabolomic Australia, AWRI (Varela et al., 2016) from wine samples after 4 months storage in bottle.

The prohibitive cost of GCMS analysis resulted in only the Chardonnay wines from all 10 yeast strains being analysed.

Summary of Outcomes

Reliable and predictable primary and secondary fermentations remain a challenge to wine makers in terms of trouble-free completion, predictable fermentation duration and product outcome. The project aimed to benefit winemakers, by providing a new 'microbial toolkit' through the generation of new yeast and bacterial strains evolved under wine-related stress conditions. We have successfully isolated several improved strains (see Section 2.0), which are the subject of further larger scale trials as part of the new bilateral agreement between Wine Australia and the University of Adelaide. We report on the outcomes of a preliminary larger scale trial whereby 5 evolved yeast (RM3_25, RM4_71, RM5_15, RM7_71 and C7H_B4) were compared to Uvaferm 43 in 20 kg Cabernet Sauvignon and ~20 L Chardonnay fermentations. The Chardonnay wines from RM5_15 and C7H_B4 were received favourably by consumers in a preliminary sensory trial. These 2 strains, together with the parent strains and EC1118 (an industry benchmark) are currently under further evaluation (2018 vintage; 50 kg Merlot ferments undertaken by WIC Wine Making Services).

Winery trials of our improved lactic acid bacteria have met with mixed results depending upon the size of the fermentation (250 mL, 5 L or 50L). In the smaller scale fermentations, the Shiraz isolates (G55 and G71) and evolved strains, A90, JJ2-49 and JJ3-83 performed better than the reference strains SB3 and VP41. In large scale (50 L) MLF no improvement was observed for A90, JJ2-49 and JJ3-83. It is too early to conclude whether the improved fermentation phenotype is confined to smaller scale MLF, as we are yet to test G55, G71 and other 'natural' isolates in our collection. At the very least several promising candidate strains are available for further study or enhancement.

Results and Discussion

Outputs 2E and 3E Lead strains evaluated (kinetic and sensory) for practical suitability

These outputs were deferred until the second half of 2016 and 2017 since suitably evolved yeast and bacterial strains were not available for vintages 2015 and 2016. Delays were due to technical and equipment issues (refurbishment of 3 out of 4 bioreactors), and extended generations of batch and continuous cultures (for both yeast and LAB DE) being required.

This resulted in a 12 month delay of phenotypically different candidate strains available for screening in laboratory scale fermentations.

In the interim, the fermentation performance of Q7 (an EMS mutant with enhanced proline utilisation in laboratory trials; GWRPh1302) and its parent, QA23 (Lallemand) were used to ferment ~80 kg of Mataro grapes in vintage 2016. Duplicate fermentations were supplemented with proline (0.6 g/Kg) and performed with and without aeration (i.e. 8 ferments). This initial round of trials by WIC Winemaking yielded mixed results and highlighted issues of process control and scale-up. Technical difficulties with the oxygenation system resulted in vigorous foaming issues and sample loss. These industry-like fermentations unfortunately did not match results previously obtained in the laboratory and no significant differences in fermentation kinetics or duration were observed between the strains and/or treatments. Further investigation of the wine metabolites was not performed. This preliminary trial did however resulted in improvements to future industry scale fermentations.

The malolactic fermentation kinetics of A90 and SB3 (parent) were examined in a Mataro Merlot blended wine. Unfortunately only minor differences in kinetics were observed between the strains for L-malic acid utilisation between the strains. However, the trial also highlighted the difficulty in growing LAB in sufficient amounts for trials of this scale. As a consequence, A90 was inoculated at a lower rate than SB3, as determined from retrospective plate counting (colony forming units, CFUs). The results of the large scale ferments to correlate with those found for SB3 and A90 in RFCDGJM and this highlights the reason for choosing A90 as the parent strain for improvement via DE.

During the 2017 vintage, the fermentation performance of five evolved yeast strains (best performing from laboratory scale fermentation profiling, Output 3B: RM3_25, RM4_71, RM5_15, RM7_71 and C7H_B4) together with their parent strains (**Table 3.1**) were evaluated in 20 L white (Chardonnay) and 20 kg scale red (Cabernet Sauvignon) wine fermentations. The strains were compared to Uvaferm 43, the industry benchmark strain of a 'rescue' yeast. Fermentation performance varied with juice type. During the first 96 hours of the Chardonnay fermentation (where ~80% of sugars had been catabolised), 3 of the 5 evolved strains (RM5_15, RM7_71 and C7H_B4) had the most rapid fermentation kinetics of all strains (**Figure 3.1**). This may be reflective of their superior capability to efficiently adapt to these specific conditions. However after 120 hours Uvaferm 43 had catabolised a similar amount of sugar. Whilst Uvaferm 43 had the fastest kinetics in Cabernet Sauvignon (in the first 168 hours) compared to RM3_25 and RM7_71, similar to the Chardonnay fermentation, they all required the same overall duration to catabolise all sugars (**Figure 3.2**). The performance of RM3_25 and RM5_15 was dependent on juice type, with RM3_25 behaving poorly in Chardonnay but well in Cabernet Sauvignon, and RM5_15 the reverse.

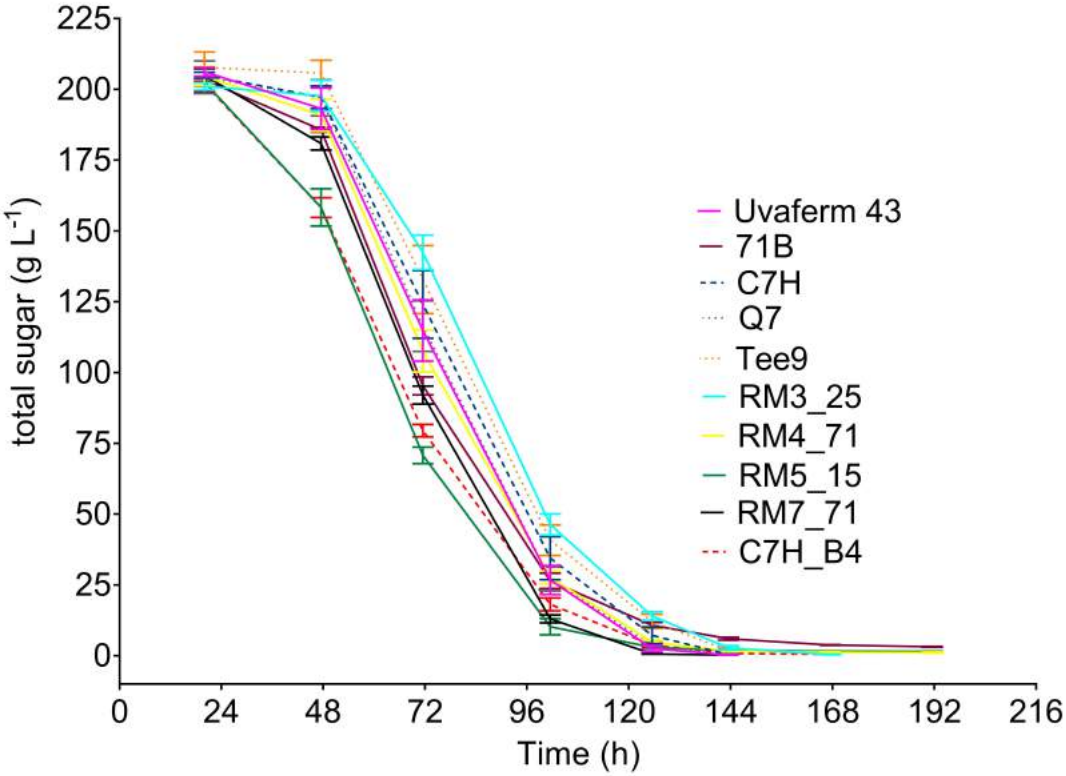


Figure 3.1: Sugar utilisation profiles of wine yeast during Chardonnay (20 L) fermentations.

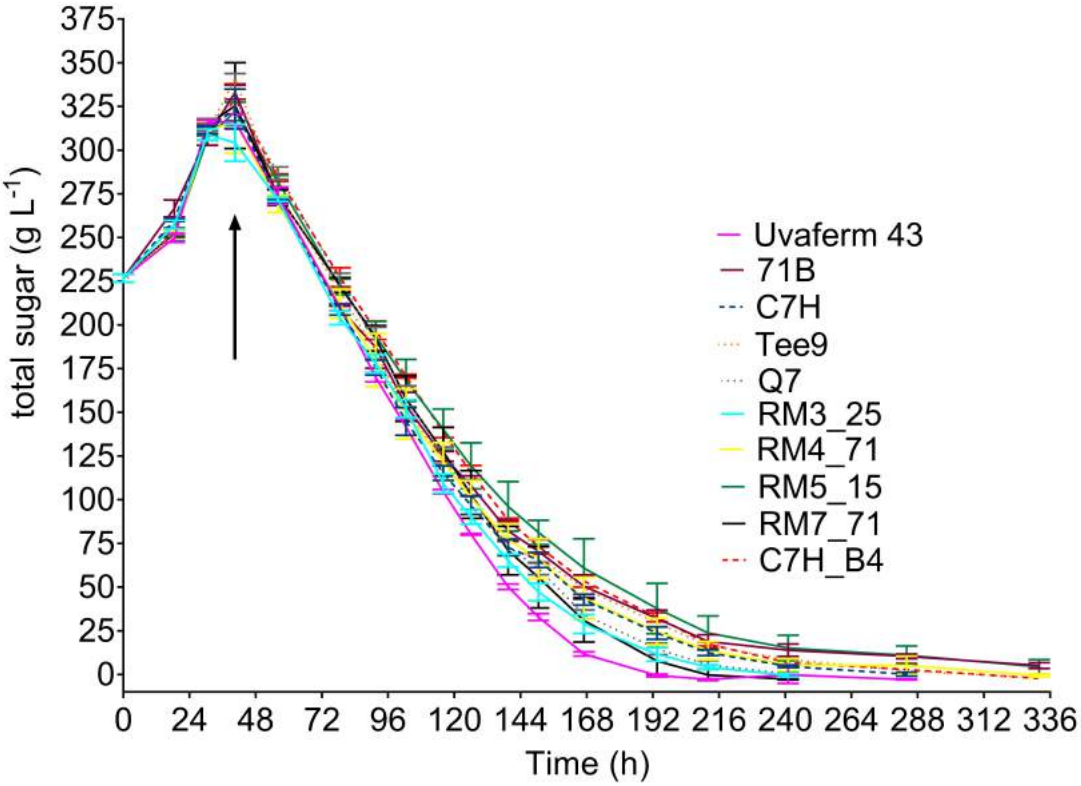


Figure 3.2: Sugar utilisation profiles of wine yeast during Cabernet Sauvignon (20 kg) fermentations.
The peak in total sugar corresponds to when the sugar addition (sucrose) become fully dissolved represented by the arrow.

The red wine fermentations were co-inoculated with Lallemand O-MEGA™, enabling MLF, chosen for suitability to co-inoculation and tolerance to high alcohol and sulfur dioxide (http://www.scottlab.com/ uploads/ documents/ technical documents/1204/Omega%20TDS %202014.pdf). MLF was of mixed success, with 50 ppm sulfur dioxide being prematurely added to the RM3_25 and C7H fermentations, when malic acid was still 2.02 and 1.45 g L^{-1} respectively. The remainder were allowed to complete MLF before bottling where no sulfur dioxide was added.

Key metabolites and aroma compounds were measured using HPLC (**Table 3.3**) and GCMS analysis (**Figure 3.3**) for the Chardonnay and Cabernet fermentations. The Cabernet wines from RM5_15 and 71B still had 5.39 g L^{-1} and 6.70 g L^{-1} residual sugar, mainly as fructose. MLF conducted by O-MEGA™ was not complete, with malic acid content varying between the strains (from 0.46 g L^{-1} for RM4_71 to 2.42 g L^{-1} for Q7). In the Chardonnay, the malic acid content varied between 3.08 g L^{-1} (71B) and 3.74 g L^{-1} (CH_B4). Overall, metabolic differences were minor with the greatest variation noted in acetic acid content, with almost all evolved strains producing approximately half the acetic acid than Uvaferm 43 in both juices (0.12 to 0.47 vs 0.68 g L^{-1}). If this is transferrable to industry, it would be of great benefit as the formation of volatile acidity in wines is a major contributor to wine spoilage.

Table 3.3. HPLC analysis of major metabolites in 2017 Chardonnay and Cabernet Sauvignon wine produced by different wine yeast strains

Chardonnay (g L^{-1})	Tartaric	Glucose	Fructose	Malic	Lactic	Glycerol	Acetic	Ethanol
Uvaferm 43	2.02	0	0	3.18	0.94	6.72	0.68	102.77
71B	2.2	0.01	2.27	3.08	0.72	6.57	0.29	96.07
Tee9	2.42	0.05	0	3.53	0.6	7.35	0.12	100.78
FM16 C7H	2.32	0.05	0	3.5	0.73	6.01	0.23	103.74
Q7	2.01	0.06	0	3.68	0.84	6.5	0.39	100.68
RM3_25	2.04	0.05	0	3.73	0.61	6.34	0.28	101.5
RM4_71	2.11	0.01	0.99	3.39	0.88	5.8	0.21	102.36
RM5_15	2.39	0.01	1.31	3.53	0.88	6.03	0.2	99.19
RM7_71	2.22	0.06	0	3.35	0.9	6.75	0.47	98.27
C7H_B4	2.27	0.02	0.45	3.74	0.71	5.72	0.33	101
Cabernet Sav. (g L^{-1})	**Tartaric**	**Glucose**	**Fructose**	**Malic**	**Lactic**	**Glycerol**	**Acetic**	**Ethanol**
Uvaferm 43	1.9	0.08	0	0.66	2.55	11.61	0.85	132.08
71B	1.61	0.21	6.69	0.53	3.44	11.99	0.52	128.01
Tee9	0.81	0.03	0.44	0.66	3.03	14.21	0.37	130.85
FM16 C7H	1.8	0.05	0.22	1.45	3.2	11.69	0.87	133.85
Q7	1.91	0.07	0	2.42	4.15	12.02	0.54	130.59
RM3_25	1.72	0.05	0	2.02	3.24	12.52	0.59	131.39
RM4_71	1.95	0.05	1.52	0.46	3.26	10.83	0.47	133.25
RM5_15	1.85	0.1	5.39	0.75	3.83	11.38	0.73	129.98
RM7_71	1.86	0.07	0	0.51	2.61	11.89	0.82	128.51
C7H_B4	1.82	0.04	0.28	1.6	3.27	10.61	0.45	130.73

Single replicates of a blend of wines from 3 replicate fermentations per strain was analysed. Values highlighted in blue and yellow are > or <10% of the value of Uvaferm 43 respectively.

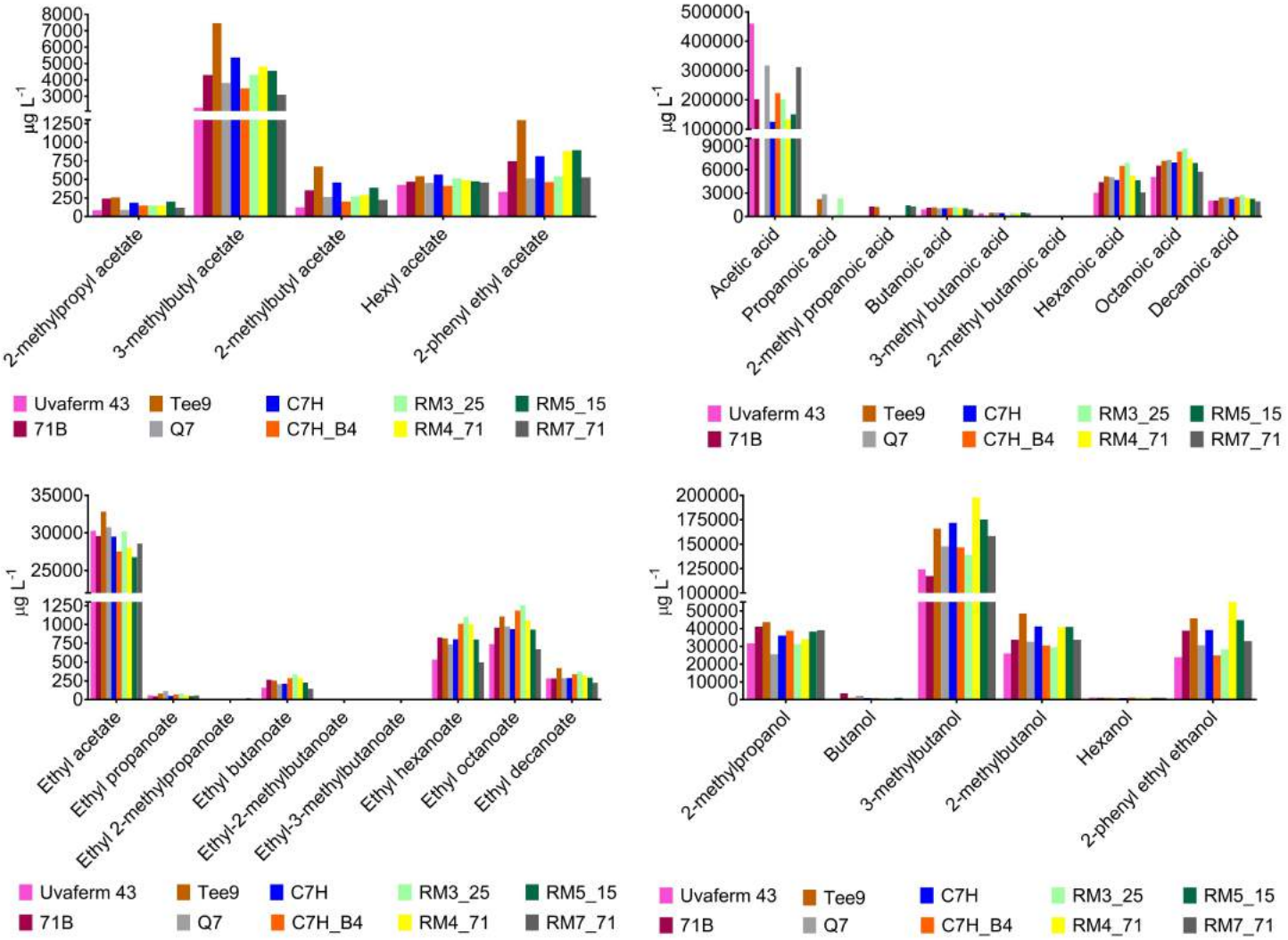


Figure 3.3. GCMS analysis of Chardonnay wines produced by evolved yeast. Analysis was conducted on wines which were bottled and stored at 4°C, 1 month.

GCMS analysis was conducted on Chardonnay fermentation samples, where in general, fermentation metabolites were in greater abundance than Uvaferm 43 in all strains. For example, octanoic and hexanoic acid, ethyl hexanoate, ethyl ocatanoate, 3-methyl butanol and 3-methylbutyl acetate which contribute to wine sensory and flavour profile (such as sweaty, green apple, sweet soap, nail polish and banana aromas). Clearly this analysis reveals the potential aromatic differences these evolved strains could contribute to industrial fermentations. This analysis was also supportive of the significant differences in wine aroma and flavour as revealed by the sensory analysis performed on two strains C7H_B4 and RM5_15 in comparison to Uvaferm 43 (as described below).

Given that we had limited resources to undertake a comprehensive sensory analysis of all wines made with evolved yeast a preliminary sensory analysis with 5 wine researchers was undertaken. The wines that this group considered as the most obviously different and in preference to the parent strains were chosen for further sensory analysis, these being Chardonnay made with two evolved strains, RM5_15 and C7H_B4 in comparison to Uvaferm 43. Brief tasting notes of all wines were provided by winemaker/wine researcher Dr Jennifer Gardner (descriptions for Chardonnay, **Supplementary Table 3.1**). Differences between wines made with Cabernet were less obvious due to their elevated alcohol (data not shown).

A comprehensive sensory analysis was conducted by Dr Yaelle Saltman to firstly compare whether consumers could pick the difference between 2 wines when provided with 3 samples (triangle test; 25 participants) and which wines did consumers prefer when given 2 wines (preference test; 44 participants). Male and female participants (aged 18-59 y) recruited from the Waite Campus, were asked to smell and taste the wines. Whilst the triangle test results were not significant at a p value of 0.05, with less than 18 out of 25 participants picking the correct wine (**Supplementary Table 3.2**), the preference test results revealed that participants preferred wine made with C7H_B4 in comparison to Uvaferm 43 ($p<0.05$, **Supplementary Table 3.3**). Thus sensory analysis of C7H_B4 warrants a repeat/further analysis of the trial to include a more comprehensive sensory analysis. C7H_B4, represents a further adaption of our previously reported evolved wine yeast strain FM16 C7H (McBryde et al., 2006) and may provide an alternative to Uvaferm 43 as a rescue strain. In comparison, sensory analysis of wines made with RM5_15 and Uvaferm 43 were not significant, with only 26 of 44 participants preferring the wine made with the evolved strain RM5_15 (29 were needed for a significant result at 0.05).

We intend to follow up on these strains in larger scale fermentations as part of a new bilateral agreement proposal, particularly when they perform as well as Uvaferm 43 or better (depending on the condition), and if they do overcome some of the empirically reported aroma shortcomings of Uvaferm 43.

Outputs 2E and 3E LAB lead strains evaluated (kinetic and sensory) for practical suitability

Bacteria: 5 L scale MLF

Five LAB strains were selected for larger scale trials in vintage 2014. These promising LAB candidates were tested for MLF performance alongside two commercial strains in 5 L triplicate ferments of two wines. The wines tested were a Shiraz (pH 3.43,14.18% ethanol) and a Red (Shiraz/Grenache) Blend (pH 3.39, 16.05% ethanol). The strains tested were *O. oeni* strains VP41 (commercial strain), SB3 (commercial strain; parent to A89 and A90), A89, A90, G55, G71 and *Lactobacillus hilgardii* strain G103. Fermentation kinetics were determined for all samples and HPLC analysis of organic acids performed for all samples. The wines have were bottled (330 mL crown sealed bottles) for later sensory analysis. Strains G71 and G55 completed fermentation 8 days faster than the other strains in Shiraz, and VP41 finished last. In the Shiraz/Grenache blend all strains were slow to complete fermentation, taking 70 days. VP41 was the slowest (90 days). G71 and *O. oeni* A90 finished fastest under the increased ethanol conditions in the red blend (16.05% ethanol). Informal sensory analysis (in-house) suggested that sensory differences in the wine were subtle (data not shown). It was decided to formally analyse the wines using the rapid descriptive analysis method of free choice profiling using post-graduate oenology students as tasters.

Bacteria: Winery scale MLF

Winery-scale fermentations were undertaken in March 2016 and March 2017. In vintage 2016 one *O. oeni* strain, AB90, from DE of SB3 (from GWRPh0901) was assessed in winery scale (200 L) fermentations, alongside the parent (SB3), with the assistance of WIC Winemaking Services (**Figure 3.4**).

In order to assess the impact of a wine matrix and potentially other organisms (e.g. yeast), a lab-scale MLF (250 mL) in a Shiraz was performed with all bacterial strains pre-cultured from glycerol stocks. Strain 2-49 finished MLF by Day 32 whilst 3-83, A90 and SB3 suffered from stuck fermentation from Day 24 (**Figure 3.5**). Trials were also conducted in 50 L kegs to more closely mimic industrial fermentation conditions.

In March 2017 four *O. oeni* strains: SB3, its evolved strain A90 and subsequently evolved strains JJ2-49 and JJ3-83, were evaluated for fermentation performance in replicated fermentations (50 L) of Waite Shiraz wines. JJ2-49 and JJ3-83 (isolated at 220 and 350 generations, respectively) completed MLF in the shortest time in lab-scale (50 mL) ferments in 4 wines (Mataro, Shiraz, Merlot and Pinot Noir) when compared to SB3 and A90 (**Figure 3.5**). Fermentation by A90 became stuck when viable cell numbers dropped below 10^6 CFU/mL from Day 23. SB3 was highly viable and completed MLF by Day 78 while the evolved strains 2-49 and 3-83 took an additional 14 days to nearly complete (**Figure 3.5**). Microscopic observation of Day 56 samples showed that yeast was the dominant microbe in all samples even though alcoholic fermentation was completed approximately two months

earlier (data not shown). It was difficult to find *O. oeni* cells in any of the samples, which is in accord with plating results. The results of the 50 L ferments are available in full in Jiao Jiang's thesis (2017).

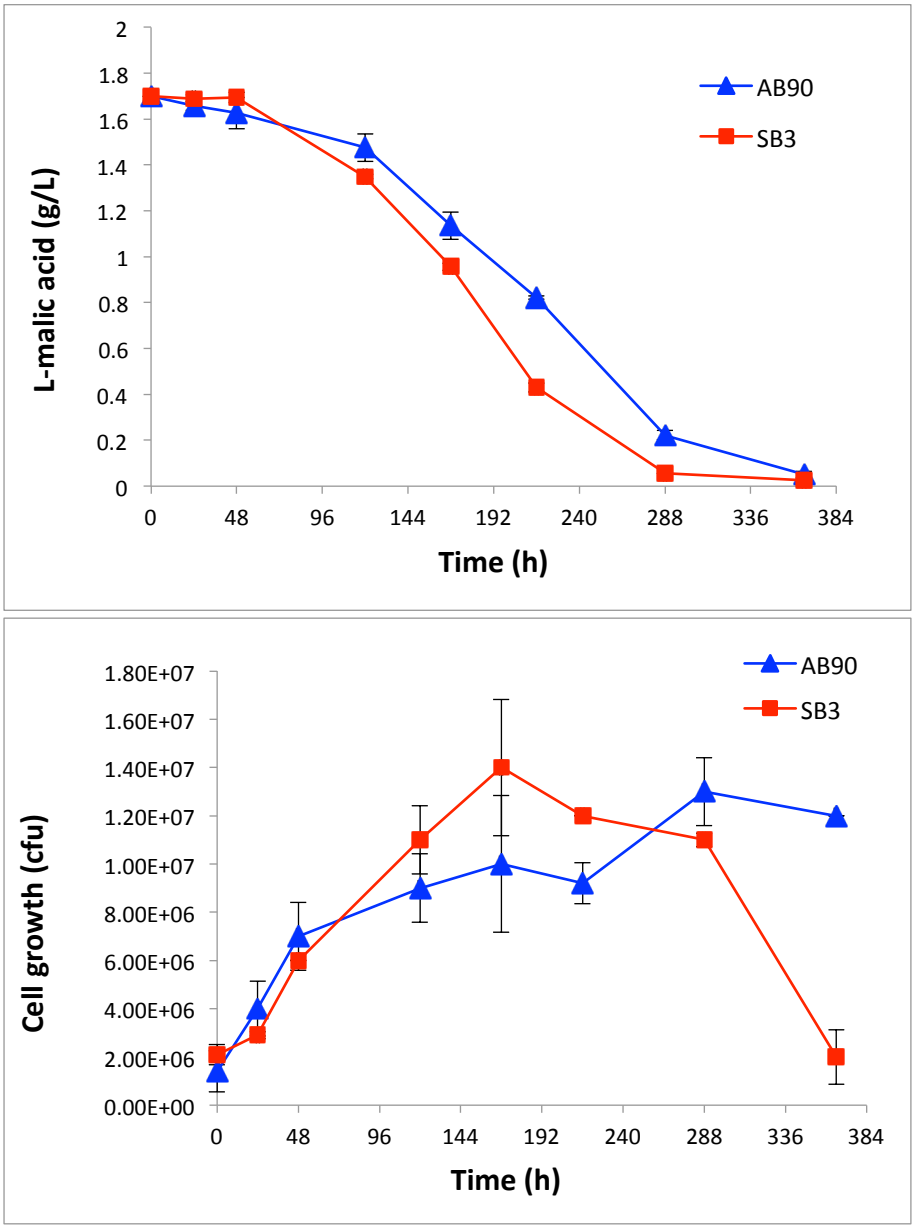


Figure 3.4: Winery-scale MLF (2016) of SB3 and A90. 200 L fermentations of a Mataro Merlot blend, 14.8% (v/v) ethanol, pH 3.5

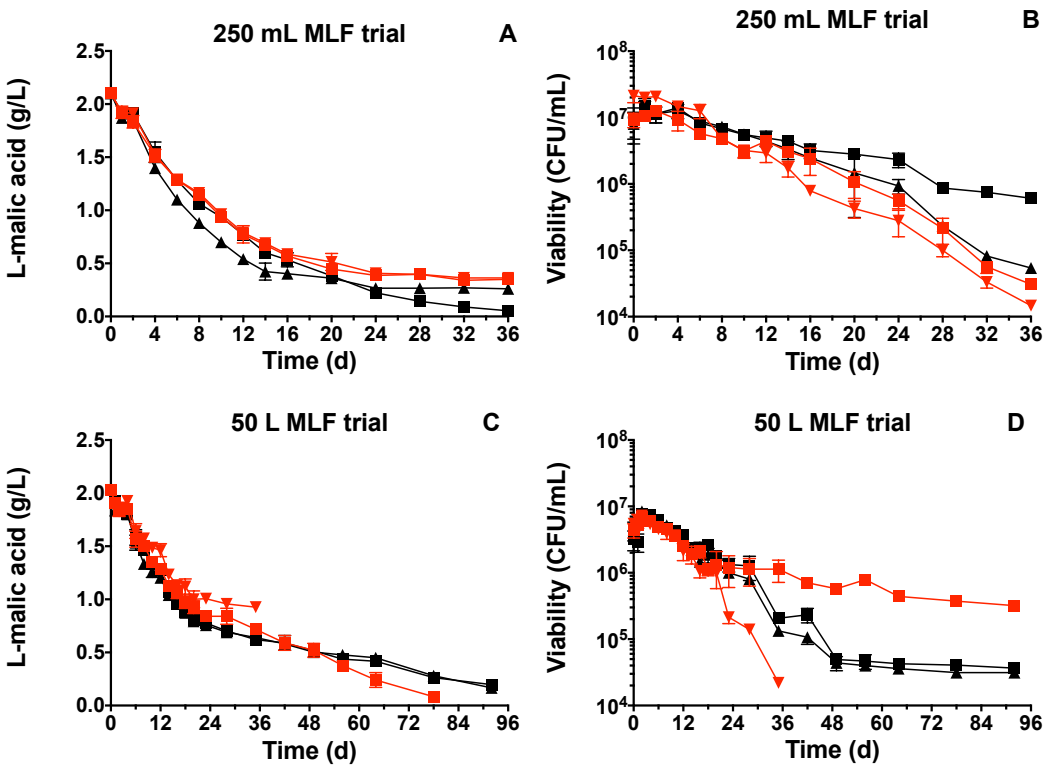


Figure 3.5: L-malic acid consumption and population of *O. oeni* strains in un-filtered Shiraz.
SB3 (■), A90 (▼), 2-49 (■) and 3-83 (▲). Values are the mean of three biological replicates ± SD.

4.0 Extension of the Fermentome to include genes of protrophic lab and wine yeast. The effect of overexpression of key genes on fermentation progression and analysis of yeast deletion mutant effects on wine colour.

Output 1B: Wine yeast Fermentome (database of fermentation essential genes): Stage 1 - preliminary screening (up to 300 genes).

- Source and screen wine yeast and prototrophic lab yeast deletion libraries (~7500 deletants) under standard and/or low nutrient availability at the micro-titre scale in order to broadly identify key genes/processes/potential stresses for further investigation and exploitation.

Output 1F: Wine yeast Fermentome (database of fermentation essential genes): Stage 2 - secondary screening (up to 100 genes).

- Evaluation of candidate yeast deletants in lab-scale fermentations under variable conditions to confirm the roles of the deleted genes in fermentation performance and process.

Output 2A: Wine yeast Fermentome (database of fermentation essential genes): Stage 3 - final selection (up to 20 genes).

- Bioinformatic analysis of wine yeast Fermentome and comparison to laboratory yeast Fermentome (from UA1101) in order to identify up to 20 yeast specific fermentation essential genes/processes.

Contributors

PhD students: Josephine J. Peter, Chien-Wei (Max) Huang
Postdoctoral fellows: *Drs Michelle Walker, Jennie Gardner, Tommaso Watson and Professor Vladimir Jiranek*

Background

We have previously utilised a laboratory yeast deletion library (BY4743 ORF::KanMX (Winzeler et al., 1999) to identify 93 genes essential for the timely completion of high sugar fermentation referred to as Fermentation Essential Genes (Walker et al., 2014). 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. These stresses change over the course of fermentation, occurring consecutively from early through to late stage fermentation. 93 genes were identified, which when deleted, either gave rise to protracted fermentation (84 mutants) or fermentation arrest (9 mutants) under conditions of high sugar (200 g L^{-1}) and optimal nitrogen (450 mg L^{-1} FAN) and did not affect cell growth. Supplementation was

successfully used, allowing fermentation performance to be assessed under optimal nitrogen conditions (Walker et al., 2014). Nonetheless, the effect of supplementation on metabolism through the blockage of pathways, or by influencing metabolic networks cannot be discounted (Pronk, 2002, Mulleder et al., 2012).

Fermentation under limited or altered assimilable nitrogen conditions remains topical amongst winemakers, because of challenges in rectifying sluggish or stuck fermentations and potential reductions in wine quality and value. The current project (UA1302) aims firstly to identify new genes that when deleted significantly improve fermentation under nitrogen limiting conditions (High Nitrogen Efficiency genes). These deletants (Δorf) in the haploid wine yeast AWRI 1631 are additional to those identified in UA1101. The second aim of the project was to elaborate on the laboratory yeast Fermentome. Specifically, were the identified FEGs (Walker et al 2014) and perhaps others, required by wine yeast for fermentation completion? We screened two prototrophic libraries - a prototrophic version of the laboratory yeast library - BY4741P (Mulleder et al., 2012) and a wine yeast deletion library in AWRI 1631 (Varela et al., 2012) under both high (450 mg L^{-1} FAN) and low nitrogen (75 mg L^{-1} FAN) conditions. Genes whose deletion result in shortened fermentation duration may be ideal targets for later strain development. We began to investigate a number of these genes linked to fermentation, with a view to understanding their role in yeast adaptation to fermentation stress.

Methods

M 4.1 Yeast strains and culture

The yeast strains used for this study are listed in **Table 4.1**. YPD medium (10 g L^{-1} yeast extract, 20 g L^{-1} peptone and 20 g L^{-1} glucose) was used for standard yeast propagation at 28 °C. The prototrophic library collection in BY4741P (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 (pHLUM) was purchased from Euroscarf. The wine yeast deletion library (WYDL) in AWRI 1631 (MATa) was provided by the AWRI. The libraries were replicated in YPD liquid medium before cryostorage. Chemically Defined Grape Juice Medium (CDGJM_450) containing 200 g L^{-1} sugar as equimolar amounts of glucose and fructose and high nitrogen (450 mg L^{-1} FAN as a mixture of amino acids and ammonium chloride) was used. CDGJM_75 medium had low nitrogen content (75 mg L^{-1} FAN). The CDGJM included 3 g L^{-1} polyphenol extract (Cat: Tppr, OenoProd, Sarl) or 30 mL L^{-1} GrapEX tannin extract (Cat: GSkinEx, Tarac Technologies, Barossa Valley) (Walker et al. 2014).

Table 4.1: Strains used in this study

Strain	Genotype	Source
BY4741P	MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0 (pHLUM)	Euroscarf (Mulleder et al., 2012).
LYDL	MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, orfΔ::KanMX (pHLUM)	Euroscarf (Cat No: COMP-SET-pHLUM) (Mulleder et al., 2012).
AWRI 1631	MATa	AWRI (Varela et al., 2012)
WYDL	MATa, orfΔ::KanMX	AWRI (Varela et al., 2012)

M 4.2 Screen of prototrophic laboratory yeast deletion library (LYDL) and wine yeast deletion library (WYDL) in CDGJM under high and low nitrogen conditions

The prototrophic laboratory yeast deletion library (LYDL) and wine yeast deletion library (WYDL) were screened for fermentation performance as microscale (0.6 mL) fermentations in CDGJM_450 (4 replicates) according to Walker et al. (2014). One replicate was removed at each timepoint for monitoring of fermentation progress (Liccioli et al., 2011). Residual glucose and fructose was measured by enzymatic analysis (Walker et al., 2014) and free amino nitrogen (FAN) (Dukes and Butzke 1998).

A second micro-fermentation screen was undertaken using the WYDL in CDGJM with low nitrogen (75 mg L^{-1} FAN) content. Conditions were as for LYDL screen.

M 4.3 Evaluation of identified yeast deletants in laboratory scale (100 mL) fermentations in CDGJM under high and low nitrogen conditions

A custom built 96-vessel fermentation platform (Tecan Freedom Evo) was designed and constructed by Dr Tommaso Watson to permit automated (i.e. 24/7) sampling of 96 fermentations and storage for 'off line' analysis (Watson and Jiranek 2015).

Trials were conducted as single (100 mL) fermentations on 90 of the best performing strains from the micro-fermentation screen for each nitrogen condition. Yeast starter cultures were grown in YPD overnight at 28 ^{o}C prior to inoculation of fermentations with 1% of culture. Fermentation progress was monitored as for micro-fermentation screens. The best 30 strains were evaluated in triplicate 100 mL fermentations in CDGJM_75, with the fermentations inoculated from YPD starter cultures at 5 x 10^6 cells mL^{-1}.

100 mL shake flask fermentations were conducted as described in the final report UA1101 'Innovative microbials for winemaking excellence'.

M 4.4 Confirmation of clonal identity of gene deletions in laboratory yeast strains

Genomic DNA was isolated from parent and deletant strains (Adams et al., 1998). Gene deletions were confirmed by Polymerase Chain Reaction (PCR) using the primer sets below, and PCR cycling conditions as described on the *Saccharomyces* Genome Deletion Project website http://www-sequence.stanford.edu/group/yeast_deletion_project/deletions3.html.

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

DNA sequencing was performed by the Australian Genome Research Facility (AGRF). DNA sequence alignment was performed using BLAST genome alignment software on the EMBL-EBI website (http://www.ebi.ac.uk/Tools/sss/ncbiblast/nucleotide.html) and Saccharomyces Genome Database (http://www.yeastgenome.org/blast-sgd). For incorrectly labelled deletants, the barcode region of the *KanMX* deletion cassette was amplified using primers U1 (GATGTCCACGAGGTCTCT) and D1 (CGGTGTCGGTCTCGTAG) and the query sequence compared to the up and down tags of the deletion library using the tool FASTAbarcodes (http://www.ttuhsc.edu/som/cbb/FASTAbarcodes/).

Summary of Outcomes

Identification of the genes required to successfully complete fermentation is fundamental to understanding which biochemical pathways are essential in this process. We have termed the collection of these genes “The Fermentome”. Mutations that lead to more efficient fermentation are also of interest as these represent potential targets for strain improvement. Previously we identified such genes using a laboratory yeast deletion library, since none were available for wine yeast. In this project we took advantage of the recent availability of a partial wine yeast deletion library. Previous work in the laboratory yeast library was also complicated by laboratory strains having mutations, which result in growth deficiencies or auxotrophies, unless supplemented with specific amino acids. Thus studies where conditions of low nitrogen (such as a typical low nitrogen juice) until recently, could not be undertaken. The wine yeast library and recent release of a prototrophic laboratory yeast deletion library do not require such supplementation and are thus suited for this study where both high and low nitrogen conditions were examined.

All clones from the prototrophic lab yeast deletion library (~6000) and the wine yeast deletion library (~2200) were analysed for fermentation performance and nitrogen utilisation in a defined medium of either sufficient or deficient nitrogen. We were interested in examining low nitrogen conditions since these are common to Australian juices and one of the prime causes of stuck fermentation. We identified 671 genes from the laboratory and 201 from the wine yeast library that when deleted resulted in fermentation protraction when only limiting nitrogen was available. These genes thus all play a role in the successful and timely completion of fermentation in the typical low nitrogen condition. Comparison between datasets has also provided insight into how the superior fermentation performance of wine yeast may be determined genetically.

842 genes from the laboratory and 299 from the wine yeast library resulted in shortened fermentation duration when deleted and sufficient nitrogen was available. These are prime targets for future yeast improvement. The fermentation performance of a wine yeast library allowing overexpression of 1-5 genes per strain was also examined in similar conditions. In this ongoing analysis the overexpression of ~60 genes are also implicated in the reduction of fermentation duration.

These datasets are an extremely valuable resource since they directly identify genes required for and mutations which enhance efficient fermentation in conditions typical of Australian winemaking, allowing future yeast improvement projects to specifically target known affected biochemical pathways.

Results and Discussion

Our initial report on the 'Fermentome' was based on the screening of the lab yeast deletion library (in BY4743) for fermentation performance (Walker et al., 2014). This screen focused on identifying those gene deletions in yeast, which resulted in either protracted or arrested 'stuck' fermentation under high sugar conditions (200 g L^{-1}) and optimal nitrogen (450 mg L^{-1}).

Output 1B. 4.1: Preliminary micro-fermentation screen of prototrophic yeast deletion libraries

In the current project, the use of prototrophic yeast gene deletion libraries allowed the screening for fermentation performance under optimal and limited nitrogen conditions. The genome-wide screen was to firstly identify yeast strains with known gene deletions, resulting in improved fermentation efficiency under both high (450 mg N L^{-1}) and low nitrogen (75 mg N L^{-1}). Secondly, to identify yeast deletants which conversely, led to protracted fermentation under both conditions, so as to elaborate on the laboratory yeast fermentome. These datasets will contribute to our current knowledge of the yeast 'wine fermentome', i.e. the genes/processes involved in wine fermentation.

A micro-fermentation screen of the prototrophic laboratory yeast deletion library (LYDL) in BY4741P (Mulleder et al., 2012) was conducted under high nitrogen conditions (cf UA05/06 and UA05/01). Samples were taken throughout fermentation at 102 h (A), 118 h (B), 134 h (C) and 146 h (D), and stored for later analysis. Given the large numbers of samples (~5400 per time-point) to analyse; 2 time-points (A and B) were chosen for analysis of residual sugar and nitrogen.

In the LYDL, 842 genes were identified from the fermentation screen conducted under high nitrogen conditions (450 mg N L^{-1}), whereby the deletion resulted in shortened fermentation duration. Screening of the LYDL under limited N was unsuccessful because of plasmid instability issues of pHLUM (used to complement auxotrophy) when cultured in YPD prior to cryostorage. Whilst recovery of the library was possible by plating on selective medium (without the specific amino acids) to isolate the strains retaining the plasmid, this was not undertaken because of time constraints. Instead, the screen was performed in a wine yeast

deletion library (WYDL) made in AWRI 1631; a stable MATa haplotype of N96 (Varela et al., 2012). The WYDL was provided by the AWRI, in accords to a materials transfer agreement, for use in UA1302 and related projects (GWRPh1313 and GWRPh1314).

The wine yeast deletion library (a partial library of ~2200 clones) was screened under identical conditions to the prototrophic lab yeast deletion library. Data was collated for fermentation duration, sugar and nitrogen utilisation. The wine yeast fermentome was widened to include not only genes whose deletion caused protracted fermentation but also those hastening fermentation. Similar to the LYDL screen, two data points were chosen for analysis: 43 and 67 h for high N screen, and 121 and 146 h for low N screen. Preliminary screening identified 299 genes (non-limiting nitrogen; 450 mg N L^{-1}) and 257 genes (limiting nitrogen; 75 mg N L^{-1}) whose deletion resulted in out-performance of the corresponding wildtype. These genes are of particular interest as targets for future strain improvement. Comparison of the high (non-limiting) N datasets from both prototrophic library screens, further confirmed the authenticity of the identified genes. A short list of the best 92 candidate strains from each condition was selected for further evaluation, and included 15 strains with shortened fermentation duration in both nitrogen conditions. The 184 selected strains were evaluated in 100 mL cultures via the "T-bot" fermentation system developed for the project. The findings of this study can be found in Josephine J. Peter's PhD thesis (Peter, 2017) which was provided to Wine Australia.

Output 2A. 4.2: Comparison of the Fermentome between data sets

Cross-comparison between the 3 micro-fermentation datasets to the original screen (Walker et al., 2014) was undertaken to identify those genes, when deleted, leading to protracted fermentation. In the LYDL, 671 yeast deletants were considered to have protracted fermentations (with a residual sugar content of > 70 g L^{-1} at 118 hour). A more stringent cut-off point was chosen for the WYDL, that of > 50 g L^{-1} sugar at 67 h (high N) and 146 h (low N). As the WYDL represents 30% of the clones in the LYDL, the number of deletants identified was smaller: 139 (high N) and 201 (low N) datasets. Twenty five genes (as yeast deletants) were found to be identified in all four library screens. These genes were enriched from processes such as negative regulation of biosynthetic processes, cellular ion homeostasis, and cellular metabolic compound salvage. These genes were identified in our previous study (336 genes; micro-fermentation data; Walker et al (2014)) as required for timely fermentation completion. These were evaluated as 100 mL fermentations (in triplicate) for fermentation protraction or in extreme cases, fermentation arrest.

Output 1F. 4.3. Laboratory scale (100 mL) evaluation of selected candidate wine yeast deletants

In the case of the yeast deletants screened for improved fermentation performance, the number of candidate genes to be evaluated was short listed to the best 92 performers from each library. In doing so, we were confident that the genes chosen would have a role in fermentation performance; which would be confirmed by evaluation in 100 mL fermentations

under various conditions. The strains were evaluated using the automated T-bot system under high and low N conditions. From 'area under the curve' data (Liccioli et al., 2011), the top 30 strains from each condition was chosen for further evaluation (in triplicate) in CDGJM (75 mg N L^{-1}). A total of 15 were selected for further characterisation. The work has been reported as a PhD thesis (Ms Josephine J. Peter; GWRPh1313) as well as 2 peer-reviewed articles (in preparation).

The 25 slowest fermenting mutants (in AWRI 1631 background) were evaluated in triplicate (100 mL) fermentations according to Walker et al., 2014. Twelve of the 25 genes identified, are present in the 93 Fermentation Essential Gene or FEG dataset, classified as affecting fermentation but not growth. Fermentations were considered protracted, when the duration was 20% or greater or the 'area under the curve' (Liccioli et al., 2011) was 20% or more greater than the parent. Growth was measured during fermentation, as optical density at 600 nm. Fourteen genes were identified as resulting in protracted fermentation, when deleted in this haploid wine yeast (**Table 4.2**). With the exception of *YPR074C* (*TKL1*) encoding transketolase, growth in the mutants was similar to the parental strain or only minimally affected. These 14 genes are representative of the wine yeast fermentome. Given that the WYDL only represents 30% of the non-essential genes in the LYDL, several of the FEG genes missing in this library still require construction. The function of the genes in relation to faster fermentation (15) and protracted fermentation (14) still requires further investigation.

Table 4.2: Identification of wine yeast Fermentome: genes required for alcoholic fermentation in chemically defined grape juice.

Growth	Ferment Protraction (AUC)	Ferment Protraction (duration)	Systematic Name	Standard Name	Brief Description
1.0	1.1	1.3	*YBR125C*	*PTC4*	Cytoplasmic type 2C protein phosphatase (PP2C)
1.0	1.1	1.3	*YCR106W*	*RDS1*	Putative zinc cluster transcription factor
0.9	1.3	1.2	*YHR194W*	*MDM31*	Mitochondrial protein - may have role in phospholipid metabolism
1.0	1.1	1.2	*YKR007W*	*MEH1*	Component of EGO & GSE complexes
1.0	1.6	1.6	*YFR053C*	*HXK1*	Hexokinase isoenzyme 1
1.0	1.2	1.3	*YDR247W*	*VHS1*	Cytoplasmic serine/threonine protein kinase
1.0	2.0	1.5	*YGR063C*	*SPT4*	Component of Spt4/5 complex (DSIF complex)
0.8	1.8	1.2	*YGR159C*	*NSR1*	Nucleolar protein - binds nuclear localization sequences
0.9	1.7	1.5	*YJR033C*	*RAV1*	Subunit of RAVE complex (Rav1p, Rav2p, Skp1p)
1.0	2.1	1.8	*YLL007C*	*LMO1*	Homolog of mammalian ELMO (Engulfment & celL MOtility)
0.9	1.7	1.4	*YMR263W*	*SAP30*	Component of Rpd3L histone deacetylase complex
0.7	7.5	3.0	*YOR209C*	*NPT1*	Nicotinate phosphoribosyltransferase
1.0	1.3	1.2	*YOR265W*	*RBL2*	Protein involved in microtubule morphogenesis
0.6	3.1	2.1	*YPR074C*	*TKL1*	Transketolase

Fermentations (100 mL) were conducted in CDGJM (450 mg N L^{-1}, 200 g L^{-1} sugar pH 3.5) as described in Materials and Methods. Fermentation Essential Genes were identified as gene deletants which resulted in protracted fermentation with no or minimal effect on growth. Mutant/parent ratio calculated for OD_{600} values (growth), AUC and duration (for fermentation protraction.

Output 1F. 4.4. Effect of overexpressed genes on fermentation duration

We reported on the micro-fermentation screening of the wine yeast overexpression library in the final report for UA1101 (Innovative microbials of winemaking excellence). The library screen identifying yeast genes (and their proteins) that contribute to the wine aromatic profile was disseminated in Haggerty (2015). Analysis of the sugar data (a measure of fermentation progress) identified genes when expressed that resulted in increased and decreased sugar consumption. 60 yeast strains were identified as having reduced fermentation duration compared to the wild type. 14 yeast strains were observed as having extended fermentation both in micro- and 100 mL lab fermentations. However, these results regarding the strains

with protracted fermentation (**Table 4.3**) must be taken with caution, because of concerns of plasmid instability. Plasmid retention in the control strain (isoC9D(pGP654) was 59% whilst that of the 14 yeast strains varied between 16 - 52% (32.1 ± 10.5%).

We have yet to complete evaluation in 100 mL ferments in order to confirm the identity of those clones that are consistently faster than the parent. To date, 25 strains have been evaluated under non-limiting nitrogen conditions (450 mg N L^{-1}). Three strains outperformed the parental strain by more than 10% - YGPM4f16 (0.88), YGPM11a08 (0.86), YGPM20l18 (0.77). The remainder were comparable to the parent strain in fermentation performance. Another 49 clones, together with the 25 already tested, are currently under evaluation in the newly configured T-bot as 30 mL fermentations (to be reported as part of the bilateral agreement). Key identified genes are yet to be overexpressed individually and evaluated for fermentation performance. Identification of such genes enhance the value of the Fermentome and represent potential targets for yeast strain improvement.

Table 4.4: Evaluation of 14 yeast over-expression strains in laboratory-scale fermentations.

Yeast Clone	Fermentation duration as ratio (mutant/parent)	Growth as percentage of parent	Plasmid retention as percentage
YGPM30k24	1.4	83	21
YGPM25l07	1.5	82	16
YGPM27p19	1.3	91	43
YGPM9l10	1.4	98	52
YGPM12i06	1.4	98	45
YGPM4a13	1.5	77	38
YGPM6d14	1.4	93	38
YGPM3p16	1.5	85	33
YGPM26i17	1.4	90	26
YGPM26n01	1.4	88	31
YGPM8i08	1.4	88	26
YGPM8b16	1.5	83	28
YGPM26i3	1.4	81	18
YGPM9d09	1.4	87	34
isoC9D(pGP654)	1	100	59

Fermentations were performed in CDGJM under FAN-unlimited conditions (450 mg L^{-1}) as described in Materials and Methods. Fermentation protraction was denoted where the fermentation duration ratio (mutant/parent) was ≥1.2. Plasmid retention (%) was calculated from CFU on non-selective YPD medium vs selective minimal drop out medium for the parental and overexpression yeast strains.

5.0 Identification of SNPs unique to evolved microbes via genome sequencing and comparison to the Fermentome

Output 3A: Genome sequence of 1-3 promising isolates (yeast or bacteria).

- Genome sequencing and assembly of up to 2 LAB and 1 yeast isolate initiated as a route to determining the basis of their phenotype and to support IP protection. Final numbers will be determined by state of progress of DE and selection experiments.

Output 3C: Summary of comparison of Fermentome data with genome sequences of evolved strains.

- Performance of a comparison of Fermentome vs genome sequence data to identify genes/processes etc relevant to fermentation and as the basis for possible strategies for better fermentation management or as additional opportunities for DE experiments. Prepare a manuscript for submission for publication in a peer-reviewed journal if appropriate.

Output 3D: Genome sequences for additional yeast and bacterial isolates (up to 8)

- Genome sequencing continued from initiation in Output 3A and used to characterise isolates (up to 4 LAB and 4 yeast across the project) as strains become available and data deemed useful.

Contributors (Yeast genome sequencing)

PhD students: Federico Tondini
Casual staff: Mr Miguel Roncoroni
Postdoctoral fellows: *Drs Tommaso Watson, Michelle Walker, Jennie Gardner and Professor Vladimir Jiranek*

Contributors (LAB genome sequencing)

PhD students: Jiao Jiang
Postdoctoral fellows: *Drs Krista Sumby, Joanna Sundstrom, Associate Professor Paul Grbin and Professor Vladimir Jiranek*

Background

The primary aim of this project was to use a Directed Evolution approach with selected wine microbes (pioneered in 2002 via UA01/04 and GWR-Ph0901) to generate superior (non-recombinant) yeast and bacteria in batch or continuous culture with key fermentation stressors. The Directed (Adaptive) Evolution technique does not require prior genetic

knowledge and is applicable to genetically complex attributes. Our 2006 paper (McBryde et al., 2006) and recent updates on GWR-Ph0901 provide proof-of-concept for its use with wine microbes and highlight its immediate applicability. Moreover, the optimisation targets highlighted by Wine Australia (resistance to stresses/inhibitors or enhanced utilisation of selected juice/wine constituents) are supremely suited to the Directed Evolution approach - ie extended cultivation of microbes under growth-suppressing conditions to allow superior strains to dominate the culture. We have taken a 'guided' approach to the design of the DE experiments, incorporating findings from relevant academic literature as well as our own unpublished experiences in DE and screening of yeast deletion libraries (used to identify genes and pathways associated with fermentation reliance). DE experiments in yeast and LAB have been undertaken simultaneously in several stress conditions using sequential batch and continuous culture approaches, in order to generate evolved strains with improved phenotypes within the time frame of the project. Genome sequencing and assembly of selected strains has been undertaken as a means to determine the basis of their phenotype and to support IP protection **(Outputs 3A and 3E)**. Additionally, comparison of the genome sequences of parental and evolved yeast to the 'yeast fermentome' will enable better understanding of the genes/processes relevant to fermentation, which has potential application in future DE and breeding strategies as well as fermentation management **(Output 3C)**.

Methods

M 5.1 Genome sequencing

M 5.1.1 Whole genome sequencing - 454 technology

NGS sequencing using Roche 454 technology was undertaken by the University of Cambridge Sequencing Centre, Cambridge, UK. Genomic DNA was extracted from overnight YPD cultures using Qiagen kits (as per manufacturer's instruction). Library construction and sequencing was performed using the GS FLX Titanium series (Roche Diagnostics, Branford, USA) shotgun and non-paired end protocols. Half a titanium plate was used for each strain (L2056 and FM16 C7H) with 275 and 230 Mb read length, generated respectively. This equates to approximately a 20-fold coverage of chromosomal regions corresponding to 11.9 and 11.8 Mb total length, respectively. The L2056 sequence files are deposited in GenBank ("Genome sequencing of wine yeast L2056" accession number AHZG00000000).

M 5.1.2 Whole genome sequencing - Illumina

NGS sequencing using MiSeq technology was outsourced to University of Queensland (UQ) and Australian Genome Research Facility (AGRF, Melbourne). Sequence coverage for yeast and bacteria genomes was 50x minimum, with 150 bp (UQ) or 300 bp (AGRF) paired end reads. Image analysis was performed in real time by the MiSeq Control Software (MCS) v2.6.2.1 and Real Time Analysis (RTA) v1.18.54, running on the instrument computer. RTA performs real-time base calling on the MiSeq instrument computer. Then the Illumina

bcl2fastq 2.20.0.422 pipeline was used to generate the sequence data. Details of recent sequencing from AGRF are shown in **Supplementary Table 5.1**.

M 5.2 Yeast genome assembly

Yeast Genomes were assembled using MIRA_4 sequence assembler (https://sourceforge.net) for yeast *Saccharomyces cerevisiae* (12.49 Mb).

A bioinformatic pipeline for the identification of Single Nucleotide Polymorphisms (SNPs) for yeast was developed by Mr Miguel Roncoroni using 454 data (**Supplementary Figure 5.1**). The alignment based program SIFT (Sorting Intolerant From Tolerant) enables prediction of amino acid substitution on structure/function of protein using GenBank protein GI number and identified SNPs. Web based SIFT was found at (http://sift.jcvi.org/ and http://sift.bii.a-star.edu.sg/). For single protein analysis, SIFT Blink option was used. Gene ontology enrichment analysis was undertaken using YeastMine in *Saccharomyces* Genome Database (http://yeastmine.yeastgenome.org/yeastmine/begin.do). An alternative program FunSpec (acronym for "Functional Specification") was also used (2. utoronto.ca/).

For Illumina data (yeast genome), reads were quality trimmed with Trimmomatic to remove possible Illumina adaptor and low quality base reads (phred score 33) (Bolger et al., 2014). The output reads were aligned to the yeast S288c reference genome (NCBI) with the BWA-mem algorithm (Li and Durbin, 2009). Differences between the parental and evolved strains were investigated visualising the mapped reads with the Integrative Genomics Viewer at chosen gene coordinates (Robinson et al 2011).

M 5.3 Bacterial genome assembly

A number of methods were used to analyse the LAB sequence data (8 *O. oeni*, 6 *Lb. plantarum* and 3 *Lb. hillgardii*) including; Geneious 8.1 software (www.geneious.com), UQ curated Galaxy Genomics Virtual Laboratory (Afgan et al. 2015) (https://galaxy-qld.genome.edu.au/galaxy/) and a customised pipeline (see below) to test the validity of our assemblies.

M 5.3.1 Bacterial genome assembly with Geneious

For the initial LAB assemblies Geneious was used as this is a graphical interface that is quick and easy to use. Sequence reads were first paired and low-quality ends were trimmed using the default setting. Two methods were then applied to assemble the LAB genomes. The paired reads were mapped to the appropriate reference genome using the Geneious assembler and Bowtie2 (Version 2.3.0). The *O. oeni* reference genome was PSU-1 (NC_008528), *Lb. plantarum* reference genome was WCFS1 (NC_004567) and *Lb. hillgardii* reference genome was ATTC8290 (ACGP00000000.1). Finally, gene annotations were transferred from the reference sequence to the consensus sequences or contigs of all assembled genomes using Geneious 8.1, with similarity set at 95%.

Whole genome alignments of parent and evolved strains were performed using LASTZ (7.0.1). Specifically, the genomes of strains 2-49 and 3-83 were aligned against the genome of A90; the genomes of strains A90, 2-49 and 3-83 together were aligned against the genome of SB3 and SNPs were preliminarily identified using the "Find variation/SNPs" function of Geneious R10. Criteria for SNP identification were at least 50-fold coverage with 100% variant frequency.

In order to obtain more complete sequence information, primer walking was employed to fill the gaps within the sequencing of genomes of strains A90, 2-49 and 3-83. Primers complimentary to sequences 200–300 bp outside of the gaps were designed using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0). PCR was conducted using Velocity DNA polymerase (BIO-21098, Bioline USA) or Ranger DNA polymerase (BIO-21121, Bioline USA) depending on the anticipated size of each gap. PCR reaction and setup was as per the manufacturer's instruction for each polymerase with modification only of extension time (2–10 min). For confirmation of SNPs between strains 2-49, 3-83 and A90, primers for amplification of fragments containing each SNP identified by genome comparison were designed using Primer3. The primers were used to amplify target regions of the DNA sequence using Velocity DNA polymerase (Bioline USA). All PCR products were sent to the AGRF (Adelaide) for Sanger sequencing.

M 5.3.2 Bacterial genome assembly with Galaxy

Sequence reads were checked with FastQC and Trimmomatic was used to remove possible Illumina adaptor and low quality base reads (phred score 33; Bolger et al., 2014). Genomes were then assembled *de novo* using SPAdes (3.5.0) with increasing kmer lengths to optimise the assemblies via the UQ curated Galaxy Genomics Virtual Laboratory. Contigs were then quality trimmed, to remove low coverage and/or short contigs (<1000 bp), using 'filter SPAdes output' function (https://galaxy-qld.genome.edu.au/galaxy/). Additionally three *O. oeni* isolates, G20, G55 and G71 isolated from high alcohol content Grenache wine have been sequenced using MiSeq with 100x coverage. The genomes are currently being assembled both *de novo* and against PSU-1 (NC_008528) as the reference. SNP variations from comparisons to other wine *O. oeni* sequences from NCBI will be identified in the coming months. Lalvin 4X (VL92) and Lallemand nuovi Ceppi Oo4, chosen for previously characterised esterase (Matthews et al. 2006) and beta-glucosidase activities (Grimaldi et al. 2005) have also been evolved by DE and sequencing data for the parent and two improved isolates from Lallemand nuovi Ceppi Oo4 is due to arrive in December. Lalvin 4X (VL92) is yet to be tested for improvement as DE experiments (the same conditions as Lallemand nuovi Ceppi Oo4) resulted is loss of cell viability and glycerol backups will need to be investigated to determine if this strain should be continued with.

M 5.3.3 Re-assembly of *O. oeni* A90 DE strains and assembly of *Lb. plantarum* DE strains with customised pipeline

All of the sequenced A90 DE isolates and parents were re-assembled with the help of the Bioinformatics Hub, School of Biological Sciences, University of Adelaide, as a part of core

infrastructure provided by the DVCR. Additionally NGS sequencing using Roche 454 technology previously undertaken for *O. oeni* strain SB3 (Betteridge, A PhD thesis) was also used as a test to improve the assembly. Three different pipelines were used to confirm the validity of our assembly and to check that gaps have actually been filled. Below is a summary of three pipelines used to conduct bacterial genome assembly of *O. oeni and Lb. plantarum*.

The pipelines are as follows:

1) Redundans_partial: This is a custom pipeline that uses SPAdes to conduct genome assembly, Pilon to correct missassemblies and the scaffolding and gapfilling tools from redundans to complete the assembly.
2) Redundans: Note that this pipeline includes the whole Redundans assembly process. This is a test pipeline, and is still in the process of development. For diploid organisms, sites can have multiple alleles. A normal assembly tool will reduce the homologous sequences around the allele, but can't reduce the heterozygous site. Often, this will result in two paths in an assembly graph which can't be resolved, resulting in two contigs which actually represent the same region. Haploid organisms don't have the multi-allele problem, however assembly programs can have trouble with repetitive regions and thus generate miss-assemblies, especially with short-read data. This can result in non-informative, redundant scaffolds. This pipeline was a test to see how the Redundans reduction step deals with redundant scaffolds in haploid organisms.
3) Unicycler: This is a standalone high quality genome assembler for bacteria. The developers state that this sacrifices contiguity for genome accuracy.

For each of the LAB variant comparisons, variants were called using BWA mem to align the trimmed sequence data to assemblies, and freebayes to call the variants. The variants were then filtered by quality ≥ 30 and depth ≥ 10. The remaining variants were annotated using the software snpEff using custom annotation databases generated from the annotation output (Annotation conducted with PROKKA).
snpEff appends a new field to the final column of the VCF file called ANN= (annotation).

Summary of Outcomes

The genomes of three wine yeast and five lactic acid bacteria strains, generated in this project using directed evolution (and their parent strains), as well as a number of other isolates that have the most desirable and improved fermentation attributes were sequenced (22 whole genome sequences in total, yeast: FM16 C7H, 71B, Q7, C7H_B4, RM5_15, RM3_25 and LAB: A90, SB3, A89, JJ2-49, JJ3-83, K45, KS72, KS78, KS78_E3, KS72_26, KS72_87, G20, G55, G71, Oe16, KS21, KS21_6, KS21_Dec1). Identification of the genetic differences between the parent and evolved strains will determine which genes have been mutated during the evolutionary process and thus which genes are the likely candidates to confer the improved phenotype. This information will highlight which biochemical processe(s) have changed and thus which are likely to enable the desirable attribute, for instance more rapid and reliable fermentation. Prototype or industry ready strains (legislation permitting) can now be constructed with this information.

Since the annotation and interpretation of genome sequences of microbes is a highly technical undertaking, with each species and in some case strain requiring specific methods, appropriate pipelines (chain of methods) were designed. This has allowed some automation of subsequent analyses and thus offered time savings. Identification of the specific mutations within each of these strains is on-going.

In the first group of *O. oeni* strains sequenced (generated from SB3 and subsequently A90) a total of 19 genetic mutations referred to here as single nucleotide polymorphisms (SNPs) were found in strains 2-49 and it's further evolved isolate 3-83 in comparison to A90. Ten SNPs were found in regions that code genes (coding regions) and previous reports suggest that modifications of these genes could impact the cell envelope, fatty acids biosynthesis, DNA translation and homeostasis of internal pH. We expect it is the modification of these pathways that leads to the improved performance of these evolved bacterial strains. Interestingly six SNPs are common to both strains with only two of these in coding regions. These genes encode acyltransferase and a membrane protein. Further studies will investigate how modification of these particular genes could confer robust and efficient malolactic fermentation.

The SNPs from a previously evolved wine yeast isolate (FM16-7, also investigated in project UA 11/01) have been defined. We found 207 in regions that are predicted to affect protein function. Of these no specific processes are highlighted as being central to this genotype, thus we hypothesise that the fermentation efficiency of this particular strain is conferred by multiple genes, fine-tuning multiple processes.

Results and Discussion

Outputs 3A and 3E. 5.1. Genome sequencing and assembly of evolved LAB and yeast isolates

These outputs are reliant on the completion of Outputs related to the performance of promising yeast and/or LAB in laboratory scale fermentations. To date, yeast DE has been problematic in terms of the time taken to obtain sufficient generations to see the effect of beneficial mutations resulting in decreased fermentation duration. Continuous culture was introduced late in 2015, as part of the Yeast DE program, to reduce the generation time required to achieve the planned target of ~250 generations, considered sufficient to stably incorporate beneficial mutations into the genome (McBryde et al., 2006). It is only now that several yeast isolates have been selected from a pool of 2,200 isolates evaluated in micro- (0.2 mL) and lab- (100 mL) scale fermentations (Outputs 2B and 2D). These strains displayed markedly improved performances compared to their parents in 20 L white juice, and 15 kg red must ferments (Output 4C). However, they did not typically outperform the Uvaferm 43 reference.

Six yeast strains from Output 2E and 3E (FM16 C7H, 71B, Q7, C7H_B4, RM5_15 and RM3_25) have been sequenced using Illumina technology (AGRF Melbourne; 31 Nov 2017) as part of the 3 month WA project extension (Oct – Dec 2017). DNA was extracted and used for Shotgun Illumina Library Preparation (with bead size selection). NGS was undertaken by Illumina MySeq sequencing with 300 bp paired end reads. The sequences will be assembled with the view of comparing the genomes of the parent(s) and evolved strains to identify the

genetic basis of their improved fermentation phenotype. The identified genes will be compared to the gene datasets associated with the updated Fermentome database in order to get a better understanding of how yeast are able to adapt to the juice environment and successfully complete fermentation.

In the interim, construction of a bioinformatics 'pipeline' enabling routine analysis of genomic data (**Supplementary Figure 5.1**) was used to sequence the genomes of one yeast strain (Tee 9) previously isolated from DE experiments (Liccioli et al., 2010), its parent (AWRI 796) and a commercially available, fructose-efficient strain, Fermichamp®. SNP variant analysis was performed on a family of hexose transporters (Hxt1-17 and Gal2) and glucose sensors, Rgt2 and Snf3 (Reifenberger et al., 1995, Guillaume et al., 2007, Karpel et al., 2008, Perez et al., 2005, Verwaal et al., 2002). It was thought that mutations in these proteins would directly impact the efficiency of hexose intake (as shown by use of radiotracer experiments) into the glycolytic pathway, ultimately increasing the fructophilicity of the evolved strain. An additional 53 genes related to glycolysis and glucose sensing were examined (data not shown), of which only *YLR377C* (*FBP1*) encoding Fructose-1,6-bisphosphatase was shown to have a C to T transition, resulting in an amino acid substitution ($G^{I34}S$) which is predicted to be tolerated (SIFT analysis). The findings of this investigation will be reported in a drafted manuscript to be submitted to a peer-reviewed journal for publication ("Isolation of an improved fructophilic yeast strain").

5.2. *O. oeni* genome assembly

The projects UA 1302 and GWRPh1308 (Ms Jiao Jiang; supervised by Drs Sumby, Sundstrom and Jiranek) have generated several evolved bacteria that show great promise in MLF (Outputs 2B and 2D). AB90, isolated from a previous DE experiment (Betteridge, 2015) was the starting material for initial DE experiments. Initial characterisation with increasing ethanol and decreasing pH established that LAB to be tolerant of pH as low as 3.1 in the presence of 10-12% ethanol, but did not survive at pH 2.8. Continuous culture of AB90 in Red Fermented Chemically Defined Grape Juice Medium (RFCDGJM, pH 3.5) with increasing alcohol was undertaken for 250 generations. Based on Minimum Inhibitory Concentration (MIC) results in RFCDGJM with added stressors (Output 3B), two *O. oeni* isolates (JJ2-49 and JJ3-83) were selected for whole genome sequencing (WGS), together with their parent AB90 and commercial strain SB3 (which is the parent of the original evolved LAB, AB90). WGS was outsourced to the University of Queensland, where MiSeq was undertaken with 100-150 x coverage. Jiao Jang has assembled the genomes both *de novo* and against the reference PSU-1 (NC_008528.1). Some of the gaps identified during the assembly (relative to PSU-1) have been covered by Sanger sequencing (AGRF). SNP variant analysis was undertaken to identify the genetic mutations within individual genes, which are specific to the evolved strains. A number of the single nucleotide polymorphisms (SNPs) identified by variant analysis were confirmed by Sanger sequencing. This work comprises of a large section of Jiao's PhD thesis and will be reported in a peer-reviewed journal article (Output 4E).

In order to generate a better parental reference of *O. oeni* SB3, this strain has been re-sequenced by AGRF to allow us to validate the sequence using this additional database. Additionally, all of the sequenced A90 DE isolates and parents were re assembled using three

different pipelines (M 5.3.3) to confirm the validity of our assembly and to check that gaps have actually been filled. A simple bar plot of the assembled genome lengths using different assembly pipelines (**Figure 5.1**) reveals that on average, Unicycler results in a smaller assembly in comparison to Redundans_partial and Redundans. This is unexpected, as the Unicycler development team believe that Unicycler will generally generate an accurate genome assembly at the cost of contiguity. If we are to believe that the length of PSU-1 is accurate, Unicycler varies from the expected genome of 1.74 Mb considerably. This suggests that either there are (i) considerable genomic differences between each of the strains, resulting in considerably different genome lengths, (ii) that the PSU-1 reference length is inaccurate or (iii) that Unicycler is struggling to consistently assemble the data.

The Redundans pipeline generates assemblies of an expected size relative to PSU-1 for 2.49, 3.83, A90 and A89, however shrinks significantly for both the Illumina only and Illumina + 454 (SB3) assemblies. This suggests that the reduction step which is designed for diploid organisms, for the most part, succeeds at removing redundant scaffolds from the assembly without affecting the overall assembly. It appears as though SB3 is an outlier in this instance. In comparison, the parital Redundans pipeline produces consistent genome assemblies with respect to size. The lengths of the genomes have had low-coverage scaffolds accounted for (i.e. coverage ≤1).

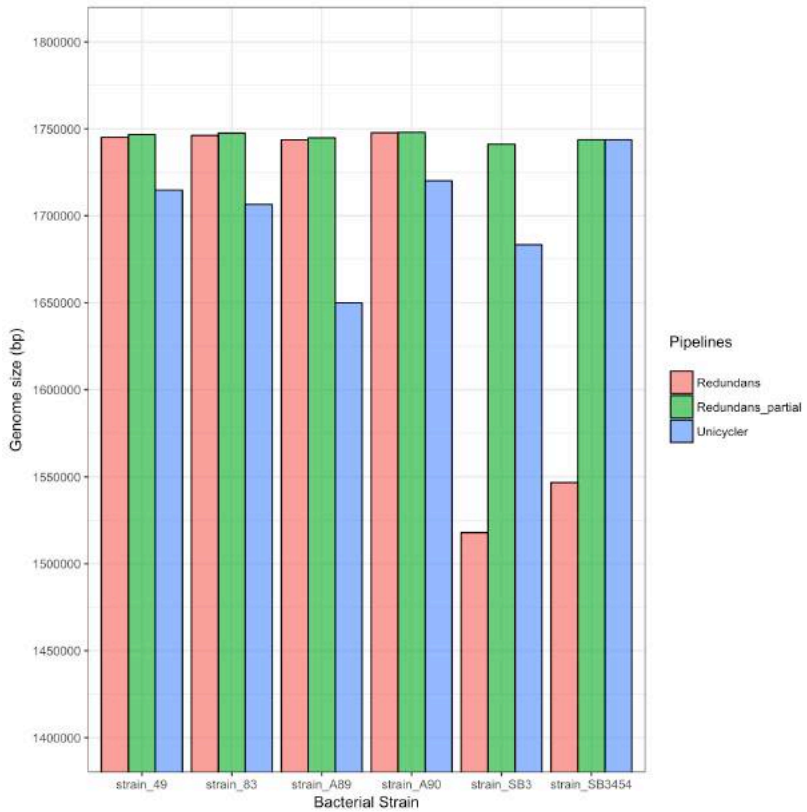


Figure 5.1: A bar plot of the assembled genome lengths using 3 assembly pipelines. The bar plot represents the genome size for each sequenced *O. oeni* strain as determined by 3 tested pipelines.

The number of scaffolds/contigs generated by each of the assembly pipelines for the individual strains was plotted (**Figure 5.2**). In general, Unicycler appears to result in a less

contiguous assembly, which is as expected. When accounting for genome size, the partial Redundans pipeline outperforms both Unicycler and the full Redundans pipeline.

The full Redundans pipeline only performs better than the partial Redundans pipeline on the SB3 assemblies, however this is likely due to a significant portion of the genome being lost in the reduction process, and should therefore likely be ignored.

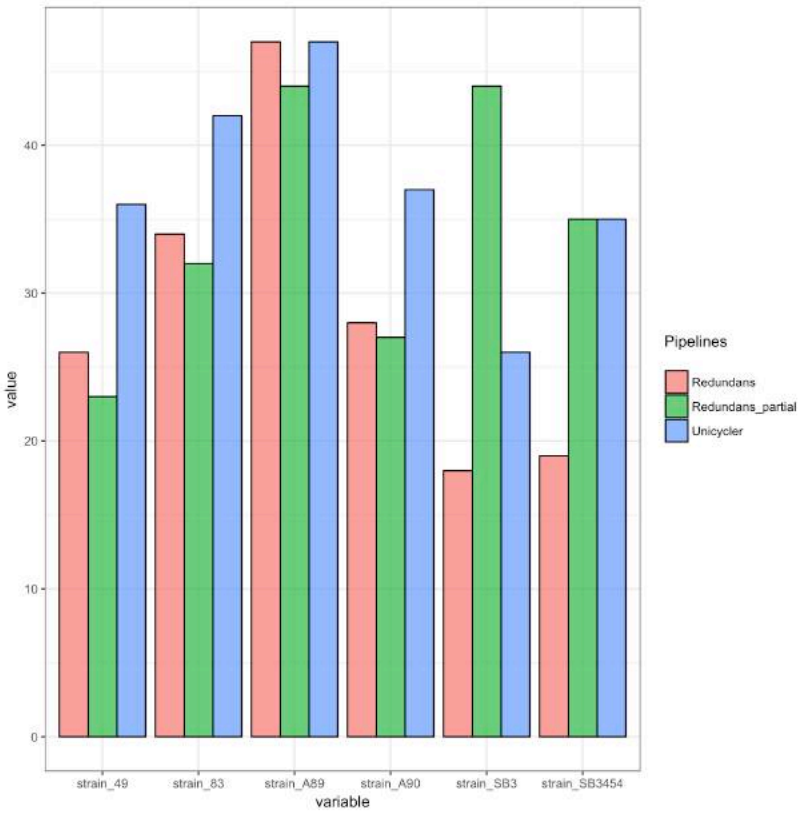


Figure 5.2: Number of contigs for each sequenced *O. oeni* strain, using the three tested pipelines.

To investigate the N50 values by strain, the assembly N50 values produced by each assembly pipeline were plotted as a box plot (**Figure 5.3**). Here, it can be seen that there is considerable N50 variation between the strains, with strain A89 being a noticeable outlier. Piecing this information with the previous bar plot, it becomes obvious that the A89 dataset was considerably more problematic to assemble, evident by the higher scaffold/contig number and lower N50 across assemblies. When N50 values are compared between pipelines (**Figure 5.4**), the Redundans pipeline reports a higher median N50 value, with Unicycler reporting the lowest.

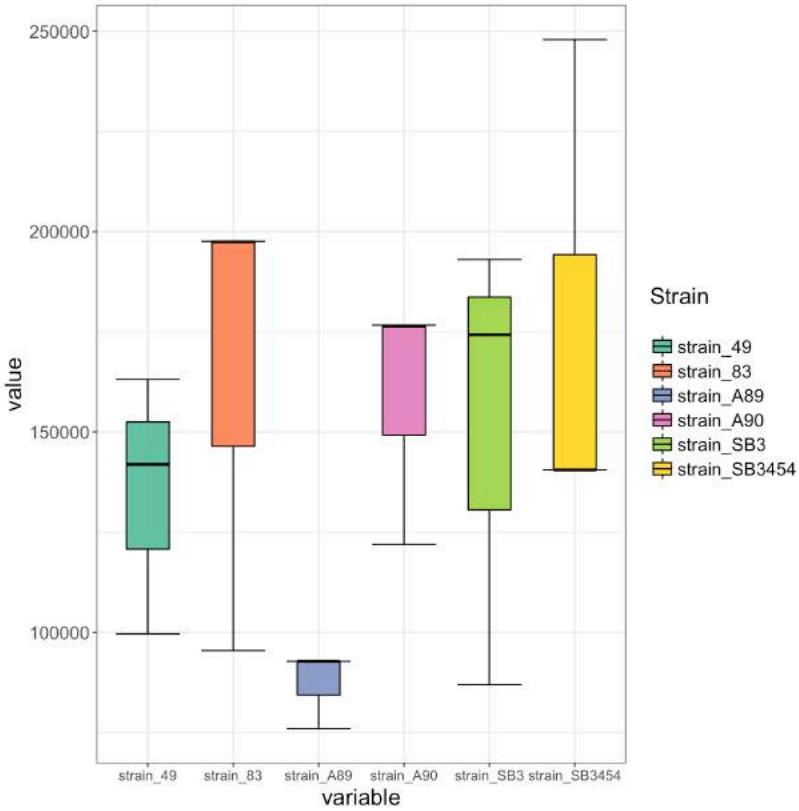


Figure 5.3: Comparison of N50 across the *O. oeni* strains.

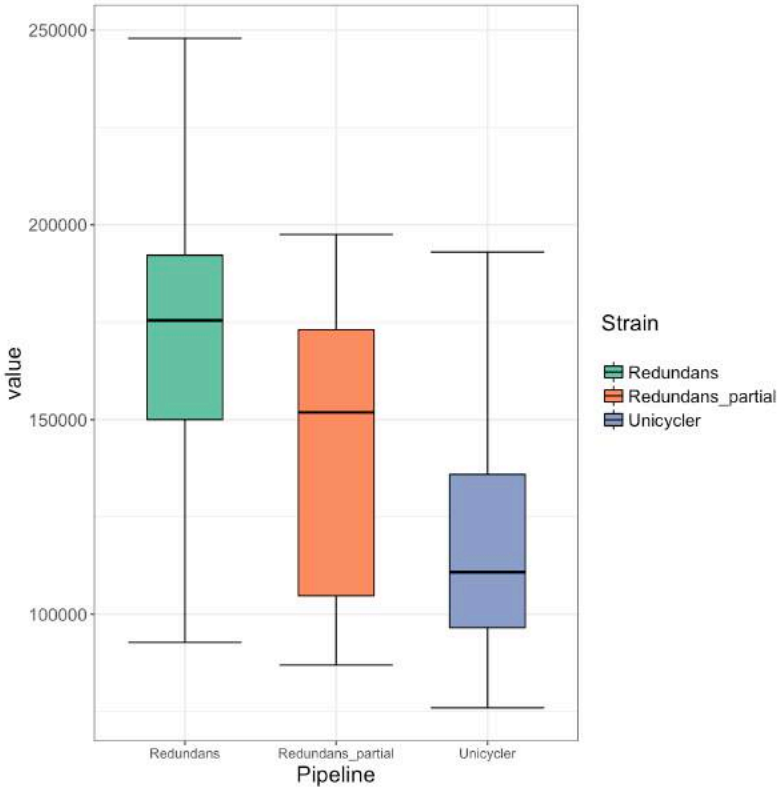


Figure 5.4: Comparison of N50 values between pipelines for *O. oeni*, where the N50 values of each strain is plotted by pipeline.

For the current datasets, the partial genome assembly appears to be performing the best, however there are some interesting observations from these analyses. Specifically, why does the full Redundans pipeline perform well on only 4 of the 5 bacterial strains, especially when the assemblies all have similar genome sizes and scaffold numbers. Further, considerable variation in reported genome size is observed when using Unicycler, even though many of the internal processes are the same as the custom Redundans pipelines (such as using SPAdes for assembly and PILON for miss-assembly correction).

Here, only variants reported as HIGH, MODERATE or LOW are kept to simplify assessment (**Figure 5.5**).

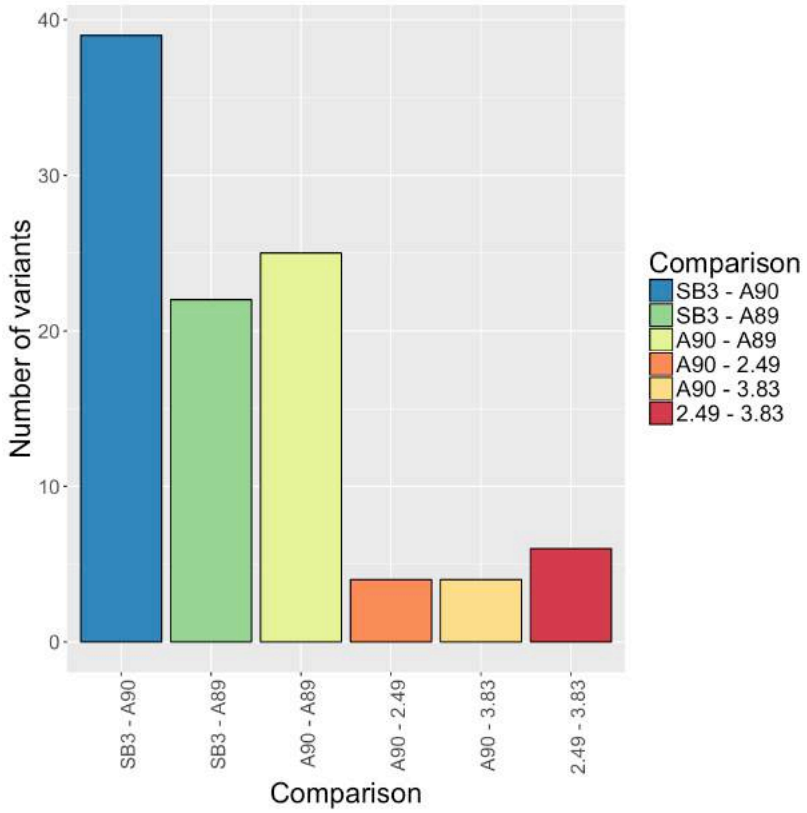


Figure 5.5: Total number of variants by strain for *O. oeni* SB3 and A90.

In order to generate a better parental reference of *O. oeni* SB3, this strain has been re-sequenced by AGRF to allow us to validate the sequence using this additional database. This will be reported on in June 2018 as part of the bilateral agreement.

5.3 Whole genome sequencing *Lb. plantarum*

DE experiments with *Lb. plantarum*, have only shown modest improvement when single isolates were evaluated in lab scale. Genomic sequencing was initiated for these strains with a view toward better understanding the genes needed for successful adaptation of *Lb. plantarum* to wine. This will also allow us to better direct any new DE experiments with this organism. The three pipelines that were used for assembly of *O. oeni* isolates were also used to conduct bacterial genome assembly of *Lb. plantarum*. The assembled genome sizes are

consistent across the assemblers (**Figure 5.6**). Unicycler reports a slightly smaller genome size across the board, however all are between 3.3-3.4 Mb in length. Unicycler reports a greater number of contigs across all bacterial strains, which fits with the developer notes of more accurate genome representation at the cost of contiguity. The Redundans pipeline consistently reports the fewest contigs, while the partial Redundans pipeline generates a slightly less contiguous assembly. Overall, there is little difference between the three (**Figure 5.7**). The only outlier is strain KS72_87, which has a significantly higher number of scaffolds across all three pipelines compared to the other bacterial strains. This may be an indication of the quality of the data.

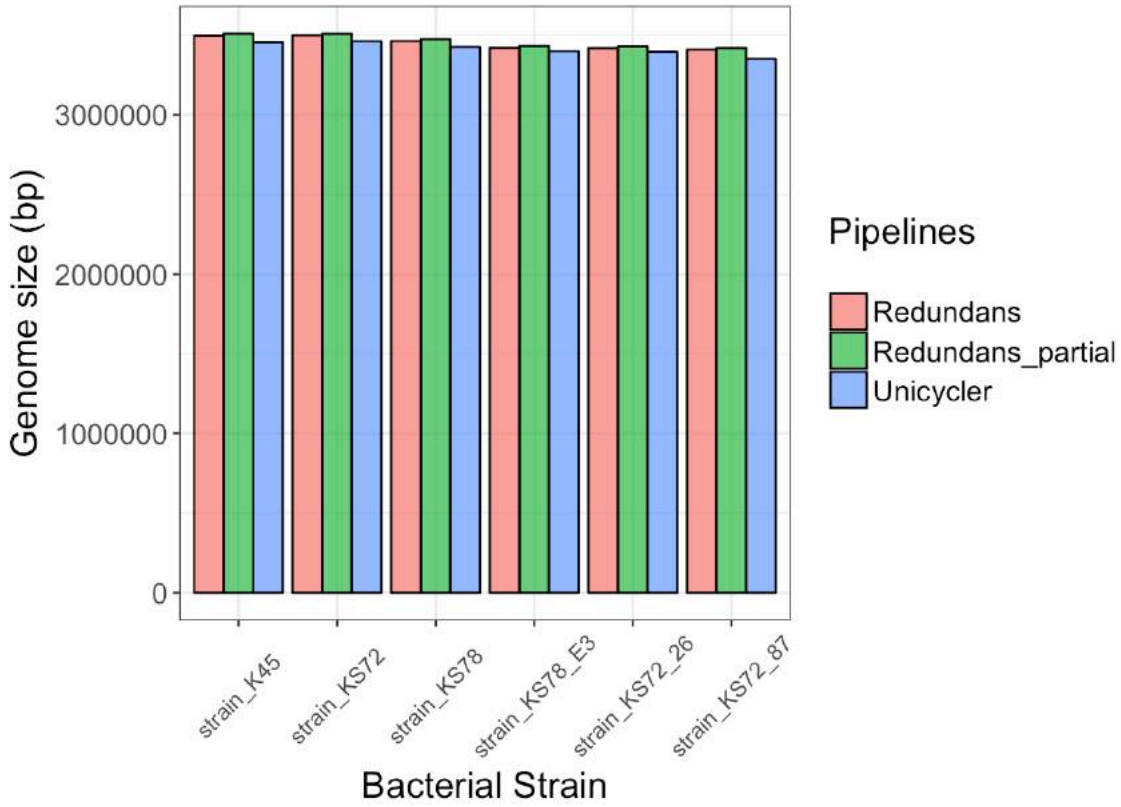


Figure 5.6: Genome size for each sequenced *Lb. plantarum* strain, as determined by the three tested pipelines.

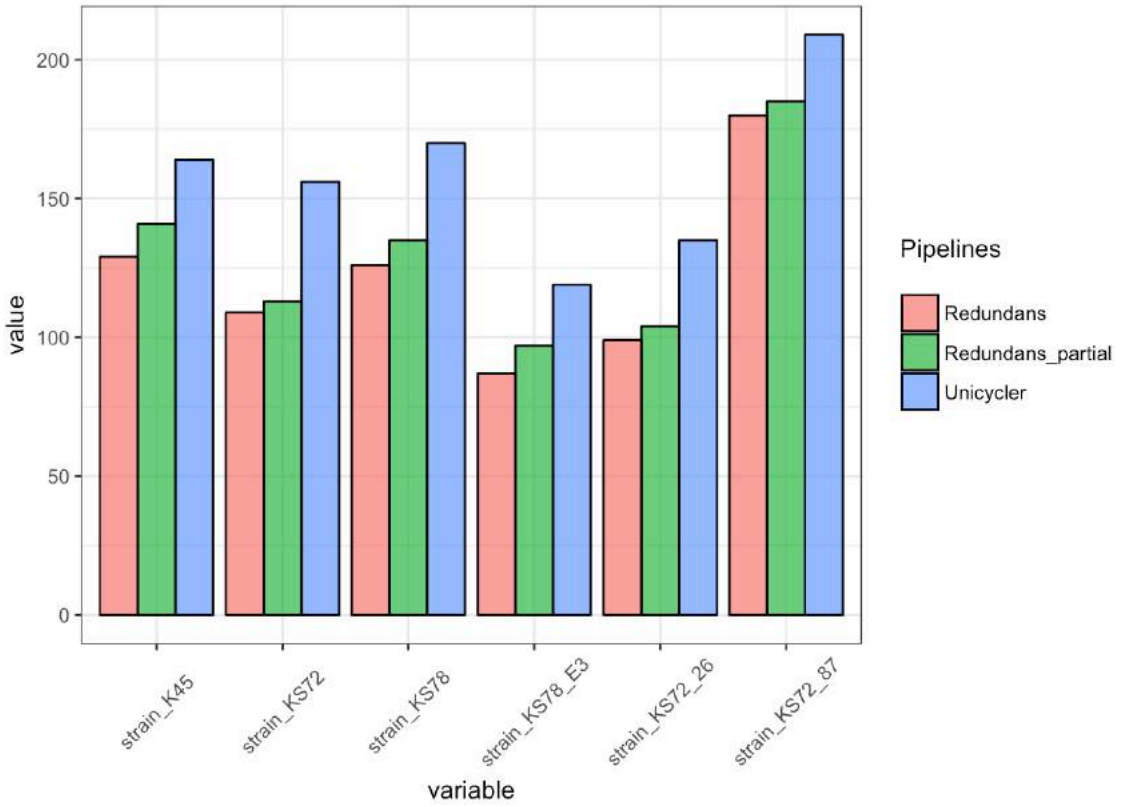


Figure 5.7: Number of contigs for each sequenced *Lb. plantarum* strain, as determined by the three tested pipelines.

Investigating *Lb. plantarum* N50 size by pipeline (**Figure 5.8**) it appears as though the two Redundans dependent pipelines generate similar assemblies when averaged across all bacterial strains. Important to note is the extreme outliers present at the ~36kb mark for both Redundan pipelines. The Unicycler pipeline appears to be far more variable in regards to the resulting N50 values for its different assemblies. Plotting N50 values by strain rather than pipeline (**Figure 5.9**) reveals how variable and contiguous assemblies were for each strain relative to assembly pipeline. Tighter boxplots indicate consistent genome assemblies across assembly pipelines, while spread box plots indicate inconsistent assemblies. The reasonably similar N50 values between bacterial strains, with KS78 being slightly lower than the rest and KS72_87 being an extreme outlier. As seen above, KS72_87 was considerably more fragmented than the other assemblies, so it is not surprising that the N50 value is considerably lower than the other strains.

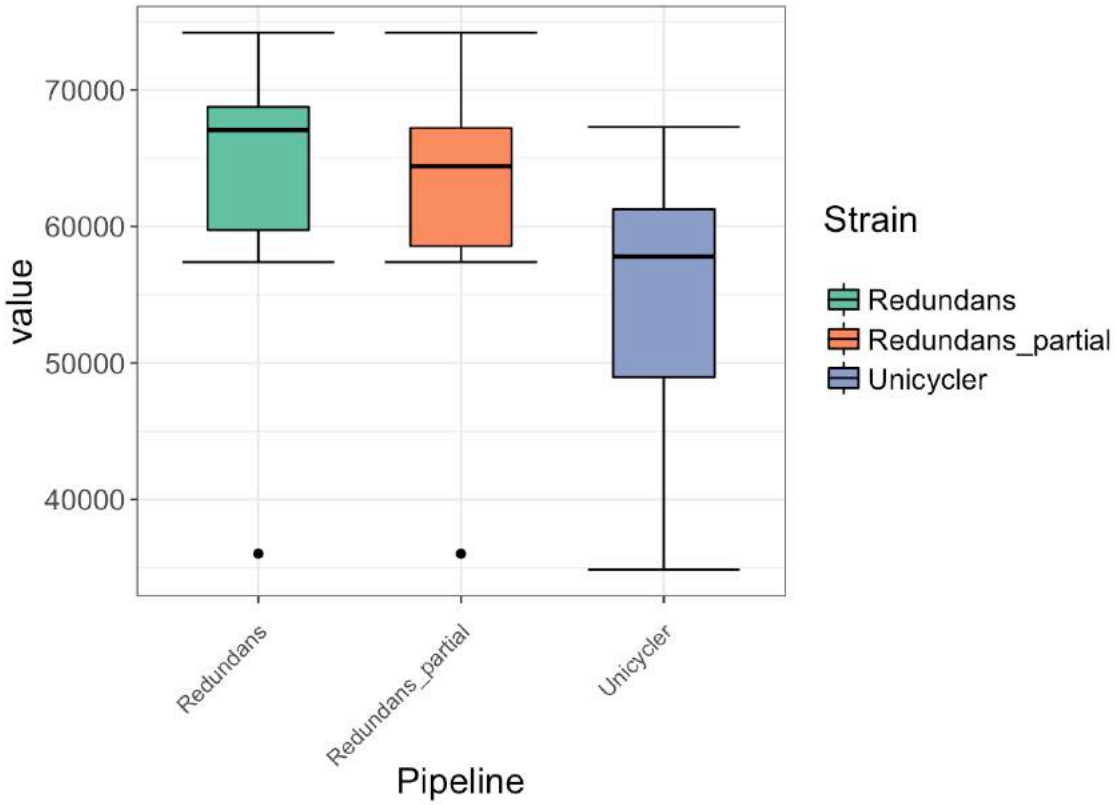


Figure 5.8: Comparison of N50 across the three tested pipelines for *Lb. plantarum*.

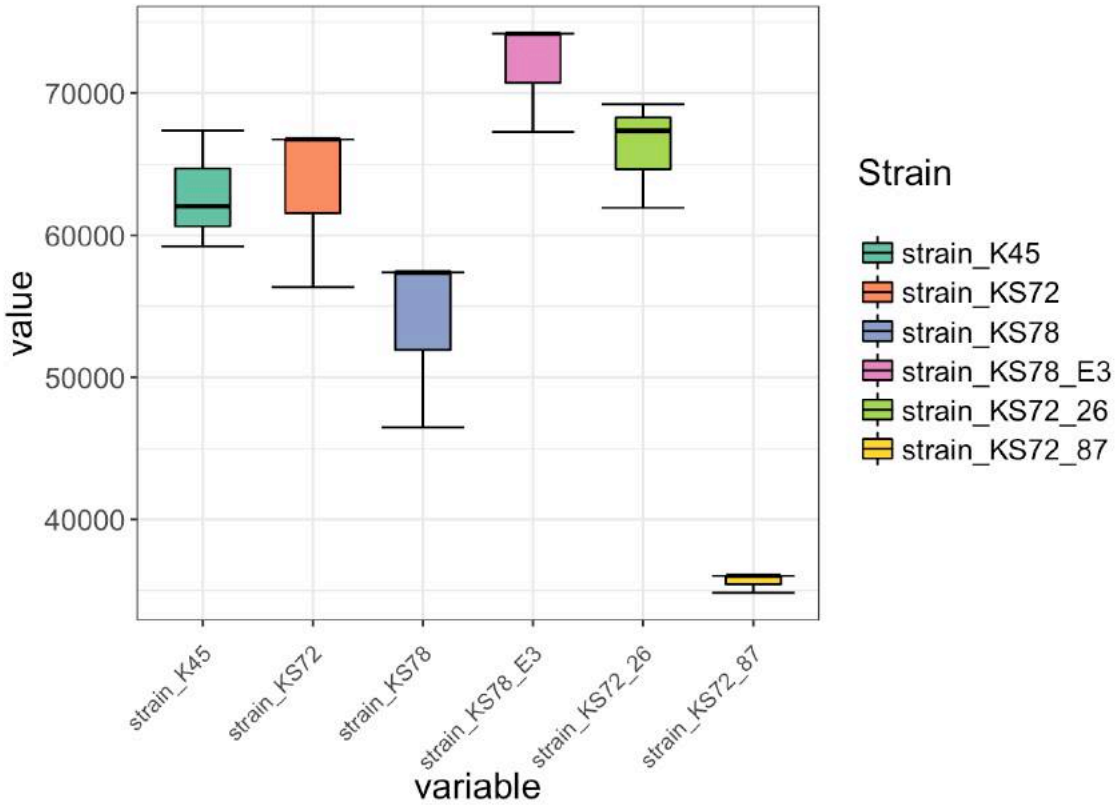


Figure 5.9: Comparison of N50 across the *Lb. plantarum* strains.

In this case of *Lb plantarum*, the assembly pipelines appear to perform very similarly with respect to assembling a genome of an expected size. As expected, Unicycler produced more fragmented assemblies, however all assemblies were fragmented due to the coverage-to-genome size ratio. Sample KS72_87 was an outlier with respect to its higher fragmentation level and lower N50 value. These two statistics are heavily dependent on each other.

Overall, the use of the partial Redundans pipeline or Unicycler would be warranted. In this analysis, we see that the reduction step in the Redundans pipeline doesn't affect genome size and results in a more contiguous assembly. This suggests that whilst designed for heterozygous diploid genomes, the reduction step is a valid approach for removing redundant scaffolds in bacterial assemblies. Here, only variants reported as HIGH, MODERATE or LOW are kept to make assessment easier (**Figure 5.10**).

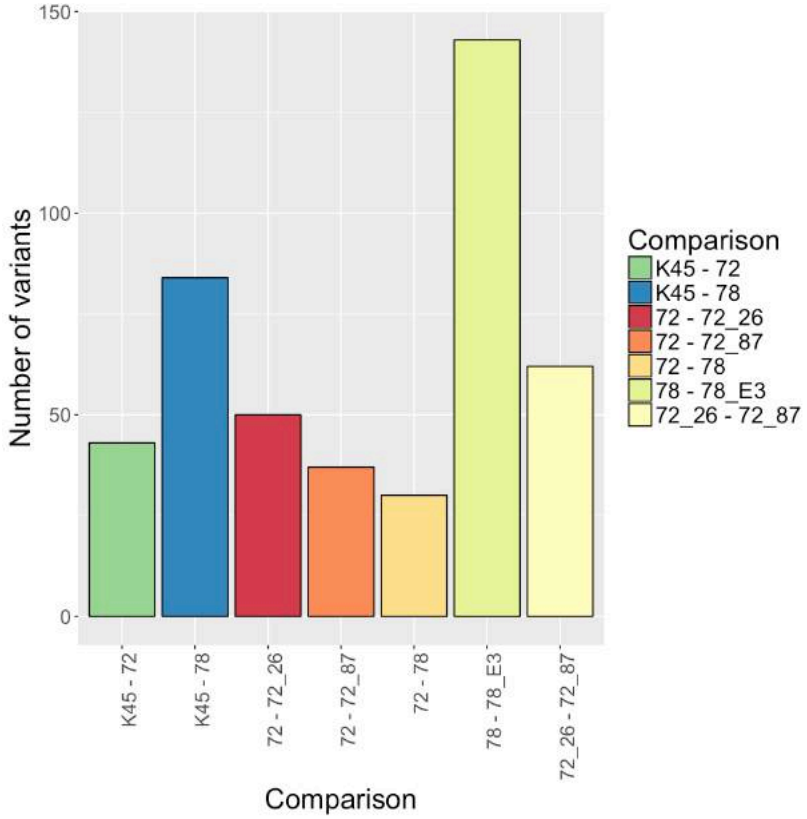


Figure 5.10: Total number of variants by Strain for *Lb. plantarum.*

We are currently analysing *Lb. plantarum* strains using LASTZ to check for chromosome rearrangements and SNP's that potentially impact MLF performance will need to be confirmed by Sanger sequencing. Now that bioinformatics 'pipelines' for yeast and LAB genomic analysis have been developed, it will enable routine comparisons between evolved strains and reference strains. Further, SNP variant analysis will allow the monitoring of the genetic mechanisms driving the evolution of specific wine-making traits. Knowledge of the molecular basis of the phenotypic diversity will further facilitate the refinement of improvement techniques used in future projects.

Output 3C. 5.6 Comparison of Fermentome with SNPs of key evolved strains

As described, the fermentome comprises yeast genes found to contribute to fermentation efficiency largely identified through screening of yeast deletion and overexpression libraries. Since the majority of evolved strains generated in this project are only currently now being sequenced we are not yet able to perform this comparison. However we have analysed the SNPs of one of our previously evolved, fermentation efficient yeast isolates FM16 C7H. SNPs of FM16 C7H that were predicted to affect protein function (SIFT score <0.5) were cross-referenced with genes of the Fermentome identified as negatively influencing fermentation efficiency (when deleted yeast are fermentation efficient). This comprised of 3 data sets, generated from 3 separate experiments. Of the 207 non-synonomous SNPs of FM16_C7H, 31 were common to one and seven were to at least two other data sets. Defects in these genes thus result in fermentation proficiency across a range of strains and/or conditions and consequently are excellent targets for future yeast modifications. We were also interested in determining how these SNPs compared to those already published. Payen and colleagues (2016) created a database compiled of a total of 1,167 mutations in 1,088 genes from 106 long-term evolution experiments conducted in 11 different conditions from nine previous studies. Comparison of our SNPs revealed 15 which had previously been reported in other studies. These will be further explored in future studies to direct strain modifications and inform how fermentation efficiency can be conferred.

6.0 Construction of recombinant strains to confirm importance of highlighted gene deletion mutants.

Output 4A: Recombinant 'proof-of-concept' strains.

- Construction of recombinant strains to confirm importance of any observed gene modifications or highlighted deletion mutants. Findings prepared for submission for publication in a peer-reviewed journal (subject to any IP restrictions).

Contributors

PhD students: Mrs Jasmine Peter, Mr Chen-Wei Huang, and Mr Tom Lang
Postdoctoral fellows: *Drs Tommaso Watson, Michelle Walker, Jennie Gardner and Professor Vladimir Jiranek*

Background

To understand how genetic mutations may confer a particular phenotype, it is useful to construct recombinant strains as 'proof of concept' where a particular genetic change identified through genome comparisons of the parent and evolved strains is reconstructed. The resultant strains can be evaluated for the same phenotype in order to determine whether one gene is sufficient or several genes are required. Gene expression studies using a targeted (QPCR) or global (RNA Seq) approach can be used to determine how a particular gene modification effects other genes and associated pathways which interact with the gene. Together, these findings help to provide a better understanding of how yeast are able to adapt to varying juice conditions and successfully complete fermentation.

Methods

Methods to be found in peer-reviewed publications and completed thesis publications.

Summary outcomes

The original intention was to reconstruct gene deletions or nucleotide substitutions (called single nucleotide polymorphisms or SNPs) identified through sequence comparison of the evolved yeast to their original parents, as single modifications in a wine yeast and confirm whether the similar 'improved' phenotype was possible. In hindsight, this output was unrealistic because of the time taken to generate and evaluate the evolved strains as well as the time needed for genome sequence analysis. Instead, we have focused on the use of 'proof-of-concept' strains from three PhD projects closely related to UA1302, to better understand the genetic and biochemical pathways involved in measurable fermentation parameters such as sugar and nitrogen utilisation, hydrogen sulfide and thiol production and colour modulation. We have identified 16 genes whose deletion in wine yeast result in faster fermentation in nitrogen-limited juice-like media. In addition, new genes, other than *MET5*

and *MET10* (encoding sulfite reductase), have been shown to affect hydrogen sulfide and thiol (3MHA/3MH) production when deleted or overexpressed. Earlier work (UA1405) identified 3 genes related to modulation of colour in model red wines though a genetic mapping study of 96 hybrid yeast obtained from mating two commercial wine yeast, Enoferm M2 and Zymaflore F15. The current project has focused on the evaluation of these genes as single and double deletions in the parent and F1 hybrid (M2 x F15) strains, in order to confirm their role in decolourisation through pigment adsorption to yeast cell biomass.

All of these genes are potential targets for strain improvement in breeding programs whereby natural genetic variants (mutations) giving similar phenotypes could be selected and crossed into current commercial strains to further improve them. Such breeding programs would be facilitated by PCR tracking of the 'targeted' genes from individual strains, similar to that of 'marker assisted' breeding of plants.

Several of these genes are the focus of further study in better understanding how the yeast can undergo successful fermentation in 'difficult' juices. New frontier technologies are being adapted, e.g. CRISPR gene editing with the view of making beneficial genetic changes without the addition of bacterial DNA sequences to the genome.

Results and Discussion

Output 4A. 6.1. Recombinant 'proof-of-concept' strains

The original aim of **Output 4A** was to construct recombinant strains to confirm the importance of any gene modifications (SNPs) or gene deletions identified during sequencing of the evolved yeast in comparison to their original parents. However, this output was not achieved because of the constant delay with regards to the isolation of the evolved yeast strains with improved fermentation performance. Instead, we report on 'proof-of-concept' strains from three PhD projects, which are closely related to UA1302 in terms of research focus, and the contribution towards supervision.

Jasmine Peter (GWR PhD 1313) has identified 15 genes which upon deletion result in shorter fermentation duration in a chemically defined grape juice under limited nitrogen conditions. These findings are reported in Jasmine's PhD thesis (2017) already provided to Wine Australia and a peer-reviewed article under review (see Output 2F).

To further examine gene modifications identified within this project as potential targets for improvement of fermentation efficiency (identified in Output 3C) the performance of key gene deletion mutants was analysed. This group of genes deletants were selected since they were common to genes identified as non synonomous SNPs in FM16_C7H (fermentation efficient evolved yeast strain) and to the database of genes that when deleted lead to shortened fermentation duration (Output 1B). Seven deletants (in the wine strain AWRI 1631) were fermented in CDGJM with low nitrogen (75 mg N L^{-1}). Cultures completed fermentation in a similar duration to the parent (data not shown). This reflects that these SNPs may be acting in concert to confer the phenotype of fermentation efficiency in FM16_C7H.

Chen-Wei Huang (GWR PhD 1314) recently published the first report of the overexpression of *TUM1* leading to the increase in thiols 3MH and 3MHA when grown in CDGJM in the presence of excess cysteine (Huang et al., 2017). Several genes which regulate cysteine uptake were identified as affecting production of hydrogen sulfide from cysteine during fermentation (Huang et al., 2017). Yeast deletion libraries via a colony colour assay on media resembling grape juice were screened for H_2S production. Both Δ*lst4* and Δ*lst7* formed lighter coloured colonies and produced significantly less H_2S than the wild-type on high concentrations of cysteine, probably because of inefficient uptake of cysteine. Nine known cysteine permeases were also examined, as yeast deletants. Deletion of *AGP1*, *GNP1* and *MUP1* led to reduced production of H_2S from cysteine, as did *STP1* and *DAL81* which are involved in the SPS-sensing pathway. These results confirm that Agp1p, Gnp1p and Mup1p are the major cysteine permeases and that they are regulated by the SPS-sensing and target of rapamycin (TOR) pathways under the grape juice-like, cysteine-supplemented, fermentation conditions. Furthermore, cysteine transportation could be a limiting factor for yeast to generate H_2S from cysteine and therefore selecting wine yeasts without defects in cysteine uptake could maximise thiol production potential. Max's research is reported in his PhD thesis and has been provided to Wine Australia.

The genetic basis of colour modulation of a model red wine (UA1405) identified two chromosomal regions (or QTLs) which may be responsible for colour modulation of model red wine. Three candidate genes have been deleted in Zymaflore F15 and Enoferm M2, as single and double deletions. Evaluation as 100 mL fermentations using CDGJM with added grape skin polyphenols allude to two genes which when deleted resulted in an increase in L* in the clarified model red wine. The influence of biomass on decolourisation was not conclusive as the colour of the biomass of the deletion strains was not statistically different from the parent (data not shown).

Deletion of the 3 genes in the F1 hybrid has been undertaken, and the resultant strains evaluated in 100 mL fermentations in CDGJM with added grape skin polyphenols. Colour data are currently being analysed and will be reported in the interim report as part of bilateral agreement. The intension is for the colour findings to be published in a paper on QTL mapping of oenological traits with Dr Miguel Roncoroni and Professor Richard Gardner, formerly of the University of Auckland. These genes would be targets for strain improvement either through the screening of naturally occurring variants, the genetic transfer of the genes into current commercial wine yeast or reconstruction using genome editing.

Tom Lang (GWR PhD 1603) has developed Crispr/Cas 9 editing technology to construct gene disruptions in industrial *S. cerevisiae* yeast. He has tested the method by introducing an in-frame insertion into the *LEU2* gene, which has resulted in leucine auxotrophy (i.e. inability to grow in the absence of leucine). Tom is currently developing the technique to create point mutations within specific genes. The aim is to reconstruct various gene modifications in industrial yeast, which have been previously identified by us and others, to enhance fermentation performance or stress tolerance. Crispr/Cas9 will be feature in the new bilateral

agreement between Wine Australia and the University of Adelaide (Wine Microbiology) as a paradigm for strain improvement.

7.0 Upgrade of the automated fermentation platform (T-bot) to allow for 384 fermentations.

A custom built 96-vessel fermentation platform (T-bot) was commissioned in Feb/Mar 2014, permitting the automated (i.e. 24/7) sampling of 96 fermentations and storage for 'off line' analysis. The T-bot platform has been in high demand, as the system offers expediency in high-throughput evaluation of candidate strains. At any given time up to 31 individual yeast strains can be evaluated (in triplicate) against a reference strain for fermentation performance.

Due to the constant demand for high throughput fermentation analysis by staff and students, the T-bot was upgraded early 2017, funded by a variation to UA1302, to enable analysis of 384 parallel (30 mL) fermentations. The platform was modified to 4 (96 x 30 mL) units, including the stirrer platforms, which can be operated at 2 independent temperatures (using 2 new chillers). As four chillers were originally purchased, the others will be used for the second T-bot which is on order.

The 384-configuration (as a single unit) has been successfully used to evaluate 76 strains whereby yeast were fermented alone or with LAB as co-inoculated fermentations, as part of a Yeast-LAB interaction project (Louise Bartle, *pers. comm*.) and screening of 95 *Lachancea thermotolerans* strains for fermentation performance (Ana Hranilovic, *pers. comm*.).

The platform was tested as 4 single operating units, with independent sampling of different blocks using the robotic arm and 2 sampling needles (data not shown). The T-bot is operated from a single 'sampling schedule' program, which allows the operator(s) to sample the blocks independently. The operation of the platform other than a single 384 unit is reliant on the coordinated timing of experimental set up (between sampling when the cabinet housing the platform can be accessed) and during sampling (such that the time interval is not less than 3 hours between sampling for 2 blocks and 6 hours for 4 blocks.

There will be a high demand for the T-bot (384) in the work for the upcoming bilateral agreement, with the screening of non-*Saccharomyces* and bacteria for novel oenological properties. The ability to interconvert between the 2 configurations (384 and 96) is useful in this context, where larger volumes with uniform cell enumerations required (T-bot (96)) when evaluating few numbers of candidate strains.

8.0 Annual review of UA1302 and AWRI project funded by Wine Australia

Outputs 1C, 2C, and 4D: Documented outcomes from Annual Project Meeting.

- Hold Annual Project Meeting to review and discuss results and suggest future project plans (with relevant staff from AWRI and GWRDC).

Output 3F: Documented outcomes from Annual Project Meeting and stop/go review point with GWRDC.

- Hold Annual Project Meeting to review and discuss results and suggest future project plans (with relevant staff from AWRI and GWRDC). Review progress with GWRDC and stop/continuation/initiation decision made on specific strain attribute targets or newly arisen target priorities.

An Annual Project meeting has been held each June between the Jiranek group (AU) and Australian Wine Research Institute (AWRI) as part of fulfilling the requirements of **Outputs 1C, 2C, 3F** and **4D**. The purpose of these meetings was to review and discuss results from our respective projects and discuss future directions. Dr Liz Waters (RD & E Portfolio Manager, Wine Australia) attended the initial meeting (Yr 1) with Dr Keith Hayes (R&D Manager, Wine Australia) attending subsequent meetings (Yr 2 and 3).

Each year, two key staff members from each institution have given powerpoint presentations (15-20 min) summarising project results to date, followed by an informal discussions with the attending participants. The meetings were beneficial in providing an opportunity to disseminate up-to-date findings on research in a confidential manner, discuss project outcomes, exchange relevant experiences, knowledge and provide advice.

The projects reviewed were:

1. **UA1302: 'Fit-for-purpose yeast and bacteria via directed evolution'**
   Dr Krista Sumby (LAB DE), Dr Michelle Walker and Dr Tommaso Watson (Yeast DE). Presentations are available upon request.

2. **GWRDC 1302 (AWRI Project 3.2.3): 'Defining the nutritional drivers of yeast performance and matching yeast to must'**
   Dr Simon Schmidt

3. **GWRDC 1303 (AWRI Project 3.2.4) - Efficient and reliable malolactic fermentation to achieve specification wine style.**
   Dr Eveline Bartowsky and Dr Peter Costello

In Yr 3 (Output 3F) the review process resulted in no marked changes to the project plan

being proposed or deemed necessary. The final annual report meeting between AU and AWRI as held on 20 June 2017 (Output 4D), prior to the completion of the individual projects (30 September 2017). Based on the success of these reports, it has been proposed that such meetings are continued on a regular basis between the AWRI and the University of Adelaide.

9.0 Communication with yeast and bacteria manufacturers regarding commercialisation of evolved strains

Output 4B: Documented outcomes from discussion with yeast and bacteria manufacturers for purposes of strain commercialisation.

- Discussions with various yeast and bacteria manufacturers (e.g. Lallemand, Mauri Australia, Christian Hansen and Laffort) with a view to commercialisation and to facilitate industry-wide trials from vintage 2017.

Laffort (Bordeaux) recently invited a student from the Jiranek lab on a research placement at the University of Bordeaux (Ana Hranilovic, GWR Ph 1407) to present her work on non-*Saccharomyces* yeasts as well as the broader activities of the group. Included in the discussion was the LAB DE work of Dr Alice Betteridge (formerly GWR Ph 0901) and now Ms Jiao Jiang (GWR Ph 1308). On the strength of these results Laffort have formerly requested to have access to the most promising strains for their in-house and field evaluation. We have provided *O. oeni* strains A90, JJ2-49 and JJ3-83 to Laffort for evaluation, which is currently ongoing.

Laffort have also expressed a keen interest in a *Metschnikowia* strain which we isolated through various vineyard and winery survey exercises via previous student projects.

In addition, we have 5 DE strains that are able to complete MLF faster than the parent strain in low ethanol (pH 3.4, 13%) RFCDGJM and complete MLF at the same time as the parent in RCDGJM. Discussions with Lallemand (17/08/17) have revealed that they would be interested to see if the strains retain stamina and performance in red juice ferments for an extended time (>3 days). Following on from this Lallemand are eager to evaluate the best strains.

10.0 Dissemination of Project findings to industry and academia

Output 2F: Data from Years 1 and 2 (DE experiments and characterisation of 'Fermentome' database) disseminated through at least two extension mechanisms.

- Subject to any IP restrictions, preparation of papers for submission to peer-reviewed journals, and poster presentations at wine industry relevant conferences (e.g. CRUSH), describing properties of the initial DE strains generated/isolated and the wine yeast Fermentome.

Output 4E: Project findings disseminated to Australian Wine Industry and researchers.

- Preparation and submission of at least 2-3 manuscripts in academic peer-reviewed journals, and at least 2 manuscripts in industry trade magazines. Where available, and subject to acceptance, at least 2 posters, at least 1 oral presentations, and at least 1 workshop (tastings) at suitable industry conferences.

The Jiranek group has disseminated project findings through written media, magazines, invited seminar presentations and poster and oral presentations at a number of national and international conferences. Vladimir Jiranek (Project Supervisor) has publicised the project to visitors to the Department of Wine and Food Science and during discussions with yeast suppliers (Laffort, Lallemand, Christian Hansen) regarding newly generated bacterial strains. Vladimir Jiranek and individual group members visiting international labs (NAIST, Japan, Cambridge University, UK and University of Bordeaux, France) have had informal discussions on the group's activities including this project.

The following presentations were given as part of fulfilling the requirements of Outputs 2F and 4E:

Conference, workshop and seminar presentations

2014 Society for Industrial Microbiology & Biotechnology Annual Meeting, St Louis (20-24 July 2014).
Liccioli, T.
S79. Hide and seek: improved microbial strains vs. researchers. *Oral presentation*.

The University of Adelaide, School of Agriculture, Food and Wine Postgraduate Symposium (23-24 September, 2014)
Jiang, J., Sumby, K., Sundstrom, J., Grbin, P. and V. Jiranek.
Directed evolution as a tool to improve *Oenococcus oeni* for optimised malolactic fermentation. *Oral presentation.*

Peter, J. J., Liccioli, T., Walker, M. and V. Jiranek.
Use of prototrophic deletion libraries as a tool to identify nitrogen related genes in lab and wine yeast. *Oral presentation.*

Crush 2014: The Grape & Wine Science Symposium, Adelaide (25-26 September, 2014).
Peter, J. J., Liccioli, T., Walker, M. and V. Jiranek.
Use of prototrophic deletion libraries as a tool to identify nitrogen related genes in lab and wine yeast. *Oral presentation.*

31st International Specialised Symposium on Yeast, Slovenia (9 -12 October 2014)
Peter, J. J., Liccioli, T., Walker, M. and V. Jiranek.
Use of prototrophic deletion libraries as a tool to identify nitrogen related genes in lab and wine yeast. *Oral presentation.*

The University of Adelaide, School of Agriculture, Food and Wine Research Day (7 November 2014)
Liccioli, T., Grbin, P. and V. Jiranek.
Do research!! No, we make robots. *Oral presentation.*

Oeno 2015: 10th International Symposium of Oenology of Bordeaux, Bordeaux, France (29 June - 1 July 2015)
Jiranek, V., Liccioli, T., Betteridge, A., Sumby, K., Walker, M., Gardner, J. and J. Sundstrom.
Generation and characterisation of improved wine microbes. *Oral presentation.*

Australasian conference on Yeast: Products and Discovery, Adelaide (2 - 4 December 2015)
Walker, M. E., Watson, T., Long, D., Lang, T., Gardner, J. M., Zhang, J. and V. Jiranek
Can further improvements be made in oenological traits of wine yeast? *Oral presentation.*

Peter, J. J., Watson, T., Walker, M., Gardner, J. and V. Jiranek
Screening for wine yeast mutants, whereby gene deletion results in faster fermentation under limited nitrogen conditions. *Oral presentation.*

15th International Wine Conference (Sinkiang, China, 12-15 August 2015)
Watson, T. Microbial modulation for increasing wine quality. *Oral presentation.*

ACPFG Waite Campus Seminar (20 Aug 2015):
Watson, T. Microbial modulation for increasing wine quality. *Invited seminar.*

16th Australian Wine Industry Technical Conference (Adelaide, 24 - 28 July 2016)
Walker, M. E., Liccioli Watson, T., Grbin, P. and V. Jiranek
Directed evolution: limiting problematic fermentation through microbial improvement. *Poster presentation.*

Sumby, K. M., Grbin, P. R. and V. Jiranek
More efficient malolactic fermentation by directed evolution of *Lactobacillus plantarum*. *Poster presentation.*

Peter, J., Watson, T., Walker, M., Gardner, J. and V. Jiranek
Identification of wine yeast genes influencing fermentation duration under limited nitrogen

conditions. *Poster presentation.*

Jiang, J., Betteridge, A., Sumby, K., Sundstrom, J., Grbin, P. and V. Jiranek
Oenococcus oeni: can we improve its malolactic fermentation performance? *Poster presentation.*

Schlank, R., Jeffery, D., Sumby, C. and T. Watson
Monitoring wine fermentation progress through metabolite analysis using low-field proton NMR. *Poster presentation.*

Valentine, G., Walker, M., Gardner, J. and V. Jiranek
Indicator genes for the genetic response to transient temperature changes in wine yeast. *Poster presentation.*

Jiranek, V.
Applied science meets wine microbiology: ecology, directed evolution and winemaking I*nvited presentation - Workshop 38*.

14th International Congress on Yeasts. Awaji Island, Japan (11-15 September 2016)
Huang, M., Gardner, R., Fedrizzi, B., Walker, M. and V. Jiranek
Identification of yeast genes responsible for the production of hydrogen sulfide (from cysteine) and volatile thiols in wine. *Oral and Poster presentations*

CSIRO Waite Campus Seminar (8 Nov 2016):
Walker, M. E.
Generating 'Fit for purpose' yeast for today's Wine Industry. *Invited seminar.*

International Conference on Yeast Genetics and Molecular Biology (ICYGMB) Prague, Czech Republic (27 Aug-1 Sept 2017)
Bartle, L., Sundstrom, J., Sumby, K., Mitchell, J., Jiranek, V.
Compatibility of industrial *Saccharomyces uvarum* and *Saccharomyces cerevisiae* with *Oenococcus oeni* during synthetic red juice fermentation. *Poster presentation.*

12th International Symposium on Lactic Acid Bacteria, Egmond aan Zee, The Netherlands (27-31 Aug 2017)
Jiang, J., Sumby, K.M., Sundstrom, J.F., Grbin, P.R., Jiranek, V.
Directed evolution of *Oenococcus oeni* for enhanced malolactic fermentation. *Poster presentation.*

Magazines

Walker, M.E., Sundstrom, J.F., Liccioli, T., Dillon, S., Long, D., Nguyen, T., Tek, E-L. and J. Zhang (2014) Adelaide researchers head 'over the ditch' for Australisian yeast meeting. Grapegrower and Winemaker March 2014 - Issue 603. www.winebiz.com.au

Walker, M.E., Gardner, J., Liccioli, T. and V. Jiranek (2014) Tackling microbial failure in problematic wine fermentations using a holistic approach. Grapegrower and Winemaker April 2014 - Issue 604. www.winebiz.com.au

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(2015) A personal touch for wine microbiology. Tecan Journal 3/2015 22-23

Watson, T.L. and Jiranek, V. (2017)
Carbon parts make wine research 60% more economical and 67% faster. Case Study, Carbon 3D. 23 Aug 2017. https://www.carbon3d.com/stories/tthandadelaide/

Refereed journal articles

Copies are included with this report.

Sumby, K., Grbin, P. and V. Jiranek (2014) Implications of new research and technologies for malolactic fermentation in wine. Appl Micro Biotech 98(19):8111-8132 [Impact factor 3.337]

Betteridge A., Grbin P. and V. Jiranek (2015) Improving *Oenococcus oeni* to overcome challenges of wine malolactic fermentation. Trends Biotech 33(9):547-53. doi: 10.1016/j.tibtech.2015.06.008. Epub 2015 Jul 18. [Impact factor 11.96]

Betteridge, A. L., Sumby, K. M., Sundstrom, J. F., Grbin, P. R. and Jiranek, V. (2017)
Application of directed evolution to develop ethanol tolerant *Oenococcus oeni* for more efficient malolactic fermentation. Appl Micro Biotech 102(2), 921-932. doi.org/10.1007/s00253-017-8593-x [Impact factor 3.376]

Huang, C-W., Walker, M.E., Fedrizzi, B., Roncoroni, M., Gardner, R.C. and V. Jiranek (2016) The yeast *TUM1* affects production of hydrogen sulfide from cysteine treatment during fermentation. FEMS Yeast Res 16(8). doi: 10.1093/femsyr/fow100. Epub 2016 Dec 2. [Impact factor 2.479]

Huang, C-W., Walker, M., Fedrizzi, B., Roncoroni, M., Gardner, R. and Jiranek, V. (2017)
Yeast genes involved in regulating cysteine uptake affect production of hydrogen sulfide from cysteine during fermentation. FEMS Yeast Res 17(5): doi: 10.1093/femsyr/fox046.

Huang, C-W., Walker, M., Fedrizzi, B., Roncoroni, M., Gardner, R. and Jiranek, V. (2017)
Hydrogen sulfide and its roles in *Saccharomyces cerevisiae* in a winemaking context. FEMS Yeast Res. 17(6): doi: 10.1093/femsyr/fox058.

Jiang, J., Sumby, K.M., Sundstrom, J.F., Grbin, P.R., and V. Jiranek (2018) Directed evolution of *Oenococcus oeni* strains for more efficient malolactic fermentation in a multi-stressor wine environment. Food Microbiology 73,150-159. doi.org/10.1016/j.fm.2018.01.005.

Nguyen, T. D., Walker, M. E., Gardner, J.M. and Jiranek. V. (2018) Appropriate vacuolar acidification in *Saccharomyces cerevisiae* is associated with efficient high sugar fermentation. Food Micro 70, 262-268 https://doi.org/10.1016/j.fm.2017.09.021 [Impact factor 3.759]

Peter, J.J., Watson, T., Walker, M.E., Gardner, J.M., Lang, T.A., Borneman, A., Forgan, A., Tran, T. Chambers, P. and V. Jiranek. Use of wine yeast deletion collection reveals genes that

influence fermentation performance under low nitrogen conditions. FEMS Yeast Research (FEMSYR-17-07-0122; submitted 26 Jul 2017)

Zhang, J., Astorga, M.A., Gardner, J.M., Walker, M.E., Grbin, P.R. and V. Jiranek. (2018) Disruption of the cell wall integrity gene *ECM33* results in improved fermentation performance by wine yeast. Metabolic Engineering (in press).

Honours Thesis

Schlank, R. (2016) Monitoring Wine Fermentation Progress. University of Adelaide, Hons thesis.

Lin, M. (2017) Characterisation of yeasts isolated from the field. University of Adelaide, Hons thesis.

McGilton, J. (2017) Growth and characterisation of yeast isolates from *Eucalyptus gunnii*. University of Adelaide, Hons thesis.

Masters Thesis

Wang, Q. (2016) Screening of *Oenococcus oeni* and *Lactobacillus hilgardii* isolates for malolactic activity in both sequential and co-inoculated fermentations. University of Adelaide, Masters of Viticulture and Oenology thesis.

Mi, X. (2016) Selecting new malolactic fermentation starter cultures. University of Adelaide, Masters of Viticulture and Oenology thesis.

Liu, Y. (2017) Developing new starter cultures of lactic acid bacteria for co-inoculation and sequential malolactic fermentation in high alcohol wine. University of Adelaide, Masters of Viticulture and Oenology thesis.

PhD Thesis

Zhang, J. (2014) Investigation and characterisation of highly nitrogen efficient yeast. University of Adelaide, PhD thesis. Jan 2014)
https://digital.library.adelaide.edu.au/dspace/bitstream/2440/103973/1/01front.pdf

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Betteridge, A.L. (2015) Enhanced wine-making efficiency through fool-proof malolactic fermentation: Evolution of superior lactic acid bacteria. University of Adelaide, PhD thesis. Jan 2015)
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Haggerty, J. (2016) Characterisation of the wine meta-metabolome: linking sensory attributes to yeast genotype University of Adelaide, PhD thesis.

Peter, J.J. (2017) Identification of yeast genes enabling efficient oenological fermentation under nitrogen-limited conditions. University of Adelaide, PhD thesis. Academically qualified for award of PhD degree 04-Oct-2017.

Jiang, J. (2017) Use of directed evolution to generate multiple-stress tolerant *Oenococcus oeni* for enhanced malolactic fermentation. Academically qualified for award of PhD degree 28 Nov 2017.

Refereed journal articles (under review)

Copies will be made available to Wine Australia, following publication.

Valentine, G.D.S., Walker, M.E., Gardner, J.M., Schmid, F., Jiranek, V. Brief temperature extremes during wine fermentation: effects on yeast viability and fermentation progress (submitted to Australian Journal of Grape and Wine Research).

Referred Journal Articles (In preparation)

Peter, J.J., Watson, T.L., Walker, M.E., Gardner J.M., Jiranek V. Investigation of the effect of an *MFA2* deletion on fermentation duration.

Watson, T.L., Chambers, P.J., Tondini, F.A., Jiranek, V. Isolation of an improved fructophilic yeast strain (final check before submission).

11.0 Outcomes and Conclusion

Reliable fermentation is essential for process efficiency and to prevent spoilage in the final product. This project was undertaken to generate 'fit-for-purpose' yeast and lactic acid bacterial strains better suited to problematic juice and wine conditions.

The primary aim of this project was to use a Directed Evolution (DE) approach with selected wine microbes (pioneered in 2002 via UA01/04 and GWR-Ph0901) to generate superior (non-recombinant) yeast and bacteria in batch or continuous culture with key fermentation stressors. The optimisation targets highlighted by Wine Australia (resistance to stresses/inhibitors or enhanced utilisation of selected juice/wine constituents) were supremely suited to the DE approach - ie extended cultivation of microbes under stressful conditions to allow superior strains to dominate the culture.

The ambitious nature of this project and the extensive time needed to obtain improved strains meant that there were often delays throughout the project. However on the whole performance targets were met. We have taken a 'guided' approach to the design of the DE experiments, incorporating findings from relevant academic literature as well as our own unpublished experiences. DE experiments in yeast and LAB were undertaken simultaneously in several stress conditions using sequential batch and continuous culture approaches, in order to generate evolved strains with improved phenotypes within the time frame of the project. This project succeeded in generating superior strains of both yeast and LAB (in terms of fermentation and MLF performance) via directed evolution (DE). By the end of the project DE of *Saccharomyces cerevisiae* and lactic acid bacteria (LAB), along with evaluation of un-inoculated isolates of LAB generated 5 improved *Saccharomyces cerevisiae* (yeast) strains, 8 LAB strains.

In addition to the above we have further defined the yeast 'Fermentome' (i.e. a database of fermentation-essential genes) by using AWRI's partial wine yeast deletion library and comparing it to the *laboratory* yeast Fermentome (from UA1101). This enabled us to identify wine yeast specific fermentation-essential genes/processes. Genome sequencing and assembly of selected strains was also undertaken to determine the genetic basis of isolates with improved phenotypes and to support IP protection. The final output (**Output 4A**) was to construct recombinant strains to confirm the importance of any gene modifications (SNPs) or gene deletions identified during sequencing of the evolved yeast in comparison to their original parents. This output was not achieved because of the constant delay with regards to the isolation of the evolved yeast strains with improved fermentation performance. Instead, we report on 'proof-of-concept' strains from three PhD projects, which are closely related to UA1302 in terms of research focus, and the contribution towards supervision.

This project has the potential to generate many benefits for winemakers primarily by decreasing the risk of problem fermentations. By decreasing this risk, profit margins will improve, not only via preservation of the annual loss in potential value or product, but also in significant production costs associated with management of the problematic fermentations. More reliable (shorter) fermentations will require less inputs (energy, additives, labour, etc.)

and result in less wastage with the salvage of poorer quality fruit, thereby reducing overall environmental impact. Greater profitability and sustainability will assist the wine industry maintain its critical employment role, particularly in regional areas. This will be achieved by enhancing profitability and expanding markets domestically (through new products), as well as helping provide sought after, ideally high value wine to emergent overseas markets such as India and China.

12.0 Recommendations

Many of the recommendations from this project will be taken up by the bilateral agreement between Wine Australia and The University of Adelaide. Wine microbiology has a large effect on both wine processing and composition and we are well placed to use our knowledge base to develop new and novel uses for wine microbes. Future work should include extending work on improving the wine microbial tool kit available for fermentation. This will be done by providing the industry with robust yeast and LAB specifically targeted to address industry requirements. We recommend development of methods to produce and/or select enhanced yeast starter cultures with novel attributes and greater robustness along with novel yeast attributes such as the ability to enhance wine flavour, colour, and mouthfeel. Successful DE could be extended to include new attributes and novel strains from environmental samples.

Future work should also investigate inoculation with selected (or enhanced) strains of non-*Saccharomyces* yeasts. This could include identification of more robust non-*Saccharomyces* (able to ferment alone) and improved knowledge of their activities during winemaking. Additionally, further comparison of the genome sequences of parental and evolved yeast to the 'yeast fermentome' will enable better understanding of the genes/processes relevant to fermentation, which has potential application in future DE and breeding strategies as well as fermentation management.

Not all these recommendations will be able to be accommodated into the bilateral agreement and as such we also intended to continue to attract research students to work on relevant and related topics.

Appendix 1: Communication

Communication and dissemination of findings from this research have been achieved through; publications, conference, workshop, seminar presentations, annual meetings (with AWRI and Wine Australia) and discussions with various yeast and bacteria manufacturers (including Lallemand and Laffort). This was for Outputs 1C, 2C, 2F, 3F, 4B, 4D and 4E and is detailed in sections 8.0, 9.0, and 10.0. 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

IP of commercial significance is linked to strains generated through the project (see Output 4B). We aim to seek the commercialisation of such IP via our established links with key suppliers of yeast and bacterial cultures. IP generated by the project will be co-owned by the University of Adelaide and Wine Australia in proportions determined. Wine Australia will share in intellectual property and royalties, according to the contribution of each party to the creation of the project intellectual property, including financial contributions and un-reimbursed in-kind contributions of background intellectual property, expertise, materials, equipment, infrastructure and/or labour to the project. Management of commercialisation and IP licensing will be undertaken by the University of Adelaide division 'University of Adelaide Enterprise' (replacing the former Adelaide Research and Innovation Pty Ltd as from July 2016).

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Appendix 4: Staff

The following staff contributed to this project:

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Appendix 5: Supplementary Data

1. Micro-fermentations:

Synthetic starter medium

Yeast extract	1 g
Glucose	20 g
KH_2PO_4	1 g
NH_4Cl	1 g
$CaCl_2.2H_20$	0.5 g
NaCl	0.5 g
$MgSO_4.7H_2O$	0.73 g
DL Malic Acid	3 g

pH to 6.0 with 10 M NaOH, and make up to 1 L. Filter sterilise and store at ambient temperature.

2% malic acid medium pH 3.5 (per litre):

Yeast extract	1 g
Glucose	3 g
KH_2PO_4	1 g
NH_4Cl	1 g
$CaCl_2.2H_20$	0.5 g
NaCl	0.5 g
$MgSO_4.7H_2O$	0.73 g
DL Malic Acid	20 g

pH to 3.5 with 10 M NaOH, and make up to 1 L. Filter sterilise and store at ambient temperature.

2% malic acid medium pH 6.0 (per litre):

Yeast extract	1 g
Glucose	3 g
KH_2PO_4	1 g
NH_4Cl	1 g
$CaCl_2.2H_20$	0.5 g
NaCl	0.5 g
$MgSO_4.7H_2O$	0.73 g
DL Malic Acid	20 g

pH to 6.0 with 10 M NaOH, and make up to 1 L. Filter sterilise and store at ambient temperature.

2. Growth assays: Malic Acid Indicator Plates

GMIA and MIA agar was a modification of the recipe described by Volschenk et al (2004). Washing of agar: ~23g bacto-agar was resuspended by swirling in 500 mL milliQ water, allowed to settle for 20 min, before the water was decanted. The process was repeated once. Afterwards the agar was resuspended in milliQ water as a 2x stock, and autoclaved. The remainder of the components were combined in solution as 2x stock, adjusted to pH 3.3 prior to addition of bromocresol green and filter sterilisation. The stock was added to molten 2x agar, mixed and poured into plates.

Glucose Malic Acid Indicator Agar or GMIA: 0.17% YNB, 0.5% ammonium sulfate, 1% L-malic acid, 5% glucose, 200 mg L-1 bromocresol green, pH 3.3, 2% bactoagar (washed 2x in sterile milliQ water).

Malic Acid Indicator Agar or MIA: 0.17% YNB, 0.5% ammonium sulfate, 1% L-malic acid, 200 mg L-1 bromocresol green, pH 3.3, 2% bactoagar (washed 2x in sterile deioinised water).

3. Yeast DE experiments:
Stress medium was based on CDGJM (Walker et al 2014) and varied in composition (see below). Medium was made within 24 hours of use, filter sterilised (0.22 μM membrane) and kept at room temperature.

Starter Medium 1: 450 mg L^{-1} FAN as ammonium chloride and FAA, 50 g L^{-1} glucose, 50 g L^{-1} fructose, 3 g L^{-1} DL-malic acid, pH 3.5

Starter Medium 2: YPD and Starter Medium 1 mixed together in equal volumes.

Stress Medium A*: 300 mg L^{-1} FAN as ammonium chloride and FAA, 160 g L^{-1} glucose, 160 g L^{-1} fructose, 5 g L^{-1} L-malic acid, pH 2.8 (with L-tartaric acid)

Stress Medium A: 350 mg L^{-1} FAN as ammonium chloride and FAA, 130 g L^{-1} glucose, 130 g L^{-1} fructose, 5 g L^{-1} L-malic acid, pH 2.8 (with L-tartaric acid)

Stress Medium B: 350 mg L^{-1} FAN as ammonium chloride and FAA, 100 g L^{-1} glucose, 100 g L^{-1} fructose, 5 g L^{-1} L-malic acid, 5% (v/v) ethanol, pH 3.5

Stress Medium A + MCFA: 350 mg L^{-1} FAN as ammonium chloride and FAA, 130 g L^{-1} glucose, 130 g L^{-1} fructose, 5 g L^{-1} L-malic acid, pH 2.8 (with L-tartaric acid), 6 mg mL^{-1} MCFA

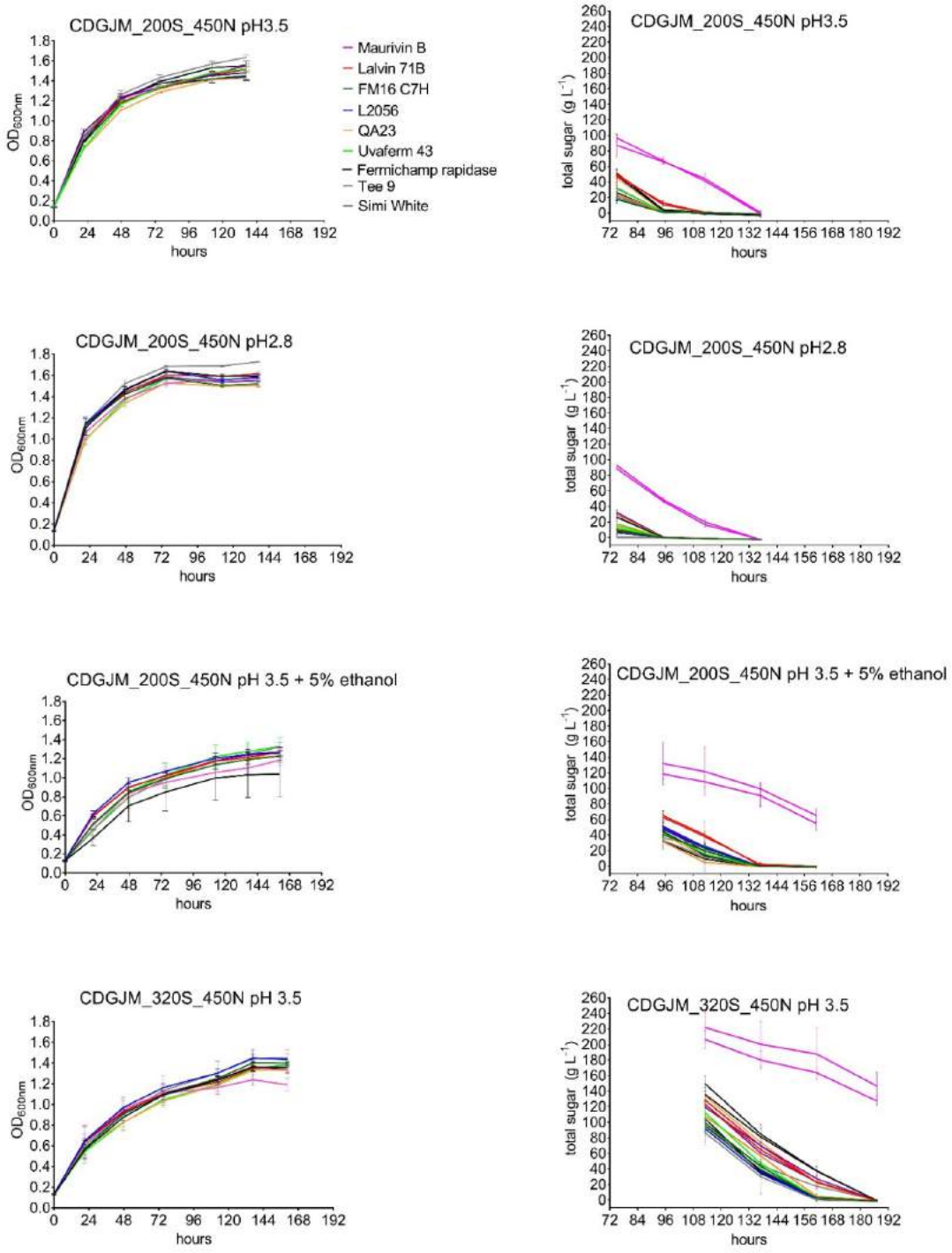
Stress Medium B + MCFA: 350 mg L^{-1} FAN as ammonium chloride and FAA, 100 g L^{-1} glucose, 100 g L^{-1} fructose, 5 g L^{-1} L-malic acid, 5% (v/v) ethanol, pH 3.5, 6 mg mL^{-1} MCFA

Stress Medium C: 350 mg L^{-1} FAN as ammonium chloride and FAA, 105 g L^{-1} glucose, 105 g L^{-1} fructose, 3 g L^{-1} L-malic acid, 6% (v/v) ethanol, pH 3.1, 3 mg mL^{-1} MCFA

Medium chain fatty acids (MCFA):
Octanoic acid and decanoic acid (10 mg mL^{-1}) stock solutions were freshly prepared using 100% ethanol. The MCFA were added together at 3 to 6 mg mL^{-1} to individual fermentation flasks containing 100 mL medium

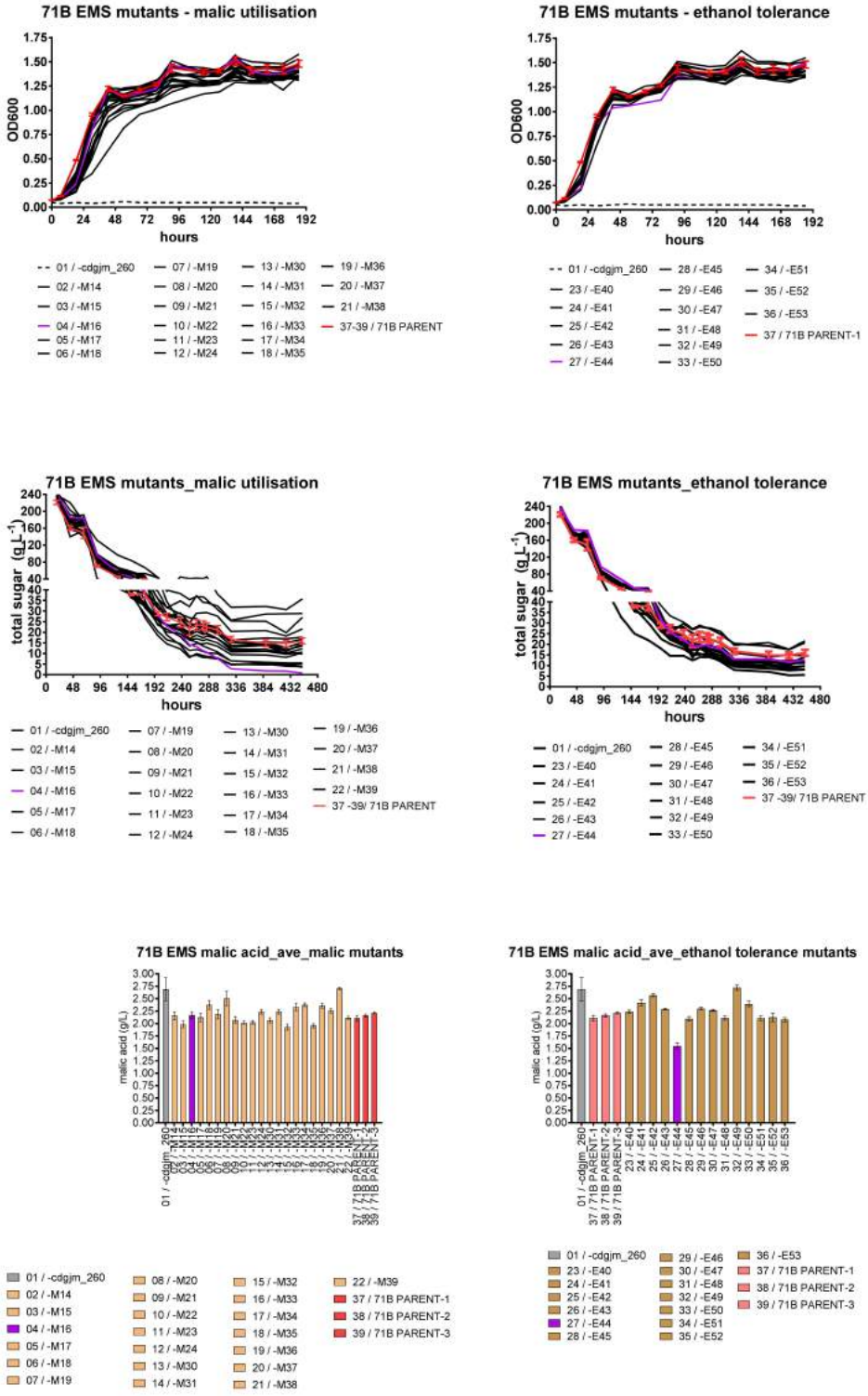
CDGJM for batch fermentations in bioreactor:
CDGJM composition was essentially that of Henscke and Jiranek (1993) except viatmins and trace minerals were at half concentration; 150 mg L^{-1} FAN as ammonium chloride and FAA, 10 g L^{-1} glucose, 30 g L^{-1} fructose, 3 g L^{-1} DL-malic acid, ethanol (8% to 12

(v/v)), pH 3.5.



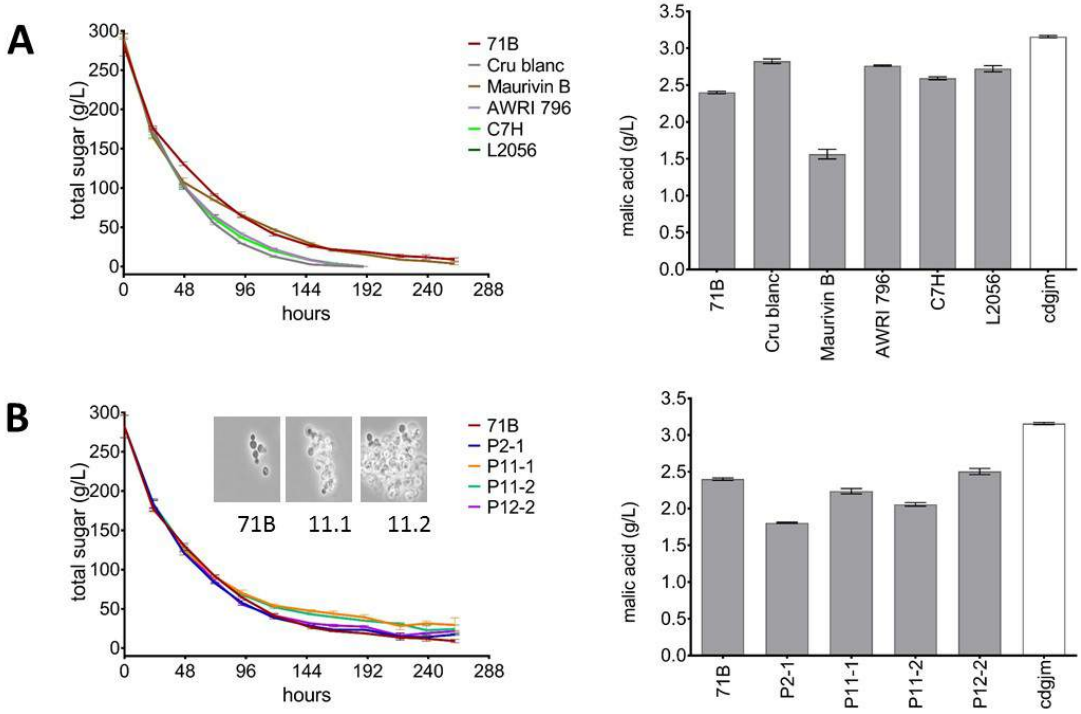
Supplementary Figure 1.1. Sugar consumption by nine wine yeast in chemically defined grape juice medium (CDGJM) with different stress factors.

Nine wine yeast (2 clonal isolates of each) were evaluated in 0.2 mL micro-fermentations. Fermentation progress and growth was monitored as residual sugar in clarified fermentation sample and optical density (OD_{600}) of culture respectively. CDGJM was modified to include either 200 g L^{-1} sugar (200S) or 320 g L^{-1} sugar (320S), and/or pH3.5 and 2.8. Nitrogen content was 450 mg FAN L^{-1} (450N). Ethanol was added at 5% (v/v). Values represent means of 4 replicates ± SD.



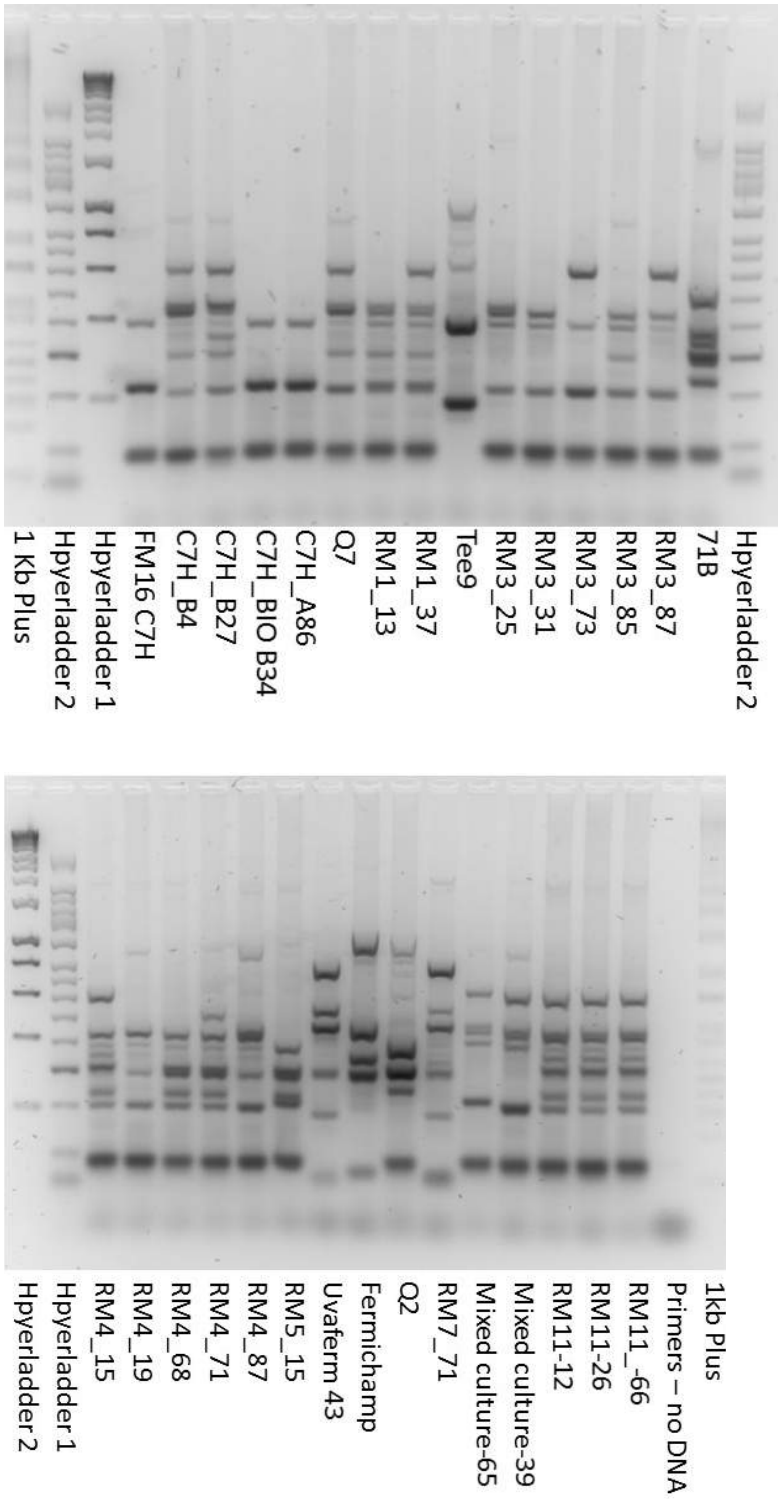
Supplementary Figure 1.2. Evaluation of EMS mutants of 71B selected for malic acid utilisation and ethanol tolerance.

EMS mutants of 71B were evaluated as single 100 mL fermentations in CDGJM (260 g L^{-1} sugar, 350 mg FAN L^{-1}, 3 g L^{-1} malic acid, pH 3.5) at 22°C. Ferments were inoculated at 1% from starter cultures (Starter Medium 2). Fermentation progress and growth was monitored as residual sugar in clarified fermentation sample and optical density (OD_{600}) of culture respectively. Malic acid utilisation was determined by MDH/GOT enzyme assay. (n = 4).

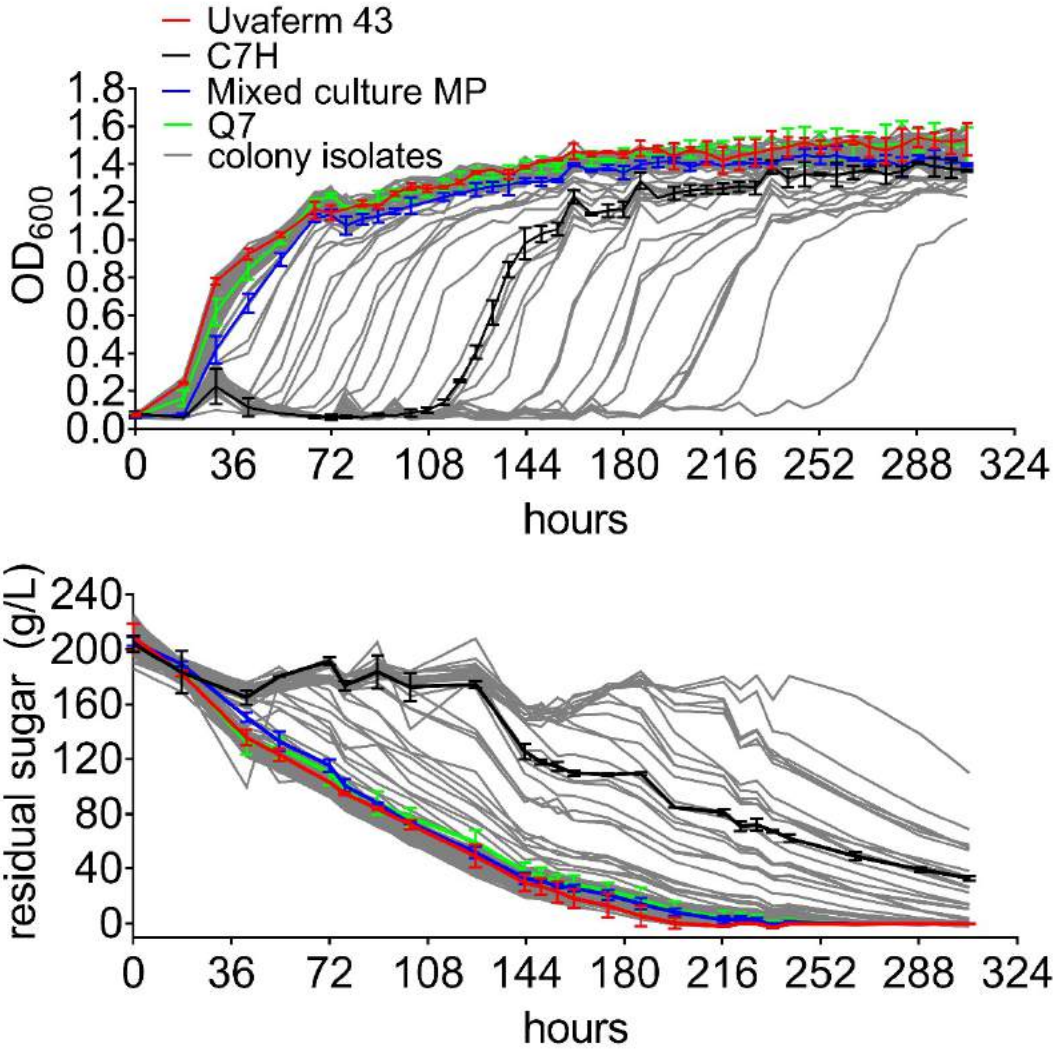


Supplementary Figure 1.3. Evaluation of commercial wine strains and EMS mutants of 71B for fermentation performance and malic acid utilisation.

A: Commercial wine strains and B: EMS mutants of 71B were evaluated as triplicate 100 mL fermentations in CDGJM (260 g L^{-1} sugar, 350 mg FAN L^{-1}, 3 g L^{-1} malic acid, pH 3.5) at 28°C. Ferments were inoculated at 5 x 10^6 viable cells/mL from starter cultures (Starter Medium 2). Fermentation progress and growth was monitored as residual sugar in clarified fermentation sample and optical density (OD600) of culture respectively. Malic acid utilisation was determined by MDH/GOT enzyme assay. Values are average of 3 replicates ± SD.



Supplementary Figure 1.4: Strain identification of evolved yeast strains using Delta PCR.
Strains from Table 2.5 were characterised by Delta PCR amplification using δ2 and δ12 primers.



Supplementary Figure 2.1. Fermentation performance of 84 colony isolates of the mixed culture DE experiment at ~130 generations.

Eighty-four colony isolates were fermented as single 100 mL cultures. The reference strains Uvaferm 43, FM 16 C7H, Q7 and the mixed population were fermented in triplicate. Fermentations were conducted at 22°C in sterile Riesling juice (Clare Valley 2016 vintage) with 4.5% ethanol.

Supplementary Table 1.1. Evaluation of growth of 12 wine yeast in the presence of a range of stressors.

FM16 C7H	10+E07	10+E06	10+E05	10+E04	10+E03
YPD	5	5	5	5	5
300mM concanamycin A	4	4	3	2	
400mM concanamycin A	3	2			
500mM concanamycin A	2				
12.5 mg KMS	5	5	5	5	5
25 mg KMS					
8% Ethanol	4	4	4	2	2
10% Ethanol	3	2	1		
12% Ethanol	1				
YPD 40◦C	4	4	4	4	4
YPD 30◦C	5	5	5	5	5
YPD 17◦C	5	5	5	5	5
YPD 8◦C	3	0.5			
GMIA	4	4	4	4	4
MIA	2	1			

L2056	10+E07	10+E06	10+E05	10+E04	10+E03
YPD	5	5	5	5	5
300mM concanamycin A	3	1	1	0.5	
400mM concanamycin A	3	1	1	0.5	
500mM concanamycin A	2	0.5	0.5		
12.5 mg KMS	5	5	5	5	5
25 mg KMS	5	5	5	5	5
8% Ethanol	4	4	2	1	0.5
10% Ethanol	3	2			
12% Ethanol	0.5				
YPD 40◦C	5	5	5	5	5
YPD 30◦C	5	5	5	5	5
YPD 17◦C	5	5	5	5	5
YPD 8◦C	3	0.5			
GMIA	4	4	4		
MIA	2	2	1	0.5	

Maurivin B	10+E07	10+E06	10+E05	10+E04	10+E03
YPD	5	5	5	5	5
300mM concanamycin A	2	0.5			
400mM concanamycin A	1				
500mM concanamycin A	1				
12.5 mg KMS	0.5				
25 mg KMS					
8% Ethanol	4	4	2	1	
10% Ethanol	2	1			
12% Ethanol	1				
YPD 40◦C	4	4	4	4	4
YPD 30◦C	5	5	5	5	5
YPD 17◦C	5	5	5	5	5
YPD 8◦C	3	0.5			
GMIA	4	4	4	4	4
MIA	2	1			

Simi White	10+E07	10+E06	10+E05	10+E04	10+E03
YPD	5	5	5	5	5
300mM concanamycin A	4	2	2	1	1
400mM concanamycin A	1				
500mM concanamycin A	1				
12.5 mg KMS	3	3	2		
25 mg KMS					
8% Ethanol	4	4	2	1	0.5
10% Ethanol	3	2			
12% Ethanol	0.5				
YPD 40◦C	4	2	0.5		
YPD 30◦C	5	5	5	5	5
YPD 17◦C	5	5	5	5	5
YPD 8◦C	2	0.5			
GMIA	4	4	4	4	4
MIA	2	1			

Lalvin 71B	10+E07	10+E06	10+E05	10+E04	10+E03
YPD	5	5	5	5	5
300mM concanamycin A	4	4	4	4	4
400mM concanamycin A	4	3	1	0.5	
500mM concanamycin A	4	3	0.5		
12.5 mg KMS	0.5				
25 mg KMS					
8% Ethanol	4	4	2	2	1
10% Ethanol	2	1			
12% Ethanol	1				
YPD 40◦C	5	5	5	5	5
YPD 30◦C	5	5	5	5	5
YPD 17◦C	5	5	5	5	5
YPD 8◦C	3	1			
GMIA	4	4	4	4	4
MIA	2	1			

QA23	10+E07	10+E06	10+E05	10+E04	10+E03
YPD	5	5	5	5	5
300mM concanamycin A	4	4	4	4	4
400mM concanamycin A	4	0.5			
500mM concanamycin A	4	0.5			
12.5 mg KMS	5	5	5	5	5
25 mg KMS					
8% Ethanol	4	4	4	2	2
10% Ethanol	3	2	1	0.5	
12% Ethanol	2				
YPD 40◦C	4	4	4	4	4
YPD 30◦C	5	5	5	5	5
YPD 17◦C	4	4	4	2	2
YPD 8◦C	2	0.5			
GMIA	4	4	4	4	4
MIA	2	1			

Uvaferm 43	10+E07	10+E06	10+E05	10+E04	10+E03
YPD	5	5	5	5	5
300mM concanamycin A	4	4	3	2	
400mM concanamycin A	0.5				
500mM concanamycin A	0.5				
12.5 mg KMS	5	5	5	5	5
25 mg KMS					
8% Ethanol	4	4	4	2	2
10% Ethanol	3	2	1	0.5	
12% Ethanol	2	0.5			
YPD 40◦C					
YPD 30◦C	5	5	5	5	5
YPD 17◦C	4	4	4	1	1
YPD 8◦C	0.5				
GMIA	4	4	4	4	4
MIA	2	1			

Q7	10+E07	10+E06	10+E05	10+E04	10+E03
YPD	5	5	5	5	5
300mM concanamycin A	4	2	2	1	
400mM concanamycin A	3	0.5			
500mM concanamycin A	3	0.5			
12.5 mg KMS	4	4	4	4	4
25 mg KMS	4	4	2	1	
8% Ethanol	4	4	2	1	
10% Ethanol	3	2			
12% Ethanol	0.5				
YPD 40◦C	3	3	3	3	3
YPD 30◦C	5	5	5	5	5
YPD 17◦C	4	3	0.5		
YPD 8◦C	0.5				
GMIA	4	4	4		
MIA	0.5				

Fermichamp rapidse	10+E07	10+E06	10+E05	10+E04	10+E03
YPD	5	5	5	5	5
300mM concanamycin A	4	1	0.5		
400mM concanamycin A	0.5				
500mM concanamycin A	0.5				
12.5 mg KMS	3	3	0.5	0.5	
25 mg KMS					
8% Ethanol	4	4	4	2	2
10% Ethanol	3	2	1	0.5	
12% Ethanol	2				
YPD 40◦C					
YPD 30◦C	5	5	5	5	5
YPD 17◦C	4	4	4	1	1
YPD 8◦C	2	0.5			
GMIA	4	4	4	4	4
MIA	2	1			

Q2	10+E07	10+E06	10+E05	10+E04	10+E03
YPD	5	5	5	5	5
300mM concanamycin A	4	4	4	4	4
400mM concanamycin A	3	0.5			
500mM concanamycin A	1	0.5			
12.5 mg KMS	4	4	4	4	4
25 mg KMS	3	3	3		
8% Ethanol	4	4	2	1	
10% Ethanol	0.5	0.5			
12% Ethanol	0.5				
YPD 40◦C	3	3	3	3	3
YPD 30◦C	5	5	5	5	5
YPD 17◦C	4	4	4	4	
YPD 8◦C	2	0.5			
GMIA	4	4	3	3	
MIA	2	1			

Tee9	10+E07	10+E06	10+E05	10+E04	10+E03
YPD	5	5	5	5	5
300mM concanamycin A	4	1	0.5	0.5	
400mM concanamycin A	2	0.5			
500mM concanamycin A	2	0.5			
12.5 mg KMS	5	5	5	5	5
25 mg KMS	5	1	0.5	0.5	
8% Ethanol	4	4	2	2	1
10% Ethanol	2	1			
12% Ethanol	1				
YPD 40◦C	4	4	4	4	4
YPD 30◦C	5	5	5	5	5
YPD 17◦C	4	4	4	4	
YPD 8◦C	3	0.5			
GMIA	4	4	4	4	4
MIA	2	1			

Cross Evo	10+E07	10+E06	10+E05	10+E04	10+E03
YPD	5	5	5	5	5
300mM concanamycin A	4	4	4	4	
400mM concanamycin A	3	0.5			
500mM concanamycin A	1				
12.5 mg KMS					
25 mg KMS					
8% Ethanol	4	4	2	1	
10% Ethanol	0.5	0.5			
12% Ethanol	0.5				
YPD 40◦C	4	4	4	4	4
YPD 30◦C	5	5	5	5	5
YPD 17◦C	5	5	5	5	5
YPD 8◦C	2	0.5			
GMIA	4	4	4	4	
MIA	2	1	1		

Twelve strains were evaluated on different selective medium as solid medium. Yeast cultures (2.5 x 10^6 cells mL^{-1}) were grown to 1 x10^7 cells mL^{-1} and serially diluted from 1 x 10^7 to 1 x 10^1 cells mL^{-1}in 10 uL, spotted onto agar plates and incubated 3 days at 20°C (except for temperature treatments). Growth was qualitatively scored from 5 (very good growth) to 0.5 (minimal growth). Malic utilisation as observed by green colony colour (from increase in pH - yellow to green). Black depicts no growth

Supplementary Table 1.2 DE strategies for yeast strain improvement.

Total generations	DE stage 1										
Batch DE	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**A**
FM16 C7H	6	6	12								
L2056	6	12	18								
Lalvin 71B	6	12	18								
Q2	6	12	18								
FM16 C7H_A				18.3	28.5	36.6	45.5	53.2	61.8	69.8	69.8
L2056_A				24	34.4	42.7	51.7	59.7	66.9	74.9	74.9
Lalvin 71B_A (REINOCULATED FROM 71B_B)						52.2	60.3	68.1			68.1
Q2_A			18								18
BIO C7H_A (90 generations from batch chemostat)											
FM16 C7H_B				18.2	28.3	38.4	48.2	56.1	64.4	72.4	72.4
L2056_B				23.3	33.1	40.6	49	57.1	65.4	73.4	73.4
Lalvin 71B_B (71B_B* from batch 6)				23.9	26.5	32	40.1				40.1
Q2_B			18								18
Lalvin 71B_B (from A culture)						52.2	60.3	68.1			68.1
BIO C7H_B (90 generations from batch chemostat)											
Q1 (EMS isolate QA23 1.9M NaCl)											
E3 (C7H x Q7 hybrid)											
Mixed culture: C7H_A, C7H_B, C7HBIO_A, and C7HBIO_B, Q1, E3, E44											

Total generations	DE stage 2							DE stage 3					
Batch DE	**DE11 (A)**	**DE12**	**DE13**	**DE14**	**DE15**	**DE16**	**DE17**	**DE18**	**DE19**	**DE20**	**DE21**	**DE22**	**DE23**
FM16 C7H_A	78.7	86.1	92.1		96.5	102	108.5	115.0	119.8	125.7	130.7	134.4	138.2
L2056_A	82.3	90	96.2										
Lalvin 71B_A (REINOCULATED FROM 71B_B)	75.5	83.4	90.6										
Q2_A	26.9	34.5	42.4										
BIO C7H_A (90 generations from batch chemostat)		90	94.2	100.2	100.2								
FM16 C7H_B	80.7	88.7	96.6	102.6	107.5	112.7	118.5	128.4	134.1	139.3	145.0	151.4	158.3
L2056_B	81.4	89.1	94.5										
Lalvin 71B_B (71B_B* from batch 6)	48.8	56.8	63.6										
Q2_B	26.6	34.7	42.7										
Lalvin 71B_B (from A culture)	77	84.7											
BIO C7H_B (90 generations from batch chemostat)		90	95.4	101.4	107.9	114.1	120.5	126.5	131.7	137.6	140.7	144.8	149.2
Q1 (EMS isolate QA23 1.9M NaCl)					9.7	17.3	24.6	32.8	40.5	46.7	51.9	57.9	64.5
E3 (C7H x Q7 hybrid)					8.9	16.6	23.0	31.0	36.4	42.0	45.8	51.1	55.3
Mixed culture: C7H_A, C7H_B, C7HBIO_A, and C7HBIO_B, Q1, E3, E44					1.9	8.4	14.8	21.8	28.2	33.7	37.1	41.9	46.1

Incorporation of MCFA to medium	DE stage 2							DE stage 3					
	DE11 (A)	**DE12**	**DE13**	**DE14**	**DE15**	**DE16**	**DE17**	**DE18**	**DE19**	**DE20**	**DE21**	**DE22**	**DE23**
C7H_A + MCFA(6mg/L decanoic acid/12 mg/L octanoic acid)	78.7	84.4											
71B_A + MCFA (6 mg/L octanoic acid/6 mg/L decanoic acid)	75.5	83.7	88.5										
Q2_A + MCFA (6 mg/L octanoic acid/6 mg/L decanoic acid)	26.9	34.7											
I1 + MCFA (6 mg/L octanoic acid/6 mg/L decanoic acid)		7.2											
Q7 + MCFA (6 mg/L octanoic acid/6 mg/L decanoic acid)		7.4											
Tee9+ MCFA (6 mg/L octanoic acid/6 mg/L decanoic acid)		7.5											
71B_A (in CDGJM B)+ MCFA (6 mg/L octanoic acid/6 mg/L decanoic acid)	75.5	83.7	88.9										
E44_B+ MCFA (6 mg/L octanoic acid/6 mg/L decanoic acid)			0	5		14.6	14.6						

Mass sporulation/mating of strains then culture in medium	DE stage 2							DE stage 3					
	DE11 (A)	**DE12**	**DE13**	**DE14**	**DE15**	**DE16**	**DE17**	**DE18**	**DE19**	**DE20**	**DE21**	**DE22**	**DE23**
RM1: C7H X Q7								9.8	16.2	23.4	27.5	33.4	39.1
RM2: C7H X TEE9								3.5					
RM3: Q7 X TEE9								7.9	14.7	21.3	26.1	32.5	38.6
RM4: Q7 X 71B								9.5	15.8	22.3	28.6	35.1	41.2
RM5: C7H X 71B								9.8	16.3	21.4	26.6	31.8	37
RM6: TEE9 X 71B								2.8					
RM7: UVAFERM 43 X Q2								9.8	15.9	22	27.9	33.8	39.1
RM8: FERMICHAMP X Q2								9.8	16.4	23.3	28.4	29.8	29.8
RM9: UVAFERM 43 X MAURIVIN B								0.1					
RM10: FERMICHAMP X MAURIVIN B								9.3	17.7	24.9	31	37.1	43.3
RM11: C7H, Q7, TEE9, 71B, UVAFERM 43, Q2, FERMICHAMP, MAURIVIN								6.1		12.9	18.2	24.3	29.2

Yeast populations and DE senarios are shown, together with the fermentation batches and total (cumulative) generations. Samples of each batch are stored at -80°C in glycerol. Black shading represents batch cultures where strains are have not been introduced into the DE program or have been discontinued.

Supplementary Table 3.1. Tasting notes of Chardonnay fermented with evolved yeast strains

Strain	Aroma	Palate
Uvaferm	Freshly sawn pine/wood shop, lemon and cucumber, simple, slight sulfide?, flat	Good viscosity, falls short mid palate, preserved lemon, clean but lacks fruit complexity
Q7	Honey suckle, bees wax, preserved lemon, fresh, lively clean, aromatic	Lemon, slate, very fine, fresh and lively lacks mid palate weight and low viscosity
71B	Gin, alcoholic, cucumber, slightly wheaty	Sweet apricot mid palate weight, medium to long persistence, acidity falls away mid palate
Tee9	Butanol, over ripe apricots, sweet and sour sauce, most intense nose of all, glue	Apricot kernel, low viscosity, acid driven, lacks fruit weight and short persistence
FM16 C7H	Malt, savoury, slightly sulphidic	Quite simple, lemon, acid falls flat mid palate, medium persistence
RM3_25 (evolved isolate of Q7 x Tee 9)	Slightly sulfidic, wet cardboard	Similar to Tee9
*RM5_15 (evolved isolate of FM16 C7H x 71B)	Lavender, apricot nectar, almond oil	Similar to 71B, slightly more mid palate fruit weight, better extension of acidity toward back palate more complexity
RM4_71 (evolved isolate of Q7 x 71B)	Slightly brandied, fresh straw, apricot nectar	Oily, round, medium viscosity and med-long persistence
RM7_71 (evolved isolate of Uvaferm 43 x Q2)	Sulfide, old watermelon, wet grass, walnuts, elevated SO_2?	Sweet wet grass, rye, medium palate length and some alcoholic heat
*C7H_B4 (evolved isolate of FM16 C7H)	Honey suckle, lime, fresh and lifted, peach stone	Similar to FM16 C7H

Wines were tasted blind (without knowing their identity) provided by winemaker / wine researcher Dr J Gardner. *evolved isolate that was preferred over parent strains.

Supplementary Table 3.2. Sensory analysis of Chardonnay wines from Uvaferm 43 and newly evolved yeast RM5_15 or C7H_B4: Triangle Test

Taster	1	2	3	Yes = 1, No = 0
1	905	349	725	1
2	905	725	272	0
3	349	272	905	1
4	349	905	725	1
5	725	349	272	1
6	349	905	272	0
7	349	272	725	0
8	272	905	725	0
9	349	905	272	0
10	905	725	349	0
11	905	272	725	0
12	725	349	272	0
13	725	349	272	0
14	349	905	272	0
15	905	725	272	0
16	349	272	905	0
17	349	905	725	0
18	905	349	725	0
19	725	349	272	0
20	272	905	725	1
21	349	272	725	0
22	905	725	349	1
23	349	905	272	0
24	905	272	725	0
25	349	272	905	1
total				**7**

Wines tested: RM5_15 (905 & 725); Uvaferm 43 (349 & 272)

Taster	1	2	3	Yes = 1, No = 0
1	112	870	686	1
2	686	870	575	0
3	870	112	686	1
4	112	686	575	1
5	870	575	112	0
6	870	112	575	0
7	112	575	686	1
8	112	686	870	1
9	575	112	686	1
10	870	575	686	0
11	686	870	575	0
12	870	112	575	1
13	686	870	575	1
14	870	112	575	1
15	870	575	112	0
16	870	112	686	1
17	112	870	686	1
18	112	686	575	1
19	870	112	575	0
20	870	575	686	1
21	575	112	686	1
22	112	686	870	1
23	686	870	575	0
24	112	575	686	0
25	112	686	575	1
total				**16**

Wines tested: C7H_4 (112 & 686); Uvaferm 43 (870 & 575)

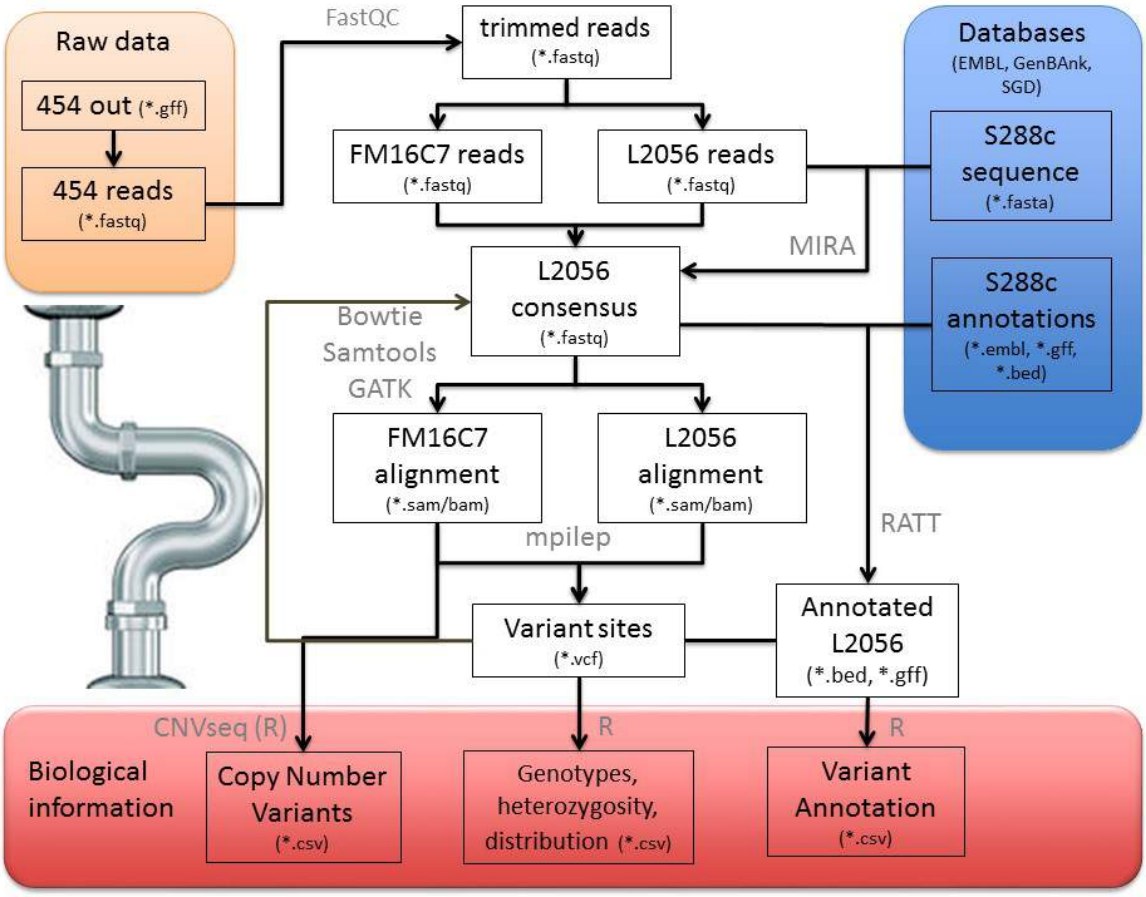
Supplementary Table 3.3. Sensory preference analysis of Chardonnay wines from Uvaferm 43 and evolved yeast RM5_15 or C7H_B4.

Taster	Preference 1		RM5_15 = 1 Uvaferm43 = 0
1	991	154	1
2	154	991	0
3	991	154	0
4	991	154	1
5	154	991	1
6	154	991	1
7	154	991	1
8	991	154	1
9	154	991	0
10	154	991	1
11	154	991	0
12	991	154	1
13	154	991	0
14	991	154	0
15	991	154	0
16	991	154	1
17	154	991	0
18	154	991	1
19	991	154	1
20	991	154	1
21	154	991	0
22	154	991	1
23	154	991	0
24	154	991	0
25	991	154	1
26	154	991	0
27	991	154	1
28	154	991	1
29	991	154	1
30	991	154	0
31	991	154	1
32	154	991	1
33	154	991	1
34	154	991	0
35	991	154	0
36	154	991	1
37	154	991	0
38	154	991	1
39	991	154	1
40	154	991	1
41	991	154	0
42	991	154	1
43	991	154	0
44	154	991	1
Total			26
Wines tested: RM5_15 = 154 or Uvaferm 43 = 991			

Preference 2		C7H_B4 = 1 Uvaferm 43 = 0
600	512	1
600	512	1
600	512	1
600	512	0
600	512	1
512	600	1
512	600	1
600	512	1
600	512	1
600	512	1
512	600	1
512	600	0
600	512	1
512	600	0
600	512	0
512	600	0
600	512	1
600	512	1
600	512	0
512	600	1
512	600	1
512	600	1
600	512	0
600	512	1
512	600	1
512	600	1
512	600	1
512	600	0
600	512	1
600	512	0
512	600	1
600	512	1
512	600	1
512	600	1
512	600	1
512	600	0
600	512	0
512	600	0
Total		31
Wines tested: C7H_4 = 512 or Uvaferm 43 = 600		

Supplementary Table 5.1. 300bp Paired end sequence data for MiSeq Illumina sequencing of genomes from evolved and parent bacterial and yeast strains.

AGRF: Flowcell ID: 000000000-BFFGF			
Sample Name	**Paired End**	**Data Yield(bp)**	**Genome Coverage**
KS10_KS21_Dec1_No2	2,019,654	1.22 Gb	
KS11_KS21_illumina2	1,780,241	1.07 Gb	
KS12_K45	1,643,983	0.99 Gb	
KS7_SB3	2,035,270	1.23 Gb	
KS8_KS21	2,002,869	1.21 Gb	
KS9_KS21_6_No2	1,682,854	1.01 Gb	
MW1_C7H	1,853,740	1.12 Gb	89x
MW2_71B	1,912,032	1.15 Gb	92x
MW3_RM5_15	1,903,484	1.15 Gb	92x
MW4_C7H_B4	1,966,214	1.18 Gb	94x
MW5_Q7	1,771,744	1.07 Gb	86x
MW6_RM3_25	1,982,596	1.19 Gb	95x
Total	**22,554,681**	**13.58Gb**	



Supplementary Figure 5.1: Pipeline for *de novo* and reference guided assembly of yeast genomes.
Reference guided assembly of the trimmed reads was undertaken using MIRA 4 software to S288c genome. Bowtie, Samtools and GATK was used to form the L2056 (parent) consensus sequence. Samtools 'mpileup function' was used for detecting variant sites in alignments. Interactive Genome Viewer (IGV) enabled visual inspection of alignments. The Rapid Annotation Transfer Tool (RATT) was used to transfer the annotations from S288c to the L2056 consensus sequence. Variant annotation was then undertaken using R Variant Annotation package. The program CNV.Seq was used for detection of copy number variation (CNV).

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MINI-REVIEW

Implications of new research and technologies for malolactic fermentation in wine

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Abstract The initial conversion of grape must to wine is an alcoholic fermentation (AF) largely carried out by one or more strains of yeast, typically *Saccharomyces cerevisiae*. After the AF, a secondary or malolactic fermentation (MLF) which is carried out by lactic acid bacteria (LAB) is often undertaken. The MLF involves the bioconversion of malic acid to lactic acid and carbon dioxide. The ability to metabolise L-malic acid is strain specific, and both individual *Oenococcus oeni* strains and other LAB strains vary in their ability to efficiently carry out MLF. Aside from impacts on acidity, LAB can also metabolise other precursors present in wine during fermentation and, therefore, alter the chemical composition of the wine resulting in an increased complexity of wine aroma and flavour. Recent research has focused on three main areas: enzymatic changes during MLF, safety of the final product and mechanisms of stress resistance. This review summarises the latest research and technological advances in the rapidly evolving study of MLF and investigates the directions that future research may take.

Keywords *Oenococcus oeni* · *Lactobacillus* · Malolactic fermentation · Wine

Introduction

Wine is a complex mixture of hundreds of compounds, many of which contribute substantially to the colour, mouthfeel or aromatic properties of this beverage. Many variables affect the characteristic aroma of wine including the grape variety, viticultural and winemaking practices along with wine maturation and storage conditions. The initial conversion of grape must to wine is an alcoholic fermentation (AF) largely carried out by one or more strains of yeast, typically *Saccharomyces cerevisiae*. After the AF, a secondary or malolactic fermentation (MLF) is often undertaken, depending on the style of wine that is being produced. MLF is carried out by lactic acid bacteria (LAB), most commonly *Oenococcus oeni* (Carr et al. 2002), which is acidophilic and indigenous to wine and similar environments and is generally thought to be best suited to the harsh environment of wine. *Lactobacillus* spp. and *Pediococcus* spp. can also carry out MLF, but not always to completion (Renouf et al. 2008). However, recent red wine trials have shown that strains of *Lactobacillus plantarum* have the potential to conduct an efficient MLF and also produce desirable sensory attributes in red wines (Lerm et al. 2011; Bravo-Ferrada et al. 2013).

The MLF involves the bioconversion of malic acid to lactic acid and carbon dioxide and improves the biological stability of wine by preventing the utilisation of malic acid by other microorganisms in bottled wine (Davis et al. 1985). LAB do not use the Krebs cycle and a terminal electron transport system for metabolism and energy generation but instead obtain their energy by carbohydrate fermentation coupled to substrate level phosphorylation. *O. oeni* is an obligate heterofermentative bacterium (Kandler 1983; Papagianni 2012). The ability to metabolise L-malic acid is strain specific, and both individual *O. oeni* strains and other LAB strains vary in their ability to efficiently carry out MLF (Sieiro et al. 1990; Vailiant et al. 1995; Zapparoli et al. 2004). Aside from impacts on acidity, LAB can also metabolise other precursors present in wine during fermentation and, therefore, alter the chemical composition of the wine resulting in an increased complexity of wine aroma and flavour. The best described example is the compound diacetyl; however, the production of esters, alcohols and other carbonyl compounds results in buttery, fruity, spicy, vanilla and smoky notes, as well as a softer mouthfeel

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(Swiegers et al. 2005; Malherbe et al. 2012). As a consequence of this, strains selected for commercial use are usually chosen for their ability to both efficiently metabolise malic acid and to confer desirable sensory properties on the wine. Different strains, both in nature and available commercially, show various properties in terms of sensory compounds formed (Malherbe et al. 2012). This has led to a great number of studies in recent years attempting to unravel the complex interaction between LAB and the wine environment with a view to developing superior MLF starter cultures and obtaining more tailored and consistent outcomes.

MLF can be one of the most problematic processes that a winemaker has to manage, but is nonetheless considered a vital process in the production of many wines, impacting on safety, quality, microbial stability and operational efficiency. Being conducted in almost all red wines, some white wines and in sparkling wine bases, it is a process of great significance. For example, red wine varieties made up 52 % of the 2013 production in Australia with a recorded crush size of 945,586 tonnes (http://www.wfa.org.au/resources/1/Reports/WFA_Vintage_Report_2013.pdf) and 57 % of the 4 million tonnes crushed in California in 2012 (CDFA and California Agricultural Statistics 2013). An understanding of MLF and how to improve it are therefore of great importance to the wine industry. The increase in studies in this area over the past few years indicates that the changes occurring in wine during the MLF are more complex than first thought. Our recent unpublished survey of Australian winemakers revealed that most were concerned with the ability of a LAB strain to rapidly conduct MLF (24 %) and its ability to ferment efficiently in the presence of high ethanol concentrations (20 %). Tolerance to the normal stresses found in wine, such as low pH and high sulfur dioxide (SO_2) concentrations, was the next key concern (16 %). The specific attributes of selected LAB strains deemed to be the most important were low acetic acid production (27 %), enhancement of wine mouthfeel (26 %) and not contributing to the aromatic profile (i.e. neutrality) (22 %). Only 10 % of winemakers were interested in enhancement of wine colour (5 %) or contribution to the aromatic profile (5 %).

The research community has focused on three main areas: enzymatic changes during MLF, safety of the final product and mechanisms of stress resistance. It is now widely accepted that LAB strains carrying out MLF can affect the wine aromatic profile and final wine colour in a strain-specific manner. Indeed, some companies that supply starter cultures for MLF also include general predictions of each strain's ability to alter certain compounds in the wine that are regarded as being linked to quality. For example, low diacetyl production is often considered a desirable trait. There has also been an increasing number of publications on the ability of LAB to withstand wine-like stresses and the mechanisms for such resistance, with the final aim being to aid selection of superior LAB strains to conduct a more reliable MLF. Finally, the safety of the final product is always of importance when intended for human consumption. Accordingly, there is much interest in this topic, primarily concerned with biogenic amine and ethyl carbamate formation during or after MLF. This review summarises the latest research in the rapidly evolving study of MLF and investigates the directions that future research may take.

Enzyme activity and sensory changes during MLF

LAB have a considerable array of metabolic pathways and enzymes that are capable of altering wine aroma during MLF by hydrolysing and synthesising volatile secondary compounds at concentrations above their odour detection threshold. Examples of secondary compounds that have been shown to change during MLF include ethyl and acetate esters, higher alcohols, carbonyls, volatile fatty acids and sulfur compounds (Bartowsky 2005; Siebert et al. 2005; Sumby et al. 2013). As previously described, strain-specific variation in metabolic capabilities can impact on both the types and concentration of the compounds produced (Matthews et al. 2004; Bartowsky 2005; Lee et al. 2009; Sumby et al. 2013). The following section summarises the current knowledge of enzymatic activities of LAB in wine.

Malolactic enzyme

The first report of cloning of the malolactic (*mleA*) gene of *O. oeni* was in 1996 and expression was performed in both *Escherichia coli* and *S. cerevisiae* (Labarre et al. 1996). However, the MleA protein expressed in *E. coli* was only active against L-malic acid after protein fractionation and concentration. Labarre et al. (1996) also reported the presence of a malate carrier protein (*mleP*) downstream of *mleA* suggesting that in *O. oeni* the genes that encode for MLF are clustered and that malic acid is actively transported into the cell. As evidence, the *mleP* gene encoding a 34-kDa protein with characteristics of a carrier protein was cloned into dicarboxylic acid transport-deficient *E. coli* mutants revealing energy-dependent L-malate transport (Labarre et al. 1996). Schümann et al. (2013) also characterised the *mleA* gene from *O. oeni* via expression into *E. coli* with the crude extract having a specific activity of 14.9 U/mg of protein and the purified recombinant protein having a specific activity of 280 U/mg of protein, much higher than that previously reported (Labarre et al. 1996). But the exact reaction mechanism of MleA remains in question and needs to be studied in further detail (Schümann et al. 2013).

Recognising the often poor growth of *O. oeni*, further work with the *O. oeni* malolactic enzyme involved expression in *L. plantarum* to determine if MLF could be improved above

the level of the wild-type strain (Schümann et al. 2013). However, induction of the expression vector at low pH and even in the presence of diluted wine decreased the expression level. At pH 4.0, activity was undetectable and conversion of the added L-malic acid (5 g/l) was the same as the wild-type strain (Schumann et al. 2012). This is probably at least partly due to expression vector loss at low pH, as the selective maker used had minimal activity at low pH, and therefore, the bacteria likely dispose of the plasmid. To overcome this, *L. plantarum* cells were cultured and induced in a medium at pH 6.0, where cells showed highest malolactic activity and then were harvested and directly inoculated to L-malic acid solutions. The authors used pre-induced cells as starter cultures and reported that the recombinant strain converted L-malic acid more efficiently than the wild-type strain (Schumann et al. 2012). Nevertheless, the malolactic activity of the recombinant *L. plantarum* was only slightly above that of adapted wild-type cells and the modified wine solution used was at a pH of 5.0, which is well above typical wine pH. The authors suggested using a food-grade selection marker, such as the pSIP vectors with the alanine racemase (*alr*) gene as the plasmid selection marker instead of the erythromycin gene (Schumann et al. 2012). This system has successfully been applied for overexpression of a β-galactosidase in a modified *L. plantarum* strain (Nguyen et al. 2011). This expression vector is however unstable when induced in the presence of D-alanine. As wine contains this amino acid and other inhibitory factors such as low pH, further study will be needed to determine if *alr* can be utilised as a selection marker in *Lactobacillus* spp. for the purpose of overexpression of *O. oeni* genes in wine. Gene expression studies of *mle* in LAB have also added to the knowledge of how *mle* functions when LAB are in the wine environment. For example, Augagneur et al. (2007) reported that the presence of L-malate in low pH media resulted in an increase in *mleP* expression leading to the suggestion that *mleP* encodes a carrier protein responsible for L-malate uptake. Following on from this, Miller et al. (2011) investigated the effect of pH, ethanol and malic acid concentration on expression of the structural *mle* gene of *L. plantarum* to find induction at low pH values and when malic acid was present in the medium. Increasing ethanol concentrations and/or pH decreased *mle* expression with increased pH appearing to have a greater effect than high ethanol concentrations. These results suggest that under certain conditions such as high pH (wine pH can exceed 4.0), the added effect of high ethanol concentrations will have a negative impact on the completion of MLF. However, the normalisation of messenger RNA (mRNA) quantity in this experiment was conducted with ribosomal RNA (rRNA) and could contain a bias, as 16S rRNA transcripts do not reflect the overall mRNA within *O. oeni* and should therefore not be used as an internal control to study gene expression (Desroche et al. 2005). Beltramo et al. (2006) have also previously reported that low pH resulted in increased *mle* expression. MLF plays a role in the regulation of the intracellular pH; thus, LAB could achieve a biological advantage by increasing *mle* expression in acidic conditions (Miller et al. 2011). It will be of interest to investigate if low pH causes increase in *mle* expression due to an increase in the undissociated form of malic acid, which would enable it to pass through the cellular membrane, or whether the previously reported increase in mleP activity (Augagneur et al. 2007) and therefore increased active transport of malic acid across the membrane are more important. At pH 3.2, 64 % of L-malic acid is undissociated and the initial rate of passive diffusion represents 50 % of the total uptake rate (Tourdot-Maréchal et al. 1993). However, *O. oeni* cells respond to ethanol by increasing membrane rigidity (Tourdot-Marechal et al. 2000; Chu-Ky et al. 2005), and this could potentially affect passive diffusion of L-malic acid into the cell.

Glycosidases

Glycosylated aroma and flavour compounds can be liberated enzymatically by microbial glycosidases. Wine-related LAB have been shown to possess the ability to hydrolyse various synthetic glycosides (Grimaldi et al. 2000, 2005a, b), and high variations in glycosidase activities have been reported amongst isolates of *O. oeni* (Ugliano and Moio 2006; Gagne et al. 2011). The activity of these enzymes can aid release of numerous aroma compounds, including monoterpenes, norisoprenoids and aliphatic compounds, all of which contribute to the fruity and floral attributes of wine. Capaldo et al. (2011a, b) reported the cloning and characterisation of two phospho-β-glucosidases from *O. oeni*. The first gene (*bglD*) is found in a putative β-glucosidase operon encoding four genes designated *bglA* to *bglD*. The *bglA*, *B* and *C* genes are thought to be phosphoenolpyruvate-dependent phosphotransferase system components IIC, IIA and IIB, which regulate the uptake, phosphorylation and translocation of β-glucosides across the cytoplasmic membrane (Capaldo et al. 2011a). High activity towards the phosphorylated β-glucoside *para*-nitrophenol-β-D-glucopyranoside-6-phosphate substrate was observed with purified BglD. The enzyme was not active against non-phosphorylated β-glucosides. The second gene characterised was phospho-β-glucosidase *celD*, which is in a putative PEP-PTS operon in *O. oeni* (Capaldo et al. 2011b). CelD is proposed to be an intracellular enzyme that also acts on phosphorylated β-glucosides. More recently, a purified recombinant glucosidase and an arabinosidase from *O. oeni* were reported to release monoterpenes from natural substrates but under optimal conditions. This indicates that these intracellular enzymes might hydrolyse aroma precursors during MLF (Michlmayr et al. 2012), but information about strain variation in these genes will likely prove more useful than

direct application of these enzymes, as their activity was very low in wine.

Wine LAB, in particular *O. oeni*, are capable of releasing attractive aroma compounds during MLF, and LAB might be a promising source of novel glycosidases with oenological potential. Whilst numerous fungi and bacteria produce glycosidases, there is variation in the abilities of these microbes to function efficiently under the high alcohol and low pH encountered in winemaking. Given the potential impact of glycosidases on the sensory profile of wine, it is envisaged that further characterisation of glycosidase systems from LAB will provide information to aid winemakers in tailoring wine aroma, colour and overall complexity.

Esterases

Sumby et al. (2009, 2012a, 2013) reported the characterisation of esterase enzymes from wine LAB. These esterases were demonstrated to retain activity under conditions relevant to winemaking (Sumby et al. 2009, 2012a). The *O. oeni* Ooeni28 esterases EstB28 and EstA2 were also shown to have both hydrolysis and synthesis activity with natural substrates (Sumby et al. 2013). Further investigation of these enzymes would provide a better understanding of the role they play in ester profile modification during MLF. The reason for strain-specific changes in esters during MLF was also investigated, and strains could be characterised in a manner that concurred with their previously observed activity against artificial ester substrates (Sumby et al. 2013). The strength of this correlation, however, will need to be investigated further. Additional experiments on these esterase genes from a strain designated as having high esterase hydrolysis activity (Sumby et al. 2012b) revealed that they are induced in the presence of substrates. More detailed investigation of these activities in wine will benefit winemaking by allowing a more informed choice of MLF culture to be made based on activities other than just the decarboxylation of malic acid.

The study of these enzymes under 'wine-like' conditions is currently only of academic value as direct application of bacterial enzymes in winemaking is not presently possible. The major obstacle to this is the necessity for recombinant enzyme production. Consumer preferences preclude the use of recombinant techniques in the food industry. However, an interesting alternative could be the use of LAB as GRAS/food-grade expressions systems, which are also recombinant but generally more acceptable. There has been much interest in designing LAB 'food-grade' gene expression systems over the past decade (Gosalbes et al. 2000; Sørvig et al. 2003; Maischberger et al. 2010; Nguyen et al. 2011). In order for these systems to be accepted for use in food products, they are designed with specific attention to self-cloning strategies, food-grade selection markers, plasmid replication and chromosomal gene replacements (Peterbauer et al. 2011). Even though *O. oeni* is not easily genetically manipulated due to poor transformability, it could be possible to express the genes of interest in other more easily manipulated LAB such as *Lactobacillus* spp. This would enable winemakers to utilise the benefits of these characterised *O. oeni* malolactic, esterase and glycosidase genes.

Potential negative impacts of MLF

Volatile phenols

Volatile phenols are aromatic compounds that are reported to be mainly produced by the yeast genus *Brettanomyces* (Curtin et al. 2013). However, there is some evidence that LAB can also produce these compounds (Chatonnet et al. 1995, 1997; Silva et al. 2011; Fras et al. 2014). The formation of volatile phenols in wine is the result of the sequential activity of two enzymes. The first enzyme, hydroxycinnamate decarboxylase, catalyses the decarboxylation of non-volatile, odourless hydroxycinnamic acid precursors (*p*-coumaric, ferulic and caffeic acids) forming hydroxystyrenes (4-vinylphenol, 4-vinylguaiacol and 4-vinylcatechol). The second enzyme a vinylphenol reductase can then sometimes reduce the hydroxystyrenes to their corresponding ethyl derivative forms (4-ethylphenol, 4-ethylguaiacol and 4-ethylcatechol) (Heresztyn 1986; Chatonnet et al. 1992; Harris et al. 2009; Carrillo and Tena 2007; Godoy et al. 2008; Tchobanov et al. 2008; Buron et al. 2012). Both the ethyl and vinyl derivatives of hydroxycinnamic acids are of concern in wine and are considered to be the most significant molecules linked to olfactory defects in wine. If the concentration of volatile phenols is greater than 425 μg/l, they can potentially mask any desirable aromas and the corresponding wine can often be described as smelling of 'animal' or 'horse sweat' (Heresztyn 1986; Chatonnet et al. 1995).

LAB produce volatile phenols via the activity of decarboxylase enzymes, which can be enhanced with increased phenolic composition of the medium (Silva et al. 2011). Couto et al. (2006) investigated the ability of 35 strains of LAB (20 species including two *O. oeni* strains) to produce volatile phenols in a culture medium. It was shown that 13 strains produced volatile phenols from *p*-coumaric acid and only three (9 %; *Lactobacillus brevis*, *Lactobacillus collinoides* and *L. plantarum*) of these could produce 4-ethylphenol (Cuto et al. 2006). There is so far only limited evidence that LAB can produce 4-ethylphenol from 4-vinylphenol and this evidence is also strain dependent (Silva et al. 2011). Buron et al. (2012) reported that four different *L. collinoides* strains were able to produce 4-ethylphenol, 4-ethylguaiacol and 4-ethylcatechol in synthetic media and in cider with amounts increasing when caffeic acid and *p*-coumaric acid were added

to the cider. More recently, Fras et al. (2014) investigated the ability of *L. plantarum* (strain NCFB 1752) to produce volatile phenols in the presence of *p*-coumaric and ferulic acids in de Man–Rogosa–Sharpe media (MRS) and in MRS supplemented with wine (red and white). There was no evidence of ferulic acid metabolism by *L. plantarum*, but there was production of 4-vinylphenol and 4-ethylphenol in varying amounts depending on the ratio of wine in the medium, suggesting that the presence of wine leads to the accumulation of 4-vinylphenol and potentially inhibits the production of 4-ethylphenol (Fras et al. 2014). The conversion yields of hydroxycinnamic acids were also still lower than those seen with *Brettanomyces*. The ability of LAB to produce phenolic off-flavours in real wine is therefore still to be investigated.

Indole

High levels of indole are mainly only reported in wines under sluggish fermentation conditions and are linked to 'plastic' off-aromas (Capone et al. 2010). Indole formation in wine still is not fully understood, but is thought to be linked to tryptophan metabolism. Four strains of LAB (*O. oeni*, *Lactobacillus lindneri*, *Pediococcus cerevisiae* and *Pediococcus parvulus*) have been reported to generate indole during MLF in defined media supplemented with 100 mg/l tryptophan and only minimal amounts without tryptophan supplementation (Arevalo-Villena et al. 2010). Indole has an aroma detection threshold of 23 mg/l in white wine (Capone et al. 2010), and all four tested LAB strains had accumulated indole at the end of MLF at concentrations ranging from 70 to 370 mg/l. It has previously been reported that yeast can release tryptophan back into wine in the later stages of AF (Henschke and Jiranek 1993) at which point it might act as a substrate for bacteria to produce indole at concentrations detrimental to wine quality. A fuller investigation of indole formation during MLF in wine by LAB is required.

Biogenic amine formation

Biogenic amines are low molecular weight organic nitrogenous bases that can be formed in fermented foods and beverages by decarboxylation of free amino acids. In wine, this can be facilitated by the action of microbial decarboxylase enzymes, from the corresponding amino acid precursors. This reaction is usually strain dependent rather than species specific, and it has been suggested that horizontal gene transfer is responsible for the variation in decarboxylation abilities amongst LAB (Marcobal et al. 2006; Coton and Coton 2009). High concentrations (i.e. 1–100 mg/l) of biogenic amines can cause undesirable physiological effects in sensitive humans, especially when alcohol and acetaldehyde are present (Maintz and Novak 2007; Spano et al. 2010). Putrescine, histamine, tyramine and cadaverine have been identified as the most abundant biogenic amines found in wine (Beneduce et al. 2010; Bartowsky and Stockley 2011; Patrignani et al. 2012; Costantini et al. 2013; Martuscelli et al. 2013). It is now widely believed that the main source of biogenic amines in wine is from LAB metabolism either during or after MLF (Lonvaud-Funel 2001; Polo et al. 2011; Smit et al. 2012). For a review of the source of amines in wine, including during MLF, refer to Ancín-Azpilicueta et al. (2008).

Choosing MLF starter culture strains that do not produce biogenic amines and/or can degrade biogenic amines formed in wine is therefore desirable. Whilst *O. oeni* has been reported to rarely degrade wine biogenic amines (García-Ruiz et al. 2011), recent studies have pointed to the potential of *Lactobacillus* strains in particular *L. plantarum* (Capozzi et al. 2012) and *L. casei* (García-Ruiz et al. 2011) to do so through the production of amine oxidase enzymes. García-Ruiz et al. (2011) reported that in a model system, the greatest biogenic amine-degrading ability was exhibited by nine strains belonging to the lactobacilli and pediococci groups, with most able to simultaneously degrade to some extent at least two of the three studied biogenic amines. The ability of LAB to reduce biogenic amines was negatively affected by the wine matrix (Capozzi et al. 2012; García-Ruiz et al. 2011). Only one LAB strain (*L. casei*) was able to significantly degrade histamine, tyramine and putrescine in a contaminated wine but at lower percentages than in culture media. Additionally, the ability of these strains to complete MLF is yet to be tested, and it is possible that they may need to be inoculated alongside *O. oeni* to ensure MLF completion. Only one of the 42 *O. oeni* strains tested showed 10 % or more degradation of biogenic amines in control media, but this strain showed no significant degradation during wine MLF.

It has also been reported that co-inoculation of *O. oeni* starter cultures at the beginning of AF simultaneously with yeast has been more effective in avoiding the production of biogenic amines than either spontaneous or conventional inoculation (Cañas et al. 2012; Smit and du Toit 2013). This is most likely due to the inoculated starter culture dominating the fermentation and reducing the impact that indigenous lactobacilli have on biogenic amine formation (Smit et al. 2012; Smit and du Toit 2013). This highlights the need to screen any potential starter culture for decarboxylase activity to minimise the production of biogenic amines. Physiochemical factors such as pH, temperature, SO_2 and alcohol concentration, along with the nutrients present in wine, influence bacterial development and, as a consequence, amine production. Complete MLF is necessary to avoid the risk of increased biogenic amines during aging (Polo et al. 2011). Smit et al. (2013) reported that cold maceration (when compared to conventional maceration) during red wine

production inhibited the formation of high levels of biogenic amines under the conditions tested. However, further investigations are necessary to determine the reasons for this.

Ethyl carbamate

Wine, like most fermented foods and beverages, can contain trace amounts of ethyl carbamate (EC), a known animal carcinogen (Canas et al. 1994). EC is formed by a reaction between ethanol and N-carbamyl compounds at acidic pH and can be due to the production of certain precursors during AF (such as urea) and MLF (citrulline and carbamyl phosphate). Its formation is dependent on reactant concentration and is favoured by high temperature and low pH (Ough et al. 1988). The EC content is therefore higher in wines that have been stored for a long time without temperature control. Some LAB can degrade the arginine present in must and wine via the arginine deiminase pathway (Liu et al. 1995). When arginine is not completely catabolised, intermediate products of the pathway, citrulline and carbamyl phosphate, can accumulate in the medium. Both compounds can react with ethanol and produce EC. EC-producing LAB strains include all heterofermentative lactobacilli, *O. oeni*, *Pediococcus pentosaceus* and some strains of *Leuconostoc mesenteroides* and *L. plantarum* (Araque et al. 2009).

In most LAB, the three genes involved in arginine catabolism are clustered in an operon-like structure (Fig. 1). The catabolic enzyme arginine deiminase, encoded by *ArcA*, converts arginine into citrulline and ammonia. Citrulline can then be further metabolised by ornithine transcarbamylase (*ArcB*) into carbamoyl-P, which can then be utilised by carbamate kinase (*ArcC*) to produce CO_2, ammonia and ATP (Tonon et al. 2001; Spano et al. 2004; Araque et al. 2009). The timing of MLF induction on EC accumulation in wine has no influence (Masqué et al. 2011). EC levels at the end of MLF were quite low (<3 μg/l) and increased after storage. It is therefore difficult to determine if the increase in EC is from remnants of either yeast or bacterial metabolism and, hence, making it important to quantify citrulline and carbamyl phosphate levels in studies investigating EC production during MLF (Masqué et al. 2011). Significant differences between two LAB species (*L. brevis* and *P. pentosaceus*) in citrulline production in the presence of ethanol have been reported (Araque et al. 2013). *ArcC* expression (carbamate kinase) was repressed in *L. brevis* making this the basis for the suggestion that this species is more likely to produce EC precursors.

Patrignani et al. (2012) reported that there was no significant difference observed in EC concentration after MLF between samples of wine produced by uninoculated fermentation and wine that was inoculated. However, citrulline and carbamyl phosphate levels were not quantified in the tested wines and the wines were not accessed for EC accumulation after storage. Arena et al. (2013) analysed citrulline production in Malbec wine and reported that formation was correlated with its low glucose, fructose, citric and phenolic acid concentrations. Further analysis of other wines with lower concentrations of these sugars and acids in relation to the formation of ethyl carbamate precursors could provide more information on wines at risk of increased EC formation.

Chemical impediments to successful MLF

One of the most important requirements of MLF is that the process is reliably completed in a timely manner therefore reducing the risk of the proliferation of spoilage microorganisms. A potential cause of stuck or sluggish MLF is the fastidious nutritional requirements of malolactic bacteria whose growth typically depends on the availability of nutrients left after AF. This is an area that has however not received much attention in the literature, and a study of the minimum nutritional needs of LAB species in wine would help eliminate the need for costly nutrient supplementation. When *O. oeni* is added to wine, it encounters multiple stressors, amongst which are low pH and temperature and high ethanol concentrations. The tolerance to ethanol varies from strain to strain, and it is generally accepted that all *O. oeni* strains grow in a medium containing 10 % ethanol and that small quantities of ethanol [3–5 (Britz and Tracey 1990) or 7 % (Alegría et al. 2004)] can stimulate their growth. Sulfur dioxide is an additional stressor that is often added to wine as an antioxidant and

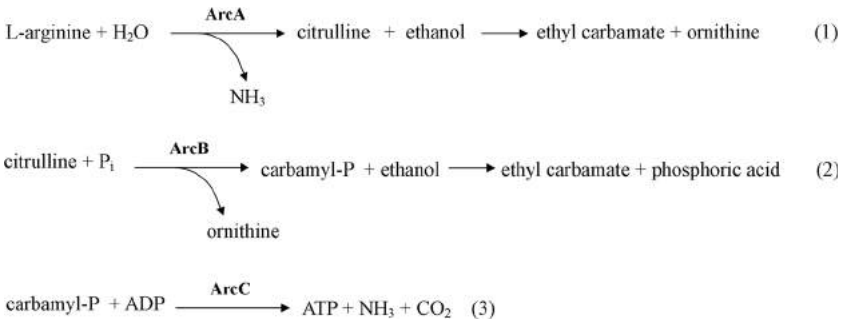


Fig. 1 Pathways for ethyl carbamate (EC) formation in wine during MLF and via acid catalysed alcoholysis. ArcA (arginine deiminase), ArcB (ornithine transcarbamylase) and ArcC (carbamate kinase) adapted from Tonon et al. (2001), Spano et al. (2004) and Araque et al. (2009)

to reduce the risk of microbial spoilage during primary fermentation. The yeast carrying out the primary fermentation can also produce it. The presence of any one of these stressors in the wine may inhibit the LAB and cause a sluggish or stuck MLF.

Other factors in the wine that might cause a slow or stuck MLF include medium chain fatty acids, phenolic compounds (e.g. phenolic acids, tannins), pesticides, metal ions (such as copper), bacteriocins produced by other LAB species (e.g. nisin, pediocin and plantaricin), inhibitors produced by yeasts during fermentation, bacteriophage infections and lysis of the malolactic bacteria. Recent research has indicated that certain grape phenolic compounds including phenolic acids that can increase membrane permeability in LAB (Campos et al. 2003, 2009) and tannins can have a negative influence on *O. oeni* (Figueiredo et al. 2008). Tannins are naturally occurring high molecular weight (M_r>500) polymers of phenolic compounds flavonoids and non-flavonoids, which form stable complexes with proteins and other plant polymers (Ribereau-Gayon et al. 2000). Tannins derived from flavonoids are naturally present in the grape skins, seeds and stems. Non-flavonoid tannins are derived from the pulp of the grape and can also be extracted from oak sources (barrels, staves, chips) during fermentation and aging. Certain red cultivars can have more difficulty undergoing MLF due to tannins (Vivas et al. 2000). Research is ongoing into this observation, as the exact nature of these tannins and their concentrations are still not well defined.

In years when the incidence of *Botrytis* infection of grapes is high, residues of systemic fungicides/pesticides have the potential to be problematic for LAB thereby influencing MLF success. Cabras et al. (1994) reported that the presence of dichlofluanid reduced the ability of *O. oeni* to conduct MLF in proportion to its concentration, but the other pesticides tested (benalaxyl, carbendazim, triadimefon and vinclozolin) did not influence MLF. The insecticide dicofol has also been reported to reduce the activity of *O. oeni*, whilst other fungicides and insecticides had only a minor effect (Vidal et al. 2001; Ruediger et al. 2005). Whilst the addition of yeast hulls or ghosts may help relieve this toxicity, inhibition of MLF by pesticides is increased in the presence of ethanol (Vidal et al. 2001). The commonly used fungicide, copper, apparently does not affect malolactic activity directly (Vidal et al. 2001), but rather through the decrease in cellular viability that accompanies increasing copper. There is also a synergistic effect when LAB are grown in media containing copper and other stressors of wine such as ethanol, SO_2, tartaric acid or low pH (Vidal et al. 2001). These findings aside, the interactions between LAB and metal ions in wine are not fully investigated.

There have been a number of reports that compounds of peptidic or proteic nature produced by yeasts can inhibit LAB growth (Comitini et al. 2005; Osborne and Edwards 2007; Mendoza et al. 2010; Nehme et al. 2010; Branco et al. 2014). For example, Mendoza et al. (2010) found that the *S. cerevisiae* strain tested inhibited wine LAB growth by a synergistic effect of a peptidic compound of low molecular size (3–10 kDa) and fermentation metabolites. Although this yeast inhibited *O. oeni* growth, it did not affect the malolactic activity. More extensive fractionation studies are called for to determine the full range of inhibitory compounds and mechanisms affecting LAB grown in newly fermented wines.

Sulfur dioxide

Sulfur dioxide is used as both an antimicrobial and antioxidant agent. Winemakers can add it at different stages during wine production. When it is added in the form of either sodium or potassium metabisulfite, these compounds release SO_2, which reacts with water to form sulfites. Yeast will also commonly produce some bisulfite during fermentation through the reduction of sulfate as part of the production of essential sulfur-containing compounds such as cysteine and methionine (Dott et al. 1976).

In solution, the different forms of sulfites are at equilibrium; however, the lower the pH, the more heavily the reaction shifts towards molecular SO_2 and bisulfite (together termed free SO_2), which make up the vast majority (99.99 % at pH 3.4) of the sulfite compounds present in wine. Sulfites will also react with other wine components such as sugars, carbonyl compounds (such as acetaldehyde) and phenolic compounds. Such ‘bound’ sulfites can no longer take part in the equilibrium reaction. Only the molecular form of SO_2 can enter through the cell membrane causing disruption to enzyme activity. It is therefore the concentration of molecular SO_2 that is considered to control microbial growth in wine.

Bacterial inhibition by SO_2 bound to other wine components is often reported as being as a consequence of the release of SO_2 following degradation of the binding compound by the bacteria (Osborne et al. 2000, 2006; Jackowetz and Mira de Orduña 2012, 2013). Larsen et al. (2003) reported that *O. oeni* was inhibited in wines with high bound SO_2 concentrations and suggested that inhibition of MLF by bound SO_2 is more significant than previously thought. The authors also reported that inhibition of *O. oeni* was stronger when SO_2 was bound to compounds other than acetaldehyde (Larsen et al. 2003). More recently, Wells and Osborne (2011) investigated the impact of the production of SO_2 and SO_2-binding compounds by wine yeast on MLF. Samples were taken from the fermentations at different time points, filter sterilised and inoculated with *O. oeni* to induce MLF. Significant differences between the yeast strains in the amount of SO_2, acetaldehyde and pyruvic acid produced were reported and high total SO_2 concentration inhibited MLF. However, for all yeast strains tested, insignificant free SO_2 was measured, indicating that bound SO_2 rather than free SO_2 was responsible for MLF

inhibition (Wells and Osborne 2011). At almost all time points of the AF, acetaldehyde-bound SO_2 was determined to be the dominant species of bound SO_2 present, suggesting that MLF inhibition by bound SO_2 was due to this species. In a subsequent investigation of the degradation of SO_2 bound to acetaldehyde and pyruvic acid, by several LAB species including *O. oeni*, no correlation with growth of the bacteria was observed (Wells and Osborne 2012). The bacteria were still metabolically active in media containing bound SO_2 even when no growth was observed, indicating that bound SO_2 is bacteriostatic rather than bacteriocidal (Wells and Osborne 2012). In accordance with the results reported by Larsen et al. (2003), the authors also reported that *O. oeni* was the most sensitive of the LAB tested against pyruvic acid-bound SO_2. Therefore, is it now evident that SO_2 bound to acetaldehyde or pyruvic acid is inhibitory to the growth of wine LAB and must be considered both when choosing a yeast strain for conducting AF and, subsequently, when choosing a LAB strain to conduct MLF.

Response of *O. oeni* to stress

There are now a number of tools available to study the effect of either individual or multiple stresses on *O. oeni* and other LAB cells, including metabolomic analysis, proteomics, genomics and transcriptomics. These techniques generate large datasets, making it possible to perform comprehensive analyses of biological systems to generate new knowledge and help to address research questions when applied to any organism. For example, there is a large amount of omics data available for wine yeast, and a detailed metabolic map of wine yeast in a model wine fermentation is currently being developed (Chambers 2011). This approach is yet to be applied to wine LAB and could provide valuable insight into how they function in wine during MLF and could also help develop informed models for the in silico design of new and/or improved industrial strains. As an example, Bon et al. (2009) used comparative genome subtractive hybridization to propose that the presence of eight stress-responsive genes was associated with high MLF performance thereby suggesting a relationship between genome variation and malic acid metabolism. The eight genes were annotated and encode for the Dps gene homolog, *fnr* (DNA binding protein), copper chaperone, maltose phosphorylase, oxo-acyl carrier protein reductase, P-type ATPase, YP_809753 (hypothetical protein) and NP_786185 (hypothetical protein). They also identified six different regions of plasticity, resulting from recombination or InDel events and suggested that IS*30*-related elements play a role in *O. oeni* genome plasticity.

In order to overcome the multiple stressors that are encountered during MLF in wine, LAB display numerous adaptive responses. In recent years, analysis of gene expression under different stress conditions have led to reports of many potential survival strategies and metabolic properties that may enable LAB to effectively compete in the wine environment (Table 1). For example, Maitre et al. (2014) proposed a model whereby *O. oeni* can adapt to ethanol stress through the synthesis of the small heat shock protein Lo18 and the subsequent modification of phospholipid content of the cell membrane. Other potentially interesting stress responses include carotenoid production, which has been shown to reduce membrane fluidity in *Staphylococcus aureus* (Clauditz et al. 2006) and increase multistress tolerance when carotenoid biosynthesis genes are overexpressed in *Lactococcus lactis* (Hagi et al. 2013). This is worthy of further investigation in *O. oeni* as geranylgeranyl pyrophosphate synthase (Table 1), an enzyme involved in carotenoid biosynthesis, is overexpressed in *O. oeni* in response to ethanol (Cafaro et al. 2014). The existence of such unique features can be viewed as evolutionary adaptation to the wine environment (Beltramo et al. 2006). The response of LAB to stress relies on the coordinated expression of genes that alter specific cellular processes (e.g. membrane biogenesis, transport, DNA metabolism, etc.). A network of regulators allows the cell to react to complex environmental changes and achieve a coordinated response. Although several studies have analysed mechanisms that enable LAB to withstand stress conditions (Table 1), more information about the mechanisms of LAB adaptation to stress conditions found in wine is required. A better understanding of the regulatory networks involved will help explain how particular LAB strains adapt to wine and help predict a strain's ability to complete MLF in a given wine. Methods allowing for identification of gene expression changes associated with differential microbial behaviour under different stress conditions include fluorescent differential display (FDD) (Sico et al. 2009), microarray analysis and RNA sequencing. For a detailed review of stress response in *O. oeni*, see Wen-ying and Zhen-kui (2013).

Genetic diversity and molecular approaches to identify LAB

Within, LAB there is a large degree of phenotypic heterogeneity and different strains vary in their capacity to adapt to wine and degrade malic acid. An increasing interest in the use of malolactic starter cultures in winemaking has produced numerous publications on this subject. LAB species may be differentiated by several DNA fingerprint profiling methods, such as restriction endonuclease analysis pulsed-field gel electrophoresis (REA-PFGE), patterns of low-frequency restricted genomes (Viti et al. 1996; Zapparoli et al. 2000; Lopez et al. 2008b; González-Arenzana et al. 2013b), random amplification of polymorphic DNA (RAPD) (Reguant and

Table 1 Studies reporting genes involved in the adaptation and stress response in wine

Gene	Function	Stress					
		Ethanol	Heat shock	Oxidative (H_2O_2)	Acid	Osmotic shock	Synthetic wine
arcR	Regulatory protein, arginine deiminase pathway		Bourdineaud (2006)			Bourdineaud (2006)	
atp—β and α subunits	H^+-ATPase				Fortier et al. (2003), Beltramo et al. (2006)		
cfa	Cyclopropane fatty acid synthase	Beltramo et al. (2006), Grandvalet et al. (2008)			Grandvalet et al. (2008)		Beltramo et al. (2006)
citE	Citrate lyase β subunit	Olguín et al. (2009)					
clpL	ATP-dependent protease	Beltramo et al. (2004a), Grandvalet et al. (2005)	Beltramo et al. (2004a), Desroche et al. (2005), Grandvalet et al. (2005)		Grandvalet et al. (2005), Beltramo et al. (2006)		
clpP	ClpP protease	Beltramo et al. (2004a)	Beltramo et al. (2004a), Desroche et al. (2005)				Beltramo et al. (2006)
clpX	ATPase regulation component of ClpP		Guzzo et al. (2000), Desroche et al. (2005)				Beltramo et al. (2006)
ctsR	CtsR, heat shock transcriptional regulator	Grandvalet et al. (2005)	Desroche et al. (2005), Grandvalet et al. (2005)		Grandvalet et al. (2005)		Beltramo et al. (2006)
ftsH	Membrane protease		Bourdineaud et al. (2003)			Bourdineaud et al. (2003)	
ggpps	Geranylgeranyl pyrophosphate synthase	Cafaro et al. (2014)					
groES	GroES, heat shock chaperone class I	Grandvalet et al. (2005)	Desroche et al. (2005), Grandvalet et al. (2005)				Beltramo et al. (2006)
grpE	GrpE, heat shock chaperone class II	Grandvalet et al. (2005)	Desroche et al. (2005), Grandvalet et al. (2005)		Grandvalet et al. (2005)		Beltramo et al. (2006)
hsp18	Small heat shock protein (Lo18)	Fiocco et al. (2007), Maitre et al. (2014)	Jobin et al. (1997), Guzzo et al. (2000), Desroche et al. (2005), Fiocco et al. (2007)		Beltramo et al. (2006)		
maeP	Putative citrate transporter	Olguín et al. (2009)					
mleA	Malolactic enzyme				Beltramo et al. (2006)		Beltramo et al. (2006)
omrA	ABC-type transporter		Bourdineaud et al. (2004)			Bourdineaud et al. (2004)	
rmlB	DtdP-glucose-4,6-dehydratase	Da Silveira et al. (2004)					
trxA	Thioredoxin		Jobin et al. (1999), Guzzo et al. (2000)	Jobin et al. (1999)	Beltramo et al. (2006)		Beltramo et al. (2006)

Bordons 2003; Solieri and Giudici 2010; Sánchez et al. 2012), amplified fragment length polymorphism (AFLP) (Cappello et al. 2008) or ribotyping analyses (Zavaleta et al. 1997; de las Rivas et al. 2004; Rodas et al. 2005). More recent methods include a combination of PFGE and multilocus sequence typing (MLST) (González-Arenzana et al. 2014), PCR-RFLP (Ilabaca et al. 2014) and variable number of tandem repeat analysis (VNTR) (Claisse and Lonvaud-Funel 2012, 2014).

Advances in DNA sequencing technology have led to rapid increases in sequencing throughput and a decrease in sequencing cost leading to more comparative studies of the whole genomes of many related species. This is also driving an interest in utilising this knowledge to design metabolic pathways in many LAB used in the production of fermented foods. This generally involves targeting the synthesis of specific compounds to produce the desired sensorial, textural and nutritional attributes. Genome analyses can provide valuable insights into key trait variations including evolutionary mechanisms, diversity and adaptability of life to environmental changes. Intra-species genetic diversity and the genomic basis of strain-specific differences amongst industrially relevant LAB have received much attention. Direct comparisons of complete genome data, subtractive hybridizations and array-based comparative hybridization have detected numerous insertion and deletion events (InDels) (Boekhorst et al. 2004; Bon et al. 2009; Borneman et al. 2010; Bartowsky and Borneman 2011; Borneman et al. 2012a, b). Genomic diversity can be increased by the presence of prophages, which generate large-size structural polymorphisms among *O. oeni* genomes (Ze-Ze et al. 2008; Jaomanjaka et al. 2013) and insertion sequences (IS) (Ze-Ze et al. 2008; El Gharniti et al. 2012). IS are autonomous transposable elements which encode a transposase gene mediating their transposition to other loci in the genome (Mahillon and Chandler 1998). IS are widely distributed in both eukaryotic and bacterial genomes and may disrupt genes, but may also activate downstream genes and fine tune gene expression through transposition-mediated genome inversions.

Inoculation, strain selection and optimisation

In order to achieve better control over MLF, many wineries now inoculate with a commercial MLF starter culture. They do this with the hope that the commercial strains will be more reliable than the indigenous microbiota, and indeed, many strains are now promoted as performing well under different stress conditions. However, there is still some contention over this as even with the use of commercial starter cultures, MLF is not always successful. Allowing the indigenous microbiota to conduct MLF is considered to have the advantage that these organisms have previous adaptation to wine. Much of the work on wine LAB in recent years has focused on *O. oeni* as it is historically considered to be the best adapted to the wine environment, but this is not always the case. Of 40 indigenous strain isolates evaluated, two of the three strains possessing desirable traits were *L. plantarum* and only one was *O. oeni* (Izquierdo et al. 2004). Further screening will undoubtedly reveal additional strains with desirable properties. In addition to strain selection, strain improvement and optimisation strategies use two broad approaches, recombinant and non-recombinant methods. Recombinant methods have the advantage of being highly specialised and controlled but are often focused on the addition or deletion of specific genes. This requires an intricate knowledge of gene identity, function and interactions prior to manipulation. Non-recombinant approaches require little prior knowledge of the genetic basis of a trait, but can be time consuming, often random and with pleotropic effects.

Co-inoculation of *O. oeni* and yeast

Prolonged or delayed MLF can increase the risk of spoilage by other microorganisms and the production of undesirable compounds such as biogenic amines (discussed above). In recent years, co-inoculation of bacterial cultures with yeast into the must at the beginning of fermentation has been proposed as a solution for obtaining fast and reliable MLF, particularly for grape juices or musts with high acidity or sugar concentration with a concomitant high level of ethanol on the completion of AF (Jussier et al. 2006; Zapparoli et al. 2009). The justification for this approach is that bacteria better adapt to the physiochemical conditions of the must rather than the wine (Abrahamse and Bartowsky 2012), and the limiting factors increase gradually according to the evolution of AF. There is, however, still some reluctance amongst winemakers to use co-inoculation based on the belief that there is a risk of producing acetic acid in high sugar juices through the heterofermentative metabolism of *O. oeni*. Also, it is though undesirable metabolites are produced because of interactions between the yeast and inoculated malolactic bacteria. Indeed, many studies report negative interactions between yeast and inoculated malolactic bacteria (Bisson 1999; Comitini et al. 2005; Comitini and Ciani 2007; Rodriguez and Thornton 2008).

More recent studies indicate, however, that co-inoculation is a viable option with multiple effects on wine composition. As summarised in Table 2, acetate and ethyl esters, acids and alcohols can show increase, decreases or no change. Abrahamse and Bartowsky (2012) investigated the influence of co-inoculation on Shiraz composition and reported that the presence of bacteria during AF did not affect the efficiency of primary fermentation and produced a distinct volatile profile along with differences in anthocyanin and pigmented polymer composition when compared with wines produced with bacteria inoculated late or post-AF. The authors also reported that

Table 2 Volatile compounds reported to change when yeast and malolactic bacteria are co-inoculated, compared to inoculation post-AF

Volatile compound measured	Shiraz[a]	Tempranillo[b]	Merlot[b]	Riesling[c]	Cabernet Franc[d]
Acetate esters					
Ethyl acetate	⇔	⇑	⇑/⇔	⇓	⇑
2-Methylpropyl acetate	⇔	⇓	⇑	NR	NR
2-Methylbutyl acetate	⇑	NR	NR	⇔	NR
3-Methylbutyl acetate	⇑	⇔	⇓/⇔	⇑	⇔
Hexyl acetate	⇑	⇓	⇔	⇑/⇔	⇑
Phenylmethyl acetate	NR	⇓	⇓	NR	NR
2-Phenylethyl acetate	⇔	⇓/⇑	⇑/⇓	⇓/⇔	⇓
Ethyl esters					
Ethyl propanoate	⇓	NR	NR	⇓	NR
Ethyl 2-methylpropanoate	⇓	NR	NR	NR	NR
Ethyl butanoate	⇔	⇑	⇑/⇔	⇑	⇑
Ethyl hexanoate	⇔	⇔	⇑/⇔	⇔/⇑	⇑
Ethyl lactate	⇑	⇑	⇑	⇑	⇑
Ethyl ocatnoate	⇑	⇔/⇑	⇑/⇔	⇔	⇑
Ethyl decanoate	⇔	⇔	⇔	⇔	⇔
Diethyl succinate	NR	⇑	⇑	⇔/⇑	⇑
Acids					
Hexanoic Acid	NR	⇑	⇑/⇔	⇔/⇑	⇔
Octanoic Acid	NR	⇔/⇑	⇑/⇔	⇔	⇔
Decanoic Acid	NR	⇔	⇔	⇔/⇑	⇔
Alcohols					
2-Methylpropanol	⇔	NR	NR	⇑	⇔
Butanol	⇔	NR	NR	⇔	NR
2-Methylbutanol	⇔	NR	NR	⇑/⇔	NR
3-Methylbutanol	⇔	NR	NR	⇔	⇔
Hexanol	⇔	⇑	⇑/⇔	⇔	⇔
2-Phenylethanol	⇔	⇑	⇑/⇓	⇔	⇓

⇑/⇔ = increased in one strain, but no difference with the other yeast strain

NR not reported

[a] Abrahamse and Bartowsky (2012)

[b] Cañas et al. (2012)

[c] Knoll et al. (2012)

[d] Cañas et al. (2014)

the time point at which bacteria were inoculated into the must or wine did not affect the final concentration of acetic acid. Differences in yeast and bacterial metabolism at various stages in fermentation were purported to be the drivers for changes in volatile chemical composition (Abrahamse and Bartowsky 2012).

In a large study, Guzzon et al. (2012) compared co-inoculation with post-AF inoculation using five pairs of commercial oenological starters, in four red musts with low nitrogen content. Whilst co-inoculation caused a slowdown in the activity of yeasts, the MLF was completed, whereas some failures in the degradation of malic acid were observed in the sequentially inoculated fermentations. The authors did note an increase in acetic acid production with some of the pairs tested, but in most cases, this was minor (only two of the 20 combinations were above the sensory threshold of acetic acid in wine of 700 mg/l) and not in all four of the tested wines.

By contrast, Cañas et al. (2012, 2014) noted that co-inoculation of yeast and *O. oeni* in Tempranillo, Merlot and Cabernet Franc resulted in reduced fermentation time with no or minimal increase in volatile acidity. A lower concentration of the volatile phenol 4-vinylguaiacol was also observed in the Cabernet Franc (Cañas et al. 2014), whilst volatile phenols were lower in Tempranillo wines but higher in some Merlot wines produced by co-inoculation (Cañas et al. 2012). The

pairing of yeast strain VRB and *O. oeni* C22L9 in Merlot wine was consistently linked with an increase in volatile phenols (Cañas et al. 2012) highlighting the strain and juice dependence of this outcome. Wines produced by co-inoculation were also lower in biogenic amines, particularly cadaverine and tyramine, whereas significant increases in hexanoic and octanoic acid production occurred in co-inoculated Tempranillo fermentations whilst only some of the Merlot fermentations had increased octanoic acid (Cañas et al. 2012). These latter increases may be biologically significant as medium chain fatty acids are known inhibitors of LAB growth and malolactic activity and fatty acids can have a synergistic inhibitory effect when present together (Lonvaud-Funel et al. 1988; Capucho and San Romão 1994).

Delving further into the interactions between yeast and bacteria, Rossouw et al. (2012) studied co-inoculations of *S. cerevisiae* and *O. oeni* in synthetic must. Many genes were differentially expressed when the yeast transcriptome was compared between co-inoculated and yeast-only fermentations. Some of these genes appeared to be responding to chemical changes in the fermenting must that were linked to bacterial metabolic activities. In general, there was an up-regulation of genes involved in nutrient uptake such as transporters for amino acids (*DIP5*), sulfate (*SUL1*), hexoses (*HXT13*, *HXT17*) and ammonium (*MEP1*, *MEP2*) (Rossouw et al. 2012). Up-regulation of several stress response genes might indicate a direct response of the yeast to the presence of a competing organism (Rossouw et al. 2012). *O. oeni* may also release antagonistic chemicals since the yeast gene *FYV12*, required for survival upon exposure to K1 killer toxin, was also up-regulated in the yeast during co-inoculation (Rossouw et al. 2012). It is possible that the bacteria inhibit yeast metabolism and/or physiology or that there is a direct competition for nutrients.

Further research is required to determine how best to minimise acetic acid production whilst utilising the benefits of co-inoculation of LAB with yeast during AF. An examination of a large matrix of yeast and bacterial combinations and juices will help delineate the propensity of given combinations to yield undesirable and desirable processing, sensory and compositional outcomes and thereby provide some guidance for winemakers in strain selection.

LAB other than *O. oeni*

In recent years, there has been an increased interest in other LAB species and their ability to carry out MLF either alone or alongside *O. oeni*. Although *O. oeni* is by far the most studied LAB species of oenological origin and the most used initiator of MLF, several studies have stated the potential of the facultatively homofermentative *L. plantarum* (du Toit et al. 2011; Lerm et al. 2011; Bravo-Ferrada et al. 2013) and more recently *Pediococcus damnosus* (Juega et al. 2014) as starter cultures. Although LAB other than *O. oeni* are generally still considered to be spoilage organisms, they have been isolated from wines that were not considered spoiled (Lerm et al. 2011; Bravo-Ferrada et al. 2013; Juega et al. 2014). *L. plantarum* strains can grow in wines (du Plessis et al. 2004; Lopez et al. 2008a); however, in a mixed culture fermentation, *O. oeni* often takes over as ethanol content increases (Lopez et al. 2008a; González-Arenzana et al. 2013a, b). There are some *L. plantarum* isolates that also display the ability to survive harsh wine conditions and commercial cultures of this LAB species are available. More recently, a mixed bacterial starter culture (*O. oeni* and *L. plantarum*) has also been released (Lerm et al. 2011). The use of *Lactobacillus* sp. increases the pool of potential desirable traits that can be selected to tailor the end result, such as an increase or decrease in individual esters (Sumby et al. 2010). More in-depth studies of viability during MLF as well as the oenological properties of *L. plantarum* and alternative LAB isolates at industrial scale vinifications, therefore, have the potential to improve MLF outcomes.

Strain improvement

Recombinant methods

This technology involves the expression of foreign genes or overexpression or deletion of native genes in the organism of interest. Such expression is often achieved via introduction of a plasmid; however, unlike other LAB, transformation is problematic in *O. oeni* and researchers have had only limited success. The first report of successful electroporation of the plasmid pGK13 into *O. oeni* strains PSU-1, ML-34 and 19CI (Dicks 1994) is yet to be confirmed in other laboratories. Another method which used ethanol as a membrane-fluidising agent prior to transformation led to the successful introduction of a foreign vector (pGID052) encoding a truncated form of the ClpL2 protein into *O. oeni* ATCC BAA-1163 (Assad-García et al. 2008). However, pGID052 has a low copy number and this work has also not led to increased publication of molecular transformations in *O. oeni*. A further plasmid, pCB42, was isolated and found to be capable of replicating successfully within *O. oeni*; however, the transformation frequency was low (Eom et al. 2010). It is clear that novel strategies are needed for successful transformation in *O. oeni*. For example, strains of *O. oeni* have been shown to contain several native plasmids (Shareck et al. 2004), some of which may have higher copy numbers and are able to replicate themselves within *O. oeni*. Generation of a custom expression vector using the origin of replication from these native plasmids, genetic markers and the genes of interest may generate a plasmid more effective for overexpression.

Another method of expression of foreign genes is transduction, which is the process by which bacteriophages carry bacterial genes from one cell to another. As bacteriophages

can be one of the causes of a failed MLF (Davis et al. 1985), this is theoretically possible in *O. oeni*; however, the mechanisms of infection have not yet been fully elucidated and this method needs further research. The final method of genetic manipulation is the use of conjugative transposons. Problematically, the current methods of conjugation for *O. oeni* do not allow for gene replacement, as the transfer frequency is lower than the recombination frequency (Zúñiga et al. 2003; Beltramo et al. 2004b).

It is difficult to apply any of these techniques to *O. oeni*, as they are most beneficial when allowing the removal or addition of genes, and therefore, the individual genes associated with optimization of stress resistance would first need to be identified. For example, stress resistance during MLF involves multiple genes at multiple loci (Table 1), which are broadly distributed throughout the genome making targeted genetic manipulation highly complex. The main stresses affecting MLF interact at a physical level and potentially also at a genetic level. If strains are improved for only a single stress, it is possible that their ability to survive other stresses will be adversely affected.

Directed evolution

Non-recombinant methods of improving bacterial strains for industrial processes are gaining increased attention. One method, directed evolution (DE) also known as adaptive evolution, has been applied successfully in the species of *Lactobacillus* (Teusink et al. 2009; Bachmann et al. 2012; Zhang et al. 2012; Wu et al. 2014). DE is a genome-wide method that manipulates and diversifies a population without the necessity for detailed knowledge of gene networks. The premise of DE is that an organism will adapt to its environment when placed under continuing stress conditions. Mutations that allow the organism to thrive and propagate under the specific stress are selected for by a gradual increase in the stress condition (Dragosits and Mattanovich 2013). The process by which this occurs is not yet fully understood with three possible models presented in the literature: firstly, the directed mutation model, in which mutations might target specific genes to relieve the stress factors; secondly the hypermutation model, in which mutation rates increase genome-wide so that both adaptive and non-adaptive mutations are stimulated; and finally, the cryptic growth model suggests that mutation rates do not increase at all, but that extra DNA replications simply let the normal rate of mutation acting on multiple DNA copies to give the appearance of an enhanced mutation rate (Foster 1999; Rosenberg 2001). An advantage of DE is that when an organism is adapted within its primary niche, it lacks the deleterious side effects of modern recombinant techniques (Sauer 2001). Genetic changes or mutations within a given gene will potentially affect the viability and productivity of that organism in unforeseen ways. Therefore, the major benefit of DE is that the organism must stay viable and functional throughout the process or it will simply vanish from the population.

O. oeni has been shown to be a rapidly evolving organism (Yang and Woese 1989), and this, combined with the inhibitory properties of wine, may in fact make it a perfect candidate for this method of optimization. Genome data from *O. oeni* PSU-1 (Mills et al. 2005) has revealed that *mutS* and *mutL*, which encode two key enzymes in the mismatch repair (MMR) pathway (Makarova et al. 2006; Makarova and Koonin 2007), are absent in *O. oeni*. The correction of mismatches by MutS and MutL decreases the spontaneous mutation rate of a species; therefore, a defect in the MMR system leads to an increase in the mutation frequency. One possible reason for the loss of MMR is that a high mutation rate generated beneficial mutations during adaptation to a restrictive environment such as wine (Marcobal et al. 2008). An increased mutation rate is initially beneficial; however, once an organism has adapted to a particular environment, an increased mutation rate can lead to a loss of fitness due to an increased load of deleterious mutations within the population (Taddei et al. 1997). In DE, mutations that cause deleterious effects on fitness arise due to genome erosion more often than advantageous mutants (Perfeito et al. 2007). When this occurs, a population remains viable within its specific environment, but its fitness is reduced for growth in any other environment and is therefore highly specialised. The application of this approach to improve *O. oeni* growth and MLF ability has been recently investigated by growing a continuous culture of *O. oeni* with increasing ethanol concentration over many generations (Betteridge et al. 2013). The evolved population not only survived under higher ethanol conditions but completed MLF in MRS with 15 % ethanol in almost half the time of the parent strain. This study confirmed that DE can be successfully used as a technique for developing new strains and is a promising method to improve other LAB strains for use in wine.

Technological advances

Immobilised cells

The use of high densities of immobilised cells to carry out MLF has been suggested as a method to combat the effect of high ethanol or low pH (mainly in cool climate wines) on the bacterial cells. Another advantage of using immobilised cells is that they can be reused for several cycles. The study of immobilisation methods for conducting MLF has increased over the years as an awareness of the impact that climate change is having on grape and wine quality grows. Increased heat during grape maturation has being linked to an increase in sugar content and decomposition of acid (Jones

et al. 2005; Mira de Orduña 2010; Bock et al. 2011). This leads to higher pH and also higher ethanol levels in the final wine. High temperatures may also inhibit malolactic bacterial growth particularly in high alcohol wines. Many matrices for bacterial immobilisation have been proposed, and the advantages and disadvantages of each are still under debate.

Two main immobilisation methods have been utilised: encapsulation of the bacterial cells (Spettoli et al. 1982; Crapisi et al. 1987; Guzzon et al. 2012; Rodríguez-Nogales et al. 2013) and attachment/adsorption onto a support (Maicas et al. 2001; Agouridis et al. 2008; Genisheva et al. 2013). Two recent encapsulation studies have used Ca-alginate microbeads coated with an organo-silica membrane (Guzzon et al. 2012) and polyvinyl alcohol-based hydrogel 'Lentikats®' (Rodríguez-Nogales et al. 2013). Evaluation of the MLF capabilities of entrapped *O. oeni* cells in Lentikats® matrix showed that the relative percentage of malic acid degradation at 14 % ethanol was increased by using immobilised cells. Relative degradation at 14 % ethanol was also increased using immobilised cells with the added challenge of either low pH or increased temperature. Furthermore, the cells could be used five times without loss of activity. Further studies will determine the full scope of the impact on wine composition and sensory properties when using this method to immobilise cells and to conduct MLF.

Immobilisation does not always prove advantageous when applied to wine. Guzzon et al. (2012) performed 50 l MLF trials using cells encapsulated in Ca-alginate microbeads. Both the effect of yeast and bacterial co-fermentation and sequential fermentation were tested, and in each case, the free cells degraded malic acid faster than the immobilised ones. However, one advantage when lysozyme was added to control the growth of unwanted LAB was that the immobilised cells could still degrade the malic acid, whereas the free cell fermentations were inhibited (Guzzon et al. 2012). More recently, Servetas et al. (2013) have reported the use of a two-layer composite biocatalyst containing both yeast and LAB for simultaneous AF and MLF in wine. The yeast and LAB species were physically separated by the use of delignified cellulosic material and a starch gel as a method to reduce species competition. Whilst improved performance was reported, further investigation comparing wines fermented with free cells vs. immobilised cells was not reported (Servetas et al. 2013) and is necessary before this technique can be adopted.

Immobilised MleA enzyme

Although numerous studies have reported the use of free or immobilised LAB to conduct MLF, direct bioconversion with free or immobilised MleA has scarcely been studied. This is most likely because the process requires the availability of both manganese (Mn^{2+}) and NAD^+, which act as cofactors (Schümann et al. 2013). Immobilised enzymes allow for continuous processing, which in turn can lead to lower production costs and energy consumption. A further benefit when compared with free enzymes in solution is that immobilised enzymes are more robust and are often more stable and resistant to environmental changes (Krajewska 2004). Wine naturally contains Mn^{2+} at a concentration that is considered sufficient for enzyme activity (Formisyn et al. 1997). However, the optimal pH for MleA activity is 6.0 (Schümann et al. 2013), and the enzyme still requires the addition of NAD^+ to the reaction system.

A membrane reactor using free *O. oeni* enzyme has also been reported (Formisyn et al. 1997). The design involved the use of two compartments separated by a polysulfone membrane, one for MleA and NAD^+ cofactor and one for the wine. This arrangement generated a pH gradient to ensure activity of the enzyme; however, this also resulted in a strong dilution of wine in the system. One potential solution to this problem is the use of synthetic enzyme cascades. For example, Köhler et al. (2013) reported a method for efficient concurrent tandem catalysis that could potentially be used to enable utilisation of Mle catalysis with regeneration of NAD^+. Given the significant degree to which the physical and chemical properties of the support influence catalytic performance, an appreciation of materials science, beyond the scope of this review, is required for the successful development of an immobilised enzyme catalyst. For more information on microcapsules, refer to Dähne and Peyratout (2004), and for the one-step multicomponent encapsulation method, see Chen et al. (2008).

Monitoring MLF

Many winemakers regard the timely determination of the completion of MLF to be of primary importance so that the wine can be promptly stabilised. There is concern that any LAB remaining in wine after the end of MLF could promote the production of undesirable volatile compounds and the formation of biogenic amines. Therefore, following on from MLF, the winemaker will often remove or inhibit the bacterial biomass, typically through the addition of sulfites. Several methods are available to monitor and identify the completion of MLF, such as enzymatic analysis kits, paper chromatography, thin layer chromatography and high-performance liquid chromatography. As all of these methods require time and resources in the winery for periodic sampling of tanks or barrels and for analysis, there is increased interest in monitoring MLF in real time. Various methods have been proposed including the use of electrochemical biosensors (Gamella et al. 2010), a wireless sensor bung (Di Gennaro et al. 2013) and ultrasonic velocity measurements (Novoa-Díaz et al. 2014).

Electrochemical biosensors can provide up to 500 measurements using the same biosensor; however, the need for manual sampling and dilution of the wine sample to ensure a liner response make this system more time consuming (Gamella

et al. 2010). A real-time solution is proposed through the use of a wireless sensor bung (WSB) system based on the detection of an increase in pH during MLF (Di Gennaro et al. 2013). Whilst the results of this preliminary study are very promising by showing good agreement between malic acid degradation and pH increase, the means by which the endpoint of MLF is defined, other than a plateau in the rise in pH, needs to be determined. This is particularly important in order to rule out the possibility that pH has stopped rising merely because the MLF has stuck. The same can be said of the use of ultrasonic velocity measurements to monitor changes in malic acid and lactic acid concentrations during MLF (Novoa-Díaz et al. 2014). Whilst possibly more sensitive, the ultrasonic approach also relies on a cease in the change of the measurement to indicate an end of MLF or a stuck MLF. In each case, these methods do not obviate the need for the winemaker to check the MLF and perhaps conduct traditional malic/lactic acid determinations off-line, but they do at least reduce the number of barrel samplings to potentially a single one. Both systems show potential for use as decisional support systems (DSS), indicating to the winemaker the correct time to conduct the final laboratory analysis. Further investigation of both of these methods will also ensure that they are flexible and accurate enough to be applied to a range of wine styles, fermentation and cellar conditions.

Expression of malolactic enzyme in yeast

In yeast, the malic enzyme predominately converts malic acid into pyruvic acid, which is further metabolised to ethanol and carbon dioxide under fermentative conditions via the malo-ethanolic (ME) pathway (Volschenk et al. 2003). Due to the lack of a specific malate transporter, uptake by *S. cerevisiae* is considered to occur by simple diffusion. In addition, the *S. cerevisiae* malic enzyme has low substrate affinity (Husnik et al. 2007) and is subject to catabolite repression (Redzepovic et al. 2003), resulting in this yeast having a limited ability to metabolise extracellular malate (Volschenk et al. 1997a, b). Two tactics have been utilised to express malolactic enzyme in *S. cerevisiae*: co-expression of the malate permease gene and surface display of malolactic enzyme. The malate permease gene (mae1) of the fission yeast *Schizosaccharomyces pombe* has been co-expressed with either the *L. lactis* malolactic gene (*mleS*) or the *O. oeni* malolactic gene (*mleA*) in *S. cerevisiae* (Volschenk et al. 1997a; Husnik et al. 2006, 2007). The industrial *S. cerevisiae* wine strain ML01 was so constructed by insertion of a malolactic cassette containing the malate transport (*MAE1*) gene from the yeast *S. pombe* (Grobler et al. 1995) and the malolactic enzyme (*mleA*) from *O. oeni* (Husnik et al. 2006). Both genes were constitutively expressed under control of the *S. cerevisiae* PGK1 promoter and terminator sequences. ML01 can complete MLF within the first 9 days of AF.

Main et al. (2007) investigated malic acid reduction using ML01 and two naturally selected yeast (ICV-GRE and 71B). Malolactic bacteria were also added at the end of an additional fermentation with ICV-GRE and general wine parameters were measured. Although ML01 successfully converted all the malic acid to lactic acid, there was also an increase in SO_2 production when compared to the other fermentations (Main et al. 2007). The other yeast investigated reduced the malic acid content in wine by 18 % (ICV-GRE) and 33 % (71B). The wine fermented with ICV-GRE+*O. oeni* had lower lactic acid and titratable acidity than ML01 because of malic consumption by the yeast before MLF, presumably via the ME pathway (Volschenk et al. 2003) as no L-lactic acid was produced at the end of AF and the final ethanol concentration was increased when compared to ML01. Expression of *MleA* in a low SO_2-producing strain has not been reported. Additionally, expression of malolactic enzyme in yeast affected the flavour profile of the wine resulting, for example, in decreased mouthfeel due to decreased ethyl lactate metabolism (Husnik et al. 2007).

In an alternative approach, the α-factor secretion signal encoding sequence has been used to display the mleA enzyme from *O. oeni* on the surface of *S. cerevisiae* cells (Zhang et al. 2013). The resulting yeast strain could degrade 21 % of L-malate after only 12 h; however, no details were provided on the capabilities of this strain over a longer period of time. The authors also used the laboratory yeast strain AH109 whose oenological properties have not been specified. Thus, although this is a promising solution, it requires further validation in the genetic background of a wine yeast.

Each of these methods offer promise but need to have their full impact on wine quality assessed. Of course, a further feature of these systems, which may be unattractive to some producers, is the fact that they rely on recombinant organisms.

Conclusion

Through ongoing research, a better understanding of the important changes occurring during MLF and how to use MLF to influence wine style will be achieved. Both the choice of LAB strain and timing of bacterial inoculation can be used to modulate modifications in wines from MLF aside from deacidification such as buttery or fruity-berry aromas and improved mouthfeel attributes. The ability of LAB to conduct MLF and produce desirable aroma compounds has been shown to differ between both LAB genera and across strains of the same species. An increased understanding of the enzymes and how they are involved in changes in wine composition during MLF along with increased understanding of the genes and metabolic pathways involved in adaptation to the stressful wine environment will lead to novel approaches for the characterisation and development of starter cultures.

Additional new strategies such as co-inoculation, using mixed LAB cultures, directed evolution of LAB strains, immobilised cells or immobilised enzymes have the potential to reduce the duration of MLF and risks associated with sequential MLF. It is becoming clear that these techniques can contribute positively to the aroma of wine without the excessive production of acetic acid. Increased knowledge of safety considerations will also impact strain selection and how we view MLF, and careful selection and use of selected strains will enable more reliable and safe MLF. Comparative genomics and transcriptomics will increase our understanding of regulatory mechanisms and, in turn, help improve MLF performance and success. Increased analysis of the coordinated expression of genes that alter specific cellular processes will help identify molecular markers for the selection of improved strains. Additionally, an increased understanding of the genes involved in production of undesirable aromas or compounds that are of safety concern to consumers, such as biogenic amines and ethyl carbamate, will enable improved screening of new strains prior to industrial use.

Future studies may involve the use of enzyme cascades to enable direct application of malolactic enzyme to wine. As is evident by recent studies, the use of different genera of LAB has the potential for future use in starter culture preparations. This will increase the genetic diversity of inoculated LAB and, if properly tested, could provide novel and desirable outcomes. Alternatively, the use of DE to evolve LAB strains shows much promise to develop new strains better suited to growth in wine and to provide an increased understanding of the metabolic processes that are important during MLF, thereby informing new strategies for generating and screening evolved strains. Additionally, new technologies for monitoring MLF in real time will enable winemakers to monitor the process more efficiently and reduce the incidence of wine production delays. This will have a knock-on effect in allowing subsequent fermentations to proceed as scheduled without the need to delay fruit harvest or wine processing. Overall, cost reductions associated with reliable MLF and more efficient wine production will ensure a better quality product.

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Review

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Improving *Oenococcus oeni* to overcome challenges of wine malolactic fermentation

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***Oenococcus oeni* is crucial for winemaking, bringing stabilization, deacidification, and sensory impacts through malolactic fermentation (MLF) to most wine styles. The poor nutritional make-up of wine together with typically low processing temperatures and pH and high ethanol content and sulfur dioxide (SO_2) hinder *O. oeni* growth and activity. Production delays and interventions with starter cultures and nutritional supplements have significant cost and quality implications; thus, optimization of *O. oeni* has long been a priority. A range of optimization strategies, some guided by detailed characterization of *O. oeni*, have been exploited. Varying degrees of success have been seen with classical strain selection, mutagenesis, gene recombination, genome shuffling, and, most recently, directed evolution (DE). The merits, limitations, and future prospects of each are discussed.**

The benefits and current limitations of MLF

The removal of L-malic acid, one of the major carbon sources in wine, during MLF (Box 1) reduces the risk of the growth of spoilage microorganisms. Also, MLF ameliorates acidity and further contributes compounds that result in wine of increased aroma and flavor complexity. Most well described is diacetyl; however, the production of esters, alcohols, and other carbonyl compounds contribute to the buttery, spicy, vanilla, and smoky notes as well as a softer, fuller mouthfeel seen in wines post-MLF [1,2]. Different strains, both in nature and available commercially, produce different profiles of sensory compounds [2]. *O. oeni* generally occurs naturally in wines and thus spontaneous MLF during or after alcoholic fermentation is common. Many wineries also inoculate with commercial starter cultures of bacteria after alcoholic fermentation is complete, to help ensure an efficient and timely MLF; however, even with starter cultures the growth of lactic acid bacteria (LABs) is often inhibited and thus MLF stalled (Box 2).

Malolactic fermentation and the growth of *O. oeni* are clearly inhibited by several of the physiochemical properties of wine. The four main wine parameters inducing stress and affecting MLF are ethanol (can exceed 16% v/v), low pH (typically less than 3.5), SO_2 (over 10 mg/l), and low temperature (can be below 12°C) (Table 1). These stressors have various cellular targets and mechanisms, which often work in combination to produce a more severe impact on growth or the enzymes involved in MLF. For example, in exploring the individual impacts of acid (pH 5.5 to pH 3.5), ethanol [0–10% (v/v)] or cold shock (30°C to 14°C) on membrane fluidity [3], near-total loss of cell viability could be demonstrated after only 30 min of exposure to a combined wine-like acid (pH 3.5) and ethanol [10% (v/v)] environment.

Improved tolerance of such abiotic stress would appear to be beneficial in increasing the efficiency of MLF. Experimental evidence supports this, since *O. oeni* strains performing faster MLF also show increased relative expression of several stress response genes [4]. Similarly, the better-performing strains also showed an increased expression of *mleA* (the gene encoding malolactic enzyme), albeit its importance was greatest for determining the initial MLF velocity.

Strategies to improve the tolerance of *O. oeni* to the harsh physiochemical properties of wine

Methods for strain improvement can be divided into two main approaches, recombinant and nonrecombinant, with each having its own advantages and disadvantages. Recombinant techniques are usually of high precision, often being focused on the addition or deletion of specific genes. Their use requires an intricate knowledge of gene identity as well as an understanding of functions and interactions before manipulation. By contrast, nonrecombinant approaches often require little prior knowledge of the genetic basis of a trait; however, they can require time-consuming screening and are random or can have pleiotropic effects. In evaluating these approaches, it is important to consider applications of LABs beyond the wine industry. LABs are widely used in the production of fermented foods and constitute most of the volume and value of bacterial starter cultures [5]. This review draws on examples from other research in addition to the work available for wine, to suggest possible strategies for improving the stress tolerance of *O. oeni*.

Molecular genetics

Overexpression of native genes or expression of foreign genes in *O. oeni* may be achieved via the introduction of

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Box 1. LAB and MLF

LAB are Gram positive, microaerophilic, and characterized by the formation of lactic acid as a primary metabolite of sugar (glucose) [48]. The most common isolates from wine are in the genera *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Oenococcus*. The latter is named from the Greek *oinos*, meaning wine. Of the three *Oenococcus* species, *O. oeni* is associated with wine, is non-motile and asporogenous with ellipsoidal-to-spherical cells usually arranged in pairs or short chains, and has an optimal growth range between 20°C and 30°C and pH 4.8 and pH 5.5 [49]. While lactobacilli predominate on grape skins, the *O. oeni* population increases throughout alcoholic (yeast) fermentation to typically become the only species found in wine at the completion of MLF. For this reason and because of its desirable flavor effects, *O. oeni* is the preferred species for this process, which is applied to most red, aged white, and sparkling wine styles [50,51].

MLF is technically not a fermentation but the enzymatic decarboxylation of the dicarboxylic L-malic acid to the monocarboxylic L-lactic acid by LAB (Figure I) in a reaction requiring NAD^+ and Mn^{2+} as cofactors and devoid of free intermediates [52]. Although MLF increases the pH of the wine, this increase does not stimulate the growth of *O. oeni*. The three genes responsible for this fermentation are present in a single cluster, with *mleA* (encoding malolactic enzyme) and *mleP* (encoding malate permease) on the same operon and *mleR* encoding the regulatory protein transcribed in the opposite direction. Maximal activity of MleA is seen at pH 5.0 and 37 °C and is noncompetitively inhibited by ethanol, underscoring the less-than-ideal nature of the wine environment.

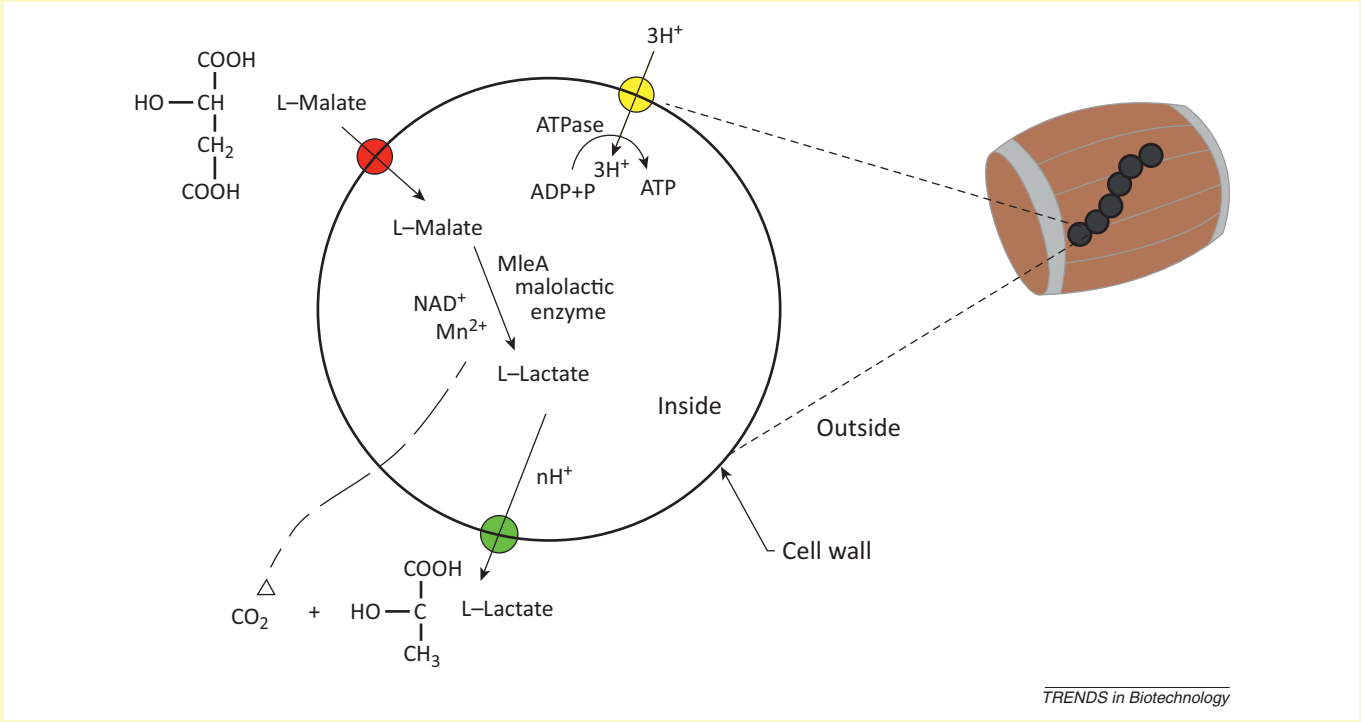


Figure I. Malolactic fermentation (MLF) involves the active transport of L-malic acid into the cell by malate permease (*MleP*; red). Decarboxylation of L-malic acid is facilitated by the malolactic enzyme (*MleA*) and requires NAD^+ and Mn^{2+} as cofactors before lactate is finally transported out of the cell (green). This process is regulated by a regulatory protein, MleR. The increase in the intracellular pH by MLF confers an energy advantage to the cell. The resulting increase in the proton motive force across the cell membrane combined with specific ATPases (yellow) facilitates the production of ATP. Adapted from [53].

plasmids, as is used widely in other microbes. Transformation requires either the chemical generation of competent cells or the forced transfer of DNA via, for example, electroporation. Unlike in other LABs, transformation is difficult in *O. oeni*. Although electroporation was used successfully to transform the plasmid pGK13 into *O. oeni* strains PSU-1, ML-34, and 19CI [6], this transformation has not been confirmed in other laboratories. A later electroporation protocol using ethanol as a membrane-fluidizing agent succeeded in the introduction of a foreign vector encoding a truncated form of the ClpL2 protein into *O. oeni* ATCC BAA-1163 [7]. However, this result has not yet led to an increase in published accounts of molecular transformations of this bacterium, possibly due to the low copy numbers of this plasmid (pGID052) [8]. Plasmid copy number is important in gene replication as an increased number increases gene dosage and therefore product yield [9].

As a way forward, *O. oeni* contains several native plasmids [10], some of which may have higher copy numbers and are able to more successfully replicate themselves within *O. oeni*. Using the origin of replication from such native plasmids, modification and the inclusion of genes of interest and markers may generate a plasmid more effective for future overexpression work [11].

Another method of expression of foreign genes in *O. oeni* is transduction, the process by which bacteriophages carry bacterial genes between cells. Certainly bacteriophages can infect *O. oeni*, where they can be the cause of failed MLF [12], but the mechanisms of infection have not yet been fully elucidated. Further research is needed to fully assess the potential of this method and see it developed to a stage where it can be routinely used for this bacterium.

The final method of genetic manipulation, conjugation, is the direct horizontal transfer of genetic material between two cells, usually on a plasmid or other mobile

Box 2. Problems caused by stuck MLF

Even before MLF commences, problems can arise. In an effort to avoid protracted or 'stuck' MLF or encourage spontaneous MLF, the addition of protective amounts of SO_2 may be delayed, thereby increasing the risk of spoilage by yeast or bacteria or oxidation of the juice/wine. Because of their more precarious state, such batches demand closer monitoring by the winemaker. Post-alcoholic (primary) fermentation there are many factors that can inhibit MLF (see text). Such inhibition can result in a fermentation process that is protracted, in some cases for months, or becomes stuck and fails to achieve complete catabolism of the malic acid present in the wine. This delays the stabilization and preparation of the wine for sale. Since wines are rarely sterile filtered, packaging wine with residual malic acid carries a risk of spoilage organisms growing to produce haze, off-odors, and/or dissolved CO_2 in the bottle. Solutions include one or more re-inoculations with fresh bacterial starter culture, addition of nutrients, removal of inhibitors (e.g., SO_2), warming of the wine, or abandonment of the MLF with stability sought through greater SO_2 addition, which itself compromises quality.

genetic element. Conjugative transposons are mobile genetic elements capable of independent replication and insertion of a copy within the genome. An example is the conjugative transposon Tn*6098*, which encodes the capacity to utilize α-galactosides in *Lactococcus lactis* strains isolated from plants [13]. The transposon was characterized and transferred into a strain of *L. lactis* derived from milk, enabling the recipient strain to grow well in soy milk (a substrate rich in α-galactosides) but retaining the flavor-forming capabilities important in dairy *L. lactis* [13]. Problematically, the current methods of conjugation for *O. oeni* do not allow gene replacement, as the transfer frequency is lower than the recombination frequency [14].

If applying any of these molecular techniques to *O. oeni*, it is clear that they would be most beneficial when allowing the removal or addition of genes. Consequently, the individual genes associated with enhanced stress resistance need to be identified first. The main stresses affecting MLF (high ethanol, low pH, low temperature and SO_2) interact at a physical level and potentially also at a genetic level. This is likely to make targeted genetic manipulation highly complex. Improvement for an individual stress may also adversely affect the organisms' ability to survive a different stress. A more detailed characterization of *O. oeni* is still needed.

While the technical problems associated with molecular genetic manipulation of *O. oeni* are numerous, another important issue relates to the purported opposition and legislative blocks to the use of genetically modified organisms (GMOs) in food and beverage production. To genetically modify organisms for use within the food industry, certain strictures typically apply. Selection markers must be food grade and not based on antibiotics [15] and the genetic elements introduced should be derived from plasmids or genes of the same bacterial species to constitute a 'self-cloning' system [15]. However, even when these conditions are met the pressure from some consumer groups as well as government regulations mean that even food-grade GMOs can be difficult to apply industrially [16]. Methods of strain optimization that take advantage of the diversity of existing microflora and improve strains by nonrecombinant techniques are therefore more practical at the present time for industrial applications.

Classical strain development

The oldest and simplest method of identifying superior strains is to take advantage of natural diversity, isolating strains from nature and screening them for desired traits. Originally fermentations were typically optimized through inoculation via a small quantity of a previously performed, successful fermentation. These successive inoculations have created populations of LABs that are suited specifically to the particular fermentation environment. *O. oeni* is a prime example of a LAB evolved to occupy a very specific ecological niche, explaining its relative tolerance to the fluctuating environment of alcoholic fermentation and the harsh conditions of wine in which it must survive. Intraspecific diversity among different strains isolated from wineries worldwide has been observed [17,18], implying diversity among the specific tolerances to different stressors facing these strains.

With the advent of the 'omics' era, whole-genome sequencing has produced a torrent of genomic information. An increasing library of LAB genomes has allowed accurate representations of evolutionary pathways of the LABs as well as comparative and functional genomics [19]. According to the NCBI, at time of publication there are 58 *O. oeni* genomes publically available and in various stages of completeness. This has allowed a modern twist on this age-old method of classical strain selection. Strains are sequenced and the genetic traits shown to be beneficial for specific fermentations can be identified and strains chosen for a given application based on this.

Bioinformatics tools for sequence analysis can identify specific components such as genes encoding enzymes

Table 1. Key inhibitors in wine of MLF and their mechanisms of inhibition

Inhibitor	Comment	Optimal condition	Typical wine conditions	Inhibitory mechanism	Refs
Ethanol	Produced during alcoholic fermentation	Up to 5% stimulates growth	12–15% (v/v)	Disrupts cell membrane structure and alters fluidity	[54]
Low pH	Acidity from grape berries and winemaker intervention	4.8–5.5	2.5–3.5	Reduces growth and malolactic activity	[55]
Low temperature	Wineries often rely on ambient temperature for MLF	25°C	12–20°C	Affects growth rate and increases lag phase	[48]
SO_2	Produced by yeasts and added to prevent spoilage during processing	0 mg/l	10–70+ mg/l	Reduces ATPase activity, decreases cell viability	[56]

required for the biosynthesis of amino acids. Strains can then be selected based on their ability to form amino acids that are the precursors of desirable volatile aroma compounds [20,21]. *O. oeni* AWRIB429, consistently shown to impart more fruit-driven characters to wines, possesses novel genes that are potential glycosidases [22]. This finding could lead to strain selection based on specific desired flavor attributes. In addition, the specific genes linked to high performance in MLF were sought by interrogation of the genome sequences of *O. oeni* that exhibit faster MLF. Although no definitive trends were found, some statistical evidence suggested that changes in a specific portion of the genome may be responsible for such attributes [23]. The other major benefit of an increasing library of genomes is the possibility of metabolic modeling. Metabolic modeling takes advantage of the comprehensive data available on metabolism and genetics to engineer specific changes in metabolic pathways. This approach has been reviewed recently elsewhere [24,25].

While these signs are positive, it is unlikely that the technological traits sought will be found only by relying on the natural diversity in target phenotypes. If possible, such strains would already have been identified over the centuries that *O. oeni* has been adapting to winemaking. Based on the difficulties still encountered with MLF, it is clear that the commonly used strains are not optimal but instead provide a platform on which to build with genetic improvement programs.

Mutagenesis

A simple method by which to improve the existing genetic diversity is through the use of mutagens. Mutagenesis has the potential to alter genes responsible for undesirable characteristics, flavor properties, or stress responses. Mutagenesis requires no specific genetic knowledge, just an effective screening process that can be applied after treatment with the mutagenizing agent (Box 3). The first reported instance of this method being applied to *O. oeni* has recently been published, using UV irradiation to generate a strain that has an increased fermentation rate compared with its parent [26]. Mutagenesis involves the random mutation of a genome and can lead to the possible loss of desirable properties. Mutagenesis is also hampered by its need for extensive screening of a population after a mutagen has been applied. A more efficient way to improve strains is to combine these steps. Strains can be screened and selected simultaneously via the method of directed evolution (DE).

Box 3. Mutagenizing agents and screening strategies useful in strain optimization

Rather than rely on a desired phenotype to have arisen through spontaneous mutation, the likelihood of identifying a strain bearing the property in question can be increased through the application of mutagens followed by screening of the surviving population. Mutagens include various physical (e.g., UV radiation) or chemical agents. The most common methods of chemical mutagenesis involve the use of compounds such as 1-methylsulfonyloxyethane (EMS) or 1-methyl-3-nitro-1-nitrosoguanidine (NTG). The resulting mutations are caused by DNA deletions, frameshifts, base substitutions, or rearrangements. Such mutations can be randomly distributed throughout the genome and are typically not singular, particularly with high doses or exposure times. Significant rates (e.g., ~50%) of cell death are typically sought and are likely to be the result of a combined effect of multiple deleterious mutations of nonessential genes or the mutation of one of more essential genes. Survivors are screened directly or after a period of recovery under nonselective conditions for the presence or absence of the attribute in question. Thus, bacterial strains of increased ethanol tolerance might be sought by plating or culture in the presence of appropriate concentrations of ethanol. Promising mutants are often also evaluated for the stability of their mutation as well as to ensure that no desirable traits have been lost or undesirable ones introduced.

Directed evolution

DE (Box 4) is also known as adaptive evolution, stationary phase mutation, adaptive mutation, or stress response mutation [27]. The best-known application of this technique is the long-term experimental evolution of *Escherichia coli*, ongoing for more than 55 000 generations [28]. DE has been used successfully to change the function of many organisms, including LABs. The growth of *Lactobacillus plantarum* on glycerol under anaerobic conditions is too slow to be accurately measured; however, after approximately 500 generations under continuous selection using glycerol as a limiting factor, growth rate improved by more than an order of magnitude [29]. A second example involves adaptation within 1000 generations under laboratory conditions of *L. lactis* from a natural plant niche to growth in milk [30].

Being based on natural evolutionary processes, DE lacks some disadvantages of modern recombinant techniques [31]. In addition, *O. oeni* may be ideally predisposed to exploitation of this strain improvement technique given its rapidly evolving nature and the inhibitory properties of wine. The genome sequence of strain PSU-1 [32] has

Box 4. Directed evolution (DE)

This process involves an organism mutating spontaneously and potentially adapting to a high-stress (selective) environment. Desired mutations are those that allow the organism to prosper and proliferate under the specific stress [57]. The process by which organisms adapt is not fully understood. Three possible models of adaptation are presented in the literature. The first is the directed mutation model, in which mutations might target specific genes to relieve the stress. The second is the hypermutation model, in which mutation rates increase genome wide so that both adaptive and non-adaptive mutations are stimulated. The final model is the cryptic-growth model, which suggests that mutation rates do not increase but that extra DNA replications simply let the normal rate of mutation acting on multiple DNA copies give the appearance of an enhanced mutation rate [57,58].

Within an evolving population, the organism must stay viable and functional throughout the process or it will vanish from the population. Mutations that cause deleterious effects on fitness arise more often than advantageous mutants [59]. Deleterious mutations accumulate over time within a population due to genome erosion, in which genes that are not necessary for a specific environment are lost. Thus, a population remains viable within its specific environment but the accumulation of deleterious mutants becomes sufficient that the strain is effectively crippled for growth in any other environment and is therefore highly specialized [60]. This outcome is more damaging in the case of nonrecombinant organisms, since without sexual reproduction they will not contain fewer mutations than their predecessor. Therefore, once DE yields a strain adapted to the environment further cultivation should be minimized to avoid an increase in the load of deleterious mutations that cause loss of fitness [61].

revealed a lack of the genes *mutS* and *mutL*, which encode key enzymes of the mismatch repair (MMR) pathway [19,33]. The MMR pathway is an excision-repair system that corrects base pair mismatches and the presence of *mutS* and *mutL* homologs is required for it to function [34,35]. The correction of mismatches by MutS and MutL decreases the spontaneous mutation rate of a species; therefore, a defect in the MMR system leads to an increase in the mutation frequency. A study comparing spontaneous mutation rates of *O. oeni* with those of the closely related LAB species *Leuconostoc mesenteroides* and *Pediococcus pentosaceus*, which do contain the relevant encoding genes, reveal a 100-fold increase in the rate of spontaneous mutations in response to rifampin and erythromycin [36]. One possible reason for the loss of MMR was that a high mutation rate generates beneficial mutations during adaptation to a restrictive environment such as wine [36].

There is currently no evidence of the generation of *de novo* functions via DE; however, the functionality of a pseudogene was restored in *L. lactis* through DE [30]. A common way of monitoring an evolving population is to monitor insertion sequences, generally small mobile genetic elements. Monitoring of insertion elements in a batch culture of *L. lactis* with a deleted *ldh* gene revealed that transposition of the insertion sequence IS*981* activated a second lactate dehydrogenase gene (*ldhB*) to restore lactic acid production under anaerobic conditions [37]. These insertion sequences and other mobile genetic elements found during sequencing demonstrate high plasticity within the genome, which contributes to the ongoing gene decay process, allowing easier external manipulation of the genome [38].

As a first example of the application of DE to *O. oeni*, our group has succeeded in evolving the commercial strain SB3 (Laffort Oenology) to withstand higher concentrations of ethanol [39]. Over the course of 290 days and approximately 260 generations the ethanol concentration of a continuous culture of SB3 was gradually increased from 5% to 15% (v/v). In laboratory MLF trials, key isolates catabolized 3 g/l of malic acid in ~70 h, while the parent SB3 catabolized only one-third of this amount in the same time before becoming stuck. Evolved isolates were also more ethanol tolerant, surviving 48 h of exposure to 22% ethanol, which eliminated SB3.

Genome shuffling

A possibility for removal of neutral or deleterious mutations while preserving useful mutations is genome shuffling (Box 5). Changes throughout an entire genome are possible without genome sequence information or knowledge of the genetic basis of desired traits [40]. Accordingly, genome shuffling is suited to the improvement of poorly understood and/or complex phenotypes and has major advantages over metabolic engineering [41].

Several instances of successful whole-genome shuffling in *Lactobacillus* spp. have been reported. *Lactobacillus delbrueckii* has been fused with *Bacillus amyloliquefaciens*, yielding a strain that produces more L-lactic acid from starchy wastes [42]. Additionally, three rounds of genome shuffling in *Lactobacillus* produced a strain able to attain 70% higher culture density at pH 3.8 and generate 40% more lactic acid than the wild type [43]. In work with *Lactobacillus rhamnosus* [44,45], two or three rounds of genome shuffling reduced the limit of growth from pH 4.4 to pH 3.8, increased cell growth by 45%, increased glucose consumption by 62.2%, and enhanced lactic acid production by 26% or 71%. Such results equate to improvements that previously required 20 rounds of classical strain improvement techniques [46], which is the difference between 20 years of mutagenesis and selection on the one hand and 1 year of selection and genome shuffling on the other hand. There are no published reports of genome shuffling being applied to *O. oeni*, but the potential of this approach remains high. A possible shortcoming, however, may be the need to screen millions of candidate hybrids, for which a stringent screening process is required. In the case of a trait expressed over an extended time, such as efficient MLF, screening would be likely to be time consuming and is best performed utilizing high-throughput approaches [47].

Box 5. Genome shuffling

This method seeks to combine elements from each of two parents that exhibit subtle phenotypic improvements. In brief, protoplast fusion is used to merge the cells and their genomes and the desired phenotype is sought in the new hybrid strains [41,43,46] (Figure I). Protoplasts are usually obtained via chemical treatment [polyethylene glycol (PEG)] [62–64]. Protoplast formation involves the removal of the cell wall, leaving a fatty membranous sac containing the genetic material of the cell. These sacs are combined or fused together allowing genetic material from multiple cells to combine. More recently, femtosecond laser and optical tweezers have been applied to more efficiently pair cells [65]. A microfluidic device has been patented that can trap and pair fusions [66]. The hybrid cell, containing genetic material from more than one cell, is then allowed to regenerate. After one round of genome shuffling, 10% of the cells are pair-wise recombinants (contain genetic material from each parent) and after two rounds at least 2% of the cells have genetic information from any four parental strains [66]. Since the strains undergo natural homologous recombination they are not considered to be genetically modified [46].

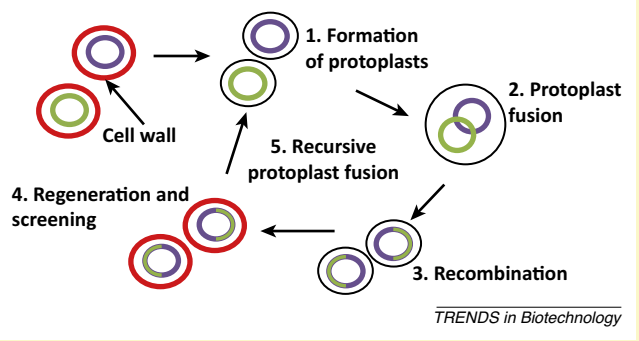


Figure I. Genome shuffling by recursive protoplast fusion. (1) Protoplasts are prepared, cell walls are removed in a process encouraged by polyethylene glycol (PEG) leaving the DNA within a fatty membranous sac. (2) Cells are fused together. (3) Recombination of the genetic components occurs. (4) Cells regenerate their walls and are screened for the desired phenotypes. (5) The process can then be repeated. Adapted from [66].

Concluding remarks and future perspectives

MLF remains an important step in the production of wine and *O. oeni*, the bacterium responsible for it, is a fastidious and recalcitrant microorganism. Slow or incomplete MLF due to failure of *O. oeni* to successfully implant, remain

viable, or metabolize all malic acid can have significant financial and quality implications. Superior strains are needed. Methods that are currently applied to other LABs to optimize their performance have been or could also be applicable to this bacterium. The options vary in feasibility and specificity. Molecular methods of recombination typically require in-depth knowledge of the target genes and also an organism amenable to transformation, which, currently, *O. oeni* is only poorly. Resistance to the use of GMOs in food and beverage production is a further strike against recombined approaches. Nonrecombinant techniques such as strain selection and mutagenesis have been and continue to be used widely and successfully. Their limitations arise from the time-consuming or impractical nature of screening large numbers of candidates, especially for an extended attribute such as MLF under wine-like conditions.

Genome shuffling offers the opportunity to introduce or combine attributes whose genetic basis is more complex in being linked to multiple genes. A disadvantage, of course, is that with the potential exchange of large amounts of genetic material, many attributes may be changed or undesirable properties are introduced along with desirable ones. Again, stringent screening processes are required. DE stems from Darwin's theory of natural selection and has clear advantages over the other nonrecombinant methods. Given its extended nature, the accumulation of desirable mutations (and simultaneous removal of isolates bearing detrimental ones) is possible. In contrast with genome shuffling, the process works with a single genome; thus, many of the resulting desirable traits of the starting strain are likely to be retained without the undesirable side effects seen when combining genomes or parts thereof. Our successful generation of an evolved *O. oeni* that is more ethanol tolerant and faster in catabolizing malic acid underscores the potential utility of DE. A key limitation of DE is the ability to link a growth advantage to traits that do not have an obvious link to growth (e.g., increase aroma compound formation). Nevertheless, DE is a method of importance in the optimization of *O. oeni*, an organism for which transformation systems are of limited efficacy and a general reluctance exists for the use of genetically modified derivatives in food and beverage production.

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RESEARCH ARTICLE

The yeast TUM1 affects production of hydrogen sulfide from cysteine treatment during fermentation

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ABSTRACT

The undesirable rotten-egg odour of hydrogen sulfide (H_2S) produced by yeast shortly after yeast inoculation of grape musts might be an important source of desirable varietal thiols, which contribute to tropical aromas in varieties such as Sauvignon Blanc. In this study, we observed that *Saccharomyces cerevisiae* strains produce an early burst of H_2S from cysteine. Both Δ*met2* and Δ*met17* strains produce a larger burst, likely because they are unable to utilise the H_2S in the sulfate assimilation pathway. For the first time, we show that *TUM1* is partly responsible for the early production of H_2S from cysteine. Overexpressing *TUM1* elevated production of H_2S, whilst its deletion yields only half of the H_2S. We further confirmed that yeast convert cysteine to H_2S by analysing growth of mutants lacking components of the transsulfuration pathway. High concentrations of cysteine overcame this growth block, but required *TUM1*. Collectively, the data indicate that *S. cerevisiae* does not convert cysteine to sulfate or sulfite, but rather to sulfide via a novel pathway that requires the action of Tum1p. The findings of this study may allow the improvement of commercial yeasts through the manipulation of sulfur metabolism that are better suited towards production of fruit-driven styles.

Keywords: *Saccharomyces cerevisiae*; *TUM1*; hydrogen sulfide; varietal thiols; sulfate assimilation pathway; transsulfuration pathway

INTRODUCTION

Volatile sulfur compounds produced by yeast are important contributors to wine aroma. Some sulfur compounds such as hydrogen sulfide (H_2S) are responsible for an unpleasant, rotten-egg aroma (Rauhut 1993). On the other hand, the other class of sulfur compounds, such as 3-mercapto-hexanol (3MH) and 3-mercaptohexylacetate (3MHA), contribute positive tropical fruity aromas (Swiegers and Pretorius 2007).

(*E*)-2-Hexenal, also known as green leaf volatile, in grape musts is generated by the enzymatic oxidation of unsaturated lipids when the grapes are crushed during the prefermentation treatment (Drawert 1974). Depending on the grape variety and

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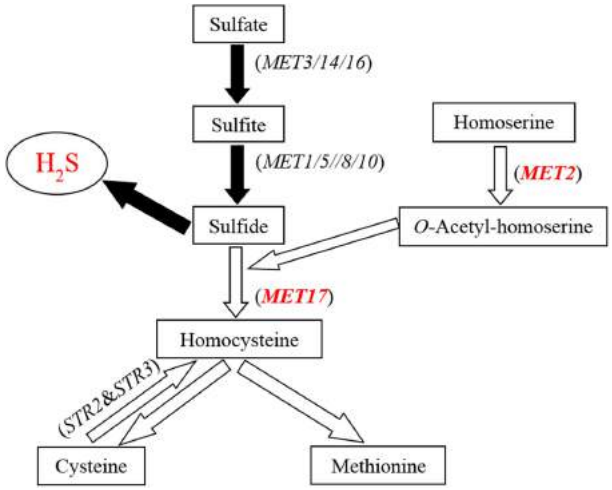


Figure 1. The SAP in *S. cerevisiae*. The black arrows indicate the major route for H_2S production during fermentation. Deletion of *MET2* or *MET17* increases H_2S production (adapted from Ugliano and Henschke 2009; Harsch and Gardner 2013).

prefermentation treatment, the concentrations of (*E*)-2-Hexenal can vary from a few to hundreds of micrograms per litre (Schneider *et al.* 2006).

Interestingly, (*E*)-2-Hexenal can react with H_2S to form 3MH. However, studies have indicated that <1% of 3MH in wine is produced via this H_2S–C6 pathway (Schneider *et al.* 2006; Subileau *et al.* 2008). Harsch *et al.* (2013) proposed that the lack of significant amounts of early H_2S produced by yeast to react with (*E*)-2-Hexenal (which is rapidly metabolised by yeast during the first 24 h post-inoculation) is responsible for the low conversion efficiency.

Most H_2S produced by yeast during fermentation is from the sulfate assimilation pathway (SAP) (Fig. 1) in which sulfate or sulfite is ultimately reduced to sulfide by sulfite reductase encoded by *MET10* (α subunit) and *MET5* (β subunit) (Thomas and Surdin-Kerjan 1997). H_2S is usually not released until yeast assimilable nitrogen becomes limited in grape juice during fermentation, as under replete nitrogen conditions, the sulfide is further metabolised to form methionine, cysteine and glutathione (Jiranek, Langridge and Henschke 1995).

Studies have shown that yeast can generate H_2S from cysteine (Tokuyama *et al.* 1973; Jiranek, Langridge and Henschke 1995). However, cysteine is not considered to be a significant source of H_2S under winemaking conditions given the small amounts of cysteine in grape must (<20 mg L^{-1}), e.g. Bordeaux musts (3 mg L^{-1}) (Pripis-Nicolau *et al.* 2001; Ugliano and Henschke 2009).

Winter and coworkers demonstrated that the supply of rehydration nutrients led to increased 3MH and 3MHA and decreased H_2S levels (Winter *et al.* 2011). They observed that H_2S production was shifted to an earlier stage of fermentation and proposed that cysteine, derived from glutathione—a component of the rehydration nutrient, is used by yeast for the early H_2S production. Therefore, a potential strategy to enhance thiol formation is to develop a yeast strain capable of generating significant amounts of early onset of H_2S from cysteine, which would be available to react with the transient (*E*)-2-Hexenal.

Santiago and Gardner (2015) established that the yeast *IRC7* gene encodes a cysteine desulfydrase which cleaves cysteine to generate H_2S. Interestingly, whilst most yeast strains possess a 38 bp deleted, non-functional as a ß-lyase *IRC7* variant (Roncoroni *et al.* 2011), they are still able to produce H_2S when grown with cysteine (Winter, Cordente and Curtin 2014), alluding to a novel pathway for H_2S formation, involving genes not yet annotated for this function.

In this study, we have confirmed that an early burst of H_2S production is induced by high concentrations of cysteine and show that there is a delayed burst of H_2S produced in strains deleted for either *MET17* or *MET2*. We then attempted to identify genes involved using both quantitative trait locus (QTL) and candidate gene approaches. Candidate genes tested were based on those previously identified in a genome-wide screen using the BY4742 deletion library as affecting H_2S production from cysteine (Winter, Cordente and Curtin 2014), but in several genetic backgrounds. This study represents the first report of the role of Tum1p in the production of H_2S from cysteine; the *TUM1* gene has not been previously annotated to this biological process.

METHODS

Yeast strains and culture

The yeast strains used for this study are listed in Table 1. YPD media (10 g L^{-1} yeast extract, 20 g L^{-1} peptone and 20 g L^{-1} glucose) was used for standard yeast propagation at 28°C. Synthetic dextrose minimal media (SD) (6.7 g L^{-1} yeast nitrogen base without amino acids and 20 g L^{-1} glucose) was used to select against uracil auxotrophic strains. Strains transformed with *KanMX*, *HphMX* or *NatMX* deletion cassettes were selected on Geneticin or G418 sulfate (200 mg L^{-1}; Astral, NSW, Australia), Hygromycin (300 mg L^{-1}; Astral, NSW, Australia) or Nourseothricin sulfate (ClonNAT; Bioscientific Pty. Ltd, Australia) (100 mg L^{-1}), respectively (Goldstein and McCusker 1999).

Yeast growth was measured using a Tecan Infinite M200 microplate reader, whereby the absorbance of 0.2 mL cultures was read every 24 h at 600 nm (OD 600 nm) with 1 min shaking prior to measurement. Yeast starter cultures (2% sugar, non-sulfate CDGJM plus 0.15 mM methionine) were centrifuged, washed twice and resuspended in sterile water. The starter cultures (~1.5×10^5 cells mL^{-1}) were inoculated in a 96-well plate (Costar 3596, Sigma-Aldrich, NSW, Australia) containing 150 μL Sulfur-Free Chemically Defined Grape Juice Medium (SFCDGJM; CDGJM lacking $MgSO_4.7H_2O$, methionine and cysteine) supplemented with 5 mM L-cysteine (168149, Sigma-Aldrich, NSW, Australia), and incubated for 72 h at 28°C in triplicate. The composition of CDGJM medium was identical to Henschke and Jiranek (1993) except amino acid and diammonium phosphate supplementation was altered to reflect Marlborough Sauvignon Blanc juice (Harsch *et al.* 2010; Santiago and Gardner 2015).

Genetic manipulation and strain construction

Polymerase chain reactions (PCR) were performed using Velocity DNA polymerase (Bioline, Australia). Yeast deletion strains were confirmed using Kan B or Kan C primers together with gene-specific primers as reported in Table S1 (Supporting Information). Yeast transformation was performed using the lithium acetate method (Gietz *et al.* 1992).

Plasmid pJC1, a 2-μm-based plasmid containing the *PGK1* promoter and *URA3* selectable marker (Crous, Pretorius and Van Zyl 1995; Martin *et al.* 2003), was used to overexpress the *TUM1* gene. *TUM1* was amplified from BY4743 genomic DNA using *TUM1-EcoRI*-F and *TUM1-XhoI*-R primers (Table S1). The PCR product was digested with restriction enzymes *Eco*RI and *Xho*I, purified (Wizard Plus SV Minipreps, Promega, USA)

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Table 1. Yeast strains used in this study.

Strain	Genotype, phenotype and comments	Origin
Zymaflore F15	Wild-type diploid; a commercial wine yeast	Laffort, France
Oenoferm M2	Wild-type diploid; a commercial wine yeast	Lallemand, Australia
M2xF15 progeny (1 ~ 96)	Wild-type diploid progeny of M2xF15; 23 tetrads, plus 4 random spores, 96 individuals	Huang, Roncoroni and Gardner (2014)
BY4743	*MATa/α, his3-Δ1/his3-Δ1, leu2-Δ0/leu2-Δ0, LYS2/lys2-Δ0, met15-Δ0/MET15, ura3-Δ0/ura3-Δ0*	Euroscarf
BY4743 Δ*met3*	*met3::KanMX*	Euroscarf
BY4743 Δ*met5*	*met5::KanMX*	Euroscarf
BY4743 Δ*met10*	*met10::KanMX*	Euroscarf
BY4743 Δ*met17*	*met17::KanMX*	Euroscarf
BY4743 Δ*tum1*	*tum1::KanMX*	Euroscarf
BY4743 Δ*uba4*	*uba4::KanMX*	Euroscarf
BY4743 Δ*ncs2*	*ncs2::KanMX*	Euroscarf
BY4743 Δ*ncs6*	*ncs6::KanMX*	Euroscarf
BY4743 Δ*urm1*	*urm1::KanMX*	Euroscarf
BY4743 Δ*ahp1*	*ahp1::KanMX*	Euroscarf
BY4743 Δ*str2*	*str2::KanMX*	Euroscarf
BY4743 Δ*yhr112c*	*yhr112c::KanMX*	Euroscarf
BY4743 Δ*yll058w*	*yll058w::KanMX*	Euroscarf
BY4743 Δ*yml082w*	*yml082w::KanMX*	Euroscarf
BY4743 Δ*ygr012w*	*ygr012w::KanMX*	Euroscarf
BY4743 Δ*bna3*	*bna3::KanMX*	Euroscarf
BY4743 Δ*irc7*	*irc7::KanMX*	Euroscarf
BY4743 Δ*vam7*	*vam7::KanMX*	Euroscarf
BY4743 Δ*fra1*	*fra1::KanMX*	Euroscarf
BY4743 Δ*fra2*	*fra2::KanMX*	Euroscarf
BY4743 Δ*mrs3*	*mrs3::KanMX*	Euroscarf
BY4743 Δ*isu1*	*isu1::KanMX*	Euroscarf
BY4743 (pJC1)	BY4743 with (pJC1)	This study
BY4743 (*TUM1*ox)	BY4743 with (pJC1+*TUM1*)	This study
BY4742	*MATα, his3-Δ1,leu2-Δ0, ura3-Δ0, lys2-Δ0*	Euroscarf
BY4742 Δ*met2*	*met2::NatMX*	This study
BY4742 Δ*met3*/Δ*str2*/Δ*str3*	*met3::KanMX; str2::HphMX; str3::NatMX*	This study
BY4742 Δ*met14*/Δ*str2*/Δ*str3*	*met14::KanMX; str2::HphMX; str3::NatMX*	This study
BY4742 Δ*met16*/Δ*str2*/Δ*str3*	*met16::KanMX; str2::HphMX; str3::NatMX*	This study
BY4742 Δ*met5*/Δ*str2*/Δ*str3*	*met5::KanMX; str2::HphMX; str3::NatMX*	This study
BY4742 Δ*met10*/Δ*str2*/Δ*str3*	*met10::KanMX; str2::HphMX; str3::NatMX*	This study
BY4742 Δ*met1*/Δ*str2*/Δ*str3*	*met1::KanMX; str2::HphMX; str3::NatMX*	This study
BY4742 Δ*met8*/Δ*str2*/Δ*str3*	*met8::KanMX; str2::HphMX; str3::NatMX*	This study
BY4742 Δ*met17*/Δ*str2*/Δ*str3*	*met17::KanMX; str2::HphMX; str3::NatMX*	This study
BY4741	*MATa, his3-Δ1, leu2-Δ0, met15Δ0, ura3Δ0*	Euroscarf
BY4741Δ*met3*	*met3::KanMX*	Euroscarf
BY4741Δ*met5*	*met5::KanMX*	Euroscarf
BY4741Δ*met10*	*met10::KanMX*	Euroscarf
AWRI1631	Haploid wine strain	AWRI
AWRI1631 Δ*tum1*	*tum1::KanMX*	AWRI
AWRI1631 Δ*cys3*	*cys3::KanMX*	AWRI
AWRI1631 Δ*cys4*	*cys4::KanMX*	AWRI
AWRI1631 Δ*irc7*	*irc7::KanMX*	AWRI
AWRI1631 Δ*str3*	*str3::HphMX*	This study
AWRI1631 Δ*vps25*	*vps25::KanMX*	AWRI
AWRI1631 Δ*vps36*	*vps36::KanMX*	AWRI
AWRI1631 Δ*fra1*	*fra1::KanMX*	AWRI
AWRI1631 Δ*tum1*/Δ*str3*	*tum1::KanMX; str3::HphMX*	This study
Sigma 1278b (pJC1)	Lab strain, *MATa*, *ura3Δ0* (pJC1)	This study
Sigma 1278b (*TUM1*ox)	Lab strain, *MATa*, *ura3Δ0* (pJC1+*TUM1*)	This study
Oenoferm M2 *ura3Δ* (pJC1)	Wine strain, *MATa*, *ura3Δ0*, ho::*HphMX* (pJC1)	This study
Oenoferm M2 *ura3Δ* (*TUM1*ox)	Wine strain, *MATa*, *ura3Δ0*, ho::*HphMX* (pJC1+*TUM1*)	This study
AWRI796 *ura3Δ* (pJC1)	Wine strain, *ura3::kanMX* (pJC1)	This study
AWRI796 *ura3Δ* (*TUM1*ox)	Wine strain, *ura3::KanMX* (pJC1+*TUM1*)	This study
Zymaflore F15 *ura3Δ* (pJC1)	Wine strain, *MATa*, *ura3Δ0* (pJC1)	This study
Zymaflore F15 *ura3Δ* (*TUM1*ox)	Wine strain, *MATa*, *ura3Δ0* (pJC1+*TUM1*)	This study
Lalvin L2056 *ura3Δ* (pJC1)	Wine strain, *ura3Δ::KanMX* (pJC1)	This study
Lalvin L2056 *ura3Δ* (*TUM1*ox)	Wine strain, *ura3Δ::KanMX* (pJC1+*TUM1*)	This study
Maurivin B *ura3Δ* (pJC1)	Wine strain, *ura3Δ::KanMX* (pJC1)	This study
Maurivin B *ura3Δ* (*TUM1*ox)	Wine strain, *ura3Δ::KanMX* (pJC1+*TUM1*)	This study

ox = overexpression.

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and ligated with pre-digested pJC1 plasmid (*Eco*RI and *Xho*I). Transformation, plasmid propagation and verification of the recombinant plasmid (pJC1+*TUM1*) by restriction digestion were performed according to Sambrook, Fritsch and Maniatis (1989). The plasmids pJC1 (as control) and the (pJC1+*TUM1*) were transformed into the uracil-minus yeast strains described in Table 1. Transformants were selected on synthetic dextrose (SD) uracil drop-out plates (Amberg, Burke and Strathern 2005).

Triple deletants of *STR2*, *STR3* and individual *MET* genes were constructed by sequential deletion of *STR2* followed by *STR3* in the individual *MET* gene deletants available from the haploid BY4742 deletion library (Table 1). The deletion of *STR2* was achieved by amplifying the *HphMX* cassette in plasmid pAG32 plus ∼100 bp of homologous untranslated sequence flanking *STR2* (using the primers pair Del-*str2*-F and Del-*str2*-R; Table S1). The PCR products were used for yeast transformation with selection of transformants on YPD agar plates containing hygromycin (300 mg L^{-1}). *Str2::HphMX* deletants were confirmed by PCR using primers *Hph*-I-F and RC*str2* (Table S1).

The *STR3* and *MET2* genes were deleted using a similar approach. The NatMX cassette in plasmid pAG25 was amplified together with ∼100 bp of homologous untranslated sequence flanking *STR3* or *MET2* using Del-*str3*-F and Del-*str3*-R or Del-*met2*-F and Del-*met2*-R primers, respectively (Table S1). Yeast transformants were selected on YPD agar plates containing nourseothricin (100 mg L^{-1}). *Str3::NatMX* and *Met2::NatMX* deletants were verified using primer pairs *Nat*-I-F and RC*str3* and *Nat*-I-F and RC*met2* (Table S1).

Fermentations and H_2S quantification

Yeast starter cultures were prepared by inoculating a single yeast colony into starter medium (2% sugar, non-sulfate CDGJM plus 0.15 mM methionine) for 24 h at 28°C. The starter culture was centrifuged, washed and resuspended in sterile water to inoculate 100 mL of medium (non-sulfate CDGJM plus 5 mM cysteine and 0.15 mM methionine) at 2.5×10^6 cells L^{-1}. The additional amounts of histidine (200 mg L^{-1}), leucine (300 mg L^{-1}), uracil (100 mg L^{-1}) and lysine (300 mg L^{-1}) were added for auxotrophies (Harsch *et al*. 2010). Fermentations were conducted in triplicate in 250 mL flasks at 28°C with shaking at 100 rpm. Fermentation progress was monitored daily as weight loss due to CO_2 evolution (Bely, Sablayrolles and Barre 1990). H_2S produced by yeast during fermentation was detected by either lead acetate (4H: 1–2000 ppm; GASTEC, Japan) or silver nitrate (120SF: 1–1000 ppm; KITAGAWA, Japan) H_2S detector tubes that tightly fitted into the glass airlock of the flask (Park 2008).

Data analysis

The mean, standard error of the mean (SEM) and *t* test (two samples assuming unequal variances) were performed using Microsoft Excel 2013 (Microsoft, Redmond, Washington, USA). Analysis of variance (ANOVA) and Tukey's honestly significant difference test were performed using JMP software (SAS Institute, Cary, NC, USA). Statistical significance was set at the confidence level of 5%.

QTL mapping

The R/QTL package (Broman *et al*. 2003) was used for mapping of QTLs. Single QTL analysis for quantitative traits was performed using the Haley–Knott regression. Significance thresholds were generated for every trait by 1000 permutations of the phenotype data. Data were normalised between runs, by making the centre of the distribution 0 and the standard deviation 1.

RESULTS

High cysteine causes an early burst of H_2S production and deletion of *MET17* or *MET2* leads to an additional delayed burst

The *MET* genes associated with the SAP are well known for their roles in affecting H_2S production during fermentation (Cordente *et al*. 2009; Linderholm *et al*. 2010; Huang, Roncoroni and Gardner 2014; Noble, Sanchez and Blondin 2015). However, their roles in H_2S production from cysteine are less well understood. Here, the *MET* gene deletants were fermented in sulfate-free CDGJM supplemented with 5 mM cysteine and 0.15 mM methionine to examine their roles in H_2S production from cysteine.

The concentration of 5 mM (605.8 mg L^{-1}) cysteine was chosen because a distinguishable amount of H_2S could be detected by H_2S detector tubes in initial trials (data not shown). In addition, a similar amount of cysteine (4.1 mM or 500 mg L^{-1}) was applied in a previous study, without biomass formation being affected (Winter, Cordente and Curtin 2014). A minimal concentration (0.15 mM) of methionine was supplemented to facilitate cell growth. No H_2S was detectable when yeast strains were fermented in non-sulfate CDGJM plus 0.15 mM methionine (Fig. 2A). Furthermore, no H_2S was detected in uninoculated, non-sulfate CDGJM plus 5 mM cysteine and 0.15 mM methionine (Fig. 2A). Therefore, this medium was considered ideal for studying H_2S production by yeast from cysteine.

BY4743 produced an early burst of H_2S when high concentrations of cysteine are added to the fermentation (Fig. 2A). Because this yeast strain lacks an active *IRC7* gene, this known cysteine desulfydrase activity cannot be responsible for the H_2S that is produced. All of the *MET* gene deletant strains also produced a similar burst of H_2S after 48 h of fermentation (Fig. 2A). However, the Δ*met17* strain produced a significant amount of additional H_2S in a more prolonged time. In addition, the *MET2* deletant of the related BY4742 strain also produced elevated H_2S compared to the corresponding wild type (Fig. 2B).

This early burst of H_2S production from cysteine must come from an as-yet-unidentified pathway, independent of both *IRC7* and most of the SAP, as strains lacking the individual *MET* genes did not affect H_2S production. The late H_2S observed for Δ*met17* and Δ*met2* strains could be derived from the SAP, as sulfate, sulfite or sulfide.

The addition of 5 mM cysteine did transiently slow the growth of BY4743 on the first day compared to BY4743 when fermented in non-cysteine containing CDGJM (Fig. S1, Supporting Information). However, BY4743 was able to overcome the toxic effect of cysteine by the second day and the overall fermentation kinetics of BY4743 grown on 0 or 5 mM cysteine were similar. The fermentation kinetics were also similar for the BY4743 *MET* gene deletants, suggesting that the additional H_2S observed for the Δ*met17* strains was not related to growth.

QTL mapping of genes linked to H_2S from cysteine

Zymaflore F15 was observed to produce more H_2S than Oenoferm M2 when fermented in non-sulfate CDGJM plus 5 mM cysteine and 0.15 mM methionine (Fig. 3A). Genome sequencing of the parental strains enabled single nucleotide polymorphisms at individual chromosomal loci to be identified. To map genes responsible for H_2S production from cysteine, a set of 96

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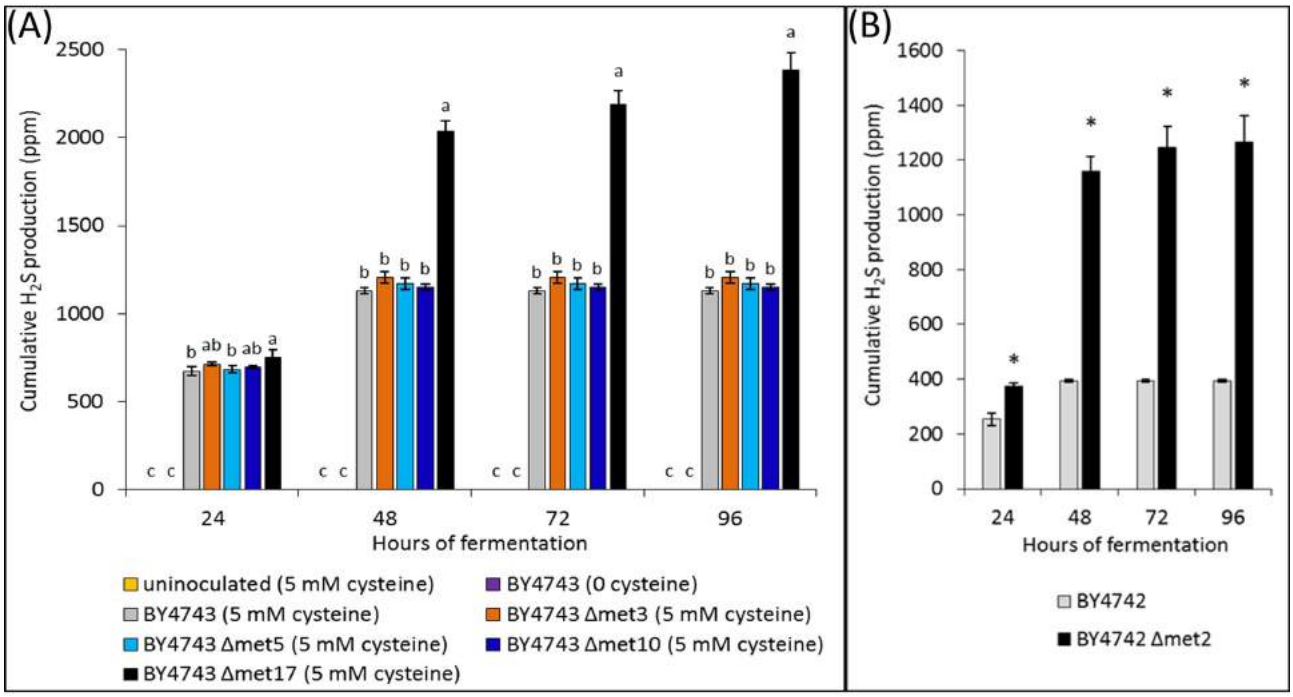


Figure 2. **(A)** Cumulative H_2S production from BY4743 and its *MET* gene deletants **(B)** BY4742 and BY4742 Δ*met2* during 24 h–96 h of fermentation. Fermentations were performed in 100 mL of non-sulfate CDGJM and 0.15 mM methionine plus or minus 5 mM cysteine at 28°C ($n = 3$) with shaking at 100 rpm. H_2S was measured with silver nitrate (120SF: 1–1000 ppm; KITAGAWA, Japan) H_2S detector tubes **(A)**, which were replaced at regular intervals. H_2S was measured by lead acetate (4H: 1–2000 ppm; GASTEC, Japan) H_2S detector tubes for **(B)** BY4742 and BY4742 Δ*met2* strains. Data represent mean values of triplicate fermentations ± standard error of the mean (SEM). Samples not connected by the same letter are significantly different (ANOVA, Tukey's HSD). Asterisks above bars represent significant differences compared to the wild types (*$P < 0.05$, two-tailed Student's *t* test).

fully sequenced M2xF15 progeny (Huang, Roncoroni and Gardner 2014) was fermented in triplicate in non-sulfate CDGJM supplemented with 5 mM cysteine and 0.15 mM methionine. H_2S production was measured over the course of the ferment using H_2S detector tubes. The final reading on the tubes (averages of replicates) was used as input for QTL analysis.

The logarithm of the odds scores for the individual chromosomes revealed that none of the peaks were significant even at the 5% level (Fig. 3C) despite variation for H_2S production among the 96 progenies (Fig. 3B). This indicates that there was no readily identifiable correlation between H_2S production from cysteine and a specific gene or DNA region.

Deletion of *TUM1* gene yields only half of the H_2S from cysteine

The inability to identify a specific QTL associated with H_2S production from cysteine may be due to insufficient sample sizes or indicate the presence of multiple genes affecting the trait (Bloom *et al.* 2013; Winter, Cordente and Curtin 2014). It was therefore decided to investigate this process by testing candidate gene deletants, based on results from previous studies (Winter, Cordente and Curtin 2014; Santiago and Gardner 2015), in order to decipher the genetic basis behind how H_2S is formed during fermentation on high cysteine. Deletants used in this study are in the homozygous diploid auxotrophic laboratory strain BY4743 background (Euroscarf) and the genes of interest identified were further validated in the haploid wine yeast strain AWRI1631 background (AWRI Wine Yeast Deletion Library collection) (Varela *et al.* 2012).

The ß-lyase gene, *IRC7*, has been previously identified as responsible for H_2S production from cysteine (Santiago and Gardner 2015). Therefore, several ß-lyase candidate gene deletants (Holt *et al.* 2011; Harsch and Gardner 2013) from two yeast gene deletion libraries were evaluated in this study for their roles on H_2S production from cysteine.

Deletion of *TUM1* gene in the laboratory strain BY4743 resulted in half of the H_2S production (Fig. 4A) and most importantly, deletion of *TUM1* did not affect the fermentation performance (Fig. S2, Supporting Information). The other ß-lyase candidate gene (YML082W) deletants did not have as big and impact on H_2S formation compared to Δ*tum1* and the lower H_2S production observed such as from BY4743 Δ*yml082w* may be related to its slower growth rate (data not shown). Deletion of *TUM1* in the AWRI1631 background also reduced H_2S production from cysteine significantly (Fig. 4B).

Overexpression of *TUM1* elevated production of H_2S from cysteine

The reduction in H_2S production to half in yeast strains lacking *TUM1* clearly demonstrated the importance of *TUM1* in H_2S production from cysteine. The impact of *TUM1* was also tested by overexpressing the *TUM1* gene originating from BY4743 in different genetic backgrounds. In all cases, overexpression of *TUM1* led to increased H_2S production at the early stage of fermentation (Fig. 5). Both BY4743 and Sigma 1278b are laboratory yeast strains whereas the others are *ura3* auxotrophic strains derived from commercial wine yeast strains. As no H_2S was detected in the normal sulfate-containing CDGJM (data not shown), it can be

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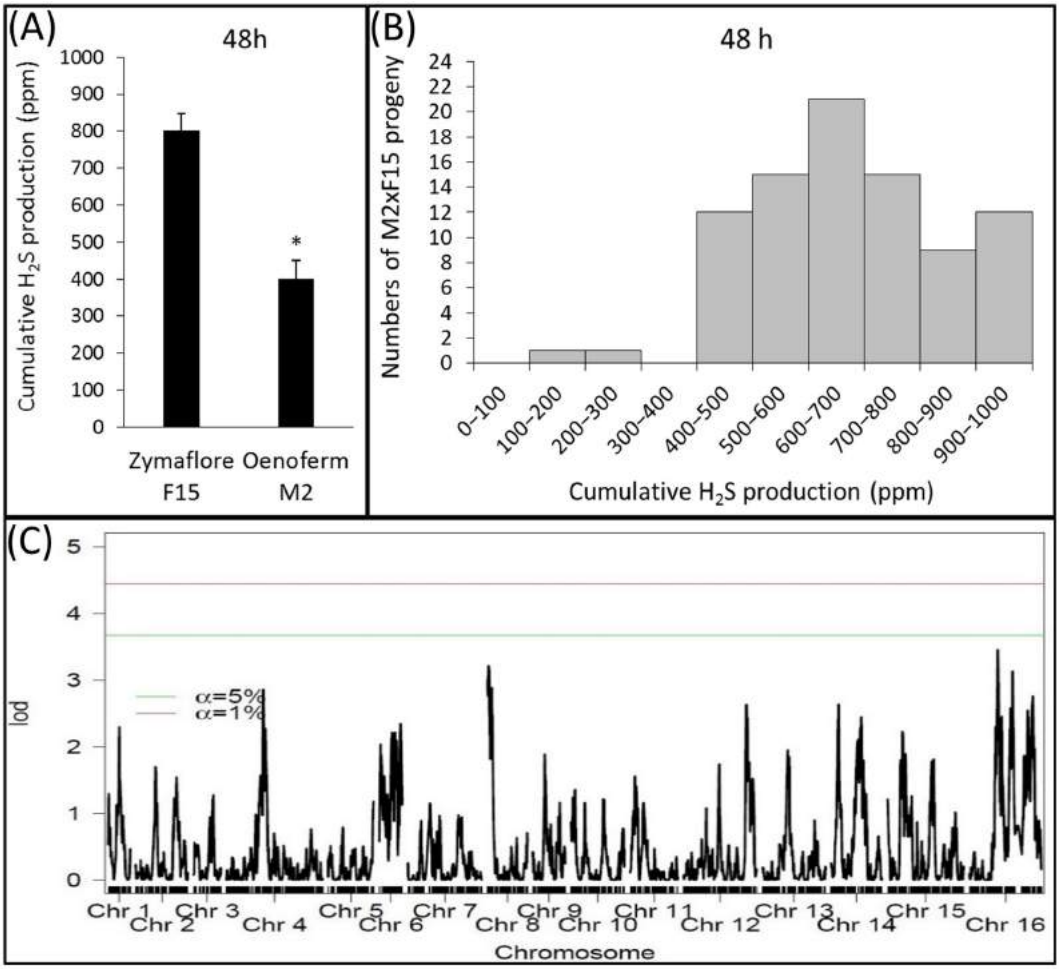


Figure 3. (A) Cumulative H_2S production from Zymaflore F15 and Oenoferm M2, **(B)** the 96 M2xF15 progenies, **(C)** LOD scores for H_2S production in 96 M2xF15 progenies plotted along the 16 yeast chromosomes. Green horizontal line indicates the 5% and red indicates the 1% significance cut-off. Fermentations were performed in 100 mL of non-sulfate CDGJM and 0.15 mM methionine plus or minus 5 mM cysteine at 28°C ($n = 3$) with shaking at 100 rpm. H_2S was measured with silver nitrate (120SF: 1–1000 ppm; KITAGAWA, Japan) H_2S detector tubes, which were replaced at regular intervals. The mean H_2S released is shown and error bars show SEM. Asterisks above bars represent significant differences compared to the wild types (*$P < 0.05$, two-tailed Student's *t* test).

concluded that the effect of *TUM1* is specific to production from cysteine.

Fate of cysteine

The observed increase in H_2S production in the BY4742 Δ*met2* and BY4743 Δ*met17* deletants, when grown in high cysteine (5 mM) medium, alluded to the formation of an unknown intermediate, derived from the degradation of cysteine, which is able to be metabolised through SAP. A series of experiments were carried out to determine the fate of cysteine.

The presence of sulfate and sulfite was measured by ion chromatography (OIV-MA-AS313-16), in high-cysteine medium prior to and after fermentation with BY4743 and BY4743 Δ*met17* strains. However, no sulfate or sulfite was detectable (data not shown). The aspiration method (Rankine and Pocock 1970; Fujita *et al.* 1979) was also used to determine if any SO_2 (detection limits ~0.5 mg L^{-1}) was present but again no sulfite could be detected (data not shown).

Hydrogen sulfide production from cysteine catabolism was further investigated in double deletants of *MET17* and the individual *MET* genes of the SAP. BY4741 was chosen because of the pre-existing Δ*met17* mutation. It was proposed that if cysteine is converted to sulfate then deletion of *MET3* would prevent the reduction of sulfate so that less H_2S would be expected than in BY4741 (Δ*met17* alone). If cysteine is converted to sulfite, then deletion of *MET3* would not affect H_2S production, but deletion of *MET1*, *MET5* or *MET10* would reduce H_2S, as sulfite could not be further assimilated. However, amounts of H_2S produced by these double deletants were similar to each other indicating that yeast does not catabolise cysteine to sulfate or sulfite (Fig. S3, Supporting Information).

Yeast can bypass the known *STR2/STR3* transsulfuration pathway and grow on high cysteine, but require both *MET17* and *TUM1*

It has been shown that Δ*str2* and Δ*str3* strains are unable to grow on a medium containing 0.2 mM cysteine or glutathione as sole sulfur source (Hansen and Johannesen 2000). However, overexpression of a full-length functional *IRC7* gene in a Δ*str3* deletant of the wine yeast F15 restored growth on 0.5 mM cysteine as the only sulfur source (Santiago and Gardner 2015). The authors proposed that extra H_2S produced by Irc7p action on cysteine could

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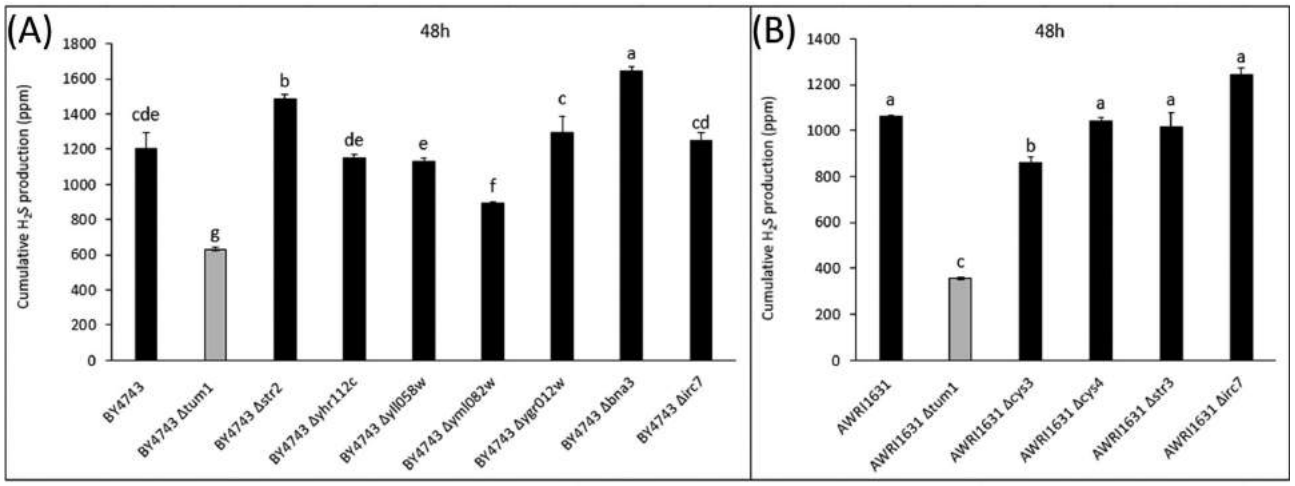


Figure 4. Cumulative H_2S production from the *ß*-lyase candidate gene deletants in **(A)** laboratory strain BY4743 **(B)** and wine yeast strain AWRI1631 backgrounds. Fermentations were performed in 100 mL of non-sulfate CDGJM and 0.15 mM methionine plus 5 mM cysteine at 28°C ($n = 3$) with shaking at 100 rpm. H_2S was measured with silver nitrate (120SF: 1–1000 ppm; KITAGAWA, Japan) H_2S detector tubes, which were replaced at regular intervals. The mean H_2S released is shown and error bars show SEM. Samples not connected by the same letter are significantly different (ANOVA, Tukey's HSD).

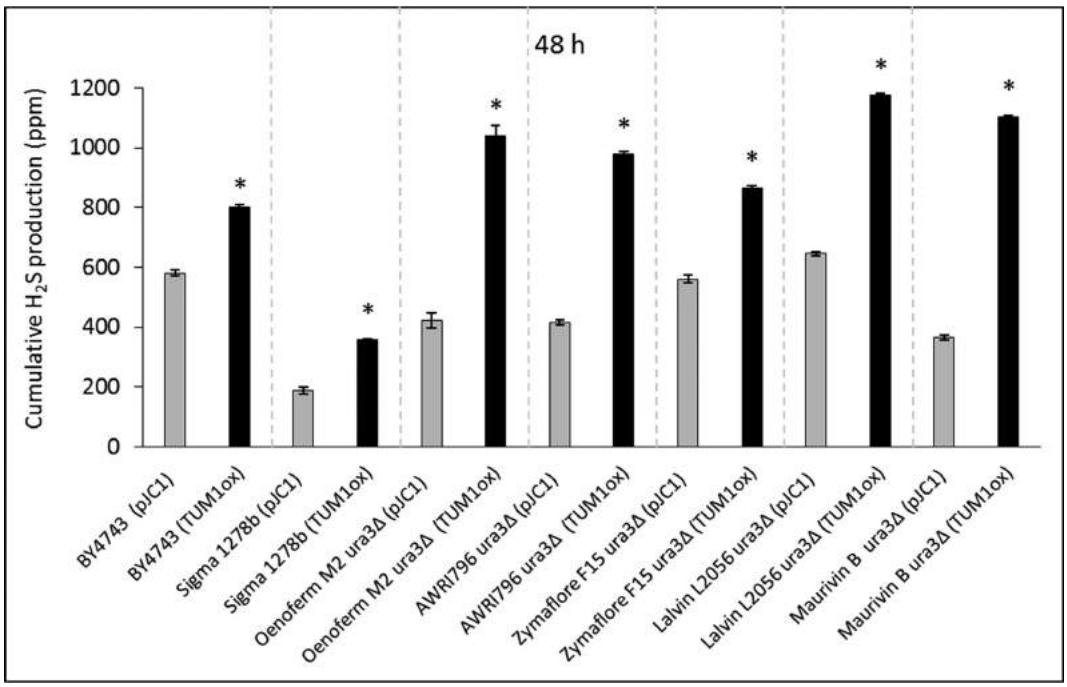


Figure 5. Cumulative H_2S production from the wild types and *TUM1* overexpression strains in different yeast genetic backgrounds (separated by dotted lines). Fermentation was carried in 100 mL of non-sulfate CDGJM plus 5 mM cysteine and 0.15 mM methionine at 28°C with shaking at 100 rpm. H_2S was measured by lead acetate (4H: 1–2000 ppm; GASTEC, Japan) H_2S detector tubes and the mean H_2S released is shown. Error bars indicate SEM. Fermentations by BY4743 and Oenoferm M2 strains were performed in triplicate, whilst only duplicate fermentations were done for the other strains. ox denotes overexpression. Asterisks above bars represent significant differences compared to the wild types (*$P < 0.05$, two-tailed Student's *t* test).

be metabolised via the SAP to produce methionine, enabling the bypass of the transsulfuration pathway and growth of the Δ*str3* strain on cysteine.

The growth of the Δ*str2* and Δ*str3* strains on high concentrations of cysteine as sole sulfur source media has not been previously investigated. The observation that yeasts are capable of generating additional H_2S from cysteine led to the speculation that Δ*str2* and Δ*str3* strains could also bypass the transsulfuration pathway, allowing growth under excess cysteine conditions. Furthermore, the ability of individual triple deletants of *STR2*, *STR3* and various *MET* genes to grow would reveal whether cysteine was converted to sulfate, sulfite or sulfide, prior to synthesis of methionine via SAP.

Triple deletants were constructed in the BY4742 genetic background (wild type for *MET17*), whereby individual *MET* gene deletants were further modified such that coding sequences of *STR2* and *STR3* were replaced with the *str2::HphMX* and *str3::NatMX* deletion cassettes (see the section 'Material and Methods'). The growth of the triple deletants was tested in Sulfur-Free Chemically-Defined Grape Juice Medium (SFCDGJM;

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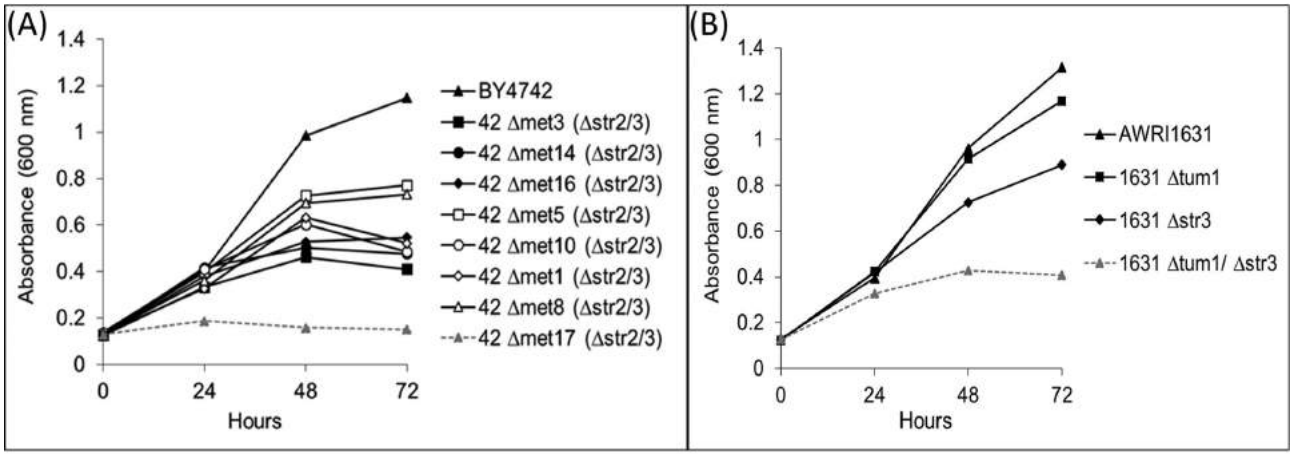


Figure 6. (A) Yeast *MET17* and **(B)** *TUM1* genes are necessary to bypass the *STR2/STR3* transsulfuration pathway and grow on high concentration of cysteine as sole sulfur source. Strains were grown at 28°C for 72 h in SFCDGJM plus 5 mM cysteine. The absorbance was measured by microplate reader every 24 h with 1 min shaking at OD600 nm. Values are means of triplicate wells.

CDGJM lacking $MgSO_4.7H_2O$, Met and Cys) supplemented with 5 mM cysteine (Santiago and Gardner 2015). Cysteine was essential for growth, as all strains showed only minimal growth in SFCDGJM, which lacked this amino acid (data not shown). The strains also failed to grow on low cysteine (0.1 mM), confirming that the deletion of *STR2* and *STR3* had been successful and that the transsulfuration pathway was inactive in these mutants (data not shown).

All of the triple deletants could grow with 5 mM cysteine as sole sulfur source, with the exception of Δ*str2*/Δ*str3*/Δ*met17* strain (Fig. 6A). These findings suggest that Δ*str2* and Δ*str3* strains can bypass the transsulfuration pathway when cultured in medium containing high cysteine. The data show that cysteine is not being converted to sulfate, as the deletion of *MET3*, *MET14* or *MET16*, which would block flow of sulfate through the SAP, did not affect growth. Sulfite was also not an intermediate, as the deletion of *MET1*, *MET5*, *MET8* or *MET10*, which block the reduction of sulfite to sulfide, did not prevent growth.

BY4742 Δ*met17*/Δ*str2*/Δ*str3* was the only strain that could not grow, consistent with the hypothesis that sulfur derived from supplementation with high cysteine most likely enters the SAP as sulfide.

We have already alluded to *TUM1* gene as playing a key role in H_2S production from cysteine and therefore the growth of Δ*tum1*/Δ*str3* double deletant was investigated. Deletion of *STR3* in wine strain AWRI 1631 was clearly able to bypass the transsulfuration pathway, as it could grow in a high cysteine medium (Fig. 6B). A slower growth rate was observed for the Δ*tum1*/Δ*str3* double deletant. The reduced ability to generate H_2S from cysteine in this strain therefore correlates with reduced growth on cysteine as sole sulfur source, consistent with the interpretation that cysteine enters the SAP as sulfide.

Genes involved in tRNA thiolation, vacuolar maintenance and iron–sulfur homeostasis have limited effect on H_2S production from cysteine

A number of other candidate genes were screened for their role in the conversion of cysteine to H_2S. In *Saccharomyces cerevisiae*, the gene *TUM1* together with *URM1*, *UBA4*, *NCS2* and *NCS6* has been identified as being involved in the wobble modification in tRNAs. Tum1p is a sulfur transferase that accepts persulfide sulfur from Nfs1p and transfers it to Uba4p (Noma, Sakaguchi and Suzuki 2009). The yeast deletants (BY4743 Δ*urm1*, Δ*uba4*, Δ*ncs2*, Δ*ncs6*, Δ*ahp1*), which represent the other genes involved in tRNA thiolation, were investigated to determine whether they also affected H_2S production from cysteine. Interestingly, none of the deletants tested showed a decrease in H_2S production (Fig. S4, Supporting Information).

Winter, Cordente and Curtin (2014) showed that the vacuole-related gene deletants in the BY4742 laboratory strain background reduced H_2S production from cysteine significantly, while mutants in iron–sulfur homeostasis increased production. However, BY4742 is auxotrophic for lysine and has been reported to ferment much more slowly in grape juice than BY4743 unless 10-fold lysine (300 mg L^{-1}) was supplemented (Harsch *et al.* 2010). Therefore, we decided to reexamine that some of the low H_2S producers (vacuole deletants) and high H_2S producers (iron–sulfur homeostasis deletants) in the auxotrophic laboratory strain BY4743 and the prototrophic wine yeast strain AWRI1631 backgrounds. Our results show that the vacuole mutant, BY4743 Δ*vam7*, did produce less H_2S than BY4743 (780 vs 1200 ppm), but it also fermented more slowly than the wild type. Surprisingly, Δ*vps25* and Δ*vps36* in the AWRI1631 background did not produce less H_2S, even they did ferment relatively slower than the wild type (Fig. S5A and B). None of the iron–sulfur homeostasis deletants (Δ*fra1*, Δ*fra2*, Δ*mrs3* and Δ*isu1*) in BY4743 background or AWRI1631 Δ*fra1* produced more H_2S from cysteine (Fig. S6, Supporting Information).

DISCUSSION

In this study, we have confirmed that *Saccharomyces cerevisiae* is capable of producing an early burst of H_2S from high concentrations of cysteine during fermentation and revealed that *TUM1* is a crucial gene affecting this H_2S production. Part of the H_2S produced is normally utilised by yeast cells as a sulfur source for growth.

High intracellular concentrations of cysteine are cytotoxic, and therefore there are mechanisms to prevent its accumulation (Stipanuk 2004). In human cells, this is achieved by converting cysteine to sulfite and sulfate using cysteine dioxygenase and sulfite oxidase (Sörbo and Ewetz 1965; Lombardini, Singer and Boyer 1969). In other species such as tobacco, cysteine is converted to sulfate (Smith 1975), whilst *Candida albicans*

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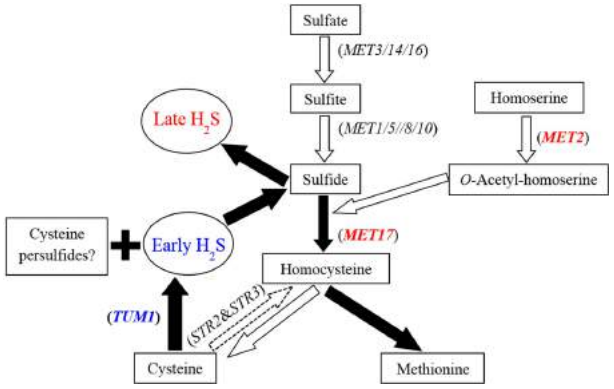


Figure 7. Model for cysteine catabolism in *S. cerevisiae*. *TUM1* (blue font) is proposed to be the principal gene responsible for the early burst of H_2S production, which is observed during fermentation. Cysteine persulfides may also be involved in this pathway. Part of the sulfide produced then enters the sulfur assimilation pathway, and this gives an additional burst of (late) H_2S if the *MET2* or *MET17* gene (red font) is deleted. The black arrows indicate the possible route used by the Δ*str2* and Δ*str3* deletants, when the *STR2/STR3* transsulfuration pathway is blocked (white dashed arrow), enabling growth on high concentrations of cysteine as the sole sulfur source (adapted from Harsch and Gardner 2013; Fräsdorf, Radon and Leimkühler 2014).

converts cysteine to sulfite through cysteine dioxygenase, encoded by the *CDG1* gene (Hennicke *et al*. 2013). This study confirms earlier findings that high cysteine is toxic to yeast (Kumar *et al*. 2006; Santiago and Gardner 2015) and that yeast responds to high cysteine in fermentation conditions by producing a burst of H_2S (Tokuyama *et al*. 1973; Jiranek, Langridge and Henschke 1995; Winter and Curtin 2012).

In this study, a larger delayed burst of H_2S was produced from cysteine in strains with deletions of either *MET17* or *MET2*. We propose that this elevated burst of H_2S occurs because these strains are unable to utilise H_2S in the SAP; hence, all of the H_2S produced from cysteine is diffused into the medium in these strains, rather than a proportion being reincorporated into the SAP. In support of this explanation, we present experimental evidence that deletants of *STR2* and *STR3* can bypass the transsulfuration pathway when grown in media containing high concentrations of cysteine as the sole sulfur source. Thus, yeast cells must possess an alternative pathway to obtain methionine in media with high concentrations of cysteine. Our genetic data using combinations of trans-sulfuration mutants with deletions of individual *MET* genes suggest that it is the H_2S generated from cysteine catabolism that is fed directly into the SAP and is utilised for the synthesis of methionine.

In addition, we identified a role for the *TUM1* gene in this novel yeast pathway of cysteine catabolism. Deletion of *TUM1* reduced H_2S production from cysteine, while overexpressing *TUM1* increased H_2S in different yeast genetic backgrounds. The requirement for *TUM1* for full growth of transsulfuration pathway mutants on high cysteine confirmed the role of this yeast gene in the production of H_2S from cysteine.

A new model (Fig. 7) for cysteine catabolism in *S. cerevisiae* is proposed in which the *TUM1* gene plays a key role. The precise role played by *TUM1* in yeast cysteine catabolism remains to be determined. However, because *TUM1* was the only gene involved in tRNA thiolation to effect H_2S production from cysteine, it seems unlikely that the cellular process of tRNA thiolation plays a role in cysteine catabolism in *S. cerevisiae*. Our data further suggested that neither sulfate nor sulfite is produced as part of this yeast pathway.

In humans, cysteine is first metabolised by cysteine aminotransferase to 3-mercaptopyruvate, which is then converted to pyruvate and protein-bound persulfide by TUM1 (sulfurtransferase). The H_2S is then released from protein-bound persulfide when reducing systems such as glutathione or thioredoxin are present (Shibuya *et al*. 2009; Mikami *et al*. 2011). The human TUM1 protein and yeast Tum1p are orthologues (Mathew, Schlipalius and Ebert 2011). In addition, alignment of the Tum1p sequences from several yeast strains, available from the *Saccharomyces* Genome Database (SGD), indicates that Tum1p are highly conserved among *S. cerevisiae* strains (http://www.yeastgenome.org/cgi-bin/FUNGI/alignment.pl?locus=tum1&submit=Submit&rm=display_result). Whilst the crystal structure of yeast Tum1p has been solved at 1.90 A resolution (Qiu *et al*. 2012), it remains to be confirmed as to whether the protein can act like a human sulfurtransferase.

In mammals, cystathionine β-synthase (CBS, EC 4.2.1.22) and cystathione γ-lyase (CSE aka CGL, EC 4.4.1.1) (Kashfi and Olson 2013) have also been found to degrade cysteine to release H_2S. However, the equivalent genes (*STR3* and *CYS3*) in yeast do not produce H_2S (Linderholm *et al*. 2008; Winter, Cordente and Curtin 2014), which was also confirmed in this study by demonstrating that deletion of *STR3* in the wine yeast AWRI1631 background did not affect H_2S production (Fig. 4B).

In humans, the biological roles of the TUM1 protein range from thiolation of cytosolic tRNAs to the generation of H_2S as a signaling molecule both in mitochondria and the cytosol (Fräsdorf, Radon and Leimkühler 2014). To our best knowledge, *S. cerevisiae* Tum1p has only been annotated to the thiolation of cytosolic tRNAs (Noma, Sakaguchi and Suzuki 2009). The biological role of the H_2S generated from cysteine by Tum1p in *S. cerevisiae* remains unknown but it is tempting to speculate that it may also have the similar function as 3MST in humans, in generating H_2S and cysteine persulfide and thus glutathione polysulfides, as signalling molecules (Ida *et al*. 2014; Santiago and Gardner 2015). This may explain why most yeast strains possess a 38 bp deleted, non-functional as a *ß*-lyase *IRC7* variant (Roncoroni *et al*. 2011) as an alternative pathway(s) exists for H_2S formation from cysteine. The Δ*tum1* deletant did not completely eliminate H_2S formation from cysteine therefore further studies are needed to investigate the involvement of other genes, pathways and polysulfides in the yeast cysteine catabolism process.

Winter, Cordente and Curtin (2014) proposed that vacuole plays a central role in H_2S production from cysteine, based on the phenotypes of several vacuole mutants such as BY4742 Δ*vam7*, which grew more slowly but produced less H_2S than wild type at the same growth stage. Surprisingly, our results show that the vacuole mutants, Δ*vps25* and Δ*vps36* in AWRI1631 backgrounds, did not reduce H_2S production. Our results do show that BY4743 Δ*vam7* produced less H_2S than wild type; however, we suspected that the poor growth of the strain is the major factor contributing to the lower H_2S production. This is because poor growth will result in less vigorous production of CO_2, which is required to sparge H_2S into the H_2S detector tube during fermentation (Park 2008). We also did not observe elevated H_2S production for the iron–sulfur homeostasis deletants (Δ*fra1*, Δ*fra2*, Δ*mrs3* and Δ*isu1*) in BY4743 and Δ*fra1* in AWRI1631 backgrounds. At this stage, we could not conclude whether the vacuole and iron–sulfur homeostasis related genes affect formation of H_2S from cysteine during fermentation and we agree with Winter and coworkers that further investigations using a more sensitive detection methods for H_2S inside the yeast cells are required to better understand the roles of these genes in yeast cysteine catabolism.

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The findings of this study greatly facilitate our knowledge of the process of yeast cysteine catabolism. Polysulfides as possible signalling molecules have attracted much attention lately, but the majority of studies have been performed in mammalian and plant cells (Mishanina, Libiad and Banerjee 2015; Höfler *et al.* 2016). The identification of the new important role played by yeast Tum1p in the generation of H_2S (and possibly polysulfides) from cysteine suggests that yeast could also be a useful model organism for studying human diseases such as Parkinson and Alzheimer, which involve a defect in cysteine catabolism (Heafield *et al.* 1990). The yeast *TUM1* also allows the improvement of commercial yeast strains for wide range of applications in the food industry. *TUM1* overexpression strains that produce H_2S from cysteine more efficiently could potentially increase 3MH/A in wine via the H_2S–C6 pathway. This would be useful to boost thiol aromas in grape juice, characteristic of the fruit-driven wine styles such as Sauvignon Blanc (Harsch *et al.* 2013). Wort can contain high levels of cysteine (up to 35 mg L^{-1}); therefore, *TUM1* deletion strains, which produce less H_2S from cysteine, might be valuable to the brewing industry in managing H_2S, and creating the fruit-driven styles of beers such as American pale ale (Lawrence and Cole 1972; Priest and Stewart 2006).

SUPPLEMENTARY DATA

Supplementary data are available at FEMSYR online.

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Conflict of interest. None declared.

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RESEARCH ARTICLE

Yeast genes involved in regulating cysteine uptake affect production of hydrogen sulfide from cysteine during fermentation

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ABSTRACT

An early burst of hydrogen sulfide (H_2S) produced by *Saccharomyces cerevisiae* during fermentation could increase varietal thiols and therefore enhance desirable tropical aromas in varieties such as Sauvignon Blanc. Here we attempted to identify genes affecting H_2S formation from cysteine by screening yeast deletion libraries via a colony colour assay on media resembling grape juice. Both Δ*lst4* and Δ*lst7* formed lighter coloured colonies and produced significantly less H_2S than the wild type on high concentrations of cysteine, likely because they are unable to take up cysteine efficiently. We then examined the nine known cysteine permeases and found that deletion of *AGP1*, *GNP1* and *MUP1* led to reduced production of H_2S from cysteine. We further showed that deleting genes involved in the SPS-sensing pathway such as *STP1* and *DAL81* also reduced H_2S from cysteine. Together, this study indirectly confirms that Agp1p, Gnp1p and Mup1p are the major cysteine permeases and that they are regulated by the SPS-sensing and target of rapamycin pathways under the grape juice-like, cysteine-supplemented, fermentation conditions. The findings highlight that cysteine transportation could be a limiting factor for yeast to generate H_2S from cysteine, and therefore selecting wine yeasts without defects in cysteine uptake could maximise thiol production potential.

Keywords: *Saccharomyces cerevisiae*; hydrogen sulfide; varietal thiols; cysteine permease; SPS-sensing pathway; target of rapamycin (TOR) pathway

INTRODUCTION

An early burst of hydrogen sulfide (H_2S) produced by *Saccharomyces cerevisiae* during fermentation could potentially elevate the levels of varietal thiols, 3-mercapto-hexanol (3MH) and 3-mercaptohexylacetate (3MHA), and therefore enhance pleasant, tropical aromas in varieties such as Sauvignon Blanc (Schneider *et al.* 2006; Winter *et al.* 2011; Harsch *et al.* 2013; Araujo *et al.* 2016, 2017). However, the majority of H_2S liberation does

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not occur until yeast assimilable nitrogen in grape juice becomes depleted during fermentation (Jiranek, Langridge and Henschke 1995a).

Yeast could be legally induced to produce an early burst of H_2S during winemaking by supplementation with rehydration nutrients that are rich in glutathione. It was proposed that the sulfur-containing amino acid, cysteine, a constituent of glutathione (γ-L-glutamyl-L-cysteinylglycine), is degraded by yeast to generate this early H_2S production (Winter *et al.* 2011). Whilst, cysteine is naturally present in grape juice at very low concentrations (<20 mg L^{-1}) (Ugliano and Henschke 2009), commercial yeast nutrient products containing cysteine e.g. Laffort FreshArom, could be supplemented during fermentation to boost the concentration of antioxidant glutathione and therefore, preserve thiols (O'Kennedy 2013).

Yeast has been known since the 1970s to be capable of releasing H_2S from cysteine (Tokuyama *et al.* 1973). Since then, several yeast genes and mechanisms have been suggested to explain H_2S production from cysteine. Winter, Cordente and Curtin (2014) identified several vacuole-related genes, whose deletion resulted in less H_2S production from cysteine. Their findings suggest that the yeast vacuole, which is functionally similar to the mammalian lysosome, may play a crucial role in relieving cysteine toxicity by degrading cysteine to H_2S. Santiago and Gardner (2015a) demonstrated that the full-length *IRC7* gene encodes a cysteine desulfhydrase, which cleaves cysteine to generate H_2S. However, most yeast strains have a 38-bp deletion within the *IRC7* gene, which results in a truncated protein of 340 amino acids, lacking β-lyase activity (Roncoroni *et al.* 2011). Recently, yeast *TUM1* was reported to affect H_2S from cysteine during fermentation, with yeast Tum1p thought to have enzyme activity similar to its human orthologue, sulfurtransferase, which is responsible for generating H_2S from cysteine (Huang *et al.* 2016). Despite recent progress in our understanding of yeast cysteine catabolism, much work is still required to identify other genes involved in this process and to fill knowledge gaps.

A genome-wide screen of thousands of yeast deletants from yeast single-gene deletion libraries for their H_2S production has proven to be a powerful approach to identify yeast genes responsible for H_2S formation (Linderholm *et al.* 2008; Yoshida *et al.* 2011; Winter, Cordente and Curtin 2014). To date, yeast deletion libraries have been screened using a range of H_2S screening assays including (i) BiGGY (Bismuth Glucose Glycine Yeast) agar plates (Linderholm *et al.* 2008), (ii) YPD plus lead nitrate agar plates (Yoshida *et al.* 2011) and (iii) the methylene blue reduction method (Winter, Cordente and Curtin 2014). However, studies have suggested that deletion of the genes, identified by these assays, does not necessarily have similar impacts on H_2S in a fermentation setting (Linderholm *et al.* 2008; Yoshida *et al.* 2011; Huang *et al.* 2016).

In this work, we aimed to identify other yeast genes required for H_2S production from cysteine during fermentation. Our screen differs from others, in that the AWRI1631 Wine Yeast Deletion Library (WYDL) collection (Varela *et al.* 2012) was screened using a colony colour assay that mimics a typical grape juice (Jiranek, Langridge and Henschke 1995b; Santiago and Gardner 2015a). The genes identified in this study as affecting H_2S formation from cysteine expand not only our current understanding of the cysteine transport process in yeast in a grape juice-like, cysteine-supplemented fermentation condition, but could also be valuable for the breeding of wine yeasts with potential to preserve (enhance) varietal thiols.

METHODS

Yeast strains and culture

The yeast strains used for this study are listed in Table 1. YPD media (10 g L^{-1} yeast extract, 20 g L^{-1} peptone and 20 g L^{-1} glucose) was used for standard yeast propagation at 28°C. Strains transformed with *KanMX* and *HphMX* deletion cassettes were selected on geneticin or G418 sulfate (200 mg L^{-1}; Astral, NSW, Australia) and hygromycin (300 mg L^{-1}; Astral, NSW, Australia),

Table 1. Yeast strains used in this study.

Strain	Genotype, phenotype and comments	Origin
BY4743	*MATa/α, his3-Δ1/his3-Δ1, leu2-Δ0/leu2-Δ0, LYS2/lys2-Δ0, met15-Δ0/MET15, ura3-Δ0/ura3-Δ0*	Euroscarf
BY4743 (pGP564)	BY4743 with (pGP564)	This study
BY4743 (*STP1*ox)	BY4743 with (pGP564+*YDR459C*+*TFB3*+*MFA*+*MRPL28*+***STP1***+*SPP41*)[a]	This study
BY4743 (*IRC7*ox)	BY4743 with (pGP564+*YFR056C*+***IRC7***+*YFR057W*)[b]	This study
BY4743 (*GAP1*ox)	BY4743 with (pGP564+*KAE*+*tD(GUC)K* +***GAP1***+*tA(AGC)K2*+*YKR040C*+*YKR041W*+*UTH1*+*YKR043C*+*UIP5*+*YKR045C*+*PET10*)[c]	This study
BY4743 (*LST4*ox)	BY4743 with (pGP564+*COY1*+*STE3*+*YKL177W*+***LST4*** +*ZRT3*+*YKL174C*+*SNU114*)[d]	This study
BY4743 (*GNP1*ox)	BY4743 with (pGP564+*tL(CAA)D* +*GIN4*+***GNP1***+*YDR509W*+*SMT3*+*YDR510CA*+*ACN9*+*EMI1*+*TTR1*+*YDR514C*)[e]	This study
BY4743 (*MUP1*ox)	BY4743 with (pGP564+*FMP48*+*YGR053C*+*YGR054W*+***MUP1***+*RSC1*+***LST7***+*YGR058W*)[f]	This study
AWRI1631	Haploid wine strain	AWRI
AWRI1631 Δ*lst4*	*lst4::KanMX*	AWRI
AWRI1631 Δ*tum1*	*tum1::KanMX*	AWRI
AWRI1631 Δ*lst7*	*lst7::HphMX*	This study
AWRI1631 Δ*lst4*/Δ*lst7*	*lst4::KanMX; lst7::HphMX*	This study
AWRI1631 Δ*tum1*/Δ*lst4*	*tum1::KanMX*; *lst4::HphMX*	This study
AWRI1631 Δ*tum1*/Δ*lst7*	*tum1::KanMX*; *lst7::HphMX*	This study
F15 (*IRC7*^{F}ox)	*ho*::P_{PGK}-*IRC7*^{F} in F15-h(*α*)	Roncoroni *et al.* (2011)

ox denotes overexpression. Genes of interests are denoted by bold font.

Identities of plasmids from the YSC4613 Yeast Genomic Tiling Collection (Jones *et al.* 2008) are as follows: [a]YGPM-10d14; [b]YGPM-25o08; [c]YGPM-27p10; [d]YGPM-25b18; [e]YGPM-25o09; [f]YGPM-14k19.

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respectively (Goldstein and McCusker 1999). Non-sulfate CDGJM medium (Huang et al. 2016), used in agar plates for screening H_2S production, was prepared by combining filter-sterilised 2x stock and molten 2x bacteriological agar (RM250, Amyl Media, Melbourne, Australia).

Screening of yeast deletants for effect on H_2S formation from cysteine

Cultures of the AWRI1631 WYDL collection (Varela et al. 2012) and BY4741 deletion collection (Euroscarf), stored at –80°C, were thawed and transferred to 96-well plates (Costar 3596, Sigma-Aldrich, NSW, Australia) containing 200 μL YPD using a 96-channel pipette (Gilson PlateMaster P220, John Morris Scientific, Australia) and incubated for 48 h at 28°C. The yeast precultures (5 μL) were then spot-inoculated using a 96-channel pipette onto Nunc OmniTrays (O0764–1CS; 128 mm × 86 mm; Sigma-Aldrich, NSW, Australia) containing non-sulfate chemically defined grape juice agar medium. The media composition contained magnesium chloride rather than magnesium sulfate (Harsch et al. 2010; Santiago and Gardner 2015b; Huang et al. 2016), 5 g L^{-1} bismuth, 0.15 mM methionine plus or minus 5 mM cysteine (non-sulfate CDGJM agar + Bi ± 5 mM Cys + 0.15 mM methionine). It should be noted that the AWRI1631 deletion library was screened on non-sulfate CDGJM agar + Bi ± 5 mM Cys + 0.15 mM magnesium sulfate + no methionine. The minimal concentration (0.15 mM) of magnesium sulfate that was initially supplemented to facilitate cell growth was later replaced with 0.15 mM methionine (non-sulfate CDGJM agar + Bi ± 5 mM Cys + 0.15 mM methionine) for the screening of BY4741 deletion library, in order to minimise H_2S production from the sulfate assimilation pathway. The screenings were conducted in duplicate. The plates were incubated at 28°C for 96 h, and colony colour was assessed visually against the wild-type strains.

Fermentations and H_2S quantification

The selected candidate genes identified from the screening were further validated in lab-scale fermentations. Yeast starter cultures were prepared by inoculating a single yeast colony into starter medium (2% sugar, non-sulfate CDGJM plus 0.15 mM methionine) for 24 h at 28°C. The starter culture was centrifuged, washed and resuspended in sterile water to inoculate 100 mL of medium (non-sulfate CDGJM ± 5 mM Cys + 0.15 mM methionine) at 2.5 × 10^6 cells L^{-1}. Elevated amounts of histidine (200 mg L^{-1}), leucine (300 mg L^{-1}) and uracil (100 mg L^{-1}) were included for auxotrophies (Harsch et al. 2010). Fermentations were conducted in triplicate in 250 mL flasks at 28°C with shaking at 100 rpm. Fermentation progress was monitored daily as weight loss due to CO_2 evolution (Bely, Sablayrolles and Barre 1990). Ferments were considered finished when weight loss was ≤ 0.1 g per 24 h. H_2S produced by yeast during fermentation was measured by lead acetate H_2S detector tubes (4H: 1–2000 ppm; GASTEC, Japan) that tightly fitted into the glass fermentation airlock (Park 2008).

Genetic manipulation and strain construction

PCR was performed using Velocity DNA polymerase (Bioline, Australia). Yeast deletion strains were confirmed using Kan B or Kan C primers together with gene-specific primers as reported in Table S1 (Supporting Information). Yeast transformation was conducted using the lithium acetate method (Gietz et al. 1992).

The deletion of *LST7* was achieved by amplifying the *HphMX* cassette in plasmid pAG32 plus ~100 bp of homologous untranslated sequence flanking *LST7* (using the primers pair Del-*lst7*-F and Del-*lst7*-R; Table S1, Supporting Information). The PCR products were used for yeast transformation with selection of transformants on YPD agar plates containing hygromycin (300 mg L^{-1}). *LST7::HphMX* deletants were confirmed by PCR using primers *Hph*-I-F and RC*lst7* (Table S1, Supporting Information). The *LST4* was deleted through a similar approach.

The overexpression of genes involved in regulating cysteine uptake was achieved by using the Yeast Genomic Tiling Collection (Jones et al. 2008) purchased from Open Biosystems (YSC4613, Thermo Fisher Scientific, Lafayette, CO). The leucine auxotrophic strain BY4743 was individually transformed with the plasmid pGP564 (*LEU2* selectable marker and 2-μm plasmid, as control) and plasmids containing the cloned ORFs of the candidate genes. The transformants were selected on synthetic complete leucine drop-out plates (SC-leu) (Sherman 2002). It should be noted that these overexpression strains not only overexpressed the gene of interest but also the adjacent three to four genes.

Data analysis

The mean, standard error of the mean (SEM) and *t* test (two samples assuming unequal variances) were performed using Microsoft Excel 2013 (Microsoft, Redmond, Washington, USA). Analysis of variance (ANOVA) and Tukey's honestly significant difference test were conducted using JMP software (SAS Institute, Cary, NC, USA). Statistical significance was set at the confidence level of 5%.

RESULTS

Validation of yeast deletion library genotype and screening assay for H_2S production

Several yeast genes responsible for H_2S formation from cysteine have been successfully identified from a genome-wide screen using a laboratory BY4742 yeast deletion library (Winter, Cordente and Curtin 2014). In order to identify other candidate genes affecting H_2S formation from cysteine, we conducted a genome-wide screen but using other yeast deletion libraries and an alternative screening assay.

Two yeast deletion libraries were screened in this study: (i) the AWRI1631 wine yeast deletion library (~2000 deletants) (Varela et al. 2012) and (ii) the laboratory BY4741 (Δ*met17*) yeast deletion library (~5000 deletants). The AWRI1631 wine yeast deletion library was selected because the deletion library was constructed using a prototrophic wine strain and the strain background is more representative of that used to conduct wine fermentations. It also has not previously been screened for H_2S production from cysteine. Deletion of *MET17* was observed to produce an additional delayed burst of H_2S from cysteine (Huang et al. 2016). Interestedly, this delayed burst of H_2S was not detected for the *MET5* deletant in the BY4741 (Δ*met17*) background during our initial trials (Fig. S1A, Supporting Information). Therefore, to identify other genes affecting this delayed H_2S production caused by the deletion of *MET17*, we decided also to screen H_2S production by deletants in the BY4741 (Δ*met17*) yeast deletion library.

A modified version of bismuth-containing indicator agar resembling grape juice (Jiranek, Langridge and Henschke 1995b; Santiago and Gardner 2015a) was employed in this work to

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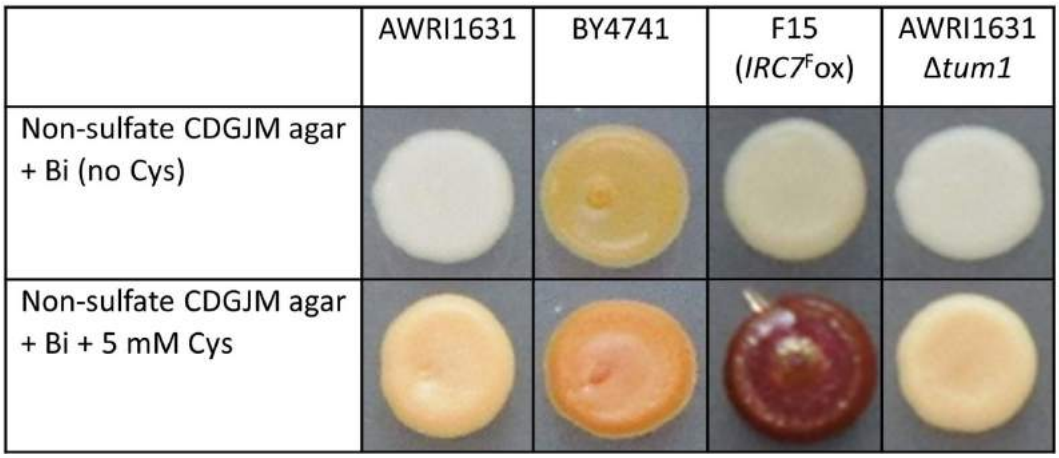


Figure 1. Colony colours of the wild-type strains on non-sulfate chemically defined grape juice agar plates containing 5 g L^{-1} bismuth, 0.15 mM methionine plus or minus 5 mM cysteine. The known high H_2S producer from cysteine, F15 (*IRC7*^{F}ox), was used as positive control. The known low H_2S producer from cysteine, AWRI1631 Δ*tum1*, was used as negative control. Images were taken after 96 h incubation at 28°C.

screen H_2S formation from cysteine. The assay is sensitive enough to detect H_2S formed this way as both AWRI1631 and BY4741 strains were observed to form darker coloured colonies when cysteine (5 mM) was added (Fig. 1). The principle of the assay is based on bismuth reacting with sulfide to form dark coloured precipitates of bismuth sulfide (Nickerson 1953). We were interested in those deletants that could still form light coloured colonies on cysteine supplemented media, as this would indicate that the genes deleted were involved in the generation of H_2S from cysteine. Since BY4741 already formed quite darkly coloured colonies on non-sulfate CDGJM agar + Bi + 5 mM Cys + 0.15 mM methionine (Fig. 1), we were primarily interested in deletants that produced light colony colours when screening the BY4741 deletion library.

Deletion of *LST4* or *LST7* resulted in lighter coloured colonies and reduced production of H_2S from cysteine

Both Δ*lst4* and Δ*lst7* deletants were identified to produce somewhat lighter coloured colonies than the wild-type strains from the screening experiment (Fig. 2A). Interestingly, the Δ*lst4* deletant was also identified as a low H_2S producer from cysteine in the previous genome-wide study using the BY4742 deletion library (Winter, Cordente and Curtin 2014). A further laboratory-scale fermentation experiment using non-sulfate CDGJM and 0.15 mM methionine plus or minus 5 mM cysteine (Huang *et al.* 2016) was thus performed to confirm the screening results.

In addition, the deletion strains in the BY4743 laboratory background have been successfully used to identify genes affecting H_2S production from cysteine during fermentation and they were also observed to ferment much better than strains in the BY4742 laboratory background (Harsch *et al.* 2010; Huang *et al.* 2016). Therefore, we decided to include deletion strains in the BY4743 background to further verify the results obtained from the screens and use them to explore other candidate genes affecting H_2S production from cysteine.

The fermentation experiment showed that H_2S was only produced by wild-type strains when cysteine was supplemented, indicating that cysteine is the most likely source of H_2S (Fig. 2B). It also confirmed that the Δ*lst4* and Δ*lst7* deletants did in fact produce significantly less H_2S on high concentrations of cysteine than the wild-type strains in different genetic backgrounds (Fig. 2B).

Although the Δ*lst4* and Δ*lst7* deletants were observed to ferment relatively slower than the wild type (Figs S2A and S2B, Supporting Information), this minor decrease in fermentation rates is unlikely to result in the observed ∼70% reduction in H_2S formation (Fig. 2B: BY4743 and AWRI1631 background). Therefore, we decided to further investigate the roles of *LST4* and *LST7* in cysteine catabolism.

Deletion of *LST4* or *LST7* further decreased H_2S in a Δ*tum1* deletant

Previous studies have suggested that multiple genes are responsible for H_2S production from cysteine (Winter, Cordente and Curtin 2014; Huang *et al.* 2016). The yeast *TUM1* gene has been recognised as one of the key genes responsible for the production of H_2S from cysteine during fermentation (Huang *et al.* 2016). We were therefore interested in the additive effect of deleting both *TUM1* and *LST4* or *LST7* on H_2S from cysteine. The amount of H_2S produced by an AWRI1631 Δ*tum1* deletant was further reduced by deleting either *LST4* or *LST7*. As shown in Fig. 3, there was no difference in H_2S production between the double deletants (Δ*lst4*/Δ*lst7*) and the single deletants of Δ*lst4* and Δ*lst7*.

Deletion of *GNP1*, *AGP1* and *MUP1* reduced H_2S production from cysteine

The yeast Lst4-Lst7 GTPase-activating protein complex is responsible for activating Gtr2p, in response to the presence of amino acids and the target of rapamycin (TOR) pathway (Péli-Gulli *et al.* 2015). This complex mediates the transport of the general amino acid permease Gap1p from the Golgi to the cell surface, with mutations in *LST4* and *LST7* leading to a decrease in Gap1p activity (Roberg *et al.* 1997). Therefore, the reduced H_2S production from cysteine observed for Δ*lst4* and Δ*lst7* is most likely as a result of reduced cysteine uptake. *GAP1* and the cysteine permease genes *AGP1*, *GNP1*, *BAP2*, *BAP3*, *TAT1*, *TAT2*, *MUP1* and *YCT1* (During-Olsen *et al.* 1999; Kosugi *et al.* 2001; Kaur and Bachhawat 2007) were chosen for further investigation. To our surprise, deletion of *GAP1* did not reduce

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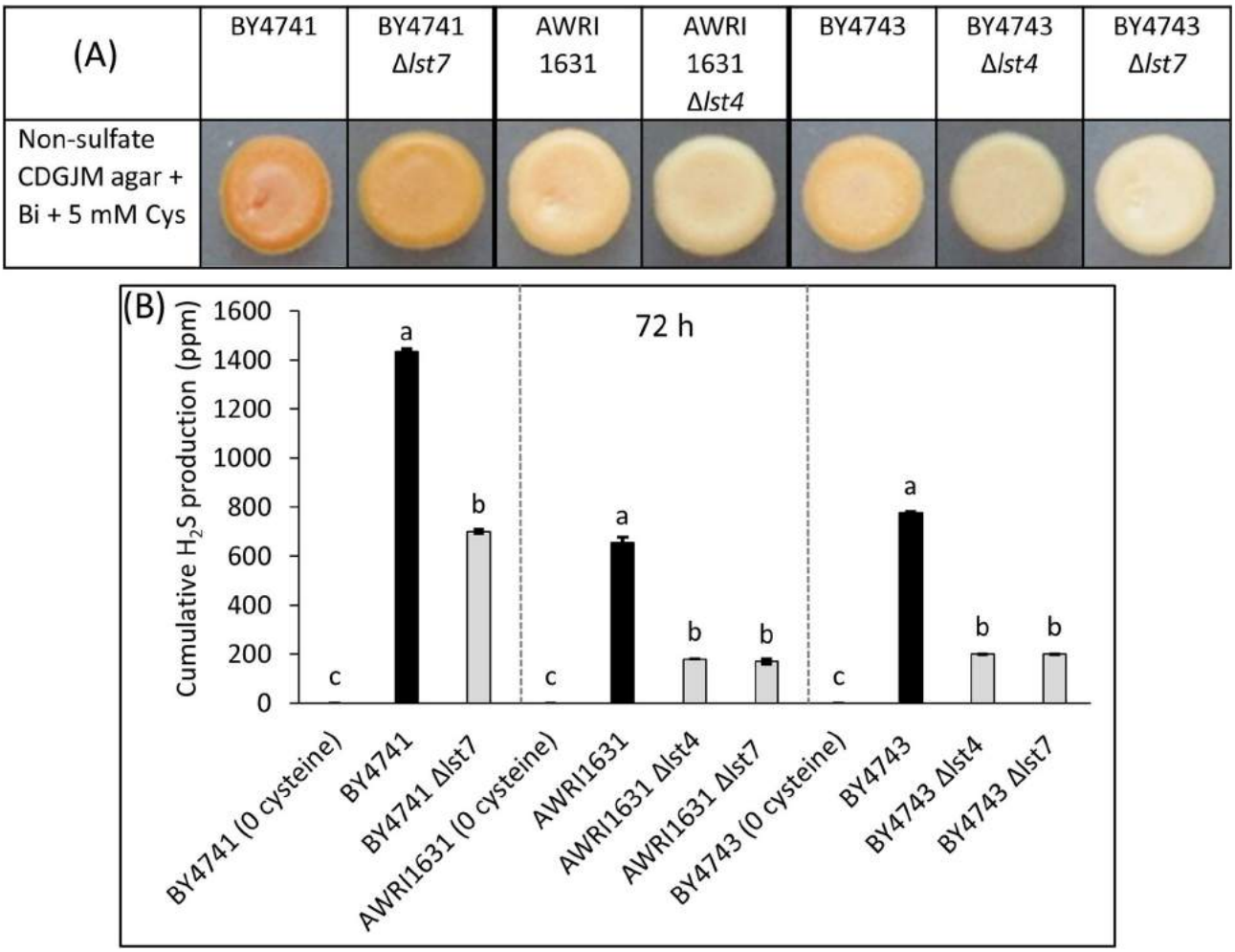


Figure 2. **(A)** Colony colours of the wild-type strains and the *LST* gene deletants on non-sulfate chemically defined grape juice agar plates containing 5 g L^{-1} bismuth, 5 mM cysteine and 0.15 mM methionine. Images were taken after 96 h incubation at 28°C. **(B)** Cumulative H_2S production of the wild-type strains and the *LST* gene deletants in different yeast genetic backgrounds (separated by dotted lines). Fermentations were performed in 100 mL of non-sulfate CDGJM and 0.15 mM methionine plus or minus 5 mM cysteine at 28°C with shaking at 100 rpm. H_2S was measured by lead acetate H_2S detector tubes (4H: 1–2000 ppm; GASTEC, Japan). Data represent mean values of triplicate fermentations ± standard error of the mean (SEM). Samples not connected by the same letter are significantly different (ANOVA, Tukey's HSD).

H_2S from cysteine but deletion of *GNP1*, *AGP1* and *MUP1* did in the laboratory strain BY4743 background (Fig. 4). A reduction in production of H_2S from cysteine was also observed for Δ*mup1* deletant in the AWRI1631 background (Fig. 4).

Deletion of *STP1* and *DAL81* involved in the SPS-sensing pathway reduced H_2S production from cysteine

The expression of yeast *GAP1* has been shown to be regulated by nitrogen (nitrogen catabolite repression) (Hofman-Bang 1999). On the other hand, *AGP1* and *GNP1* are regulated by Ssy1-Ptr3-Ssy5 (SPS) sensor (Forsberg *et al.* 2001). We were interested in knowing whether deletion of genes involved in the SPS-sensing pathway would also lead to a reduction in H_2S production from cysteine. Our results showed that deletion of *STP1* and *DAL81* did reduce H_2S from cysteine significantly (Fig. 5), and this indirectly demonstrated that the SPS-sensing pathway did play a significant role in cysteine uptake under the fermentation conditions.

Other deletants identified from the screening of AWRI1631 wine yeast deletion library

A few deletants other than Δ*lst4* and Δ*lst7* were also observed to form lighter coloured colonies than the wild-type on non-sulfate CDGJM agar + Bi ± 5 mM Cys + 0.15 mM magnesium sulfate + no methionine (Table 2); an indication that they produced less H_2S from cysteine. However, these deletants either had smaller colonies (e.g. AWRI1631 Δ*mct1*, Δ*ktr1*) or are involved in the sulfate assimilation pathway (e.g. AWRI1631 Δ*met5*, Δ*met16*; Fig. S3, Supporting Information). The smaller colony-forming deletants such as AWRI1631 Δ*mct1*, Δ*ktr1* fermented slower than the wild type (data not shown), suggesting that their inability to generate H_2S (dark colonies) from cysteine was likely due to their growth defect. Deletion of *MET* genes (e.g. *MET1*, *MET5*, *MET8* and *MET10*) has been shown to result in the formation of white colonies on BiGGY agar (Linderholm *et al.* 2008) and here, the *MET* gene deletants were also observed to form lighter coloured colonies in the presence of cysteine, although they also had lighter colouration even when cysteine was not added (Fig. S3, Supporting

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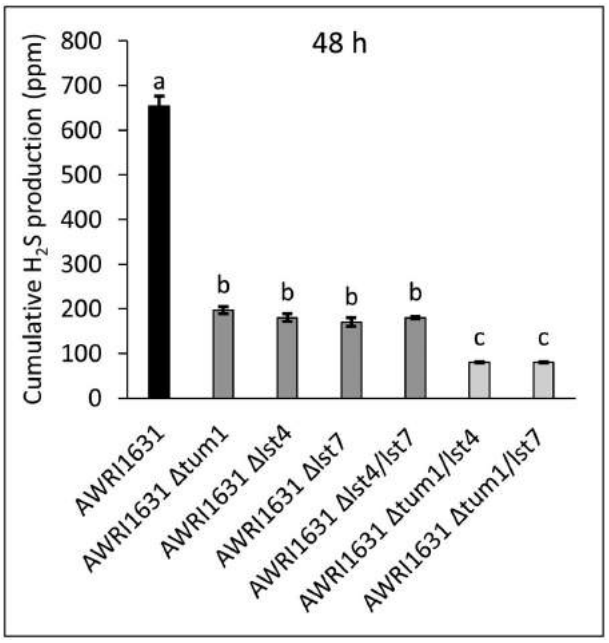


Figure 3. Cumulative H_2S production from AWRI1631 and its double *TUM1/LST* gene deletants. Fermentations were performed in 100 mL of non-sulfate CDGJM plus 5 mM cysteine and 0.15 mM methionine at 28°C with shaking at 100 rpm. H_2S was measured by lead acetate H_2S detector tubes (4H: 1–2000 ppm; GASTEC, Japan) and the mean H_2S released is shown ($n = 3$). Error bars indicate SEM. Samples not connected by the same letter are significantly different (ANOVA, Tukey's HSD).

Information). Since *MET3*, *MET5* and *MET10* do not affect H_2S from cysteine during fermentation (Huang *et al.* 2016), a significant amount of the H_2S detected by the assay conditions (0.15 mM sulfate) was likely to be from the sulfate assimilation pathway.

Some of the yeast deletants formed darker coloured colonies than the wild-type on non-sulfate CDGJM agar + Bi ± 5 mM Cys + 0.15 mM magnesium sulfate + no methionine (Table 2), indicating that they produced more H_2S from cysteine. However, most of these already formed darker coloured colonies even when cysteine was not supplemented (Fig. S3, Supporting Information) and some of the deletants (e.g. AWRI1631 Δ*hom2*, Δ*hom6*) have been identified to form darker coloured colonies on BiGGY agar (Linderholm *et al.* 2008). Therefore, their effects on colony colouration might not be cysteine specific. Interestingly, yeast deletants with smaller colonies (e.g. AWRI1631 Δ*pps1*) were observed to produce darker colonies than the wild type.

The formation of darker coloured colonies than the wild type by vacuole-related gene deletants (e.g. AWRI1631 Δ*vps4*, Δ*vps25* and Δ*vps36*) (Fig. S3, Supporting Information) is consistent with the BiGGY agar results obtained by Linderholm *et al.* (2008). However, Winter, Cordente and Curtin (2014) proposed that this elevated production of H_2S by vacuole-related gene deletants (seen as darkly coloured colonies on BiGGY agar) is not related to cysteine catabolism, and it has also been observed that deletion of *VPS25* or *VPS36* in AWRI1631 had limited effect on H_2S from cysteine during fermentation (Huang *et al.* 2016).

Deletion of *HEM25* may reduce H_2S from cysteine

The AWRI1631 Δ*hem25* deletant was observed to form lighter coloured colonies than the wild type in the initial screen (Fig. S3, Supporting Information); however, the colonies were also lighter when cysteine was not supplemented. Fermentation

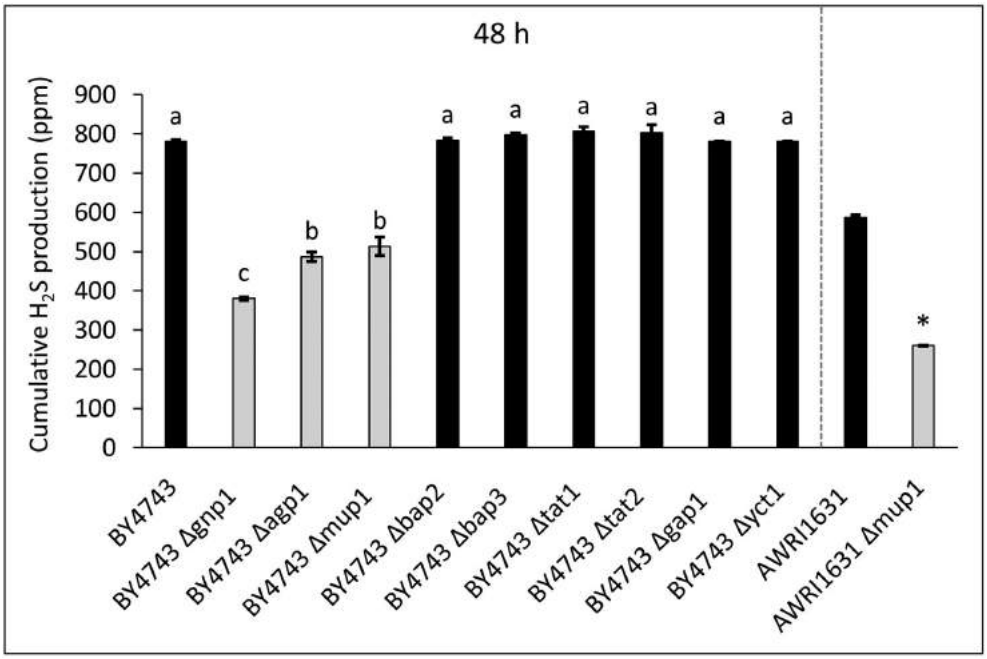


Figure 4. Cumulative H_2S production from the cysteine permease gene deletants in laboratory strain BY4743 and wine yeast strain AWRI1631 (separated by dotted lines). Fermentations were performed in 100 mL of non-sulfate CDGJM plus 5 mM cysteine and 0.15 mM methionine at 28°C with shaking at 100 rpm. H_2S was measured by lead acetate H_2S detector tubes (4H: 1–2000 ppm; GASTEC, Japan) and the mean H_2S released is shown ($n = 3$). Error bars indicate SEM. Samples not connected by the same letter are significantly different (ANOVA, Tukey's HSD). Asterisks above bars represent significant differences compared to the wild types (*$P < 0.05$, two-tailed Student's *t* test).

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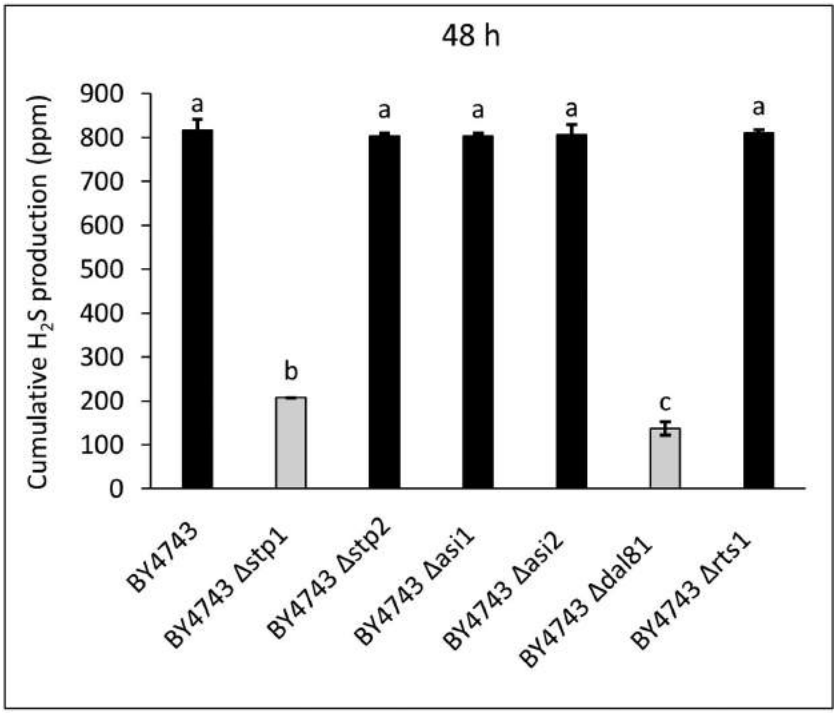


Figure 5. Cumulative H_2S production from the deletants involved in SPS-sensing pathway in laboratory strain BY4743. Fermentations were performed in 100 mL of non-sulfate CDGJM plus 5 mM cysteine and 0.15 mM methionine at 28°C with shaking at 100 rpm. H_2S was measured by lead acetate H_2S detector tubes (4H: 1–2000 ppm; GASTEC, Japan), and the mean H_2S released is shown ($n = 3$). Error bars indicate SEM. Samples not connected by the same letter are significantly different (ANOVA, Tukey's HSD).

Table 2. AWRI1631 deletants that formed lighter or darker colony colours than the wild type on non-sulfate CDGJM agar + Bi ± 5 mM cysteine + 0.15 mM $MgSO_4$ + no methionine.

AWRI1631 deletants that formed lighter coloured colonies	Functional name/group
Δ*met5*, Δ*met16*	Methionine biosynthesis
Δ*hem25*	Mitochondrial glycine transporter
Δ*mct1*	Phospholipid remodelling
Δ*lih1*	Lipase homologue
Δ*ktr1*	Kre two related
Δ*trp4*	Tryptophan biosynthesis
AWRI1631 deletants that formed darker coloured colonies	**Functional name/group**
Δ*hom2*, Δ*hom6*	Methionine biosynthesis
Δ*vps4*, Δ*vps25*, Δ*vps36*, Δ*snf8*, Δ*bro1*	Vacuolar protein sorting
Δ*ino2*, Δ*ino4*	Phospholipid synthesis
Δ*fre3*	Cellular iron ion homeostasis
Δ*npr1*	Nitrogen permease reactivator
Δ*pps1*	Protein phosphatase S phase
Δ*tom5*	Translocase of the outer mitochondrial membrane
Δ*stp22*	Sterile pseudoreversion
Δ*rrd1*	Resistant to rapamycin deletion
Δ*gdh1*	Glutamate biosynthetic process
Δ*yhl026c*, Δ*yjr120w*	Unknown

[a]Function information was obtained from *Saccharomyces* Genome Database (http://www.yeastgenome.org/).
[b]Deletants denoted by bold font indicate they were also identified to form dark coloured colonies on BiGGY agar (Linderholm *et al.* 2008).
[c]Deletants in underlined font indicate they had obvious smaller colonies (growth defects).

trials revealed that Δ*hem25* did produce less H_2S than the wild type in both BY4743 and AWRI1631 backgrounds, but deletion of *HEM25* only resulted in ~30% reduction in H_2S production in the AWRI1631 background (Fig. 6). Therefore, it is unclear whether the *HEM25* gene plays a significant role in H_2S formation from cysteine, given that the Δ*hem25* deletants were observed to ferment at a slightly slower rate relative to the wild-type strains (Fig. S4, Supporting Information). This slight growth defect was also observed by Lunetti *et al.* (2016) for BY4742 Δ*hem25* grown on YPD media.

The yeast *HEM25* gene (Heme synthesis by SLC25 family member) has recently been identified as encoding a

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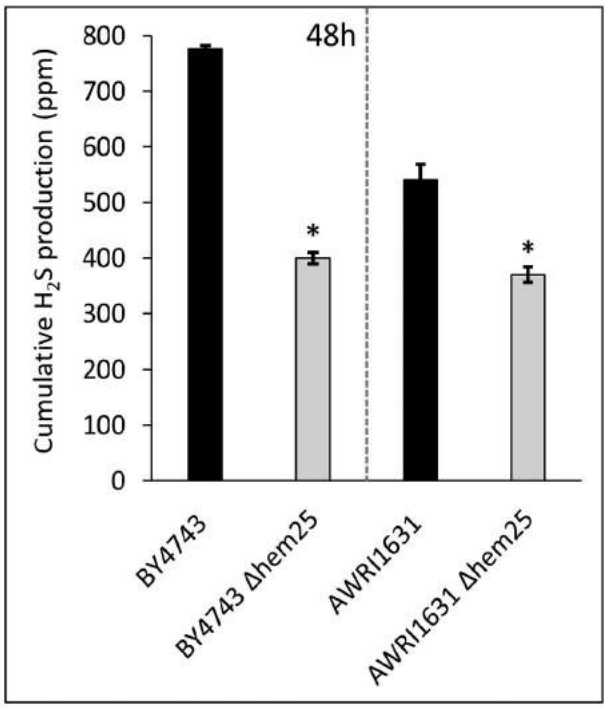


Figure 6. Cumulative H_2S production of the wild-type strains and the Δ*hem25* deletants in laboratory strain BY4743 and wine yeast strain AWRI1631 backgrounds (separated by dotted lines). Fermentations were performed in 100 mL of non-sulfate CDGJM plus 5 mM cysteine and 0.15 mM methionine at 28°C with shaking at 100 rpm. H_2S was measured by lead acetate H_2S detector tubes (4H: 1–2000 ppm; GASTEC, Japan). Data represent mean values of triplicate fermentations ± standard error of the mean (SEM). Asterisks above bars represent significant differences compared to the wild types (*$P < 0.05$, two-tailed Student's *t* test).

mitochondrial glycine transporter and is required for heme synthesis (Fernández-Murray *et al.* 2016). Given that H_2S from cysteine was affected by some of genes involved in iron–sulfur homeostasis when deleted (Winter, Cordente and Curtin 2014), makes it perhaps further investigation of the role of *HEM25* in H_2S formation from cysteine by more sensitive H_2S detection methods is worthwhile, in order to decipher the mechanism involved.

Other deletants identified from the screening of laboratory BY4741 (Δ*met17*) yeast deletion library

To identify the candidate genes affecting the delayed burst of H_2S from cysteine (Δ*met17*), the BY4741 (Δ*met17*) yeast deletion library was screened on non-sulfate CDGJM agar +Bi +0.15 mM methionine ±5 mM cysteine. However, most of the deletants that formed lighter coloured colonies (e.g. BY4741 Δ*tre1*, Δ*irc10* and *MET* genes deletants) also produced lighter coloured ones in the absence of cysteine (Table 3, Fig. S5, Supporting Information). These findings suggest that a substantial amount of the H_2S detected was from the sulfate assimilation pathway even though non-sulfate CDGJM was used. BY4741 Δ*tre1* and Δ*irc10* were observed not to affect the delayed burst of H_2S from cysteine (Fig. S1A, Supporting Information). Yeast deletants such as **Δ***mac1*, Δ*ado1* that formed smaller colonies or those noted as slower growers (e.g. Δ*coa1*, Δ*mdm32*) tended to form lighter coloured colonies. Interestingly, most of the deletants that formed lighter coloured colonies identified in this study have been observed to form lighter coloured colonies on lead nitrate agar plates (Yoshida *et al.* 2011). Collectively, the results suggested that most of the light coloured colonies identified did not specifically affect H_2S formation from cysteine.

BY4741 Δ*met5**, Δ*qdr2**, Δ*msl1** and Δ*coa1** were observed to affect the delayed burst of H_2S from cysteine (Fig. S1A, Supporting Information). However, they turned out to have a functional *MET17* gene (as identified by PCR; Fig. S1B: Δ*met5**, Supporting Information), so whilst they have decreased H_2S production, it is not related to the delayed burst of H_2S as described by Huang *et al.* (2016).

The yeast Dbf2p is a serine-threonine protein kinase that has been shown to be required for transcription, sorbic acid stress tolerance and vacuolar acidification (Liu *et al.* 1997; Makrantoni *et al.* 2007). The vacuolar acidification deletants have been

Table 3. BY4741 deletants that formed lighter or darker colony colours than the wild type on non-sulfate CDGJM agar + Bi ± 5 mM cysteine + 0.15 mM methionine.

BY4741 deletants that formed lighter coloured colonies	Functional name/group
(**Δ***cbf1*), **Δ***met1*, **Δ***met3*, **Δ***met10*, **Δ***met14*, **Δ***met16*, (**Δ***met18*), **Δ***met28*, **Δ***met5**	Methionine biosynthesis
(**Δ***ada2*)	Transcription
Δ*tre1*	Metal transporter degradation
Δ*mdm32*	Mitochondrial inner membrane protein
Δ*rsm25*	Mitochondrial ribosomal protein of the small subunit
Δ*sds3*	Component of the Rpd3L histone deacetylase complex
Δ*ldb18*	Component of the dynactin complex
Δ*paf1*	RNA polymerase II associated factor
Δ*irc10*, (**Δ***mac1*), (**Δ***ado1*), (Δ*pex4*), (Δ*ume6*), (Δ*rtg2*), (Δ*idh1*), (**Δ***fyv5*), (**Δ***fen2*), (**Δ***trk1*), **Δ***suv3*, **Δ***dfg10*, **Δ***tps2*, **Δ***srv2*, **Δ***ccs1*, **Δ***rtc6*, **Δ***qdr2**, **Δ***msl1**, **Δ***coa1**, Δ*lst7*, Δ*ssd1*	Others
BY4741 deletants that formed darker coloured colonies	**Functional name/group**
Δ*dbf2*	Ser/Thr kinase

[a]Function information was obtained from *Saccharomyces* Genome Database (http://www.yeastgenome.org/).
[b]Deletants in underlined font were also observed to form light brown colonies on YPD plus lead nitrate agar plates (Yoshida *et al.* 2011).
[c]Deletants denoted by an asterisk indicated that the *MET17* in BY4741 was not deleted within the library.
[d]Deletants denoted by bracket indicated that these were defective in growth, having smaller colonies on either 0 or 5 mM cysteine.
[e]Deletants denoted by bold font indicate they already had obvious lighter coloured colonies on plate without cysteine.

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shown to produce less H_2S from cysteine (Winter, Cordente and Curtin 2014) but, interestingly, here, deletion of *DBF2* gene in the BY4741 background was observed to form darker coloured colonies when cysteine was supplemented (Table 3, Fig. S5, Supporting Information). It has been proposed that the slower fermentation rate is the major contributor to the lower levels of H_2S from cysteine produced by the vacuolar acidification deletants (Huang *et al*. 2016). The fact that these mutants form light brown colonies on YPD plus lead nitrate agar plates in the absence of cysteine (Yoshida *et al*. 2011), whilst other, vacuole-related, mutants have darker coloured colonies on bismuth-containing plates (Linderholm *et al*. 2008), suggests that the darker coloured colonies produced by Δ*dbf2* are also not directly related to cysteine. Nevertheless, the possibility that the colony colour is related to other functions of Dbf2p cannot be excluded.

DISCUSSION

In this work, we have shown that either deletion of yeast *LST4* or *LST7* reduced production of H_2S from high concentrations of cysteine (5 mM) during fermentation. This led to the identification of a new set of genes involved in regulating cysteine uptake that, in turn, impact H_2S production from cysteine during fermentation. Whilst our study is not the first to report identifying Δ*lst4* as a low H_2S producer from cysteine (Winter, Cordente and Curtin 2014), the mechanism by which *LST4* gene affects H_2S from cysteine remains to be elucidated. Yeast *LST4* and *LST7* have long been known for their important roles in the transport of the general amino acid permease Gap1p from the Golgi to the cell surface (Roberg *et al*. 1997), but it was not until recently that the Lst4-Lst7 complex was also revealed to be involved in the regulation of amino acid signalling by mediating the activity of target of rapamycin complex 1 (TORC1), a central regulator of eukaryotic cell growth (Schmelzle and Hall 2000; Péli-Gulli *et al*. 2015). Our findings suggest that for Δ*lst4* and Δ*lst7*, the reduction in H_2S derived from cysteine is most likely due to a defect in cysteine uptake/sensing, and unrelated to the degradation process, by which H_2S is formed within the yeast cell.

The deletion of *TUM1* has been observed to reduce the H_2S formed from cysteine by half during fermentation, and *TUM1* was proposed to act like a sulfurtransferase when generating H_2S from cysteine (Huang *et al*. 2016). The observation that deletion of *TUM1* further reduced the amount of H_2S produced from cysteine by the Δ*lst4* or Δ*lst7* single deletants by ∼54% (Fig. 3) is consistent with the interpretation that the effects of *LST4*/*LST7* and *TUM1* genes on H_2S formation from cysteine are caused by two different mechanisms. The observation that the Δ*lst4*/Δ*lst7* double deletant produced similar amount of H_2S as the Δ*lst4* or Δ*lst7* single deletants (Fig. 3) indicates that a disruption of either *LST4* or *LST7* genes is enough to cause a loss of Lst4–Lst7 complex function.

To date, nine cysteine permease genes *GAP1*, *AGP1*, *GNP1*, *BAP2*, *BAP3*, *TAT1*, *TAT2*, *MUP1* and *YCT1* have been identified. The effect of these genes is dependent on growth condition and yeast genotype (During-Olsen *et al*. 1999; Kosugi *et al*. 2001; Kaur and Bachhawat 2007). For example, Yct1p (yeast cysteine transporter) is the main cysteine-specific transporter at low-cysteine (<200 μM) concentrations. Its role becomes less prominent when other permeases start to take up cysteine at high-cysteine (>500 μM) concentrations (Kaur and Bachhawat 2007). In addition, the cysteine permeases Agp1p, Gnp1p and Mup1p have been reported as the major cysteine transporters in media containing ammonium and limited amino acids. Deletion of *GNP1* and *MUP1* was shown to decrease cysteine uptake by at least 40% (minimal ammonium medium + 0.3 mM L-[^{35}S] cysteine; During-Olsen *et al*. 1999) and 60% (synthetic medium + 50 μM L-[^{35}S] cysteine; Kosugi *et al*. 2001), respectively. In our study, a reduced amount of H_2S from cysteine was also observed for Δ*gnp* (∼51%), Δ*agp1* (∼38%) and Δ*mup1* (∼34%) in BY4743 but not the other cysteine permease deletants tested (Fig. 4). The results indirectly demonstrated that Agp1p, Gnp1p and Mup1p are the dominant cysteine permeases in grape juice-like, cysteine-supplemented fermentation conditions and also showed that the cysteine uptake process could be a limiting factor for yeast to generate H_2S from cysteine. Interestingly, deletion of *MUP1* led to a ∼56% reduction in H_2S from cysteine in AWRI1631, whilst in BY4743, H_2S production was only reduced by 34% (Fig. 4). It would appear that the effects of these permease genes are strain dependent, which may partly explain why different cysteine permeases have been implicated as the main cysteine permease in other studies (During-Olsen *et al*. 1999; Kosugi *et al*. 2001; Kaur and Bachhawat 2007).

The expression of yeast cysteine permease genes such as *AGP1* and *GNP1* is regulated by the plasma membrane Ssy1p-Ptr3p-Ssy5p (SPS) sensor (Forsberg *et al*. 2001). The SPS sensor is activated in response to extracellular amino acids, and induces endoproteolytic processing of the N-terminal regulatory domain of the transcription factors Stp1p and Stp2p (located in the cytoplasm). Following targeting to the nucleus, the shortened forms of Stp1p and Stp2p activate transcription of amino acid permease genes (Andréasson and Ljungdahl 2002). TORC1 is also activated by the Lst4-Lst7 complex on the vacuolar membrane (Péli-Gulli *et al*. 2015), whereby TORC1 controls the expression of the amino acid permeases through stabilisation of the transcription factor Stp1p (Shin, Kim and Huh 2009). Expression of the permease genes is further enhanced by the action of Dal81p (Boban and Ljungdahl 2007).

This study took advantage of yeast being able to generate H_2S from cysteine; the amount produced by individual yeast gene deletants related to cysteine regulation, a rough indicator of the amount of cysteine taken up. The observation that deletion of genes (in BY4743) involved in regulating amino acids uptake such as *LST4*, *LST7*, *STP1* and *DAL81* reduced H_2S formation from cysteine indirectly confirmed the findings of previous studies (Boban and Ljungdahl 2007; Shin, Kim and Huh 2009; Péli-Gulli *et al*. 2015). Figure 7 shows the model proposed regarding the regulation of cysteine uptake in *S. cerevisiae* during fermentation in a grape juice-like medium supplemented with cysteine, in which genes involved the SPS-sensing pathway and TOR pathway play central roles.

Deletion of amino acid sensor-independent genes, e.g. Δ*asi1*, has been shown to result in constitutive expression of SPS sensor-regulated genes, e.g. *AGP1* and *GNP1*, as a result of the unprocessed transcription factors Stp1p and Stp2p leaking from the cytoplasm into the nucleus and inducing the transcription of amino acid permease genes (Boban and Ljungdahl 2007). Deletion of *RTS1* was also observed to lead to constitutive amino acid signalling as the result of the increased Stp1p processing (Eckert-Boulet *et al*. 2006). Interestingly, deletion of *ASI1* or *RTS1* in BY4743 did not affect H_2S production from cysteine (Fig. 5). Results from a preliminary trial (Fig. S6, Supporting Information), whereby the genes were overexpressed using plasmids from the Genome Tiling library (Jones *et al*. 2008), suggested that overexpression of genes involved in regulating cysteine uptake may not elevate production of H_2S from cysteine. This result is not surprising, given that Kosugi *et al*. (2001) observed that overexpressing *MUP1* in a Δ*mup1* strain only restored the rate

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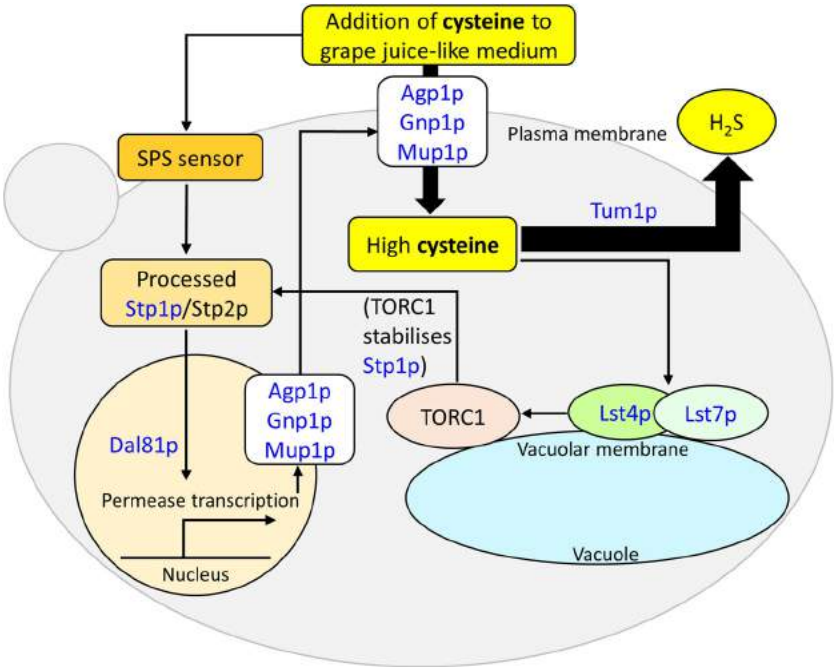


Figure 7. A model proposed for the regulation of cysteine uptake in *S. cerevisiae* under the grape juice-like, cysteine-supplemented fermentation conditions. Yeast responds to increased extracellular cysteine by activating the SPS-sensing pathway, which then leads to the transcription factors, Stp1 and Stp2 being processed and migrating to the nucleus to induce transcription of amino acid permease genes. Dal81p is required to enhance the expression of permease genes and Agp1p; Gnp1p and Mup1p are the major cysteine permeases. The TOR pathway is activated as well, with TORC1 being activated by Lst4-Lst7 complex, localised on the vacuolar membrane. TORC1 controls the expression of amino acid permeases through stabilising the transcription factors, Stp1. Disruption of proteins in the blue font resulted in reduced H_2S in this study. Whilst other permease genes may expressed, only the major cysteine permease genes identified *AGP1*, *GNP1* and *MUP1* are shown (adapted from Boban and Ljungdahl 2007; Shin, Kim and Huh 2009; Ljungdahl and Daignan-Fornier 2012; Péli-Gulli *et al.* 2015; González and Hall 2017).

of cysteine uptake to levels similar to wild type. Together, with the findings of inconsistent improvements in 3MH levels, when *MUP1* is overexpressed in yeast undergoing fermentation (Santiago 2014), we conclude that overexpression of genes, involved in regulating cysteine uptake, has limited effect on H_2S production from cysteine and on 3MH production.

Overall, most of the genes noted in this study have previously been identified in screens for genes responsible for H_2S from the sulfate assimilation pathway (Linderholm *et al.* 2008; Yoshida *et al.* 2011). This result suggested that whilst efforts to remove most of the sulfate content in the liquid medium used in the assay (non-sulfate CDGJM), a substantial amount of H_2S was still generated from the sulfate within the bacteriological agar, masking the effects of cysteine-related genes. The sulfate content in the assay could potentially be minimised by testing other solidifying agents that contain less sulfate. Alternatively, the methylene blue reduction method for H_2S detection could be an option as it does not require addition of solidified agents. However, as evaporation and oxidation may affect the colour of methylene blue, (the basis of H_2S detection), this method requires further evaluation (Winter and Curtin 2012). Of note, Δ*tum1* (a known low H_2S producer, under high cysteine conditions) only formed slightly lighter coloured colonies to the wild type on the bismuth plate assay shown in Fig. 1. Furthermore, we cannot exclude the possibility that the somewhat lighter coloured colonies produced by Δ*lst4* and Δ*lst7* deletants were due to their slightly slower growth. In fact, the identification of genes affecting H_2S formation from cysteine, other than *LST4* and *LST7*, which were identified in this study, was through a candidate gene approach and therefore, a better H_2S detection assay that accurately reflects the actual H_2S production during fermentation would be helpful.

Whilst the deletants identified as having lighter coloured colonies do not specifically affect the H_2S formed from cysteine, this study still provides interesting findings that may provide new insight into yeast sulfur metabolism. For example, BY4741 Δ*mdm32* (mitochondrial inner membrane protein), Δ*rsm25* (mitochondrial ribosomal protein of the small subunit), Δ*sds3* (component of the Rpd3L histone deacetylase complex), Δ*ldb18* (component of the dynactin complex) and Δ*paf1* (RNA polymerase II associated factor), which form lighter coloured colonies on our bismuth plate assay, exhibited an increased sulfur(1+) accumulation phenotype (Yu *et al.* 2012). The fact that these genes, having different functions can all affect the process of sulfur(1 +) accumulation and form lighter coloured colonies on bismuth plates, raises questions as to whether the colour is a result of increased sulfur(1+) accumulation. This aspect of sulfur metabolism is clearly worthy of further investigation.

In the initial screen, BY4741Δ*met5** unexpectedly produced lighter colony colours and less H_2S (Figs S1A and S5, Supporting Information). However, PCR analysis confirmed that whilst BY4741 Δ*met5** was haploid, and the *MET5* gene correctly deleted, it turned out to be a *MET17* wild type (Fig. S1B, Supporting Information). *MET17* was chosen as a selectable marker (in BY4741) because deletion of *MET17* led to excess H_2S production and resulted in dark-brown colonies on lead-containing plates (Brachmann *et al.* 1998). Interestingly, a few Δ*met17* deletants have been observed to be able to grow slowly on synthetic dextrose minimal plates but they are not considered true revertants (Brachmann *et al.* 1998). The BY4741 deletion library in our laboratory was obtained more than 10 years ago, so whilst there may be some cross-contamination, we are not sure how these *MET17* revertants may have arisen.

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This work illustrated the complexity of the yeast cysteine uptake processes, with many questions still to be answered. First, it is well established that yeast synthesise glutathione (Grant and Dawes 1996), convert cysteine to methionine through the transsulfuration pathway (Hansen and Johannesen 2000) and degrade cysteine to H_2S (Tokuyama *et al.* 1973). However, high extracellular cysteine is toxic to yeast (Kumar *et al.* 2006), and therefore it is intriguing that yeast can take up excess amounts of cysteine into the cell and still maintain cysteine homeostasis. We observed that yeast is rather cysteine tolerant, being able to grow at CDGJM containing 30 mM cysteine (data not shown). Our screening results showed that none of the deletants were unable to grow as a result of the high (5 mM) concentration of cysteine. Those that did not grow already grew poorly on agar plates without cysteine.

Second, the deletants with defects in the SPS or TOR pathways tested in this study still produced limited amounts of H_2S from cysteine. Moreover, Santiago and Gardner (2015b) also showed that a yeast strain with all nine known cysteine transporters deleted could still grow on cysteine as sole nitrogen source. These findings indicate that there are more, yet unidentified, cysteine transporters or mechanisms by which yeast take up extracellular cysteine.

Another interesting question is how the Lst4-Lst7 complex is able to sense extracellular amino acids (Hatakeyama and De Virgilio 2016). The mammalian folliculin/folliculin interacting proteins (FLCN/FNIP) complex and yeast Lst7/Lst4 complex are orthologues (Péli-Gulli *et al.* 2015). The mutations in FLCN/FNIP complex have been linked to cancer (Birt-Hogg-Dubé syndrome) but the role of the FLCN/FNIP complex in cancer is not well understood currently (Nickerson *et al.* 2002; Pacitto *et al.* 2015; Péli-Gulli *et al.* 2015). The reduced production of H_2S from cysteine for Δ*lst4* and Δ*lst7* deletants, likely due to their inability to sense and transport extracellar cysteine, may provide some insight into the role of the FLCN/FNIP complex in cancer given that H_2S from cysteine has been shown to inhibit the growth of breast and prostate cancer cells (Chattopadhyay *et al.* 2012). Moreover, yeasts with heterozygous deletion of *TUM1* (the gene affects H_2S from cysteine) have also been observed to have an abnormal cell cycle phenotype, which is associated with cancer (de Clare and Oliver 2013). Further research is needed to complete the yeast cysteine uptake/sensing puzzle, and this could potentially help us understand the mechanisms behind a human cancer.

This study was initially intended to identify yeast genes that are responsible for the degradation of cysteine to H_2S. Instead, the main group of genes affecting H_2S formation from cysteine identified here are those involved in regulating cysteine uptake. Previous researches on varietal thiols have focused mainly on the transport of cysteinylated and glutathionylated thiol precursors (Cordente, Capone and Curtin 2015; Santiago and Gardner 2015b). Deletion of yeast *GAP1* (general amino acid permease gene) has been shown to reduce production of 3MH and 3MHA significantly in synthetic media, likely because it may involve in the uptake of S-3-(hexan-1-ol)-L-cysteine (Cys-3MH) (Subileau *et al.* 2008). Although it is obvious that yeast will not produce any significant amount of H_2S from cysteine, if it cannot take up the extracellular cysteine in the first place, cysteine uptake is seldom considered to have any significant impact on varietal thiol production as there are at least nine yeast cysteine transporters. But here we demonstrate that deleting genes involved in cysteine uptake reduced production of H_2S from cysteine significantly and therefore could potentially influence thiol production. To our best knowledge, this is the first time that Agp1p, Gnp1p and Mup1p (in BY4743) were indirectly shown to be the main cysteine transporters in a grape juice-like medium supplemented with high concentrations of cysteine, and that the regulation of cysteine uptake involves both the SPS-sensing and TOR pathways.

To successfully complete wine fermentation, wine yeast strains are required to respond to the rapidly changing fermentation conditions, sense and uptake the nutrients swiftly when supplemented. The polymorphisms of *AGP1* gene in wine yeasts cause differences in amino acid utilisation (Jara *et al.* 2014). In addition, polymorphisms in the promoter region of *MUP1* were observed for BY4716 and RM11 (a vineyard isolate) (Fehrmann *et al.* 2013). The new set of genes identified here would therefore be useful as markers to screen and breed yeast strains that are capable of taking up supplemented cysteine efficiently and therefore preserve (enhance) tropical fruity aromas. Most of the deletants that produced less H_2S from cysteine identified here could still ferment at similar rates as the wild type (except for Δ*lst4* and Δ*lst7*). Wort can contain excess amounts of cysteine (up to 35 mg L^{-1}) (Lawrence and Cole 1972). Therefore, these genes could be the alternative targets for reducing H_2S from cysteine, which might be desired for the brewing industry (Duan *et al.* 2004).

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSYR* online.

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Conflict of interest. None declared.

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Minireview

MINIREVIEW

Hydrogen sulfide and its roles in *Saccharomyces cerevisiae* in a winemaking context

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ABSTRACT

The rotten-egg odour of hydrogen sulfide (H_2S) produced by the yeast *Saccharomyces cerevisiae* has attracted considerable research interest due to its huge impact on the sensory quality of fermented foods and beverages. To date, the yeast genetic mechanisms of H_2S liberation during wine fermentation are well understood and yeast strains producing low levels of H_2S have been developed. Studies have also revealed that H_2S is not just a by-product in the biosynthesis of the sulfur-containing amino acids, but indeed a vital molecule involved in detoxification, population signalling and extending cellular life span. Moreover, polysulfides have recently emerged as key players in signalling and the sensory quality of wine because their degradation leads to the release of H_2S. This review will focus on the recent findings on the production of H_2S and polysulfides in *S. cerevisiae* and summarise their potential roles in yeast survival and winemaking. Recent advances in techniques for the detection of H_2S and polysulfides offer an exciting opportunity to uncover the novel genes and pathways involved in their formation from different sulfur sources. This knowledge will not only provide further insights into yeast sulfur metabolism, but could potentially improve the sensory quality of wine.

Keywords: *Saccharomyces cerevisiae*; hydrogen sulfide; population signalling; polysulfides

INTRODUCTION

The rotten-egg odour of hydrogen sulfide (H_2S) produced by yeast during fermentation is well known for its ability to significantly reduce the sensory quality of wine (Swiegers and Pretorius 2007; Franco-Luesma *et al.* 2016). Extensive research over the past decade has led to the identification of several yeast genes responsible for H_2S production in wine, which have now been used to breed commercial low H_2S-producing yeast strains (Cordente *et al.* 2009; Linderholm *et al.* 2010; Huang, Roncoroni and Gardner 2014; Noble, Sanchez and Blondin 2015).

Hydrogen sulfide is now recognised as not merely an intermediate of the biosynthesis of the sulfur-containing amino acids, but it has important functions in detoxification, population signalling and extending life span in yeast (Gadd and Griffiths 1977; Sohn, Murray and Kuriyama 2000; Hine *et al.* 2015). In addition, polysulfides have recently been recognised as potential mediators of signalling in mammalian cells because their degradation results in the release of H_2S (Ida *et al.* 2014; Kimura 2015; Olson and Straub 2016). The effects of polysulfides on the sensory quality of wine have also attracted a lot of attention

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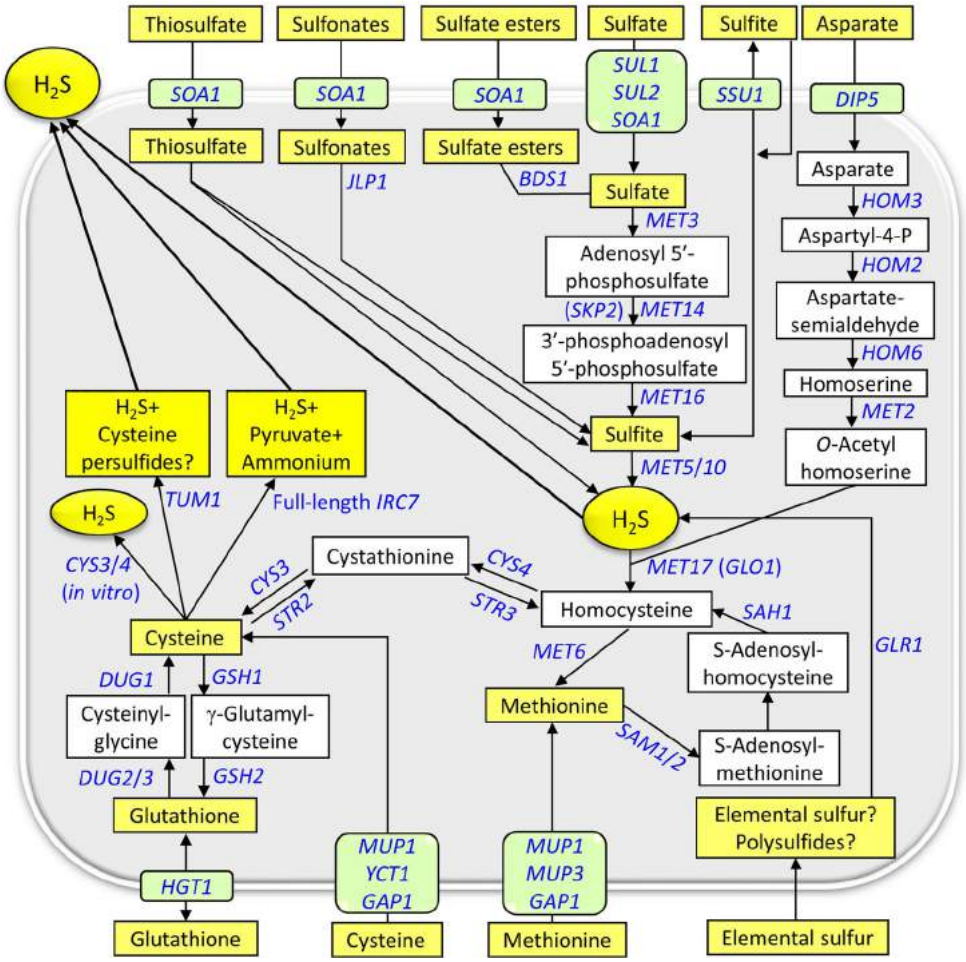


Figure 1. Sulfur metabolism in the yeast *S. cerevisiae*. Yeast can utilise a variety of sulfur sources to produce H_2S and sulfur-containing amino acids, cysteine and methionine (according to Thomas and Surdin-Kerjan 1997; Hogan, Auchtung and Hausinger 1999; Hall, Brachat and Dietrich 2005; Singh *et al.* 2009; Ugliano and Henschke 2009; Sato *et al.* 2011; Yoshida *et al.* 2011; Harsch and Gardner 2013; Hopwood, Ahmed and Aitken 2014; Santiago and Gardner 2015; Huang *et al.* 2016; Holt *et al.* 2017; Kinzurik *et al.* 2017).

lately (Kreitman *et al.* 2017) and hydropolysulfides such as H_2S_2 and H_2S_3 have been shown to contribute to the flint and mineral odour in wine (Starkenmann *et al.* 2016).

There have been numerous recent excellent reviews about the formation of H_2S and polysulfides, and their potential roles in mammals (Filipovic 2015; Kimura 2015; Olson and Straub 2016; Cuevasanta, Möller and Alvarez 2017), plants (Quirós-Sauceda *et al.* 2016) and microbes (Findlay 2016). However, there are few related reviews specifically for the model organism, *Saccharomyces cerevisiae*, which may reflect the fact that this aspect of sulfur metabolism in *S. cerevisiae* is still relatively unexplored. The goal of this review is to summarise recent work related to the production of H_2S and polysulfides in *S. cerevisiae*, to bring to the attention of researchers this emerging aspect of *S. cerevisiae* sulfur metabolism.

H_2S PRODUCTION FROM THE SAP DURING WINE FERMENTATION

The majority of H_2S produced by yeast during wine fermentation is from the sulfate assimilation pathway (SAP), where sulfate is taken up and progressively reduced to sulfide (using 2 ATPs and 4 NADPHs), the precursor of the sulfur-containing amino acids cysteine and methionine, which are required for yeast growth (Fig. 1) (Thomas and Surdin-Kerjan 1997). Grape juice usually contains plenty of sulfate (~160 to 700 mg L^{-1}) but very low concentrations of cysteine and methionine (<20 mg L^{-1}), and therefore the SAP is triggered during fermentation to support yeast growth (Rauhut 2009; Ugliano and Henschke 2009). The mechanisms by which H_2S is released from the SAP are well studied and reviewed (refer to Henschke and Jiranek 1993; Thomas and Surdin-Kerjan 1997; Swiegers and Pretorius 2007; Rauhut 2009; Ugliano and Henschke 2009).

In short, sulfur assimilation starts with extracellular sulfate entering yeast cells through the sulfate transporters, Sul1p and Sul2p (Cherest *et al.* 1997), where sulfate is first activated by the ATP sulphurylase (Met3p) to 5′-adenylylsulfate (APS), which is then phosphorylated to 3′-phospho-5′-adenylylsulfate (PAPS) by the APS kinase (Met14p). In the next step, PAPS is reduced by the PAPS reductase (Met16p) to sulfite, which is further reduced by sulfite reductase (Met5p/Met10p) to sulfide. The sulfide produced is subsequently integrated into the nitrogenous precursor, *O*-acetyl homoserine (OAH), to form homocysteine with the help of *O*-acetylhomoserine sulfhydrylase (Met17p). Finally, homocysteine is converted into cysteine, methionine and glutathione (Fig. 1) (Thomas and Surdin-Kerjan 1997). The liberation of excessive H_2S during fermentation usually occurs as a result of insufficient assimilable nitrogen (OAH) in grape juice

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to combine with the sulfide generated (Jiranek, Langridge and Henschke 1995).

SULFATE TRANSPORT

Sulfate uptake is the first step of the SAP; however, the sulfate-sensing mechanisms in yeast remain poorly understood. Recent work has revealed that the yeast sulfate transporters, Sul1p and Sul2p, can also function as independent extracellular sulfate receptors (Transceptor: **trans**port and re**ceptor**) (Kankipati *et al.* 2015). The authors observed that addition of the sulfate analogue, D-glucosamine 2-sulfate, whilst not transported into the cell, triggered the activation of Sul1, Sul2-dependent PKA signalling pathway, measurable as increased trehalase activity. Furthermore, the PKA signalling pathway was activated in the two yeast mutants, $Sul1^{E427Q}$ and $Sul2^{E443Q}$, incapable of uptaking exogenous sulfate added to the medium (Kankipati *et al.* 2015).

The yeast Sul1p and Sul2p are the major sulfate transporters, but their deletion does not completely block yeast growth on high concentrations of sulfate as the sole sulfur source (~30 mM), indicating the existence of unknown sulfate transporters (Cherest *et al.* 1997; Kankipati *et al.* 2015). Recently, a third sulfate transporter, Soa1p (**s**ulf**o**n**a**te transport; low affinity for sulfate and broad substrate specificity), was identified (Holt *et al.* 2017). The authors demonstrated that deletion of all three genes (*SUL1*, *SUL2* and *SOA1*) resulted in an inability to grow on high concentrations of sulfate as the sole sulfur source, alluding to no other sulfate transporters.

Jennings and Cui (2012) proposed that Sul2p may also act in the efflux of sulfate, based on the observed transient efflux of sulfate mediated by Sul2p in an S-starved *met3* deletant when sulfate was added. The authors suggested that this mechanism could potentially protect yeast from an excessive sulfate influx (Jennings and Cui 2012). The inability of Δ*met3*, Δ*met14* and Δ*met16* to take up sulfate gives credence to the speculation that the sulfate transporters, Sul1p and Sul2p, may form complexes with the proteins downstream of the SAP (Met3p, Met14p and Met16p) and participate in the process of activation of sulfate (Breton and Surdin-Kerjan 1977; Thomas and Surdin-Kerjan 1997). However, there is currently insufficient evidence to support this hypothesis.

SKP2, *MET2* AND *GLO1* AFFECTING H_2S PRODUCTION FROM THE SAP

SKP2 encodes an F box protein, which is predicted to be part of an SCF (Skp1p-Cullin-F-box) ubiquitin protease complex involved in biosynthesis of sulfur amino acids required for growth and H_2S and SO_2 production (Yoshida *et al.* 2011). The authors observed that the *skp2* deletant produced more H_2S and SO_2 than the wild type. As Skp2p is involved in the degradation of Met14p (APS kinase), the increase in H_2S and SO_2 production in the *skp2* deletant is likely due to a more stable Met14p, allowing extended sulfate assimilation and therefore increased H_2S and SO_2 levels.

Recently, two *SKP2* allele variants, $SKP2_{I350V}$ and $SKP2_{T357I}$, resulting in low H_2S and SO_2 production were identified by Noble, Sanchez and Blondin (2015) using a quantitative trait locus mapping strategy. It is thought that these mutational changes in Skp2p may increase its efficiency of targeting and degradation of Met14p, resulting in less sulfate through the SAP and so, reduced H_2S and SO_2.

The yeast *MET2* encodes homoserine *O*-acetyl transferase (HTA), which catalyses the conversion of homoserine to *O*-acetyl homoserine. OAH is subsequently incorporated into H_2S to yield homocysteine (Baroni *et al.* 1986) (Fig. 1). Genetic variation in *MET2* can lead to differences in H_2S production. The Δ*met2* has been shown to produce more H_2S during beer production (Hansen and Kielland-Brandt 1996). Recently, the $R_{301}G$ mutation in *MET2* was identified to be responsible for low H_2S production (Noble, Sanchez and Blondin 2015), in agreement with our findings (Huang, Roncoroni and Gardner 2014). It has been proposed that the $R_{301}G$ mutation in *MET2* may increase the activity of HTA, hence the efficiency of converting homoserine into OAH. Because of the ample supply of OAH to combine with sulfide, less sulfide will be released into wine as H_2S (Huang, Roncoroni and Gardner 2014; Noble, Sanchez and Blondin 2015).

The recently identified *SKP2* and *MET2* allele variants (Huang, Roncoroni and Gardner 2014; Noble, Sanchez and Blondin 2015), together with the *MET5* and *MET10* allele variants, identified earlier by Cordente *et al.* (2009) and Linderholm *et al.* (2010), have now been successfully employed to breed low H_2S-producing strains for winemaking.

The yeast *GLO1* encodes glyoxalase I, which is essential for the detoxification of methylglyoxal, a toxic metabolite of glycolysis (Inoue and Kimura 1996). Recently, *GLO1* has been added to the growing list of genes affecting H_2S production from the SAP. Kinzurik *et al.* (2017) showed that the *glo1* deletant grew more slowly than the wild type on sulfate as the sole sulfur source, produced more H_2S only when sulfate was added and decreased the expression level of Met17p. The authors therefore suggested that Glo1p is critical for a fully functional Met17p (*O*-acetylhomoserine sulfhydrylase). This finding is exciting in the sense that a gene not previously linked to the SAP could affect H_2S production from this pathway, illustrating the complexity of the SAP and the possibility of the existence of other unknown genes affecting the SAP (Kinzurik *et al.* 2017).

H_2S PRODUCTION FROM ELEMENTAL SULFUR

Elemental sulfur is frequently sprayed in the vineyard to fight grapevine powdery mildew, and the residual sulfur on grape has been observed to contribute to the formation of H_2S during fermentation (Thomas *et al.* 1993; Araujo *et al.* 2017). Although it has been suggested that elemental sulfur may be spontaneously converted to sulfide under the anaerobic and low pH fermentation conditions (Linderholm *et al.* 2008), Araujo *et al.* (2017) showed that no H_2S could be detected when elemental sulfur was added to grape juice model solutions (sparged with nitrogen). These findings indicate that yeast is likely to be responsible for most of the H_2S production from elemental sulfur during fermentation.

Sato *et al.* (2011) observed that a yeast with deletion of *GLR1* (glutathione reductase) produced less H_2S than wild type on sulfur (e.g. colloidal elemental sulfur and powdery elemental sulfur). Moreover, Sato *et al.* (2011) proposed that insoluble elemental sulfur is most likely converted into more soluble polysulfides first, which could then enter into yeast cells, where they are reduced by the glutathione reductase to sulfide.

H_2S PRODUCTION FROM GLUTATHIONE AND CYSTEINE

The tripeptide, glutathione (L-γ-glutamyl-L-cysteinyl-glycine), is naturally present in grape juice (~1.3 to 102 mg L^{-1}) and can also be synthesised by yeast through the SAP (Rauhut 2009). Recently, glutathione has been permitted as an additive (up to 20 mg L^{-1}) to grape juice to protect aromatic compounds from

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oxidation according to the 2015 OIV resolutions (Wegmann-Herr *et al.* 2016). The addition of glutathione to grape juice has been observed to increase H_2S production (Rauhut 2009; Winter *et al.* 2011; Wegmann-Herr *et al.* 2016). The mechanism is not yet fully understood but it is generally assumed that glutathione is first hydrolysed to cysteine, which is then degraded by cysteine desulfhydrase to release H_2S under nitrogen-limited conditions (Rauhut 2009).

Adding cysteine to grape juice has long been observed to induce H_2S production (Jiranek, Langridge and Henschke 1995; Winter and Curtin 2012), and today several yeast genes affecting the formation of H_2S from cysteine have been identified. Both yeast Cys4p (cystathionine β-synthase, CBS) and Csy3p (cystathionine γ-lyase, CSE) have been reported to cleave cysteine and release H_2S *in vitro* (Singh *et al.* 2009; Hopwood, Ahmed and Aitken 2014). However, other *in vivo* studies suggested that deletion of yeast *CYS4* or *CSY3* did not reduce the production of H_2S (Linderholm *et al.* 2008; Winter, Cordente and Curtin 2014; Huang *et al.* 2016).

Santiago and Gardner (2015) demonstrated that the full-length *IRC7* gene encoding cysteine desulfhydrase was responsible for cleaving cysteine to release H_2S. However, in many yeast strains Irc7p has no functional cysteine desulfhydrase activity, as the strains carry a 38-bp deleted, non-functional version of *ß*-lyase *IRC7* (Roncoroni *et al.* 2011).

The importance of the vacuole-related genes in H_2S production from cysteine was revealed by Winter, Cordente and Curtin (2014), who showed that deletants of such genes produced less H_2S from cysteine compared to the wild type. It has been suggested that the yeast vacuole could have a vital function in the cysteine detoxification process, which involves degrading cysteine to H_2S (Winter, Cordente and Curtin 2014).

Deletion of yeast *TUM1* gene has recently been shown to reduce H_2S production from cysteine during fermentation, and it is suspected that yeast Tum1p may act like its human orthologue, sulfurtransferase, an enzyme involved in the production of H_2S from cysteine (Huang *et al.* 2016).

OTHER SULFUR SOURCES

Thiosulfate can be cleaved to sulfite and sulfide by thiosulfate reductase and utilised as a sulfur source by yeast. However, the yeast gene encoding thiosulfate reductase remains unknown (Chauncey and Westley 1983; Thomas *et al.* 1992). Funahashi *et al.* (2015) showed that yeast grew better and produced ethanol more efficiently on thiosulfate than sulfate because less energy was required for yeast to assimilate sulfite (cleaved from thiosulfate) than sulfate.

The alternative sulfur sources such as sulfonates and sulfate esters that are generally abundant in soil can also be utilised by *S. cerevisiae* (Linder 2012). Hogan, Auchtung and Hausinger (1999) demonstrated that yeast *JLP1* encodes a sulfonate dioxygenase, which is required for yeast to utilise sulfonates (e.g. isethionate and taurocholate) as a sulfur source by degrading sulfonates to sulfite. A strain with deletion of *JLP1* was shown to grow more slowly than wild type on sulfonates (Hogan, Auchtung and Hausinger 1999). The yeast *BDS1* encodes a sulfatase, which is essential for yeast to release the sulfate from the sulfate esters (e.g. sodium dodecyl sulfate), and whose deletion reduced the ability of yeast to use sulfate esters as a sulfur source (Hall, Brachat and Dietrich 2005).

There has been some doubt as to whether the alternative sulfur sources play an important part in *S. cerevisiae* sulfur utilisation as this yeast generally does not grow well on the alternative sulfur sources compared to other ascomycetes (Linder 2012). To date, only two yeast genes, *JLP1* and *BDS1*, have been associated with the utilisation of these alternative sulfur sources (Linder 2012). Recently, yeast Soa1p was found to be capable of transporting a range of sulfur compounds including sulfate, sulfite, thiosulfate, sulfonate and choline sulfate (Holt *et al.* 2017). The identification of the sulfonate and choline sulfate transporter, Soa1p, in yeast highlights that sulfonates or sulfate esters could be important sulfur sources, at least for *S. cerevisiae* living in soil. Further studies to investigate other yeast genes involved in utilisation of these sulfur sources are clearly worthwhile.

H_2S AND HEAVY METAL DETOXIFICATION

H_2S has been shown to play a critical role in detoxification of heavy metals and yeast strains that produce more H_2S were observed to be more resistant to copper (Kikuchi 1965) and methylmercury (Ono *et al.* 1991). The detoxification effect of H_2S is likely through the formation and precipitation of insoluble metal sulfides (e.g. copper sulfide) (Gadd and Griffiths 1977). Interestingly, genes (e.g. vacuole-related genes) that were recently identified to affect H_2S production from cysteine (Winter, Cordente and Curtin 2014) were similar to those previously reported to be essential for metal (e.g. cadmium, nickel) detoxification (Ruotolo, Marchini and Ottonello 2008; Arita *et al.* 2009), highlighting the central role of H_2S in detoxification.

H_2S AND POPULATION SYNCHRONY

Hydrogen sulfide's role in cell signalling was first demonstrated by Sohn, Murray and Kuriyama (2000), who established that the gas was responsible for the synchronisation of the yeast population, when grown in aerobic continuous culture. Periodic changes in H_2S production (an inhibitor of respiration) were connected to an ultradian oscillation in respiration, with H_2S being highest when respiration decreased, before declining with the onset of respiration (Sohn, Murray and Kuriyama 2000). The H_2S produced during these oscillations was shown to be generated from sulfate or sulfite by sulfite reductase through the SAP and not from cysteine or glutathione in the media (Sohn and Kuriyama 2001a). It has been suggested that the cyclic changes in H_2S production (a strong reducing agent) are a protection mechanism against oxidative stress and are triggered by the periodic depletion of glutathione and cysteine used by yeast to detoxify toxic respiration metabolites (e.g. reactive oxygen species) (Sohn and Kuriyama 2001b; Kwak *et al.* 2003). The importance of glutathione homeostasis for H_2S production and respiratory oscillation was also highlighted by Sohn *et al.* (2005), who observed that deletion of *GLR1*, encoding glutathione reductase (responsible for the reduction of oxidised glutathione), led to the yeast no longer being able to produce H_2S and undergo respiratory oscillation.

DOES H_2S GIVE *SACCHAROMYCES* A COMPETITIVE ADVANTAGE?

The ethanol and heat produced by *S. cerevisiae* during wine fermentation have been reported to give *S. cerevisiae* a significant advantage to dominate over other competing non-*Saccharomyces* species (Goddard 2008; Salvadó *et al.* 2011). Given that H_2S is also produced during fermentation and its important role in synchronising yeast populations, it has been suggested by Linderholm *et al.* (2010) that H_2S produced by *S. cerevisiae* may inhibit

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respiration and oxidative metabolism of their microbial competitors, allowing *S. cerevisiae* to dominant in wine fermentation. Therefore, it seems reasonable that yeast capable of efficiently producing H_2S may confer a selective advantage under certain conditions and this may explain the difference in H_2S production across commercial and natural wine strains (Spiropoulos *et al.* 2000; Linderholm *et al.* 2008). However, whether H_2S plays a role in modifying the environment (niche construction), killing other competitors and contributing to competitive advantage of *S. cerevisiae* remains to be elucidated.

ROLE OF H_2S AND OTHER VOLATILES IN YEAST–INSECT INTERACTIONS

How yeasts survive in the wild between vintages and are transferred between the vineyard and winery is still not clearly understood. Recent studies have shown that yeast can reside in the gut of social wasps (*Polistes dominula*), alluding to the role of insects as a means of survival between vintages (Stefanini *et al.* 2016). Furthermore, the authors' ability to isolate new intraspecific (*S. cerevisiae* x *S. cerevisiae*) and interspecific (*S. cerevisiae* x *S. paradoxis*) hybrids from the wasp's gut is particularly exciting in relation to the maintenance of genetic diversity within a wild yeast population. From the many studies in yeast–insect ecology, insects are now regarded as one of the main vectors for yeast dispersal. These include bees (Goddard *et al.* 2010), *Drosophila* (Chandler, Eisen and Kopp 2012) and social wasps (Stefanini *et al.* 2012).

Another question remains as to how insects are attracted to yeast, as this relationship is clearly beneficial to both organisms: the yeast as a food source and for insects who in turn provide an efficient dispersal mechanism. Certain volatiles produced by yeast during fermentation act as attractants for insects, for example, acetate esters and acetic acid (Becher *et al.* 2012; Palanca *et al.* 2013; Buser *et al.* 2014; Günther *et al.* 2015; Dapporto *et al.* 2016). Moreover, Christiaens, Franco and Cools (2014) showed that a yeast strain with deletion of *ATF1* (encoding alcohol acetyl transferase that is responsible for acetate ester production) was less attractive to *Drosophila*, hence reducing its dispersal via this vector.

H_2S, well known for its repulsive rotten-egg smell, has also been implicated in yeast–insect interactions (Sundstrom *et al.* 2016). The preliminary results suggest that *Drosophila* fruit flies were slightly less attracted to a medium supplemented with H_2S (12.5 μM sodium sulfide); however, *Drosophila* were also seen to lay more eggs on yeast colonies supplemented with H_2S (Sundstrom *et al.* 2016). Interestingly, H_2S has been demonstrated to react with (*E*)-2-hexenal in grape juice to form the fruity varietal thiols 3-mercapto-hexanol and 3-mercaptohexylacetate (Harsch *et al.* 2013). However, only tiny amounts of thiols (<1%) are produced through this pathway as (*E*)-2-hexenal is rapidly metabolised by yeast during fermentation (Schneider *et al.* 2006; Subileau *et al.* 2008; Harsch *et al.* 2013). Nevertheless, whether H_2S exerts a repulsive or attractive effect on the insect carriers and thus affects the dispersal and survival of *S. cerevisiae* remains to be investigated.

H_2S AND YEAST LIFE SPAN

Yeast produced more H_2S on 0.5% glucose than on 2% glucose and this may explain why glucose restriction can extend yeast lifespan (Hine *et al.* 2015). Life span was also extended following supplementation with external H_2S (5 μM NaHS). The H_2S produced by yeast during glucose restriction was shown to come from cysteine or methionine and not from sulfate (SAP). This is based on the observation that *MET* gene deletants (Δ*met5*, Δ*met14* and Δ*met16*) that block sulfate assimilation (see Fig. 1 for pathway) were still capable of producing H_2S and had extended longevity on 0.5% glucose, whilst removal of cysteine or methionine from the media resulted in decreased H_2S production in a *met14* deletant.

In addition, methionine restriction has been reported to increase yeast lifespan, and interestingly, that yeast deletants Δ*met2* and Δ*met17*, known to be high H_2S producers, were observed to have a longer lifespan than wild type (Johnson and Johnson 2014; Ruckenstuhl *et al.* 2014; Hine and Mitchell 2015). Autophagy-mediated vacuolar acidification has been proposed to play a vital role in yeast longevity as deletion of genes involved in autophagy (Δ*atg5*, Δ*atg7* and Δ*atg8*) led to shortened chronological lifespan (Ruckenstuhl *et al.* 2014). Interestingly, these observations coincide with the recent findings that both Δ*met2* and Δ*met17* produce more H_2S from cysteine than wild type (Huang *et al.* 2016) and yeast vacuoles play a crucial role in formation of H_2S from cysteine (Winter, Cordente and Curtin 2014).

POLYSULFIDES IN YEAST

Polysulfides (S_x^{2-}; $x > 2$) and protein persulfides such as glutathione persulfide (GSSH) and cysteine persulfide (CysSSH) have lately emerged as potential mediators of sulfide signalling in mammalian cells (Ida *et al.* 2014; Kimura 2015; Olson and Straub 2016). To our knowledge, the formation of polysulfides in yeast remains largely uninvestigated; therefore, we have referred to studies using mammalian cells as a means to uncover any potential analogies.

Melideo, Jackson and Jorns (2014) proposed that the first step of mammalian H_2S metabolism is the conversion of H_2S (with sulfite) to thiosulfate by sulfide:quinone oxidoreductase (SQOR). In the next step, thiosulfate (with glutathione) is converted by the thiosulfate:glutathione sulfurtransferase (TST) into glutathione persulfide (GSSH), which is further converted to sulfite by sulfur dioxygenase (SDO). The sulfite produced is either oxidised to sulfate by sulfite oxidase (SO) or to thiosulfate (with H_2S) by SQOR (Fig. 2). Although mammalian TST has not been isolated, its yeast orthologue (Rdl1p) has been identified, being capable of acting like a TST and converting glutathione (with thiosulfate) to GSSH (Melideo, Jackson and Jorns 2014). Furthermore, cysteine persulfide was detected in yeast Rdl1p after its reaction with thiosulfate (Melideo, Jackson and Jorns 2014). However, whether yeast has other enzymes (e.g. SQOR, SDO) involved in the other steps of the mammalian H_2S metabolism pathway is currently unknown. Therefore, the formation of persulfides in yeast through this pathway, for example, during fermentation, remains to be explored.

Ida *et al.* (2014) showed that both mammalian H_2S-producing enzymes, CBS and CSE, are capable of converting cystine to cysteine polysulfides (e.g. CysSSSH and CysSSSCys)—precursors in the formation of glutathione polysulfides (e.g. GSSSH and GSSSG). It is proposed that the polysulfide species could be the real mediators of signalling with H_2S being released as part of persulfide degradation (Ida *et al.* 2014). Moreover, polysulfide species are much more abundant than H_2S in mammalian cells. Recently, reduced levels of protein persulfide in yeast Δ*cys4* (CBS) and Δ*cys3* (CSE) were observed using a novel persulfide detection method, suggesting that yeast *CYS4* and *CSY3* could also have roles in persulfidation (Dóka *et al.* 2016).

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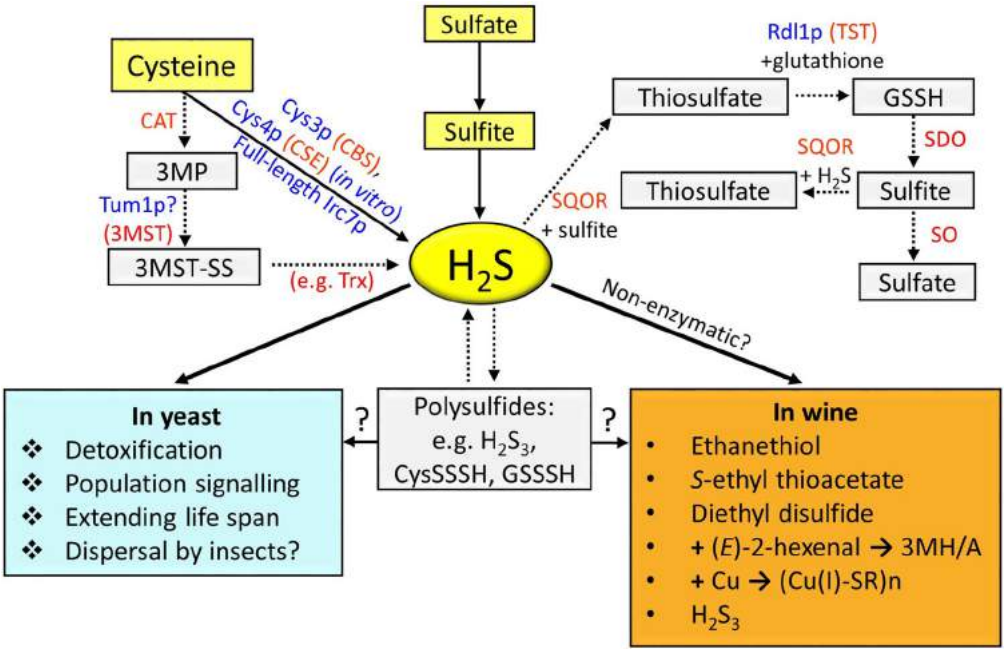


Figure 2. Potential pathways for H_2S and polysulfides production and their functions in the yeast *S. cerevisiae*. H_2S is produced from the sulfate/sulfite (SAP) and cysteine in yeast. Polysulfides are generated by CBS, CSE and 3MST in mammalian cells but currently little is known in yeast. Yeast Rdl1p can convert thiosulfate to GSSH (with glutathione). H_2S can react with Cu and (*E*)-2-hexenal to form $(Cu(I)\text{-}SR)_n$ and 3MH/A that affect the sensory quality of wine, respectively. Enzymes in *S. cerevisiae* (blue font) and in mammalian cells (red font). Dashed arrows indicate that pathways have been shown in mammalian cells but not in *S. cerevisiae*. Abbreviations: CAT: cysteine aminotransferase; 3MST: 3-mercaptopyruvate sulfurtransferase; Trx: thioredoxin; CBS: cystathionine β-synthase; CSE: cystathionine γ-lyase; SQOR: sulfide:quinone oxidoreductase; TST: thiosulfate:glutathione sulfurtransferase; SDO: sulfur dioxygenase; SO: sulfite oxidase. (according to Gadd and Griffiths 1977; Sohn Murray and Kuriyama 2000; Singh *et al.* 2009; Harsch *et al.* 2013; Hopwood, Ahmed and Aitken 2014; Ida *et al.* 2014; Melideo, Jackson and Jorns 2014; Kimura 2015; Kimura *et al.* 2015; Santiago and Gardner 2015; Hine *et al.* 2015; Huang *et al.* 2016; Kinzurik *et al.* 2016; Olson and Straub 2016; Starkenmann *et al.* 2016; Sundstrom *et al.* 2016; Kreitman *et al.* 2017).

Another mammalian H_2S-producing enzyme, 3-mercaptopyruvate sulfurtransferase (3MST), is implicated in the generation of pyruvate and protein persulfides from 3-mercaptopyruvate (3MP), which is produced by cysteine aminotransferase from cysteine. H_2S could be released from protein persulfides, once reducing systems such as glutathione or thioredoxin are available (Shibuya *et al.* 2009; Mikami *et al.* 2011). In addition, Kimura *et al.* (2015) showed that 3MST could catalyse the formation of H_2S_3 and H_2S directly from 3MP in mammalian brain cells. The human TUM1 (or 3MST) protein and yeast Tum1p are orthologues (Mathew, Schlipalius and Ebert 2011), and deletion of yeast *TUM1* has recently been observed to reduce H_2S production from cysteine during fermentation (Huang *et al.* 2016). However, whether yeast Tum1p can act like its human orthologue, 3MST, to produce H_2S and polysulfides from 3MP, is unknown. Additional indirect evidence for the potential existence of polysulfides in yeast (*S. cerevisiae*) is the ability of the yeast mitochondrial ATP-binding cassette transporter, Atm1p, to transport glutathione polysulfides (e.g. glutathione trisulfide) *in vitro* (Schaedler *et al.* 2014).

H_2S AND POLYSULFIDES RESPONSIBLE FOR 'OFF-ODOURS' IN WINE

It has long been suspected that H_2S produced by yeast during fermentation may lead to the formation of other unpleasant volatile sulfur compounds such as dimethyl tri- and tetrasulfide (reviewed by Waterhouse, Sacks and Jeffery 2016). Recently, Kinzurik *et al.* (2016) demonstrated that ^{34}S-labelled sulfate when fed to a Δ*met17* (a high H_2S producer, unable to synthesise sulfur-containing amino acids) was incorporated into ethanethiol, S-ethyl thioacetate and diethyl disulfide. This work was the first validation of H_2S (produced during fermentation) contributing to the formation of the 'cooked onion' odour of these volatile sulfur compounds. However, whether yeast enzymes are required for this process remains to be determined.

Copper fining is commonly practiced to remove H_2S responsible for the rotten-egg 'off-odour' in wine. However, growing evidence suggests that copper treatment leads to increased H_2S formation during bottle storage (Ugliano *et al.* 2011; Viviers *et al.* 2013). One of the proposed mechanisms is that H_2S reacts with copper to form copper sulfide complexes, which may then release H_2S under anaerobic storage conditions (Franco-Luesma and Ferreira 2014, 2016). The formation of (Cu(I)-SR)n complexes in wine has also been proposed (Kreitman *et al.* 2016). Furthermore, the same group showed that reaction of H_2S, copper, cysteine or glutathione led to the formation of cysteine or glutathione polysulfides in model wine, which may also contribute to H_2S production during bottle storage (Kreitman *et al.* 2017).

Hydropolysulfides such as H_2S_2 and H_2S_3 have also been reported to be associated with the flint and mineral odour in wine (Starkenmann *et al.* 2016). However, as these hydropolysulfides are unstable in wine, their effects on the sensory quality of wine are not clear.

METHODS FOR THE DETECTION OF H_2S AND POLYSULFIDES

Several detection methods for H_2S and polysulfides have been developed (see review by Takano, Shimamoto and Hanaoka

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2016). Fluorescent probes for H_2S and polysulfides are particularly useful for investigating the biological functions of these highly reactive molecules because the technique is simple, non-destructive and allows real-time monitoring (imaging) of H_2S and polysulfides inside cells (Takano, Shimamoto and Hanaoka 2016). The fluorescent probes for monitoring H_2S (e.g. WSP-1; Peng *et al.* 2014) and polysulfides (e.g. SSP4; Chen *et al.* 2013) are now commercially available and have been extensively used to study the roles of H_2S and polysulfides in mammalian cells (Ida *et al.* 2014; Monti *et al.* 2016). Whilst there are no current reports on the use of these probes in yeast, preliminary data from our group suggest these probes can be used to detect polysulfides within yeast cells (unpublished results). The development of other novel and improved versions of fluorescent probes (Takano, Shimamoto and Hanaoka 2016) will allow polysulfides to be detected much earlier and more accurately, thus enabling yeast researchers to decipher the yeast genes and pathways involved in the formation of polysulfides.

Polysulfides are unstable at the low pH (Kamyshny, Borkenstein and Ferdelman 2009), and therefore it can be challenging to analyse and quantify these transient species in wine. Detection methods include sensitive but simple HPLC detection of polysulfides (Kamyshny, Borkenstein and Ferdelman 2009) to more complex ones such as reversed-phase ultrafast liquid chromatography coupled with quadrupole-time-of-flight mass spectrometry (Kreitman *et al.* 2017). Both are worthy of investigation as analytical tools for the determination of polysulfide species in wine.

CONCLUSION AND FUTURE PERSPECTIVES

Over the past years, much has been learnt about how H_2S is produced by *Saccharomyces cerevisiae* during wine fermentation but there are still aspects of H_2S in yeast waiting to be explored. For instance, low H_2S-producing yeast strains have been selected and bred for the wine industry, and whilst they have similar fermentation kinetics to the wild type (Dahabieh *et al.* 2015), it is tempting to speculate (given that H_2S may play an important role in population signalling) that the reduced ability to produce H_2S may affect the overall microbial (population) dynamics during fermentation. Further studies assessing whether H_2S provides a competitive advantage (or disadvantage) to yeast, through investigating the interactions of both low and high H_2S-producing strains with lactic acid bacteria, non-*Saccharomyces* species or *Drosophila* may be worthwhile. Other gas molecules such as nitric oxide (NO) have also been suggested to play crucial roles in signalling and various stress responses (e.g. oxidative stress) in yeast, and modulation of production of NO could improve fermentation ability of yeast (see review by Astuti, Nasuno and Takagi 2016). There is substantial evidence that NO may interact with H_2S in mammalian cells (Fago *et al.* 2012; Olson and Straub 2016). Therefore, future studies on the synergistic effect of H_2S and NO in yeast would be valuable for the fermentation industry.

Polysulfide species have recently been proposed as the central players in signalling in mammalian cells (Ida *et al.* 2014; Kimura 2015; Olson and Straub 2016) and they have been detected in the fungus *Aspergillus nidulans* (Wróbel *et al.* 2009). However, the roles of polysulfides or even their existence in *S. cerevisiae* remain largely unknown. Polysulfide production and metabolism has been suggested to be the next frontier in sulfide biology in eukaryotes (Olson and Straub 2016). Here, we propose that *S. cerevisiae* could be an excellent model organism. For such research, *S. cerevisiae* gene deletion collections and the recently developed polysulfide detection techniques will likely lead to the identification of novel genes and pathways involved in the formation of polysulfides, providing a greater understanding of the essential role of polysulfides in a wide variety of biological processes. This knowledge will not only give a more complete picture of yeast sulfur metabolism but also could potentially improve the sensory quality of wine.

Saccharomyces cerevisiae is present everywhere, being found in a wide range of habitats, including wineries, soil, oak trees, insects and human gut and not only the laboratory (Liti 2015). It has been proposed that investigation of the natural history (ecology) of *S. cerevisiae* through a population genomics approach is necessary to fully understand the *S. cerevisiae* metabolism that may have been shaped during evolution (Jouhten *et al.* 2016). A huge difference in H_2S production has been observed across yeast strains (Spiropoulos *et al.* 2000), and Olson and Straub (2016) have suggested that H_2S is likely to play a pivotal role in evolution. Future studies considering the ecological aspect of H_2S metabolism might be the key to uncovering the actual role of this exciting molecule. Finally, we would finish this review with a slightly modified version of Dobzhansky' (1973) famous quote that 'Nothing in biology (H_2S production) makes sense, except in the light of evolution.'

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APPLIED MICROBIAL AND CELL PHYSIOLOGY

Application of directed evolution to develop ethanol tolerant *Oenococcus oeni* for more efficient malolactic fermentation

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Abstract Malolactic fermentation (MLF) is an important step in winemaking, which can be notoriously unreliable due to the fastidious nature of *Oenococcus oeni*. This study aimed to use directed evolution (DE) to produce a more robust strain of *O. oeni* having the ability to withstand high ethanol concentrations. DE involves an organism mutating and potentially adapting to a high stress environment over the course of extended cultivation. A continuous culture of *O. oeni* was established and exposed to progressively increasing ethanol content such that after approximately 330 generations, an isolate from this culture was able to complete MLF in high ethanol content medium earlier than its parent. The ethanol tolerance of a single isolate, A90, was tested to confirm the phenotype and its fermentation performance in wine. In order to investigate the genotypic differences in the evolved strain that led to the ethanol tolerance phenotype, the relative expression of a number of known stress response genes was compared between SB3 and A90. Notably, there was increase in *hsp18* expression in 20% (*v*/*v*) ethanol by both strains with A90 exhibiting a higher degree of expression. This study is the first to use directed evolution for *O. oeni* strain improvement and confirms that this technique can be used successfully for the development of new candidate strains for the wine industry. This study also adds to the current knowledge on the genetic basis of ethanol tolerance in this bacterium.

Keywords *Oenococcus* · Wine · Ethanol · Stress response · Directed evolution · Malolactic fermentation

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Introduction

Oenococcus oeni is a vital microorganism for the wine industry; the stabilisation, deacidification and organoleptic changes that it facilitates in wine are an integral part of the production of many wine styles. More specifically in most red wines and some white wines, a secondary or malolactic fermentation (MLF) is carried out following alcoholic fermentation by lactic acid bacteria (LAB), most commonly *O. oeni*. MLF involves the conversion of L-malic acid to L-lactic acid and carbon dioxide, resulting in deacidification and improvement of microbial stability of the wine. Wine is typically a nutritionally poor environment and has physicochemical properties that can hinder the growth and malolactic activity of *O. oeni*. Common inhibitors in wine include, high ethanol content, low pH, low temperature, low nutrient availability, the presence of sulfur dioxide (SO_2), phenolic compounds and acetic acid (Bauer and Dicks 2004). Furthermore, the alcohol concentration in wines has been rising in recent decades (Godden et al. 2015) and MLF in wines with a high ethanol content commonly stalls (Zapparoli et al. 2009). Consequently increased LAB ethanol tolerance is a desirable trait. To alleviate ethanol stress, a number of strain improvement methods are available that encompass both recombinant and non-recombinant protocols (Betteridge et al. 2015). To produce strains suitable for use in the wine industry, non-recombinant methods are

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preferred since recombinant techniques are still not favoured by some consumers. Even if the use of recombinant strains was favoured by the wine industry, there are still no reliable methods of genomic manipulation for use in *O. oeni*.

Directed evolution (DE) is a process based on Darwin's theory of natural selection and is also known as adaptive evolution, stationary phase mutation, directed mutation or stress response mutation (Rosenberg and Hastings 2003). The process involves adaptation to a high stress environment through spontaneous DNA mutations. Those organisms that have gained advantageous mutations prosper, proliferate and dominate under the specific stress (Foster 1999). DE has been successfully used with several organisms. *Lactobacillus plantarum* growth on glycerol under anaerobic conditions is too slow to be accurately measured; however, after approximately 500 generations under continuous selection using glycerol as a limiting factor, growth rate was improved by over 10-fold (Teusink et al. 2009). Another example is the replication of the natural adaptation of *Lactococcus lactis* from a plant niche to growth in milk in 1000 generations under laboratory conditions (Bachmann et al. 2012).

There are as yet no reports of the successful application of this method to *O. oeni*. Previous studies have shown that *O. oeni* is a rapidly evolving organism (Yang and Woese 1989). This is most likely due to the absence of the genes *mutS* and *mutL*, which encode two key enzymes in the methylated mismatch repair (MMR) pathway (Makarova et al. 2006; Makarova and Koonin 2007). The MMR pathway is a DNA excision-repair system, which corrects nucleotide base pair mismatches during DNA synthesis. The presence of *mutS* and *mutL* homologues is required for MMR to function (Miller 1996; Oliver et al. 2002). The correction of mismatches by MutS and MutL decreases the spontaneous mutation rate of a species; therefore, a defect or absence of the MMR system leads to an increase in mutation frequency (Miller 1996). A recent study comparing spontaneous mutation rates of *O. oeni* to those of closely related LAB species *Leuconostoc mesenteroides* and *Pediococcus pentosaceus*, which contain the relevant mismatch repair genes, showed a 100-fold greater rate of spontaneous mutation of *O. oeni* in response to antibiotics rifampin and erythromycin (Marcobal et al. 2008). One possible reason for the loss of MMR in *O. oeni* is that a high mutation rate generates beneficial mutations during adaptation to a restrictive environment such as wine (Marcobal et al. 2008). The high rate of mutation combined with the inhibitory properties of wine, therefore make *O. oeni* a perfect candidate for DE.

Stress response in *O. oeni* is controlled by a number of genes that alter several cellular processes. Genes reported to be involved in the cellular response to ethanol stress and contributing to biostability of wine include *ggpps*, *clpX*, *clcP*, *trxA*, *hsp18*, *ftsH*, *omrA*, *groESL*, *dnaK*, *clpA*, *ctrS* and *rmlB* (Sumby et al. 2014). The physiological response of *O. oeni* to ethanol stress has been well studied (Guzzo 2011; Wen-Ying and Zhen-Kui 2013). Ethanol perturbs the bacterial cell membrane leading to leakage of intracellular proteins, which affects the proton gradient and subsequently influences cellular processes, including ATP synthesis and the transport of amino acids (Guzzo et al. 2000). The physiological response of the cell to ethanol stress consists firstly of an increase in the proportion of cyclopropane fatty acids (CFAs) in the membrane, through conversion of *cis* vaccenic acid to lactobacillic acid (Teixeira et al. 2002), to counteract the effect of ethanol on membrane fluidity (Grandvalet et al. 2008), followed by an increase in membrane protein/phospholipid ratio. The precise mechanism by which *O. oeni* might be changed through a DE approach to improving its robustness during MLF might not be predictable, but once isolated, such strains offer a subject for further investigation of enhanced tolerance.

The aim of this study was to investigate the suitability of DE as a method for improving the robustness and hence MLF efficiency of an *O. oeni* strain that might subsequently be applied to winemaking. Specifically, the focus was on improving ethanol tolerance, a common wine stress known to inhibit MLF.

Materials and methods

Bacterial strains and maintenance

The strain used in this study, *Oenococcus oeni* SB3, was isolated from a commercial packet of this readily available wine starter culture (Laffort Oenology, Bordeaux, France). All bacterial isolates were cultured in de Man, Rogosa and Sharpe media (Cat. # AM103, Amyl Media, Victoria, Australia), supplemented with 20% apple juice (MRSAJ). When solid media was required, 2% agar was added to MRSAJ. Unless otherwise stated, all isolates were incubated at 30 °C for 5 days. Strains were stored at − 80 °C as glycerol stocks (culture grown for 5 days in MRSAJ and then supplemented with glycerol (final concentration 25% *v*/*v*)).

Directed evolution of *O. oeni* strain SB3

A continuous culture was established in a Biostat A plus bioreactor (Sartorius BBI System GmbH, Goettingen, Germany) and controlled using the MFCS/DA A plus 2.1 software (Sartorius BBI System GmbH). The empty bioreactor was autoclaved at 121 °C for 30 min and filled with 1100 mL of MRSAJ with 5% (*v*/*v*) ethanol. The medium was inoculated with a 10 mL culture of SB3 at an optical density measured at 600 nm to be greater than one ($OD_{600} > 1.0$). The volume in the bioreactor remained stable via a level sensor, feed/effluent pumps and control software. The culture temperature was maintained at 30 °C and stirred constantly at 100 rpm. The feed medium was MRSAJ via one pump starting at 54 mL h^{-1}.

Ethanol was added via a second pump throughout the course of the experiment at a constant rate of 2.4 mL h^{-1}. As the maximum specific growth rate (μ) of the population slowed in response to the ethanol addition, the feed rate of MRSAJ was also slowed over the course of the experiment to a final rate of 13.5 mL h^{-1}. In this way, ethanol concentration gradually increased to 15% (*v*/*v*) over the course of 290 days.

The culture was sampled weekly to measure optical density (OD_{600}) and determine viability (colony forming units) by plating on to MRSAJ agar. Once the bioreactor culture had surpassed 13% (*v*/*v*) ethanol, the *O. oeni* population was sampled and screened for improvement in L-malic acid consumption in the presence of ethanol compared to the parent SB3 strain, in a sacrificial microplate experiment described below.

Evaluation of the bioreactor population compared to the parent strain SB3

A replicated 96–well microtitre plate experiment was designed to measure growth and L-malic acid consumption of the evolved, potentially non-uniform population in MRSAJ with 15% (*v*/*v*) ethanol supplemented with 1 g L^{-1} L-malic acid. Screening was performed in quadruplicate 200 μL fermentations. Eight identical plates were generated, from an individual source plate, in order to enable sacrificial sampling over an 8–10-day period. This allowed a fresh plate to be analysed at each time point for monitoring of the depletion of L-malic acid, without influencing the performance of the small volume fermentations. Each plate was sealed with a membrane (Excel Scientific, 100-SEAL-PLT) and incubated at 30 °C in an anaerobic vessel (BD Diagnostic Systems, 260671, 260001). At each time point, the L-malic acid concentration was determined using the enzymatic method described below.

L-malic acid assay

L-Malic acid concentration was determined using a modified version of the Boehringer-Mannheim UV method (Boehringer-Mannheim 1995). Each well of a 96-well microtitre plate was filled with 150 μL of solution 1 (0.1 M glycylglycine, 0.1 M L-glutamate (adjusted to pH 10.0 with 5 M NaOH)) and 30 μL of solution 2 (0.0164 g mL^{-1} NAD^+) using a liquid-handling robot (Corbett Robotics CAS3800). The undiluted sample (4 μL) was then pipetted into each well except for the final column, which contained eight standards of L-malic acid solutions ranging from 0 to 3.5 g L^{-1} in 0.5 g L^{-1} increments. The plate was inserted into a plate reader (Infinite 200 PRO, Tecan, Männedorf, Switzerland), and 10 μL of glutamate oxaloacetate transaminase (GOT) solution (0.0269 U μL^{-1}) was added to each well of the plate using the injectors. The plate was shaken for 3 min before measurement at 340 nm (A0). Finally, 10 μL of the enzyme L-malate dehydrogenase (L-MDH) (0.0261 U μL^{-1}) were added to each well. The plate was shaken for 25 min and then read again at 340 nm (A1). The change in absorbance (ΔA) was calculated (A1–A0) for each of the standards and a standard curve generated for each individual plate. This equation was then applied to the ΔA for each of the samples within the plate to calculate L-malic acid concentration.

Characterisation of single isolates from the evolved bioreactor population

A replicated 96-well microtitre plate experiment, allowing sacrificial sampling, was designed to measure growth and L-malic acid consumption of single isolates from the evolved bioreactor population in MRSAJ with 15% (*v*/*v*) ethanol supplemented with 2 g L^{-1} L-malic acid. Each isolate was analysed in quadruplicate. A sample of the DE culture taken at 332 days (15% (*v*/*v*) ethanol) was plated onto MRSAJ agar and 180 individual colonies were isolated and inoculated into individual wells of two deep-well plates (Cat. # P-DW-20-C-S, Axygen, NY, USA) each containing 1 mL of MRSAJ. Deep-well plates were sealed with a breathable membrane (Cat. # AXY BF-400, Axygen, NY, USA) and incubated statically at 30 °C for 3 days. These liquid cultures were then diluted 1:5 in MRSAJ containing 15% (*v*/*v*) ethanol to generate source plates (Fig. 1). The source plates were arranged in quadrants, with each sample replicated once per quadrant, so that for each quadruplicate fermentation, the influence of the position of the sample in the plate was minimised. From the source plates, samples were diluted 1:20 into a 96-well plate (each well containing 190 μL of sterile MRSAJ with 15% (*v*/*v*) ethanol), using a liquid-handling robot (Corbett Robotics, Model # CAS3800). These six identical cell culture plates (Cat. # CLS3596, Corning, NY, USA) were prepared from each source plate and incubated statically and anaerobically in anaerobic vessels (Cat. # 260671, 260001, BD Diagnostic Systems) at 30 °C. One unique replicated set of the six identical plates was sampled at each of six time points. The plates were sampled regularly and monitored by determining L-malic acid concentration (as above) and therefore consumption over time.

Based on this analysis to determine the fastest L-malic acid consumption, a subset of isolates (27) were selected from the above screening to be evaluated in small-scale laboratory fermentations. Fermentations (10 and 50 mL) were performed in triplicate in MRSAJ with either 15% (*v*/*v*) ethanol at 22 °C or 13% (*v*/*v*) ethanol at 30 °C, respectively. Strains were grown in MRSAJ for 7 days to an $OD_{600} > 1.0$ and inoculated to fresh MRSAJ, with either 13 or 15% (*v*/*v*) ethanol, at a dilution of 1 in 50. Samples were taken regularly and L-malic acid concentration was determined enzymatically (as above). The strain that completed fermentation first, A90, was selected for further characterisation.

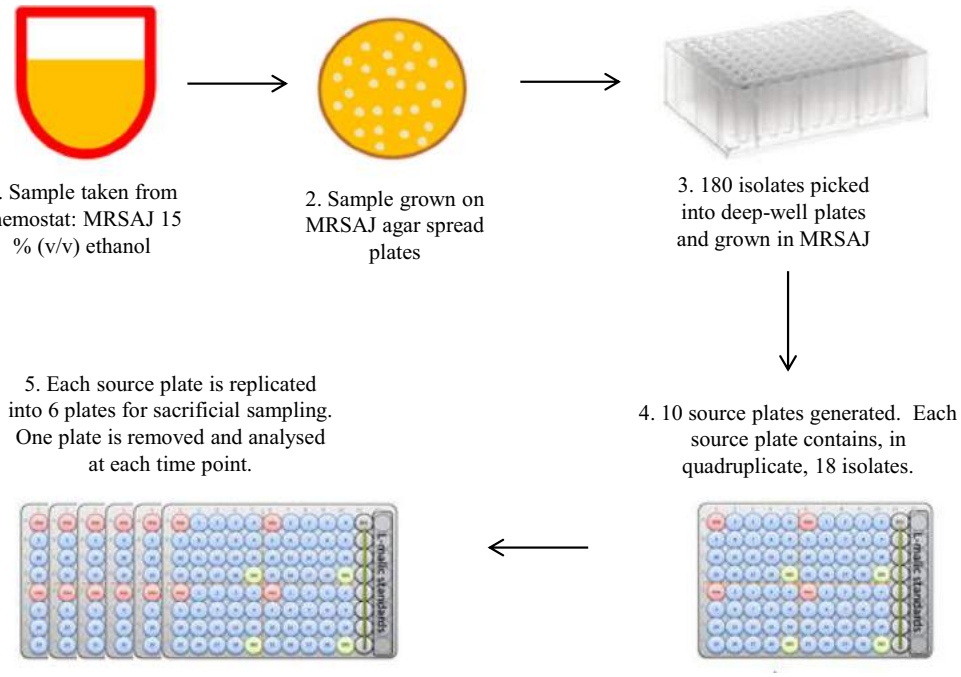


Fig. 1 Flow diagram representing the method for isolating 180 clones

Determination of survival from exposure to high ethanol

DE strain A90 and the parent SB3 were grown in MRSAJ to an OD_{600} > 1.0. An aliquot (200 μL) of each was then inoculated into 10 mL of MRSAJ with 0 or 20% (*v*/*v*) ethanol and incubated at 22 °C. Fermentations were sampled at 1, 12, 24, 36 and 48 h after inoculation. A 10-fold dilution series was performed (to 10^{-5}) and 2 μL samples were spotted onto MRSAJ agar. Plates were incubated in anaerobic vessels at 30 °C for 7 days prior to visualisation.

Malolactic fermentation performance of an evolved strain in wine

The MLF performance of A90 and parental strain SB3 was evaluated in a fermentation of a Pinot Noir wine (Tasmania, 13% (*v*/*v*) ethanol) to confirm that the ethanol tolerance phenotype of the isolate was not restricted to MRSAJ fermentations. A90 and SB3 were grown to an OD_{600} > 1.0 and inoculated into 10 mL of 0.22 μm filtered Pinot Noir wine. In addition, A90 and SB3 were also inoculated into the same Pinot Noir wine supplemented with ethanol to 15% (*v*/*v*). Samples (200 μL) were taken regularly and analysed for L-malic acid consumption (as above).

Strain passaging and ethanol tolerance

Single colonies of A90 and SB3 were isolated from ethanol-containing MRSAJ agar (20% *v*/*v* ethanol, 48 h) prepared during the generation of RNA for QPCR analysis (see below). Strains were cultured in 10 mL of MRSAJ in a CO_2 incubator at 30 °C until turbid. Optical density (OD_{600}) was adjusted to 1.2 and 250 μL of culture were used to inoculate fresh 10 mL MRSAJ. Strains were cultured for between 3 and 5 days, before re-adjusting culture OD_{600} to 1.2 and subsequently inoculating fresh 10 mL MRSAJ media. This process was repeated ten times for both A90 and SB3. To determine the number of generations that had occurred during each culture, a sample was taken immediately after each inoculation and at the end of each culture, prior to optical density adjustment. A 10-fold dilution series was performed (to 10^{-6}) and spot-plated onto MRSAJ agar to determine colony forming units. A minimum of 50 generations were achieved for both A90 and SB3. Passaged A90 and SB3 populations were inoculated (as above) into MRSAJ with 0, 18 and 21% (*v*/*v*) ethanol in triplicate and incubated at 22 °C. A 10-fold dilution series was performed (to 10^{-6}) on samples taken at 1, 24 and 48 h and spot-plated onto MRSAJ agar.

Transcriptional analysis

RNA extraction

Total RNA was extracted from six biological replicates of 50 mL cultures of A90 and SB3 under 4 conditions: (1) 1 h after inoculation into MRSAJ, (2) 1 h after inoculation into MRSAJ + 20% ethanol, (3) 24 h after inoculation into MRSAJ and (4) 24 h after inoculation into MRSAJ + 20% ethanol. *O. oeni* cells were harvested by centrifugation (5000 × *g*, 15 min), and the cell pellet was resuspended in 1 ml of cold 100% ethanol (− 30 °C) and transferred to a fresh 2 ml tube. The cells were then centrifuged (3800 × *g*, 30 s), the supernatant removed and the cell pellet

resuspended in 1 ml of TRIzol (Cat. # 15596026, Invitrogen, Australia) and frozen in liquid nitrogen. These were then stored at − 80 °C prior to RNA extraction.

RNA was extracted as follows: glass beads (425–600 μm, Cat. # G8772S, Sigma-Aldrich, Australia) were added to the cell suspension to 50% of the 1 ml volume. Cells were lysed by a bead-beater for 2 min and then incubated at 65 °C for 3 min. Chloroform (200 μL) was added and the tubes were shaken vigorously for 15 s and incubated at 20 °C for 5 min. Samples were centrifuged (12,800 × *g*, 10 min) at 4 °C, and the aqueous phase transferred to a fresh tube along with 500 μL of isopropanol. Samples were inverted six times and incubated at 20 °C for 10 min, followed by centrifugation (12,800 × *g*, 10 min, 4 °C). The supernatant was discarded and the RNA pellet washed in 1 ml of 75% cold ethanol (− 30 °C), all ethanol was then removed and the pellet air dried for 5–10 min. Purified RNA were dissolved in 50 μL of 0.1% dimethyl pyrocarbonate-treated water. RNA concentrations and purity were calculated by measuring absorbance at 260 and 280 nm using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific).

Reverse transcription and real-time qPCR

Prior to reverse transcription, total RNA was treated with 2 U of Turbo DNase (Cat. # AM1907, Invitrogen) as described by the manufacturer. Then, cDNA was synthesised using the iScript cDNA Synthesis Kit (Cat. # 1708891, Bio-Rad) as recommended. The absence of chromosomal DNA contamination was checked by real-time PCR. Initial experiments evaluated reference genes from previous studies (Sumby et al. 2012). The performance of these was analysed using qbase_plus software (Biogazelle) with *gmk*, *recA*, *gapA* and *ldhD* chosen as the most stable reference genes under the conditions of this study.

Primers for real-time PCR were designed with a length of about 20–25 bases, a G/C content of over 50% and a T_m of approximately 60 °C. Primer design software (Primer3) was used to select primer sequences. The length of the PCR products ranged between 107 and 200 bp. Potential primers were checked for gene-specific binding using NCBI primer design tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primers were purchased from Sigma-Aldrich. All real-time PCR was carried out on a Bio-Rad CFX96 real-time PCR system with the SsoFast EvaGreen Supermix (Cat. # 1725203, Bio-Rad) in 96-well plates. After 1:5 dilution of cDNA, 1 μL was added to 14 μL of PCR mixture (7.5 μL EvaGreen Supermix, 1 μL each primer at 10 pmol μL^{-1} and 4.5 μL RNase-free water). cDNA was amplified by real-time PCR with specified primers (Table 1). In each run, a no RT-PCR and a non-template control were included. Thermal cycling conditions were as follows: initial denaturation at 95 °C for 30 s, followed by 38 cycles of denaturation at 95 °C for 5 s and annealing/extension at 60 °C for 5 s. A melt curve was included in the end of the run from 65 to 95 °C to verify the specificity of the real-time PCR reaction for each primer pair. Efficiency of amplifications was determined by running a standard curve for each tested primer set with serial dilutions of cDNA. qPCR efficiency and normalisation was carried out using qbase_plus software (Biogazelle). For each gene, the fold changes between the two samples were generated by calculating the ratio of the average of the normalised signals for all 20% ethanol replicates to the average of the normalised signals for all 0% ethanol replicates and are represented as log2-fold-changes.

Statistical analysis

Data analysis was performed using Graphpad Prism 6. The qPCR results are presented as the mean of six replicates ± SEM (standard error of the mean). Significant differences between the two strains were calculated using paired *t* tests, and the significance level was set at a *p* value of 0.05.

Results

Directed evolution of an industrially relevant *O. oeni* strain

The commercially available *O. oeni* strain SB3 was cultivated over 290 days in a bioreactor having progressively increased ethanol concentration in the feed from 0 to 15% (*v*/*v*) decreasing the growth rate from 0.10 to 0.05 h^{-1}. The bioreactor contents were sampled at appropriate intervals and screened for improvement in L-malic acid consumption compared to the parent. For samples taken from the evolving population at 290 days, there was little difference to the fermentation profile of the parent (SB3) in the absence of ethanol (Fig. 2). However, in the presence of 15% (*v*/*v*) ethanol the evolved population consumed all available L-malic acid at least 69 h earlier than SB3 (89.5 vs 158.5 h, respectively) a 56% improvement in fermentation time, thereby showing an improved ability to perform MLF under higher ethanol conditions.

Isolation and characterisation of evolved clones with improved MLF performance

Individual isolates were obtained after 332 days from the mixed population that showed improved fermentation performance when grown in MRSAJ with 15% (*v*/*v*) ethanol. Through microscale fermentations of 180 individual isolates, 27 were selected as being significantly improved performers. These were reassessed in larger 10 and 50 mL fermentations at 13 and 15% (*v*/*v*) ethanol and at 22 °C and 30 °C, respectively (Fig. 3). Strain A90 showed an ethanol tolerant phenotype and completed MLF in the shortest time.

Table 1 Primer sequences for the putative reference and target genes used in this study

Target gene	Description	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)	Reference
ldhD	d-Lactate dehydrogenase	GCCGCAGTAAAGAACTTGATG	TGCCGACAACACCAACTGTT	(Beltramo et al. 2006)
rpoA	Sigma α factor	GATGGAACTTGGCATTGGTG	AAGACTCGCTTGGACTAACAGAAC	(Sumby et al. 2012)
gyrB	Gyrase subunit B	CGTCCGGCTATGGAAGCTGT	ACAACCGAAGCGCCAACAC	(Sumby et al. 2012)
gapA	d-Glyceraldehyde-3-phosphate dehydrogenase (GA3PDH)	TCCACGCTTACACATCGACTCA	CGCTGAGCATGACCATTCAAC	(Sumby et al. 2012)
gmk	Guanylate kinase	CGCAGGAATGAGGTTGATGGT	CTGGCGCTGCTCGTCTATCC	(Sumby et al. 2012)
recA	Recombinase A	ACTTGCCGGTACACTGAATAGAAC	GAGCAGATCGACGCACTTCA	(Sumby et al. 2012)
rpoB	Subunit beta of DNA-directed RNA polymerase	GCTTGTTGACCGATGATGAG	ACCGTTAGGAGCAGCCATT	(Sumby et al. 2012)
ftsZ	Filamenting temperature-sensitive mutant Z—GTPase	TGCCGGATCGACACCTGA	CGGACGAGTAACAACGCCAAC	(Sumby et al. 2012)
pta	Phosphotransacetylase	CATGGCTGAGATTGCCGTTC	TCTCCTGCGCCAGCTTAGT	(Sumby et al. 2012)
ggpps	Geranylgeranyl pyrophosphate synthase	AACAGTTCGGACAAAGTACAG	CGACCATCTGTGTCATTTCA	(Cafaro et al. 2014)
citE	Citrate lyase	CCGCACGATGATGTTTGTTCC	GCTCAAAGAAACGGCATCTTCC	(Olguín et al. 2009)
hsp18	Stress protein Lo18	CGGTATCAGGAGTTTTGAGTTC	CGTAGTAACTGCGGGAGTAATTC	(Beltramo et al. 2006)
cfa	Cyclopropane fatty acid synthase	TGGTATTACATTGAGCGAGGAG	CGTCTTTGAGATCACGATAATCC	(Beltramo et al. 2006)
clpP	ClpP protease	CGGTACCAAAGGCAAGCGTTTTAT	CTCTTCCGAGTCTTCAAAAGTTGAT	(Beltramo et al. 2006)
atpB	OEOE_0665 F0F1-type ATP synthase, beta subunit	ATACTGATCCGGCTCCGGC	CAGCGGGATAAATACCTTG	(Olguín et al. 2015)
RS07535	S-ribosylhomocysteine lyase	AATTCCATCAAGCCGGTCCC	CCCAGCCAAACCAAACTTCG	This study
mleP	Malate permease	TCACGAAGAGCACCGTTGAA	GCGGATCAATGCCAAAGCTT	This study
mleR	Malolactic regulator	TGTCCTGATCGAAGACGCTG	ACCCTGGAATTGAGCAACGA	This study
mleA	Malolactic enzyme	CCGACAATTGCTGATACAATTGAA	GGCATCAGAAACGACCAGCAG	(Beltramo et al. 2006)
DDCarb	D-alanyl-D-alanine carboxypeptidase EAV39051.1	TTCAACATAGCAGTTGCGTC	AGGAACTTCACCCGATACGG	This study
RS06010	(OEOE_1247) glucosaminidase	AACCAATGCTTTAGCGGCAG	GCTGGCGCATGCTAAATTCA	This study
RS06975	(OEOE_1444) peptidoglycan interpeptide bridge formation protein	AAGGCTGTCAACAAGCTCGA	GAAAGCGGCCAGATTGATGC	This study
RS05625	(OEOE_1168) amino acid transport	CTAACCGGAAAGGGAGCGTT	TTCAGCGTAACAGAGACCGG	This study
ppk	(OEOE_1629) ppk	CTTAGCGCAGGCAAACGATC	TGATGAATCGAGGCCAGACG	This study
RS07625	(OEOE_1581) SAM-dependent methyltransferase	ACAAAGTCCAAGCCGGTGAA	GGCTGTCTCAACTTCCGGAA	This study
RS04370	(OEOE_0911) SAM-dependent methyltransferase	GTTCTGGCAAACTGGCTGTT	CGTTGCTTTGCCAGAGATAACA	This study
RS03525	(OEOE_0738) ABC-type multidrug transport system, ATPase component	TTGATCGGTCCATCAGGCAC	GACTTCTCGGTTTGGCATGC	This study
ddl	d:alanine–d:alanine ligase	CCTAAGCGAGCCTAATACAC	CCTGTTGGCTTCGATTCATTCC	(Olguín et al. 2010)
rmlB	dtdP-glucose-4,6-dehydratase	TATCCGCAATGCGCAATTGG	GAACGGTCAACATGCGATTCAG	(Olguín et al. 2010)
ctsR	Master regulator of stress response	GGGCCATGGCAGAAGCTAATATTTCAG	AAACGGGTGTTGATTACATAATT	(Beltramo et al. 2006)
clpX	Clp ATPase protein	GAAGCGTGTTAACGAGTCC	CAGCGACTGAGCCAAATAAG	(Olguín et al. 2010)
trxA	Thioredoxin	GCCACTTGGTGTACCCCTTGT	TCCATTTGCCGTTTCCTGGTTT	(Beltramo et al. 2006)
groES	GroES, heat shock chaperone class I	GCCACAACAGAACCCATCACTGGTT	GGCGATCGAATTGTTCTTAGTAT	(Beltramo et al. 2006)
grpE	GrpE, heat shock chaperone class II	CGCAGGCAGAAAAGAACAATC	GCTGAAGACGAAGCAGTTGC	(Beltramo et al. 2006)
gsh	Glutathione reductase	GGCATTATCACCGAGCTGTT	TCCCGAAGAAGCAAAGAAGA	(Bordas et al. 2015)
pyrB	OEOE_0258 pyrB aspartate carbamoyltransferase	GGCAGGTTGTTGCCAATC	TTGTTGCCGAGGACTTGTTGGG	(Olguín et al. 2015)
trhD	OEOE_0394 threonine dehydrogenase	AGAGTTCTTGCGCGAGAC	CCGGTGCCACTCATATTCTTAG	(Olguín et al. 2015)
amt	OEOE_0411 aminotransferase	TTGGACAGCGAAGGAAGAGT	GTTTATCTTCGGCCGTCAAC	(Olguín et al. 2015)

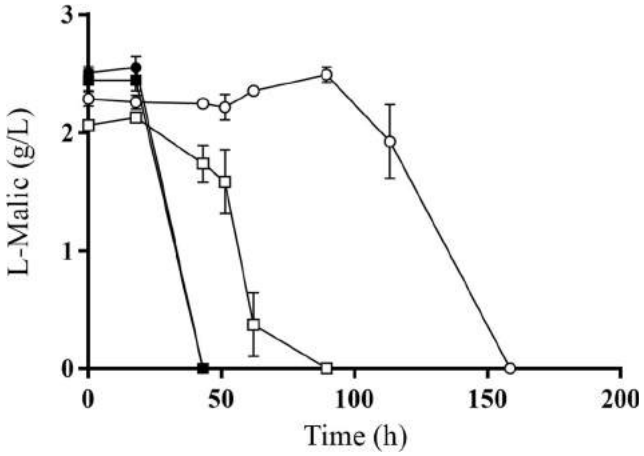


Fig. 2 Evaluation of MLF performance of an evolved population and the parent strain SB3 at 30 °C in MRSAJ supplemented with 1 g L^{-1} L-malic acid. Samples were screened in 200 μL microplate fermentations. L-malic acid consumption of the parent SB3 (●,○) and the evolved population (■,□) in 0% (*v*/*v*) (●,■) and 15% (*v*/*v*) (○,□) ethanol. The evolved population completed the consumption of available L-malic acid 69 h earlier than the parent in the presence of 15% (*v*/*v*) ethanol

Enhanced ethanol tolerance of evolved strain A90

The relative ethanol tolerance of strain A90 and the parent (SB3) was investigated by analysing growth and viability in the presence of high concentrations of ethanol. As a control, both strains were incubated in MRSAJ without ethanol and sampled at set time points for the duration of the experiment. No difference in viability between SB3 and strain A90 was observed at any of these times (Fig. 4). However, when exposed to 20% (*v*/*v*) ethanol for 48 h, the viability of parent (SB3) was reduced by at least four orders of magnitude when compared to A90.

Improved MLF performance of evolved strain A90 in Pinot Noir

MRSAJ is a highly nutritious medium especially when compared to wine. As the isolates may have been evolved specifically for the MRSAJ medium, the ability to conduct MLF in wine may have been lost or reduced. To test this, the MLF performance of A90 was monitored in Pinot Noir wine alongside the parent (SB3). Both strains were inoculated into Pinot Noir wine with an ethanol concentration of 13% (*v*/*v*) as well as the same wine with ethanol supplementation to 15% (*v*/*v*). At 13% (*v*/*v*) ethanol, A90 completed MLF of the wine in 75% of the time required by the parent (Fig. 5). This difference was more pronounced at 15% (*v*/*v*) ethanol with A90 finishing in 40% of the time of the parent (Fig. 5).

Enhanced ethanol tolerance of evolved strain A90 persists after 50 generations of passaging

Evolved strain A90 and the parent (SB3) were passaged in MRSAJ ten times, resulting in a minimum of 50 generations for each strain (data not shown). Ethanol tolerance of the resulting

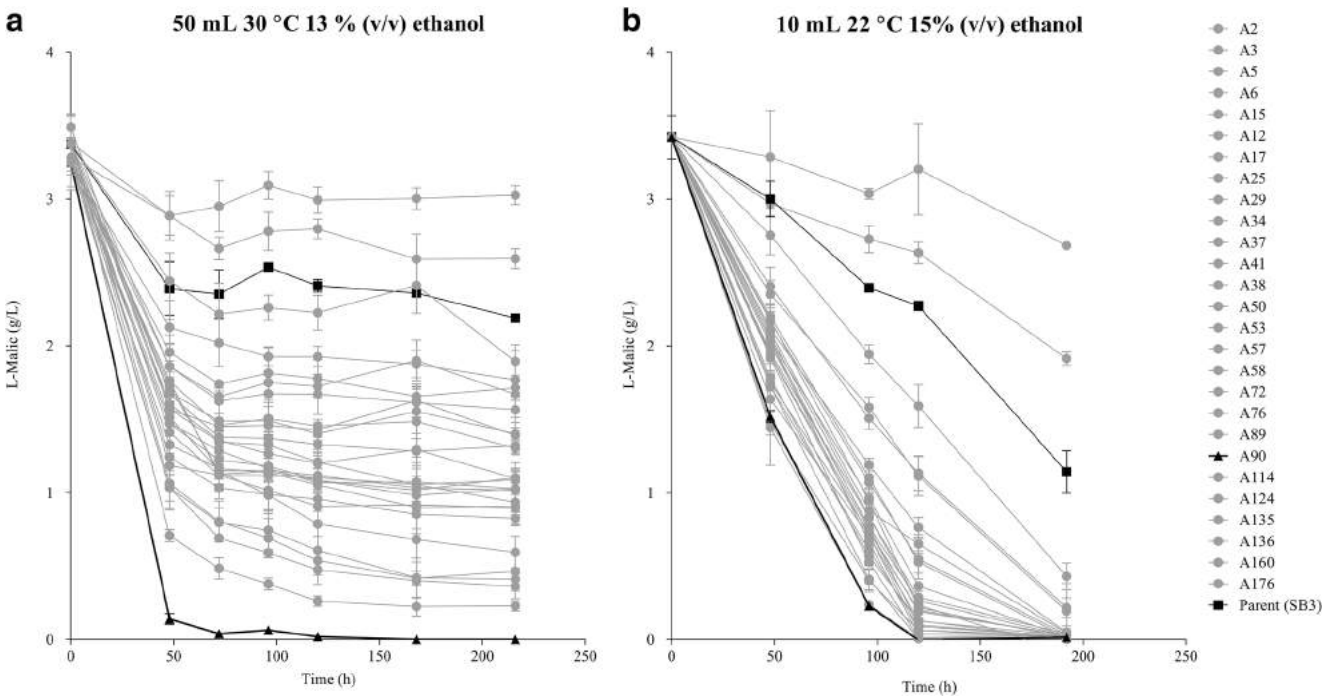


Fig. 3 Malic acid utilisation of 27 clonal isolates selected through the microplate screening and the parent (SB3). Samples were screened in either 50 mL MRSAJ at 30 °C (**a**) or 10 mL MRSAJ at 22 °C (**b**) supplemented with 2 g L^{-1} L-malic acid and 13 or 15% (*v*/*v*) ethanol, respectively. Values are the average of three biological replicates and error bars indicate the standard deviation. Strain A90 was selected as the most improved strain for further study

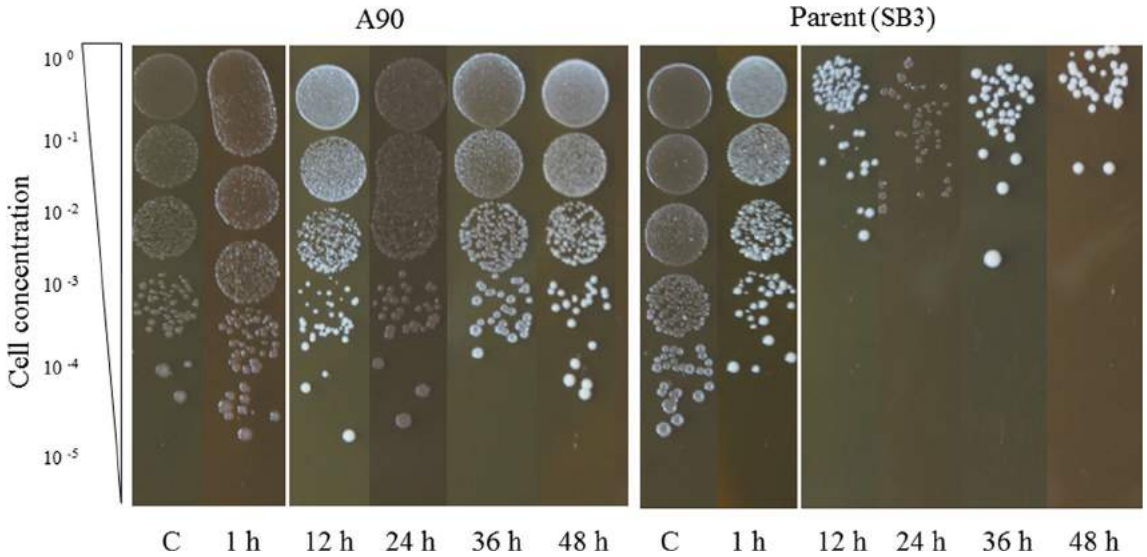


Fig. 4 Ethanol tolerance of evolved strain A90 and the parent SB3 exposed to 20% (*v*/*v*) ethanol at 20 °C. The impact of exposure to 20% (*v*/*v*) ethanol, for up to 48 h, on culturability of strains A90 and the SB3 was assessed by spot-plating on MRSAJ. Strains were sampled at 1, 12, 24, 36 and 48 h after inoculation in MRSAJ containing 20% (*v*/*v*) ethanol. Images are a composite of MRSAJ agar made at each time point of a 10-fold dilution series with the top sample an undiluted 2 μL sample. C is the inoculation control sampled after 1 h in 0% (*v*/*v*) ethanol, indicating a viable population

populations was re-evaluated to determine if the passaged A90 (p-A90) was still more ethanol tolerant than the passaged SB3 (p-SB3). After exposure to 21% (*v*/*v*) ethanol for either 24 or 48 h, p-A90 retained a higher percentage of viable cells, indicating a greater ethanol tolerance, compared to p-SB3 (Fig. S1). No difference in ethanol tolerance was observed at 18% (*v*/*v*) ethanol after either exposure duration. This data suggests a degree of stability of the ethanol tolerance attribute.

The addition of ethanol altered the expression of several targeted genes

In an effort to begin to understand the basis for the superior performance of A90, the expression of a selection of genes was examined. Several of the targeted genes changed expression due to ethanol exposure in both SB3 and A90. Genes that increased in expression after 1 h of ethanol exposure in both strains were *hsp18*, *trxA*, *grpE*, *groES*, *trhD*, *amt*, DDCarb, RS03525 and *clpP* (Table S1). Genes that decreased in expression following 1 h of ethanol exposure in both SB3 and A90 were *ddl*, *ppk*, RS06010, *atpB* and RS07625 (Table S1). Genes that displayed increased expression after 24 h of ethanol exposure in both SB3 and A90 were *rmlb*, *hsp18*, *trxA*, *gshR*, *ctsR*, *clpX*, *grpE*, *groES* and *clpP* (Table S1), while RS06975, *pyrB*, *mleP*, *mleR*, *ppk*, RS06010, *ggpps*, *DDCarb*, RS07625 and RS04370 showed decreased expression in both strains under the same conditions (Table S1). There were several genes showing significant difference between strains A90 and SB3 at both time points (Table 2).

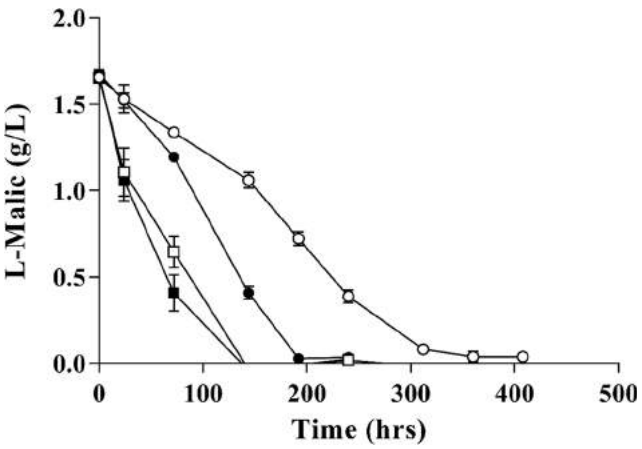


Fig. 5 Evaluation of MLF performance of evolved strain A90 and its parent, SB3, in Pinot Noir wine at 22 °C. Residual L-malic acid content for fermentations conducted by the parent SB3 (●,○) and evolved strain A90 (■,□) in Pinot Noir wine with 13% (●,■) and 15% (○,□) (*v*/*v*) ethanol

Discussion

Directed evolution was performed on the commercial *O. oeni* wine strain SB3, and preliminary characterisation of the mixed population (grown for 290 days, reaching 15% (*v*/*v*) ethanol) revealed a culture that fermented L-malic acid more rapidly than the parent in MRSAJ supplemented with 15% (*v*/*v*) ethanol and L-malic acid. The faster MLF phenotype of the DE strain A90 was only observed when conducted in the presence of ethanol. This is the first study using DE to target *O. oeni* and ethanol tolerance and shows that DE can be used to generate a population with improved MLF performance. A90 clearly outperformed its parent in terms of tolerance to high ethanol concentrations and MLF performance in the presence

Table 2 Relative expression of genes that were differentially expressed 1 and 24 h after ethanol addition to SB3 (parent strain) and A90 (DE strain) MRSAJ cultures

Target gene[a]	Description[b]	Relative expression[c]			
		1 h		24 h	
		SB3	A90	SB3	A90
Cell envelope biogenesis, outer membrane					
RS06975	Peptidoglycan interpeptide bridge formation protein	0.74 ± 0.19	− 1.10 ± 0.20*	− 2.51 ± 0.44	− 0.60 ± 0.15*
ddl	D-alanine D-alanine ligase	− 3.18 ± 0.24	− 2.06 ± 0.20*	0.20 ± 0.28	0.85 ± 0.20
Stress response					
trxA	Thioredoxin	2.58 ± 0.16	3.47 ± 0.27*	3.20 ± 0.42	4.00 ± 0.42
hsp18	Heat shock protein Lo18	3.43 ± 0.27	4.34 ± 0.34*	3.57 ± 0.45	5.40 ± 0.55*
Posttranslational modification, protein turnover, chaperones					
clpX	ATP-binding subunit of Clp protease and DnaK/DnaJ chaperones	0.98 ± 0.16	1.83 ± 0.21*	1.59 ± 0.35	3.20 ± 0.37*
groES	GroES, heat shock chaperone class I	2.23 ± 0.24	2.88 ± 0.20	4.39 ± 0.61	2.78 ± 0.33*
Amino acid transport and metabolism					
trhD	Threonine dehydrogenase-like Zn-dependent dehydrogenase	3.28 ± 0.27	2.71 ± 0.15	−2.54 ± 0.55	1.01 ± 0.21*
pyrB	Aspartate carbamoyltransferase	− 0.40 ± 0.18	1.38 ± 0.18*	− 3.19 ± 0.69	− 1.80 ± 0.24
RS05625	Amino acid permease	1.87 ± 0.17	0.46 ± 0.14*	− 0.64 ± 0.28	1.25 ± 0.19*
MLF/citrate metabolism					
mleR	MLF system transcription activator	− 0.35 ± 0.19	0.87 ± 0.20*	− 3.35 ± 0.71	− 2.26 ± 0.43
mleA	Malolactic enzyme	0.74 ± 0.20	− 1.41 ± 0.28*	− 0.71 ± 0.24	0.04 ± 0.18
citE	Citrate lyase beta subunit	1.49 ± 0.17	0.60 ± 0.21*	0.12 ± 0.31	− 1.09 ± 0.19*
Cellular biosynthetic process, secondary metabolism, energy metabolism					
ppk	Polyphosphate kinase	− 0.71 ± 0.15	− 0.72 ± 0.27	− 5.25 ± 1.06	− 2.38 ± 0.19*
RS06010	Glucosaminidase	− 1.53 ± 0.18	− 0.87 ± 0.21*	− 5.39 ± 1.60	− 2.29 ± 0.36*
ggpps	Geranylgeranyl pyrophosphate synthase	− 0.12 ± 0.18	0.17 ± 0.25	− 5.75 ± 1.67	− 2.73 ± 0.23*
amt	Aspartate aminotransferase	1.56 ± 0.22	2.44 ± 0.18*	−1.08 ± 0.40	1.51 ± 0.22*
Nucleotide binding					
atpB	F0F1-type ATP synthase, beta subunit	− 2.04 ± 0.22	− 0.86 ± 0.21*	− 1.38 ± 0.25	− 0.39 ± 0.15
DDCarb	D-alanyl-D-alanine carboxypeptidase EAV39051.1	1.37 ± 0.26	1.90 ± 0.19	− 4.98 ± 1.09	− 2.58 ± 0.52*
RS03525	ABC-type multidrug transport system, ATPase component	2.95 ± 0.18	2.57 ± 0.17	− 2.86 ± 0.58	0.07 ± 0.20*
Transferase					
cfa	Cyclopropane-fatty-acyl-phospholipid synthase	0.01 ± 0.16	0.18 ± 0.20	− 0.43 ± 0.28	1.71 ± 0.21*
RS07625	SAM-dependent methyltransferase	− 1.40 ± 0.17	− 0.86 ± 0.19	− 5.31 ± 1.15	− 2.75 ± 0.24*
RS04370	SAM-dependent methyltransferase	− 0.65 ± 0.21	− 0.59 ± 0.28	− 3.19 ± 0.58	− 1.17 ± 0.18*

[a] The gene names are taken from the NCBI database for *Oenococcus oeni* PSU-1 complete genome (http://www.ncbi.nlm.nih.gov)

[b] Gene descriptions were taken from NCBI database (http://www.ncbi.nlm.nih.gov) and UniProt database (http://www.uniprot.org)

[c] Relative expression of gene is described as the log2 of the fold change after ethanol addition with respect to 0% ethanol. A positive value for fold change indicates a higher expression while a negative value indicates a lower expression in 20% ethanol when compared with 0% ethanol in each strain

*Significant difference between the strains ($p < 0.05$)

of ethanol, additionally A90 formed a greater number of CFUs when plated on MRSAJ agar after 48 h exposure to 20% (*v*/*v*) ethanol compared to its parent (SB3). Importantly, during the DE process A90 did not lose its ability to conduct MLF. In a Pinot Noir wine containing 15% (*v*/*v*) ethanol, strain A90 completed MLF in 40% of the time required by the parent. This result is potentially significant to the wine industry. The alcohol content in wines has been increasing (Godden and Muhlack 2010) resulting in sluggish or failed MLF. More effective strains of *O. oeni* will reduce production time costs to the wine industry.

Stability of the phenotypic and presumably genetic changes were evaluated by passaging under non-ethanol selective conditions (MRSAJ). After 50 generations, the stability of the high ethanol tolerance phenotype of A90 was still apparent at 21% (*v*/*v*).

To determine the basis for the observed high ethanol tolerance phenotype, expression of several genes from both strains was investigated by qPCR in high (20% *v*/*v*) and 0% (*v*/*v*) ethanol media. Previous studies have suggested that *O. oeni* increases expression of many stress response genes in order to survive the harsh conditions found in wine. Olguín et al. (2010) showed that strains with higher relative expression of *hsp18*, *clpP*, *ctsR* and *rmlB* in high ethanol conditions showed better MLF performance in a modified FT80 medium. Bordas et al. (2015) also reported that *citE*, *hsp18* and *clpP* showed higher levels of expression, in ethanol tolerant strains compared to a low ethanol tolerant strain, in a 'wine-like' media. In this study, A90 showed higher expression of *hsp18* compared to SB3, but this was not the case for any other of the above genes. Maitre et al. (2014) proposed a model whereby *O. oeni* can adapt to ethanol stress through the synthesis of the small heat shock protein Lo18 encoded by *hsp18* and the subsequent modification of phospholipid content of the cell membrane. The existence of such unique features can be viewed as an evolutionary adaptation to the wine environment (Beltramo et al. 2006). The response of LAB to stress relies on the coordinated expression of genes that alter specific cellular processes (membrane biogenesis, transport, DNA metabolism, etc.). A network of regulators allows the cell to react to complex environmental changes and achieve a coordinated response.

When relative expression changes are compared between the two strains after 1 h of ethanol exposure, A90 showed an increased expression of eight of the tested genes compared to SB3: *ddl*, *trxA*, *hsp18*, *clpX*, *pyrB*, *mleR*, *amt* and *atpB* (Table 2). A90 also showed decreased expression of four of the tested genes compared to SB3: RS06975, RS05625, *mleA* and *citE*. When relative expression changes are compared between the two strains after 24 h of ethanol exposure, A90 showed higher expression of 15 of the tested genes compared to SB3: RS06975, *hsp18*, *clpX*, *trhD*, RS05625, *ppk*, RS06010, *ggpps*, *amt*, atpB, DDCarb, RS03525, *cfa*, RS07625 and RS04370 (Table 2). A90 also showed lower expression of two of the tested genes compared to SB3: *groES* and *citE*.

Our results show that in A90, *hsp18*, *amt*, *clpX* and *trxA* expression was increased after 24 with 20% (*v*/*v*) ethanol addition, and not only as a transient shock response. After growth in MRSAJ 20% (*v*/*v*) ethanol, the mRNA levels of these genes were higher than in the control (MRSAJ 0% ethanol). This result suggests that an improved basal level of gene expression followed the initial increase. This was only true in SB3 for *hsp18* and *trxA*, where the increases were not as great as A90. As these are two important stress response genes, they could be an important indicator of the reason for the improved phenotype of A90. The final gene showing consistent response to ethanol independent of time was *atpB*, where expression decreased relative to the control at both 1 h and 24 h. *atpB* encodes the beta subunit of a F0F1-type ATP synthase in which the primary function in most organisms is ATP synthesis. It is important to control ATP-driven proton pumping activity of ATP synthase in order to avoid wasteful ATP hydrolysis under conditions when no proton motive force can be generated (e.g. leaky damaged membrane). To avoid exhaustion of the intercellular ATP pool, expression of ATP synthases may be down-regulated to suppress ATPase activity. Additionally, a recent study of stress response in a wine-like medium reported that expression of enzymes encoding ATPase activity were down-regulated in *O. oeni*; however, the expression of *atpB* was not reported (Margalef-Catalá et al. 2016).

The genes that were down-regulated were dependent on time. For example both *mleA* and RS06975 initially decreased (1 h), but then increased slightly to levels that were not significantly different. Additionally, RS05625 expression was down-regulated in SB3 only after 24 h and A90 expression went up. RS05625 encodes an amino acid putative amino acid transporter, and the reason for its differential expression is currently unclear and warrants further investigation. A decrease in *citE* expression over time was also seen by Olguín et al. (2009); they however used media supplemented with citrate so it is hard to make a direct comparison. SB3 showed the same upregulation of *citE* as reported by Margalef-Catalá et al. (2016), whereby expression initially increased and then decreased slightly over time; they however only measured up to 8 h after inoculation. Even though A90 showed much lower initial expression of *citE* the general trend was the same as SB3 expression. Although *ddl* showed an initial down regulation after 1 h, expression was at similar levels to the no ethanol control at 24 h. Expression of *pyrB* decreased over time, in both strains; down regulation of *pyrB* over time was also observed by Margalef-Catalá et al. (2016). pyrB encodes an aspartate carbamoyltransferase which catalyses the first step in the pyrimidine biosynthetic pathway. The patterns of expression described above suggest a complex response to ethanol during the first 24 h of exposure.

Olguín et al. (2015) reported that genes involved in amino acid transport and metabolism, cell envelope biogenesis and transcription were most affected by ethanol addition (12% (*v*/

v)) in a modified MRS medium. That study, like many others, uses PSU-1, which has been shown to be a poor performer under stress conditions and during MLF. Our results showed that for both SB3 and A90, expression of genes involved in stress response, protein turnover proteolysis, chaperones, amino acid transport and metabolism, and nucleotide binding increased after 1 h exposure to ethanol. However, after 24 h, only genes involved in stress response, cell envelope biogenesis, protein turnover and chaperones, and proteolysis had higher relative expression in 20% (*v*/*v*) ethanol relative to the 0% (*v*/*v*) ethanol control. Additionally, Cafaro et al. (2014) reported that the gene encoding geranylgeranyl pyrophosphate synthase (ggpps), an enzyme involved in carotenoid biosynthesis, is overexpressed in *O. oeni* in response to ethanol. Carotenoid production has been shown to increase multistress tolerance when carotenoid biosynthesis genes are overexpressed in *Lactococcus lactis* (Hagi et al. 2013). However Cafaro et al. (2014) used late-exponential phase cells and an increase in *ggpps* expression was not observed in this study.

To investigate this further, future work would include knockout of individual genes of interest to show cause and effect relating to the ethanol tolerance phenotype. However, systems to easily make targeted genetic modifications to the *O. oeni* genome are not yet readily available. As discussed above, there are several studies reporting regulation of genes under stress conditions in *O. oeni*. However, it would appear that apart from a few generalisations, these changes are highly strain specific and warrant a more detailed investigation. This is outside the scope of the current project, but there is a need for analysis of multiple strains using RNA seq to provide a more holistic overview of *O. oeni* stress response.

This study is the first of its kind to use DE as a tool for the non-recombinant modification of *O. oeni*. We have successfully used DE to modify wine strain SB3, to have a higher ethanol tolerance in MRSAJ. Importantly, the ability to ferment L-malic acid was not lost during DE. Under high ethanol stress conditions the evolved strain showed superior L-malic acid consumption compared to the parent SB3, in both MRSAJ media and Pinot Noir. The DE strain A90 completed MLF in Pinot Noir in 40% of the time of SB3, suggesting this strain could be of value to the wine industry. Additionally, there were clear differences in the expression of several stress response genes. Most notably, the relative expression of *hsp18*, which encodes the small heat shock protein Lo18, was significantly higher in A90 compared to SB3 in 20% (*v*/*v*) ethanol. This result is in keeping with the enhanced ethanol tolerance phenotype of A90. The findings also highlight the opportunities for the use of DE as a method of non-recombinant strain improvement for the wine industry.

Acknowledgements VJ conceived the project. ALB performed the DE, screening of isolates and fermentation experiments, and analysed the data. KMS performed qPCR experiments, and analysed and interpreted the data. JFS performed strain passaging and phenotype stability experiments and analysed the data. ALB, KMS and JFS wrote and edited the manuscript. PRG and VJ revised and edited the manuscript.

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Compliance with ethical standards

Conflict of interest ALB was supported by scholarships from AGWA (UA1101/P3102/GWR Ph 0901) and the University of Adelaide. KMS and JFS are supported by Wine Australia project funding (UA1302). The authors declare that they have no conflicts of interest.

Human and animal rights and informed consent This article does not contain any studies with human participants or animals performed by any of the authors.

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Appropriate vacuolar acidification in *Saccharomyces cerevisiae* is associated with efficient high sugar fermentation

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ABSTRACT

Vacuolar acidification serves as a homeostatic mechanism to regulate intracellular pH, ion and chemical balance, as well as trafficking and recycling of proteins and nutrients, critical for normal cellular function. This study reports on the importance of vacuole acidification during wine-like fermentation. Ninety-three mutants (homozygous deletions in lab yeast strain, BY4743), which result in protracted fermentation when grown in a chemically defined grape juice with 200 g L^{-1} sugar (pH 3.5), were examined to determine whether fermentation protraction was in part due to a dysfunction in vacuolar acidification (VA) during the early stages of fermentation, and whether VA was responsive to the initial sugar concentration in the medium. Cells after 24 h growth were dual-labelled with propidium iodide and vacuolar specific probe 6-carboxyfluorescein diacetate (6-CFDA) and examined with a FACS analyser for viability and impaired VA, respectively. Twenty mutants showed a greater than two-fold increase in fluorescence intensity; the experimental indicator for vacuolar dysfunction; 10 of which have not been previously annotated to this process. With the exception of Δhog1, Δpbs2 and Δvph1 mutants, where dysfunction was directly related to osmolality; the remainder exhibited increased CF-fluorescence, independent of sugar concentration at 20 g L^{-1} or 200 g L^{-1}. These findings offer insight to the importance of VA to cell growth in high sugar media.

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1. Introduction

The fermentative performance of yeast (*Saccharomyces cerevisiae*) is one of the most crucial factors for successful sugar fermentation processes, including winemaking. During oenological fermentations, yeast growth and fermentation kinetics are affected by many factors such as temperature, pH, concentration of sugar, ethanol and nutrients (Cardona et al., 2007; Ribereau-Gayon et al., 2006). The capability of wine yeast to adapt to these conditions or stressors, and their capacity to efficiently complete alcoholic fermentation are primary considerations when choosing a strain for winemaking.

Typically there are multiple stressors present during grape juice fermentations, especially those with initially high concentrations of sugar (200–270 g L^{-1} or more *i.e.* High Sugar Fermentation). Importantly, yeasts undergoing this type of batch fermentation must also tolerate a progressively changing environment and are required to adapt to a combination of stressors at any one time. For instance, during the initial stage of fermentation these stressors include high osmolarity (brought about by high concentrations of sugar), acidity (pH ~3.5) and often low temperatures. Later in fermentation, yeast must contend with the transition to nutrient depletion, temperature upshift and increasing alcohol content.

To understand how the yeast overcome these stresses, we have previously used a genome-wide screening approach to identify 93 genes, designated Fermentation Essential Genes (FEGs), which upon deletion, had minimal effect on biomass yield but result in protracted, and in extreme cases, arrested fermentation in high sugar

Abbreviations: CDGJM, Chemically Defined Grape Juice Medium; 6-CFDA, 6-carboxyfluorescein diacetate; CF, 6-carboxyfluorescein; FACS, Flow Activated Cell Sorting; FAN, free amino nitrogen; FEGs, Fermentation Essential Genes; OD, optical density; VA, vacuolar acidification.

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medium (Walker et al., 2014). Several processes were identified as vital for adaption to conditions typical of industrial fermentations; with the clustering of genes into specific groupings, i.e. Gene Ontology (GO) terms describing the gene product's biology. The GO term 'vacuolar acidification' significantly enriched with 20 of the 26 genes annotated to this process, identified amongst the 93 FEGs.

The acidification of the vacuole and endosomal system is important in the regulation of intracellular pH, namely the cytosol. Cystolic pH is controlled through the coordinated activity of the plasma membrane proton pump, Pma1p, and the vacuolar ATPase complex (Cyert and Philpott, 2013; Orij et al., 2012). Cytosolic pH, itself, is considered a signal transducer, sensing nutrient status and pH and affecting growth (Orij et al., 2012), whilst vacuolar pH is responsive only to environmental pH (Brett et al., 2011). The coordination of the two pumps enables yeasts to be unaffected in growth and fermentation at pHs ranging from 3.5 to 6 (reviewed in Orij et al., 2011). The vacuolar ATPase is an adaptive mechanism enabling yeast to tolerate several different conditions including high glucose (Teixeira et al., 2010); ethanol stress (Fujita et al., 2006; Teixeira et al., 2009), weak acid stress (Mira et al., 2010) and the ability to complete high sugar fermentations (Walker et al., 2014). In this study, we have assessed vacuolar acidification (VA) using flow cytometry and the fluorescent probe 6-carboxyfluorescein diacetate (6-CFDA), where measurement of the hydrolysed 6-carboxy fluorescein (CF), is dependent upon pH and therefore indirectly to vacuole acidification. We analysed the 93 FEG mutants (Walker et al., 2014) grown in 'juice like' conditions (200 g L^{-1} sugar, pH 3.5) to determine whether these genes, when deleted, also result in defective VA, and therefore are involved in the maintenance of vacuolar acidification and pH regulation. This study, specific to high sugar (wine-like) fermentation, builds on previous studies of pH regulation in response to the external environment.

2. Materials and methods

2.1. Strains and media

Saccharomyces cerevisiae BY4743 (*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/LYS2 MET15/met15Δ0 ura3Δ0/ura3Δ0*) and a collection of 93 derived diploid homozygous deletion mutants (deletants) described in Walker et al. (2014) were used in this study. Strains were routinely grown on yeast peptone dextrose (YPD) medium (Guthrie et al., 1991). G418 sulphate (Amresco®) at 200 mg L^{-1} was included for deletant strains.

2.2. Fermentations

Fermentations were performed at 28 °C, in 100 mL of Chemically Defined Grape Juice Medium (CDGJM_200) according to (Walker et al., 2014). CDGJM_200 contained 100 g L^{-1} each of glucose and fructose and 450 mg L^{-1} free amino nitrogen (FAN) as a mixture of amino acids and ammonium chloride at pH 3.5. Uracil (150 mg L^{-1}) was included to meet the uracil auxotrophic requirements of BY4743. For the low sugar condition, the sugar content in the CDGJM was reduced to 20 g L^{-1}, as equimolar glucose and fructose (CDGJM_20). Fermentations were considered dry when the total residual sugar was less than 2.5 g L^{-1}.

2.3. Evaluation of fermentation performance in the presence of V ATPase inhibitor, concanamycin A

Fermentation trials of BY4743 were conducted in triplicate as described above, in CDGJM_200 (control) or with the addition of the vacuole inhibitor concanamycin A. A 10 μM stock solution of concanamycin A (concA, Cat # BVT-0237, AdipoGen®) was prepared in DMSO and stored at −20 °C. Concanamycin was added to individual cultures 24 h post-inoculation at a final concentration of 30 nM and 300 nM (in 300 μL DMSO). DMSO (300 μL) was added to the control fermentation.

2.4. Quantitative assessment of vacuolar acidification and cell viability using flow cytometry

Flow cytometry analysis was performed using two fluorescent dyes: propidium iodide (PI; Cat #P3566; Molecular Probes®) to measure cell membrane damage and therefore cell viability and 6-carboxyfluorescein diacetate (6-CFDA; Cat #C1362; Molecular Probes®) to measure vacuolar acidification as the fluorescence of 6-carboxyfluorescein (6-CF) derived from the enzymatic hydrolysis of 6-CFDA is directly related. Yeast cells were sampled 24 h after inoculation in 100 mL CDGJM_200. Cells were centrifuged at 5000 rpm (2655 × g) for 5 min, washed twice in 5 mL of sterile 0.9% sodium chloride and resuspended to 2×10^7 cells mL^{-1}. One mL of cells was labelled with the addition of 1 μL of 5 mM 6-CFDA (final concentration of 5 μM) and incubated at 28 °C for 4 min 30 s. One μL of PI (1 mg mL^{-1} stock) was then added and cells incubated for a further 30 s. Label reproducibility was maintained by holding samples on ice. Dual-labelled cells were analysed on the FACSCalibur E0250 instrument (BD Biosciences). Excitation was set at 488 nm and emission was monitored separately through a 530/30 nm bandpass filter for fluorescence 1 (FL1), 585/42 nm bandpass filter for fluorescence 2 (FL2) and a 661/16 nm bandpass filter for fluorescence 3 (FL3). Fluorescence data, acquired for 10,000 events per sample, was initially analysed with BD CellQuest™ Pro (www.bdbiosciences.com) and subsequently with FCS5 Express software (www.denovosoftware.com). Fluorescence attributed to PI (PI positive and negative) was gated on FL3 whilst 6-CFDA (vacuolar probe) was gated on FL1. Total events (10,000), cell viability (number of PI negative events), and 6-carboxyfluorescein intensity in PI positive cells and PI negative cells was tabulated using Microsoft Excel 2013 software.

2.5. Qualitative assessment of vacuolar acidification by microscopy

Yeast vacuoles were labelled *in situ* according to Preston et al. (1989) with minor modifications. A 5 mM 6-CFDA stock solution was prepared in DMSO. Labelling medium was freshly made by dilution of 6-CFDA to a final concentration of 5 μM in YPD (pH 3.5; adjusted with 5 mM citric acid). Cells were harvested by centrifugation at 5000 rpm (2655 × g) for 5 min and resuspended at 2×10^7 cells mL^{-1} in labelling medium and incubated for 30 min at 28 °C. Label reproducibility was maintained by performing subsequent steps at 4 °C (centrifugation) or on ice. Cells were washed twice in sterile 0.9% sodium chloride, pelleted as before by centrifugation and resuspended at 1×10^8 cells mL^{-1} in ice-cold YPD. Images of cellular fluorescence were taken within 4 h, using a Nikon Eclipse 50i microscope at 100× magnification with a GFP-B filter, at an excitation of 450–500 nm and an emission at 520 nm. Fluorescence emission was collected with a digital Nikon camera (NIKON Digital sight DS-2MBWs).

2.6. Gene Ontology (GO) analysis

Classification of genes to specific GO terms was performed using Slim Mapper (http://www.yeastgenome.org), whilst LAGO (http://go.princeton.edu/cgi-bin/LAGO) (Boyle et al., 2004) was used to look for enrichment of genes within the identified dataset to specific GO terms (biological processes, function or cellular component).

3. Results and discussion

3.1. Inhibition of V-ATPase resulted in protracted fermentation

To investigate the importance of vacuolar acidification to fermentation performance, concanamycin A, an inhibitor of VA in eukaryotes (Hughes and Gottschling, 2012), was added to cultures of the VMA^+ parental strain BY4743, 24 h post-inoculation at final concentrations of 30 nM and 300 nM, and fermentation progress i.e. sugar consumption was monitored over time. Addition of concancamycin A at 300 nM resulted in fermentation protraction (350 h) compared to the control (167 h); the treated fermentation taking an additional 183 h or 109% to finish (Fig. 1). Whilst no inhibition of VA (measured as increased fluorescence) could be demonstrated after 4 h post-treatment (data not shown), the concanamycin A treatment resulted in decreased cell viability as determined by PI staining and FACS analysis. The percentage of viable cells was 50 ± 19% and 10 ± 1.6% lower than the control in the 300 nM and 30 nM treatments, respectively (data not shown). These results were consistent with the degree of protraction of the fermentation depicted in Fig. 1.

3.2. Assessment of vacuolar acidification using pH sensitive probe 6-carboxyfluorescein

We assessed whether the protracted fermentation demonstrated by the 93 yeast deletants (including the 20 describe above) was related to the yeast's inability to properly acidify the vacuole. The 93 yeast deletants were used to ferment CDGJM containing 200 g L^{-1} sugar (high sugar conditions). Vacuolar acidification was measured using pH-sensitive fluorescent probe 6-carboxyfluorescein diacetate or 6-CFDA in the wild type BY4743 and the 93 yeast deletants. Cells labelled with 6-CFDA were initially examined by fluorescence microscopy to determine whether under the experimental conditions, fluorescence due to hydrolysis of 6-CFDA was detectable, and therefore also by flow cytometry. Carboxy fluorescein (CF) has an excitation at 490 nm and the highest fluorescence is observed when the pH is neutral. Fluorescence intensity decreases as the vacuolar acidity increases (Preston et al., 1992). Fig. S1: Supplementary data depicts images of cells of BY4743 (wild type) and Δvma1 (a typical vacuolar acidification mutant) sampled during fermentation in CDGJM_200 (pH 3.5). For labelling, the cells were buffered at pH 3.5 to keep the cells at the same pH as the CDGJM_200 since external pH and weak acid stress is known to influence vacuolar pH (Brett et al., 2011). The labelled cells were buffered with 5 mM citric acid to maintain the acidic stress imposed by organic acids in CDGJM (1.15 mM citric acid and 11 mM malic acid). At 24 h, wild type cells exhibited minimal fluorescence resulting from hydrolysis of 6-CFDA, alluding to an acidic pH in the vacuole. The Δvma1 cells demonstrated increased fluorescence (indicating increased vacuolar pH from impaired acidification) at 24 h, which decreased, as the vacuolar pH gradually recovered with extended growth (sampled at 72 and 125 h, data not shown).

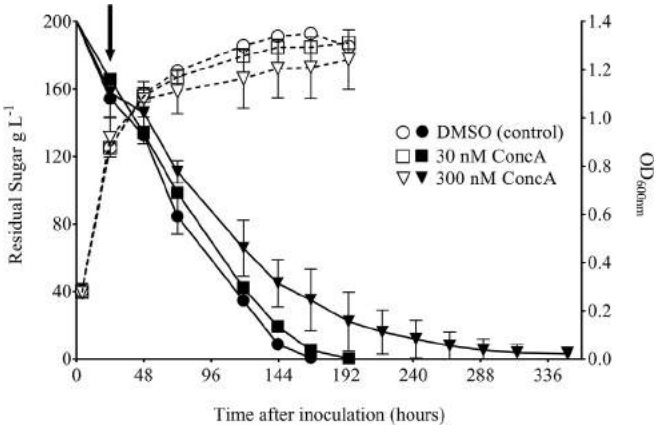


Fig. 1. Fermentation profile of VMA^+ strain BY4743 after treatment with specific vacuolar acidification inhibitor concanamycin.
Fermentations were performed at 28 °C, in 100 mL of Chemically Defined Grape Juice Medium (CDGJM_200). Concanamycin A (300 μL) was added to the fermenting cultures 24 h after inoculation (solid arrow), to give the final concentrations of 30 mM or 300 mM. The control included 300 μL DMSO. Sugar catabolism is shown by solid symbols, whilst growth as measured by optical density at 600 nm (OD_{600nm}), is represented by open symbols. Error bars represent standard deviation of the mean for 3 replicates per treatment.

It was decided to measure vacuolar acidification (as CF fluorescence) in the 93 FEG mutants at 24 h of growth in CDGJM_200, when the sugar consumption rates between the mutants were expected to be similar, given that maximum sugar catabolism occurs after 72 h. Initial experiments on the wild type and vma mutants previously reported to affect VA (Δvma1 and Δvma3, which lack V_1 subunit A and V_0 subunit c, respectively (Forgac, 2004)) were conducted in triplicate, to determine the biological variation for each strain when analysed by flow cytometry. A single FACS analysis (10,000 events) was conducted on each of three fermentation replicates per strain. The distribution of fluorescence intensity is mathematically represented as the centre of the distribution or geometric mean of the logarithmically displayed data for each replicate (Ormerod, 2013), as reported in Fig. S2: Supplementary data. The average of geometric means (including the standard deviation) of the three replicates measured the biological variation for each strain, which was ~20%. The same strains were grown under low sugar conditions (20 g L^{-1}) and analysed in an identical manner, whereby the biological variation was found to be ~17% (data not shown).

Single fermentations were conducted for the 93 FEG mutants and parent strain BY4743, with 10,000 events per strain analysed to minimise heterogeneity within the sample population. Dual-labelling of cells with propidium iodide (PI) and 6-CFDA and subsequent analysis of the PI negative and PI positive cell populations, enabled the exclusion of cells with membrane damage (PI positive cells) in the 6-CFDA analysis (vacuole dysfunction). The actual percentage of cells with membrane damage was <5% of the total population as shown in Table S1: Supplementary data, which is not considered to have a significant impact on fermentation outcome (Davey and Hexley, 2011). The CF fluorescence intensity of each mutant was reported as the geometric mean. The ratio of the geometric mean of each mutant to the parent was calculated to indicate the relative fluorescence intensity of each strain. An arbitrary cut off value of two-fold difference was chosen as a measure of impaired VA which more than accounted for the 20% variation between biological replicates as shown in Fig. S2: Supplementary data.

3.3. FEG yeast deletants fail to maintain vacuolar acidification under high sugar conditions

We have defined Fermentation Essential Genes or FEGs as those genes required for fermentation. Their deletion extends sugar catabolism (fermentation duration) but has little effect on growth (Walker et al., 2014). The mutants achieved ≥70% of the growth of the parent BY4743 as estimated by OD. A total of 20 mutants (21% of the original 93 FEGs) were observed to have a fluorescence intensity significantly higher (≥2-fold) than the parent (Table 1, Table S1) including 10 which been previously annotated to terms

linked to the vacuole (Teixeira et al., 2010; Walker et al., 2014). Our study and dataset differs from previous studies on vacuolar and cytosolic pH, where vacuolar dysfunction was linked to impaired growth (Brett et al., 2011; Orij et al., 2012). In our study, the genes affecting vacuolar pH, typically did not affect cell viability and subsequent growth (Table 1, Table S1).

Twenty out of the 27 genes annotated to vacuolar acidification and cellular pH, were originally identified in a genome-wide screen for protracted fermentation phenotype in the BY4743 homozygous diploid deletion collection (Walker et al., 2014). These genes (*DBF2, MEH1, NHX1, RAV1, RAV2, VMA1, VMA2, VMA3, VMA4, VMA5, VMA6, VMA7, VMA8, VMA10, VMA11, VMA13, VMA16, VMA22, VPH1,* and *VPH2*) were amongst 93 FEGs identified.

Ten of the 20 genes annotated to V-ATPase (either as a component or involved in assembly), resulted in vacuolar dysfunction, when deleted, correlating to the increased 6-CF fluorescence intensity (Table S1). The remainder were either unaffected (Table S1; Δrav1 (1.07), Δrav2 (0.74), Δmeh1 (0.62), Δnxh1 (0.83), Δvma5 (1.09), Δvma8 (0.63) or showed a <2 fold increase in relative fluorescence compared to the parent (Δvma2 (1.29), Δdfb2 (1.53), Δpkr1 (1.56), Δvma6 (1.60) and Δvma16 (1.84)). Our results are similar to Brett and coworkers (2011). Those authors screened the BY4742 deletion library for growth and vacuolar pH following 19 h of growth in media with varying pH. Increased vacuolar pH was measured in the following mutants: Δpkr1, Δvma1, Δvma3, Δvma4, Δvma5, Δvma7, Δvma9, Δvma11, Δvma12, and Δvma22. *RAV1* and *RAV2* (together with *PKR1*) are involved in V-ATPase assembly and activity (*VMA8*) of the V-ATPase complex (Graham et al., 1995). The fact that these mutants have protracted fermentation but are able to complete sugar catabolism suggests that retention of acidic conditions in the vacuole is either through passive diffusion or ammonium acting as a protonophore, which would support growth and fermentation, albeit at a slower rate (Plant et al., 1999).

Deletion of *NXH1,* encoding a Na^+/H^+ and K^+/H^+ exchanger failed to increase vacuolar pH, as did *TRK1*, a high affinity potassium transporter, involved in cation homeostasis. Orij et al. (2012) demonstrated that when Δnhx1 and Δtrk1 were grown at pH 3.0, there was no affect on cytosolic pH. Similarly, we could only demonstrate a 1.5 × increase in CF fluorescence in Δdbf2, which does not support earlier work (Makrantoni et al., 2007), where Dbf2p (a Ser/Thr kinase involved in transcription and stress response) was shown to be required for the phosporylation of subunits A (Vma1p) and B (Vma2p) of vacuolar ATPase. Interestingly, those authors linked vacuolar acidification to cell cycle (Makrantoni et al., 2007), as Dbf2p was already known to participate in exiting of mitosis and cytokinesis (Surana et al., 2002). It is likely that the mutants Δdfb2 (1.53), Δpkr1 (1.56), Δvma6 (1.60) and Δvma16 (1.84) are actually impaired in VA after 24 h growth, but they have been excluded because of the stringent cut-off value of ≥2. It is probable from the differences between our findings and others that experimental design is important, whether a pH sensitive probe (Brett et al., 2011), or a pH sensitive reporter gene construct (Orij et al., 2012) is used, and the method to detect fluorescence; a plate-reader (Orij et al., 2012; Brett et al., 2011) or flow cytometry, where vacuole (and cell) morphology and percentage of cells affected can affect the fluorescence signal (Fig. S3).

3.4. FEG yeast deletants fail to maintain vacuolar acidification under low sugar conditions

In order to determine whether vacuolar acidification was affected by external sugar concentration, namely glucose and fructose, alcoholic fermentation was conducted in CDGJM_200 (200 g L^{-1}; high sugar) and CDGJM_20 (20 g L^{-1}; low sugar), and samples taken for FACS analysis after 24 h growth. The intensity of CF fluorescence calculated as the ratio of the geometric mean value of mutant to the parent for the two sugar conditions is represented in Fig. 2. Whilst the 20 deletants exhibit increased fluorescence under both conditions, a slightly larger effect was observed under high sugar conditions with most deletants. In the case of Δhog1, Δpbs2 and Δvph1, CF in viable (PI negative cells) was related to sugar content, with increased 6-CF fluorescence observed under high sugar conditions (Fig. 2, Table S1). Whilst cell viability in the wild type strain was 98% for both sugar conditions, the number of viable (PI negative) cells was greatly reduced in CDGJM_200 for Δhog1 (39.0%), Δpbs2 (21.4%) and Δvph1 (57.3%), compared to low sugar (CDGJM_20): Δhog1 (96.7%), Δpbs2 (96.0%) and Δvph1 (88.9%) (Table S1). Therefore, for Δhog1 and Δpbs2, it is likely that

Table 1
Identification of 20 genes affecting vacuolar acidification when grown in CDGJM_200 (high sugar conditions).

Systematic name	Standard name	Gene deletant	CF_GEO mean	PI^- (% population)	Description
YML008C	*ERG6*	Δerg6	129	79.66	Delta(24)-sterol C-methyltransferase
YJL128C	*PBS2*	Δpbs2	111.6	13.9	MAP kinase kinase of HOG signaling pathway
YLR113W	*HOG1*	Δhog1	108.8	40.65	Mitogen-activated protein kinase involved in osmoregulation
YCL007C	*YCL007C*	Δycl007c	81.81	96.64	Dubious open reading frame
YJL056C	*ZAP1*	Δzap1	75.19	84.52	Zinc-regulated transcription factor
YDR069C	*DOA4*	Δdoa4	45.08	80.31	Ubiquitin hydrolase
YAL047C	*SPC72*	Δspc72	42.19	84.05	Gamma-tubulin small complex receptor
YOR080W	*DIA2*	Δdia2	35.48	92.88	Origin-binding F-box protein
YFL023W	*BUD27*	Δbud27	31.41	98.02	Unconventional prefoldin protein involved in translation initiation
YDR369C	*XRS2*	Δxrs2	29.05	93.31	Protein required for DNA repair
YOR270C	*VPH1*[a]	Δvph1	245.89	96.64	Subunit a of V0 domain of vacuolar-ATPase
YHR060W	*VMA22*[a]	Δvma22	198.24	94.55	Protein required for vacuolar ATPase function
YKL119c	*VPH2*[a]	Δvph2	92.84	96.82	Integral membrane protein required for vacuolar ATPase
YGR020C	*VMA7*[a]	Δvma7	77.38	96.37	Subunit F of V1 membrane domain of vacuolar ATPase
YOR332W	*VMA4*[a]	Δvma4	57.91	98.69	Subunit E of V1 domain of vacuolar ATPase
YPL234C	*VMA11*[a]	Δvma11	50.96	98.87	Subunit c' of V0 domain subunit of vacuolar ATPase
YHR039C-A	*VMA10*[a]	Δvma10	46.04	98.21	Subunit G of V1 membrane domain of vacuolar ATPase
YDL185W	*VMA1*[a]	Δvma1	35.54	97.26	Subunit A of V1 membrane domain of vacuolar ATPase
YEL027W	*VMA3*[a]	Δvma3	33.86	97.04	Proteolipid subunit c of V0 domain of vacuolar ATPase
YPR036W	*VMA13*[a]	Δvma13	29.35	99.11	Subunit H of V1 membrane domain of vacuolar ATPase

CF_GEOmean represents intensity of carboxy fluorescein fluorescence of the PI^- population. CF_Geomean for BY4743 was 14.86 ± 3.45 (n = 4).
PI^- (% population) represents the percentage of the yeast population (of 10,000 events) not stained with propidium iodide; i.e. PI negative. These cells are considered viable. PI^- (% population) for BY4743 was 98.77 ± 0.47 (n = 4).
[a] Denotes genes known to be annotated to vacuolar acidification.

impaired fermentation is due to pleiotropic effects of primary defects in the processes related to normal growth and cell division, namely adaption to osmotic stress (Saito and Posas, 2012).

Teixeira et al. (2010) have used a similar pH-sensing fluorescent probe, 5- (and 6-) carboxy-2,7-dichlorofluorescein diacetate, succinimidyl-ester or (5(6)-CDCFDA,SE) together with fluorescence microscopy to measure vacuolar pH. Vacuolar acidification was observed to decrease (as demonstrated by an increase in fluorescence) when wild type cells were grown in 300 g L^{-1} glucose. The authors proposed that perturbation of VA by high glucose was mediated by a dysfunctional proton pump, the vacuolar H^+-ATPase, given that Δvma16 and Δvma4 mutants displayed increased growth susceptibility to high sugar. We have found similar growth defects with Δvma4, Δvma6, Δvma11 and Δvma22 when fermented in CDGJM_200, which resulted in protracted fermentation (Walker et al., 2014). Our analysis of vacuolar acidification of deletants in low and high glucose further suggest that whilst VA is responsive to sugar content in these mutants, vacuolar pH is still affected when grown in low sugar (CDGJM_20; Fig. 2).

3.5. Identification of genes previously not associated with vacuolar acidification

Ten genes, previously not annotated to vacuolar acidification, were demonstrated to have at least a 2-fold increase in 6-CF fluorescence intensity as compared to the parent strain (Table S1). The 93 FEGs were originally classified as those genes which, upon deletion, extended sugar catabolism (fermentation duration) but grew similarly (≥70%) to the parent BY4743 (Walker et al., 2014). However, we found that for Δhog1 and Δpbs2 the fermentation defect in CDGJM_200 could be attributed to a loss in viability. Interestingly, the intense CF fluorescence exhibited by Δhog1 and Δpbs2 alludes to vacuolar acidification being associated with the high-osmolarity glycerol (HOG) response pathway. This adaption response results in the induction of glycerol-3-phosphate dehydrogenase (*GPDH*) upon hyperosmotic stress, resulting in the increased production and retention of glycerol. In the case of ion-related osmotic stress induced by salt, activation of the HOG pathway results in the activation of cation transporters and increased vacuole fragmentation, presumably to increase the uptake of Na^+ into the vacuole (Li et al., 2012). V-ATPase and the HOG pathway are proposed to act in parallel to combat osmotic stress by salt. Li et al. (2012) demonstrated haploinsufficiency in diploid yeast with only single copies of both *VMA3* and *PBS2* or *VMA2* and *HOG1*. Those authors showed that loss of V-ATPase activity led to activation of Hog1p under low levels of salt, suggesting that in the absence of V-ATPase, the HOG pathway had a compensatory role. It is unlikely that vacuolar acidification can compensate for a dysfunction in the HOG pathway through the up-regulation of specific genes and their proteins, as indicated by the impaired acidification in the Δhog1 and Δpbs1 mutants. How these pathways act during fermentation where both osmotic and ion (acid) stress are prominent remains to be determined.

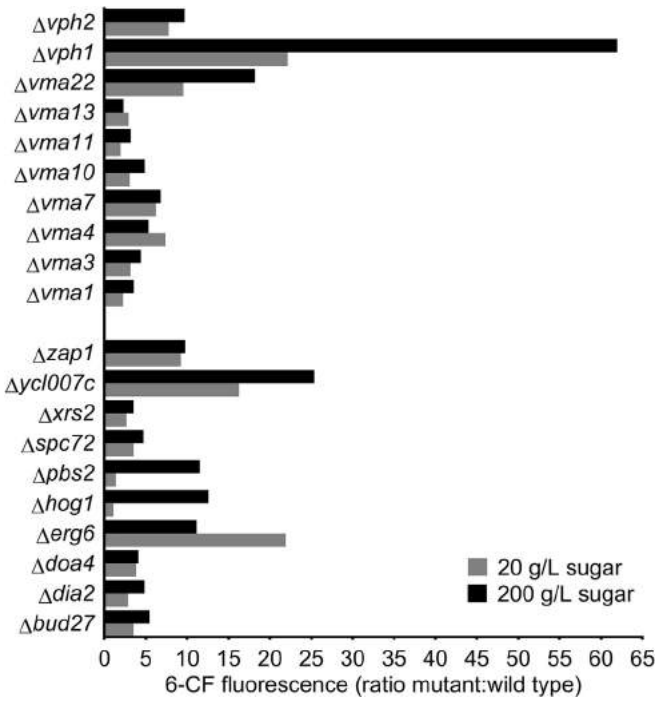


Fig. 2. 6-CF fluorescence of 20 deletants in high sugar (200 g L^{-1}) and low sugar (20 g L^{-1}) conditions. Bar graph of 6-CF fluorescence intensity calculated as a ratio between each deletant and the wild type strain of the Geomean values derived from analysis of single fermentation cultures after 24 h growth. 10,000 events were analysed per culture using FCS5 Express software.

YCL1007c is of interest as the strain Δycl007c exhibited good viability (87%) whilst showing increased CF fluorescence due to reduced vacuolar acidification. Whilst this gene is annotated as a dubious open reading frame, its deletion in yeast (alias Δcwh36) results in at least one subunit of vacuolar ATPase being missing (Davis-Kaplan et al., 2004; Sambade et al., 2005). This supports the proposal that the deletion overlaps the functional gene *YCL005W-A* (*VMA9*) on the opposite DNA sense strand, which encodes for subunit e of the V0 domain of vacuolar ATPase (Davis-Kaplan et al., 2004; Hirosawa et al., 2004; Sambade et al., 2005). Another gene associated with alkaline vacuoles is *ERG6* (involved in lipid metabolism). Similar to that of Brett et al. (2011), who demonstrated Δerg6 to have alkaline vacuoles and 70% growth at pH 2.7 (2% glucose), we also found the mutant to have high CF fluorescence and slightly reduced cell viability, dependent upon sugar content (Table S1). For three mutants, where cell viability was largely unaffected (Δbud27 (97%), Δdia2 (86%) and Δdoa4 (80%)), further work is required to determine whether the effect on vacuolar acidification of deletion of these FEGs is a secondary consequence of impaired cell division and cell proliferation.

3.6. GO analysis of identified 20 genes which upon deletion result in impaired acidification

It is evident from several genome-wide screens for mutants affecting acidification and intracellular pH (Brett et al., 2011; Orij et al., 2012; Sambade et al., 2005) that there are many genes in addition to the classical *VMA* genes linked to these processes. Gene Ontology (GO) analysis (Table S1) demonstrated a statistical over-representation of genes annotated to vacuolar acidification (10 out of 20; 50%) compared to the rest of the genome (27 out of 7165; 0.4%). The genes *VMA1, VMA3, VMA4, VMA7, VMA10, VMA11, VMA13* and *VPH1* encode components of the vacuolar ATPase complex, whilst *VMA22* and *VPH2* are involved in its assembly (Table 1). With the exception of *VMA22* and *VPH2*, these and *ERG6* and *DOA4* are annotated to organelle membranes. Sterol alteration in the membrane bilayer in Δerg6 mutants has been shown to increase sensitivity to small lipophilic drugs through altered passive diffusion (Emter et al., 2002). It is feasible that Δerg6 similarly may affect the permeability of the vacuole, causing proton leakage or altered V-ATPase activity. Doa4p has a role in deubiquination and endomembrane trafficking of proteins en route to the vacuole (Amerik et al., 2000), as such in the Δdoa4 mutant, the lack of the ubiquitin hydrolase may result in vacuole dysfunction. Perhaps not surprisingly, GO analysis did not identify any enrichment of the 10

newly identified genes which resulted in increased CF fluoresence, given their diversity in function. Some of the categories to which the ten genes were classified using GO Slim Mapper are summarized in Table 2 (see Table S1 for complete analysis). How these new genes are related to vacuolar acidification is unclear given the complexity of the genetic and physical interactions within the yeast cell (Costanzo et al., 2010; Elbaz-Alon et al., 2014).

4. Conclusion

Efficient completion of fermentation in high sugar media is a complex phenotype and dependent on many biological processes, some of which involve vacuolar acidification (Anraku et al., 1989; Corbacho et al., 2012; Cyert and Philpott, 2013; Klionsky et al., 1990; Makrantoni et al., 2007; Nass and Rao, 1999; Plant et al., 1999; Ungermann et al., 1999; Yamashiro et al., 1990). Cells adapt to ethanol stress through the action of V-ATPase, in response to ethanol-induced intracellular acidification arising from increased membrane permeability to protons (Charoenbhakdi et al., 2016). However, V-ATPase may also protect yeast cells from ethanol induced oxidative and cell wall stress, although the mechanism(s) is unclear (Charoenbhakdi et al., 2016).

In this study we looked at the role of vacuolar acidification in fermentation using conditions similar to wine making – low pH (~3.5) and high sugar (200 g L^{-1}). We have previously identified 93 genes, whose deletion resulted in fermentation protraction or arrest, but with little impact on growth (Walker et al., 2014). This study explores the implied link between genes required for efficient high sugar fermentation and proper maintenance of vacuolar acidification. Vacuolar acidification seems to play a role in early adaption to high osmotic and low pH conditions at the start of fermentation, as well as cell maintenance and survival during the extended period of fermentation, when the cell is exposed to fermentation stresses (oxidative, nutrition limitation and ethanol toxicity). This study provides further insight into the sensing and adaptive mechanisms that allow the yeast to survive and successfully complete wine fermentation. Practical applications include better choice of stress conditions in directed evolutionary strategies, as well as selection (e.g. mutants resistant to concanamycin A), with the goal of generating new 'industry ready' strains, better suited to low pH, high sugar juice fermentations.

Author contributions

Experimental conception and design: TDN, MW, JG, VJ. Experimental work: TDN. Data analysis: TDN, MW, VJ. Manuscript revision: MW, JG, VJ.

Table 2
Classification of 10 genes not previously annotated to the GO term 'vacuolar acidification' using SGD Gene Ontology Slim Mapper.

GO term	Gene(s)
regulation of DNA metabolic process	*DOA4, DIA2, HOG1*
regulation of cell cycle	*HOG1, SPC72*
cytoskeleton organisation	*SPC72, PBS2*
organelle fission	*SPC72, XRS2*
response to osmotic and heat stress	*PBS2, HOG1*
response to starvation	*ZAP1*
mitotic cell cycle	*SPC72*
meiotic cell cycle	*XRS2*
translational initiation	*BUD27*
lipid metabolic process	*ERG6*
biological process unknown	*YCL007C*

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.fm.2017.09.021.

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Tackling microbial failure in problematic wine fermentations using a holistic approach

Researchers Vladimir Jiranek**,** Michelle Walker **together with** Jennie Gardner**,** Joanna Sundstrom **and** Tommaso Liccioli **are tackling the issue of problematic primary (alcoholic) and secondary (malolactic) fermentation from a wine microbiology angle, using a multi-pronged approach to generate superior microbial strains better which exhibit fermentation robustness i.e. fermentation reliability, vigour and reduced nutritional needs.**

AT A GLANCE:

- Efficient and cost-effective production of quality wine is critical to the sector's viability, and is inherently linked to the microbiology of the winemaking process
- Approximately 8% of fermentations encounter problems due to microbial failure (http://www.agwine.adelaide.edu.au/winemicro/news/Winemaker_survey_2010_summary.pdf). Whilst some is made unsaleable because of quality losses, the value of the salvageable remainder maybe reduced through the wine being downgraded.
- Improved microbial process efficiency specifically within wine fermentation is possible through the production of robust yeast and bacteria and application protocols and use of fundamental scientific knowledge.

THE QUALITY of Australian wine is dependent upon predictable fermentation outcomes in terms of consistent trouble-free completion and where yeast and/or bacterial supplement requirements are easily forecast. 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 $35 million. Clearly research aimed at microbial process efficiency specifically within wine fermentation is of great value to the Australian wine industry.

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.

In our previous GWRDC funded projects we have produced several improved yeast and lactic acid bacteria with robust fermentation properties under high stress conditions relevant to wine fermentation as well as much related new knowledge. To date, we have a collection of candidate improved strains awaiting allocation to specific wine making applications.

This project (UA11/01: 'Innovative Microbials') focused on industry specific outcomes outlined in the GWRDC's Program 2 – Winemaking Excellence (GWRDC Five Year R&D Plan 2007–12). Specifically, the development of superior microbial strains and applications of these and new knowledge on fermentation management, so to secure and improve Australian wine quality.

We have used a multi-pronged approach to generate candidate yeast and lactic acid bacteria based on earlier work, in what is essentially a holistic approach combining yeast and bacterial genomics, strain optimization and high-throughput robotic screening systems for strain evaluation. Broadly, the research encompasses molecular and '-omic' technologies, microbiology, biochemistry and physiology in support of non-recombinant improvement by mutagenesis, directed evolution (DE) and classical breeding. A bioinformatic interrogation of extensive datasets of genome sequences of novel strains, mutant fermentation performance and relevant online datasets has helped to identify genes and hence targets or strategies for strain improvement. Genes associated with stress tolerance; nutrient efficiency and fermentation performance are of particular interest. Ultimately we seek to deliver superior yeast and LAB to enable more reliable and/or distinct winemaking outcomes.

The key findings of this study were related to the broad objectives:

Yeast genes and biological processes relevant to fermentation

1 Two Fermentation Relevant Yeast Gene (FRYG) databases were compiled, based on in silico analysis (data-mining) of databases specifically related to winemaking (wine yeast genomic, transcriptomic and metabolomics data). The first, LIT_FRYG, is a collection of genes identified in previously published studies of single stress conditions that are relevant to fermentation. The second, SGD_FRYG, is a collection of genes associated with fermentation relevant phenotype terms reported in the Saccharomyces Genome Database (www.yeastgenome.org).

2 Together with data from yeast library screens conducted under winemaking conditions we have defined the laboratory 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

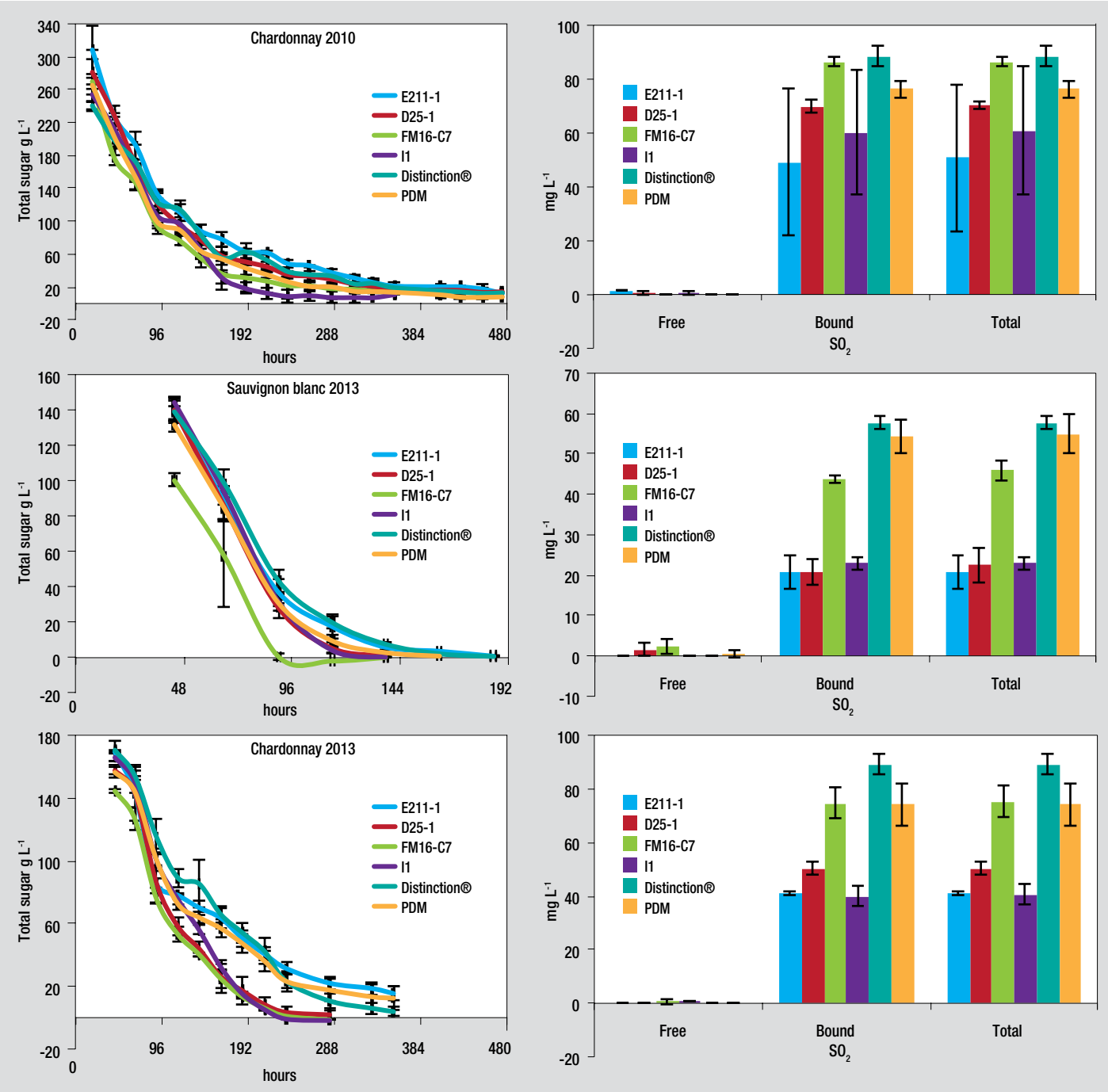


Figure 1: Evaluation of low H2S producing wine yeast in 10 L white juice fermentations
Three low H_2S producing strains were evaluated in 10 L white juice fermentations at 22°C. Strains were compared to PDM, Distinction® and the evolved strain FM16-C7. Strains were initially propagated in YPD, followed by Juice Starter Media with DAP (JSM_DAP). Fermentations were inoculated at 5 x 10^6 cells mL^{-1}. 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).

complete and efficient fermentation. The lab yeast "Fermentome" whilst incomplete and needing verification in a wine yeast background, includes a 93 gene Fermentation Essential Genes (FEG) dataset, which are necessary for fermentation completion in high sugar (Walker et al., 2013).

3 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.

4 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). Laboratory scale (100 mL) evaluation of the putative candidate strains are being conducted as part of our new GWRDC funded project UA1302 'Fit-for-purpose yeast and lactic acid bacteria'.

5 Vacuolar acidification of the yeast cell is implicated in high sugar fermentation, with 20 FEG genes

associated with this biological process. In this study we have examined whether any of the other FEG genes, when deleted in yeast, lead to vacuolar acidification defects. Surprisingly, a further 14 genes not previously associated with vacuolar acidification were identified when vacuolar acidification was monitored during fermentation. These results further support vacuolar acidification as an important biological process necessary for efficient fermentation in high sugar conditions (Nguyen et al. 2013).

The data from this study provides a valuable source of genetic information for future work which will focus on understanding 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.

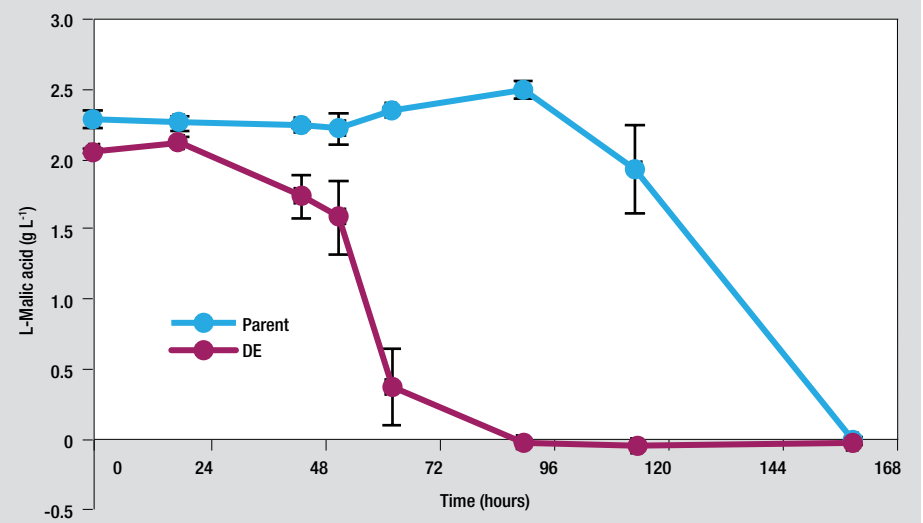


Figure 2: Malolactic fermentation (L-malic acid utilisation) by either a mixed evolved culture (Mix) from the Directed Evolution SB3 culture at 15% ethanol or the original SB3 parent.

Characterisation of adaptively evolved (AE) strains of yeast and LAB on lab- and pilot-scale, for improved performance in specific problem fermentations

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

6 Sporulation and subsequent mass mating of ~80 wine yeast strains, followed by ~190 generations of adaptive evolution were performed to generate yeast strains. A relatively small number of novel strains were analysed for fermentation vigour (~120 strains in micro-scale and seven in 10 L fermentations). An extensive glycerol collection is available for future screening in either juice or chemically defined media for strains of interest.

7 The adaptively evolved FM16 mixed population reported by McBryde et al (2006) was rescreened in juice and chemically defined media to identify new single isolates. 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.

8 Yeast strains with low H_2S and SO_2 production were 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 (Figure 1). This work was funded in part by a University of Adelaide Wine 2030 award to Dr Michelle Walker.

9 Four malolactic fermentation efficient bacterial strains derived from SB3 were generated by Directed Evolution (DE). Figure 2 shows MLF (L-malic acid utilisation) by parent SB3 and the mixed evolved culture. The four isolates are still to be completely characterised and genome sequencing of these and the parent is underway.

10 Our most promising evolved strain of interest FM16-C7, for which a provisional patent has been applied, has undergone preliminary trials as a rescue strain for sluggish/stuck fermentations. In the 2000 – 14000 L trials, whilst FM16-C7 was not able to complete fermentation of these stuck fermentations, 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.

MISCELLANEOUS

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During the course of this project, we have modified current spectrophotometric methods for sugar, FAN and malic acid determination to allow for high-throughput, cost and time efficient and reliable determination of these. Potential applications include 'on-going' monitoring of fermentations and screening of candidate yeast and bacteria strains.

This 18 month project was funded by the Grape and Wine Development Corporation (Project GWRDC UA11/01 'Innovative Microbials for Winemaking Excellence') which concluded in December 2012. 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. Eight students have been trained and have contributed to this research during this project as part of the University's commitment as a teaching and research facility. Research findings are disseminated through course-base lectures, conference presentations, electronic magazines (e-science) and academic and industry journals.

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Drs Jennie Gardner, **Joanna Sundstrom** and **Tommaso Liccioli** are Research Fellows in Wine Microbiology and Wine Biotechnology (headed by Professor Jiranek) at the School of Agriculture, Food and Wine, University of Adelaide.

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Adelaide researchers head "over the ditch" for Australasian yeast meeting

University of Adelaide Research Fellows Michelle Walker, Joanna Sundstrom **and** Tommaso Liccioli **together with students** Simon Dillon, Danfeng Long, Trung Nguyen, Ee Lin Tek **and** Jin Zhang **report on the "Yeasts: Products, Discovery and more".**

YEASTS: Products, Discovery and More 2013 (YPD) was held at Massey University, Albany, New Zealand last November 2013.

The combined meeting of the Australasian Yeast Network and the New Zealand Microbiological Society Special Interest Group attracted 63 delegates, predominantly from Australia and New Zealand, but also invited speakers from Europe and the US.

The meeting program incorporated seven plenary speakers, 30 seminar presentations and a research poster display.

Conference themes covered cell and systems biology of both medically and industrially relevant yeast with many reports specific to alcoholic fermentations, including wine, beer and bioethanol production. Wine ecology was also well represented.

Specific topics included yeast breeding, stress adaptation, ecology and evolution, genetic and protein inheritability, genome organisation and cellular metabolism.

The meeting's small size strongly encouraged networking and lively discussion with other researchers and delegates were able to keenly engage with conference exhibitors.

Jan Singer, the proprietor of Singer Instruments, came from the UK to demonstrate the latest cutting edge equipment for yeast micro-dissection, an essential tool for yeast geneticists.

GENETICS, BREEDING AND TOOLS FOR YEAST STRAIN IMPROVEMENT

Professor Richard Gardner (University of Auckland) gave an overview of conventional yeast breeding, in his seminar "Breeding better wine yeast".

He discussed his group's strategy of using standard yeast breeding methods in conjunction with molecular markers (pieces of DNA known to be associated with particular characteristics or traits), to develop novel yeast strains with improved oenological properties.

This group's focus is on using these techniques to improve white wine yeast strains, with particular emphasis on improvements to yeast cold tolerance as well as improved yeast derived aroma characteristics of the final wine from Sauvignon Blanc juice.

Presentations from Professor Gardner's group were also made in Winemaking focus sessions. For example, Margarita Santiago highlighted the link between IRC7 gene expression and thiol release during Sauvignon Blanc fermentation in her presentation "4MMP production by Saccharomyces cerevisiae".

Dr Rebecca Deed's poster "Improvement of commercial wine strains for Sauvignon Blanc fermentation through backcrossing" illustrated how commercial wine yeast strains, identified as tolerant to low temperature and high sulfite conditions, were successively bred to introduce desirable traits for Sauvignon Blanc fermentation.

This type of performance targeting resulted in novel yeast strains with reduced 'off' aromas and increased thiol production.

Genes (fragments of DNA that code for proteins) targeted included IRC7450, encoding a ß-lyase for increased production of 4MMP (boxwood aroma) and pad1, a variant of PAD1 that does not result in the production of phenolic off-flavours such as the aroma of clove.

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Miguel Roncoroni (PhD student: Gardner group) presented a highly relevant and informative talk on "Mapping genes of oenological importance in commercial winemaking yeast".

This talk described the generation of 96 wine yeast strains derived from breeding together two commercial wine yeast strains, Enoferm M2 (similar to AWRI 796) and Zymaflore F15.

The complete DNA sequences (genomes) of these 96 strains were subsequently determined. The 96 strains were then used to ferment Sauvignon Blanc juice and the production of various aroma compounds in the finished wines was measured.

Comparison of the 96 genomes to data collected from the fermentation, allowed the identification of links between specific DNA sequences (from either Enoferm M2 or Zymaflore F15) and particular oenological traits (e.g. the level of production of a particular aromatic compound).

This is known as mapping of quantitative trait loci (QTLs).

By understanding which DNA sequences will give yeast particular desirable traits, researchers can more easily select strains likely to have these desired traits rather than needing to test hundreds – or thousands – of strains.



Team yeast: University of Adelaide researchers visit Soljan Wine Estate (from left): Simon Dillon, Tommaso Liccioli, Ee Lin Tek, Michelle Walker, Danfeng Long, Jin Zhang and Joanna Sundstrom).

For example in this presentation, the genes linked to traits such as hydrogen sulfide (H2S) release and the production of several beneficial volatile compounds was identified.

Chien-Wei Huang, of the same group, gave a presentation entitled "Identification of yeast genes responsible for low H2S production in wines" which also utilised the 96-strain set, along with a second cross between Zymaflore F15 and E4.

To illustrate the power of this QTL technique in more detail, this research identified three genes (DNA sequences), MET2, MET5 and MET 10, as contributors to low H_2S production.



The MET2 gene from strain M2 (or E4) was responsible for low H2S production and normal levels of SO2.

H2S levels could be reduced even further when the MET5 gene from strain M2 (or E4) was also present.

Inheritance of the MET10 gene from strain E4 resulted in no H2S during fermentation and reduced 'off-aromas' methional, carbon disulphide and methyl thioacetate in the finished wine.

However, strains carrying this gene inherited from strain E4 produced high levels of SO2 and grew slowly on sulfate as the sulfur source.

In summary, these 96 yeast strain populations will prove to be very useful resources for further research to identify DNA sequences responsible for conferring additional traits aiding yeast breeding programs to specifically 'tailor' yeast to meet the requirements of winemakers.

Professor Ed Louis (University of Leicester, Leicester, UK) gave a plenary seminar "Exploring genetic and phenotypic variation in Saccharomyces yeasts" describing how whole-genome sequencing of hundreds of isolates from different geographical areas around the world, has revealed an enormous degree of genetic variability in wild isolates, including new gene families which are absent in laboratory strains.

Specific combinations of genetic variants were found within distinct populations, which may be adapted to their respective geographical/ecological niches.

Professor Louis highlighted the potential application for generating new industrial yeast strains, by breeding different yeast species together, to develop strains with attributes which do not occur in the current population.

Also highlighted was the recent identification of QTLs responsible for various phenotypic traits relevant to industrial applications.

Examples included ethanol tolerance (MKT1, SWS2, APJ1) and ethanol production (SSK1, GPD1, HOT1, SMP1).



Taste of research: Danfeng Long, Ee Lin Tek and Jin Zhang sampling a pint of Hallertau No 2 beer.

Whilst some phenotypic traits are linked to single QTLs or genes, others are multi-gene traits which are more difficult to analyse.

Further studies into wild yeast isolates and understanding 'genotype to phenotype' (how DNA sequences influence a strains particular good and bad attributes), will also help to develop improved yeast strains for industry.

WINE MICROBE ECOLOGY A HOT TOPIC

Wine ecology is currently very topical, with the role of fruit flies as 'vectors' and yeast-derived volatiles (which are similar to those found in rotting fruit), as a mechanism behind the mutualistic (beneficial) coevolution of these two species being explored in a number of presentations.

Dr Catrin Guenther (Dr Matthew Goddard's group, University of Auckland) presented a fascinating seminar entitled "Does chemical attraction drive the coevolution of yeasts and fruit flies?"

The group is curious to know whether yeast volatile production has evolved as a means of attracting fruit flies (Drosophila) to grapes that are spontaneously fermenting.

The theory is that this would be beneficial to the yeast as they could 'piggy back' on the flies (which feed on the yeast), and be transported to other regions thereby dispersing the yeast more widely.

The flies also benefit as they are reportedly better able to reproduce in fermenting fruit. Dr Guenther showed differing levels of attractiveness between flies and various Saccharomyces cerevisiae strains, with those more 'attractive' yeast more successfully dispersed than 'unattractive' yeasts.

Work is continuing to identify which particular volatiles, including the combinations and concentrations, which are the attractants and repellents of Drosophila.

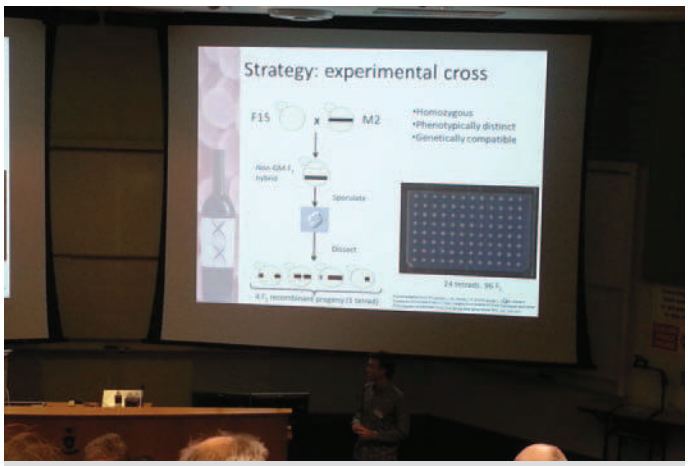
So far more than 200 volatiles have been identified by dynamic headspace GCMS as fly attractants.

This area of research was also touched on by Professor Kevin Verstrepen, whereby he speculated on the biological role of esters, such as ethyl acetate and isoamyl acetate, in beer. He showed an intriguing video of virgin female Drosophila being attracted to beer volatiles. Furthermore, yeast derived ester fly attraction, relied upon the presence of the yeast alcohol acetyl-transferase Aft1p, which is involved in ester production.

A seminar presentation by Sarah Knight (PhD student: Goddard group) and a poster presentation by Samuel Lam (Honours student: Dr Kate Howell's group, University of Melbourne) discussed their research into microbial populations associated with vineyards and/or Drosophila from within vineyards and wineries.

Sarah Knight's presentation focused on the identification of yeast microbial





Cross words: Miguel Roncoroni (PhD student: Gardner group, University of Auckland) giving his presentation at the YPD 2013 conference.

populations, using microsatellite genotyping, isolated from vineyard soil, spontaneous fermentations and native plants, from three geographically distinct regions of New Zealand.

No differentiation between species was found from the different biological samples taken from within one geographic region.

She proposed that insects move species between locations within a region.

Conversely, when comparing differentiation between geographic regions, population differentiation was observed.

This directional migration of some populations was suggested as a consequence of transporting grapes between the different regions.

Sam Lam's poster entitled "Yeasts associated with Drosophila in Australian vineyards" involved collecting Drosophila from Victorian vineyards and wineries and isolating yeast attached to the Drosophila, to identify the yeast species located in these vineyards and wineries.

The two most common species of fly, identified morphologically, from the vineyards were D. melanogaster and D. simulans and the most commonly associated yeasts, identified by DNA sequencing of the ITS rDNA region, were Metschnikowia pulcherrima, Pichia membranifaciens and Hanseniaspora valbyensis.

The yeasts Hanseniaspora uvarum and Torulaspora delbrueckii were also found, associated with large numbers of Drosophila located near grape waste piles near wineries.

Interestingly, the common fermentation yeast Saccharomyces cerevisiae was not isolated from any Drosophila in the study.

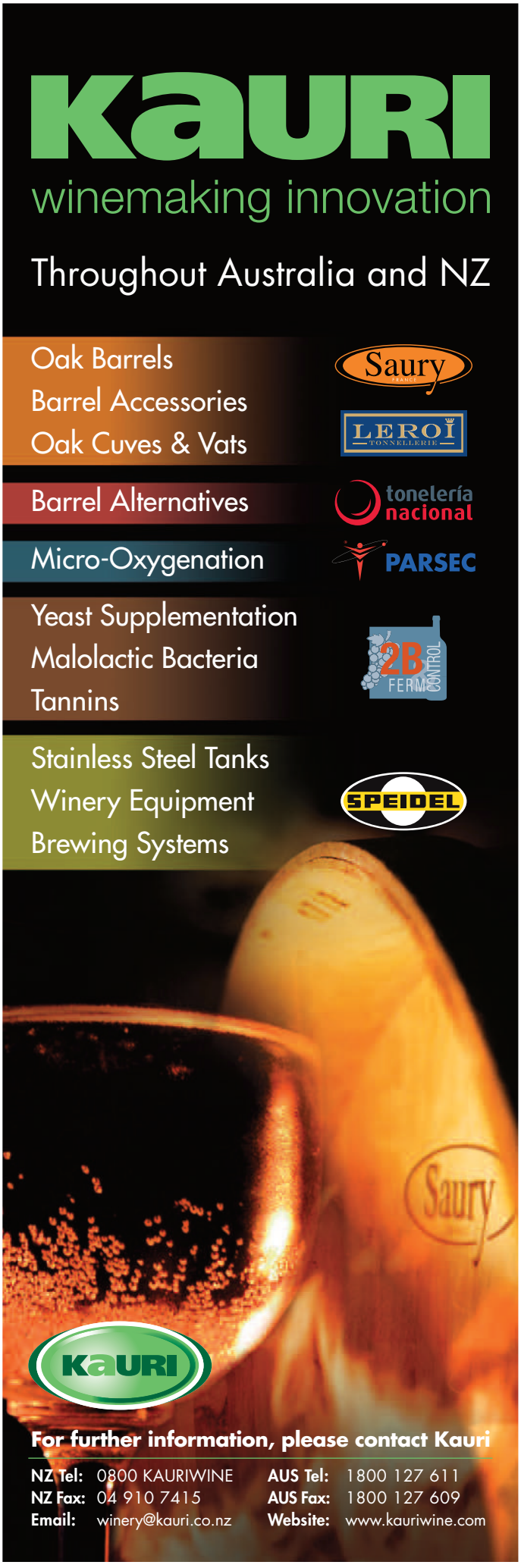
YEAST PRODUCTS, A THEME CONTINUED BEYOND THE CONFERENCE SCENE

vThe theme of 'yeast products' was continued in the social program with Dr Richard Gardner (University of Auckland) exhibiting a Sauvignon Blanc wine with enhanced varietal character, produced from a newly bred wine Saccharomyces strain.

Delegates were also treated to an in-depth tour of the Soljans Estate Winery, a third generation winery in Kumeu, and the Hallertau Brewbar.

In conclusion, conference attendance is invaluable for Australian research. Discussions, particularly those informally, enable the development of new research ideas and promote sharing of knowledge and new collaborations which enhances scientific research.

Members of the WMMB group thank the Grape and Wine Research and Development Corporation and YPD 2013 for financial assistance to enable attendance at this conference.



TJ

A personal touch for wine microbiology

The Wine Microbiology and Microbial Biotechnology Laboratory at the University of Adelaide has developed a customized solution offering fully automated sampling for yeast fermentations. Based on a Freedom EVO® 200 platform, this system frees researchers from the need to manually aliquot samples day and night for up to three weeks.



Members of the Wine Microbiology and Microbial Biotechnology Laboratory team with the customized Freedom EVO

The Wine Microbiology and Microbial Biotechnology Laboratory is part of the University of Adelaide and the Wine Innovation Cluster. Led by Professor Vladimir Jiranek and Associate Professor Paul Grbin, the group aims to improve the winemaking process by developing new yeast strains offering characteristics such as faster fermentation rates, higher tolerance to ethanol and improved nutrient utilization. Dr Tommaso Liccioli, a postdoctoral research fellow in the laboratory, explained: "Winemaking is a complex process, with grape juice undergoing many biochemical and chemical reactions to become wine. These processes are largely carried out by yeast and bacteria, which have been 'domesticated' for thousands of years to withstand the harsh conditions during fermentation and impart specific characteristics to the finished product. However, new wine styles, modern viticulture techniques and the effect of climate change are pushing fermentation conditions to extreme levels, challenging the fitness of these micro-organisms. This can result in problems during fermentation, leading to undesirable wine characteristics and financial losses for the industry."

"Our aim is to develop novel yeast and bacteria strains which improve the winemaking process. However, because the use of genetic engineering techniques is prohibited in the food and beverage industry in most global markets, we focus on more traditional approaches, such as directed evolution and mutagenesis. These methods can be equally effective, but are often very time consuming, requiring large-scale screening of hundreds or even thousands of strains, often from heterogeneous populations. Monitoring the fermentation process for large numbers of samples is also very laborious, requiring regular sampling of fermentation vessels every six, four, or even two hours over the course of several days to several weeks."

"An important part of our job is to modernize these classical microbiological techniques and develop powerful new approaches for microbial improvement. We already had a number of robotic processing systems in the lab, as well as the necessary programming and engineering expertise in house, and so we were interested in developing a system that would allow fully automated screening. We looked at the various liquid handling platforms on the market, and Tecan's Freedom EVO really stood out, enabling us to build a walkaway fermentation monitoring solution that would generate 96-well microplates ready for loading onto our various analytical instruments. The real strength of the Tecan system was its flexibility – including the software – which allowed us to integrate our own custom hardware solutions onto the platform with relative ease."

"Our first task was to create a jacketed carrier system allowing 96 fermentations to run simultaneously on the Freedom EVO's workdeck. We developed four 24-position jacketed carriers which have warm water pumped through them to maintain the optimal fermentation temperature, and each carrier has a 24-position stirrer underneath to continuously mix the fermentations. Each flask has also been



fitted with a custom-designed airlock along with a silicon septum in the top, allowing the workstation's Liquid Handling (LiHa) Arm to sample the anaerobic fermentations using fixed tips. At user-defined intervals, aliquots are transferred directly from the flasks to purpose-built cooled microplate carriers which maintain the samples at 10 °C until the user is ready to remove them from the workstation for analysis. It only takes half a day to set up a 96-fermentation experiment, then you can just remotely monitor its progress via a smartphone from another lab, from home or – because this is Australia – from the beach. Compared to having to be in the lab every few hours, day and night, this is fantastic. Everyone here loves the system, and is using it a lot."

"Although we had previous experience with Tecan's equipment and services – we already had an Infinite® M200 microplate reader in the lab – we had no real way of knowing if this system was going to work, as we weren't aware of anyone that had tried anything similar. However, the Tecan team here in Australia was extremely good. Their communication was great, and they helped us with protocol development and script writing. The platform's technical specifications were also very thorough in terms of exactly what the instrument could do, what pressures could be applied to the LiHa tips, etc., so we were fairly confident. Once we had designed and built our bespoke hardware and put everything together, it worked incredibly well first time. We got extremely good results straight away and, after a bit of fine tuning, we're very happy with the system."

"At the moment, samples are still analyzed offline, but the next step is to develop a system that monitors at least a few key metabolites – such as sugar and alcohol concentrations – during the fermentation. One option is to integrate a small, benchtop NMR system underneath the workdeck of the Freedom EVO, and we are already discussing this with an instrument manufacturer in New Zealand. We could then run the NMR analyses in virtually real time, and still collect an aliquot of each sample in microplates to perform offline assays. It would also be good to replicate our set-up in other labs in Australia or further afield, allowing direct comparison of results between platforms."

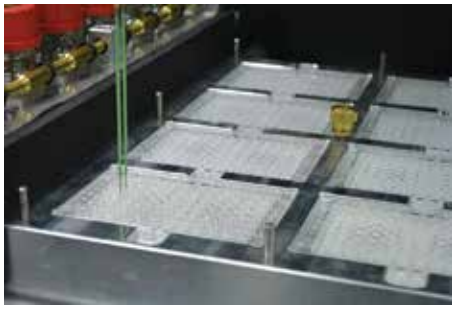
"You can just remotely monitor its progress via a smartphone from another lab, from home or – because this is Australia – from the beach."

To find out more about Tecan's food sciences solutions, visit **www.tecan.com/food**

To learn more about the Wine Microbiology and Microbial Biotechnology Laboratory, go to **www.agwine.adelaide.edu.au/winemicro**



The LiHa Arm's fixed tips allow direct transfer of samples from fermentation flasks...



...to cooled microplates, ready for offline analysis

Carbon parts make wine research 60% more economical and 67% faster

August 23 2017

Small-batch winemakers and academic researchers are always seeking new methods to further their understanding of the winemaking and yeast fermentation process. Important advances include improvements in testing equipment, as well as improved processes, including automation and robotics. In order to produce insights relevant to at-scale manufacturing, researchers must be able to simulate the kinetics of volume production in small batches. Traditionally, water-filled airlocks integrated with testing flasks have been the 'go to' solution for small-batch fermentation research that can mirror the conditions of volume production. The airlocks keep oxygen away from yeast during the fermentation process while allowing carbon dioxide to escape. This helps simulate large tank kinetics in a small-batch process.

Professor Vladimir Jiranek and Dr. Tommaso Watson, leading academic researchers at the University of Adelaide, had the vision to automate their research processes by integrating robotic sampling of key lab components—in this case, the water-filled airlock, the design of which had not changed in decades. This case study describes the airlock redesign challenges faced by this research group, their journey through various manufacturing alternatives, and their ultimate solution based on a redesign and production with Carbon's technology. Working with a leading contract manufacturer, The Technology House (TTH), the research team successfully produced a redesigned airlock using Carbon's M Series printers and high temperature resistant CE 220 resin.

CHALLENGES

The first challenge Dr. Watson and the team tackled was to improve the efficiency of the research process by introducing robotic sampling of flasks used for testing. A robotic arm would be used to draw samples from these flasks, reducing the number of manual steps in the testing process and therefore the potential for human error. Previously, a technician had to sample the flasks manually to study the fermentation kinetics. The second challenge was to change the airlock design so that a large number of flasks could fit on a static test platform. This meant that the airlock needed a complete redesign.

Figure 1 shows a traditional water-filled airlock and shake flask used by the team prior to the redesign. The purpose of this airlock is threefold:

- Allow CO_2 produced during fermentation to escape
- Maintain anaerobic conditions by preventing oxygen in the air from entering the flasks
- Prevent microbial contamination from the outside environment



FIGURE 1

A TRADITIONAL WATER-FILLED AIRLOCK ON TOP OF A SHAKE FLASK WITH CARBON DIOXIDE FLOW SHOWN IN THE INSET

A --- B --- OUT

PRODUCT DEVELOPMENT JOURNEY

From the outset, Dr. Watson and the team worked on reducing the form factor of the airlock and flasks so that they would be able to fit more flasks on the test platform, thereby enabling robotic overhead sampling. When the team embarked on the redesign, they were targeting to fit 96 flasks on the test platform. Prior to reaching the final solution based on Carbon's technology, the team explored two other manufacturing approaches; each of which required an entire product development cycle.

APPROACH 1

MACHINED STAINLESS STEEL



In the first approach, the airlock was designed and manufactured from several stainless steel parts assembled with silver soldering.

ADVANTAGES:

- Compact design to support robotic sampling
- Robust construction
- Sterilizable by autoclave at 121°C

DISADVANTAGES:

- Silver soldering can corrode due to the acidic environment during the fermentation process and by the CO_2 produced
- Heavy stainless steel can damage the glass flasks
- Lack of internal visibility for water level observation
- Expensive to produce
- Difficult to manufacture due to multiple parts

COST OF DEVELOPMENT High, due to machining of individual steel parts and soldering

TIME FROM DESIGN TO PRODUCTION Long, due to machining and soldering complexity

APPROACH 2

ADDITIVELY MANUFACTURED USING A TRADITIONAL LAYER-BY-LAYER DESPOSITION METHOD



In the second development approach, the research team tried to address the disadvantages of Approach 1 by using a different material. Due to the complex design, the team explored the design freedom offered by additive manufacturing and used two commonly available thermoplastics, ABS and PLA, to 3D print the airlocks using a layer-by-layer deposition technology. Using this approach, the team also hoped to address the high weight disadvantage, as well as the lack of internal visibility of the airlock.

ADVANTAGES:

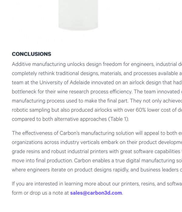
- Lightweight and does not damage the glass flasks
- Easy to prototype
- Compact design
- Refinement of design enabling complex internal airflow chambers
- See-through for water level observation
- Resistant to corrosion

DISADVANTAGES:

- Traditional layer-by-layer process materials (ABS or PLA) are temperature sensitive and are not autoclavable at 121°C
- Adhesion imperfections between deposition layers reduce functionality, for example water leaks

COST OF DEVELOPMENT Very high, due to new technology learning curve

TIME FROM DESIGN TO PRODUCTION Very long, due to challenges associated with airlock design iteration such as printing air channels, overhanging surfaces, and solving adhesion imperfections



In the third and final approach to product development, the research team looked for a solution that combined both the flexibility and design freedom of additive manufacturing with autoclavability and required functionality. The team was introduced to **Digital Light Synthesis™** technology by The Technology House (TTH), a leading contract manufacturer. With their wealth of experience in using Carbon's technology and the M Series printers, the engineers at TTH evaluated the research team's design, materials, and functional requirements, and began production using Carbon's Cyanate Ester resin (**CE 220**)—delivering the perfect airlock.

ADVANTAGES (in addition to those listed in Approach 2):

- Superior finish, compared to layer-based additive manufacturing methods
- Isotropic and homogenous part
- Mechanical characteristics combine to deliver the perfect seal against water and CO_2
- Carbon's CE 220 meets autoclavability requirement at 121°C

DISADVANTAGES:

- None

COST OF DEVELOPMENT Low, due to ease of air channel printability with Carbon technology

TIME FROM DESIGN TO PRODUCTION Short, less than one week

BENEFITS

Academic researchers at the University of Adelaide, led by Professor Jiranek and Dr. Watson, were looking to solve a process challenge by replacing an error-prone manual step with the robotic sampling of flasks. To achieve this, they had to innovate on the airlock design. Using Carbon's technology, researchers produced the most robust functional parts at the lowest cost and in the shortest development time. Table 1 shows the comparison of all three product development approaches, as tracked by Professor Jiranek and Dr. Watson.

Table 1: Comparison of cost and time for the three product development approaches

Comparison Benchmarking	Approach 1 – Machined Stainless Steel Airlock	Approach 2 – Layer-by-Layer (ABS or PLA) Airlock	Approach 3 – Carbon with CE 220 Airlock
Cost of Development ($)	2.5*X	4*X	X (lowest cost)
Time of Development (hours)	3*Y	6*Y	Y (shortest time)

The final airlock is more compact and replaces the previously bulky flasks with smaller flasks (Figure 5). This miniaturization also unlocks the ability to accommodate 384 flasks instead of the initial target of 96 flasks, enabling easy robotic sampling. The University of Adelaide with TTH's continued partnership plans to produce and use thousands of these CE 220 airlocks over the next few years. This new airlock design represents an important new contribution to small-batch winemakers and research teams worldwide, as they further their understanding of wine research.

FIGURE 5

NEWLY DESIGNED, MINIATURIZED AIRLOCK

CONCLUSIONS

Additive manufacturing unlocks design freedom for engineers, industrial designers, and researchers alike, allowing them to completely rethink traditional designs, materials, and processes available at their disposal to make final parts. The research team at the University of Adelaide innovated on an airlock design that had not changed for decades and had become a bottleneck for their wine research process efficiency. The team innovated on the part design, the material used, and the manufacturing process used to make the final part. They not only achieved a better functioning part that helped them move to robotic sampling but also produced airlocks with over 60% lower cost of development and over 67% lower time of development, compared to both alternative approaches (Table 1).

The effectiveness of Carbon's manufacturing solution will appeal to both engineering leaders and business leaders alike, as organizations across industry verticals embark on their product development cycles. Carbon's technology offers engineering grade resins and robust industrial printers with great software capabilities to help teams rethink product design and ultimately move into final production. Carbon enables a true digital manufacturing solution with shorter product development cycles, where engineers iterate on product designs rapidly, and business leaders can launch to market differentiated products faster.

If you are interested in learning more about our printers, resins, and software features, please reach out via our contact us web form or drop us a note at **sales@carbon3d.com**.