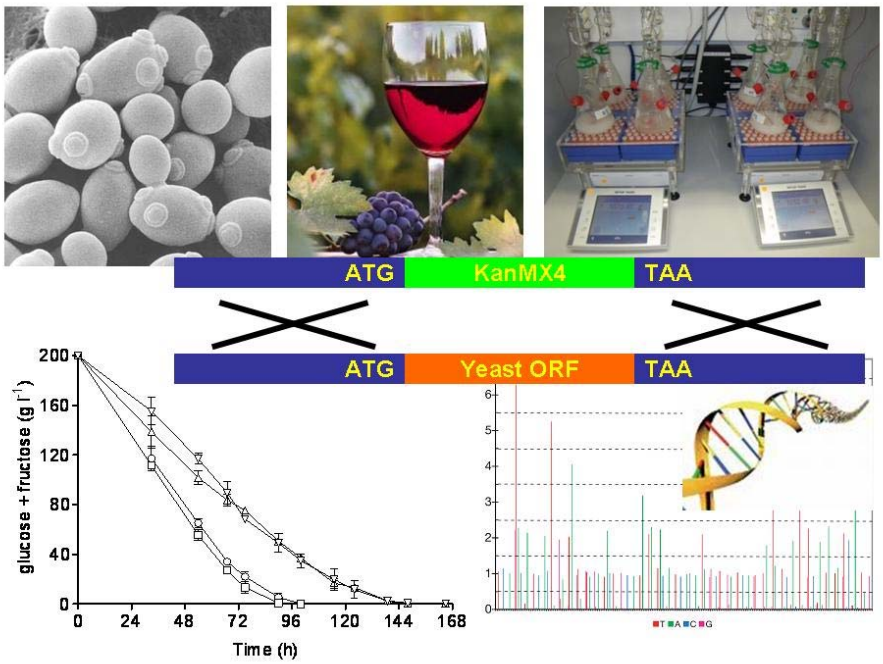
SCHOOL OF AGRICULTURE, FOOD AND WINE | THE UNIVERSITY of ADELAIDE

Australian Government
Grape and Wine Research and Development Corporation

BETTER WINE THROUGH NOVEL AND BETTER INFORMED APPLICATION OF MICROBIOLOGY



FINAL REPORT to

GRAPE AND WINE RESEARCH & DEVELOPMENT CORPORATION

Project Number: **UA 05/01**

Principal Investigator: **Assoc Prof Vladimir Jiranek**

Research Organisation: **The University of Adelaide**

Date: **30 November 2011**

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Abbreviations

AE	Adaptively evolved
APCI	Atmospheric pressure chemical ionisation
bp	base pair of DNA
CDGJM	Chemically Defined Grape Juice Medium
CDGJM+PP	CDGJM enriched with polyphenol extract
CME	Cold methanol extraction
CI	Confidence interval
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
ENDO	Endometabolome
ESI	Electrospray ionisation
EXO	Exometabolome
FACS	Fluorescence Activated Cell Sorting
FAN	Free amino acid nitrogen
FM16	Mixed culture of evolved yeast derived from commercial strain L2056
FM16-C7	Clonal isolate from FM16 and equivalent to L2056-C7
GC-MS	Gas chromatography-mass spectrometry
GO	Gene Ontology
GFH1	Glycosyl hydrolase family 1
HILIC	Hydrophobic liquid interaction chromatography
HPLC	High performance liquid chromatography
HEE	Hot ethanol extraction
HNE	High nitrogen efficiency
HSF	High sugar fermentation
kb	kilo base of DNA
LAB	Lactic acid bacteria
LC-MS	Liquid chromatography-mass spectrometry
MLF	Malo-lactic fermentation
MRS	de Man Rogosa and Sharpe media/broth
OD	Optical density
ORF	Open reading frame
PEP-PTS	Phosphoenolpyruvate-phosphotransferase system
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
QTL	Quantitative trait loci

RFLP	Restriction fragment length polymorphism
RI	Refractive index
RNA	Ribonucleic acid
RP	Reverse phase
Rpm	revolutions per minute
SIM	Selective Ion Monitoring
SNP	Single nucleotide polymorphism
SPME	Solid phase micro extraction
TAE	Tris acetate EDTA
TOF	Time of flight
UTR	untranslated region
X-gal or BCIG	5-bromo-4-chloro-indolyl-galactopyranoside

Abstract

This project sought to provide a better understanding of the potential influence of yeast and lactic acid bacteria (LAB) in fermentation and the resulting wine. We have identified a number of genes required for or enhancing fermentation using techniques such as genome sequencing, whole genome screening and metabolomic analysis and comparative fermentation. These genes are future 'targets' in directing yeast improvement programs. As part of this project we have developed high-throughput micro-fermentation systems and enzyme assays for key metabolites such as malic acid, reducing sugars and nitrogen. The project also completed the first screening of the lab yeast deletion library under oenological fermentation conditions. Again, highlighted were several genes and therefore cellular processes critical to successful fermentation completion.

We have defined the potential contribution of LAB in terms of whole cells and associated esterase and glycosidase activities to wine composition. We have biochemically characterised four esterase enzymes from *Oenococcus oeni* and heterologously expressed and purified them from *E. coli*. Action against artificial substrates indicates a greater preference for short-chain esters, such as ethyl acetate, raising the possibility of their application to wine to control this undesirable ester. Preliminary data in wine, however, reveals conflicting results with ethyl acetate unchanged or increasing, whilst significant changes were also seen for other esters. Further work will explore the opportunities for specific enzymatic modulation of wine ester profile. Three glycosidase enzymes have been cloned and heterologously expressed in *E. coli*. Insight into their importance to cell function and potential impact on wine composition, aroma and colour compounds was obtained. These findings go some way towards a more informed application of LAB or their enzyme extracts for use in wine making.

Future directions detailed herein including commercialisation plans have been incorporated into the new project: UA 11/01, *Innovative Microbials for Winemaking Excellence.*

Executive Summary

This project sought to increase the knowledge base specifically in relation to the contribution of microorganisms to fermentation kinetics and wine composition. In addition, it sought to define the basis for these contributions and to develop strategies and strains or treatments which could be used to tailor winemaking in a predictable manner. The key findings are listed below.

- Extensive expertise in yeast and bacterial functional genomics has been developed through this project along with an impressive inventory of strains and specialist research infrastructure. These form the foundation of further work to benefit the industry including, but not limited to, efforts recently initiated through project UA 11/01.

- Nutrient efficient yeast were isolated and/or characterised. The extent to which these proof-of-concept strains are also metabolically distinct (with regard to their output of sensorily significant metabolites) is still being evaluated.

    - A transposon mutagenesis strategy has successfully been applied to identify yeast genes whose manipulation alters fermentation performance. Deletion of one such gene, *ECM33*, specifically increased nitrogen efficiency (in low nitrogen media) and fermentation rate (in nitrogen sufficient media). These outcomes have been confirmed for laboratory and wine yeast strains in a chemically defined grape juice medium and grape juices. Efforts to attain analogous (non-recombinant) strains are warranted as they will offer winemakers greater reliability during fermentation, especially in low nitrogen juices.

    - Further characterisation of other High Nitrogen Efficiency (HNE) strains, namely deletants Δ*ngr1* and Δ*gid7*, confirmed their nitrogen efficiency as single deletants. However, unlike the laboratory strain background, deletion of both genes in wine strain DR4 was not synergistic in providing further reductions in fermentation duration compared to the respective single deletion mutants.

    - The *ngr1* and *gid7* mutants were further charactersised through a number of approaches including phenotypic arrays and metabolomic analysis to provide a greater understanding of the metabolic perturbation in these strains. The precise link between these genes and nitrogen efficiency remains to be determined.

    - We demonstrated for the first time the existence of haploproficiency in a wine fermentation context. Heterozygous diploid deletants (i.e. halving of the gene copy number) were found to be haploproficient for wine fermentation, completing fermentation under FAN-limited conditions in reduced time compared to the parental strain. Genes highlighted were *IRC9*, *RMD6*, *HSP150* and *RPL2B*. Further investigation exploiting these genes could yield enhanced strains according to this entirely novel concept.

    - Optimised strains previously isolated via adaptive evolution strategies were characterised to a high degree using fermentation screens, phenotypic arrays, genome sequencing and metabolomic analysis. The fermentation efficient phenotype of evolved strains FM16, FM5 and L2056-C7 is not due to a single gene modification but rather a number of gene modifications. However single deletion mutants of key candidate genes did display partial fermentation efficient phenotype. These genes can therefore form the basis of optimised strains with increased fermentation robustness.

- The evolved strains FM5 and FM16 tended toward higher glycerol yield (~10%), while the clonal isolate L2056-C7 (FM16-C7) yielded 20% more glycerol.

- Intra-specific hybridisation viz spore-spore mating of *Saccharomyces cerevisiae* wine yeast by methodologies developed 'in house', and utilising sorting of hybrids by Fluorescence Activated Cell Sorting followed by molecular typing of yeast was successfully achieved.

- Hybridisation between commercial strains (chosen for their distinct properties) produced approximately 120 hybrids. The properties of some hybrids clearly differed from the parents, however, the magnitude of these properties (at least for the small number of hybrids examined here) fell within the range observed across the entire collection of commercial strains. A screening exercise utilising a much larger set of hybrids is warranted. At the time this was not available, thus this work was abandoned. However, in the latter stages of this project we developed a high-throughput micro fermentation screening system utilising robotic liquid handling. Revisiting our collection of candidate hybrids will now likely prove rewarding.

o A comprehensive dataset pertaining to the enzymatic (glycosidase and esterase) activities of lactic acid bacteria was accumulated.

- In order to characterise these enzymic properties without interference from other activities of the cell, we sought to clone and heterologously express the structural genes before purifying and biochemically analyzing the proteins. Using a bioinformatic approach sequence information derived from the genome sequence of *Oenococcus oeni* strain PSU-1 and sequences of enzymes from related bacteria it was possible to reveal the existence of two operons (bgl and cel) involved in glycoside utilization and several putative esterase genes.

- We exceeded the planned number of strains (8 *vs* 2) to be characterized in terms of their enzymatic properties. Specifically, the growth of *Oenococcus oeni* on natural glycoside substrates (not specifically derived from grapes) was used to demonstrate the importance of these compounds to cell nutrition. Data on the expression pattern of the related genes (e.g. *celC*, *celD* and *bglD*) provided insight into the conditions and timing during growth when these substrates might of greatest importance.

- MLF trials using Cabernet Sauvignon and Chardonnay were used to investigate the possible impact of selected LAB on the volatile profile of these wines. Methods for analysis of volatile aroma compounds by GC/MS, including purification of compound standards were developed. Notable findings were that some strains were able to reduce the ethyl acetate, ethyl hexonoate and isoamyl acetate content of wines. Opportunities therefore exist for selective use of such strains to eliminate the former, at times undesirable, compound from wine.

o Many targets for the generation of optimised yeast strains were identified.

- In addition to the genes mentioned above and identified through our study of high nitrogen efficiency or haploproficiency, we have identified some 90 genes that are important for fermentation of high sugar media (see below). In addition, a population of adaptively evolved, fermentation robust strains is available to be screened using our recently developed high-throughput micro-fermentation screening systems.

o A limited number of pilot scale trials on selected LAB and/or enzymes or optimised yeast were conducted. Results of those that were conducted are nonetheless very promising

- Delays in achieving performance targets for the earlier parts of the project (due to the ambitious nature of the work, unforeseen technical difficulties and staff absences) as well as the complications of the last 3 vintages (2 x drought, 1 x excessive rain and disease pressures) greatly restricted opportunities for large scale trials. To begin to address this, trials were very recently conducted at UC Davis to take advantage of an alternate vintage. Analysis of the resulting wines is pending.

- A complete list of genes essential for the completion of fermentation of a high sugar medium i.e the High Sugar Fermentation HSF gene set has successfully been defined through use of the commercial yeast deletion library. This is the first time this gene set has been defined. It represents a novel finding since previous screens of the library have only looked at growth or short term fermentation under the influence of a single stressor. This is the first time genes needed for growth and completion of an extended, high sugar fermentation have been reported. Complementary gene sets were sought through attempts to screen the yeast over-expression library.

  - Preliminary work demonstrated the validity of using the commercially available lab yeast deletion library to conduct a high sugar fermentation screen for genes of importance. Specifically, a supplementation approach that allowed these auxotrophic strains to be grown in a defined medium was shown to be appropriate. This work preceded the availability of at least a partial deletion library in a wine yeast background as being prepared by the AWRI.

  - Using the lab yeast deletion library in supplemented media, 80 genes, when deleted, were identified as essential for the completion of fermentation under high sugar conditions. The deletants exhibited extended fermentation with most eventually completing catabolism of sugars. Even so, such mutants did not meet the criterion of a timely completion of fermentation. By comparison, nine genes, when deleted, were shown to result in 'stuck' fermentation, these genes are central to transcription (*RPA12, SIN3, ZAP1*), NAD recycling (*NPT1*), trehalose synthesis (*TPS1* and *TPS2*), signalling (*PLC1*), ubiquitin recycling (*DOA4*) and ion homeostasis (*SSQ1*). These genes and the HSF gene set as a whole highlight key cellular processes, particularly ion homeostasis, as being critical for successful fermentation.

  - In an effort to produce a complementary data set, significant efforts were made to take advantage of the over-expression library. Methos for large scale preparation of this library of some 5000 plasmids were developed. The outstanding requirement is developing a strain background in which the $GAL_{promoter}$ driven constructs will operate in the presence of glucose (as found in juice). Despite several promising leads, this work was eventually abandoned due to limited time and availability of an alternate, non-unique (i.e. multiple genes in each construct) library. In preparation of utilising this Genomic Tiling Collection, a recipient strain derived from a wine yeast and bearing a leucine auxotrophy was successfully constructed. Introduction of the collection into this strain background is underway with screening for various phenotypes (performance in high and low nitrogen or high sugar conditions, aroma compound production, etc) to be undertaken by current PhD students.

- We successfully cloned and heterologously expressed structural genes for 7 (*vs* the planned 2) hydrolytic enzymes from lactic acid bacteria. These proteins were largely produced in active form and subjected to extensive biochemical characterisation.

  - Putative genes encoding esterase and β-glucosidase (ultimately found to be phospho-β-glucosidase) enzymes were identified by sequence comparison of known enzymes from related

bacteria and the reported genome sequence for PSU-1. Two phospho-β-glucosidases and four esterases from *O. oeni* were successfully expressed in *E. coli* as soluble recombinant proteins (HIS-tagged fusion proteins) and were highly purified. The above enzymes were biochemically characterised in terms of substrate specificity (using artificial p- or o- nitrophenyl linked substrates), and effect of temperature, pH and metal ions on enzyme activity.

- Site directed mutagenesis of the characterised GHF1 phospho-β-glucosidase BglD ($S^{432}E$) demonstrated the importance of this residue for catalytic activity. Although the enzyme specificity was not altered towards non-phosphorylated substrates as expected, the modified protein lacked activity towards phosphorylated substrates. This finding adds to the knowledge about this group of enzymes and the basis for their substrate specificity.

- The activity of the esterase EstB28 was not diminished by the presence of increasing ethanol content. In fact activity was found to increase 250% in 28% ethanol (w/v) with some 80% of activity retained at enthanol concentrations as high as 50% (w/v). Although not fully characterised this enzyme demonstrates excellent potential for application in fortified wines and other high ethanol content processes, perhaps unrelated to wine making.

- The potential of esterase EstA2 as a potential winemaking additive was highlighted in that the enzyme reduced ethyl acetate. Further characterisation of EstA2 is required in terms of enzyme stability at temperatures and time frames relative to winemaking practises.

o The basis for the superior performance of the adaptively evolved strains L2056-C7 was sought through a very comprehensive analysis of these strains. Work undertaken eventually included a complete genome sequence, comprehensive metabolomic analysis and trails of various candidate deletion strains (reflecting the mutations observed in the evolved strains). In the process this work has allow significant expertise to be developed in the group and paved the way for strong linkages with expert groups around the world.

- Our pioneering work with collaborators at the Universities of Manchester and Cambridge allowed the development of sample preparation and analytical approaches for the analysis of complex, wine-like fermentation samples.

- This was achieved with great effort ahead of the now more readily available services of Metabolomics Australia (MA). In fact, ours were amongst the first sets of fermentation samples to be analysed by MA, thereby requiring the workflow and analytical protocols to be developed - in part the reason for the long delay in seeing these analyses completed.

- Metabolomic analysis revealed about 35 discriminatory metabolites across the endo- and exo-metabolome. The links between these and the observed superior fermentation phenotype of L2056-C7 are under investigation, though some tentative associations with key metabolic pathways have been found.

- Genome sequencing of L2056-C7 has reveals some 200 mutations (SNPs) in recognised open reading frames which result in an amino acid change in the encoded protein. Some of these have the potential to affect genes related to sterol and lipid metabolism (more specifically the mevalonate/isoprenoid pathway), DNA architecture (histone binding), extracellular nucleotide metabolism and methionine/cysteine biosynthesis. Genes of particular interest with SNPs unique to the evolved strain included *HIF1* (chromatin assembly), *CRC1* (mitochondrial carnitine

transporter), *HMG2* (HMG-CoA reductase), *ERG10* (Acetyl-CoA C-acetyltransferase), *ASP1* (cytosolic L-asparaginase) and *AXG1* (alanine:glyoxylate amino transferase). Hypotheses for how these mutations may contrinbute to the fermentation phenotype L2056-C7 have been developed.

- The genetic and physiological basis for the fermentation vigour exhibited by the evolved strain L2056-C7 has been extensively examined using systems biology multiplatforms: whole genome comparison, metabolomics and phenotype analysis. We believe this to be the first report of its kind for a wine yeast. This comprehensive characterisation of the evolved strains has introduced many opportunities for the targeted generation of superior strains. Many such leads will be taken up in project UA 11/01.

- The fermentation efficient phenotype exhibited by L2056-C7 was shown to be stable even after 50 generations of non-selective growth on rich media (YPD) suggesting that the desirable mutations are stably incorporated into the genome.

- Data from larger scale trials has been produced from a limited number of opportunities to conduct such work. The standout result related to L2056-C7 whose fermentation performance, along with that of the reference strains, varies depending on the medium and fermentation conditions of the trial – as is seen for most strains. Thus the precise niche for application of this strain is being identified through further trials. At very least the strain performs as well as or better than its parent, L2056, or EC1118, the strain currently considered to be the best available.

- A clear commercialisation plan has been developed for the strains and enzymes derived from this project. Potential winery and supplier partners have been identified and preliminary discussion held. The protection of IP from this project is being pursued via Plant Breeders Rights (PBR) in the first instance for our evolved yeast strains.

- The project has trained almost 20 research students in areas of strong relevance to the wine industry, thereby increasing the intellectual capital in this field. Specifically, 5 PhD students with a further 3 part-way through candidature were trained. In addition, 2 Master of Oenology and 8 Honours students undertook work connected with this project. Seven Summer Scholarship students and 2 visiting researchers participated as well.

- Our findings have been disseminated through 13 peer-reviewed publications, 4 written articles, and over 30 conference/seminar/workshop presentations (national and international).

Background

Winemaking involves at least two key microbial phases: the alcoholic and malolactic fermentations conducted by yeast and lactic acid bacteria, respectively. The scope and magnitude of their influence is considerable with regards to process efficiency, wine composition, quality and style, and shelf-life. It is clear therefore that research targets in the wine microbiology area must include both the fundamental issues such as reliability of yeasts or bacteria in making their core contribution to wine (ie sugar catabolism or MLF). Further targets must include the definition and exploitation of other contributions of microbes to wine composition and production efficiency. The key to capturing the broader benefits offered through microorganisms as biological catalysts with multiple capabilities is a greater understanding of the genetic, metabolic and biochemical basis for these properties, so that they can be deliberately and reliably applied. At present, a comprehensive understanding of how and why microbes interact with the juice during fermentation is not fully understood. As such we are some way from being able to achieve predictable outcomes let alone being able to tailor outcomes to a specific winemaking target.

This GWRDC project UA 05/01 (*Better Wine Through Novel and Better Informed Application of Microbiology*) consolidates the achievements of previous GWDRC project UA 01/04 (*Microbial Enhancement of Wine Quality and Complexity*) with the view of having outcomes which are application-ready. At the scheduled mid-point review of project UA 05/01 (3 yr) the proposal was modified in response to feedback from the GWRDC, industry trends and emerging issues, where the objectives were reiterated. Most notably, the yeast-based activities were expanded, while the work on lactic acid bacteria (i.e. 2 PhD projects) would be concluded.

We have used cutting-edge molecular biological techniques for 'proof-of-concept' work and non-recombinant and high-throughput screening strategies (previously proven to yield 'industry-ready' strains). It is anticipated that our research findings will benefit the Australian wine research community in helping to maintain its technological advantage.

Project Aims and Performance Targets

The aim of the project objectives, stated below (from the 2008 grant renewal), was to attempt to address the limited knowledge as to how and why microbes interact with the juice during fermentation. They were as follows:

- Define the genetic determinants of fermentation performance (fermentation reliability, nutrient efficiency, etc) by exploiting laboratory yeast deletion and over-expression libraries.
- Produce or isolate strains with superior fermentation reliability (by adaptive evolution, hybridization of commercial strains or using recombinant methods).
- Produce yeast that consistently make distinct and novel contributions to wine composition and flavour (i.e. through production of flavour compounds or elaboration of flavour or colour modifying enzymes and metabolites).
- (LAB Projects): Characterise oenologically important enzymes (e.g. esterases and glycosidases) from lactic acid bacteria and develop protocols of the application of strains or extracts for specific winemaking outcomes.

In most cases for this ambitious project we have successfully achieved these objectives and associated performance targets, whilst for the few exceptions, we have made significant in-roads towards achieving the goals in the new GWRDC grant (UA 11/01 *Innovative Microbials for Winemaking Excellence*).

Project Aims and Performance Targets

The Outputs and Performance Targets outlined in the original application for the first 3 years (2005-2008) of the proposed 5 year project are shown below.

A scheduled mid-point review of the project was undertaken in the 3rd year and Outputs and Performance Targets revised in light of this review, feedback from the GWRDC, industry trends and emerging issues. Updated Outputs and Performance Targets for a further 3 years of the project (2008-2011; see below) were included in the Final Project Application (8th February 2008) made to the GWRDC for support for this period.

After two approved variations (28 January 2010, 7 October 2011), the end date of the project was adjusted to 30 November 2011.

To simplify reference to these Outputs throughout the report, they have been grouped around common themes (e.g. dissemination, lactic acid bacteria, student recruitment/graduation, etc) and have been renumbered (A – M). Those Outputs with multiple components through the project are colour coded. The Performance Targets have also been renumbered (1 – 29).

Outputs and Performance Targets 2005-2006

Outputs	Performance Targets
A. Nutrient efficient yeast	**1.** Outputs from UA 01/04 consolidated to give at least 2 novel yeasts with improved efficiency of use of limited nutrient groups (e.g. nitrogen and oxygen) and hence greater fermentation reliability.
A. Metabolically distinct yeast	**2.** Outputs from UA 01/04 consolidated to give at least 2 strains via non-recombinant means (hybridisation of commercial strains, adaptive evolution or genome shuffling) with distinct and predictable yield of metabolites which impact on wine quality or processing.
L. PhD graduates	**18.** 1-3 PhD candidates (currently part of UA 01/04) to complete and submit their theses.
M. Disseminated findings	**23.** 1-3 publications/seminar presentations detailing project findings.
L. PhD students recruited	**21.** 1-2 PhD students recruited to work on aspects detailed within this project application.

Outputs and Performance Targets 2006-2007

Outputs	Performance Targets
B. Information on LAB with distinct properties	**4.** 2 LAB strains with distinct hydrolytic properties of potential oenological value identified. Enzymatic basis for these defined.
B. Optimised bacterial strains and application protocols	**5.** 1 LAB strain with greater enzymatic activities (e.g. glycosidase, tannase, esterase, anthocyanase) and a method for application in winemaking. Larger-scale/industry trials completed ('07 vintage).
C. Identification of targets for 'second generation' (G2) optimised yeast	**7.** Complete preliminary assessment of candidate properties to form basis for a 'second generation' (G2) of non-GM optimised yeast strains that have enhancements in at least two key properties.
M. Disseminated findings	**24.** 1-3 publications/seminar presentations detailing project findings.
L. Honours graduate	**19.** 1 Honours graduate from work undertaken as part of this project.

Outputs and Performance Targets 2007-2008

Outputs	Performance Targets
D. Industry or pilot scale information on selected LAB and/or enzymes	**8.** Complete analysis from larger-scale/industrial trails from the '07 vintage. Report on findings with recommendations to facilitate adoption by industry.
D. Industry or pilot scale information on properties of G2 yeast	**9.** G2 ferments completed and analysed ('08 vintage). Report prepared for dissemination to industry and yeast suppliers.
E. Interim progress review and proposal for Yrs 4 & 5	**10.** Review of progress and re-assessment of project related priority areas prepared for GWRDC.
M. Disseminated findings	**25.** 1-3 publications/seminar presentations detailing project findings.
L. Honours graduate	**20.** 1 Honours graduate from work undertaken as part of this project.

Outputs and Performance Targets 2008-2009

Outputs	Performance Targets
L. Hons/PhD student(s) recruited	**22.** 1-3 additional students recruited to the project to support emerging areas.
F. Identity of yeast genes vital to winemaking	**11.** Screening of laboratory yeast deletion and over-expression libraries completed. Data analysis. Initiate preparation of list of genes key to wine making prepared.
A. Novel hybrid yeasts	**3.** 1-2 strains developed through hybridisation of commercial yeasts. Fermentation properties defined.
M. Publication on characterisation of initial AE strain	**26.** 1 paper describing properties of initial AE strain with commentary on gross genetic differences in the strain.
G. Characterised LAB enzymes	**12.** 1-2 recombinant esterases or glycosidases purified and characterised. Action on natural substrates tested.
B. 'Special purpose' LAB identified	**6.** Strains with specific esterase or glycoside modifying properties identified. Application protocols developed.

Outputs and Performance Targets 2009-2010

Outputs	Performance Targets
M. Preliminary trial data disseminated	**27.** Initial trials of above conducted during '09 vintage. Data disseminated via 1-3 papers, seminars or workshops.
H. Basis for evolved strains defined	**13.** Genetic or physiological basis for novel properties of AE strains defined and used to guide further optimisation.
I. New yeast/LAB strains constructed	**14.** 1-2 new strains with greater robustness and reliability (e.g. sugar and/or salt tolerance) constructed via methods and expertise derived from early part of project.
J. Linkages with wineries and industry suppliers	**15.** Initiation of Phase 1 industry trials of yeast, bacteria and enzymes. Initiate commercial preparation.

Outputs and Performance Targets 2010-2011

Outputs	Performance Targets
M. Data from Phase 1 trials disseminated	**28.** 1-3 publications, seminar presentation or workshops. Benefits for industry promulgated.
K. Data from Phase 2 industry trials	**16.** Data (incl. sensory) for novel yeast, bacteria and enzyme treatments collected in '10 vintage. Wines set aside for medium-term (<2 yrs) storage trials of above.
K. Commercialisation plan	**17.** Plan for commercialisation of beneficial strains or treatments developed.
M. Final report and data dissemination	**29.** Final report on project to date. 1-3 publications, seminars or workshops for researchers and winemakers.

Outputs

Given the scope of the work reported herein, we have elected to report **Methods, Results and Discussion** according to the Output groupings detailed above.

Output A: Nutrient efficient and metabolically distinct yeast

1. **Outputs from UA 01/04 consolidated to give at least 2 novel yeasts with improved efficiency of use of limited nutrient groups (eg nitrogen and oxygen) and hence greater fermentation reliability.**
2. **Outputs from UA 01/04 consolidated to give at least 2 strains via non-recombinant means (hybridisation of commercial strain, adaptive evolution or genome shuffling) with distinct and predictable yield of metabolites which impact on wine quality or processing.**
3. **1-2 strains developed through hybridisation of commercial yeasts. Fermentation properties defined.**

Contributors:

Past PhD students: Maria Astorga and Colin McBryde
Current PhD students: Jin Zhang
Past Honours students: Thomas Parker
Postdoctoral fellows and/or student supervisors: Drs Michelle Walker, Jennie Gardner, Paul Grbin, Miguel de Barros Lopes (AWRI) and Assoc Prof Vladimir Jiranek.

Background:

Through this aspect of the project we set out to 1) progress the work initiated by Jennifer Gardner, and subsequently Maria Astorga and Jin Zhang on generating strains of High Nitrogen Efficiency 2) evaluate these and other strains (for instance evaluation of single gene manipulations highlighted from transcriptional analysis of adaptively evolved strains) for novel metabolite profiles as well as 3) explore hybridisation of commercial *Saccharomyces cerevisiae* wine yeast as a means of generating strains of increased genetic diversity and, ideally, distinct traits.

Nitrogen efficiency is defined as the ability to catabolise an increased amount of sugar when supplied with a defined amount of nitrogen. Nitrogen deficiency is known to lead to an arrest of protein synthesis, a restriction of biomass formation and also a rapid inactivation of sugar transport systems, ultimately resulting in a retarded or even stuck fermentation. An option for the prevention of retarded or stuck fermentation which is being employed industrially is the use of “nitrogen efficient” wine yeast strains. Nitrogen metabolism couples with carbon metabolism to be not only the ultimate pathways contributing to fermentation efficiency, but also those heavily contributing to metabolites extruded from the cell and influencing the quality of wine. For these reasons it is valuable to further analyse metabolic effects of HNE mutants as well as gain a better understanding of the mechanism of how each gene involved contributes to this desirable phenotype.

Maria Astorga and Jin Zhang continued the work initiated by Jennie Gardner during her PhD studies. Specifically, Maria used the transposon mutagenesis approach to generate a library of mutants in a wine yeast background to identify genes important for high nitrogen efficiency (i.e. efficient fermentation under low nitrogen availability). Jin continued on from Maria, expanding the current list of potential HNE

strains through extensive literature analysis and further analysing the phenotypes of such mutants within fermentation.
A further project area developed out of the availability of an Honours student part way through the project. Thomas Parker, examined genes highlighted to be of importance not through their complete deletion but rather their reduced dosage. The concept of haploproficiency was first described in yeast by Stephen Oliver's group (University of Cambridge) and refers to genes for which deletion of a single copy in a diploid strain produces superior strain performance. Thomas examined a subset of genes identified from a data base search performed by Dr Jiranek during an extended stay in Cambridge and in collaboration with the Oliver group. The specific focus was genes involved in the proteosome, important for nitrogen recycling in the cell and therefore with the potential to be important to efficient utilisation of nitrogen.

Through UA 01/04 we also found that a rich source of metabolic differences in spent fermentation media was with the use of evolved strains FM5, FM16 and clonal isolates of such. Transcriptional analysis of these strains at pertinent time points throughout fermentation revealed a set of genes we suspect are the major influencers upon the desirable fermentation phenotypes. Colin McBryde continued this work here in this project by construction of a panel of single gene mutants, both deletion and overexpression strains, and evaluated their fermentation and metabolite profiles.

It is well known that fermentation characteristics vary widely between industrial yeast strains, with very few possessing, what are considered to be a perfect set of attributes. Not only this, but the demand for the availability of strains with novel attributes, such as an influence on the aroma profile, palate structure or fermentation robustness in industrial winemaking is still high. We chose to exploit this natural variation by undertaking a breeding program, through hybridisation of strains with desirable traits, undertaken by Drs Michelle Walker and Jennie Gardner, followed by screening and selection of improved progeny for desirable metabolic outcomes, undertaken by Michelle Walker.

Methods:

Yeast strains and growth conditions

Strains used in this study are listed in Tables A.1 and A.2.

YPD liquid starter cultures were inoculated from YPD agar plates (in the case of the wine yeast strains, cell mass was selected from a streak rather than an individual colony, to include variation that may occur within a culture sample).

Experimental fermentations were undertaken with three biological replicates of 100 mL cultures (in 250 mL Erlenmeyer flasks) in Chemically Defined Grape Juice Medium (CDGJM, McBryde *et al.*, 2006) with 590 mg FAN/L, supplied as ammonia (except where highlighted). These media were prepared with analytical grade reagents. CDGJM was inoculated with 1×10^5 or 5×10^6 cells/mL, as highlighted in the text (from an intermediate CDGJM starter medium with half the amount of glucose at 100 g/L and also with the addition of sterols) and incubation was at 25°C, as this is representative of the temperature of a typical industrial wine fermentation. Experiments were undertaken in an automated fermentation facility (www.agwine.adelaide.edu.au/wine/facilities/msff/). Fermentation progress was monitored by cumulative weight loss (due to carbon dioxide evolution; Bely *et al.*, 1990) and refractive index of residual sugar in clarified supernatant samples. Samples were extracted from flasks where necessary with a sterile needle through a silicon seal. The samples were stored at -20°C for later analyses. Fermentations were considered finished when the residual sugar was less than 2.5 g/L as determined by Clinitest™ (Cat: 2107, Bayer).

Culture development was monitored during logarithmic phase by determining the optical density at 600 nm (OD_{600}). Culture dry weight was determined by collecting biomass from 5 mL culture aliquots on a pre-dried and pre-weighed 0.22 µm membrane filter, followed by washing, drying, and reweighing. Commercially available kits were used for enzymatic quantitation of glucose and fructose (Boehringer Mannheim, Germany). Succinic acid, acetic acid, acetaldehyde, glycerol, and ethanol were determined by HPLC analysis as described previously (Walker *et al.* 2003).

Table A.1: Strains of *Saccharomyces cerevisiae* used in this output

Yeast Strain	Genotype	Source
L2056	Industrial wine yeast	Lallemand Pty Ltd
C911D	L2056 *ho*Δ *MAT*a	(Walker *et al.*, 2003)
S288C	*MAT*α *gal2*	(Mortimer and Johnston, 1986)
BY4741	*MAT*a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	(Brachmann *et al.*, 1998)
BY4743	*MAT*a/*MAT*α *his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 MET15/met15Δ0 ura3Δ0/ura3Δ0*	(Brachmann *et al.*, 1998)
FM5	Adaptively evolved mutant of C911D (~250 generations, mixed culture)	UA 01/04
FM16	Adaptively evolved mutant of L-2056 (~350 generations, mixed culture)	UA 01/04
L2056-C7	Adaptively evolved mutant, clonal culture derived from FM16	UA 01/04
1922	BY4741 *hxt1*Δ::kanMX4	(Winzeler *et al.*, 1999)
586	BY4741 *hxt2*Δ::kanMX4	(Winzeler *et al.*, 1999)
4182	BY4741 *hxt3*Δ:kanMX4	(Winzeler *et al.*, 1999)
3913	BY4741 *gdh2*ΔkanMX4	(Winzeler *et al.*, 1999)
6388	BY4741 *glc3*Δ::kanMX4	(Winzeler *et al.*, 1999)
2767	BY4741 *ald6*Δ:kanMX4	(Winzeler *et al.*, 1999)
1272	BY4741 *ino1*Δ::kanMX4	(Winzeler *et al.*, 1999)
4959	BY4741 *hap4*ΔkanMX4	(Winzeler *et al.*, 1999)
5575	BY4741 *gph1*Δ::kanMX4	(Winzeler *et al.*, 1999)
4525	BY4741 *rck1*Δ::kanMX4	(Winzeler *et al.*, 1999)
C911D *hxt1*	L-2056 *ho*Δ *hxt1*Δ::kanMX4 *MAT*a	This study
C911D *hxt2*	L-2056 *ho*Δ *hxt2*Δ::kanMX4 *MAT*a	This study
C911D *hxt3*	L-2056 *ho*Δ *hxt3*Δ::kanMX4 *MAT*a	This study
C911D *gdh2*	L-2056 *ho*Δ *gdh2*Δ::kanMX4 *MAT*a	This study
C911D *glc3*	L-2056 *ho*Δ *glc3*Δ::kanMX4 *MAT*a	This study
C911D *ald6*	L-2056 *ho*Δ *ald6*Δ::kanMX4 *MAT*a	This study
C911D *ino1*	L-2056 *ho*Δ *ino1*Δ::kanMX4 *MAT*a	This study
C911D *hap4*	L-2056 *ho*Δ *hap4*Δ::kanMX4 *MAT*a	This study
L-2056 ald6	L-2056 *ald6*Δ::kanMX4/*ald6*Δ::kanMX4	This study
L-2056 *gph1*	L-2056 *gph1*Δ::kanMX4/*gph1*Δ::kanMX4	This study
L-2056 *rck1*	L-2056 *rck1*Δ::kanMX4/*rck1*Δ::kanMX4	This study
C911D adh2^{UP}	C911D integrant [pCMTDH3BADH2^{UP}]	This study
C911D adr1^{UP}	C911D integrant [pCMTDH3AADR1^{UP}]	This study
C911D vid24^{UP}	C911D integrant [pCMTDH3EVID24^{UP}]	This study

DR4-20a	DR4 *ho*Δ *MAT*a	UA 01/04
DR4-20c	DR4 *ho*Δ *MAT*alpha	UA 01/04
DR4-20a *ngr1*	DR4 *ngr1*Δ *ho*Δ *MAT*a	This study
DR4-20c *gid7*	DR4 *gid7*Δ *ho*Δ *MAT*alpha	This study

General molecular methods

Basic molecular methods such as DNA preparation, polymerase chain reaction (PCR), Southern blot, mating and sporulation of yeast were performed as detailed in the previous report UA 01/04.

Construction of key yeast gene deletion mutants

The entire open reading frames of a number of genes of interest, as highlighted in the text, were disrupted in C911D (wine yeast haploid), using the *kanMX* gene replacement strategy (Wach, 1996). We took advantage of the readily available yeast deletion strains from the Yeast Deletion Project (http://wwwsequence.stanford.edu/group/yeast_deletion_project/deletions3.html). PCR products corresponding to the *kanMX4* module flanked by approx. 200 – 500 bases of sequence of the gene of interest were amplified from genomic DNA purified from the corresponding yeast deletion mutants with primers *GENE*A and *GENE*D (Table A.3 or, where not listed, as described by the Yeast Deletion Project). C911D, or other parent strain, where stated, was transformed with these PCR products (Gietz *et al.*, 1992) and transformants were selected on YPD-geneticin (400 μg/mL).

Heterozygous deletions were constructed using the same protocol used for haploid gene replacement. After gene replacement of a single gene copy, strains were sporulated, dissected and allowed to rediploidise. Homozygous diploid deletion strains were then identified.

Deletion of each ORF was confirmed by Southern Blot hybridisation with a probe specific for *kanMX4* (data not shown). The *kanMX4* probe was PCR amplified from pFA6-lackanMX4 (Wach *et al.*, 1994). The probe was generated using DNA from a *KanMX4* containing plasmid; pFA6a (Wach *et al.*, 1994) and gene specific primers G418F and G418R (Table A.3).

Construction of overexpression strains of genes of interest

Over-expression strains were constructed based on the self-cloning gene expression system, with a two-step gene replacement (Hirosawa *et al.*, 2004). In brief, a 0.7 kb DNA fragment of the *TDH3* promoter was amplified using C911D genomic DNA and primers TDH3-1 through TDH3-5 (Table A.4, depending on restriction sites of gene and upstream sequences), digested with the appropriate restriction enzymes and inserted into pGG119 to form pCMTDH3A→E. Fragments of the gene of interest were also amplified from C911D gDNA, with the corresponding primers, e.g. *ADR1* (+ 1 → + 1273) with ADR1-NCO and ADR1-XMA, *ADH2* (+1 → + 987) with ADH2-NCO and ADH2-XBA and *VID24* (+ 1 → + 815) with VID24-NCO and VID24-XMA (Table A.4). The *ADR1* and *AHD2* fragments were digested with *Nco*I and *Xba*I for insertion into the corresponding sites in pCMTDH3A and pCMTDH3B respectively, the *VID24* fragment was digested with *Nco*I and *Xma*I and inserted into the corresponding sites in pCMTDH3E. All genes were located downstream of the promoter. The 5' upstream regions of each gene were also amplified using the following primer configurations, ADR1-UPEAG and ADR1-UCAAT (-1981 → -77), ADH2-UPEAG and ADH2-UCAVR (-1943 → -69), and VID24-SPE VID24-AAT (-1885 → -9). Upstream fragments and corresponding plasmids were digested with the appropriate restriction enzymes before ligation (Table A.5). Finally plasmids were restriction digested before transformation into C911D (Gietz *et al.*, 1992). The transformation mixture was spread on minimal plates containing 1.0 μg/mL cerulenin. The cerulenin-resistant colonies were streaked on minimal plates

containing 0.5 µg/mL cycloheximide as a double-check (Akada *et al.*, 2002). For counter-selection, transformants were grown at 28°C for 1 day in liquid YPGal medium and then cells were spread on YPGal plates at approximately 100 cells/plate. Colonies that grew on YPGal plates after 36–48 hours incubation at 28°C were selected. Confirmation of integration of the correct product was confirmed by sequencing (data not shown). Several other plasmid constructs were attempted, including those for genes *HSP10*, *SIP18, AHP1* and *HXT5*, however these were unsuccessful and due to time limitations were not completed.

Table A.2: High Nitrogen Efficiency deletions in haploid wine yeast C911D

Strains name	Construction	Descriptions of HNE genes (adapted from Saccharomyces Genome Database)
C911D *ptp1*::KanMX4	Maria Astorga	Phosphotyrosine-specific protein phosphatase that dephosphorylates a broad range of substrates in vivo, including Fpr3p; localized to the cytoplasm and the mitochondria; proposed to be a negative regulator of filamentation
C911D *ssn8*::KanMX4	Maria Astorga	Cyclin-like component of the RNA polymerase II holoenzyme, involved in phosphorylation of the RNA polymerase II C-terminal domain; involved in glucose repression and telomere maintenance Also known as: CycC
C911D *YNL134C*::KanMX4	Maria Astorga	Putative protein of unknown function with similarity to dehydrogenases from other model organisms; green fluorescent protein (GFP)-fusion protein localizes to both the cytoplasm and nucleus and is induced by the DNA-damaging agent MMS
C911D *leu3*::KanMX4	Maria Astorga	Zinc-finger transcription factor that regulates genes involved in branched chain amino acid biosynthesis and ammonia assimilation; positively regulated by alpha-isopropylmalate, an intermediate in leucine biosynthesis
C911D *ecm33*::KanMX4	Maria Astorga	GPI-anchored protein of unknown function, has a possible role in apical bud growth; GPI-anchoring on the plasma membrane crucial to function; phosphorylated in mitochondria; similar to Sps2p and Pst1p
C911D *ngr1*::KanMX4	Jennie Gardner	RNA binding protein that negatively regulates growth rate; interacts with the 3' UTR of the mitochondrial porin (*POR1*) mRNA and enhances its degradation; over-expression impairs mitochondrial function; expressed in stationary phase
C911D *gid7*::KanMX4	Jennie Gardner	Protein of unknown function, involved in proteasome-dependent catabolite inactivation of fructose-1,6-bisphosphatase; contains six WD40 repeats; computational analysis suggests that Gid7p and Moh1p have similar functions
C911D *psr1*::KanMX4	Jin Zhang	Plasma membrane associated protein phosphatase involved in the general stress response; required along with binding partner Whi2p for full activation of STRE-mediated gene expression, possibly through dephosphorylation of Msn2p
C911D *pst1*::KanMX4	Jin Zhang	Cell wall protein that contains a putative GPI-attachment site; secreted by regenerating protoplasts; up-regulated by activation of the cell integrity pathway, as mediated by Rlm1p; upregulated by cell wall damage via disruption of *FKS1*
C911D *syn8*::KanMX4	Jin Zhang	Endosomal SNARE related to mammalian syntaxin 8 (Syn8p)
C911D *slt2*::KanMX4	Jin Zhang; not confirmed	Serine/threonine MAP kinase; involved in regulating maintenance of cell wall integrity, progression through the cell cycle, and nuclear mRNA retention in heat shock; required for mitophagy and pexophagy; affects recruitment of mitochondria to the phagophore assembly site (PAS); regulated by the PKC1-mediated signaling pathway
C911D *sps2*::KanMX4	Jin Zhang	Protein expressed during sporulation, redundant with Sps22p for organization of the beta-glucan layer of the spore wall; *Schizosaccharomyces pombe* ortholog is a spore wall component

Phenotype Microarrays

Phenotype microarrays were performed by BIOLOG (www.biolog.com, Hayward, CA, USA) as described by Bochner *et al.* (2001).

Generation of hemizygous deletion mutants by mating

Haploid strains were grown in 0.4 mL YPD at 28°C (approximate inoculum: 5 x 10^8 cells mL^{-1}) for 6 hours in 96-well plates. An equal volume of each mating type was combined in the same well of a 96-well plate, centrifuged at 405 x *g* for 2 min and incubated statically for 20 hours at 28°C. Resulting diploids were selected using two rounds of growth in 0.6 mL synthetic minimal galactose medium containing inositol (SC MIN I+ Gal: Sherman, 2002) at 28°C for 20 hours and streaked out on solid SC MIN I+ Gal for selection of single colonies. The ability to sporulate was confirmed on solid potassium acetate media. Ploidy was determined by mating type (MAT) PCR according to Huxley *et al.* (1990).

Generation of hemizygous mutants by gene disruption

5' and 3' templates of gene-specific deletion cassettes were provided by Professor Charles Boone (University of Toronto, Canada). The primers DC-FWD and KanC5, and DC-REV and KanB2 were used in separate PCRs to amplify the 5' and 3' ends of the deletion constructs respectively (Figure A.1 and Table A.6). The PCRs were performed in 50 µl reactions with 0.25 mM dNTPs, and 0.5 µM of each primer using 0.5 U Phusion Hot Start DNA polymerase (Finnzymes), over 40 cycles at 95°C, 30 s; 55.5°C, 30 s; 72°C, 45 s. The PCR products were separated by gel electrophoresis and the paired products combined and purified together using the Wizard® SV Gel and PCR Clean-Up System (Promega). The purified DNA was used for yeast transformation as described by Gietz *et al.* (1992). Transformants were selected on YPD agar containing 200 mg/L geneticin (G418 sulfate; Amresco). PCR analysis was used to confirm the identity of the gene disruptions (Table A.6).

Table A.3: PCR primers for construction of yeast deletion strains

Primer	Sequence (5' → 3')
G418F	AAAAGACTCACGTTTCGAGGC
G418R	CGAGCATCAAATGAAACTGC
HXT1A	TGAGAAAAAGAATATTCATCAAGTGC
HXT1D	GAACTATGTATAGCGCCTATGTGGT
HXT2A	CGACAACAATAAGTCGCCCATTTTTTAT
HXT2D	TCAATACCTCGAAGCAGCGTTTCA
HXT3A	GGAGAAAGAAACAACTCAATAACGA
HXT3D	GTTTTCTAACTCTACTGCGCACTT
GDH2A	GCAATTGGGGTAAAAATACACATTA
GDH2D	AGTTTTTATTGATTTTCGATCGTTG
GLC3A	CTGATGAGCTTTCACTATCTCTTCC
GLC3D	AACCAATGCTTCGTTTTAGACATAC
ALD6A	CATCCAGCTTCTATATCGCTTTAAC
ALD6D	AATATGATTTTGTGTGGGATGTTTT
INO1A	CTTTTTCTTCGTTCCTTTTGTTCTT
INO1D	TTGTGTTTTTCAGCTTCTTCTTTCT
HAP4A	TTAATTCCTTCACCTCTCTAAACCC
HAP4D	AACGGATATGTGAAAATGCTCTTAG
GPH1A	GAGGAAATAAGTAAGGGGTGGATAA
GPH1D	TTCGAGTAAGTGATAATAAGGACCG
RCK1A	GATTTTCAGTTATATGGAGTGGGTG
RCK1D	CAAGTTTTTCCGTGTTTCTAGGATA

Table A.4: PCR primers for the construction of over-expression plasmids

Primer	Sequence (5' → 3')*
TDH3-1	GGGCGGCCGGACGTCTTTATCATTATCAATACTGCCATTTC
TDH3-2	GGGCGGCCGCCTAGGTTTATCATTATCAATACTGCCATTTC
TDH3-3	GGGACTAGTGACGTCTTTATCATTATCAATACTGCCATTTCA
TDH3-4	GCATCTAGACCATGGTTGTTTGTTTATGTGTGTTTATTCGA
TDH3-5	GCACCCGGGCCATGGTTGTTTGTTTATGTGTGTTTATTCG
ADH2-NCO	GGGCCATGGATGTCTATTCCAGAAACTCAAAAAG
ADH2-XBA	GGGTCTAGAAATTTCTGGTAAACTGGATAAGC
ADH2-UPEAG	GAGCGGCCGTCTTTATCAACGAAGGGC
ADH2-UCAVR	GGGCCTAGGACCAAGAAGAAACAAGAAGTGA
ADR1-NCO	GGGCCATGGATGGCTAACGTAGAAAAACCAAAC
ADR1-XMA	GGGCCCGGGATCGACGAAATCAGGTTGGT
ADR1-UPEAG	ATACGGCCGGGGGAAGGTTATATCTTTG
ADR1-UCAAT	GGGGACGTCGTAGCAGAATATTTTTCTGAAGTG
VID24-NCO	GGGCCATGGATGATCAATAATCCTAAGGTAGACA
VID24-XMA	GGGCCCGGGATTCTTGATATGTTACCAG
VID24-UPSPE	GGGACTAGTATAATGAAACGGGGATGG
VID24-UCAAT	TTTGACGTCCTTGCGATATGTGTTGG

*Underlined text denotes restriction site

Table A.5: Plasmids, primers and insertion sites for construction of integrative over-expression cassettes

Plasmid	Promoter Fowardprimer	Promoter Reverse primer	5' Promoter restriction sites	Insertion of gene	3' Promoter restriction sites	Insertion of upstream sequence	Linearisation restriction site for transformation
pCMTDH3A	TDH3-1	TDH3-4	NcoIXbaI	pCMTDH3AADR1	EagIAatII	pCMTDH3AADR1^{UP}	SphI
pCMTDH3B	TDH3-2	TDH3-4	NcoIXbaI	pCMTDH3BADH2	EagIAvrII	pCMTDH3BADH2^{UP}	AocI
pCMTDH3E	TDH3-3	TDH3-5	NcoIXbaI	pCMTDH3EVID24	SpeIAatII	pCMTDH3EVID24^{UP}	AflII

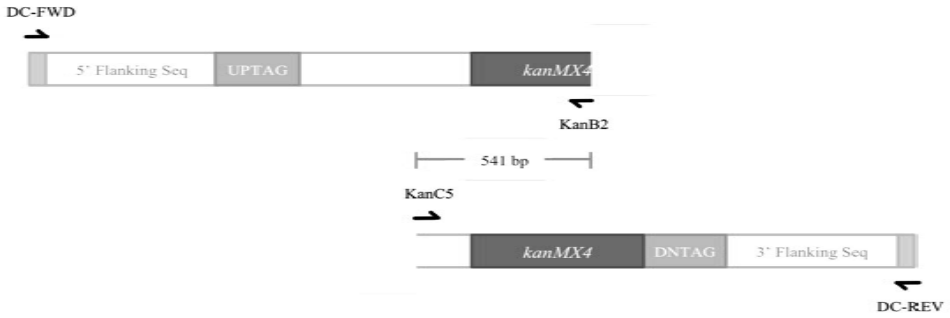


Figure A.1: Two overlapping DNA fragments used for gene disruption by homologous recombination. Separate halves of the deletion construct were amplified from 5' and 3' DNA templates provided by Professor Charles Boone (University of Toronto) using primers as indicated (see Table F.8). Note the 541 bp of overlapping sequence is smaller than the original 700 bp overlap in order to accommodate a primer with increased annealing efficiency.

Table A.6: Oligonucleotides used for PCR and expected PCR product sizes

Primer Name	Sequence (5' → 3')	Primer Pair	Expected Product Size (bp)
DC-FWD [a]	GACTGTCACTGATCGTACATGC	KanB2	*
DC-REV [a]	CCAAGTCTGACTATCGTAGTGC	KanC2	*
KanB [c]	CTGCAGCGAGGAGCCGTAAT	#	#
KanB2 [a e]	ATTACGCTCGTCATCAAAATCAC	DC-FWD KanC2	* 541
KanC [c]	TGATTTTGATGACGAGCGTAAT	#	#
KanC5 [b e]	ACAGTTCTCACATCACATCCGA	DC-REV KanB2	* 541
MAT [f]	AGTCACATCAAGATCGTTTATGG	MATa MATα	492 369
MATa [f]	ACTCCACTTCAAGTAAGAGTTTG	MAT	492
MATα [f]	GCACGGAATATGGGACTACTTCG	MAT	369
YBR112C_A [d]	ATTTTATTTTTCTTTTCTTCGGTCG	KanB YBR112C_B	627 939
YBR112C_B [d]	TGAGACCATTTACCCTGATGTTTAT	YBR112C_A	939
YGR253C_A [d]	ACTTTTGCAACTACTGCTGAAGACT	KanB YGR253C_B	624 475
YGR253C_B [d]	CTCCACACCTAGTACAACACCTTCT	YGR253C_A	475
YKL145W_A [d]	TATATGCATCTGGAACACACATACC	KanB YKL145W_B	584 808
YKL145W_B [d]	CACCTACACGCATACCTTCTTCTAT	YKL145W_A	808
YML112W_A [d]	ATTCATTTCCAGCATACCACTAAAA	KanB YML112W_B	571 620
YML112W_B [d]	AACCAAGTCTATGTCCATGAGTAGC	YML112W_A	620

[a,b] primers used for amplification of deletion cassettes. [a] primers provided by Professor Charles Boone (University of Toronto). [c,d] primers used for strain verification. [d] strain-specific primers (Winzler *et al.*, 1999; see website http://www-sequence.stanford.edu/group/ yeast_deletion_ project/deletions3.html). [e] primers used for screening of putative transformants. [f] primers used for MAT PCR (Huxley *et al.*, 1990). * primer pairs and expected product sizes of various sizes depending on individual deletion cassette.

Intra-specific hybridisation of wine yeast

Spore-spore mating of labelled cells

Sporulation was performed on solid potassium acetate media for 7 days at 30°C. The degree of sporulation was checked under the microscope, prior to overnight digestion (30°C) in 1 mL of sterile MQ water with 15 µL β-glucoronidase. The suspension was vortexed, and washed twice (centrifugation of cells, and resuspension) in YPD before a final resuspension in 1 mL YPD.

Spore labelling

Spore labelling and FACS (fluorescence activated cell sorting) was according to Attfield *et al.* (1998) with minor modification. For labelling with epicocconone, 1 x 10^7 cells were pelleted by centrifugation and resuspended in 50 µL YPD. Two µL of epicocconone (1 mg/mL) and 48 µL sterile MQ was added to give a final concentration of 4.8 µM, and thoroughly mixed before incubation at ambient temperature in the dark for 45 min.

For labelling with CT-BODIPY-Green, 1 x 10^7 cells were pelleted by centrifugation and resuspended in 950 µL YPD with 10.53 µM of CT-BODIPY-Green. The cells were thoroughly mixed and incubated at

30°C in the dark for 45 min. The unbound label was removed by washing 3 times with 1 mL YPD, before transferring to a new tube and repeating the washing step 3 times. The cells were resuspended in 0.5 mL YPD per 1 x 10^7 cells.

Mating of labelled yeast cells

0.5 mL of each of labelled strains was added to a tube, mixed by vortexing, and pelleted by centrifugation. The pellet in YPD was incubated at 25°C for 19-20 h (in the dark).

Fluorescence Activated Cell Sorting (FACS) sorting of dual-labelled yeast cells and microdissection of zygotes

The cells were spun down and resuspended in 1-2 mL phosphate buffered saline for FACS sorting with a BD FACS Aria (at IMVS). Unlabelled and singularly labelled cells of each strain (1 x 10^7 cells) were used as FACS compensation controls. The samples were vortexed prior to sorting. 40,000 to 50,000 events were sorted, of which the fraction containing dual labelled cells represented ~1%. The cells were concentrated by centrifugation and resuspended in a small volume of YPD prior to micro-dissection of the zygotes onto thin YPD agar plates, and grown 2 days at 30°C. *NB*: very few zygotes were identified with most of the cells present as doublets in the sample.

Molecular Typing of wine *Saccharomyces cerevisiae* by Delta sequence typing

Genomic DNA was prepared from the strain S288C according to Adams *et al.* (1998). Delta sequencing was performed according to Schuller *et al.* (2004) with minor modifications to the primers and protocol (see Table A.7). Amplification reactions were performed using 100 pmols each of primers δ1A/T and δ2 (primer set 1) or δ2 and δ12 (primer set 2) in 50 µL reactions with 1U DyNAzyme Ext DNA polymerase (Finnzymes), 3.5 mM $MgCl_2$ and 0.2 mM dNTPs. After initial denaturation (95°C, 2 min), the reaction mix was cycled firstly for 2 cycles (95°C: 30 s, 42°C: 30 s, 72°C: 2 min) followed by 29 cycles (95°C: 30 s, 45°C: 30 s, 72°C: 2 min), followed by a final extension of 72°C for 10 min. The amplification products were separated on a 1.5% (w/v) agarose gel containing ethidium bromide or Biotium Gel Red™ (Biotum), and visualised under UV light.

Table A.7: Primers used in Delta sequence typing

Primer	Sequence (5'-3')
δ1A/T	CAAAATTCACCTAA(A/T)TCTCA
δ2	GTGGATTTTTATTCCAACA
δ12	TCAACAATGGAATCCCAAC

Mitochondrial DNA preparations

Yeast were grown in 100 mL of 2% lactate medium (Supplementary Table 1) for 24 h at 30°C. The cells were pelleted by centrifugation (5,000 rpm for 5 min), and washed in 0.6 M KCl, and centrifuged again. The pellet was washed in 10 mL 0.9 M sorbitol, 0.1 M EDTA pH 7.5, and re-centrifuged before final resuspension in 3 mL of fresh media. The cells were incubated with 33 µL of 3 mg/mL lyticase (Sigma) at 37°C for 60 minutes before re-centrifugation. The cell pellet was resuspended in 5 mL 50 mM Tris pH 7.4, 20 mM EDTA to which 0.5 mL 10% SDS was added.

After 30 minutes incubation at 65°C, 1.5 mL of 5 M potassium acetate was added prior to incubation on ice for 45 min. The suspension was centrifuged in a Beckman centrifuge (JA17 rotor), 10,000 rpm for 10 min at ambient temperature, and the supernatant transferred into a clean tube. The DNA was precipitated with 2 volumes of ethanol at ambient temperature (minimum 1 hr), and subsequent centrifugation (5,000 rpm for 15 min at ambient temperature). The DNA pellet was air-dried and resuspended in 0.4 mL TE (1

mM Tris pH 8.0, 0.1 mM EDTA) and digested with 5 µl RNAse cocktail (Ambien) for 1 hr at 37°C. DNA was precipitated with 1 mL ethanol (10 min, ambient temperature), followed by centrifugation (10 min, ambient temperature, 14,000 rpm). The DNA pellet was air-dried for 20 min, and resuspended in 0.5 mL sterile MQ water.

Mitochondrial restriction fragment length polymorphism (mRFLP) analysis

20 µL DNA was digested overnight in a 50 µL reaction, with 20 units of the following restriction enzymes: *EcoR*V, *Rsa*I or *Hinf*I (according to manufacturer's instructions). The total digest was loaded onto a 0.8% (w/v) agarose gel in TAE buffer, and separated by gel electrophoresis.

Microsatellite analysis

Methods are described in Output F.

Results and Discussion:

Identification of High Nitrogen Efficiency genes: Enhanced fermentation performance of wine yeast deletion mutants under variable nitrogen conditions

A number of genes have been identified as High Nitrogen Efficiency genes: *GID7, NGR1* (Gardner *et al.*, 2005), *LEU3, ECM33, SSN8, PTP1, MIP6, YDL133W, YNL134C* (a selection of these were reported in UA 01/04) and *PUT4* (Poole *et al.*, 2009 and UA 00/5). The HNE phenotype (shortened fermentation duration) was evident for yeast expressing a modified *PUT4* gene, which resulted in constitutive expression during fermentation. This was due to increased uptake of proline and increased tolerance to osmotic stress (Poole *et al.*, 2009).

Fermentation profiles of a number of the *HNE* mutants (in wine yeast) were reported in UA 01/04: Δ*ssn8*, Δ*leu3*, Δ*ptp1*, Δ*ecm33*, Δ*ynl134c*. The fermentations were conducted in nitrogen-limited CDGJM (55 mg FAN/L as amino acids and ammonia). The parental strain of these deletants was not C9, as reported, but a derivative C911D (Walker *et al.*, 2003). Several new deletants (Δ*slt2*, Δ*psr1*, Δ*pst1*, Δ*syn8*, Δ*sps2*) were recently constructed in the same background (C911D; Table A.2). Fermentation performance was evaluated under high and low nitrogen conditions in CDGJM for some of the mutants (Δ*ssn8*, Δ*leu3*, Δ*ptp1*, Δ*ecm33*, Δ*ynl134c*) as shown in Table A.8. All of the mutants conducted fermentation within 10 % of the parent strain under both high and low nitrogen containing CDGJM, with the exception of Δ*ecm33*. This mutant performed significantly faster in both high (69.9 ± 4.8%) and low nitrogen (57.8 ± 1.8%) when compared with the parent strain C911D. The ability of this mutant to retain the HNE phenotype in unsupplemented white juice (~125 - 150 mg FAN/L) is promising given the fermentation duration was shortened by ~2 days, which has obvious economic implications (efficient turnover of tank space). Ecm33p, a GPI-anchored protein of unknown function, is only functional when anchored to the plasma membrane (Terashima *et al.*, 2003). Although similar to Pst1p, the latter does not have the same HNE phenotype in fermentation (Table A.8) and sensitivities to stress inducing compounds such as calcofluor white, hygromycin B and caffeine (Pardo *et al.*, 2004). Ecm33p is the current focus of our research due to dramatic improvement of fermentation when it is deleted. We would like to understand how deletion of this protein enables the yeast to withstand the stresses imposed during fermentation, concomitant in a reduction in fermentation duration. We speculate that the HNE phenotype is a secondary effect from the suppression of the growth defect (resulting from abnormal cell wall structure) by the high external sugar content of the juice. The sugar, acts as an external osmo-protectant, whilst initiating the cell wall damage response pathway, similar to protoplasts being maintained in hyper-osmotic concentrations of sugar prior to regeneration of the cell walls (Guthrie and Fink, 1991).

Screening of the over-expression library in a wine yeast (currently still under construction, Output F) will be useful in identifying other genes with the HNE phenotype, specifically under nitrogen-limited conditions. Currently, this is not possible with the commercially available deletion libraries (in lab yeast) because of the auxotrophic requirement. Recently, the AWRI has offered to make available their deletion library in the wine yeast AWRI 1631, for purposes of conducting fermentation screens to look at specific fermentation traits such as nitrogen utilisation. Utilisation of this library for these purposes would clearly be very useful and may be possible via UA 11/01.

Table A.8: Fermentations conducted by C911D and HNE mutants in CDGJM, Riesling and Chardonnay Juice

Triplicate 100 mL scale ferments were performed in CDGJM (Henschke & Jiranek, 1993; McBryde *et al.*, 2006) and two white grape juices; Chardonnay and Reisling in shake flasks, as described in Methods. Tabulated results represent the time taken to complete fermentation (< 2.5 g/L residual sugar), expressed relative to that of the parent (as a percentage).

CDGJM fermention				
	High nitrogen (450 mg FAN/L)		**Low nitrogen (55 mg FAN/L)**	
Strain	**Duration (hrs)**	**% of parent**	**Duration (hrs)**	**% of parent**
C911D	150.50 ± 0.00	100	590.92 ± 14.43	100
C911D *ssn8*::*KanMX4*	129.97 ± 7.23	86.35 ± 4.80	559.25 ± 26.41	94.64 ± 4.48
C911D *ecm33*::*KanMX4*	105.17 ± 7.22	69.88 ± 4.79	341.75 ± 10.83	57.84 ± 1.83
C911D *leu3*::*KanMX4*	153.94 ± 5.96	102.29 ± 3.96	559.08 ± 26.27	94.61 ± 4.45
C911D *ptp1*::*KanMX4*	138.00 ± 0.00	91.69 ± 0.00	590.92 ± 14.43	100.00 ± 2.44
C911D *ynl134C*::*KanMX4*	141.33 ± 2.89	93.91 ± 1.92	599.58 ± 22.88	101.47± 3.87

Grape juice fermentation				
	Fermentation Duration Time		**% of parent**	
Strain	**Chardonnay** (152 mg YAN/L)	**Riesling** (124 mg YAN/L)	**Chardonnay**	**Riesling**
C911D	267.33 ± 8.08	341 ± 11.79	84.23 ± 5.77	80.94 ± 2.03
C911D *ecm33*::*KanMX4*	225.17 ± 15.43	276 ± 6.93		

Further analysis of high nitrogen efficient genes *NGR1* and *GID7*, construction of a wine yeast double mutant

As described, *NGR1* and *GID7* were discovered as nitrogen efficient genes (Gardner *et al.*, 2005 and UA 01/04) and as previously reported construction of a double deletion of these in a laboratory yeast strain (W303) resulted in a further reduction of fermentation duration. However, we wished to evaluate whether this further enhancement was transferrable to a wine yeast strain.

Ngr1p is an RNA binding protein that interacts with the 3' UTR of the mitochondrial porin (*POR1*) mRNA and enhances its degradation (Buu *et al.*, 2004; Lee & Moss, 1993), through the interaction of Dhh1p (Chang & Lee, 2011). It is expressed during stationary phase (Martinez *et al.*, 2004) and is thought to negatively regulate growth rate (Akada *et al.*, 1997) and overexpression impairs mitochondrial function (Buu *et al.*, 2004).

Gid7p is involved in proteasome-dependent catabolite inactivation of fructose-1,6-bisphosphatase (Regelmann *et al.*, 2003) and apart from increasing nitrogen efficiency in low nitrogen conditions, we previously have shown that the transcription of *GDH2* is dramatically increased when *GID7* is deleted (Gardner *et al.*, 2005). The predominant function of Gdh2p is to release ammonium and α-ketoglutarate from glutamate as part of Central Nitrogen Catabolism. Gdh2p is functional when cells are grown on glutamate or nitrogen sources that are glutamate precursors, thereby making ammonium available for the formation of glutamine.

Construction of a wine yeast strain harbouring whole gene deletions of *NGR1* and *GID7* proved quite troublesome, single deletions were constructed in C911D (haploid derivative of L2056, as reported in UA 01/04). However, a double deletion in this strain seemed to not be viable. We eventually managed construction in a related strain, DR4 (a *ho* mutant constructed using seamless gene modification, UA 01/04). Strains harbouring a single gene deletion of either *NGR1* or *GID7* were constructed in both mating types of DR4 (20a and 20c), as detailed previously (Gardner *et al.*, 2005). Strains DR4-20a *ngr1* and DR4-20c *gid7* were then mated, sporulated and double homozygous deletions identified by Southern blot hybridisation (Figure A.2).

The fermentation dynamics of a *NGR1 GID7* double deletion mutant in DR4 were then analysed as was in Gardner *et al.* (2005), in CDGJM with 75 mg FAN/L (as a complex mix of amino acids and ammonium). However we found that in this wine yeast strain, there was no extra benefit (no extra decrease in fermentation duration) with the deletion of both genes. For instance the DR4 parent completed fermentation in 218 hrs, DR4-20a *ngr1* in 156.5 hrs, and DR4-20c *gid7* or DR4 *gid7 ngr1* each completed in 192 hrs. It was unexpected that the double deletion did not complete in at least the same time as the fastest mutant (Δ*ngr1*). This infers that there may be some (albeit distant) interaction between these gene products. Certainly also, the effect on fermentation of mutants of *NGR1* and *GID7* appears to be different between lab and wine yeast.

To further analyse the function of these genes in fermentation, we attempted to analyse their expression profile during fermentation with the use of Real Time PCR. However, due to technical difficulties this could not be completed within the time constraints of this project.

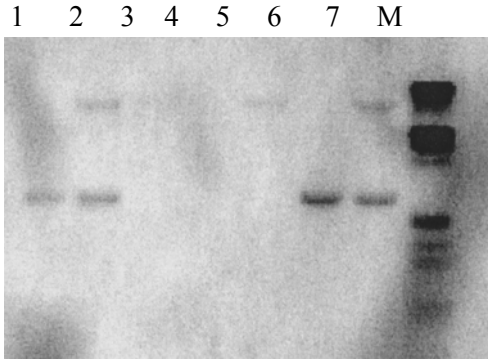


Figure A.2: Southern blot hybridisation of *EcoR*1 digested genomic DNA from DR4-20a *ngr1* (lanes 1 and 6), DR4-20c *gid7* (lanes 3 and 5) and DR4-*ngr1 gid7* double deletions (lanes 2 and 7) A G418 specific probe (generated with gene specific primers $G418^F$ and $G418^R$) was used.

Analysis of *NGR1* and *GID7* function utilising phenotype arrays

We also analysed these gene mutants in phenotype arrays and in the metabolomic studies that were underway in the laboratory (Section H). Metabolomic data is unfortunately yet to be fully analysed. However phenotype arrays (performed with the laboratory yeast strain versions W303 *ngr1* and W303 *gid7*) did reveal the following: deletion of *NGR1* resulted in resistance to piperine and 100 mM sodium nitrate, and sensitivity to D,L-serine hydroxamate and deletion of *GID7* also resulted in resistance to 100 mM sodium nitrate and a loss of the specific ability to use the dipeptides Met-Ala and Gly-Glu as nitrogen sources.

Piperine is thought to supress lipid accumulation through the action of diacylglycerol acyl transferase (Kamisaka *et al.*, 2011; Kimura *et al.*, 2006), encoded by *DGA1,* which catalyzes the terminal step of triacylglycerol formation by acylatingdiacylglycerol using acyl-CoA as an acyl donor. Triacylglycerol biosynthesis, a key component of lipid homeostasis, is coordinated with nutritional requirements in yeast (as reviewed in Kohlwein, 2010). Perhaps Ngr1p normally functions as repressor of lipid formation in the presence of piperine.

Sodium nitrate, a common food preservative, affects yeast growth. There is very little information regarding how yeast confer resistance but there are some reports of strains with decreased mitochondrial activity or in stationary phase resulting in resistance (Święciło, 2008). Since both strains display this increased resistance to sodium nitrate, it is possible that there is a common link between NGR1 and GID7, however we suspect that this phenotype may actually be due to the presence of the geneticin resistance cassette *Kan*MX4. Further studies are required to be certain.

D,L-serine hydroxamate is a serine analogue and inhibitor of yeast and bacterial seryl-tRNAsynthetases (Ahel *et al.*, 2005). Amino acyl tRNAsynthetases catalyze acylation of transfer RNAs with their cognate amino acids (in this case serine), and thus possess a central role in assuring faithful translation of RNA to protein. This phenotype may be related to the already reported RNA binding (and potential destabilisation).

Dipeptide utilisation by laboratory yeast is well known to be poor compared to that of other yeast strains (i.e. W303 and S288C *vs* wine yeast strains). Peptide transporters include Ptr2p and Dal5p, and the recently described FOT family (Damon *et al.*, 2011). These transporters are able to use

many types of dipeptides, Ptr2p is known to be able to transport over 100. The loss of the utilisation of specifically these two dipeptides in *GID7* mutant is intriguing given the seeming lack of specificity of peptide transporters. Furthermore Damon and colleagues (2011) did show that Met-Ala depends on Ptr2p and not Dal5p.

Identification of genes in hemizygous deletion mutants which confer haploproficiency during a wine fermentation

Hemizygous diploid mutants possess both an active and inactive copy of a particular gene (Winzeler *et al.*, 1999). Haploproficiency describes the phenotypic response of such mutants, which exhibit an increased fitness when compared with the wild type homozygote. Recently, Delneri and coworkers (2008) identified haploproficiency in a competition experiment using continuous culturing of the yeast hemizygous (heterozygous) diploid deletion library under four aerobic conditions: glucose limited, phosphate limited, nitrogen limited and juice-like conditions. Although the nitrogen limited and juice-like conditions are of interest, the continuous culturing of yeast is very different to the batch culturing of a wine ferment, whereby most of the fermentation is conducted by stationary cells. We asked, in a project undertaken by Thomas Parker, would the hemizygous mutants identified by Delneri *et al.* (2008) also exhibit haploproficiency in a wine context, i.e. enhanced fermentation performance rather than increased fitness?

Ninety five candidate genes were chosen to address this question (Table A.9). Of these 72 had previously been identified by Delneri *et al.* (2008) as haploproficient: 28 encoded subunits of the 26S proteosome, 33 were essential for viability (Giaever *et al.*, 2002), whilst others included 6 dubious ORFs, 2 pseudogenes, as well as *NGR1* and *GID7*, included as controls and because of our ongoing interest in these (Gardner *et al.*, 2005).

Prototrophic hemizygous mutants were constructed to enable experiments to be conducted under nitrogen sufficient and limiting conditions. The genetic background of the mutants was derived from S288C (*MAT*α, prototrophic) and BY4741 (*MAT*a, auxotrophic). For the non-essential genes, the corresponding haploid deletion mutants in BY4741 were crossed with S288C. For the essential genes, the hemizygous mutants were sourced from the heterozygous diploid deletion library in BY4743 (Invitrogen; from Steve Oliver, University of Cambridge), and compared with the parent BY4743. Attempts to generate the mutants in the BY4741/S288c background by transformation with the appropriate deletion cassette proved difficult; firstly in the construction of each of the *ORF::KanMX* cassettes by PCR amplification of two corresponding overlapping halves, and secondly in the yeast transformation, which relied on the formation of each functional cassette from the two halves *in vivo* and integration into the genome, thus conferring resistance to G418. Only two hemizygous mutants were generated, Δ*pup2::kanMX* and Δ*rpt1::kanMX.,* which were included in the initial micro-fermentation evaluation of a total of 97 mutants (sacrificial plate method; 3 time points). 375 mg FAN/L was used as a ‘standard’ concentration of FAN whereas 75 mg FAN/L was used to simulate a FAN-deficient grape must. Nineteen strains which consistently yielded a post-fermentation supernatant refractive index (RI) of less than 95 % of the parental strain ($p < 0.05$) across all three time points were selected for lab scale

Systematic Name	Standard Name	Aliases
YBR212W ^	NGR1	RBP1
YCL039W ^	GID7	MOH2
YBL009W	ALK2	
YBR036C	CSG2	CLS2
YBR084W	MIS1	
YBR112C	CYC8	SSN6 | CRT8
YBR116C		
YBR146W	MRPS9	
YDL008W *	APC11	
YDL053C	PBP4	
YDL088C	ASM4	NUP59
YDL191W	RPL35A	SOS1
YDL193W *	NUS1	
YDR026C		
YDR209C		
YDR240C *	SNU56	MUD10
YDR440W	DOT1	PCH1
YDR532C		KRE28
YEL072W	RMD6	
YER037W	PHM8	
YER048C	CAJ1	
YER061C	CEM1	
YER118C	SHO1	SSU81
YER128W		
YER130C		
YER162C	RAD4	
YER164W	CHD1	
YHL036W	MUP3	
YIL018W	RPL2B	LOT2 | RPL5A
YIL022W *	TIM44	MPI1 | MIM44 | ISP45
YIL060W		
YIL077C		
YIL093C	RSM25	
YIL167W	SDL1	
YIL171W *		HXT12
YIR008C *	PRI1	
YJL115W	ASF1	CIA1
YJL119C		
YJL142C	IRC9	
YJL159W	HSP150	PIR2 | ORF1 | CCW7
YJL208C	NUC1	
YJL214W	HXT8	
YKL099C *	UTP11	
YKR002W *	PAP1	
YLR262C-A	TMA7	RBF7
YLR279W		
YLR283W		
YLR297W		

Systematic Name	Standard Name	Aliases
YLR300W	EXG1	BGL1
YLR312C		
YLR318W	EST2	TERT
YLR414C		
YML003W		
YML069W *	POB3	
YML112W	CTK3	
YMR028W *	TAP42	
YMR139W	RIM11	MDS1 | GSK3
YMR145C	NDE1	NDH1
YMR160W		
YMR190C	SGS1	
YMR231W	PEP5	VPT11 | VPS11 | VPL9 | VAM1 | END1
YMR296C *	LCB1	TSC2 | END8
YMR306C-A		
YNL159C	ASI2	
YNL206C	RTT106	
YNR056C	BIO5	
YOL152W	FRE7	
YOR043W	WHI2	
YOR363C	PIP2	OAF2
YPL056C		
YPL072W	UBP16	
YDR394W *	RPT3	YTA2 | YNT1
YOL038W *	PRE6	
YDL007W *	RPT2	YTA5 | YHS4
YDL097C *	RPN6	NAS4
YDL147W *	RPN5	NAS5
YDR427W *	RPN9	
YER012W *	PRE1	
YER021W *	RPN3	SUN2
YFR004W *	RPN11	MPR1
YFR050C *	PRE4	
YFR052W *	RPN12	NIN1
YGL011C *	SCL1	PRC2
YGL048C *	RPT6	SUG1 | SCB68 | CRL3 | CIM3
YGR135W	PRE9	
YGR253C *	PUP2	DOA5
YHR200W	RPN10	SUN1 | MCB1
YIL075C *	RPN2	SEN3
YKL145W *	RPT1	YTA3 | CIM5
YMR314W *	PRE5	
YOR117W *	RPT5	YTA1
YOR157C *	PUP1	
YOR261C *	RPN8	
YOR362C *	PRE10	
YPR103W *	PRE2	SRR2 | PRG1 | DOA3

Table A.9: List of candidate genes selected for fermentation analysis in CDGJM under high and low N conditions to evaluate their haploproficiency. Unless shaded or marked with ^, all genes were identified as haploproficient in all four conditions evaluated by Delneri *et al.* (2008). * genes essential for viability. The genes highlighted by shading and semi-shading form part of the 26S proteasome. ^ genes previously identified as important in nitrogen efficiency during fermentation (Gardner *et al.*, 2005).

fermentation (Figure A.3). Four genes were identified as being haploproficient for wine fermentation: *HSP150, IRC9, RMD6* and *RPL2B* under FAN-limited conditions (*ca* -91.4 ± 3.4, -91.4 ± 3.4, -57.1 ± 3.4, and -57.1 ± 3.4 hours *cf* parent), and *HSP150* under FAN-sufficient conditions (*ca* -48.1 ± 3.4 hours *cf* parent). Hsp150p is an O-mannosylated heat shock protein that is secreted and covalently attached to the cell wall via beta-1,3-glucan and disulfide bridges. It is required for cell wall stability and is induced by heat shock, oxidative stress, and nitrogen limitation. *IRC9* appears not to encode a protein, although the sequence partially overlaps that of YJL141C, which encodes Yak1p, a serine-threonine protein kinase, involved in glucose-sensing (involving the RAS/PKA signalling pathway and stress responsive transcription factors Msn2p and Hsf1p) and inhibiting growth in response to glucose availability. The remaining two genes, *RPL2B* and *RMD6*, encode for Rpl2bp, a protein component of the large ribosomal subunit, and Rmd6p, a protein required for sporulation. The role of these genes in fermentation is unclear. Further work is required to determine this as well as examine ways of utilising these genes in future strain constructs and optimisation.

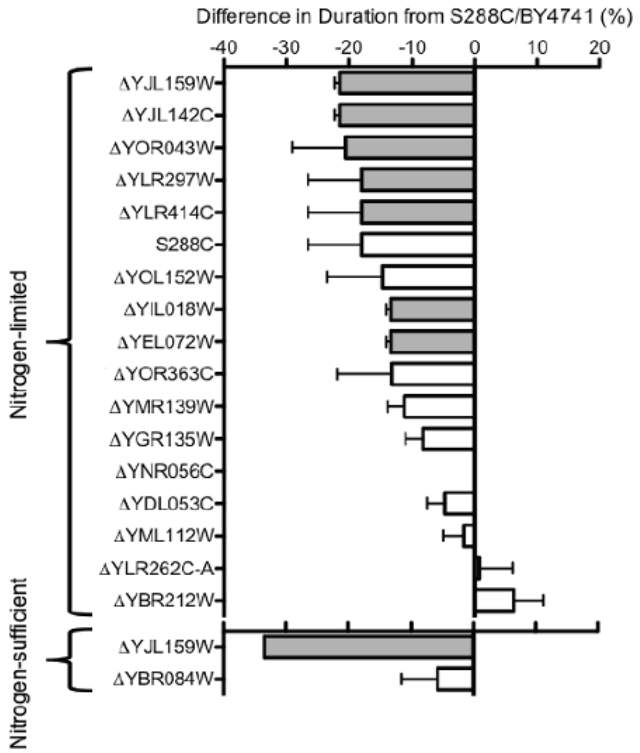


Figure A.3: Percentage difference in fermentation duration relative to the parent strain S288C/ BY4741 for hemizygous mutant strains analysed in 100 mL ferments. Fermentations were performed in CDGJM under two different FAN concentrations: FAN-sufficient (375 mg/L) and FAN-limited (75 mg/L). Shaded bars indicate significantly different values. Common names of the systematic gene names listed are found in Table A.9.

Genes encoding subunits of the 26S proteasome, previously identified as haploproficient in a FAN-limited culture (Delneri *et al.*, 2008), posed interesting candidates for haploproficiency in a wine fermentation because of the proposed conservation of proteins from having a less active 26S proteasome, under FAN-limited conditions. Most of wine fermentation is spent in stationary phase, where very little if any cellular growth occurs, and thus decreased turnover of proteins might help the yeast endure the multiple stresses of fermentation. Interestingly, deletion of many of the genes of the proteasome, as hemizygous mutants, produced behaviour similar to the parent

strain (data not shown). However, the deletants in other proteasome genes (*PUP2, RPN3, RPN9, RPN12* and *PRE6*) exhibited haploinsufficiency in FAN-limited media, in that they consistently catabolised less sugar than the parent strain (data not shown). This finding is of interest with regards to the identification of genes critical for fermentation. Halving the gene dosage for these genes in a diploid, results in decreased fermentation performance, whilst complete deletion is lethal to the cell as all five genes are essential for viability. As such, they were not present in the homozygous diploid library used for the HSF screen (Output F) and thereby an advantage of this approach is that it allowed a more comprehensive screening of the genome for genes important to fermentation. *DOA4*, encoding ubiquitin isopeptidase, was identified in the HSF screen wherein deletion of both copies of the gene resulted in stuck fermentation. Doa4p is required for recycling ubiquitin from proteasome-bound ubiquitinated intermediates, acting at the late endosome/prevacuolar compartment to recover ubiquitin from ubiquitinated membrane proteins en route to the vacuole.

Analysis of the fermentation dynamics of key gene mutants, as identified by transcriptome analysis of FM5 and FM16

As described in Final Report UA 01/04, Section 3.2.2, analysis of the transcriptome of the adaptively evolved strains FM5 and FM16 led to the identification of a number of genes with differential expression during fermentation. To follow on from this work we constructed a series of deletion and overexpression strains in an attempt to replicate some or all of the fermentation phenotypes of these strains. Where a gene was found to be down-regulated in the mutant (FM5 or FM16), the fermentation profile of a mutant harbouring a single deletion of that gene was analysed, and likewise where an increase in expression was recorded, an overexpression mutant was constructed and analysed. Overexpression studies were only carried out in haploid strains due to appropriate background strain availability and time constraints.

Analysis of the fermentation dynamics of key gene deletion mutants relevant to FM5 and FM16

Unfortunately no strains constructed in C911D with gene deletions were found to have a reduced fermentation duration (Table A.10). However, disruption of three (Δ*glc3*, Δ*ald6*, Δ*hxt3*) of the seven genes in C911D, were found to increase fermentation duration, considered as an undesirable trait. Aside from these, the remaining fermentations yielded no strain differences in the duration to catabolise available sugar.

There were a number of alterations in metabolites accumulated at the end of fermentation (Table A.10). For example four strains had altered succinic acid accumulation (Δ*hxt3*, Δ *hxt2*, Δ*gdh2* and Δ*glc3*). The lactic acid produced by Δ*hxt3* was almost double that of the parent (0.61 *vs.* 0.33 g/L), contrasting to this Δ*ald6* only produced 17 % of the lactic acid of that produced by C911D. Four strains had increased glycerol production (Δ*hxt1*, Δ*hxt3*, Δ*ald6* and Δ*ino1*), of greatest note, deletion of *ALD6* resulted in 1.1 g/L (16 %) more glycerol than that of C911D. The yield of acetic acid was significantly reduced in Δ*ald6* and Δ*hxt3*, while increased in Δ*hxt1*. With regard to acetaldehyde production and ethanol production, again significant variations were seen.

Table A.10: **Dry weight, fermentation duration and major metabolites present in terminal fermentation samples of C911D deletion strains**

	Duration hr	Succinic Acid g/L	Lactic acid g/L	Glycerol g/L	Acetic acid g/L	Acetaldehyde g/L	Ethanol g/L	Dry weight g/L
C911D	62.0 ± 2.0	0.73 ± 0.01	0.34 ± 0.01	7.09 ± 0.08	1.39 ± 0.03	0.03 ± 0.00	102.4 ± 1.2	6.2 ± 0.2
C911D *hxt1*	100 ± 2.0	95.6 ± 0.1	105.8 ± 0.1	106.5 ± 0.1	106.7 ± 0.1	120 ± 0.1	91.2 ± 4.3	104.6 ± 0.2
C911D *hxt2*	100 ± 2.0	90.5 ± 0.1	96.7 ± 0.2	104.3 ± 0.1	105.7 ± 0.1	130 ± 0.1	92.6 ± 3.9	114.5 ± 0.5
C911D *hxt3*	130.6 ± 2.0	140.7 ± 0.1	179.6 ± 0.1	106.1 ± 0.1	86.8 ± 0.1	60 ± 0.1	94.2 ± 1.4	105.4 ± 01.3
C911D *gdh2*	100 ± 2.0	90.3 ± 0.1	105.3 ± 0.1	102.4 ± 0.1	104.4 ± 0.1	120 ± 0.1	91.5 ± 4.8	111.7 ± 0.3
C911D *glc3*	104.8 ± 2.0	87.7 ± 0.1	104.0 ± 0.1	102.8 ± 0.1	101.6 ± 0.1	105 ± 0.1	88.7 ± 0.2	102.9 ± 0.1
C911D *ald6*	130.6 ± 2.0	114.4 ± 0.1	17.1 ± 0.1	116.8 ± 0.1	20.8 ± 0.1	200 ± 0.1	94.0 ± 2.0	102.4 ± 0.5
C911D *ino1*	100 ± 2.0	113.9 ± 0.1	105.5 ± 0.1	106.3 ± 0.1	102.2 ± 0.1	120 ± 0.1	91.6 ± 4.5	107.2 ± 0.1
C911D *hap4*	100 ± 2.0	95.2 ± 0.1	98.9 ± 0.1	104.2 ± 0.1	98.8 ± 0.1	120 ± 0.1	86.8 ± 4.2	106.5 ± 0.3
FM5	100 ± 2.0	97.4 ± 0.1	100 ± 0.1	110.3 ± 0.1	109.1± 0.1	120 ± 0.1	86.5± 0.8	108.6 ± 0.1

Values are the mean of triplicate experiments and are shown as a percentage of the parent (C911D) strain ± standard deviation. Absolute values for C911D are shown. Values which were increased or decreased by > 5 % and were also significantly different to the parent, i.e. $p < 0.05$ (Student's t-test) are highlighted by yellow or red respectively.

Table A.11: Dry weight, fermentation duration and major metabolites concentrations present in terminal fermentation samples of L2056 homozygous deletion strains

	Duration hr	Succinic Acid g/L	Lactic acid g/L	Glycerol g/L	Acetic acid g/L	Acetaldehyde g/L	Ethanol g/L	Dry weight g/L
L2056	99.0 ± 2.0	0.42 ± 0.05	0.22 ± 0.03	8.37 ± 0.49	1.64 ± 0.05	0.05 ± 0.00	97.0 ± 4.8	4.41 ± 0.35
L2056 *ald6*	60.6 ± 2.0	78.2 ± 0.1	105.3 ± 0.1	113.2 ± 0.1	102.6 ± 0.1	207.4 ± 0.1	107.3 ± 8.3	98.4 ± 0.1
L2056 *gph1*	71.2 ± 2.0	90.0 ± 0.1	110.5 ± 0.1	121.8 ± 0.3	98.2 ± 0.1	274.1 ± 0.1	105.3 ± 6.3	100.3 ± 0.1
L2056 *rck1*	75.8 ± 2.0	82.9 ± 0.1	104.2 ± 0.1	90.6 ± 0.1	102.0 ± 0.1	133.3 ± 0.1	107.3 ± 6.8	100.0 ± 0.3
FM16	60.6 ± 2.0	92.6 ± 0.1	93.4 ± 0.1	106.2 ± 0.1	96.1 ± 0.1	140.7 ± 0.1	101.9 ± 10.7	102.3 ± 0.2
L2056-C7	59.3 ± 2.0	105.7 ± 0.1	175.4 ± 0.1	121.1 ± 0.3	99.8 ± 0.2	94.4 ± 0.1	107.7 ± 6.7	104.3 ± 0.4

Values are the mean of triplicate experiments and are shown as a percentage of the parent (L2056) strain ± standard deviation. Absolute values for L-2056 are shown. Values which were increased or decreased by > 5 % and were also significantly different to the parent, i.e. $p < 0.05$ (Student's t-test) are highlighted by yellow or red respectively.

All three homozygous deletion strains (Δ*ald6*, Δ*gph1* and Δ*rck1*) were able to complete fermentation within a shorter duration than the parent (Table A.11; the fastest being 61 % of the duration of L2056). The glycerol concentrations at the end of fermentation by Δ*ald6* and Δ*gph1* were also increased by 1.1 g/L and 1.8 g/L (13.2 % and 21.8 %) respectively, whereas deletion of *RCK1* decreased glycerol accumulation. The other significant finding was the dramatic increase in acetaldehyde Δ*ald6* and Δ*gph1* strains. Interestingly, deletion of *ALD6*, as described here in L2056 (a diploid) has a different effect than deletion from C911D (haploid version of L2056). For instance, L2056 *ald6* has shorter fermentation duration and increased glycerol and acetaldehyde, whereas C911D *ald6* has increased duration, decreased lactic and acetic acid, and increased glycerol and acetaldehyde (Table A.10 *vs.* Table A.11).

Ald6p, which preferentially uses $NADP^+$ (Meaden *et al.*, 1997; Wang *et al.*, 1998), together with other ACDH's is responsible for oxidation of acetaldehyde to acetate during fermentation (Meaden *et al.*, 1997; Remize *et al.*, 2000; Saint-Prix *et al.*, 2004). In *ald6* mutants, the mitochondrial route of acetate formation is operative due largely to the presence of Ald4p, however the cytosolic route can be markedly decreased (thought to be due to the functionality of *ALD2* and *ALD3* - encoding only a minor form of a cytosolic, stress induced, glucose repressed ACDH (Navarro-Avino *et al.*, 1999; Norbeck & Blomberg, 1997). Deletion of *ALD6*, the main cytosolic ACDH, results in a decrease in NADPH formation (Kurita & Ito, 1994). However, the NADP/NAD ratio might also be affected in these mutants, depending on the contribution of other ACDHs that can use NAD (Ald2p and Ald3p (Navarro-Avino *et al.*, 1999)) or NAD and NADP (Ald4p). The increased glycerol production noted in C911D *ald6* and L2056 *ald6*, therefore, could reflect deregulation mechanisms.

Theoretically, an Δ*ald6* mutant may have to achieve redox balance in individual cellular compartments (i.e. cytosol or mitochondria). The compensation of the mitochondrial enzymes for the loss of *ALD6* would most likely result in redox imbalance. The low concentration of acetic acid in C911D, maybe a function of compartmentalisation of ACDH's, that is, the inability of other ACDH's to oxidise acetaldehyde. However, the somewhat unchanged concentration of acetic acid in L2056 *ald6* could be accounted for by Ald4p, as the function of this protein is thought to be strain dependent (Wang *et al.*, 1998).

GLC3 encodes the glycogen branching enzyme involved in glycogen biosynthesis (Rowen *et al.*, 1992; Thon *et al.*, 1992). Branching of glycogen is required for cellular accumulation of glycogen, thereby helping the cell cope with heat or osmotic stress (Parrou *et al.*, 1997), as well as enhancing survival upon deprivation of nutrients such as carbohydrates and assimilable nitrogen (Lillie & Pringle, 1980; Parrou *et al.*, 1999). The initiation of glycogen accumulation is a function of glycogenin, a self-glucosylating protein which initiates biosynthesis acting on UDP-glucose (formed from glucose 1-phosphate) (see review by Francois & Parrou, 2001). Consequently disruption of *GLC3* may allow for the shuffling of glucose 6-phosphate into the production of another storage carbohydrate, trehalose (Lillie & Pringle, 1980; Parrou *et al.*, 1999, 1997), proposed to play a regulatory role in glycolysis, through inhibition of hexokinases, which phosphorylate glucose and fructose (Blazquez *et al.*, 1993). Thus glycolytic capacity of the cell is limited when trehalose accumulates and this might explain the increased fermentation duration in the glc3 deletant.

RCK1 is thought to encode a protein kinase (Dahlkvist & Sunnerhagen, 1994), as well as having a general involvement in meiosis (Ramne *et al.*, 2000). Specifically, Rck1p is implicated in the high osmolarity glycerol (HOG) pathway, through Hog1p, which is thought to suppress protein synthesis after osmotic shock as well as promote intracellular osmolyte production. The change in fermentation phenotype of Δ*rck1* may be a consequence of the HOG pathway, activating a response to high osmolarity (Schwartz & Madhani, 2004). Rck1p is phosphorylated by Hog1p which is known to down-regulate protein synthesis (Teige *et al.*, 2001). Consequently loss of function of *RCK1* may alleviate the negative regulation of Hog1p under conditions of stress, thus allowing sustained protein production by *HOG1* at a sacrifice of osmolyte production, hence the reduced fermentation duration.

Analysis of the fermentation dynamics of key gene overexpression mutants relevant to FM5 and FM16

There were no clear associations between the phenotypes observed in constructed over-expression strains compared to FM5-M, with the exception of the marginal increase in acetic acid concentration in C911D vid24^{UP} (Table A.12). An increase in lactic acid concentration was noted in C911D vid24^{UP} and C911D adr1^{UP}, however, this contrasts to that of the phenotype of FM5, where no difference in production has been found. There was also a significant increase in fermentation duration observed in C911D vid24^{UP}, this too is dissimilar to reported phenotype of FM5. Further analysis is required to explain these discrepencies.

Summary of outcomes from single gene modifications, as identified from transcriptome analysis of FM5 and FM16

a) Deletion of either *HXT1*, *HXT2*, *HXT3*, *GLC3*, *GDH2*, *ALD6* or *INO1* from C911D resulted in fermentation phenotypes similar to that of FM5.
b) Deletion of either *ALD6*, *GPH1*, *RCK1* from L2056 resulted in fermentation phenotypes similar to that of FM16 and L2056-C7.
c) No single deletion resulted in all of the desirable fermentation characteristics of either FM5, FM16 or L2056-C7.
d) Deletion of *ALD6* from C911D and L2056 have different outcomes.

In summary, the complex phenotype of each of the evolved strains is not explained by a single gene modification. We certainly did not expect this to be the case, instead it is most likely that a number of cellular activities have been altered. For this reason, a systems biology approach to further characterise these strains (namely L2056-C7) was undertaken (see Output H). We have however also discovered many gross metabolite changes as direct effects of single gene manipulations, many of these of great significance to industrial winemaking, and this information will be used to further direct future yeast strain development.

Table A.12: Dry weight, fermentation duration and major metabolites present in terminal fermentation samples of C911D over-expression strains

	Duration hr	Succinic Acid g/L	Lactic acid g/L	Glycerol g/L	Acetic acid g/L	Acetaldehyde g/L	Ethanol g/L	Dry weight g/L
C911D	79.0 ± 3.5	0.36 ± 0.01	0.37 ± 0.01	7.66 ± 0.36	1.67 ± 0.02	0.05 ± 0.00	104.4 ± 0.6	5.27 ± 0.31
C911D adh2^{UP}	100.0 ± 2.0	108.3 ± 0.1	112.8 ± 0.1	103.9 ± 0.1	102 ± 0.1	96.7 ± 0.1	102.9 ± 6.2	105.5 ± 0.2
C911D adr1^{UP}	105.5 ± 2.0	93.8 ± 0.1	133.6 ± 0.1	105.4 ± 0.1	103.8 ± 0.1	100 ± 0.1	102.0 ± 4.1	89.1 ± 0.4
C911D vid24^{UP}	116.5 ± 2.0	89.8 ± 0.1	111.9 ± 0.1	105.4 ± 0.1	105.9 ± 0.1	90.9 ± 0.1	100.2 ± 7.1	96.2 ± 0.3
FM5	95.0 ± 2.0	98.6 ± 0.1	114.0 ± 0.1	107.1 ± 0.1	108.7 ± 0.1	97.0 ± 0.1	93.7 ± 0.6	112.8 ± 0.1

Values are the mean of triplicate experiments and are shown as a percentage of the parent (C911D) strain ± standard deviation. Absolute values for C911D are shown. Values which were increased or decreased by > 5 % and were also significantly different to the parent, i.e. $p < 0.05$ (Student's t-test) are highlighted by yellow or red respectively.

Generation of novel commercial wine yeast strains through hybridisation

The hybrid program was initiated as part of the outputs from UA 01/04 and initially focused on generating new strains by sporulation from a number of commercial strains, chosen for their distinct properties. As commercial wine yeast are often heterozygous (Bradbury *et al.*, 2006), it was envisaged that there would be sufficient diversity within the re-diploidised 'selfed' progeny in terms of fermentation attributes. Some of the properties of the commercial strains and their re-diploidised spores measured are reported in the final report UA 01/04.

An alternative method is intra-specific hybridisation that is hybridisation of two strains of the same species. As wine yeast are usually diploid and do not readily mate, the procedure relies on sporulation and mating of the haploid spore progeny prior to their switching mating type and re-diploidising. This form of rare mating is referred to as spore-spore mating.

Commercial strains, with distinct properties (H_2S production, increased tolerance to 12% ethanol, 0.5 M NaCl, and growth at 15°C) were evaluated for their ability to sporulate with reasonable efficiency, with the production of tetrads with 4 viable spores. Three methods for molecular typing were used to differentiate between strains: delta PCR (Schuller *et al.*, 2004), mitochondrial RFLP (Schuller *et al.*, 2004) and microsatellite analysis (Bradbury *et al.*, 2006). Rare mating was performed on sporulating cultures from commercial wine yeast identified as having sufficiently different phenotypes. For each mating set of two strains (sporulating cultures), one strain was labelled with either epicocconone (Beljian Red) and the other with CT-BODIPY Green and the two allowed to mate for 20 hours prior to sorting of dual labelled zygotes by FACS. The zygotes within the collected fraction were isolated by manual micro-manipulation using a dissecting microscope. Approximately 120 hybrids were produced and identified by molecular typing. These strains were evaluated for fermentation performance and effect on colour in both micro-fermentation and lab scale fermentation experiments (100 mL CDGJM or 1 kg lots of grape berries). A number of hybrids produced different colour outcomes (compared with their parents) following fermentation, however, the overall fermentation performance or impact on wine colour was not dissimilar from the parent strains (UA 05/06). A collection of 91 brewing yeast hybrids generated as part of this study, were genotyped by PCR amplification of delta sequences and micro-satellite analysis (UA 05/06). To date, no fermentation trials have been conducted due to limited availability of personnel.

The hybridisation program *per se* was abandoned at this point and the focus of attention was diverted to directed (adaptive) evolution as a means of generating new yeast strains which perform well under sub-optimal nutrient conditions. However, valuable knowledge and insight was gained into intra-specific hybridisation and screening of measurable phenotypes, relevant to fermentation. Firstly, wine *Saccharomyces cerevisiae* are highly evolved, adapted to the harsh environment of the juice ferment, with largely similar performance under supplemented conditions, although there may be differences in their aroma profile, nitrogen requirements, etc. As such, in order to show heterosis (hybrid vigour) within the progeny, that is, an improved phenotype such as fermentation performance under a number of conditions, compared to the parent, a large number of progeny would need to be identified (at least 100 per cross). This was a limitation of the present study as only 120 hybrids were isolated from 20 crosses between commercial wine yeast.

An improvement on the methodology would be to perform the mating with unlabelled cultures as described prior to inoculation into batch ferments; each with an individual stress condition such as low nitrogen, or high sugar. The hybridisation strategy (perhaps in combination with chemical mutagenesis) would increase the genetic diversity as compared to inoculation with only parental

strains. Adaptive evolution, a proven tool in this lab, would be used in a batch fermentation approach, over only 50-100 generations, which is considerably shorter than the usual 3-400 generations reported (McBryde *et al.*, 2006). High through-put screening (robotic handling and micro-ferments) will enable screening of hundreds of colonies under several stress conditions. Those exhibiting desired cross-tolerance phenotypes would be chosen for further evaluation in white juice or grape must (100 mL to 10 L scale).

Conclusion:

This investigation of strains that are nutrient efficient or metabolically distinct has yielded a rich source of information that is directly applicable to future yeast strain development. Also, collectively, a wealth of experience has been attained from this output, which is paramount for success in the next phase of our wine yeast generation strategies. We have produced a large collection of commercial yeast derivatives and non-GMO yeast, which will provide the raw material for future yeast improvement programs.

The list of genes, now known to contribute to nitrogen efficiency during fermentation has been expanded, with *ECM33* of particular interest due to its significant shortening of fermentation duration, being 60-70% of its parent in defined mediums and up to 2 days shorter in grape juices. Four genes have also been found to be haploproficient in both limiting (*IRC9*, *RMD6* and *RPL2B*) and limiting or sufficient (*HSP150*) nitrogen fermentation. Future studies should include evaluation of combinatory mutants, such as that done with *NGR1* and *GID7* in an attempt to further improve desirable characteristics.

So far the probable mechanisms underlying this phenotype seem to be very broad. Connections between known HNE genes are very limited and functional analysis employing phenotype microarrays revealed few obvious connections to fermentation dynamics. We expect that this phenotype is in fact brought about by small perturbations in multiple unrelated metabolic pathways. This is not surprising since nitrogen and carbon metabolism is central to life, and impacts upon many other pathways.

Further identification of more HNE genes would be extremely valuable, a complete list of all possible genes contributing to this phenotype should allow a thorough analysis of all involved pathways. This is now achievable with the recent availability of the AWRI wine yeast deletion library (albeit incomplete), which has no auxotrophies and thus can be screened for HNE genes, in the presence of limited nitrogen.

Analysis of single gene mutants, as identified from transcriptional analysis of adaptively evolved strains revealed the complex associations between genes and the consequences, be it metabolites or dynamics of fermentation. We found that alteration of single genes could result in dramatic metabolic effects, and in some cases these were strain dependent.

The yeast hybridisation program generated valuable insights in to how best to perform these types of intra-specific hybridisations. However, we found that phenotypic variability between hybrids and their parents was quite low, leading to the requirement of a larger than possible program to be undertaken for the successful generation of strains with novel attributes.

Output B: Information on LAB with distinct properties; Optimised bacterial strains and application protocols; 'Special Purpose' LAB identified

4. **2 LAB strains with distinct hydrolytic properties of potential oenological value identified. Enzymatic basis for these defined.**
5. **1 LAB strain with greater enzymatic activities (eg glycosidase, tannase, esterase, anthocyanase) and a method for application in winemaking. Larger-scale/industry trials completed ('07 vintage).**
6. **Strains with specific esterase or glycoside modifying properties identified. Application protocols developed.**

Contributors:

Current PhD students: Alana Capaldo (submitted) and Krista Sumby
Postdoctoral fellows and/or student supervisors: Drs Michelle Walker, Jennie Gardner, Chris Ford, Paul Grbin and Assoc Prof Vladimir Jiranek.

Background:

Investigations undertaken in UA 01/04 included a comprehensive screening of some 50 isolates of *Oenococcus oeni*, *Pediococci* and *Lactobacilli* for tannase, lichenase, cellulose, β-glucanase, xylanase, esterase and various glycosidic activities. With the exception of xylanases, all activities were represented across at least some isolates of all genera. The esterases and β-glucosidases were selected for further characterisation. Specifically the sequences of the structural genes were deduced from comparisons of the genome sequence of the *O. oeni* strain PSU-1 with sequences of similar enzymes from related bacteria. Such sequence information was also used to enable cloning, heterologous expression and characterisation of these enzymes free from other complicating activities (See Output F for full details). Information about the expression patterns of some of these genes was also collected in order to determine the conditions under which their influence might be maximal.

In the case of the β-glucosidases, basic information about their potential contribution to strain growth and metabolism was sought through supplementation experiments using natural glycosidic substrates (e.g. arbutin and salicin). Esterase activities were further characterised through examination of the impact of whole bacterial cells on esters and ester formation in natural substrates.

Methods:

Strains

Several strains including *O. oeni* and *Lactobacillus hilgardii* were used in this part of the project. They were selected based on their possession of high activities against glycosylated or ester substrates (UA 01/05, Grimaldi *et al.*, 2005a, Matthews *et al.*, 2006; Table B.1).

Bioinformatic analysis

Sequences with potential to be a structural gene for the enzymic activities observed in earlier work were sought. Six genes across at least two putative operons (*bgl* and *cel*) were identified (AG1, ORF 1, ORF 2, ORF 3, ORF 4 and ORF 5) based on comparative sequence analysis of the published PSU-1 *O. oeni* genome with glucosidase sequences from other lactic acid bacteria (Makarova *et al.*, 2006). Candidates were identified based on the presence of signature sequences important in GHF 1 and 3 β-

glycosidases (Table B.2). Two candidates, ORF 1 and 5, had a leucine in the place of a methionine as a start codon, the potential impact of which on activity of the gene product was not known. An analogous sequence comparison approach was used to identify putative esterase genes, *estA2*, *estA7*, *estB28* and *estCOo8* from *O. oeni* as well as *estC34* from *L. hilgardii*.

Table B.1: Lactic acid bacteria strains used in this study

Strain	Species	Comment	Origin*
Ooeni8	*Oenococcus oeni*	High esterase activity	CHR Hansen Viniflora
Ooeni28	*Oenococcus oeni*	High esterase activity	Lallemand nuovi Ceppi Oo2
Lac34 (CSCC-5489)	*Lactobacillus hilgardii*	High esterase activity	SCRC
Lalvin 4X (VL92)	*Oenococcus oeni*	High glycosidase activities	Lallemand

* Isolates from commercial starter cultures or purchased from the Australian Starter Culture Research Centre (SCRC)

Table B.2: Putative genes identified in *O. oeni* PSU-1 which may be responsible for β-glucosidase metabolism

Gene	Putative function	Gene length (bp)	Position in genome	Gene ID*
AG1	β-glucosidase/6-phospho-β-glucosidase/β-galactosidase	1332	Complement (211150..212482)	-
ORF 1	β-glucosidase/6-phospho-β-glucosidase/β-galactosidase	1443	Complement (211150..212592)	OEOE_0224
ORF 2	β-glucosidase/6-phospho-β-glucosidase/β-galactosidase	1458	(331260..332717)	OEOE_0341
ORF 3	β-glucosidase/6-phospho-β-glucosidase/β-galactosidase	1446	(329798..331243)	OEOE_0340
ORF 4	β-glucosidase/6-phospho-β-glucosidase/β-galactosidase	1323	(1143928..1145250)	OEOE_1210
ORF 5	β-glucosidase-related glycosidase	2214	Complement (1486489..1488702)	OEOE_1569

*NCBI Gene tag

Growth of *O. oeni* on glycosidic substrates

The *O. oeni* wine strain Lalvin 4X (VL92) was cultured for approximately five days in de Man, Rogosa and Sharpe (MRS) broth supplemented with 10% filter sterilized preservative-free apple juice (commercially available). This culture was used to inoculate 50 mL flasks of MRS (1:100 dilution) made up without meat extract or dextrose, supplemented with 1% (w/v) of arbutin, cellobiose, salicin, arbutin/glucose, cellobiose/glucose or salicin/glucose, which was subsequently filter sterilized. Growth was monitored by measuring the optical density at 600 nm (OD_{600}) and visual confirmation by observing the cells at a 1000 x magnification.

Transcription analysis

Samples (1 mL) were taken from a culture of *O. oeni* strain Lalvin 4X (VL92) at an OD_{600} of 0.5 and 1.0, and centrifuged at 14,000 x *g* for 10 min. After the supernatant was removed, RNA was extracted using the High Pure RNA Isolation Kit (Roche), reverse-transcribed using iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions and quantified using a NanoDrop 1000 (Thermo Scientific). Primers were synthesized by Sigma-Aldrich Australia (Table B.3). PCR amplification was carried out using DyNAzyme Ext (Finnzymes) using the cDNA as a template. The reference genes *ldhD* and *gyrA* were included as they have been validated for *O. oeni* in response to

stress conditions as a useful tool for relative transcript quantification. The resulting cDNA was validated on a 2% (w/v) agarose gel stained with GelRed™ (Biotium, distributed by Jomar Diagnostics) run in 1% TAE buffer at 100 V. The reaction conditions are described in Appendix 5: Supplementary Tables 3 and 4.

Table B.3: Primers used for transcriptional analysis of the *cel* and *bgl* operon

Gene	Primer	5' – 3'	Tm*	Amplicon length (bp)
ldhD	ldhDprobe155Fwd	ggacgtttatgaaaatgagattgg	64.50	73
ldhD	ldhDprobe155Rev	caaacgagcatcagggaact	64.55	
gyrA	gyrAprobe148Fwd	ccaaaggaatcccggttatta	63.79	70
gyrA	gyrAprobe148Rev	acatttataacggcctgaacg	62.92	
celD	celDprobe11Fwd	agcggcctgcgtaaagat	65.16	83
celD	celDprobe11Rev	cctttgccatccacatcc	64.52	
celC	celCprobe44Fwd	cagattgcgcatcgtattga	65.11	67
celC	celCprobe44Fwd	cggataaatcgggcacat	63.72	
bglD	bglDprobe61Fwd	cagcgtcgttattggtttagc	63.71	91
bglD	bglDprobe61Rev	ccggacgaaagttgttctgt	64.43	

*TM, primer melting temperature

Volatile fingerprinting

Malolactic fermentation trails were carried out to determine if the ester profile of wine could be altered in a distinct way by addition of a particular strain of LAB. Two wines were used in this study, a Cabernet Sauvignon and a Chardonnay and up to 8 strains of LAB (Table B.4). Initial and final malic acid concentrations for both wines were determined using a spectrophotometric-based enzymatic analysis kit. Wine was aliquoted into 3x 22 mL GCMS grade glass vials (sterilised by autoclaving) and inoculated according to the table below (Table B.5). Wines were incubated at 21°C without agitation for the duration of malolactic fermentation (MLF). MLF was deemed to be complete when the concentration of malic acid was less than 0.1 g/L. At the completion of MLF all samples were filter sterilised (0.22 μm) and stored in 2x 10 mL GCMS grade glass vials at 4°C to minimise changes in ester concentration due to shifts in water activity when freezing. All samples were analysed within one month.

Table B.4: Esterase activity of LAB strains used in MLF of wine

Strain	Esterase activity[#]	Origin
Ooeni8	High	CHR Hansen Viniflora
Ooeni12	High	Lalvin 3X 1Step
Ooeni28	High	Lallemand nuovi Ceppi Oo2
Ooeni2	Low	Lalvin 4X(VL92)
Ooeni3	Low	Inobacter
Ooeni6A	Low	Lalvin MT01 Standard
Lac34	High	SCRC
Lac40	Low	SCRC

SCRC strains were purchased from the Australian Starter Culture Research Centre. [#]Refers to the level of esterase activity seen by previous experiments in this laboratory.

Table B.5: Samples analysed by volatile fingerprinting

Sample #	Treatment	Wine sample
1	No inoculation	Cabernet Sauvignon
2	No inoculation	Cabernet Sauvignon
3	No inoculation	Cabernet Sauvignon
4	Strain1 (Ooeni8)	Cabernet Sauvignon
5	Strain1 (Ooeni8)	Cabernet Sauvignon
6	Strain1 (Ooeni8)	Cabernet Sauvignon
7	Strain2 (Ooeni12)	Cabernet Sauvignon
8	Strain2 (Ooeni12)	Cabernet Sauvignon
9	Strain2 (Ooeni12)	Cabernet Sauvignon
10	Strain3 (Ooeni28)	Cabernet Sauvignon
11	Strain3 (Ooeni28)	Cabernet Sauvignon
12	Strain3 (Ooeni28)	Cabernet Sauvignon
13	Strain4 (Ooeni2)	Cabernet Sauvignon
14	Strain4 (Ooeni2)	Cabernet Sauvignon
15	Strain4 (Ooeni2)	Cabernet Sauvignon
16	Strain5 (Ooeni3)	Cabernet Sauvignon
17	Strain5 (Ooeni3)	Cabernet Sauvignon
18	Strain5 (Ooeni3)	Cabernet Sauvignon
19	Strain6 (Ooeni6A)	Cabernet Sauvignon
20	Strain6 (Ooeni6A)	Cabernet Sauvignon
21	Strain6 (Ooeni6A)	Cabernet Sauvignon
22	Strain7 (Lac34)	Cabernet Sauvignon
23	Strain7 (Lac34)	Cabernet Sauvignon
24	Strain7 (Lac34)	Cabernet Sauvignon
25	Strain8 (Lac40)	Cabernet Sauvignon
26	Strain8 (Lac40)	Cabernet Sauvignon
27	Strain8 (Lac40)	Cabernet Sauvignon
28	No inoculation	Chardonnay
29	No inoculation	Chardonnay
30	No inoculation	Chardonnay
31	Strain1 (Ooeni8)	Chardonnay
32	Strain1 (Ooeni8)	Chardonnay
33	Strain1 (Ooeni8)	Chardonnay
34	Strain2 (Ooeni12)	Chardonnay
35	Strain2 (Ooeni12)	Chardonnay
36	Strain2 (Ooeni12)	Chardonnay
37	Strain3 (Ooeni28)	Chardonnay
38	Strain3 (Ooeni28)	Chardonnay
39	Strain3 (Ooeni28)	Chardonnay
40	Strain4 (Ooeni2)	Chardonnay
41	Strain4 (Ooeni2)	Chardonnay
42	Strain4 (Ooeni2)	Chardonnay
43	Strain5 (Ooeni3)	Chardonnay
44	Strain5 (Ooeni3)	Chardonnay
45	Strain5 (Ooeni3)	Chardonnay
46	Strain6 (Ooeni6A)	Chardonnay
47	Strain6 (Ooeni6A)	Chardonnay
48	Strain6 (Ooeni6A)	Chardonnay
49	Strain7 (Lac34)	Chardonnay
50	Strain7 (Lac34)	Chardonnay
51	Strain7 (Lac34)	Chardonnay
52	Strain8 (Lac40)	Chardonnay
53	Strain8 (Lac40)	Chardonnay
54	Strain8 (Lac40)	Chardonnay
55	No inoculation	Chardonnay + ethyl acetate
56	No inoculation	Chardonnay + ethyl acetate
57	No inoculation	Chardonnay + ethyl acetate
58	Strain1 (Ooeni8)	Chardonnay + ethyl acetate
59	Strain1 (Ooeni8)	Chardonnay + ethyl acetate
60	Strain1 (Ooeni8)	Chardonnay + ethyl acetate
61	Strain2 (Ooeni12)	Chardonnay + ethyl acetate
62	Strain2 (Ooeni12)	Chardonnay + ethyl acetate
63	Strain2 (Ooeni12)	Chardonnay + ethyl acetate
64	Strain3 (Ooeni28)	Chardonnay + ethyl acetate
65	Strain3 (Ooeni28)	Chardonnay + ethyl acetate
66	Strain3 (Ooeni28)	Chardonnay + ethyl acetate

Volatile fingerprinting of all treatments was carried out by Metabolomics Australia. Deliverables resulting from services carried out including preliminary PCA analysis. The volatile fingerprinting screening technique was employed in an attempt to reduce the number of samples required for quantitative analysis. Samples analysed by this method are shown in Table B.5. However due to a lack of sensitivity in this technique it was deemed necessary to carry out more in-depth analysis on a smaller sample set using SPME-GCMS.

Analysis of fermentation-derived volatiles

Standard solutions were prepared using analytical reagent grade chemicals (Sigma). Stock solutions of each compound were prepared in ethanol. A combined standard solution was prepared for each group of compounds (esters, acetates, alcohols and acids) from the stock solutions.

For volatile analysis, an Agilent CP9205 gas chromatograph (Agilent Technologies) was equipped with a Gerstel MPS2 multi-purpose sampler and coupled to a mass selective detector. The instrument was controlled and the data analysed with MSD ChemStation E.02.00.493 software (Agilent Technologies). The gas chromatograph was fitted with a 30 m x 0.25 mm Phenomenex fused silica capillary column ZB-Wax (0.25 μm film thickness). The carrier gas was helium (Air Liquide or BOC, Ultra High Purity), linear velocity 36 cm/s, flow rate 1.0 mL/min in constant flow mode. The oven temperature was started at 40 °C, held at this temperature for 5 min, then increased to 120 °C at 3 °C/min, then increased to 240 °C at 10 °C/min and held at this temperature for 10 min. The inlet was fitted with a borosilicate glass SPME inlet liner (0.75 mm I.D., Supelco) and was held at 230 °C. The mass spectrometer quadrupole temperature was set at 150 °C, the source was set at 230 °C, and the transfer line was held at 240 °C. Positive ion electron impact spectra at 70 eV were recorded in scan mode from 40 to 250. The conditions for headspace SPME sampling were as follows: a 10 mL aliquot of sample was added to a 20 mL vial containing 2 g of sodium chloride, and the vial was immediately capped (magnetic, Teflon lined rubber septum). The vial and its contents were heated to 40 °C for 10 min. The Supelco Carboxen/divinylbenzene/Polydimethylsiloxane (CAR/DVBPDMS) 50/30 μm fibre was exposed to the headspace for 10 min with agitation of the vial at 250 rpm. The SPME fibre was desorbed in the split mode, at 10:1. The fibre was allowed to bake in the inlet for 15 min.

Results and Discussion:

O. oeni was unable to grow significantly (OD_{600} < 0.1) in the absence of a carbon source (Figure B.1). When provided with the naturally occurring plant β-glucosides salicin and arbutin and the disaccharide cellobiose (with two glucose moieties) as sole carbon source, growth was observed. Glucose was added at 1% (w/v) to three growth assays to establish the impact on growth patterns, and also to determine whether glucose repressed the phospho-β-glucosidase genes characterised in Output G. The three cultures, one each containing supplementation from salicin, arbutin or cellobiose, had a greater lag (approximately 10 days) than cultures grown on salicin and cellobiose with added glucose (approximately 5 days). In the presence of arbutin plus glucose, the cells still had a 10 day lag before increasing exponentially. When glucose was additionally present, the maximum OD attained was typically twice that seen in the absence of glucose. These findings confirm the ability of this wine strain of *O. oeni* to grow on natural glycosylated substrates and thereby underline the importance of the associated utilisation systems and in turn their genes.

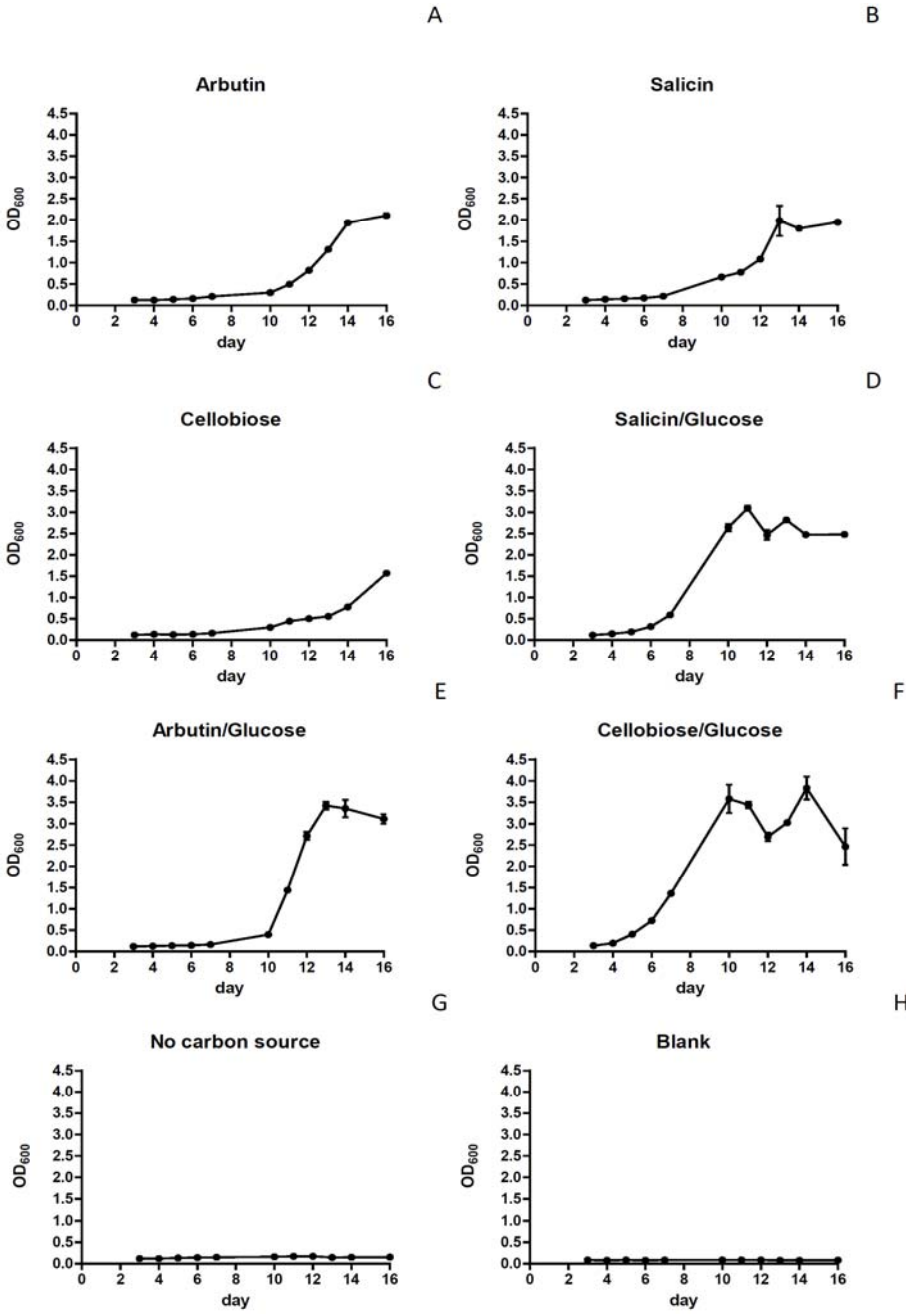


Figure B.1: Growth of *O. oeni* strain Lalvin 4X (VL92) in MRS broth lacking meat extract and dextrose at 30°C under anaerobic conditions. The MRS broth was supplemented with 1% (w/v) of A) Arbutin, B) Salicin, C) Cellobiose, D) Salicin/Glucose, E) Arbutin/Glucose, F) Cellobiose/Glucose, G) No carbon source, H) uninoculated (blank).

The cultures were examined daily under the light microscope. Figure B.2 shows images taken of cells 16 days post inoculation. The cells grown in the presence of glucose carbon source were more numerous and tended to grow in small clusters. In the absence of glucose, clusters comprised longer chains of cells, perhaps indicative of the slower growth under these conditions.

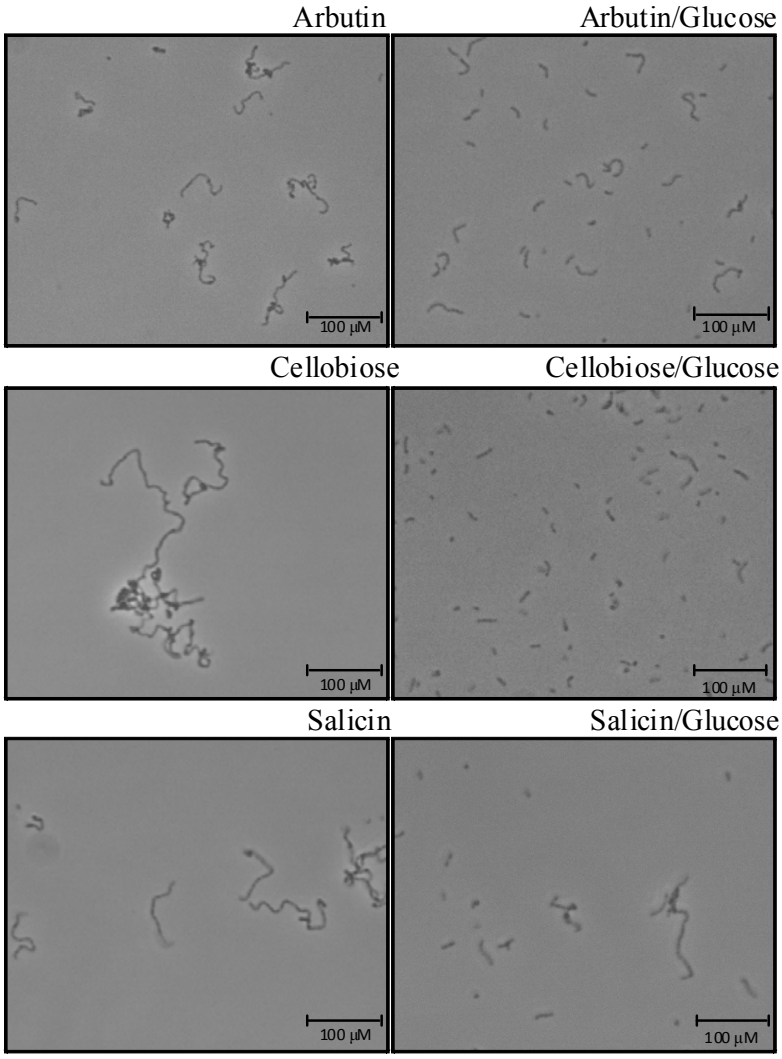


Figure B.2: Images of *O. oeni* strain Lalvin 4X (VL92) after 16 days growth in MRS broth lacking meat extract dextrose but containing various carbon sources. Taken at 1000 x magnification. **Arbutin)** growth on 1% (w/v) arbutin; **Arbutin/Glucose)** growth on 1% arbutin and 1% glucose; **Cellobiose)** growth on 1% cellobiose; **Cellobiose/Glucose)** growth on 1% cellobiose and 1% glucose; **Salicin)** growth on 1% salicin; **Salicin/Glucose)** growth on 1% salicin and 1% glucose.

To gain insight into the regulation of the glycosidase genes investigated in this project, their pattern of expression during the course of growth was examined. RNA was extracted from the cultures when at an OD_{600} of 0.5 and 1.0, reverse transcribed and used as a template for PCR. Five gene products were amplified: *celC*, *celD*, *bglD* and *ldhD* and *gyrA*. The cDNA was quantified as described, and 20 µL (over 1100 ng/µl of amplified cDNA for all the samples) was loaded into each well of the 2% (w/v) agarose gel, to allow for maximum product visibility. The cDNA from cultures sampled at OD_{600} 0.5 was only evident for the reference genes *ldhD* and *gyrA*, and no product was observed for *bglD*, *celC* and *celD* at this time point (data not shown). Figure B.3 depicts electrophoretic analysis of amplicons from cultures sampled at OD_{600} 1.0. No PCR products were visible using the *celC* primers. However, bands of approximately 80 bp corresponding to the reference genes *ldhD* and *gyrA* were observed under UV illumination when the same cDNA template was used (refer to Table B.3 for expected amplicon sizes). Different amounts of product could be identified between the different templates when amplified with the reference gene primers. This may be due to an inconsistent RNA extraction or efficiency of reverse transcription. Amplification with *celD* primers yielded a faint product approximately 100 bp in size for all assays with the exception of arbutin. Amplification using *bglD* primers produced a

faint product (just discernible, see arrow) when cells were grown in arbutin as the sole carbon source. This implies that the expression of the *bglD* and *celD* genes may be induced to different degrees depending on the carbon source. The impact of a wine-like environment on expression of these genes is therefore warranted. Notably, the residual content of glucose in wines is likely to be less than the 1% (10 g/L) used here.

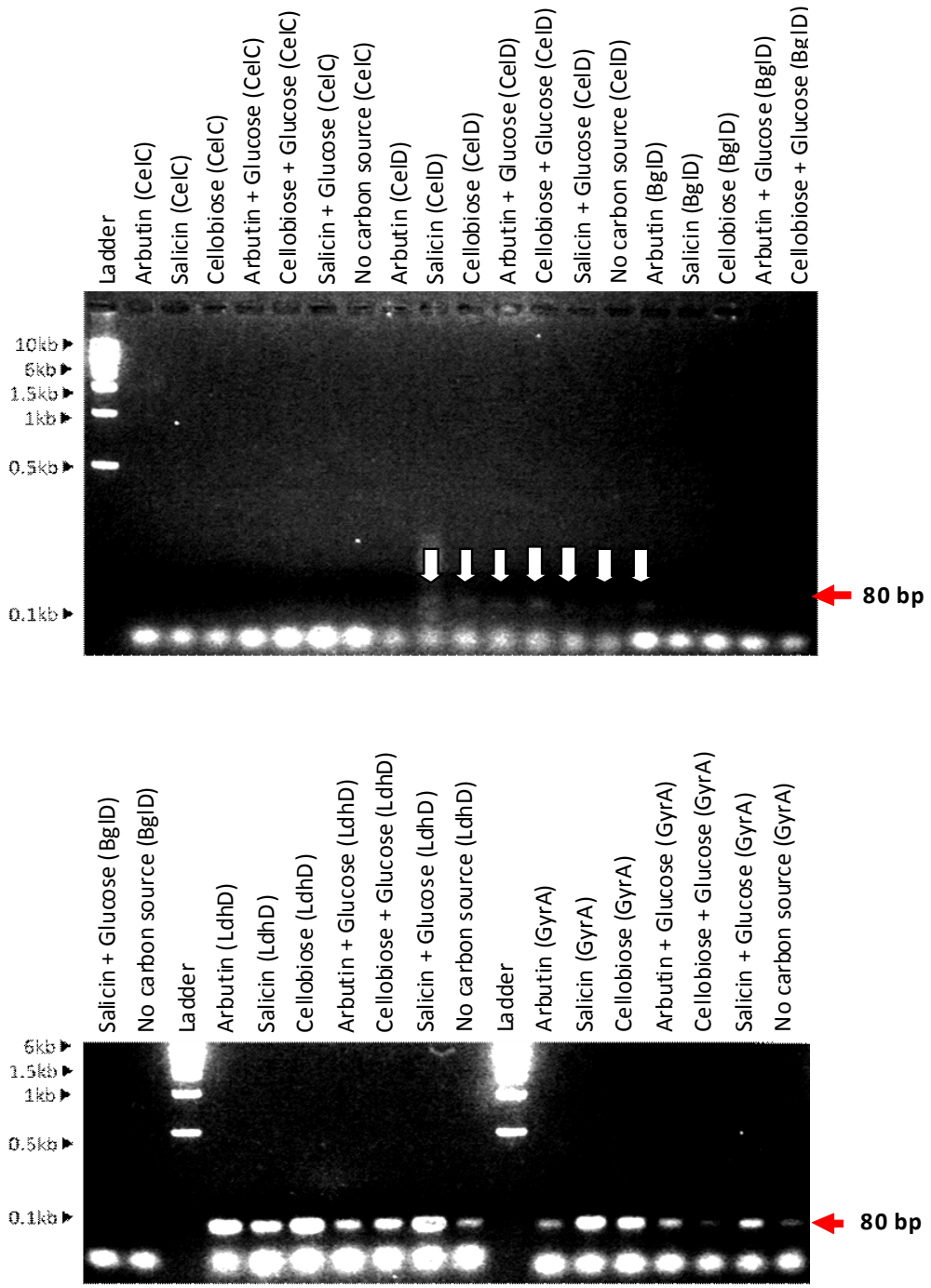


Figure B.3: Electrophoresis of amplification products derived from cDNA (as template) and gene specific primers for *celC*, *celD* and *bglD*. *ldhD* and *gyrA* genes were used as controls for quantification purposes. DNA was visualised under UV, following separation on a 2% agarose gel in the presence of GelRed.

The potential implication of esterase activities of lactic acid bacteria for the ester profile of wines was examined. A Cabernet Sauvignon and a Chardonnay wine were inoculated with up to 8 different strains as detailed in Table B.4. In some cases the wines were spiked with ethyl acetate (Table B.5). At the completion of MLF volatile fingerprinting of all treatments was carried out by Metabolomics Australia. Preliminary PCA analysis showed minimal differences between the tested treatments (e.g. see Figure B.4 and Appendix 5 – Supplementary Figures 1-3). Whilst the samples are clustered according to wine variety, there are no clear distinctions between the strains or the uninoculated samples. Some of the ethyl acetate spiked wines did form a distinct group suggesting that they contained a high residual of ethyl acetate, a derivative therof or a metabolite produced in response to this ester.

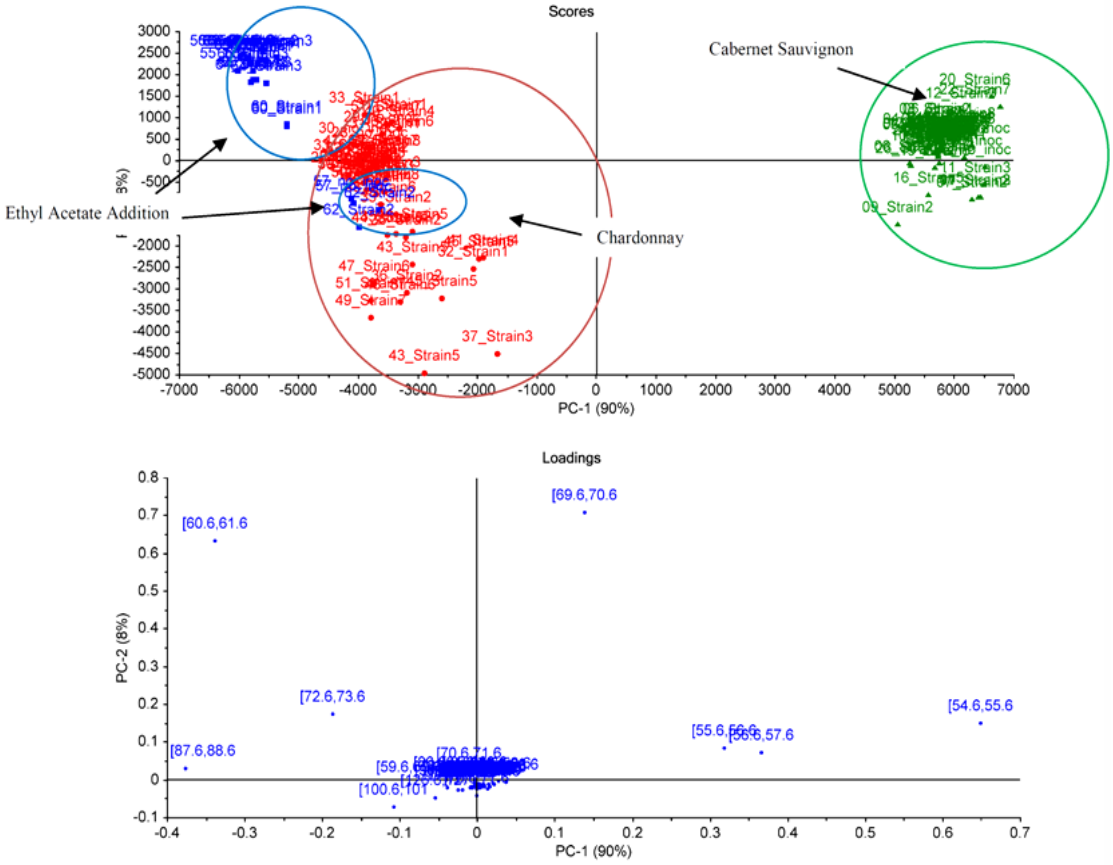


Figure B.4: Scores and Loading Plot from the PCA of all samples (including those spiked with ethyl acetate) analysed for impact of MLF on volatile profile (PC1 and PC2).

In order to gain greater resolution between the samples, some were analysed using SPME-GCMS. A summary of the results is in Tables B.6 and B.7. All strains changed the ester profile of the wine relative to the uninoculated control. In the Cabernet Sauvignon, only Ooeni8 produced a statistically significant reduction in ethyl acetate (i.e. ~61 g/L *vs* 36 mg/L). Ooeni8 also produced the greatest reduction in ethyl acetate in the Chardonnay wine (i.e. ~85 mg/L *vs* 20 mg/L; Table B.7). In this case all strains with 'high' esterase activity (reported in UA 01/04 with artificial substrates) reduced ethyl acetate to a statistically significant level. Whilst significant, the reduction in ethyl acetate by Ooeni3 was not as great, again in agreement with the low acetate-specific esterase activity of the strain. Ethyl acetate is often an important contributor to wine aroma; at low concentrations (< 100 mg/L) giving a desirable and fruity character to the wine, however, at higher concentrations it can impart a solvent/nail varnish-like aroma. Therefore it is ideal to keep this ester below this undesirable level, but at the same time it may not be desirable to reduce other esters present in the wine. Importantly, most strains could alter ethyl hexanoate content of the

Cabernet Sauvignon, at times around the aroma threshold. Isoamyl acetate in Chardonnay was reduced 10-fold by Ooeni8, although the lowest content (324 μg/L) remained above the aroma threshold (160 mg/L). Other changes in the Chardonnay were unlikely to be detected by a sensory panel when considering the contents of individual compounds, however effects arising out of interactions cannot be excluded. It would therefore be of interest to explore these results further using sensory analysis.

Conclusions:

The work undertaken as part of this Output has succeeded in identifying strains with distinct hydrolytic properties. In fact, rather than the projected two strains, up to eight have been characterised. Based on their potential contribution to wine composition and sensory properties, the enzymes behind the glycosidic and esterase activities of a selection of these 8 strains were examined. Ultimately they were investigated through a cloning of putative structural genes, heterologous production of active enzymes and their biochemical characterisation (see Output G). This approach was taken because of the high level of complexity of the enzymatic systems of these strains (i.e. multiple enzymes in whole cells). Ahead of such analyses, we began to define the basis and function of some of these activities. Thus growth of LAB on natural glycosidase supports the involvement of the glycosidases in the metabolism of these compounds. It also suggests the ability of these strains to attack glycosides in grapes. Further work will be needed to confirm this. Expression patterns for three putative glycosidase genes (*celC*, *celD* and *bglD*) reveal marked differences in terms of expression according to strain and growth conditions. Where such genes are involved in glycosidase metabolism, there remains an opportunity to selectively utilise strains to modify glycoside content of wine.

Parallel work extended our seminal observations regarding esterases in LAB. Importantly, trials with wine demonstrated that at least the strains tested have the ability to modify ester profiles. In keeping with their performance on artificial substrates, those with high activity against acetate esters also exhibited strong hydrolysis of ethyl acetate in wine. Such strains may represent an approach to reducing the ethyl acetate content of wines. Modifications to other esters were observed with future sensory analysis being called for to establish the net sensory impact of the use of these strains.

In each case, larger scale trials are planned as part of the follow-on project, UA 11/01.

Table: B.6: The concentrations of selected esters prior to and post MLF for five strains of *O. oeni* in Cabernet Sauvignon wine

Ester (μg/L)	Uninoc-ulated	Ooeni8	Ooeni12	Ooeni28	Ooeni3	Ooeni6A	Reported aroma threshold (μg/L)
ethyl acetate	61247 ± 3398	36008 ± 6091*	39409 ± 6919	39422 ± 12709	85091 ± 2251	46759 ± 14915	12270[w]
ethyl butanoate	323 ± 46	191 ± 80	204 ± 62	168 ± 90	497 ± 46	298 ± 104	20[a], 40[b]
isobutyl acetate	58 ± 14	514 ± 24	69 ± 19	69 ± 20	72 ± 21	94 ± 31	1600[b]
isoamyl acetate	1156 ± 255	616 ± 323	805 ± 395	815 ± 375	1919 ± 122	1080 ± 337	160[w]
ethyl hexanoate	381 ± 32	220 ± 11*	284 ± 22*	288 ± 20*	507 ± 8*	344 ± 29	80-850[w]
hexyl acetate	80 ± 5	91 ± 8	96 ± 6	90 ± 10	127 ± 2*	127 ± 8*	670[w]
ethyl lactate	11231 ± 841	64264 ± 11009*	66990 ± 10732*	90851 ± 24263*	37728 ± 2963	67356 ± 9652*	150000[w]
ethyl octanoate	214 ± 37	638 ± 78*	450 ± 66 *	411 ± 14*	721 ± 24*	579 ± 112*	580[w]

Values are the mean of triplicate determinations, ± standard deviation, of a single wine by analysed by SPME-GCMS. Aroma thresholds as reported previously (reviewed by Sumby *et al.* 2010). Values determined in: [a] 10% (v/v) aqueous ethanol, [b] beer, [w] wine. *Significant difference, $P < 0.05$ inoculated strain *vs.* control (uninoculated), analysed using one way ANOVA in GraphPad Prism.

Table: B.7: The concentrations of selected esters prior to and post MLF for four strains of *O. oeni* in Chardonnay wine

Ester (μg/L)	Uninoc-ulated	Ooeni8	Ooeni12	Ooeni28	Ooeni3	Reported aroma threshold (μg/L)
ethyl acetate	82538 ± 5940	19781 ± 377*	28543 ± 2412*	27380 ± 7742*	49883 ± 14774*	12270[w]
ethyl butanoate	1278 ±141	202 ± 14*	429 ± 62*	451 ± 91*	604 ± 80*	20[a], 40[b]
isobutyl acetate	21 ± 8	26 ± 7	29 ± 9	27 ± 6	30 ± 7	1600[b]
isoamyl acetate	3510 ± 392	324 ± 89*	638 ± 172*	1092 ± 107*	633 ± 41*	160[w]
ethyl hexanoate	833 ± 90	442 ± 57*	672 ± 22	575 ± 61*	807 ± 78	80-850[w]
hexyl acetate	63 ± 10	95 ± 10*	85 ± 6	101 ± 9*	83 ± 14	670[w]
ethyl lactate	ND	46708 ± 7158*	50998 ±8725*	39091 ± 4854*	18251 ± 3927*	150000[w]
ethyl octanoate	1123 ± 125	1010 ± 34	1172 ± 97	1184 ± 147	1247 ± 89	580[w]

Values are the mean of triplicate determinations, ± standard deviation, of a single wine by analysed by SPME-GCMS. Aroma thresholds as reported previously (reviewed by Sumby *et al.* 2010). Values determined in: [a] 10% (v/v) aqueous ethanol, [b] beer, [w] wine. *Significant difference, $P < 0.05$ inoculated strain *vs.* control (uninoculated), analysed using one way ANOVA in GraphPad Prism.

Output C: Identification of targets for 'second generation' (G2) optimised yeast

7. **Complete preliminary assessment of candidate properties to form basis for a 'second generation' (G2) or non-GM optimised yeast strains that have enhancements in at least two key properties.**

Contributors:

Past PhD student: Colin McBryde
Current PhD students: Jin Zhang, Jade Forrester and Trung Dung Nguyen
Past Honours students: Thomas Parker
Postdoctoral fellows and/or student supervisors: Drs Michelle Walker, Jennie Gardner, Joanna Sundstrom and Assoc Prof Vladimir Jiranek

Background:

It is recognised that the 'ideal' yeast possesses a number of preferred traits relating to fermentation performance, particularly under extreme conditions, as well as positive sensory contributions. Many genes and expression patterns underpin these traits, thereby requiring modification at a number of loci. While the Systems Biology approach is being put forward as a means of achieving multiple changes in a complex biological system, at the commencement of this project only the reductionist approach (dealing with one or a few genes at a time) was possible. Nonetheless, this approach remains highly relevant and offers significant promise for strain optimisation.

Through UA 01/04 and the present project numerous targets for modification have been identified, typically each impacting on a single target attribute e.g. nitrogen efficiency, fermentation robustness, elevated yields of specific metabolites, etc. The objective of this output was to generate a shortlist of likely candidates for co-introduction into a single strain or else (in the case of recombinant strains) to generate non-recombinant equivalents.

Certainly preliminary assessment of the properties of candidate strains has been completed and is detailed in other Outputs of this project. A short list is reported here (Table C.1), while the ongoing work by this group continues to reveal further candidates.

Methods:

The methods relevent to the identification of the genes and attributes are detailed in the relevant Outputs.

Results and Discussion:

Canditates (gene or attributes) for further investigation (including via UA 11/01), co-introduction into a single strain or else as targets for regeneration as non-recombinant strains are listed below in Table C.1.

Table C.1. Promising candidate genes or strains representing targets for generation of multi-trait optimised strains

***Gene*/Strain**	**Attribute(s)**	**Winemaking Application**
NGR1	Deletion increases nitrogen efficiency	Nitrogen deficient juices
GID7	Deletion increases nitrogen efficiency	Nitrogen deficient juices
ECM33	Deletion increases nitrogen efficiency	Nitrogen deficient juices
FM16 (series)	Adaptively evolved yeasts with robust fermentation	Shortened fermentation duration
HSP150	Haploproficient in N-limited of N-sufficient media	Nitrogen deficient juices or standard fermentations
IRC9	Haploproficient in N-limited media	Nitrogen deficient juices
RMD6	Haploproficient in N-limited media	Nitrogen deficient juices
RPL2B	Haploproficient in N-limited media	Nitrogen deficient juices
High Sugar Fermentation (HSF) genes e.g. *RPA12*, *SIN3*, *ZAP1*, *NPT1, TPS1, TPS2, PLC1, DOA4, SSQ1*	Deletion results in stuck fermentation a (not attributable to poor growth)	Standard/high sugar fermentations
Further HSF genes (~80)	Deletion results in protracted fermentation (not attributable to poor growth)	Standard/high sugar fermentations

Conclusion:

We have developed an expansive list of gene targets and actual yeast strains with novel fermentation attributes, with the highlights listed here. These now form the raw material for our ongoing efforts in yeast strain optimisation as well as contributing to the understanding of which parts of metabolism are manipulable in industrial oenological fermentation.

Output D: Industry or pilot scale information on selected LAB and/or enzymes; Industry or pilot scale information on properties of G2 yeast

8. **Complete analysis from larger-scale/industrial trails from the '07 vintage. Report on findings with recommendations to facilitate adoption by industry.**

9. **G2 ferments completed and analysed ('08 vintage). Report prepared for dissemination to industry and yeast suppliers.**

Summary:

As part of the process of assessing the effectivenes of selected LAB or else enzymes isolated from these, trials in juices/wines on a larger scale were planned. Trials with intact LAB have been postponed while the enzymatic basis for the observed activities of selected strains was defined (see Output B). The latter has progressed well with purified heterologously expressed enzymes being produced more recently (see Output G). Preliminary, lab-scale trials of these have only been undertaken in the last year (Output G). Assuming the generation of large enough quantities of enzyme is straightforward, the next opportunity to undertake large scale trials will be the coming (2012) vintage. LAB will be included in such trials, with these in fact being a planned component of UA 11/01.

Whilst not strictly G2, the most fully characterised output from this project is the adaptively evolved strain L2056-C7. This strain has undergone numerous comparative fermentation trials on a progressively increasing scale, including a trial at UC Davis, California in late October 2011. With revision of the timelines around this activity (e.g. during the review at year 3), many of the data pertaining to these trials is included in Output K.

Output E: Interim progress review and proposal for Yrs 4 & 5

10. **Review of progress and re-assessment of project related priority areas prepared for GWRDC.**

Summary:

The planned review was completed and reported to the GWRDC in the form of an application for funding for subsequent 3 years. Success of this application permitted extension of the work until late 2011.

Output F: Identity of yeast genes vital to winemaking

11. Screening of laboratory yeast deletion and over-expression libraries completed. Data analysis. Initiate preparation of list of genes key to wine making prepared.

Contributors:

Past undergraduate students: Nicholas Kalatzis, May Yong, Phylicia Toh, Damon DeSouza and Ruben Jacobs
Past Honours students: Paul Easton
Past PhD students: Tommaso Liccioli.
Current PhD students: Jade Forrester, Trung D. Nguyen and Jin Zhang
Past Research Assistant: Nga Lam
Postdoctoral fellows and/or student supervisors: Drs Jennie Gardner, Frank Schmid, Joanna Sundstrom, Michelle Walker, Kerry Wilkinson, Paul Chambers (AWRI), Prof Dennis Taylor and Assoc Prof Vladimir Jiranek.

Background:

We sought to utilise the commercially available laboratory yeast deletion library as a vehicle for the discovery of genes important to various oenological traits. No wine yeast deletion library was available at the commencement of this project (or even now). The logic behind use of a deletion library is that where a gene is important to a particular outcome (e.g. the timely completion of fermentation) then its deletion prevents this outcome from being achieved.

Apart from fermentation completion, performance in the presence of limited or selected assimilable nitrogen was a key topic of interest (based on previous projects supported by GWRDC). However, since the latter requires the withholding of assimilable nitrogen, particularly amino acids, such work was not compatible with the use of the laboratory strain background of the deletion library since such strains possess autotrophic markers and must be supplied the corresponding amino acids in order to even grow. Supplementation trials were therefore performed to determine the appropriateness of using media supplemented with the auxotrophic requirement to at least permit fermentation performance to be assessed for the library of deletants.

A deletion library allows one to look at the consequences of the loss of function of the deleted gene. Generally a screen of such a library identifies genes whose function is critical to the desired phenotype (e.g. fermentation completion). Thus by eliminating these genes the strain performs worse. Conversely, genes which are detrimental to a capability can be found as their deletion improves the phenotype of interest. In contrast, an over-expression library offers the ability to confirm findings from a deletion library screen as well as find new genes. Thus ‘good’ genes, when over-expressed, may produce an even better result, whereas over-expression of others becomes problematic for the strains.

The commercially available over-expression library is marketed as a collection of plasmids for transformation into your strain of choice. Whilst it becomes possible to insert such constructs into a wine yeast background, the limitation is that the gene sequences are driven by a *GAL* promoter, which is activated in the presence of the alternate carbon source, galactose. Unfortunately, such a promoter does not function in the presence of the preferred carbon sources, glucose or fructose, which predominate in oenological fermentations. As a consequence some effort was put into modifying a wine strain in order to

permit the effective expression of the $GAL_{promoter}$-driven constructs in this preferred background under wine conditions (i.e. high glucose and fructose concentration).

Methods:

Yeast strains

The study used the collection of yeast diploid deletion strains developed by the Yeast Genome Deletion Project (Winzeler *et al.*, 1999) which is available from Open Biosystems (www.openbiosystems.com/GeneExpression/Yeast/YKO/). The homozygous (Δ*orf::KanMX4*) gene deletions were in BY4743 (*MATa/a*, *his3Δ1/his3Δ1, leu2Δ0 /leu2Δ0, lys2Δ0/LYS2, MET15/met15Δ0, ura3Δ0 /ura3Δ0* (BY4741/BY4742)). The haploid strain BY4741 (*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0*) was used in the construction of a prototrophic derivative (BY4741P).

Media and growth conditions

Strains were maintained on YPD (1% yeast extract, 2% bactopeptone, 2% D-glucose [w/v]) containing 200 mg/L geneticin (G418 sulfate; Amresco). Solidified minimal drop out medium (Sherman, 2002) was used for the selection of the prototrophic derivative BY4741P. Potassium acetate medium (10 g/L potassium acetate, 1 g/L yeast extract, 0.5 g/L glucose) was used to induce yeast sporulation. Media was solidified with 2% (w/v) bacteriological agar (Amyl Media).

Chemically Defined Grape Juice Medium (CDGJM) containing 200 g/L equimolar glucose and fructose and 450 mg/L FAN (Henschke & Jiranek, 1993) was used in the preliminary fermentation evaluations of BY4741 and BY4741P. Supplementation of the medium with 150 mg/L uracil was required in order to attain satisfactory growth of BY4741. For the purpose of screening of the deletion library (in BY4743), the CDGJM was supplemented with 150 mg/L uracil. A further potential inhibitor of yeast under oenological conditions, polyphenols, was included as 3 g/L of a polyphenol extract (Cat: Tppr, OenoProd, Sarl), which had been dissolved in 100% ethanol (3 g powder in 5 mL, 24 h, in dark) prior to addition to the sterile medium. The enriched medium (CDGJM+PP) was stored in the dark for up to 24 h (4°C) before use.

Construction of a prototrophic derivative (BY4741P) of BY4741

Genomic DNA was prepared from the laboratory strain S288C according to Adams *et al.* (1998). The genes *HIS3*, *LEU2*, *MET15* and *URA3* were amplified using gene-specific primers (Table F.1) and Pfu Ultra II DNA polymerase (Integrated Sciences). Amplification was for 30 cycles of: 95°C, 30 s; 60°C (+/- 3°C), 30 s; 72°C, 1 min 10 s. The gel purified DNA fragments for each functional gene (*HIS3*, *LEU2*, *MET15* and *URA3*) were sequentially transformed into BY4741 and five gene deletants (Table F.2) according to Gietz and Schiestl (2007) with a minor modification. Replacement of water in the wash step with phosphate buffered saline (PBS) was shown to increase transformation efficiency 10-fold. Transformants were selected on minimal drop-out medium lacking the appropriate amino acid or uracil (Sherman, 2002).

Possible contamination during the sequential transformation process was ruled out by molecular typing using delta sequence specific primers δ1 and δ2 (See Output A). The strain-dependent sized fragments were visualised with Sybrasafe (Invitrogen) following separation on a 1% (v/w) agarose gel run in 1x TAE (Tris Acetate EDTA pH 8) buffer.

Comparative fermentations of supplemented auxotrophic *vs.* prototrophic yeast strains

The auxotrophic strain BY4741 and its prototrophic derivative BY4741P were grown overnight in YPD and inoculated at 5 x 10^6 cells/mL into starter CDGJM (with supplementation) (Henschke & Jiranek, 1993; McBryde *et al.*, 2006). Following 24 h growth, the starter cultures were used to inoculate 100 mL

Table F.1: Primers used to amplify functional genes used to restore prototrophy in yeast

Primer	Sequence (5'-3')	Fragment size (kb)
HIS3 FWD[a]	CACCCCGTAATTGGTCAA CA	2.088
HIS3 REV[a]	ATCCTCGGGGACACCAAATA	2.088
URA3F1 FWD[a]	AGGGAAGACAAGCAACGAAA	2.149
URA3R1 REV[a]	CGATTACTGCTGCTGTTCCA	2.149
MET15 FWD[a]	CGTTTTCTACTTTCTTCTGCTGCT	3.302
MET15 REV[a]	ATGGTCTCTAGCGGGATCG	3.302
LEU2 FWD[a]	CGAGACCAAGAAGAACATTGC	2.967
LEU2 REV[a]	GTGGCCAAGAGAGAGGATTG	2.967

[a] Primers for amplification

of CDGJM (with supplementation) at 5 x 10^6 cells/mL. Fermentations were conducted in triplicate at 25°C in an automated fermentation facility (www.agwine.adelaide.edu.au/wine/facilities/msff/). Fermentation progress was monitored by cumulative weight loss (due to CO_2 evolution; Bely *et al.*, 1990) and RI measurement of residual sugar in clarified supernatant samples. The samples were stored at -20°C for spectrophotometric glucose/fructose assays (Bergmeyer *et al.*, 1983). Fermentations were considered finished when the residual sugar was less than 2.5 g/L as determined by Clinitest™ (Cat: 2107, Bayer). A one-way ANOVA and a t-test (95% CI) were performed for the glucose/fructose assays, to determine whether there were significant differences between the fermentation profiles of BY4741 and BY4741P.

Table F.2: Yeast strains used in the transformation and comparative fermentation trials

Yeast strain	Deleted ORF name	Relevant genotype	Source
BY4741		*MAT* **a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Winzeler *et al.* 1999
3446	*YCL039W*	BY4741 *gid7::KANMX4*	Winzeler *et al.* 1999
3352	*YBR212W*	BY4741 *ngr1::KANMX4*	Winzeler *et al.* 1999
-2098*	*YPL154C*	BY4741 *pep4::KANMX4*	Winzeler *et al.* 1999
5351*	*YNL025C*	BY4741 *ssn8::KANMX4*	Winzeler *et al.* 1999
1922*	*YHR094C*	BY4741 *hxt1::KANMX4*	Winzeler *et al.* 1999
BY4741P		BY4741P[1]	This Project
3446P	*YCL039W*	BY4741P *ngr1*	This Project
3352P	*YBR212W*	BY4741P *gid7*	This Project

[1] P denotes prototrophic strain, * denotes strain not used in the fermentation trial

Screening of the diploid gene deletion library for fermentation performance

Liquid handling of the library and resulting fermentation samples was performed using a Corbett CAS3800 robot (Corbett Robotics, Sydney). The library was replicated (6 μL) into 96-deep-well plates (0.6 mL YPD) and incubated 24-48 h at 28°C. Each plate had 11 rows (A1-11 to H1-11) containing single samples of the deletion strains and four replicates of the parental strain (BY4743; A12-D12) and CDGJM+PP (E12-H12). Cells were resuspended by agitation on a mini-shaker and 6 μL inoculated into 0.6 mL CDGJM+PP in 96-deep-well plates, which were covered with breathable sealing film (Cat: AXYBF-400, Axygen).

The first fermentation screen (of some 5,000 deletants) was performed in duplicate. The plates were placed on pre-wet paper towel in sealed plastic containers to reduce evaporation. After 180 h at 28°C, the cells were pelleted by centrifugation (3,000 rpm, 5 min) and 0.2 mL samples were dispensed into 96-well flat bottomed plates (Cat: P-96-300F-PS, Axygen) and frozen at -20°C for sugar analysis. Residual glucose and fructose were quantified using the spectrophotometric method described by Bergmeyer *et al.*

(1983) modified to suit the 96-well plate format. The total volume of the reaction was reduced from 1.62 mL to 0.125 mL. Three times the concentration of the reaction buffer was used to provide NADP and ATP to excess. To ensure accurate pipetting of the enzymes, 10 μL of a 1:10 dilution in MQ water was used (as opposed to 1 μL). Absorbance at 340 nm was measured using a μQuant Microplate spectrophotometer (Bio-Tek Instruments). The samples were diluted 1:10 (for < 20 g/L) and 1:100 (for > 20 g/L) and determined against a set of identically diluted standards which were within the linear range of the reaction (0 - 0.08g/L).

The second screen, of a smaller subset (336 strains) of deletion strains identified in the first screen, was performed in duplicate as above. Using the sacrifical plate method (Liccioli *et al.*, 2011), a plate was used at specific time points (41, 89, 113, 137 and 190 h) to measure growth (OD_{600}) and residual sugar before subsequent disposal. Absorbance measurements (OD_{600}) were taken using a 1:10 diluted sample after agitation of the culture to resuspend the cells.

To ensure that the inability to complete fermentation was a phenotype associated with the high sugar content of the media (*vs.* some other feature of the medium), a third screen using the same subset of 336 deletion strains was performed in a low sugar medium. The method was identical to the second screen, except CDGJM+PP containing 20 g/L equimolar glucose and fructose and 450 mg/L FAN was used, with growth and residual sugar being measured at 32 h and 56 h.

Evaluation of identified deletion mutants in laboratory scale (100 mL) fermentations

Triplicate (100 mL) fermentations were performed in CDGJM enriched with polyphenols (i.e. CDGJM+PP; 200 g/L equimolar glucose and fructose; 450 mg/L FAN) in 250 mL Erlenmeyer flasks fitted with airlocks (Walker *et al.*, 2003, McBryde *et al.*, 2006). N_2 sparging was omitted as the fermentation is anaerobic within 4-6 hours of commencement (data not shown). Fermentation was monitored as described above.

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

The datasets were analysed using GOToolBox; specifically GO-Stats (http://genome.crg.es/GOToolBox/) (Martin *et al.*, 2004) and GO Finder Version 0.83 software (http://www.yeastgenome.org/cgi-bin/GO/goTermFinder.pl), which allowed the hierarchical clustering (and over-representation/enrichment or under-representation/depletion) of genes based on shared Gene Ontology (GO) terms. Both computational tools use a hypergeometric distribution with Multiple Hypothesis (Bonferroni) Correction (and additional False Discovery Rate; GO Finder) to calculate *p*-values. The clustering of genes based on their shared gene annotations was also determined using GO-Proxy (http://genome.crg.es/GOToolBox/) and SGD Gene Ontology Slim Mapper (http://www.yeastgenome.org/cgi-bin/GO/goSlimMapper.pl). The yeast genetic interaction networks between genes annotated to a particular GO term were visualised using the DRYGIN database (http://www.drygin.ccbr.utoronto.ca/) (Costanzo *et al.*, 2010).

Molecular typing of *Saccharomyces cerevisiae* strains by microsatellite analysis

The method used was described in Bradbury *et al.* (2006). Genomic DNA was prepared from a single colony resuspended in 0.3 mL 5% Chelex 100 Molecular Biology Grade Resin (Cat No. 142-1253, Bio-Rad) made up in sterile MQ water. The cell suspension was heated (100°C) for 10 min prior to vortexing for 10 sec and centrifugation (12,000 rpm, 3 min). The DNA extract is stable for 1 month at -20°C, but requires centrifugation before each use in microsatellite analysis.

Microsatellite analysis was performed using Qiagen Multiplex PCR Kit (Cat No. 206143). The primers were supplied as a 0.1 mM solution and kept in the dark at -20°C. Each primer set had a forward primer

which was labelled with a fluorescent marker and a reverse primer which was unlabelled. The markers were Fam (blue), Hex (green) or NedD (yellow). Table F.3 and F.4 describe preparation of the 10x primer stock and information of the primer sets. A 10x primer stock was made containing all the primers (refer to Table F.3 for preparation of the 10x primer stock). A 10 µl multiplex reaction was performed for each strain using 5µl 2x Qiagen multiplex PCR master mix, 1 µl 10x primer stock, 2 µl DNA, 2 µl H_2O. The PCR cycling conditions were: 95°C, 15 min (denaturation); 35 cycles of amplification (95°C, 30 s; 54°C, 90 s; 72°C, 60 s) followed by a final extension at 60°C for 30 min. The reactions were kept overnight prior to running on a capillary sequencer (ABI 3130X or ABI 3730).

Table F.3: Preparation of 10x primer mix for multiplex PCR reaction

Primer	**Concentration in reaction (µM)**	**Volume in 10x primer mix (µl)**
C3-fam	0.1	1
C3-R	0.1	1
C8-ned	0.25	2.5
C8-R	0.25	2.5
C5-hex	0.1	1
C5-R	0.1	1
AT4-fam	0.75	7.5
AT4-R	0.75	7.5
091C-ned	0.5	5
091C-R	0.5	5
C4-hex	0.75	7.5
C4-R	0.75	7.5
009C-fam	0.2	2
009C-R	0.2	2
AT2-fam	1	10
AT2-R	1	10
267C-hex	0.2	2
267-R	0.2	2
Scaat3-ned (160W)	1	10
Scaat3-R (160W)	1	10
Alpha-fam	0.1	1
a-hex	0.1	1
Mat-R	0.1	1
Total	**10 µM**	**100 µl**

Table F.4: Primer sets used in microsatellite analysis

Set A

Locus	Name	Label	Gene	Allele sizes	Alleles	Repeat*	Chromosome	Fwd primer	Rev primer
YGL139W	C3-fam	FAM	unknown	94-135	12	$(CAA)_{13}$	VII	*GTGTCT*CTTTTTATTTACGAGCGGGCCAT	AAATCTCATGCCTGTGAGGGGTAT
YLL049W	AT4-fam	FAM	unknown	270-302	11	$(TA)_{19}$	XII	GCAACATAATGATTTTGAGGT	*GTGTCT*TGTGTGAGCATAGTGGAGAA
YBR240C	AT2-fam	FAM	THI2	338-366	11	$(AT)_{18}$	II	TTTCCAACCAATCTGCGA	CCATTTGGTTCTAGTGCGG
YPL009C	ORF3-fam	FAM	unknown	403-466	16	$(GAA)_{16}$	XVI	*GTGTCT*GGGTTTTGGATTTTTATGGA	*GTGTCT*TTCAATTTTCCTCTTTTACCAC
MAT alpha	alpha-fam	FAM	MATalpha	468 (cere)	na	na	III	GGTGCATTTGTCATCCGTC	CAGCACGGAATATGGGACT
YGLO14W	C8-ned	NED	PUF4	128-157	12	$(TAA)_{13}$	VII	*GTGTCT*CAGGTCGTTCTAACGTTGGTAAAATG	GCTGTTGCTGTTGGTAGCATTACTGT
YML091C	SCAAT1-ned	NED	RPM2	243--326	26	$(AAT)_{35}$	XIII	*GTGTCT*AAGCCTCTTCAAGCATGAC	*GTGTCT*GGACAATTTTGCCACCTTA
YDR160W	SCAAT3-ned	NED	SSY1	340-465	15	$(AAT)_{23}$	IV	*GTGTCT*GAGGAGGGAAATGGACAG	GCCTGAAGATGCTTTTAG
YFR028C	C5-hex	HEX	CDC14	117-179	23	$(GT)_{30}$	VI	*GTGTCT*TGACACAATAGCAATGGCCTTCA	GCAAGCGACTAGAACAACAATCACA
YOL109W	C4-hex	HEX	ZEO1	242-361	16	$(TAA)_{13}(TAG)_5$	XV	*GTGTCT*AGGAGAAAAATGCTGTTTATTCTGACC	TTTTCCTCCGGGACGTGAAATA
YOR267C	ORF2-hex	HEX	HRK1	369-460	12	$(CAA)_{20}$	XV	*GTGTCT*CGATGTTGACGATGATTG	*GTGTCT*TGAAGCATGTGGTAGTAGACG
MATa	a-hex	HEX	MATa	492 (cere)	na	na	III	GGTGCATTTGTCATCCGTC	CAATGATTAAAATAGCATAGTCGG

Set B

Locus	Name	Label	Gene	Allele sizes	Alleles	Repeat*	Chromosome	Fwd primer	Rev primer
YGL139W	C3-fam	FAM	unknown	94-135	12	$(CAA)_{13}$	VII	*GTGTCT*CTTTTTATTTACGAGCGGGCCAT	AAATCTCATGCCTGTGAGGGGTAT
YLL049W	AT4-fam	FAM	unknown	270-302	11	$(TA)_{19}$	XII	GCAACATAATGATTTTGAGGT	*GTGTCT*TGTGTGAGCATAGTGGAGAA
YBR240C	AT2-fam	FAM	THI2	338-366	11	$(AT)_{18}$	II	TTTCCAACCAATCTGCGA	*GTGTCT*CCATTTGGTTCTAGTGCGG
YPL009C	ORF3-fam	FAM	unknown	403-466	16	$(GAA)_{16}$	XVI	GGGTTTTGGATTTTTATGGA	*GTGTCT*TTCAATTTTCCTCTTTTACCAC
MAT alpha	alpha-fam	FAM	MATalpha	468 (cere)	na	na	III	GGTGCATTTGTCATCCGTC	CAGCACGGAATATGGGACT
YGLO14W	C8-ned	NED	PUF4	128-157	12	$(TAA)_{13}$	VII	*GTGTCT*CAGGTCGTTCTAACGTTGGTAAAATG	GCTGTTGCTGTTGGTAGCATTACTGT
YML091C	SCAAT1-ned	NED	RPM2	243--326	26	$(AAT)_{35}$	XIII	AAGCCTCTTCAAGCATGAC	*GTGTCT*GGACAATTTTGCCACCTTA
YDR160W	SCAAT3-ned	NED	SSY1	340-465	15	$(AAT)_{23}$	IV	*GTGTCT*GAGGAGGGAAATGGACAG	GCCTGAAGATGCTTTTAG
YFR028C	C5-hex	HEX	CDC14	117-179	23	$(GT)_{30}$	VI	*GTGTCT*TGACACAATAGCAATGGCCTTCA	GCAAGCGACTAGAACAACAATCACA
YOL109W	C4-hex	HEX	ZEO1	242-361	16	$(TAA)_{13}(TAG)_5$	XV	*GTGTCT*AGGAGAAAAATGCTGTTTATTCTGACC	TTTTCCTCCGGGACGTGAAATA
YOR267C	ORF2-hex	HEX	HRK1	369-460	12	$(CAA)_{20}$	XV	CGATGTTGACGATGATTG	*GTGTCT*TGAAGCATGTGGTAGTAGACG
MATa	a-hex	HEX	MATa	492 (cere)	na	na	III	GGTGCATTTGTCATCCGTC	CAATGATTAAAATAGCATAGTCGG

Two sets of primers were used for the microsatellite analysis: Set A (Bradbury *et al.*, 2006) and Set B (modified from Bradbury *et al.*, 2006). Alias names are given to the following primers: Scaat1-ned (091-ned), ORF2-hex (267-hex), ORF3-fam (009-fam). The primers in coloured blocks were labelled with fluorophores HEX (green), FAM (blue) and NED (yellow). Nucleotides in italics represent 'pigtails' added to gene sequence to reduce slippage during PCR, making markers easier to score.
* the microsatellite repeat in lab strain S288c.

Construction of a *GAL1*promoter-*LACZ* reporter system to evaluate the over-expression library during fermentation in CDGJM

Plasmid pYES2-OEP (from Phunbum Park, University of Suwon, Korea) was used as the source of pYES2 vector. The plasmid has the 1053 bp *OEP1* gene (Soy Bean ononitol epimerase) cloned at the *Sac*I and *Xho*I sites in the multiple cloning site. The plasmid 'backbone' was used for the *GAL1p-LACZ* reporter construct as a 5.9 kb *Kpn*I –*Xba*I DNA fragment (these sites are external of the *Sac*I-*Xho*I sites used to clone *OEP1*). The *LACZ* gene was PCR amplified from plasmid pT161T (gift of Keith Shearwin, University of Adelaide) using Pfu Ultra II DNA polymerase (Integrated Sciences) and 25 pmol/μL primers (Table F.5). Reaction conditions were: 95°C, 2 min (denaturation) followed by 30 cycles of: 95°C, 20 s; 60°C, 45 s; 72°C, 50 s. The PCR product was gel purified and digested with *Kpn*I and *Xba*I and repurified. The *LACZ* PCR product was ligated with pYES2 (*Kpn*I-*Xba*I) fragment. *E. coli* transformants containing the plasmid pYES2-LACZ were isolated following transformation into DH5α. The identity of the plasmid from four transformants was confirmed by restriction digest and DNA sequence analysis. Plasmid DNA was transformed into haploid wine yeast 1B14 (*MATa,* Δ*ho::KanMX,* Δ*ura3::KanMX*) using the lithium acetate method (Gietz & Schiestl, 2007). Cells were plated onto minimal drop out medium without uracil (0.5 g/L glucose, 20 g/L galactose). A volume of 0.1 mL 2% (w/v) X-gal (BCIG; Astral Scientific) was spread on the plate to enable colour selection (blue colonies) of transformants expressing β-galactosidase enzyme.

Table F.5: Primers used in the construction of *GAL1p-LACZ* reporter construct

Primer	Sequence (5'-3')
KpnILACZ Fwd[a]	GGGGTACC*ATGATTACGGAATTCACTG
XbaILACZrev[a]	TGCTCTAGATTAT TTTTGACACCAGACCAACT
Fwd primer Gal1p[b]	TCTATACTTTAACGTCAAGGAGA
LacZfwd_nest[b]	CGGTTTCCATATGGGGATTGG

[a] Primers for amplification. [b] Primers for DNA sequencing. * bases (underlined) are restriction sites.

PCR amplification of GAL4(T_{859} I) gene sequence

Plasmid DNA was used as template to amplify the *Gal4c* gene sequence from pRS413-Gal using DNAzyme EXT DNA polymerase (Finnzymes). PCR amplification conditions were 94°C, 2 min (denaturation) followed by 25 cycles of: 94°C, 30 s; 60°C, 30 s; 72°C, 2 mins and a final extension at 72°C for 5 mins. The A-tailed PCR products were gel purified using the Wizard SV Gel and PCR Clean-Up System (Promega) as per manufacturer's instructions. The purified PCR product was cloned into the pGEMT Easy Vector (Promega) using TA cloning as per the manufacturer's instructions before transformation into *E. coli*. Colonies were isolated and plasmid DNA extracted using the Wizard® Plus SV Minipreps DNA Purification System (Promega) as per the manufacturer's instructions. Sequence analysis using the primers listed below (Table F.6) confirmed the presence of the mutated *Gal4c* sequence.

Generation of a leucine auxotroph of the haploid wine yeast isoC9 required for the Yeast Genomic Tiling Collection in pGP564

The *leu2::KanMX* disruption cassette was constructed in the plasmid pBS418 (Walker *et al.*, 2005). Genomic DNA was extracted from isoC9d and used as template in a PCR reaction to amplify the 5' and 3' non-coding (UTR) sequences of *LEU2* using the primers in Table F.7 and DNAzyme EXT polymerase (Finnzymes). PCR amplification conditions were 94°C, 2 min (denaturation) followed by 32 cycles of: 94°C, 30 s; 58°C, 30 s; 72°C, 30 s and final extension 72°C for 5 min. The A-tailed PCR products were gel purified before cloning into pGEM®T (Promega) using TA cloning. The 3'UTR (228 bp) and 5'UTR

Table F.6: Primers used in the PCR amplification of *GAL4c* gene

Primer	Sequence (5'-3')
GAL4c mutation_F[a]	GAC TTA AAA AGC TCA AGT GCT CCA
GAL4c mutation_R[a]	TTA CTC TTT TTT TGG GTT TGG TGG
T7[b]	TAA TAC GAC TCA CTA TAG GG
Gal4_600bp_F [b]	ATG GGT TCT TTG GCG ACG G
Gal4_1300bp_F [b]	CAT GGC ATC ATT GAA ACA GC
Gal4_1900bp_F [b]	GTA CTG GAA GAG GTA TGT GC

[a] Primers for amplification. [b] Primers for DNA sequencing.

(250 bp) sequences were verified by DNA sequence analysis. The *EcoR*I and *Xho*I 3' UTR and *Bam*HI-*Xba*I 5' UTR fragments were sequentially cloned into pBS418 using standard molecular techniques (Sambrook & Russell, 2001). The *leu2::KanMX* deletion cassette was gel purified following restriction digestion of the plasmid with *Xba*I and *Xho*I and used to transform isoC9d. Transformants were selected on YPD containing 0.2 mg/L. Leucine auxotrophy was demonstrated by lack of growth on minimal dropout medium without leucine. Fermentation trials were performed using CDGJM supplemented with leucine.

Table F.7: Primers used in construction of a leucine auxotroph of haploid wine yeast

Primer	Sequence (5'-3')
5' UTR leu2 XbaI fwd[a,b]	CCGAGCGGTCTAGA* GCGCCTGATTCAAG
5' UTR leu2 BamHI rev[a]	CCGGATCCGGCGCTCTCACCTTTCCTT
3'UTR leu2 EcoRI fwd[a]	CGGAATTCTAATGGCTCAACGTGATAAGGAA
3'UTR leu2 XhoI rev[a]	CTTTCTCGAGTATGGGAAATGGTTCAAGAAGG
KanMX3 rev[b]	CATCCATGTTGGAATTTAATCGCGGCCTCG

[a] Primers for amplification. [b] Primers for sequencing. * bases (underlined) are restriction sites.

Plasmid DNA amplification and purification: Yeast Genomic Tiling Collection in pGP564

The collection comprised of 1500 *E. coli* clones, each containing the pGP564 plasmid with a unique region of *S. cerevisiae* genomic DNA. 10 mL cultures were grown overnight in LB lennox liquid media (low salt) with the addition of kanamycin 50 mg/L for plasmid selection, as recommended by Open Biosystems. Plasmid minipreps were performed using the Wizard® Plus SV Minipreps DNA Purification System (Promega) as per the manufacturer's instructions. The plasmid DNA was transferred back into the same 96-well format as the original *E. coli* glycerol collection from Open Biosystems, and stored at -20°C.

Yeast transformation: Yeast Genomic Tiling Collection in pGP564

A 96-well plate yeast transformation method was tested using the isoC9d *leu2::KanMX* yeast strain developed in this laboratory. Each transformation used ~50 ng of plasmid DNA per well. The method is described on the "Prelich Lab Yeast Systematic Over-expression Library Methods" website (http://steelhead.aecom.yu.edu/SystematicLibrary.html#Library_transformation_and_screening.).

Results and Discussion:

Modification of yeast deletion library – Restoration of prototrophy to allow screening for fermentation traits under low nitrogen conditions

In order to allow exploitation of the commercial laboratory yeast deletion library under nitrogen-limited conditions, we sought to restore the strain background to prototrophy. Rather than eliminate all auxotrophies through sequential transformations to replace each of the auxotrophic markers *his3*Δ*1, leu2*Δ*0* and *ura3*Δ*0* (in strain BY4743) individually by homologous recombination (for each of the ~4,500 deletants in question), we sought to attempt a single transformation with a 'triple auxotrophy rescue fragment' targeted to the *his3*Δ*1/his3*Δ*1* locus. Construction of the 6.78 kb *LEU2::HIS3::URA3* fragment flanked by 1299 bases of 5' and 1166 bases of 3' *HIS3* sequence attempted by sequential PCR amplification and cloning into pGEMT (Promega). A 9.2 kb *Pvu*II digested ([5'*HIS3*]*::LEU2::HIS3::URA3::*[*3'HIS3*]) fragment was to be isolated and used for transformation (Gietz & Schiestl, 2007). Rescue of the auxotrophic markers by homologous recombination of the 9.2 kb fragment into the *his3*Δ*1/his3*Δ*1* locus would result in the ability of the transformants to grow on minimal media lacking histidine. Replica plating of the transformants onto minimal media lacking uracil and leucine would confirm the integration of the entire fragment. If successful, the complete library was to be transformed using a modified 96-well transformation procedure. In the case of BY4741, an additional transformation step would be required for the restoration of the *met15*Δ*0* allele. Unfortunately, the triple-auxotrophy construct could not be generated, likely due to its large size.

As an alternate approach a sequential transformation procedure was trialled. The individual auxotrophic markers were to be replaced by homologous recombination of the corresponding functional genes as PCR amplified DNA fragments. A protocol for high efficiency transformation was developed. Several strains were selected from the haploid library (in BY4741) for use in the preliminary trial. A comparison of how many of the functional genes (*HIS3*, *LEU*2, *MET15*, and *URA3*) could be inserted in the same transformation reaction was undertaken. The mean efficiencies of this study are shown in Table F.8: The greatest transformation efficiency was seen for the single genes, however, the efficiencies were lower than for that expected for plasmid DNA: 1×10^{-2} cells/μg DNA (Gietz & Schiestl, 2007). Double transformations occurred at a lower frequency, which in turn declined for further insertions. The inability to simultaneously insert all four functional genes was not surprising as the most successful mean efficiency for single transformations was 1.65×10^{-7} cells/μg DNA, which suggests the arithmetic probability of inserting all four genes would be 1.65×10^{-28}. It was therefore established that a multiple DNA insertion method was not feasible for transformation of the entire library of some 5,000 deletants. Furthermore, transformation of the entire library using a sequential approach to generate prototrophic strains would be far too time consuming and laborious given it would require some 20,000 successful transformations. At this point, modification of the deletion library was abandoned (as per advice to the GWRDC, 22 Dec 2009).

Despite the challenges faced in eliminating auxotrophies from the laboratory yeast deletion library, we still wished to utilise this resource to identify genes vital to winemaking. Accordingly, the influence of supplementation on the performance of the unmodified, deletant (i.e. auxotrophic) strains was examined. In order to do this, prototrophic strains were generated from BY4741 and two deletants, BY4741*ngr1*Δ and BY4741*gid7*Δ. These strains were in fact largely produced through the transformation trials described above. Comparative fermentation of the strains grown in the presence and absence of supplementation was undertaken (Figure F.1.).

Table F.8: Mean transformation efficiency for strains BY4741, BY4741*ngr1*Δ and BY4741*gid7*Δ

DNA fragments	n[a]	%[b]	BY4741	BY4741*ngr1*Δ	BY4741*gid7*Δ
			Mean Transformation Efficiency (cells/µg DNA)[c]		
Single	68	51	1.65×10^{-7}	2.88×10^{-7}	2.62×10^{-7}
Double	37	19	3.33×10^{-7}	6.67×10^{-7}	1.00×10^{-8}
All	17	0	0	0	0
96 well format	33	9	1.00×10^{-6}	Not tested	1.00×10^{-8}

[a] total number of single transformation experiments performed. [b] percentage of successful transformations (i.e. where 1 or more transformants were generated). [c] mean transformation efficiency (number of transformants / total number of cells).

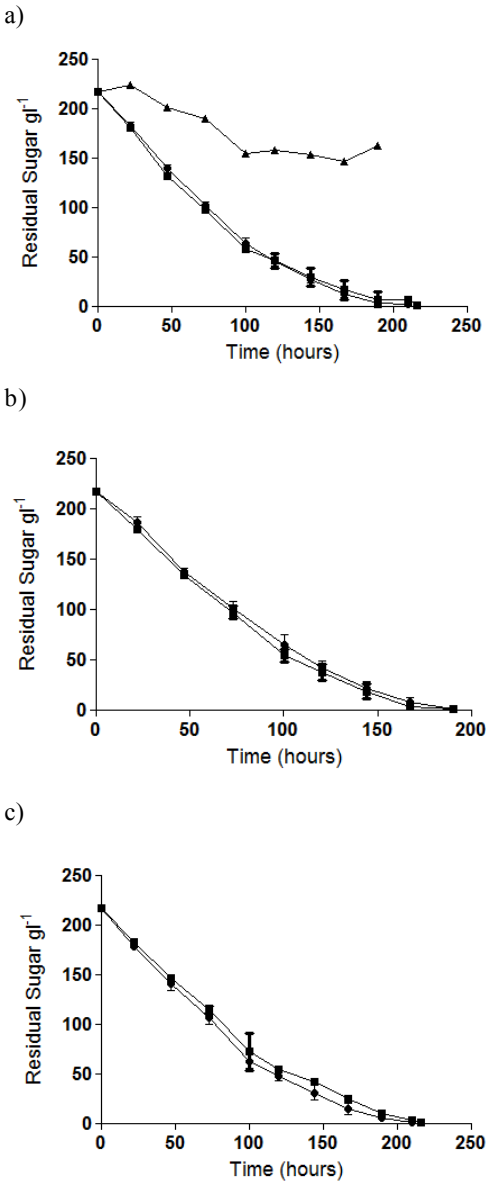


Figure F.1: Comparative fermentations by auxotrophic and prototrophic laboratory yeast strains in CDGJM. Panel a) sugar catabolism by the parental strain BY4741 (*MAT*a *his3*Δ1 *leu2Δ0 met15Δ0 ura3Δ0* with (●) or without (▲) supplementation, compared to the prototrophic strain BY4741P in unsupplemented medium (■). Panel b) sugar catabolism by deletant strain BY4741*ngr1*Δ (●) and the equivalent deletant in a prototrophic background, BY4741P*ngr1*Δ (■). Panel c) sugar catabolism by deletant strain BY4741*gid7*Δ (●) and equivalent deletant in a prototrophic background, BY4741P*gid7*Δ (■). Fermentations were performed in CDGJM containing 200 g L^{-1} of sugars (glucose and fructose), 600 mg L^{-1} FAN and supplemented with 150 mg $l^{-1}$of uracil (unless stated otherwise). NB: smaller error bars are concealed behind the data symbols.

Auxotrophic strains without nutritional supplementation failed to complete fermentation supporting the fact that auxotrophs require supplements for growth (Harsch *et al.*, 2010). Importantly, however, there were negligible differences between sugar catabolism of supplemented auxotrophic and prototrophic

strains (statistically confirmed by a t-test with a 95% CI). A recent study by Harsch *et al.* (2010) using the parental strain BY4742 (*MAT*a, *his3*Δ*1*, *leu2*Δ*0*, *lys2*Δ*0*, *ura3*Δ*0*) from a similar gene deletion library (Winzeler *et al.*, 1999) supports our findings. Harsch and coworkers demonstrated that full supplementation of the genetic auxotrophies in the laboratory yeast BY4742 had relatively minor affect on growth and fermentation compared to a prototrophic S288C strain. Both haploid strains are able to complete fermentation within a similar time (5-10 days) to the commercial wine yeast (Bisson, 1999). It can be concluded that both haploid and diploid deletion libraries can be screened with supplementation, at least in terms of attributes such as fermentation duration. We therefore proceeded to utilise the library with this specific objective.

Screening of unmodified yeast deletion library – Ability to complete fermentation under high sugar conditions

To date, the use of the collections of laboratory yeast of gene deletion mutants (deletants) to ascertain the genetic mechanisms behind wine yeast's ability to adapt to the high sugar conditions of a juice environment and complete fermentation has been limited. Several studies have focused on growth response to single parameters such as anaerobic conditions (Reiner *et al.*, 2006), high sugar content (Ando *et al.*, 2006; Teixeira *et al.*, 2010) and increasing concentrations of ethanol (van Voorst *et al.*, 2006; Teixeira *et al.*, 2009), which are implicated in fermentation. However, these studies do not require the completion of fermentation and were conducted under aerobic conditions for short periods of time (24 – 72 h).

This study used a fermentation approach to identify those genes required for the ability of yeast to complete fermentation in high sugar media (200g/L total as glucose and fructose). The distinguishing feature of this approach is that the yeast must adapt to multiple stresses consecutively or sequentially for the duration of the fermentation. Not only must the yeast grow in the presence of these, they must also complete fermentation of most of the sugar.

The collection of homozygous diploid deletion mutants in BY4743 were screened in CDGJM containing grape skin polyphenols (CDGJM+PP) for two fermentation parameters: (i) modulation of wine colour and (ii) ability to complete fermentation under high sugar conditions. The effect of gene deletions on modulating wine colour (increasing or decreasing colour intensity) is reported in the final report for UA 05/06 "The effect of wine yeast on colour extraction and stabilisation in red wine". The present report details the affect of gene deletions on the yeast's ability to complete fermentation. Where a deletant produced a protracted (or stuck) fermentation but without an effect on growth, the corresponding gene was considered fundamental for successful fermentation in the high sugar conditions as found in juice. The classification of the genes into specific groups or Gene Ontology (GO) terms related to biological process, cellular function, and cellular component was useful in gaining an understanding of the many cellular mechanisms behind yeast's adaptation to the stresses encountered during such fermentations.

The initial screen of the deletant library was undertaken for 180 h because the parental strain BY4743 was able to complete fermentation within 144 h (data not shown). 336 deletants were unable to complete fermentation after 180 h, retaining residual sugar in excess of 2.5 g/L. Any such deletants whose growth was also markedly affected, as shown by an optical density (600 nm) less than 70% of the parent, were eliminated. In this way, 100 deletants were shown to have 'normal' growth but were unable to complete fermentation. As a control, these 100 deletants could complete fermentation under low sugar conditions (CDGJM+PP; 20 g/L) within 56 h (the parent required 32 h), confirming that their impediment was specifically related to extended, high sugar fermentation. All strains exhibited similar growth to the parent.

In a subsequent screen, fermentation performance of the 100 deletants was compared with the parental strain in triplicate 100 mL ferments to see whether the fermentation phenotype was reproducible at a larger scale. The larger scale permitted greater control over parameters that can influence experimental reproducibility and fermentation outcome. In particular, inoculation rate and inoculum preparation can be standardised. In this case, 71% of the deletants were shown to have protracted fermentation. Specifically, the time it took to complete fermentation was 20% or more greater than the parent strain. An example of such ferments conducted over a series of experiments is shown in Figure F.2. The 71 genes confirmed in this way to be important in the completion of fermentation under high sugar conditions are referred to as the High Sugar Fermentation (HSF) data set.

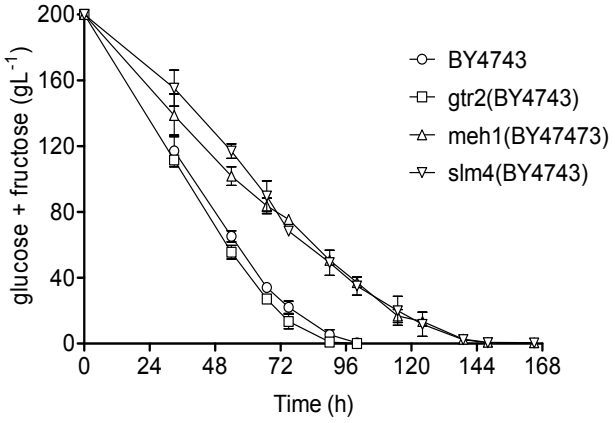


Figure F.2: Comparative fermentations with parent yeast BY4743 and deletants Δ*gtr2*, Δ*meh1*, Δ*slm4* in high sugar media. Fermentations were conducted with the parent yeast BY4743 and deletants Δ*gtr2*, Δ*meh1*, Δ*slm4* in high sugar media (CDGJM+PP, 450 mg N/L as free amino acids, 200 g/L total sugar (as glucose and fructose)). Genes *MEH1* and *SLM4* appear to be required for high sugar fermentation since their deletion results in protracted fermentation, whilst *GTR2* does not appear essential as the deletants behave similarly to the parent.

The 89 gene HSF dataset was compared with datasets from previous studies related to fermentation (Abe & Minegishi, 2008; Ando *et al.*, 2006, 2007; Fujita *et al.*, 2006; Mollapour *et al.*, 2004; Reiner *et al.*, 2006; Teixeira *et al.*, 2009, 2010; Tucker & Fields, 2004; van Voorst *et al.*, 2006; Auesukaree *et al.*, 2009). Sixty four of the genes (Figure F.3) had been reported in previous growth studies (typically single stress parameters) and a transcriptomics study looking at the gene expression profile of strain VIN13 fermenting Riesling juice in response to increasing ethanol concentrations (Marks *et al.*, 2008).

Laboratory scale ferments were also conducted on 81 genes, which were shown to be functionally related to the HFS genes, but which either had been able to complete fermentation in the initial micro-fermentation screen or were excluded because of their growth defect (< 70% of the optical density of the parent strain). Of these, 17 deletants (21%) were shown to have protracted fermentation and thus were also considered essential for fermentation. Nine deletants that gave rise to stuck fermentations are central to transcription (*RPA12*, *SIN3*, *ZAP1*), NAD recycling (*NPT1*), trehalose synthesis (*TPS1, TPS2*), signalling (*PLC1*), ubiquitin recycling (*DOA4*) and ion homeostasis (*SSQ1*).

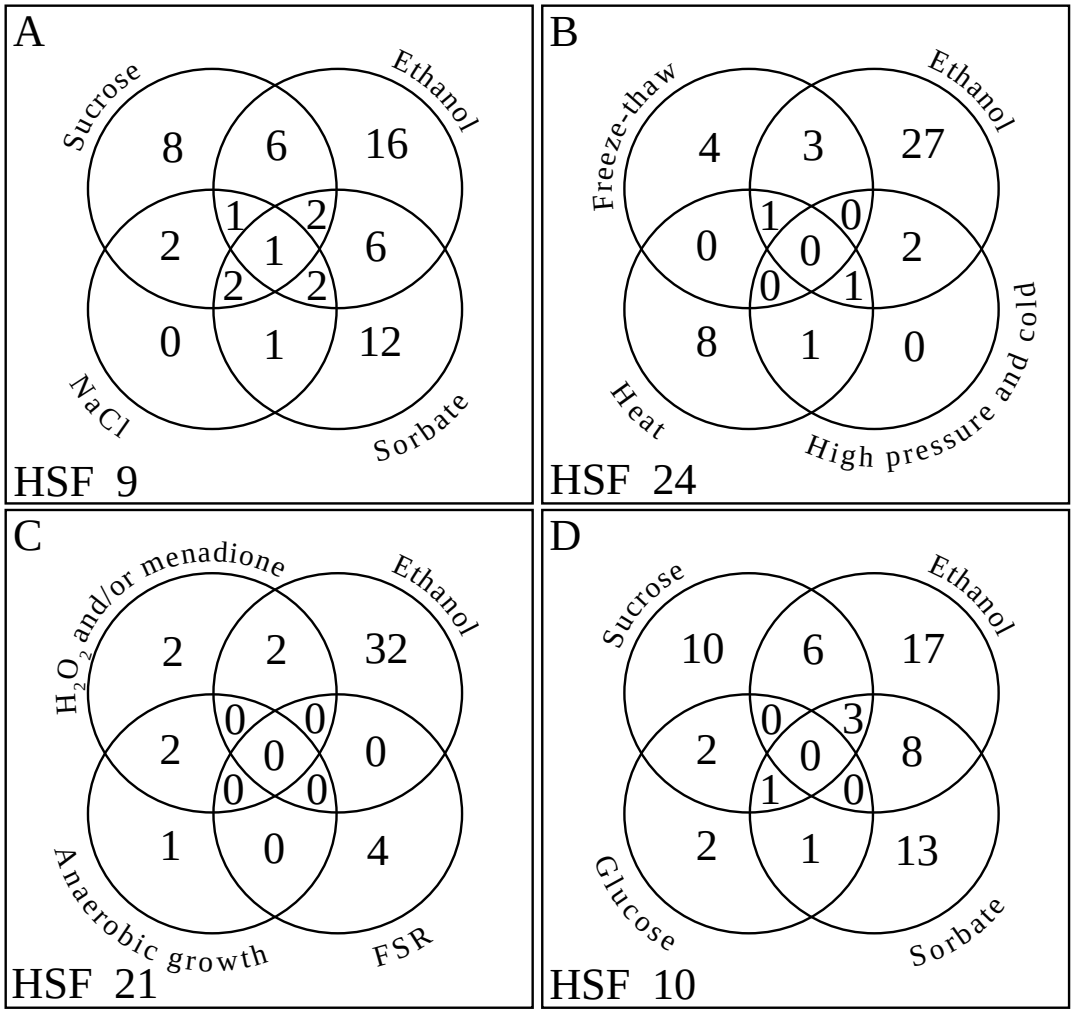


Figure F.3: Comparison of genes identified in this study with genes identified in growth studies to single stress conditions and a transcriptomics study of fermentation. Sixty four genes were shown to result in protracted fermentation when deleted. The Venn diagrams depict the number of genes withing the 64 gene High Sugar Fermentation (HSF) dataset which show cross-sensitivity to A. Ethanol, sorbate, sucrose and salt (NaCl); B. Ethanol, high pressure and cold, heat, freeze-thaw; C. Ethanol, H_2O_2 and menadione, anaerobic growth and fermentation stress response (FSR); and D. Ethanol, sorbate, sucrose and glucose.

The HSF dataset (89 genes) was classified into groups of genes annotated to distinct groups based on their role in biological process, function within the cell and cellular localisations as shown in Table F.9 (only those GO terms with statistically significant over-representation or enrichment of genes relevant to fermentation are presented). The clustering and enrichment of genes annotated as these GO terms is useful in understanding the cellular mechanisms behind the yeast's adaption to the fermentation environment. Numerical enrichment of genes within the HSF dataset annotated to these GO terms, as compared to their occurrence within the genome, is useful in highlighting the potential role of such cellular mechanisms in response to the yeast's exposure and adaptation to fermentation stress. Accordingly, HSF genes were over-represented in GO terms relating to cellular ion homeostasis, vacuolar acidification, ion transport, cellular pH reduction, autophagy and regulation of transcription. Not surprisingly, these genes were also annotated to the vacuole, and to a lesser extent, the endosome. The vacuole has a multifunctional role; trafficking of membrane proteins to the plasma membrane, proteolytic

degradation and recycling of proteins sent to the vacuole, sequestration of toxic metal ions, ion homeostasis (calcium and phosphate), osmo-regulation (Na^+/K^+ accumulation) and storage of amino acids.

Table F.9: Classification and numerical enrichment of genes annotated to biological process, function, and cellular component

GO ID	GO Term	Description
6873	**Cellular ion homeostasis*** **	
DBF2	YGR092W	Ser/Thr kinase involved in transcription and stress response; functions as part of a network of genes in exit from mitosis; localization is cell cycle regulated; activated by Cdc15p during the exit from mitosis
HRK1	YOR267C	Protein kinase implicated in activation of the plasma membrane H(+)-ATPase Pma1p in response to glucose metabolism; plays a role in ion homeostasis
MEH1	YKR007W	Component of the EGO complex, which is involved in the regulation of microautophagy, and of the GSE complex, which is required for proper sorting of amino acid permease Gap1p; loss results in a defect in vacuolar acidification
NHX1	YDR456W	Na+/H+ and K+/H+ exchanger, required for intracellular sequestration of Na+ and K+; located in the vacuole and late endosome compartments; required for osmotolerance to acute hypertonic shock and for vacuolar fusion
OCT1	YKL134C	Mitochondrial intermediate peptidase, cleaves destabilizing N-terminal residues of a subset of proteins upon import, after their cleavage by mitochondrial processing peptidase (Mas1p-Mas2p); may contribute to mitochondrial iron homeostasis
RAV1	YJR033C	Subunit of the RAVE complex (Rav1p, Rav2p, Skp1p), which promotes assembly of the V-ATPase holoenzyme; required for transport between the early and late endosome/PVC and for localization of TGN membrane proteins; potential Cdc28p substrate
RAV2	YDR202C	Subunit of RAVE (Rav1p, Rav2p, Skp1p), a complex that associates with the V1 domain of the vacuolar membrane (H+)-ATPase (V-ATPase) and promotes assembly and reassembly of the holoenzyme
DBF2	YGR092W	Ser/Thr kinase involved in transcription and stress response; functions as part of a network of genes in exit from mitosis; localization is cell cycle regulated; activated by Cdc15p during the exit from mitosis
HRK1	YOR267C	Protein kinase implicated in activation of the plasma membrane H(+)-ATPase Pma1p in response to glucose metabolism; plays a role in ion homeostasis
MEH1	YKR007W	Component of the EGO complex, which is involved in the regulation of microautophagy, and of the GSE complex, which is required for proper sorting of amino acid permease Gap1p; loss results in a defect in vacuolar acidification
SSQ1	YLR369W	Mitochondrial hsp70-type molecular chaperone, required for assembly of iron/sulfur clusters into proteins at a step after cluster synthesis, and for maturation of Yfh1p, which is a homolog of human frataxin implicated in Friedreich's ataxia
TRK1	YJL129C	Component of the Trk1p-Trk2p potassium transport system; 180 kDa high affinity potassium transporter; phosphorylated in vivo and interacts physically with the phosphatase Ppz1p, suggesting Trk1p acitivy is regulated by phosphorylation
TFP1	YDL185W	VMA1; Subunit A of the eight-subunit V1 peripheral membrane domain of the vacuolar H+-ATPase; protein precursor undergoes self-catalyzed splicing to yield the extein Tfp1p and the intein Vde (PI-SceI), which is a site-specific endonuclease
VMA2	YBR127C	Subunit B of the eight-subunit V1 peripheral membrane domain of the vacuolar H+-ATPase (V-ATPase), an electrogenic proton pump found throughout the endomembrane system; contains nucleotide binding sites; also detected in the cytoplasm
VMA3	**YEL027W**	Proteolipid subunit of the vacuolar H(+)-ATPase V0 sector (subunit c; dicyclohexylcarbodiimide binding subunit); required for vacuolar acidification and important for copper and iron metal ion homeostasis
VMA5	YKL080W	Subunit C of the eight-subunit V1 peripheral membrane domain of vacuolar H+-ATPase (V-ATPase), an electrogenic proton pump found throughout the endomembrane system; required for the V1 domain to assemble onto the vacuolar membrane
VMA7	YGR020C	Subunit F of the eight-subunit V1 peripheral membrane domain of vacuolar H+-ATPase (V-ATPase), an electrogenic proton pump found throughout the endomembrane system; required for the V1 domain to assemble onto the vacuolar membrane
VMA8	YEL051W	Subunit D of the eight-subunit V1 peripheral membrane domain of the vacuolar H+-ATPase (V-ATPase), an electrogenic proton pump found

		throughout the endomembrane system; plays a role in the coupling of proton transport and ATP hydrolysis
VMA10	YHR039C-A	Subunit G of the eight-subunit V1 peripheral membrane domain of the vacuolar H+-ATPase (V-ATPase), an electrogenic proton pump found throughout the endomembrane system; involved in vacuolar acidification
VMA13	YPR036W	Subunit H of the eight-subunit V1 peripheral membrane domain of the vacuolar H+-ATPase (V-ATPase), an electrogenic proton pump found throughout the endomembrane system; serves as an activator or a structural stabilizer of the V-ATPase
VMA16	YHR026W	PPA1; Subunit c" of the vacuolar ATPase, which functions in acidification of the vacuole; one of three proteolipid subunits of the V0 domain
VPH1	YOR270C	Subunit a of vacuolar-ATPase V0 domain, one of two isoforms (Vph1p and Stv1p); Vph1p is located in V-ATPase complexes of the vacuole while Stv1p is located in V-ATPase complexes of the Golgi and endosomes
VPH2	YKL119C	Integral membrane protein required for vacuolar H+-ATPase (V-ATPase) function, although not an actual component of the V-ATPase complex; functions in the assembly of the V-ATPase; localized to the endoplasmic reticulum (ER)
7035	**vacuolar acidification*** (VMA5,DBF2,MEH1,VMA2,NHX1,VMA13,VMA10,VMA7,VPH1,TFP1,RAV1,RAV2,VPH2,VMA8)**	
51452	**intracellular pH reduction*** (VMA5,DBF2,MEH1,VMA2,NHX1,VMA13,VMA10,VMA7,VPH1,TFP1,RAV1,RAV2,VPH2,VMA8)**	
DBF2	YGR092W	Ser/Thr kinase involved in transcription and stress response; functions as part of a network of genes in exit from mitosis; localization is cell cycle regulated; activated by Cdc15p during the exit from mitosis
MEH1	YKR007W	Component of the EGO complex, which is involved in the regulation of microautophagy, and of the GSE complex, which is required for proper sorting of amino acid permease Gap1p; loss results in a defect in vacuolar acidification
NHX1	YDR456W	Na+/H+ and K+/H+ exchanger, required for intracellular sequestration of Na+ and K+; located in the vacuole and late endosome compartments; required for osmotolerance to acute hypertonic shock and for vacuolar fusion
RAV1	YJR033C	Subunit of the RAVE complex (Rav1p, Rav2p, Skp1p), which promotes assembly of the V-ATPase holoenzyme; required for transport between the early and late endosome/PVC and for localization of TGN membrane proteins; potential Cdc28p substrate
RAV2	YDR202C	Subunit of RAVE (Rav1p, Rav2p, Skp1p), a complex that associates with the V1 domain of the vacuolar membrane (H+)-ATPase (V-ATPase) and promotes assembly and reassembly of the holoenzyme
TFP1	YDL185W	VMA1; Subunit A of the eight-subunit V1 peripheral membrane domain of the vacuolar H+-ATPase; protein precursor undergoes self-catalyzed splicing to yield the extein Tfp1p and the intein Vde (PI-SceI), which is a site-specific endonuclease
VMA2	YBR127C	Subunit B of the eight-subunit V1 peripheral membrane domain of the vacuolar H+-ATPase (V-ATPase), an electrogenic proton pump found throughout the endomembrane system; contains nucleotide binding sites; also detected in the cytoplasm
VMA3	**YEL027W**	Proteolipid subunit of the vacuolar H(+)-ATPase V0 sector (subunit c; dicyclohexylcarbodiimide binding subunit); required for vacuolar acidification and important for copper and iron metal ion homeostasis
VMA5	YKL080W	Subunit C of the eight-subunit V1 peripheral membrane domain of vacuolar H+-ATPase (V-ATPase), an electrogenic proton pump found throughout the endomembrane system; required for the V1 domain to assemble onto the vacuolar membrane
VMA7	YGR020C	Subunit F of the eight-subunit V1 peripheral membrane domain of vacuolar H+-ATPase (V-ATPase), an electrogenic proton pump found throughout the endomembrane system; required for the V1 domain to assemble onto the vacuolar membrane
VMA8	YEL051W	Subunit D of the eight-subunit V1 peripheral membrane domain of the vacuolar H+-ATPase (V-ATPase), an electrogenic proton pump found throughout the endomembrane system; plays a role in the coupling of proton transport and ATP hydrolysis
VMA10	YHR039C-A	Subunit G of the eight-subunit V1 peripheral membrane domain of the vacuolar H+-ATPase (V-ATPase), an electrogenic proton pump found throughout the endomembrane system; involved in vacuolar acidification
VMA13	YPR036W	Subunit H of the eight-subunit V1 peripheral membrane domain of the vacuolar H+-ATPase (V-ATPase), an electrogenic proton pump found throughout the endomembrane system; serves as an activator or a structural stabilizer of the V-ATPase

VMA16	YHR026W	PPA1; Subunit c" of the vacuolar ATPase, which functions in acidification of the vacuole; one of three proteolipid subunits of the V0 domain
VPH1	YOR270C	Subunit a of vacuolar-ATPase V0 domain, one of two isoforms (Vph1p and Stv1p); Vph1p is located in V-ATPase complexes of the vacuole while Stv1p is located in V-ATPase complexes of the Golgi and endosomes
VPH2	YKL119C	Integral membrane protein required for vacuolar H+-ATPase (V-ATPase) function, although not an actual component of the V-ATPase complex; functions in the assembly of the V-ATPase; localized to the endoplasmic reticulum (ER)
42625	**ATPase activity, coupled to transmembrane movement of ions***	**(VMA5,VMA2,VMA13,VMA10,VMA7,VPH1,TFP1,RAV1,VMA8)**
46961	**proton-transporting ATPase activity, rotational mechanism***	**(VMA5,VMA2,VMA13,VMA10,VMA7,VPH1,TFP1,RAV1,VMA8)**
220	**vacuolar proton-transporting V-type ATPase, V0 domain***	**(VMA3, VMA16, VPH1)**
34220	**ion transmembrane transport***	**(VMA5,VMA2,VMA13,VMA7,VPH1,TFP1,VMA8)**
TFP1	YDL185W	VMA1; Subunit A of the eight-subunit V1 peripheral membrane domain of the vacuolar H+-ATPase; protein precursor undergoes self-catalyzed splicing to yield the extein Tfp1p and the intein Vde (PI-SceI), which is a site-specific endonuclease
VPH1	YOR270C	Subunit a of vacuolar-ATPase V0 domain, one of two isoforms (Vph1p and Stv1p); Vph1p is located in V-ATPase complexes of the vacuole while Stv1p is located in V-ATPase complexes of the Golgi and endosomes
VMA2	YBR127C	Subunit B of the eight-subunit V1 peripheral membrane domain of the vacuolar H+-ATPase (V-ATPase), an electrogenic proton pump found throughout the endomembrane system; contains nucleotide binding sites; also detected in the cytoplasm
VMA5	YKL080W	Subunit C of the eight-subunit V1 peripheral membrane domain of vacuolar H+-ATPase (V-ATPase), an electrogenic proton pump found throughout the endomembrane system; required for the V1 domain to assemble onto the vacuolar membrane
VMA7	YGR020C	Subunit F of the eight-subunit V1 peripheral membrane domain of vacuolar H+-ATPase (V-ATPase), an electrogenic proton pump found throughout the endomembrane system; required for the V1 domain to assemble onto the vacuolar membrane
VMA8	YEL051W	Subunit D of the eight-subunit V1 peripheral membrane domain of the vacuolar H+-ATPase (V-ATPase), an electrogenic proton pump found throughout the endomembrane system; plays a role in the coupling of proton transport and ATP hydrolysis
VMA10	YHR039C-A	Subunit G of the eight-subunit V1 peripheral membrane domain of the vacuolar H+-ATPase (V-ATPase), an electrogenic proton pump found throughout the endomembrane system; involved in vacuolar acidification
VMA13	YPR036W	Subunit H of the eight-subunit V1 peripheral membrane domain of the vacuolar H+-ATPase (V-ATPase), an electrogenic proton pump found throughout the endomembrane system; serves as an activator or a structural stabilizer of the V-ATPase
VMA16	YHR026W	PPA1; Subunit c" of the vacuolar ATPase, which functions in acidification of the vacuole; one of three proteolipid subunits of the V0 domain
70072	**vacuolar proton-transporting V-type ATPase complex assembly**	
PKR1	YMR123W	V-ATPase assembly factor, functions with other V-ATPase assembly factors in the ER to efficiently assemble the V-ATPase membrane sector (V0)
VPH2	YKL119C	Integral membrane protein required for vacuolar H+-ATPase (V-ATPase) function, although not an actual component of the V-ATPase complex; functions in the assembly of the V-ATPase; localized to the endoplasmic reticulum (ER)
10324	**membrane invagination***	
ATG7	YHR171W	Autophagy-related protein and dual specificity member of the E1 family of ubiquitin-activating enzymes; mediates the conjugation of Atg12p with Atg5p and Atg8p with phosphatidylethanolamine, required steps in autophagosome formation
CIS1	YDR022C	Autophagy-specific protein required for autophagosome formation; may form a complex with Atg17p and Atg29p that localizes other proteins to the pre-autophagosomal structure; high-copy suppressor of CIK1 deletion
DOA4	YDR069C	Ubiquitin isopeptidase, required for recycling ubiquitin from proteasome-bound ubiquitinated intermediates, acts at the late

		endosome/prevacuolar compartment to recover ubiquitin from ubiquitinated membrane proteins en route to the vacuole
MEH1	YKR007W	Component of the EGO complex, which is involved in the regulation of microautophagy, and of the GSE complex, which is required for proper sorting of amino acid permease Gap1p; loss results in a defect in vacuolar acidification
SLM4	YBR077C	Component of the EGO complex, which is involved in the regulation of microautophagy, and of the GSE complex, which is required for proper sorting of amino acid permease Gap1p; gene exhibits synthetic genetic interaction with MSS4
SNX4	YJL036W	Sorting nexin, involved in retrieval of late-Golgi SNAREs from post-Golgi endosomes to the trans-Golgi network and in cytoplasm to vacuole transport; contains a PX phosphoinositide-binding domain; forms complexes with Snx41p and with Atg20p
VAC8	YEL013W	Phosphorylated and palmitoylated vacuolar membrane protein that interacts with Atg13p, required for the cytoplasm-to-vacuole targeting (Cvt) pathway; interacts with Nvj1p to form nucleus-vacuole junctions
VAM3	YOR106W	Syntaxin-related protein required for vacuolar assembly; functions with Vam7p in vacuolar protein trafficking; multispecificity vacuolar t-SNARE that mediates docking/fusion of multiple distinct late transport intermediates with the vacuole
VMA3	**YEL027W**	Proteolipid subunit of the vacuolar H(+)-ATPase V0 sector (subunit c; dicyclohexylcarbodiimide binding subunit); required for vacuolar acidification and important for copper and iron metal ion homeostasis
VPS41	YDR080W	Vacuolar membrane protein that is a subunit of the homotypic vacuole fusion and vacuole protein sorting (HOPS) complex; essential for membrane docking and fusion at the Golgi-to-endosome and endosome-to-vacuole stages of protein transport
6914	**autophagy********	(ATG7, CCZ1, CIS1, MEH1, SLM4, SNX4, VAC8, VAM3, VPS41)
16237	**microautophagy*****	(ATG7, CIS1, MEH1, SLM4, SNX4, VAC8, VAM3, VPS41)
34727	**piecemeal microautophagy of nucleus*****	(ATG7, CIS1, SLM4, VAC8, VAM3, VPS41)
42594	**response to starvation**	(CCZ1, VAC8, VAM3, ZAP1, ATG7)
422	**mitochondrion degradation*****	(ATG7, CIS1,SNX4, VAC8, VAM3, VPS41)
ATG7	YHR171W	Autophagy-related protein and dual specificity member of the E1 family of ubiquitin-activating enzymes; mediates the conjugation of Atg12p with Atg5p and Atg8p with phosphatidylethanolamine, required steps in autophagosome formation
CCZ1	YBR131W	Protein involved in vacuolar assembly, essential for autophagy and the cytoplasm-to-vacuole pathway
CIS1	YDR022C	Autophagy-specific protein required for autophagosome formation; may form a complex with Atg17p and Atg29p that localizes other proteins to the pre-autophagosomal structure; high-copy suppressor of CIK1 deletion
MEH1	YKR007W	Component of the EGO complex, which is involved in the regulation of microautophagy, and of the GSE complex, which is required for proper sorting of amino acid permease Gap1p; loss results in a defect in vacuolar acidification
SLM4	YBR077C	Component of the EGO complex, which is involved in the regulation of microautophagy, and of the GSE complex, which is required for proper sorting of amino acid permease Gap1p; gene exhibits synthetic genetic interaction with MSS4
SNX4	YJL036W	Sorting nexin, involved in retrieval of late-Golgi SNAREs from post-Golgi endosomes to the trans-Golgi network and in cytoplasm to vacuole transport; contains a PX phosphoinositide-binding domain; forms complexes with Snx41p and with Atg20p
VAC8	YEL013W	Phosphorylated and palmitoylated vacuolar membrane protein that interacts with Atg13p, required for the cytoplasm-to-vacuole targeting (Cvt) pathway; interacts with Nvj1p to form nucleus-vacuole junctions
VAM3	YOR106W	Syntaxin-related protein required for vacuolar assembly; functions with Vam7p in vacuolar protein trafficking; multispecificity vacuolar t-SNARE that mediates docking/fusion of multiple distinct late transport intermediates with the vacuole
VPS41	YDR080W	Vacuolar membrane protein that is a subunit of the homotypic vacuole fusion and vacuole protein sorting (HOPS) complex; essential for membrane docking and fusion at the Golgi-to-endosome and endosome-to-vacuole stages of protein transport
ZAP1	YJL056C	Zinc-regulated transcription factor; binds to zinc-responsive promoters to induce transcription of certain genes in presence of zinc, represses other genes in low zinc; regulates its own transcription; contains seven zinc-finger domains

5773	**vacuole*** **	**(**VMA5,VAM3,SLM4,MEH1,VMA2,VPS41,VMA13,VMA10,VMA7,VPH1,TCO89,TFP1,RAV1,CCZ1,VAC8,VMA8)
MEH1	YKR007W	Component of the EGO complex, which is involved in the regulation of microautophagy, and of the GSE complex, which is required for proper sorting of amino acid permease Gap1p; loss results in a defect in vacuolar acidification
NHX1	YDR456W	Na+/H+ and K+/H+ exchanger, required for intracellular sequestration of Na+ and K+; located in the vacuole and late endosome compartments; required for osmotolerance to acute hypertonic shock and for vacuolar fusion
SLM4	YBR077C	Component of the EGO complex, which is involved in the regulation of microautophagy, and of the GSE complex, which is required for proper sorting of amino acid permease Gap1p; gene exhibits synthetic genetic interaction with MSS4
TCO89	YPL180W	Subunit of TORC1 (Tor1p or Tor2p-Kog1p-Lst8p-Tco89p), a complex that regulates growth in response to nutrient availability; cooperates with Ssd1p in the maintenance of cellular integrity; deletion strains are hypersensitive to rapamycin
TFP1	YDL185W	VMA1; Subunit A of the eight-subunit V1 peripheral membrane domain of the vacuolar H+-ATPase; protein precursor undergoes self-catalyzed splicing to yield the extein Tfp1p and the intein Vde (PI-SceI), which is a site-specific endonuclease
VAC8	YEL013W	Phosphorylated and palmitoylated vacuolar membrane protein that interacts with Atg13p, required for the cytoplasm-to-vacuole targeting (Cvt) pathway; interacts with Nvj1p to form nucleus-vacuole junctions
VAM3	YOR106W	Syntaxin-related protein required for vacuolar assembly; functions with Vam7p in vacuolar protein trafficking; multispecificity vacuolar t-SNARE that mediates docking/fusion of multiple distinct late transport intermediates with the vacuole
VMA2	YBR127C	Subunit B of the eight-subunit V1 peripheral membrane domain of the vacuolar H+-ATPase (V-ATPase), an electrogenic proton pump found throughout the endomembrane system; contains nucleotide binding sites; also detected in the cytoplasm
VMA3	**YEL027W**	Proteolipid subunit of the vacuolar H(+)-ATPase V0 sector (subunit c; dicyclohexylcarbodiimide binding subunit); required for vacuolar acidification and important for copper and iron metal ion homeostasis
VMA5	YKL080W	Subunit C of the eight-subunit V1 peripheral membrane domain of vacuolar H+-ATPase (V-ATPase), an electrogenic proton pump found throughout the endomembrane system; required for the V1 domain to assemble onto the vacuolar membrane
VMA7	YGR020C	Subunit F of the eight-subunit V1 peripheral membrane domain of vacuolar H+-ATPase (V-ATPase), an electrogenic proton pump found throughout the endomembrane system; required for the V1 domain to assemble onto the vacuolar membrane
VMA8	YEL051W	Subunit D of the eight-subunit V1 peripheral membrane domain of the vacuolar H+-ATPase (V-ATPase), an electrogenic proton pump found throughout the endomembrane system; plays a role in the coupling of proton transport and ATP hydrolysis
VMA10	YHR039C-A	Subunit G of the eight-subunit V1 peripheral membrane domain of the vacuolar H+-ATPase (V-ATPase), an electrogenic proton pump found throughout the endomembrane system; involved in vacuolar acidification
VMA13	YPR036W	Subunit H of the eight-subunit V1 peripheral membrane domain of the vacuolar H+-ATPase (V-ATPase), an electrogenic proton pump found throughout the endomembrane system; serves as an activator or a structural stabilizer of the V-ATPase
VMA16	YHR026W	PPA1; Subunit c" of the vacuolar ATPase, which functions in acidification of the vacuole; one of three proteolipid subunits of the V0 domain
VPH1	YOR270C	Subunit a of vacuolar-ATPase V0 domain, one of two isoforms (Vph1p and Stv1p); Vph1p is located in V-ATPase complexes of the vacuole while Stv1p is located in V-ATPase complexes of the Golgi and endosomes
VPS41	YDR080W	Vacuolar membrane protein that is a subunit of the homotypic vacuole fusion and vacuole protein sorting (HOPS) complex; essential for membrane docking and fusion at the Golgi-to-endosome and endosome-to-vacuole stages of protein transport
5768	**endosome****	(CCZ1, DOA4, MEH1, NHX1, RAV2, SLM4, SNX4, VPS41)
CCZ1	YBR131W	Protein involved in vacuolar assembly, essential for autophagy and the cytoplasm-to-vacuole pathway
DOA4	YDR069C	Ubiquitin isopeptidase, required for recycling ubiquitin from proteasome-bound ubiquitinated intermediates, acts at the late endosome/prevacuolar compartment to recover ubiquitin from ubiquitinated membrane proteins en route to the vacuole
MEH1	YKR007W	Component of the EGO complex, which is involved in the regulation of microautophagy, and of the GSE complex, which is required for proper

		sorting of amino acid permease Gap1p; loss results in a defect in vacuolar acidification
NHX1	YDR456W	Na+/H+ and K+/H+ exchanger, required for intracellular sequestration of Na+ and K+; located in the vacuole and late endosome compartments; required for osmotolerance to acute hypertonic shock and for vacuolar fusion
RAV2	YDR202C	Subunit of RAVE (Rav1p, Rav2p, Skp1p), a complex that associates with the V1 domain of the vacuolar membrane (H+)-ATPase (V-ATPase) and promotes assembly and reassembly of the holoenzyme
SLM4	YBR077C	Component of the EGO complex, which is involved in the regulation of microautophagy, and of the GSE complex, which is required for proper sorting of amino acid permease Gap1p; gene exhibits synthetic genetic interaction with MSS4
SNX4	YJL036W	Sorting nexin, involved in retrieval of late-Golgi SNAREs from post-Golgi endosomes to the trans-Golgi network and in cytoplasm to vacuole transport; contains a PX phosphoinositide-binding domain; forms complexes with Snx41p and with Atg20p
VPS41	YDR080W	Vacuolar membrane protein that is a subunit of the homotypic vacuole fusion and vacuole protein sorting (HOPS) complex; essential for membrane docking and fusion at the Golgi-to-endosome and endosome-to-vacuole stages of protein transport
6366	**transcription from RNA polymerase II promoter**	(CCR4, DST1, HFI1, HOG1, MKS1, PAF1, RTT103, SAP30, SIN3, SPT4, ZAP1)
6360	**transcription from RNA polymerase I promoter*** **	(DST1, PAF1, RPA12, RXT13, SAP30, SIN3, SPT4)
CCR4	YAL021C	Component of the CCR4-NOT transcriptional complex, which is involved in regulation of gene expression; component of the major cytoplasmic deadenylase, which is involved in mRNA poly(A) tail shortening
DST1	YGL043W	General transcription elongation factor TFIIS, enables RNA polymerase II to read through blocks to elongation by stimulating cleavage of nascent transcripts stalled at transcription arrest sites
HFI1	YPL254W	Adaptor protein required for structural integrity of the SAGA complex, a histone acetyltransferase-coactivator complex that is involved in global regulation of gene expression through acetylation and transcription functions
HOG1	YLR113W	Mitogen-activated protein kinase involved in osmoregulation via three independent osmosensors; mediates the recruitment and activation of RNA Pol II at Hot1p-dependent promoters; localization regulated by Ptp2p and Ptp3p
MKS1	YNL076W	Pleiotropic negative transcriptional regulator involved in Ras-CAMP and lysine biosynthetic pathways and nitrogen regulation; involved in retrograde (RTG) mitochondria-to-nucleus signaling
OPI1	YHL020C	Transcriptional regulator of a variety of genes; phosphorylation by protein kinase A stimulates Opi1p function in negative regulation of phospholipid biosynthetic genes; involved in telomere maintenance
PAF1	YBR279W	Component of the Paf1p complex that binds to and modulates the activity of RNA polymerases I and II; required for expression of a subset of genes, including cell cycle-regulated genes; homolog of human PD2/hPAF1
RPA12	YJR063W	RNA polymerase I subunit A12.2; contains two zinc binding domains, and the N terminal domain is responsible for anchoring to the RNA pol I complex
RTT103	YDR289C	Protein that interacts with exonuclease Rat1p and Rai1p and plays a role in transcription termination by RNA polymerase II, has an RPR domain (carboxy-terminal domain interacting domain); also involved in regulation of Ty1 transposition
RXT3	YDL076C	Subunit of the RPD3L complex; involved in histone deacetylation
SAP30	YMR263W	Subunit of a histone deacetylase complex, along with Rpd3p and Sin3p, that is involved in silencing at telomeres, rDNA, and silent mating-type loci; involved in telomere maintenance
SIN3	YOL004W	Component of the Sin3p-Rpd3p histone deacetylase complex, involved in transcriptional repression and activation of diverse processes, including mating-type switching and meiosis; involved in the maintenance of chromosomal integrity
SPT4	YGR063C	Protein involved in the regulating Pol I and Pol II transcription, pre-mRNA processing, kinetochore function, and gene silencing; forms a complex with Spt5p
ZAP1	YJL056C	Zinc-regulated transcription factor; binds to zinc-responsive promoters to induce transcription of certain genes in presence of zinc, represses

		other genes in low zinc; regulates its own transcription; contains seven zinc-finger domains
9408	**response to heat*** **	
HOG1	YLR113W	Mitogen-activated protein kinase involved in osmoregulation via three independent osmosensors; mediates the recruitment and activation of RNA Pol II at Hot1p-dependent promoters; localization regulated by Ptp2p and Ptp3p
NUP133	YKR082W	Subunit of the Nup84p subcomplex of the nuclear pore complex (NPC), localizes to both sides of the NPC, required to establish a normal nucleocytoplasmic concentration gradient of the GTPase Gsp1p
NUP188	YML103C	Subunit of the nuclear pore complex (NPC), involved in the structural organization of the complex and of the nuclear envelope, also involved in nuclear envelope permeability, interacts with Pom152p and Nic96p
PBS2	YJL128C	MAP kinase kinase that plays a pivotal role in the osmosensing signal-transduction pathway, activated under severe osmotic stress; plays a role in regulating Ty1 transposition
SAP30	YMR263W	Subunit of a histone deacetylase complex, along with Rpd3p and Sin3p, that is involved in silencing at telomeres, rDNA, and silent mating-type loci; involved in telomere maintenance
SIN3	YOL004W	Component of the Sin3p-Rpd3p histone deacetylase complex, involved in transcriptional repression and activation of diverse processes, including mating-type switching and meiosis; involved in the maintenance of chromosomal integrity
7154	**cell communication*** **	(ASC1, GPA2, GPR1, HOG1, MKS1, OPI1, PBS2, PLC1, SLM4, TCO89, VAC8, VAM3, ZAP1)
23052	**signalling**	(ASC1, GPA2, GPR1, HOG1, MKS1, OPI1, PBS2, PEX1, PLC1, SLM4, TCO89)
9757	**hexose mediated signaling*** **	(ASC1, GPA2, GPR1)
ASC1	YMR116C	G-protein beta subunit and guanine nucleotide dissociation inhibitor for Gpa2p; ortholog of RACK1 that inhibits translation; core component of the small (40S) ribosomal subunit; represses Gcn4p in the absence of amino acid starvation
ATG7	YHR171W	Autophagy-related protein and dual specificity member of the E1 family of ubiquitin-activating enzymes; mediates the conjugation of Atg12p with Atg5p and Atg8p with phosphatidylethanolamine, required steps in autophagosome formation
CCZ1	YBR131W	Protein involved in vacuolar assembly, essential for autophagy and the cytoplasm-to-vacuole pathway
GPA2	YER020W	Nucleotide binding alpha subunit of the heterotrimeric G protein that interacts with the receptor Gpr1p, has signaling role in response to nutrients; green fluorescent protein (GFP)-fusion protein localizes to the cell periphery
GPR1	YDL035C	Plasma membrane G protein coupled receptor (GPCR) that interacts with the heterotrimeric G protein alpha subunit, Gpa2p, and with Plc1p; sensor that integrates nutritional signals with the modulation of cell fate via PKA and cAMP synthesis
HOG1	YLR113W	Mitogen-activated protein kinase involved in osmoregulation via three independent osmosensors; mediates the recruitment and activation of RNA Pol II at Hot1p-dependent promoters; localization regulated by Ptp2p and Ptp3p
MKS1	YNL076W	Pleiotropic negative transcriptional regulator involved in Ras-CAMP and lysine biosynthetic pathways and nitrogen regulation; involved in retrograde (RTG) mitochondria-to-nucleus signaling
OPI1	YHL020C	Transcriptional regulator of a variety of genes; phosphorylation by protein kinase A stimulates Opi1p function in negative regulation of phospholipid biosynthetic genes; involved in telomere maintenance
PBS2	YJL128C	MAP kinase kinase that plays a pivotal role in the osmosensing signal-transduction pathway, activated under severe osmotic stress; plays a role in regulating Ty1 transposition
PEX1	YKL197C	AAA-peroxin that heterodimerizes with AAA-peroxin Pex6p and participates in the recycling of peroxisomal signal receptor Pex5p from the peroxisomal membrane to the cystosol; induced by oleic acid and upregulated during anaerobiosis
PLC1	YPL268W	Phospholipase C, hydrolyzes phosphatidylinositol 4,5-biphosphate (PIP2) to generate the signaling molecules inositol 1,4,5-triphosphate (IP3) and 1,2-diacylglycerol (DAG); involved in regulating many cellular processes
SLM4	YBR077C	Component of the EGO complex, which is involved in the regulation of microautophagy, and of the GSE complex, which is required for proper

		sorting of amino acid permease Gap1p; gene exhibits synthetic genetic interaction with MSS4
TCO89	YPL180W	Subunit of TORC1 (Tor1p or Tor2p-Kog1p-Lst8p-Tco89p), a complex that regulates growth in response to nutrient availability; cooperates with Ssd1p in the maintenance of cellular integrity; deletion strains are hypersensitive to rapamycin
VAC8	YEL013W	Phosphorylated and palmitoylated vacuolar membrane protein that interacts with Atg13p, required for the cytoplasm-to-vacuole targeting (Cvt) pathway; interacts with Nvj1p to form nucleus-vacuole junctions
VAM3	YOR106W	Syntaxin-related protein required for vacuolar assembly; functions with Vam7p in vacuolar protein trafficking; multispecificity vacuolar t-SNARE that mediates docking/fusion of multiple distinct late transport intermediates with the vacuole
ZAP1	YJL056C	Zinc-regulated transcription factor; binds to zinc-responsive promoters to induce transcription of certain genes in presence of zinc, represses other genes in low zinc; regulates its own transcription; contains seven zinc-finger domains
51186	**cofactor metabolic process**	
SSQ1	YLR369W	Mitochondrial hsp70-type molecular chaperone, required for assembly of iron/sulfur clusters into proteins at a step after cluster synthesis, and for maturation of Yfh1p, which is a homolog of human frataxin implicated in Friedreich's ataxia
NPT1	YOR209C	Nicotinate phosphoribosyltransferase, acts in the salvage pathway of NAD+ biosynthesis; required for silencing at rDNA and telomeres and has a role in silencing at mating-type loci; localized to the nucleus
TKL1	YPR074C	Transketolase, similar to Tkl2p; catalyzes conversion of xylulose-5-phosphate and ribose-5-phosphate to sedoheptulose-7-phosphate and glyceraldehyde-3-phosphate in the pentose phosphate pathway; needed for synthesis of aromatic amino acids
9116	**nucleoside metabolic process**	
ADO1	YJR105W	Adenosine kinase, required for the utilization of S-adenosylmethionine (AdoMet); may be involved in recycling adenosine produced through the methyl cycle
10525	**regulation of transposition, RNA-mediated**	
ASC1	YMR116C	G-protein beta subunit and guanine nucleotide dissociation inhibitor for Gpa2p; ortholog of RACK1 that inhibits translation; core component of the small (40S) ribosomal subunit; represses Gcn4p in the absence of amino acid starvation
BUD27	YFL023W	Unconventional prefoldin protein involved in translation initiation; mutants have inappropriate expression of nutrient sensitive genes due to translational derepression of Gcn4p transcription factor; diploid mutants show random budding
PAF1	YBR279W	Component of the Paf1p complex that binds to and modulates the activity of RNA polymerases I and II; required for expression of a subset of genes, including cell cycle-regulated genes; homolog of human PD2/hPAF1
RTT103	YDR289C	Protein that interacts with exonuclease Rat1p and Rai1p and plays a role in transcription termination by RNA polymerase II, has an RPR domain (carboxy-terminal domain interacting domain); also involved in regulation of Ty1 transposition
6629	**lipid metabolic process**	
ERG6	YML008C	Delta(24)-sterol C-methyltransferase, converts zymosterol to fecosterol in the ergosterol biosynthetic pathway by methylating position C-24; localized to both lipid particles and mitochondrial outer membrane
RML2	YEL050C	Mitochondrial ribosomal protein of the large subunit, has similarity to E. coli L2 ribosomal protein; fat21 mutant allele causes inability to utilize oleate and may interfere with activity of the Adr1p transcription factor
MCT1	YOR221C	Predicted malonyl-CoA:ACP transferase, putative component of a type-II mitochondrial fatty acid synthase that produces intermediates for phospholipid remodelling
PLC1	YPL268W	Phospholipase C, hydrolyzes phosphatidylinositol 4,5-biphosphate (PIP2) to generate the signaling molecules inositol 1,4,5-triphosphate (IP3) and 1,2-diacylglycerol (DAG); involved in regulating many cellular processes
OPI1	YHL020C	Transcriptional regulator of a variety of genes; phosphorylation by protein kinase A stimulates Opi1p function in negative regulation of phospholipid biosynthetic genes; involved in telomere maintenance

5991	**trehalose metabolic process**	
TPS1	YBR126C	Synthase subunit of trehalose-6-phosphate synthase/phosphatase complex, which synthesizes the storage carbohydrate trehalose; also found in a monomeric form; expression is induced by the stress response and repressed by the Ras-cAMP pathway
TPS2	YDR074W	Phosphatase subunit of the trehalose-6-phosphate synthase/phosphatase complex, which synthesizes the storage carbohydrate trehalose; expression is induced by stress conditions and repressed by the Ras-cAMP pathway
46164	**alcohol catabolic process****	(HXK1, TKL1)
8645	**Hexose transport**	(HXK1)
HXK1	YFR053C	Hexokinase isoenzyme 1, a cytosolic protein that catalyzes phosphorylation of glucose during glucose metabolism; expression is highest during growth on non-glucose carbon sources; glucose-induced repression involves the hexokinase Hxk2p
TKL1	YPR074C	Transketolase, similar to Tkl2p; catalyzes conversion of xylulose-5-phosphate and ribose-5-phosphate to sedoheptulose-7-phosphate and glyceraldehyde-3-phosphate in the pentose phosphate pathway; needed for synthesis of aromatic amino acids
278	**mitotic cell cycle**	
CCR4	YAL021C	Component of the CCR4-NOT transcriptional complex, which is involved in regulation of gene expression; component of the major cytoplasmic deadenylase, which is involved in mRNA poly(A) tail shortening
CNM67	YNL225C	Component of the spindle pole body outer plaque; required for spindle orientation and mitotic nuclear migration
DCC1	YCL016C	Subunit of a complex with Ctf8p and Ctf18p that shares some components with Replication Factor C, required for sister chromatid cohesion and telomere length maintenance
SIN3	YOL004W	Component of the Sin3p-Rpd3p histone deacetylase complex, involved in transcriptional repression and activation of diverse processes, including mating-type switching and meiosis; involved in the maintenance of chromosomal integrity
SPC72	YAL047C	Component of the cytoplasmic Tub4p (gamma-tubulin) complex, binds spindle pole bodies and links them to microtubules; has roles in astral microtubule formation and stabilization
TOM1	YDR457W	E3 ubiquitin ligase of the hect-domain class; has a role in mRNA export from the nucleus and may regulate transcriptional coactivators; involved in degradation of excess histones
VHS1	YDR247W	Cytoplasmic serine/threonine protein kinase; identified as a high-copy suppressor of the synthetic lethality of a sis2 sit4 double mutant, suggesting a role in G1/S phase progression; homolog of Sks1p
910	**cytokinesis**	
CYK3	YDL117W	SH3-domain protein located in the mother-bud neck and the cytokinetic actin ring; mutant phenotype and genetic interactions suggest a role in cytokinesis
BUD31	YCR063W	Component of the SF3b subcomplex of the U2 snRNP; diploid mutants display a random budding pattern instead of the wild-type bipolar pattern
7568	**Aging**	
GPA2	YER020W	Nucleotide binding alpha subunit of the heterotrimeric G protein that interacts with the receptor Gpr1p, has signaling role in response to nutrients; green fluorescent protein (GFP)-fusion protein localizes to the cell periphery
GPR1	YDL035C	Plasma membrane G protein coupled receptor (GPCR) that interacts with the heterotrimeric G protein alpha subunit, Gpa2p, and with Plc1p; sensor that integrates nutritional signals with the modulation of cell fate via PKA and cAMP synthesis
NPT1	YOR209C	Nicotinate phosphoribosyltransferase, acts in the salvage pathway of NAD+ biosynthesis; required for silencing at rDNA and telomeres and has a role in silencing at mating-type loci; localized to the nucleus
398	**nuclear mRNA splicing, via spliceosome**	
LSM1	YJL124C	Lsm (Like Sm) protein; forms heteroheptameric complex (with Lsm2p, Lsm3p, Lsm4p, Lsm5p, Lsm6p, and Lsm7p) involved in degradation of

		cytoplasmic mRNAs
SNT309	YPR101W	Member of the NineTeen Complex (NTC) that contains Prp19p and stabilizes U6 snRNA in catalytic forms of the spliceosome containing U2, U5, and U6 snRNAs; interacts physically and genetically with Prp19p
BUD31	YCR063W	Component of the SF3b subcomplex of the U2 snRNP; diploid mutants display a random budding pattern instead of the wild-type bipolar pattern
6974	**response to DNA damage stimulus**	
DCC1	YCL016C	Subunit of a complex with Ctf8p and Ctf18p that shares some components with Replication Factor C, required for sister chromatid cohesion and telomere length maintenance
SIN3	YOL004W	Component of the Sin3p-Rpd3p histone deacetylase complex, involved in transcriptional repression and activation of diverse processes, including mating-type switching and meiosis; involved in the maintenance of chromosomal integrity
SNF5	YBR289W	Subunit of the SWI/SNF chromatin remodeling complex involved in transcriptional regulation; functions interdependently in transcriptional activation with Snf2p and Snf6p
SPT4	YGR063C	Protein involved in the regulating Pol I and Pol II transcription, pre-mRNA processing, kinetochore function, and gene silencing; forms a complex with Spt5p
XRS2	YDR369C	Protein required for DNA repair; component of the Mre11 complex, which is involved in double strand breaks, meiotic recombination, telomere maintenance, and checkpoint signaling
YDR433W	YDR433W	Dubious open reading frame unlikely to encode a functional protein
51052	**regulation of DNA metabolic process**	
DIA2	YOR080W	Origin-binding F-box protein; forms an SCF ubiquitin ligase complex with Skp1p and Cdc53p; plays a role in DNA replication; plays a role in transcription; required for correct assembly of RSC complex, correct RSC-mediated transcription regulation, and cor
CCR4	YAL021C	Component of the CCR4-NOT transcriptional complex, which is involved in regulation of gene expression; component of the major cytoplasmic deadenylase, which is involved in mRNA poly(A) tail shortening
DOA4	YDR069C	Ubiquitin isopeptidase, required for recycling ubiquitin from proteasome-bound ubiquitinated intermediates, acts at the late endosome/prevacuolar compartment to recover ubiquitin from ubiquitinated membrane proteins en route to the vacuole
SIN3	YOL004W	Component of the Sin3p-Rpd3p histone deacetylase complex, involved in transcriptional repression and activation of diverse processes, including mating-type switching and meiosis; involved in the maintenance of chromosomal integrity
226	**microtubule cytoskeleton organization**	
RBL2	YOR265W	Protein involved in microtubule morphogenesis, required for protection from excess free beta-tubulin; proposed to be involved the folding of beta-tubulin; similar to mouse beta-tubulin cofactor A
CNM67	YNL225C	Component of the spindle pole body outer plaque; required for spindle orientation and mitotic nuclear migration
SPC72	YAL047C	Component of the cytoplasmic Tub4p (gamma-tubulin) complex, binds spindle pole bodies and links them to microtubules; has roles in astral microtubule formation and stabilization
6417	**regulation of translation**	
ZUO1	YGR285C	Cytosolic ribosome-associated chaperone that acts, together with Ssz1p and the Ssb proteins, as a chaperone for nascent polypeptide chains; contains a DnaJ domain and functions as a J-protein partner for Ssb1p and Ssb2p
PAT1	YCR077C	Topoisomerase II-associated deadenylation-dependent mRNA-decapping factor; also required for faithful chromosome transmission, maintenance of rDNA locus stability, and protection of mRNA 3'-UTRs from trimming; functionally linked to Pab1p
ASC1	YMR116C	G-protein beta subunit and guanine nucleotide dissociation inhibitor for Gpa2p; ortholog of RACK1 that inhibits translation; core component of the small (40S) ribosomal subunit; represses Gcn4p in the absence of amino acid starvation

7005	**mitochondrion organization**	
MSW1	YDR268W	Mitochondrial tryptophanyl-tRNA synthetase
TPM1	YNL079C	Major isoform of tropomyosin; binds to and stabilizes actin cables and filaments, which direct polarized cell growth and the distribution of several organelles; acetylated by the NatB complex and acetylated form binds actin most efficiently
OCT1	YKL134C	Mitochondrial intermediate peptidase, cleaves destabilizing N-terminal residues of a subset of proteins upon import, after their cleavage by mitochondrial processing peptidase (Mas1p-Mas2p); may contribute to mitochondrial iron homeostasis
34394	**protein localization at cell surface**	
GSF2	YML048W	ER localized integral membrane protein that may promote secretion of certain hexose transporters, including Gal2p; involved in glucose-dependent repression
other	**Other**	
PUG1	YER185W	Plasma membrane protein with roles in the uptake of protoprophyrin IX and the efflux of heme; expression is induced under both low-heme and low-oxygen conditions; member of the fungal lipid-translocating exporter (LTE) family of proteins
YGR064W	YGR064W	Dubious open reading frame unlikely to encode a protein
8150	**biological process unknown**	
YCL007C	YCL007C	Dubious ORF unlikely to encode a protein
IES6	YEL044W	Protein that associates with the INO80 chromatin remodeling complex under low-salt conditions; human ortholog INO80C is a member of the human INO80 complex; implicated in DNA repair based on genetic interactions with RAD52 epistasis genes
YLL007C	YLL007C	Putative protein of unknown function
YFL012W	YFL012W	Putative protein of unknown function; transcribed during sporulation; null mutant exhibits increased resistance to rapamycin
DUF1	YOL087C	Putative protein of unknown function

Genes were classified using the computational tools GO Finder, Slim Mapper (SGD database; (http://www.yeastgenome.org/) and GOToolBox (Martin *et al.*, 2004) into categories based on GO terms for biological process, function and cellular component. Description of genes is taken from SGD database. Enrichment of genes annotated to particular GO terms is highlighted with asterisk as shown ***enrichment ($p < 0.01$), **enrichment ($p < 0.05$).

Key to genes:
genes in regular font were identified in all 1st and 2nd screen and lab scale ferments
genes in regular underlined font were identified in 1st screen and lab scale ferments
genes in bold font were identified through GO analysis

Role of vacuolar H^+ ATPase complex in fermentation

Interestingly, a large number of the HSF genes are involved in the assembly and function of the vacuolar H^+-ATPase (V-ATPase) complex (Kane, 2006; Kane & Smardon, 2003). The V-ATPase complex is essential in maintaining intracellular pH through acidification of the vacuolar lumen relative to the cytosol. The action of the V-ATPase in organelle acidification is essential in maintaining cellular (ion) homeostasis (through action of antiporters utilizing the electrochemical or proton gradient generated), proteolytic activation (digestion of the autophagasomes during autophagy or vacuolar proteolysis induced by starvation), endocytosis, and vacuole fusion (Graham *et al.*, 2003). Cell wall structure and function, cytoskeletal organisation and maintenance and phospholipid content are also implicated (Zhang *et al.*, 1998; Kane, 2006). Yeast vma (vacuolar membrane ATPase) deletants, through the loss of V-ATPase activity, exhibit growth sensitivity in high pH (pH 7 or higher) and extracellular calcium and are unable to grow on non-fermentable carbon sources (Kane, 2006).

The importance of the assembly and function of V-ATPase complex, was highlighted in the number of genes identified which encode for either the subunits of the V_1 peripheral membrane and V_o integral membrane domains or the assembly factors including the RAVE complex, which are required for the reversible assembly and disassembly into the free V_1 and V_o domains, in response to glucose (Figure F.4; Kane, 2006; Kane & Smardon 2003). The assembly of V-ATPases is thought to prevent cytosolic acidification when metabolism is active, whilst when energy sources are limited (glucose), the disassembly might conserve ATP. Interestingly, not all the vma mutants, where deletion of these genes leads to the loss of organelle acidification (Kane, 2006), exhibit the protracted fermentation phenotype (Table F.10). Deletion of subunits E (VMA4) c' (VMA11), d (VMA6) and subunit of the golgi/endosome located V-ATPase (STV1) did not affect fermentation. Speculation arises as to whether passive diffusion, or the V_o and V_1 domains (lacking a subunit) are able to assemble and partially associate into an unstable albeit active holoenzyme, to maintain the proton gradient essential for many cellular processes. Furthermore, the V-ATPase in the golgi apparatus/endosome may not be required, as the vacuolar V-ATPase, whilst in transit to the vacuole, could contribute to endosomal acidification under certain conditions (Kane, 2006).

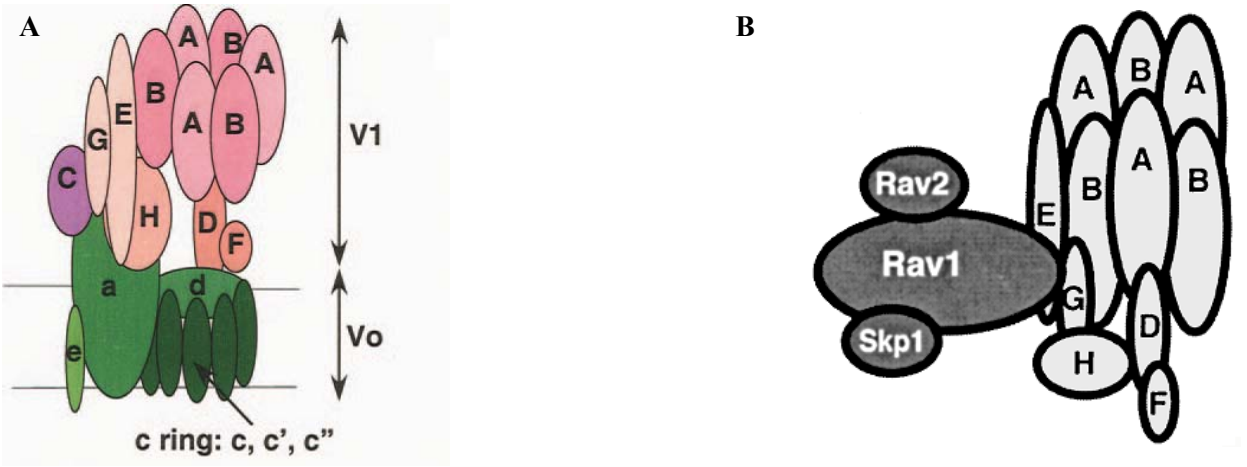


Figure F.4: Subunit composition and structural model of yeast V-ATPase and RAVE complex.
A. Structural model of yeast V-ATPase. V_1 subunits are shown in pink and purple, and V_0 subunits in green. A single stator stalk containing subunits C, E, G, H is shown (sourced from Kane, 2006). B. RAVE complex. Rav1p is central to binding of RAVE complex to V_1 domain of V-ATPase via subunits E and/or G (sourced from Kane & Smardon, 2003).

Table F.10: Genes encoding vacuolar H^+ ATPase (V-ATPase) whose deletion affects high sugar fermentation

Gene	Subunit	Proposed Function	Genes essential for fermentation	Genes non-essential for fermentation
	V_1 domain	**(eight subunit peripheral domain)**		
VMA1 (TFP1)	A	ATP hydrolysis	*VMA1 (TFP1)*	
VMA2	B	Regulatory, ATP binding?	*VMA2*	
VMA5	C	Stator	*VMA5*	
VMA8	D	Rotor	*VMA8*	
VMA4	E	Stator		*VMA4*
VMA7	F	Rotor	*VMA7*	
VMA10	G	Stator	*VMA10*	
VMA13	H	Stator	*VMA13*	
	V_o domain	**(five subunit integral domain)**		
VPH1	A	Proton pore, stator, sorting (vacuole)	*VPH1*	
STV1	a (Stv1p)	Proton pore, stator, sorting (golgi/endosome)		*STV1*
VMA3	C	Proton pore, rotor (dicyclohexylcarbodiimide binding)	*VMA3*	
VMA11 (TFP3)	c'	Proton pore, rotor		*VMA11 (TFP3)*
VMA16 (PPA1)	c"	Proton pore, rotor	*VMA16 (PPA1)*	
VMA6	D	Rotor		*VMA6*
VMA9	E	?	nd	nd
		Assembly Factors		
VPH2 (VMA12)	Vph2p	Integral membrane protein, assembly of V-ATPase	*VPH2 (VMA12)*	
VMA22	Vma22p	Peripheral membrane protein required for V-ATPase assembly		*VMA22*
VMA21	Vma21p	Integral membrane protein required for vacuolar H+-ATPase (V-ATPase) function, although not a part of V-ATPase complex; functions in V-ATPase assembly; localized to yeast endoplasmic reticulum (ER)	nd	nd
VPS3	Vps3p	CORVET tethering complex; cytoplasmic protein required for sorting & processing of soluble vacuolar proteins, acidification of vacuolar lumen, & assembly of V-ATPase		*VPS3*
PKR1	Pkr1p	V-ATPase assembly factor, functions with other V-ATPase assembly factors in ER for efficient assemble the V-ATPase membrane sector (V0)	*PKR1*	
		RAVE complex		
SKP1	Skp1p	Evolutionarily conserved kinetochore protein; part of SCF ubiquitin ligase complex, CBF3 complex binding centromeric DNA, & RAVE complex regulating assembly of the V-ATPase	nd	nd
RAV1	Rav1p	Subunit of RAVE complex (Rav1p, Rav2p, Skp1p), promotes assembly of V-ATPase	*RAV1*	
RAV2	Rav2p	Subunit of RAVE complex (Rav1p, Rav2p, Skp1p), promotes assembly of V-ATPase	*RAV2*	

nd, not determined. Deletants which completed fermentation in the initial micro-fermentation screen were *VMA21* (YGR105W); not determined for 100 mL scale, *VMA11* (YPL234C),*VMA6* (YLR447C), *VMA4* (YOR332W), *VMA22* (YHR060W), *STV1* (YMR054W), *VPS3* (YDR495C). *SKP1* (YDR328C) is essential gene. *VMA9* (YCL005W-A) was not in homozygous diploid deletion library.

The role of other cellular mechanisms in fermentation

The molecular basis of yeast resistance to ethanol and ethanol toxicity appears to be multi-factorial resulting in the interaction of many genes and their products in several key processes in cellular metabolism. Single genes such as *FPS1*, encoding a plasma membrane aquaglycoporin, which mediates controlled glycerol efflux, has been suggested to play a role in maintaining intracellular ethanol levels during fermentation and thus resulting in increasing ethanol concentrations in the wine (Teixeira *et al.*, 2009). Interestingly, this gene was not identified in the current study. Two genes involved in trehalose biosynthesis are also implicated in having a protective role against ethanol stress. The trehalose biosynthetic genes *TPS1* (trehalose-6-phosphate synthase) and *TPS2* (trehalose-6-phosphate phosphatase) were identified in our screen as having stuck fermentations. The fermentation conducted by the Δ*tps1* deletant was 1.9 times longer than the parent, having 5.36 ± 0.73 g/L residual sugar, whilst that of Δ*tps2* was extended 6-fold, with considerable residual sugar (32.87 ± 3.96 g/L). Trehalose, a non-reducing disaccharide, is known to accumulate during stationary phase, towards the end of fermentation (Majara *et al.*, 1996, cited in Gibson *et al.*, 2008), and is involved in protecting cells from several stresses. The trehalose biosynthetic genes *TPS1* and *TPS2* were previously identified through growth sensitivity of the Δ*tps1* to ethanol (van Voorst *et al.*, 2006) and oxidative stress (Tucker & Fields, 2004) and Δ*tps2* to ethanol, heat and NaCl (Auesukaree *et al.*, 2009), high acidity (Mollapour *et al.*, 2004) and oxidative stress (Tucker & Fields, 2004; Auesukaree *et al.*, 2009), respectively. V-ATPase is thought to protect yeast from alcohol stress (Fujiata *et al.*, 2006, Teixeira *et al.*, 2010), osmotic stress (Teixeira *et al.*, 2009), acid stress (Mollapour *et al.*, 2004) as well as against air-drying (Shima *et al.*, 2008). The latter is important as wine and brewers' yeast are supplied as dried yeast preparations. Ethanol, the main product of fermentation, has an inhibitory effect on growth and viability, with Marks *et al.* (2008) implicating ethanol rather than nutrient depletion as causing cells to enter stationary phase, where the majority of fermentation is conducted. Interestingly, Marks and co-workers (2008) were unable to show any up-regulation of the genes encoding V-ATPase, in response to increasing ethanol as observed in a wine fermentation.

Osmotic stress, whether salt or sugar induced, has a direct effect on cellular ion homeostasis. A number of genes involved in ion homeostasis were identified as having a role in fermentation. Hohmann (2002) showed that a proton gradient was required for increased uptake of K^+ ions, to compensate for the high external osmolarity. Interestingly, Trk1p, a high affinity K^+ transporter, is required for high-glucose (Teixeira *et al.*, 2009), high acidity (Mollapour *et al.*, 2004) stress resistance and completion of fermentation (this study). Nhx1p, an endosomal Na^+/H^+ and K^+/H^+ exchanger required for intracellular sequestration of Na^+ and K^+ was identified in the screen. Nhx1p, located in the late endosome/prevacuolar compartment (PVC), confers osmotolerance to acute hypertonic shock in postdiauxic/stationary phase cells (Nass & Rao, 1999). Teixeira and coworkers (2010) demonstrated the importance of the plasma membrane ATPAse, Pma1p, in maintenance of intracellular pH in response to high osmolarity. Although Pma1p was not identified in this study, Hrk1p, a protein kinase implicated in activation of the plasma membrane H^+-ATPase, Pma1p, in response to glucose metabolism, was identified. Genes required for cellular division and maintenance of cell structure, which were implicated in tolerance to ethanol and osmotic stress, such as *BUD31*, *CNM67*, *CYK3*, *TC089*, *TPM1*, and *RBL2* (Fujita *et al.*, 2006; Teixeira *et al.*, 2009, 2010; Ando *et al.*, 2006) not surprisingly have also been identified in this study.

Microautophagy and the EGO/GSE complex and fermentation

The EGO complex is a vacuolar membrane-associated protein complex composed of Meh1p, Gtr2p and Slm4p, which is required for activation of microautophagy (Dubouloz *et al.*, 2005). The GSE

(GAP1 Sorting in the Endosomes) complex is a GTPase complex required for intracellular sorting of Gap1p out of the endosome, for eventual delivery to the plasma membrane (Gao & Kaiser, 2006). The GSE complex is composed of two small GTPases (Gtr1p and Gtr2p), as well as three other proteins (Slm4p, Meh1p and Ltv1p) that are located in the late endosomal membrane.

Microautophagy and the EGO/GSE complex have been previously implicated as mechanisms allowing yeast cells to grow under low temperature and high pressure. Abe and Minegishi (2008) demonstrated a marked defect in uptake of histidine, leucine and lysine in the EGO/GSE complex mutants (Δ*gtr1*, Δ*gtr2*, Δ*meh1* (Δ*ego1*) and Δ*slm4* (Δ*ego3*)), following high-pressure and low temperature incubation. The authors hypothesise that the EGO/GSE complex is likely to play a primary role in cell surface delivery of amino acid permeases at low temperature, and under high pressure conditions, the complex has multiple yet undefined roles. In our study *GTR2* does not appear to have a role in fermentation, as Δ*gtr2* completed fermentation at the same time as the parental strain, BY4743. Deletion of *GTR1* and *LTV1* resulted in 7% and 5% slower fermentation (thus they are considered not essential for fermentation), whilst deletion of *MEH1* and *SLM4* severely affected fermentation (65% slower than the parent). These findings suggest that *MEH1* and *SLM4* play a role in fermentation, through vacuolar acidification (Meh1p), microautophagy and protein sorting of Gap1p. The localisation and degradation of this broad specificity permease is regulated by the quality of the nitrogen source (Roberg *et al.*, 1997; Springael & Andre, 1998).

Intracellular signalling and fermentation

Intracellular signal transduction, specifically, that related to nutrient sensing of rapidly fermentable sugar, and nutrients essential for growth (including amino acids and ammonium) is a fundamental process to enable yeast to adaptively respond to changing environments such as during fermentation. Nutrient sensing occurs through either nutrient transporters, which act as nutrient sensors, or non-transporting nutrient carrier homologues or transceptors. The high affinity phosphate transporter Pho84p, general amino acid permease Gap1p, and ammonium transporters, Mep1p, Mep2p, are examples of the first, whilst the Snf3p and Rgt2p are glucose transceptors and Ssy1p is an amino acid transceptor (reviewed by Rubio-Texeira *et al.*, 2010). Surprisingly, none of these genes were identified in the micro-fermentation screen, however, genes encoding two proteins of the sugar G Protein Coupled Receptor system; the receptor, Gpr1p, and Gα protein Gpa2p were identified as having protracted fermentation. Gpr1p is important for the rapid adaption to extracellular glucose (and not fructose), via the cAMP-PKA pathway. The latter is thought to activate cAMP signalling via a glucose phosphorylation mechanism (reviewed by Rubio-Texeira *et al.*, 2010). Hxk1p, identified in this study, is one of three protein kinases (Glk1p, Hxk1p, and/or Hxk2p) required for glucose phosphorylation, as a means of intracellular signalling via Gpr1p-Gpa2p. Further, Asc1p, a guanine dissociation inhibitor of Gpa2p, links glucose metabolism to nitrogen metabolism, as it represses Gcn4p in the absence of amino acid starvation (reviewed by Rubio-Texeira *et al.*, 2010). Another gene identified in this study was *PLC1*, deletion of which resulted in stuck fermentation. This phenotype alludes to the importance of the phosphoinositide signalling pathway (e.g. Plc1 pathway) in cellular metabolism, however, the physiological roles are not clearly understood. Phospholipase C, hydrolyzes phosphatidylinositol 4,5-biphosphate (PIP2) to generate the signalling molecules inositol 1,4,5-triphosphate (IP3) and 1,2-diacylglycerol (DAG). Plc1p has been suggested to be involved in activation of Pkc1p controlling the MAP kinase cascade, which is essential to adaptation to high osmolarity and cell wall integrity. It is also implicated in nutrient induced signal transduction via the Gpr1p-Gpa2p mediated GPCR system, coupling glucose and nitrogen signalling at the sensor level (reviewed by Wera *et al.*, 2001, Palacek *et al.*, 2002).

The high-osmolarity glycerol (HOG) response pathway is responsible for induction of glycerol-3-phosphate dehydrogenase (GPDH), in response to hyperosmotic stress, resulting in the increased production and retention of glycercol. Not surprisingly, a Hog1p and its activator, Pbs2p, were identified in the fermentation screen, whilst interestingly, the other four genes of the pathway, *OPY2*, *RCK2*, *RGD1* and *SHO1*, were not essential for fermentation completion. The signalling cascade which regulates gene expression in response to nutrient status was also identified as having a role in fermentation. This is not surprising, as the TORC1 complex mediates cell growth in response to nutrient availability and cellular stresses by regulating protein synthesis, ribosome biogenesis, autophagy, cell cycle, transcriptional activation and permease trafficking. A component of the TORC1 was identified, Tco89p, which is localised to the plasma membrane and vacuole membrane, having a role in cell wall integrity.

Many of these processes and signalling cascades are related to stress response. Recently Villa-García and coworkers (2011) implicated lipid metabolism, specifically, inositol-containing lipids in stress response signalling, as genes annotated to processes such as vacuolar acidification (V-ATPase), protein trafficking and turnover, TOR and HOG signalling pathways, when deleted, were associated with inositol auxotrophy. The nature of such lipid signal molecules has not been discerned.

It is evident that a number of mechanisms involving a myriad of genes are involved in the adaptive response to the four major stressors encountered during fermentation: high osmolarity (extracellular sugar), low pH, increasing levels of ethanol and nutrient depletion. Only some of which have been alluded to in this report. Further investigation is required to produce a comprehensive interpretation and inturn exploitation of the results of this unique experiment and dataset.

Utilisation of yeast over-expression libraries in a wine yeast background: Enabling *GAL1* promoter driven expression to allow use of the Yeast ORF over-expression collection and exploitation of the Yeast Genomic Tiling Collection

Regulation of GAL1 *under glucose repressive conditions*

Genes in the galactose utilisation pathway are tightly regulated, being induced when cells are grown on galactose and repressed when grown on glucose. Gal gene expression requires the transcriptional activator protein Gal4p, which binds to and activates a number of genes in the galactose utilisation pathway including *GAL1*. Gal4p function is inhibited by the Gal80p repressor protein, which binds directly to Gal4p, and by Mig1, which represses *GAL1* and *GAL4* expression, when cells are grown in the presence of glucose (Ostergaard *et al.*, 2000). Gal3p mediates the galactose induced release of Gal4p from Gal80p (Lohr *et al.*, 1995). Deletion of *GAL80* and *MIG1* results in growth defects due to their pleiotropic nature, and as such, were not considered good candidates for modulation of the galactose utilisation pathway. Instead, the first genes to be deleted were *GAL1* (Galactose kinase), *GAL83* (one of three beta-subunits of the Snf1 kinase complex) and *REG1* (Regulatory subunit of type 1 protein phosphatase Glc7p), with the view of affecting *GAL1* expression in the presence of inhibitory concentrations of glucose (and a small amount of galactose, which could not be utilised).

An alternative paradigm was the over-expression of Gal4p and/or Gal3p, such that the ratio of the activators Gal4p (and/or Gal3p) was greater than that of Gal80p, allowing induction of *GAL1* promoter, in the presence of glucose. Li *et al.* (2010) demonstrated that various *GAL4* mutants, encoding Gal4p with reduced interaction with the Gal80p repressor, could induce the expression of LACZ under the control of the *GAL1* promoter, in the presence of glucose. It was envisaged that if

one of these *GAL4* mutations could be recombined into the genomic DNA of a yeast strain, in place of the native *GAL4* gene sequence, this yeast strain could subsequently be used to screen the Yeast ORF Collection (see below). Such strains would allow over-expression of the yeast gene within each plasmid under the control of the *GAL1* promoter even in high glucose conditions, where expression would normally be repressed. One of these mutants was kindly provided by Li and co-workers in the plasmid pRS413. This particular *GAL4* mutant has a single base pair change in the *GAL4* coding sequence (Thr859 to Ile) and is referred to as Gal4c in this report.

Yeast ORF Collection

The Yeast ORF Collection is a library of individual open reading frames (genes) cloned behind the *GAL1* promoter in plasmid pBG1805, which has a *URA*3 selectable marker for maintenance in yeast. The collection of individual clones was amplified in *E. coli* in a 96-well format and the DNA extracted for future transformation into a haploid derivative of the diploid wine yeast Lalvin Rhone L2056 (Lallemand).

Construction of a GAL1p-LACZ reporter system to evaluate the Yeast ORF Collection during fermentation in CDGJM

A *LACZ* reporter construct was generated for purposes of testing β-galactosidase expression under the control of the *GAL1* promoter (as for the yeast ORF collection). The source of the full length *LACZ* gene (1027 aa) was plasmid pT161T (8589 bp) for expression in mammalian cells (a kind gift from Keith Shearwin, University of Adelaide). The *LACZ* sequence is shown in Appendix 5: Supplementary Figure 4. The *LACZ* gene was cloned into pYES2 (Invitrogen) by conventional cloning to give pYES2-LACZ. Plasmid pYES2 is comparable to pBG1805 having the same selective markers, *GAL1* promoter and *CYC1* terminator although it is not a GATEWAY vector (Promega).

Construction of isogenic haploid wine yeast isoC9c and isoC9d

We have previously generated three haploids of the commercial strain L2056: C9 (*MATa,* Δ*ho*), C911d (*MATa,* Δ*ho*) and C911a (*MATα,* Δ*ho*) (Walker *et al.*, 2003). The extensive heterogeneity (53%) of L2056, as shown by Bradbury *et al.* (2006) using microsatellite analysis of 17 loci, highlighted the need for an isogenic version of one of these original strains. As C911d was extensively used, an isogenic set of strains, isoC9c (*MATα,* Δ*ho*) and isoC9d (*MATa,*Δ*ho*) were constructed. Strain C911d was transformed with a plasmid-borne functional *HO*, encoding homothallic switching endonuclease, enabling mating-type switching and re-diploidisation. The diploid strain was cured of the plasmid prior to sporulation. A spore was picked for transformation with the HO plasmid and the procedure of rediploidisation, plasmid curing and sporulation was repeated. In the final (third) round of the procedure, four haploid spore colonies from a single tetrad (isoC9a to isoC9d) were evaluated for fermentation performance in CDGJM (220 g/L sugar, 125 mg N/L as ammonium sulphate, 25°C). The isogenic strains were shown to have comparable fermentation performance to L2056 and C9. The dry cell weight is less for the haploid strains than the diploid L2056 because of the smaller cell size (Appendix 5: Supplementary Figure 5).

Microsatellite data analysis of the isogenic clones and the original haploid strains C9, C911a and C911d (Walker *et al.*, 2003) showed the inheritance of the alleles for 10 loci (see Appendix 5: Supplementary Table 2). The four isogenic strains isoC9a to isoC9d were identical to C9 and C911D for these 10 loci. Microsatellite analysis was also performed on the strains used in the construction of another haploid strain using repeat DNA sequence originating from *HO* itself (reported in Walker *et al.*, 2005). This strain was referred to as 3-3a (*MAT*a*,* Δ*ho*). Neither 3-3a nor the other haploids

generated through backcrossing with 4-5a (*MAT*α*, Δho::KanMX*):- 18a (*MAT*a*, Δho*) and 18d (*MAT*α*, Δho*) were used for further strain construction. Interestingly, the L2056 isolate used in the construction of the 'C9 series' of haploids was slightly different (Keith Richards, pers. comm.) to that typed by Bradbury *et al.* (2006), and the isolate used to construct the 'DR4 series'. Nine out of 10 loci were identical, however, there was a difference noted in peak size for C5-hex (*CDC14*/*YFR028C*). The latter two isolates were identical, producing a 121 bp peak with C5-hex whilst the isolate used in the 'C9 series' of haploids resulted in a larger 161 bp peak.

Modification of isogenic haploid wine yeast strains isoC9c and isoC9d

The selection marker for the over-expression library is *URA*3 (orotidine-5'-phosphate decarboxylase) which requires a recipient yeast strain to have an auxotrophic growth requirement for uracil. Thus *ura3::KanMX* deletants of isoC9c and isoC9d were constructed by gene disruption using a 2.5 kb *Sac*II-*Kpn*I *ura3::KanMX* DNA fragment from plasmid pDR1123 (Replogle et.al., 1998). The strains were unable to grow on minimal medium lacking uracil thereby indicating success in achieving the set objective.

Gene disruptions of the genes *GAL1*/*YBR020W*, *REG1*/*YDR028C* and *GAL83*/*YER027C* were made in isoC9c and isoC9d using the deletion cassettes derived from the homozygous diploid deletion library. The individual library clones were cultured and genomic DNA extracted for PCR amplification of the deletion cassettes using Velocity DNA polymerase (Bioline) and gene specific primers (http://www-sequence.stanford.edu/group/yeast_deletion_project/deletions3.html). Both the lab yeast and wine yeast clones were confirmed by PCR analysis and DNA sequencing analysis (data not shown). Fermentation performance was evaluated for the gene deletants in CDGJM as shown in Appendix 5: Supplementary Figure 6. The Δ*gal1::KanMX* mutants were not evaluated for fermentation.

The Δ*gal83::KanMX* mutant in the lab and wine yeast behaved similarly to the parental strains. However, the Δ*reg::KanMX* mutants showed protracted fermentation when compared with the parents. Performance differences were noted for the Δ*reg1* mutants of the haploid wine yeast which was dependent on mating type. IsoC9cΔ*reg1::KanMX* completed fermentation ~101 h after the parental strain, whilst isoC9d Δ*reg1::KanMX* was unable to complete fermentation (113 g/L residual sugar after ~292 h). Colony size variation was also noted for the two mating types for the Δ*reg1* mutants (data not shown). These phenotype disparities may be due to the genetic variation between different haploid strains because of the original heterogeneity of the wine strain L2056 (Bradbury *et al.*, 2006). The Δ*reg1* mutants were not suitable for further modification for purposes of a host strain for the library.

As regulation of galactose metabolism is complex, involving a number of genes, multiple deletants were constructed for further evaluation using a *GAL1-LACZ* reporter plasmid (analogous to pBG1805). Their construction required the mating of the haploid mating types with different gene deletions. For example isoC9c Δ*gal83::KanMX* (*MAT*α) mated with strain 5.3.3 (MATa, Δ*gal1::KanMX*, Δ*ura3::KanMX*) to give a diploid which had 4 viable spores per tetrad. The desired strains (Δ*gal1::KanMX*, Δ*gal83::KanMX,* Δ*ura3::KanMX*) of both mating types were identified by PCR analysis of the spore progeny. As yet, the strains are to be tested using the *GAL1-LACZ* reporter construct in minimal media without uracil using 2% glucose and 0.5% galactose as carbon source.

Recently, Li *et al.* (2010) demonstrated that a T_{859}I variant of Gal4p was shown not to bind to Gal80p (a negative regulator of Gal4p), allowing *GAL1* expression under repressive conditions. The

GAL4(T_{859}I) gene was amplified as a DNA fragment and confirmed by DNA sequencing. As there was not selectable marker incorporated with the mutated *GAL4* gene sequence, a screening method to identify those yeast colonies with the mutated *GAL4* gene compared to the native gene was required. The plasmid pYES-LACZ, encoding the *LACZ* reporter gene under the control of the *GAL1* promoter, was developed for this purpose (see above). When transformed into a yeast strain of interest, colonies expressing *LACZ* can be identified by growth on media containing X-Gal. This substrate is cleaved by β-galactosidase, the protein encoded by the *LACZ* reporter gene, resulting in blue, rather than white colonies. Wild type yeast, transformed with the pYES2-LACZ plasmid, grown on media containing galactose and low or no glucose should turn blue due to the activation of expression from the *GAL1* promoter and hence β-galactosidase activity. In addition, yeast containing the mutated *Gal4c* gene instead of the native *GAL4* gene, grown on media containing glucose should turn blue due to the activation of expression from the *GAL1* promoter and hence β-galactosidase activity, due to the reduced interaction of GAL4p with GAL80p.

To incorporate this *GAL1::LACZ* reporter system haploid yeast strains iso C9c *ura3::KanMX* and iso C9d *ura3::KanMX* were transformed with plasmid pYES2-LACZ. Cells were plated onto minimal drop-out media lacking uracil (20 g/L glucose) to identify transformed yeast with an incorporated pYES2-LACZ plasmid. These transformants were re-streaked onto minimal drop-out media lacking uracil (0.5 g/L glucose, 20 g/L galactose), which had first been spread with 40 µL X-Gal (50 mg/mL), to identify those expressing β-galactosidase enzyme (blue colonies). It was anticipated that all yeast transformed with the pYES2-LACZ plasmid grown on these plates with a low glucose and high galactose concentration would induce expression from the *GAL1* promoter, express the *LACZ* gene resulting in β-galactosidase enzyme activity and therefore blue colonies. The haploid wine yeast 1B14 previously transformed with pYES2-LACZ was also spread onto these plates as a control. These were the only colonies to turn blue. The reason for failure of the other constructs is not clear.

This approach to utilizing an over-expression library was demoted in the list of project priorities due to difficulties in obtaining a screening system. Attention has instead focused on the yeast genomic tiling collection, which is a smaller, non-unique library (i.e. multiple yeast genes present in each construct) that permits study of the effect of over-expression of yeast genes (due to multiple gene copy number) on fermentation performance.

Generation of a leucine auxotroph of haploid wine yeast isoC9 as host for Yeast Genomic Tiling Collection in plasmid pGP564

The use of the over-expression library in a wine yeast background will allow comprehensive screening under a variety of nutritional conditions including low nitrogen, and high sugar (> 200 g/L), which would not be possible in a laboratory strain with multiple auxotrophic requirements. It was therefore decided to use the haploid wine strain, isoC9 as the host strain as it was shown to behave like L2056 in fermentation (profile and key metabolites). A leucine auxotroph of isoC9 was required for stable maintenance of the library (as independent plasmids) in the yeast. As a *leu2::KanMX* disruption cassette was not available as a deletion library clone, one was constructed in the plasmid pBS418 (a derivative of pBluescript, Stratagene). The cassette was used to transform isoC9d to leucine auxotrophy, with the transformants being selected for resistance to G418 and inability to grow on minimal drop-out media without leucine. Three isoC9Δ*leu2::KanMX* isolates were evaluated for fermentation performance in CDGJM and were able to complete fermentation in leucine supplemented media at a similar time as the parental strain isoC9d (unsupplemented CDGJM; data

not shown). IsoC9Δ*leu2::kanMX* transformant 1 was chosen as the genetic background for the Yeast Genomic Tiling Collection in plasmid pGP564.

Transformation of isoC9Δ*leu2::KanMX* with the library is in progress using the transformation procedure described on the "Prelich Lab Yeast Systematic Overexpression Library Methods" webpage http://steelhead.aecom.yu.edu/SystematicLibrary.html#Library_transformation_ and_screening. The over-expression library will be invaluable in identifying genes that are required for a number of fermentation traits including nitrogen efficiency, yeast 'robustness', ethanol tolerance, and wine quality (production of yeast derived aroma compounds). In the latter case, initial studies using isoC9 and isoC9Δ*leu2* in CDGJM show promising results, in that both are able to produce most of the 38 yeast derived metabolites chosen for their importance based on odour activity rating. The exceptions were ethyl lactate, hexyl acetate, butanol and hexanol. The 38 compounds have been synthesised and purified as standards to use in quantifying wine samples. Work in progress will modify the CDGJM to allow for these compounds to be synthesised (Jade Forrester, personal communication). The application of the over-expression library for identifying which genes play a role in the synthesis and modification of these compounds will be a first in oenological research, and the development of identification protocols will be useful in wine quality analysis.

Conclusion:

The above work has been successful in identifying a large number of genes whose deletion results in modified fermentation performance under various conditions. Such genes are designated HSF, i.e. essential to high sugar fermentation. The identification of these genes allows the critical processes in which they participate to be highlighted thereby revealing potentially new foci for research for strain optimisation or else new strategies for managing fermentation conditions and therefore outcome. Several of these genes will be incorporated into UA 11/01 as a means of generating industry-ready solutions to fermentation problems. In addition, further summer, Honours and PhD students will be sought to extend these studies.

Output G: Characterised LAB enzymes

12. 1-2 recombinant esterases or glycosidases purified and characterised. Action on natural substrates tested.

Contributors:

Current PhD students: Alana Capaldo and Krista Sumby
Past Honours students: Paul Easton and Thomas Parker
Postdoctoral fellows and/or student supervisors: Drs Michelle Walker, Jennie Gardner and Assoc Prof Vladimir Jiranek.

Background:

Work reported in UA 01/04, demonstrated the widespread occurrence of hydrolytic enzymes in LAB which may impact on winemaking and product quality. Glycosidase and esterase enzymes were selected for more detailed characterisation as an extension to Output B. In this Output the presumptive identification, cloning and heterologous expression of structural genes for these enzymes is detailed. The enzymes encoded by these genes were then purified and characterised. The performance target of this Output (1-2 enzymes) has been exceeded with the cloning of at least 7 genes and the characterisation of their enzymes. Much of this work has been published and as such these papers are included in Appendix 1. The following is largely limited to any additional or as yet unpublished findings.

Methods:

Refer to publications in Appendix 1: Sumby *et al.* (2009); Capaldo *et al.* (2011a); Capaldo *et al.* (2011b). Only the unpublished mnethods are detailed below.

BglD gene synthesis

The gene *bglD* was synthesised by Gene Oracle, Inc (Mountain View, CA 94043) with a mutation to incorporate a glutamic acid (E) residue in the place of S^{432} and *Nde*I and *Xho*I restriction sites at the 5' and 3' ends of the sequence. This mutation was selected because of its speculated importance in the affinity of GHF 1 phospho-β-glucosidases towards phosphorylated substrates. The gene was supplied cloned into a pUC based vector, pGOV4, devoid of most common restriction sites (All rights of this vector are licensed to Gene Oracle, https://www.geneoracle.com/services/pGOV4.pdf.).

Overexpression and purification of the recombinant esterases

Potential esterase genes were identified by sequence comparison between known esterases and the reported genome sequence for PSU-1 (Mills *et al.*, 2005). The putative genes, cloned into pET14.b, and once positive clones had been sequenced, one was chosen for further experiments. The *E. coli* [BL21(λDE3)] cells containing the plasmid of interest were induced and cell extracts obtained for analysis by SDS-polyacrylamide gel electrophoresis. Crude cell extracts were assayed for activity as described in Sumby *et al.* (2009). EstA2, EstCOo8 and EstC34 were purified from the whole-cell lysate to electrophoretic homogeneity using affinity chromatography. The purified enzymes could be stored without significant loss of activity for several weeks at -80°C in 0.1 M McIlvaine buffer (pH 5.0) (EstA2), or Elution buffer (EstCOo8, EstC34), supplemented with glycerol (10%, v/v).

Analysis of ester-related volatiles

Standard solutions were prepared using analytical reagent grade chemicals (Sigma). Stock solutions of each compound were prepared in ethanol. A combined standard solution was prepared for each group of compounds (esters, acetates, alcohols and acids) from the stock solutions.

An Agilent CP9205 gas chromatograph (Agilent Technologies) was equipped with a Gerstel MPS2 multi-purpose sampler and coupled to a mass selective detector. The instrument was controlled and the data analysed with MSD ChemStation E.02.00.493 software (Agilent Technologies). The gas chromatograph was fitted with a 60 m x 0.25 mm Phenomenex fused silica capillary column ZB-Wax, 0.25 μm film thickness. The carrier gas was helium (Air Liquide or BOC, Ultra High Purity), linear velocity 36 cm/s, flow rate 1.0 mL/min in constant flow mode. The oven temperature was started at 40°C, held at this temperature for 5 min, then increased to 120°C at 3°C/min, then increased to 240°C at 10°C/min and held at this temperature for 15 min. The inlet was fitted with a borosilicate glass SPME inlet liner (0.75 mm I.D., Supelco) and was held at 230°C. The mass spectrometer quadrupole temperature was set at 150°C, the source was set at 230°C, and the transfer line was held at 240°C. Positive ion electron impact spectra at 70 eV were recorded selective ion monitoring (SIM) for the dwell times shown in Table G.1. The conditions for headspace SPME sampling were as follows: a 10 mL aliquot of sample was added to a 20 mL vial containing 2 g of sodium chloride, and the vial was immediately capped (magnetic, Teflon lined rubber septum). The vial and its contents were heated to 40°C for 10 min. The Supelco Carboxen/divinylbenzene/Polydimethylsiloxane (CAR/DVBPDMS) 50/30 μm fibre was exposed to the headspace for 10 min with agitation of the vial at 250 rpm. The SPME fibre was desorbed in the split mode, at 10:1. The fibre was allowed to bake in the inlet for 15 min.

Table G.1: SIM dwell times

Compound	m/z
Ethyl acetate	61,70,73
Ethyl butanoate	71,43,88
Ethyl propanoate	29,57,27
Ethyl hexanoate	88,99,60
Ethyl octanoate	88,101,127
Acetic acid	43,45,60
Butyric acid	
Hexanoic acid	60,73,87
Octanoic acid	60,73,101

Results and Discussion:

Purification and characterisation of recombinant phospho-β-glucosidases

Alana Capaldo submitted her PhD thesis on this topic on 25 November 2011. She has published two articles on her research on the cloning and characterisation of three genes involved in β-glucoside metabolism in *O. oeni* (see Output M). The enzymes encoded by these genes (*celC*, *celD* and *bglD*) were ultimately determined to be phospho-β-glucosidases based on their near strict dependence on phosphorylated substrates. Figures G.1 and G.2 (adapted from the Capaldo papers) summarise the specific activity of the respective enzymes.

BglD possessed 480 residues and a molecular mass of 55.5 kD, His-tagged CelD had 485 residues and a MW of 55.8 kDa while celc had 481 residues and a MW of 55.7 kDa. BglD and celD exhibited high activity towards the phosphorylated β glucoside para-nitrophenol-β-D-glucopyranoside-6-phosphate (pNPβD6P), with a pH optimum of ~5. Both proteins showed maximal activity at 40°C, however little loss of activity was seen even at temperatures of ~5°C. CelC was not investigated further as it showed only minor activity towards phosphorylated and non-phosphorylated β-glucopyranosides. These proteins are proposed to function in conjunction with the phosphoenolpyruvate-phospho transferase system (PEP-PTS) to hydrolyse β-glucosides, as alternative carbon sources to glucose, by simultaneous uptake and phosphorylation of the substrate (Deutscher *et al.*, 2006). CelD and BglD exhibited affinity towards phosphorylated substrates despite showing high homology to glycosyl hydrolase family 1 (GHF 1) β-glucosidases (Cantarel *et al.* 2009). This could have been expected given that β-glucosidases and phospho-β-glucosidases share high sequence similarity in conserved regions and use the same catalytic mechanism. Although the substrate specificity differs, the phospho-β-glucosidases additionally only require a phosphate group at C6 of the glucose moiety in order to bind to the substrate (Wiesmann, *et al.*, 1995).

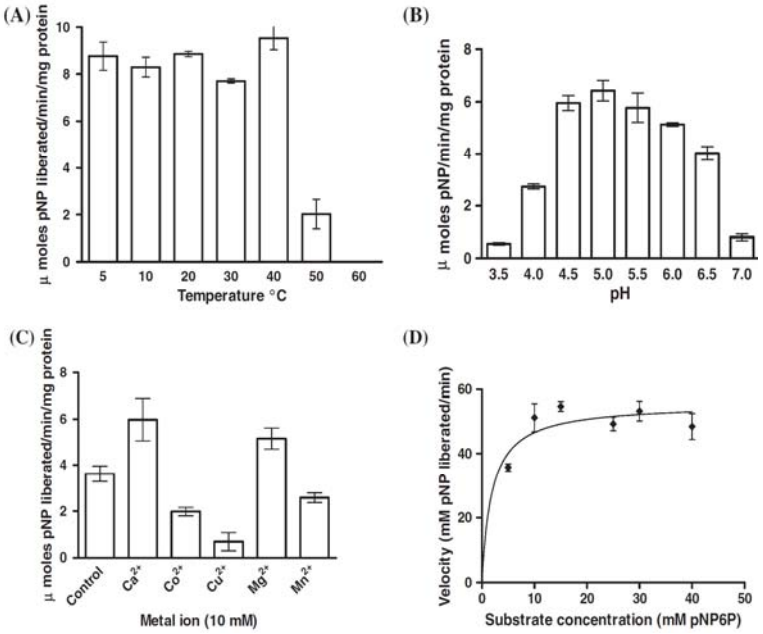


Figure G.1: Effect of pH, temperature, metal ions and substrate concentration on activity of bglD.
A) Enzyme activity was assayed at 37°C for 30 min in McIlvaine buffer of indicated pH. **B)** Effect of temperature on the specific activity of bglD. Activity was assayed at pH 5.5 for 30 min at the indicated temperature. The buffer was brought to temperature 10 min prior to commencement of the assay in a PCR machine. **C)** Effect of 10 mM addition of Ca^{2+}, Co^{2+}, Cu^{2+}, Mg^{2} and Mn^{2+} on the specific activity of bglD. Enzyme activity was assayed at pH 5.5 for 30 min. **D)** Effect of substrate concentration on enzyme kinetics, analysed with Michaelis-Menten non-linear regression analysis.

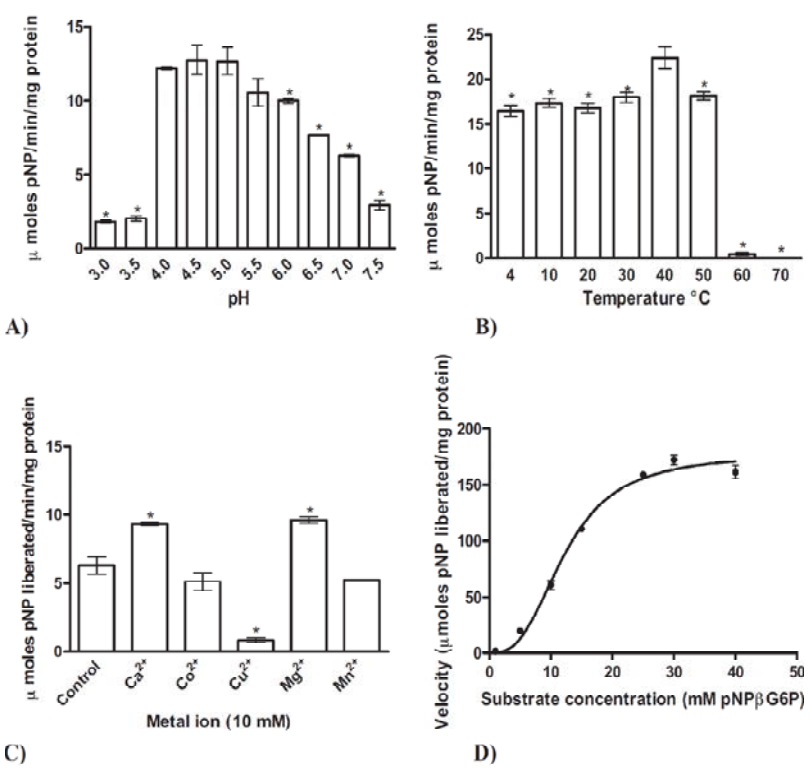


Figure G.2: Effect of pH, metal ion and temperature on CelD activity. **A)** Effect of pH on specific activity of CelD. Activity was assayed at 37°C for 30 min in McIlvaine buffers of indicated pH. **B)** Effect of temperature on specific activity of CelD. Actiivity was assayed at pH 5.5 for 30 min at the indicated temperature. The buffer was brought to temperature 10 min prior to commencement of the assay in a PCR machine. **C)** Effect of addition of 10 mM Ca^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+} or Mn^{2+} on activity of CelD. Enzyme activity was assayed at pH 5.5 for 30 min. **D)** Effect of substrate concentration on enzyme kinetics (CelD), analysed with allosteric sigmoidal non linear regression.
* Indicates significant differences compared to control on each panel (one-way ANOVA with Tukey's multiple comparison post test, $p < 0.05$).

It is proposed that the phospho-β-glucosidases associated with PEP-PTS, may contribute to some of the β-glucosidase activity detected in whole cells of wine *O. oeni* (Grimaldi *et al.*, 2000, 2005a). However, the majority of the activity is dependent on extracellular or cell wall associated β-glucosidases (Weber *et al.*, 1998). To date, two β-glucosidases have been identified in wine *O. oeni* (Spano *et al.*, 2005; Michelmyer *et al.*, 2010). The β-glucosidase isolated by Michelmyer *et al.* (2010) is a GHF-3 β-glucosidase (Cantarel *et al.*, 2009), which has the same sequence as the putative β-glucosidase (ORF5) identified but not cloned or expressed in *E. coli*, as part of this study. BglD and celC and celD are part of the β-glucoside *bgl* and *cel* operons respectively (including the PEP-PTS), based on sequence comparison of lactic acid bacteria (Capaldo *et al.*, 2011a, b).

In this study, the substrate specificity of bglB and celD towards naturally occurring substrates such as wine glycosides (which require purification or synthesis), and commercially available plant β-glucosides salicin and arbutin and disaccharide cellobiose, was not investigated because of the specificity towards phosphorylated substrates. However, given the stability of these enzymes at low temperatures and retention of some activity below a pH of 4, it may be of value to investigate the impact of purified enzyme in juice and wine – even though they possess specificity for phosphorylated substrates.

Site-directed mutagenesis of *O. oeni* GHF-1 BglD

The characterised GHF 1 phospho-β-glucosidase BglD was mutated in an attempt to alter its specificity towards non-phosphorylated substrates and thereby determine the basis for the observed preference for these. Mutations were introduced into GHF 1 β-glucosidases to target the proposed phosphate group recognition site and thereby change the specificity towards phosphorylated β-glucosides (Kaper *et al.* 2000) and broaden specificity towards β-glycosides (Hancock *et al.*, 2005,

Corbett *et al.*, 2001). No information (in reported literature) is available about mutating the phosphorylation site of GHF 1 phospho-β-glucosidases towards nonphosphorylated substrates.

A lysate of *E. coli* bearing the characterized GHF 1 phospho-β-glucosidase with the serine in position 432 mutated into a glutamic acid (plasmid pET 14.b-BglD_S-E) was assayed against pNP-β-D-glucopyranoside-6-phosphate (pNPβD6P) and pNP-β-D-glucopyranoside (pNPβG). Lysates had greatly reduced activity towards the phosphorylated substrates but did not gain marked activity against non-phosphorylated substrates (Figure G.3).

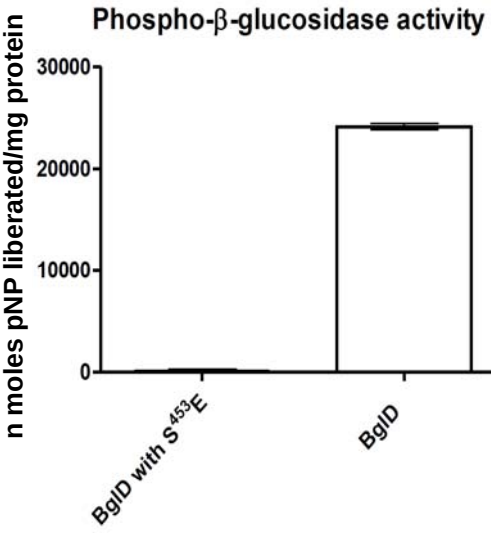


Figure G.3: Activity of crude extract from cell bearing wildtype BglD *vs* BglD carrying the $S^{453}E$ substitution against pNPβD6P. Activity was assayed at 37°C for 1 hr in McIlvaine buffer pH 5.5.

The purified protein BglD S-E was assayed against an extensive range of non-phosphorylated pNP substrates but with no observable activity (data not shown).

Elements of GHF 1 enzymes such as the N-terminal signature sequence and the catalytic sites are well characterized. In the instance of the active sites, two glutamic acid residues in positions E178 and E377 are highly conserved in GHF 1 enzymes and have been experimentally validated many times (An *et al.*, 2004, 2005, Hong *et al.*, 2007, Hong *et al.*, 2006, Wiesmann *et al.*, 1997). A third site, located inside the cavity that the two catalytic glutamic acids form, is important for the broad substrate specificity demonstrated by most GHF 1 enzymes and the phosphate group-enzyme interaction of GHF 1 phospho-β-glucosidases (Wiesmann *et al.*, 1995, Hill & Reilly, 2008). The single point mutation $E^{417}S$ in CelB demonstrated not only an increase in affinity towards phosphorylated substrates but a shift in pH optimum from 4.0 to 5.0 (Kaper *et al.*, 2000). It is interesting to note that the wild type β-glucosidase CelB was able to hydrolyse oNP-β-D-galactopyranoside-6-phosphate (oNPβD6P) whereby BglD_S-E had lost the ability to hydrolyse pNPβD6P (Kaper *et al.*, 2000). This reinforces the hypothesis that it is likely that more than one residue is important in the interaction of the phosphate group from the substrate with the phosphate receival site in GHF 1 phospho-β-glucosidase/galactosidases. However, in accordance with the conclusions made by Kaper *et al.* (2000), the equivalent BglD S432 seems to be the determining factor for the difference in substrate specificity between the two types of family 1 glycosidases.

In this study BglD was successfully modified and we observed a predicted lack of activity towards phosphorylated substrates. It would be of interest to also mutate the residues around S432 (BglD numbering) to provide insight into substrate specificities of GHF 1 enzymes and crystalize the wild type enzyme as the first crystalized GHF 1 phospho-β-glucosidase with associated characterisation data.

Esterases

We cloned, heterologously expressed and purified the following esterases: EstCOo8 from *O. oeni* strain Ooeni8; EstB28, EstA2 and EstA7 from *O. oeni* strain Ooeni28; and EstC34 from *L. hilgardii* strain LAC34. Crude extracts reveal high activity for all except EstA7 (data not shown). As such, only the remaining four were affinity-purified and characterised further.

Determination and characterization of esterase activity

The relative activity of all four purified esterase enzymes was tested with pNP butanoate. EstA2 and EstB28 had 10-fold the esterase activity of EstCOo8 and EstC34 (Figure G.4). Having established that the product of the cloned *estB28*, *estA2, estCOo8* and *estC34* genes confer esterase activity, further characterisation of these products commenced.

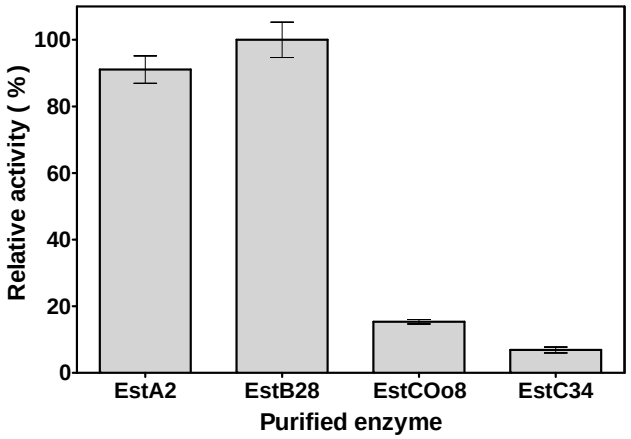


Figure G.4: The relative activity of all four purified esterase enzymes, tested with pNP butanoate.

Substrate specificity was determined using pNP-linked esters of various acyl chain lengths (C2 to C18) at 37°C, along with the impact of pH, temperature and ethanol. Results for EstB28 are shown in Figure G.5, while the data for the remaining esterases is shown in Appendix 5 (Supplementary Figures 7 – 9). Although the enzymes were active at all of the pH values tested, the highest activity was observed at pH 5.0 for EstC34 and EstB28, and pH 5.5 for EstA2 and EstCOo8. The esterases were also active at temperatures of between 10 and 60°C, with the highest activity being observed at 40°C for EstCOo8 and EstB28, and 50°C for EstA2 and EstC34. The influence of ethanol on esterase activity was tested at 37°C. EstA2 activity increased with increasing ethanol concentration, however, EstCOo8 and EstC34 did not. EstB28 was quite remarkable in that activity increased (to ~250%) with ethanol content up to 28% (v/v) and even retained some 80% of activity at ethanol contents as high as 50% (v/v). The possibilities for application of such a robust esterase likely extend beyond wine and even fortified wines to other processes outside of the industry.

EstCOo8 had highest activity at ethanol concentration of 6% and EstC34 had highest activity at ethanol concentration of 10% (Appendix 5: Supplementary Figures 7 and 8). As a consequence of this only EstA2 and EstB28 were tested with the full range of natural substrates and in wine (Table G.2).

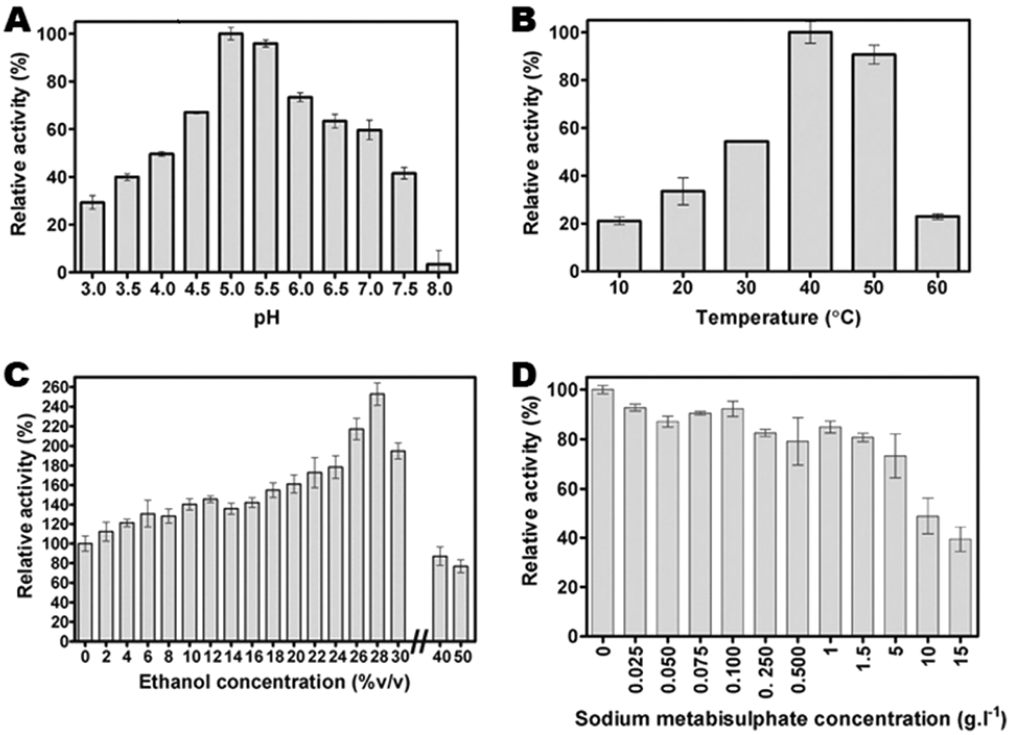


Figure G.5: Characterization of EstB28. Values are the means of triplicates standard deviations. Values are shown relative to the highest observed activity, which was arbitrarily designated 100%, except for panel C, where values are shown relative to the activity observed in the absence of ethanol, which was arbitrarily designated 100%. (A) Influence of pH on esterase activity of EstB28. (B) Influence of temperature on esterase activity of EstB28. (C) Influence of ethanol on esterase activity of EstB28. (D) Influence of sulfite on esterase activity of EstB28. Free SO_2 includes the concentrations of sulfur dioxide forms which are in equilibrium and are not bound to any compound in solution. This is defined as $[SO_2] + [HSO_3^-]$ ($[SO_3^{2-}]$ is minimal, i.e., 0.02%) at pH 3.5, where 98% of the sulfur dioxide exists as bisulfite ion (HSO_3^-).

Enzyme activity with natural substarates

Enzyme activity was detected towards all the natural substrates tested, showing potential for these enzymes to synthesise/hydrolyse a variety of wine esters (Table G.2).

Enzyme activity in wine

The concentrations of selected esters detected in wine with and without the addition of purified enzymes are reported in Table G.3. Neither enzyme significantly decreased ethyl acetate concentrations in either wine. Addition of EstB28 to the Cabernet Sauvignon wine increased ethyl acetate in both wines although the result was only significant in the Cabernet Sauvignon wine. An increase in ethyl acetate is generally undesirable as higher concentrations increase the risk of a solvent/balsamic aroma. There was no significant change in ethyl butanoate, ethyl hexanoate, hexyl acetate, and ethyl lactate for both enzymes additions in both of the tested wines. In all cases isobutyl acetate was detected below the reported threshold in wine so any changes detected would not impact on final wine quality. EstB28 increased isoamyl acetate concentrations in both wines, and EstA2

decreased isoamyl acetate concentrations in both wines (although only statistically significant in the Chardonnay wine). EstA2 decreased the high concentration of ethyl octanoate in the Chardonnay wine. There were no other changes in ethyl octanoate concentrations. Overall EstA2 is the most promising candidate of the two tested enzymes to decrease high ester concentrations in wine, whereas EstB28 consistently increases ester concentration.

Table G.2: Activity of purified enzymes towards artificial and natural substrates

		Enzyme Activity – substrates towards which activity was detected		
Purified Esterase	**Source Strain**	**pNP linked substrates hydrolysis**	**Natural substrate synthesis**	**Natural substrate hydrolysis**
EstA2	Ooeni28	*p*NP-acetate (C2)*, *p*NP-butanoate (C4), *p*NP-hexanoate (C6)*, *p*NP-octanoate (C8), *p*NP-decanoate (C10), *p*NP-dodecanoate (C12)	ethyl acetate, ethyl butanoate*, ethyl hexanoate, ethyl octanoate	ethyl acetate, ethyl butanoate*, ethyl hexanoate, ethyl octanoate
EstB28	Ooeni28	*p*NP-acetate (C2), *p*NP-butanoate (C4), *p*NP-hexanoate (C6)*, *p*NP-octanoate (C8), *p*NP-decanoate (C10), *p*NP-dodecanoate (C12)	ethyl acetate, ethyl butanoate, ethyl hexanoate*, ethyl octanoate	ethyl acetate, ethyl butanoate, ethyl hexanoate*, ethyl octanoate*
EstCOo8ϕ	Ooeni8	*p*NP-acetate (C2)*, *p*NP-butanoate (C4), *p*NP-hexanoate (C6)*, *p*NP-octanoate (C8), *p*NP-decanoate (C10), *p*NP-dodecanoate (C12)	ethyl butanoate	ethyl butanoate
EstC34ϕ	Lac34	*p*NP-acetate (C2)*, *p*NP-butanoate (C4), *p*NP-hexanoate (C6)*, *p*NP-octanoate (C8), *p*NP-decanoate (C10), *p*NP-dodecanoate (C12)	ethyl butanoate	ethyl butanoate

* detected activity was highest towards these substrates. ϕ enzyme specific activity was 10-fold lower for these enzymes

Table G.3: The concentrations of selected esters, after incubation with purified esterase for 3 weeks, in Cabernet Sauvignon and Chardonnay wine.
Values are the mean of duplicate determinations, ± standard deviation, of a single wine by analysed by SPME-GCMS.

	Cabernet Sauvignon			**Chardonnay**		
Ester	**No enzyme**	**EstA2**	**EstB28**	**No enzyme**	**EstA2**	**EstB28**
ethyl acetate	64765 ± 1136	64541 ± 358	71558 ± 1060*	89938 ±1684	89572 ±1175	93164 ±1731
ethyl butanoate	285.1 ± 1	320.9 ± 10	317.5 ± 36	1328.8 ± 82	1253.0 ± 44	1549.9 ± 31
isobutyl acetate	93.4 ± 1	106.3 ± 1*	109.3 ± 4*	12.2 ± 1	13.8 ± 2	6.4 ± 9
isoamyl acetate	924.7 ± 17	899.3 ± 19	1069.3 ± 9*	3451.5 ± 15	3020.2 ± 14*	3766.6 ± 28*
ethyl hexanoate	308.3 ± 1	301.4 ± 1	315.3 ± 3	777.3 ± 42	666.8 ± 4	810.9 ± 25
hexyl acetate	111.0 ± 7	102.6 ± 5	106.3 ± 7	171.1828 ± 5	173.0 ± 3	171.5 ± 9
ethyl lactate	12393 ± 295	11633 ± 570	12040 ± 342	793.9 ± 23	802.5 ± 15	795.3 ± 43
ethyl octanoate	165.6 ± 13	162.8 ± 14	159.9 ± 11	902.7 ± 17	725.0 ± 19*	924.7 ± 42

*Significant difference, $P < 0.05$ inoculated strain *vs* control (not inoculated), analysed using one way ANOVA in GraphPad Prism.

Conclusion:

Two phospho-β-glucosidases CelD and BglD and four esterases (EstA2, EstB28, EstCO08, EstC34) were characterised in terms of their substrate specificity, and effect of temperature, pH, metal ion and ethanol concentration on their enzyme activity.

The work on β-glucosidases resulted in the cloning and characterisation of three phospho-β-glucosidases, which share highly similar sequence homology (protein and DNA) to β-glucosidases, which are desirable as potential winemaking additives. As these intracellular enzymes participate in glycoside metabolism in conjunction with the PTS system, as a mechanism for obtaining glucose to be used in energy production, their application in the wine industry is limited. Of interest would be the characterisation of β-glucosidases such as those isolated by Michlmayr *et al.* (2010) which is identical to the ORF5 cloned but not expressed in *E. coli* in this study.

The potential industrial use of one esterase (EstA2) is highlighted in that the enzyme reduced ethyl acetate levels in wine, an outcome which would be beneficial. The other esterase enzymes tested resulted in increased levels of ethyl acetate, which is potentially undesirable as an 'off-aroma' at concentrations exceeding the aroma threshold may result. Further characterisation of EstA2 in terms of enzyme stability (time frame, temperature, etc) and situations related to wine making (fermentation, aging, and storage) is required prior to undertaking any plan to commercialise the enzyme. This would include small-scale fermentation trials with either the added enzyme or *O. oeni* strain (Ooeni28) to determine the potential action on the wine produced. Basic sensory analysis (difference testing: duo trio for aroma) as well as chemical analysis of aroma compounds found in wine would be required as part of such characterisation. Application methodologies such as selection of immobilisation matrices, free form versus bound, would be well within the scope of a future PhD project.

Output H: Basis for evolved strains defined

13. Genetic or physiological basis for novel properties of AE strains defined and used to guide further optimisation.

Contributors:

Past PhD students: Colin McBryde
Postdoctoral fellows and/or student supervisors: Drs Jennie Gardner, Michelle Walker and Assoc Prof Vladimir Jiranek.
Collaborators: Steve Oliver and Douglas Kell groups at the University of Manchester (S.O. now University of Cambridge), specifically Marie Brown, Elisabeth Bilsland and Warwick Dunn.

Background:

Analysis of both the genetic and physiological basis for the adaptively evolved strain L2056-C7 (a diploid, fermentation efficient wine yeast strain, a clonal isolate of FM16, derived from L2056 (McBryde *et al.*, 2006, generated as part of UA 01/04)) is essential for a better understanding of the mechanism(s) behind its efficient fermentation profile. A cross platform approach was undertaken incorporating genomic, metabolomic and a phenotype array analysis. Since these types of analyses involve highly specialised equipment and expertise, the initial raw data collection was either outsourced or performed by collaborators, where as preparation of biological samples and final data interpretation was done in-house in all cases. Where possible, preliminary integration of results from each of these platforms was also attempted in-house, however, due to the complexities of each dataset and time constraints, this was limited with current available capabilities.

A complete understanding of how such strains enable more reliable fermentation will allow a more specific, targeted approach in further yeast strain development as well as a better understanding of some of the main contributing metabolic pathways influenced in oenological fermentation. We plan to extend this research where possible.

Materials and Methods:

Yeast strains, growth conditions

The adaptively evolved wine stain L2056-C7 (McBryde *et al.*, 2006), its parent L2056 (Lallemand Australia), and the deletion strains W303 *ngr1*, W303 *gid7* (Gardner *et al.*, 2005) and their parent W303 were used for this study.

YPD liquid starter cultures were inoculated from YPD agar plates (in the case of the wine yeast strains, cell mass was selected from a streak rather than an individual colony, to include variation that may occur within a culture sample).

Experimental fermentations were undertaken with six biological replicates of 100 mL cultures (in 250 mL Erlenmeyer flasks). Either Footprinting medium (FM), F1-limited nitrogen medium (F1) with 97.5 mg FAN/L supplied as ammonia (Baganz *et al.*, 1997) or Chemically Defined Grape Juice Medium (CDGJM) with 590 mg FAN/L, supplied as ammonia (McBryde *et al.*, 2006) was employed. These media were prepared with analytical grade reagents.

Media were inoculated with 5 x 10^5 cells/mL (pre-washed in PBS) and cultures were incubated at 30°C. In the case of CDGJM, media was inoculated with 5 x 10^6 cells/mL (from an intermediate CDGJM starter medium with half the amount of glucose at 100 g/L and also with the addition of sterols) and incubation was at 25°C, as this is more representative of the temperature of a typical industrial wine fermentation. Exponential phase cultures were collected once the culture reached 1 x 10^7 cells/mL and stationary phase cultures were collected 48 hrs after inoculation, except in the case of CDGJM cultures where samples were collected at the time where all glucose and fructose were exhausted.

Metabolome sampling procedure

Samples for examination of the exometabolome were harvested by centrifugation (8,000 x *g* for 5 minutes) of 1.5-10 mL of culture followed by collection of the supernatant.

Quenching and extraction of internal metabolites (endometabolome) was primarily achieved using the hot ethanol extraction method (HEE) which was performed as Castrillo and colleagues described (2003), originally based on the method developed by Gonzaez *et al.* (1997). Briefly, 10 mL (equating to approximately 30 mg of dry cell weight) of each culture was sampled and quenched directly in 25 mL of methanol (60%)-tricine (10 mM; pH = 7.4) buffer at -50°C for 3 minutes. Following this, cells were collected by centrifugation (770 x *g*, 20 minutes, -20°C). Cells were then washed a further 3 times in 10 mL of the same buffer to ensure no metabolites from the growth medium remained. HEE of the cell pellet was then performed in 5 mL of ethanol (75%)-tricine (0.5 mM; pH = 7.4) at 80°C for 3 minutes. Extracted metabolites were then separated from the cell debris by centrifugation (500 x *g* for 10 minutes at 4°C).

The suitability of quenching and extraction of metabolites of samples using a cold methanol extraction method (CME) was also evaluated. In short, the method was performed as for HEE except following washing, cells were resuspended (vortexed) in 0.75 mL of pure methanol at -40°C, frozen in liquid nitrogen, thawed on ice and the supernatant collected by centrifugation, as developed by Villas-Boas (2005). The freeze-thaw procedure was repeated once and supernatants pooled to a total of 1.5 mL.

All samples for Gas Chromatography Mass Spectrometry (GC-MS) analysis (exo and endo-metabolome) were stored at -80°C until being concentrated and dried by evaporation in a Speedvac concentrator connected to a Freeze dryer (Thermo Life Sciences, UK) at the soonest possible time after collection. Samples evaluated by Liquid Chromatography Mass Spectrometry (LC-MS) were left untreated.

Metabolite detection for metabolomics analysis

GC-MS analysis was performed on an Agilent 6890N gas chromatograph and LECO Pegasus III TOF mass spectrometer at The University of Manchester as described by O'Hagan *et al.* (2005).

LC-MS analysis was performed by Metabolomics Australia on an Agilent 1200 SL HPLC coupled to a BrukermicroTOF-Q II. Both Reverse Phase (Electrospray ionisation (ESI) + and – mode and Atmospheric Pressure Chemical Ionisation (APCI; + and -) and HILIC (Hydrophilic Interaction Liquid Chromatogrpahy, ESI + and -) methods were employed. For Reverse Phase ESI + and – modes, 30 µL of neat sample was injected on to an Agilent Poroshell 120 SB-C18 150 mm x 2.1 mm column with matched guard cartridge. Two solvents were used: 0.1 % (v/v) formic acid (solvent A) and 0.1 % (v/v) formic acid in methanol (solvent B). Solvents were run at a flow rate of 0.35 mL/min according to the following timetable: 1 % solvent B, 0-55 min, 100 % solvent B, 55-70 min, 1 % solvent B 72-80 min at an oven temperature of 45°C and a DAD acquisition range of 200-400 nm. Every 5th sample was analysed in

duplicate with an equilibration time between samples. Mass Spectrometer settings for each method and mode are described in Table H.1. Calibration was achieved using Bruker'sDataAnalysis (v4.0 SP2) software, mass spectra (line) were calibrated on sodium formate cluster ions in the range 90-906 m/z for + mode and 112-928 for - mode using a sodium formate solution (5 mM sodium hydroxide, 0.2 % (v/v) formic acid, 50 % (v/v) 2-propanol), introduced immediately following sample injection via an inline post-column switching valve and sample loop. Bruker's enhanced quadratic algorithm was used for mass calibration.

For Reverse Phase APCI + and - modes, the method was the same as for ESI except for the following modifications; 100 µL of sample was injected on to the HPLC, the oven temperature was set at 50°C and DAD acquisition range 190-400 nm. Solvents were run according to the following timetable: 0 min; 0 % solvent B a flow rate of 0.5 mL/min, 0.6 min; 0 % solvent B, 0.1 mL/min, 40 min; 100 % solvent B, 0.1 mL/min, 60 min; 0 % Solvent B, 0.1 mL/min, 68 min; 0% solvent B, 0.5 mL/min. Mass spectra was calibrated on ions in the range of 121-1221 (APCI +) and 119-955 (APCI -) m/z using Agilent APCI tuning mix.

For the HILIC analysis the method was the same as for Reverse phase ESI except for the following modifications, 100 µL of sample was diluted with 900 µL of 50 % methanol, 0.05 % (v/v) formic acid and 4 µL was injected on to a Cogent Diamond Hydride 150 mm x 2.1 mm column with matched guard column. For HILIC ESI + mode Solvent B consisted of 0.1 % (v/v) formic acid in 97 % acetonitrile, where as – mode solvent A consisted of 0.1 % formic acid, 0.1 % acetic acid and solvent B consisted of 0.1 % ammonium acetate, 0.1 % acetic acid in 90 % acetonitrile. The oven temperature was 50°C and solvents were run at a flow rate of 0.4 mL/min (+ mode) and 0.3 mL/min (- mode) and according to the following timetables: + mode:0 min; 100 % solvent B, 29 min; 20 % solvent B, 31 min; 100 % solvent B and - mode: 0 min; 100 % solvent B, 11 min; 50 % Solvent B; 17 min; 100 % solvent B. Mass spectra was calibrated on sodium formate cluster ions in the range 90-634 m/z (+ mode) and 112-656 (- mode).

Table H.1: Mass Spectrometer parameters for each of the 6 analytical methods used for Liquid Chromatography (RP: Reverse Phase)

Parameter	RP-ESI +	RP-ESI -	RP-APCI+	RP-APCI-	HILIC-ESI+	HILIC-ESI-
Capillary voltage	Positive	Negative	Positive	Negative	Positive	Negative
End plate offset	200 ºC	200 ºC	200 ºC	200 ºC	200 ºC	200 ºC
Nebuliser pressure	3.0 Bar	4.0 Bar	4.0 Bar	4.0 Bar	2.0 Bar	2.0 Bar
Dry gas flow	9.0 L/min	9.0 L/min	9.0 L/min	9.0 L/min	9.0 L/min	9.0 L/min
Capillary voltage	4,000 V	3,000 V	4,500 V	4,000 V	3,500 V	2,500 V
End plate offset	-500 V	-500 V	-500 V	-500 V	-500 V	-500 V
Mass range	50 - 2000 m/z	50 - 2000 m/z	50 - 2000 m/z	50 – 1600 m/z	50 - 1000 m/z	50 - 1000 m/z
Acquisition rate	0.5 Hz	0.5 Hz	0.5 Hz	0.5 Hz	0.5 Hz	0.5 Hz
MS^nmode	Auto MS^n (top 3)	Auto MS^n (top 3)	Auto MS^n (top 3)	Auto MS^n (top 3)	Auto MS^n (top 3)	Auto MS^n (top 3)
Corona Current	N/A	N/A	10 000 nA	10 000 nA	N/A	N/A
APCI heater	N/A	N/A	400 ºC	400 ºC	N/A	N/A

Data extraction and analysis for metabolomics

GC-MS raw data analysis was performed as described by Castrillo *et al.* (2007). Discriminant metabolites, either between parent and mutant or between exponential and stationary phase were identified by Principle Component Analysis (PCA) or univariate analysis (Kruskall Wallis) where appropriate, but often both. Relative proportions of the presence of certain metabolites were determined by comparison of peak areas between related strains and conditions. Since GC-MS spectral libraries are continually evolving, updated metabolite identifications was also performed in November 2011.

LC-MS raw data analysis was performed by Metabolomics Australia. Briefly, the "Find Molecular Features" algorithm was used to extract molecular features from each sample's mass chromatogram and ProfileAnalysis v2.0 and the Advanced Bucketing algorithm (Bruker, Germany) was used to compile a data matrix, which was subsequently used as the basis for statistical analysis. Boxplots of log2 scaled bucket intensities did not reveal any gross outlier samples (data not shown), thus all were retained for further analysis. A simple ANOVA model fitted to the data for each bucket ascertained any differences in bucket intensities between parent and mutant profiles and both a Bonferroni and Benjamini and Hochberg correction was applied. Following this, the matrix was filtered such that a bucket must contain a value in 80 % of all parents and/or all mutants. Quantile normalisation and Pareto scaling were applied and Principal Component Analysis was performed.

Since a single metabolite can yield multiple signals and indeed the same metabolite is detectable across the different analytical platforms, we consolidated likely related candidates to single peak groups both within and across the 6 analytical platforms. A ranking of significant features was also performed to select individual features with low p-values. Rank 3 indicates features with false discovery corrected p value less than 0.05, rank 2 have p values less than 0.01 and rank 1 for p value less than 0.002. Additionally, features that were detected in each sample of one cohort (parent or mutant) but were not detected in any samples of the remaining cohort were scored as rank 4. Any features that were present in a peak group with a ranked feature were given a promoted ranking to the lowest number rank score for that peak group.

Genome sequencing of FM16-C7

Yeast cultures were obtained from original in-house glycerol cultures, which in the case of industrial strains were from commercially available freeze-dried preparations. Genomic DNA was extracted using Qiagen kits (as per the manufacturer's instruction).

Library construction and sequencing was performed at The University of Cambridge (Steve Oliver's laboratory), 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 L2056-C7) 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.

Sequences were assembled *de novo* using Newbler software and/or CLC Genomics workbench 4.0.2. software (www.clcbio.com) and scaffolded against the most up to date version of the S288C laboratory yeast strain genome (Goffeau *et al.*, 1996) using CLC Genomics workbench software. The reference genome was 12.1 Mb total length. The unmapped regions of L2056 were analysed using NCBI MULTIBLAST. Alignment of L2056-C7 and L-2056 was also performed against publically available whole genome sequences of the haploid derivatives (haplotypes) of wine yeast EC1118 and AWRI 1631 and bioethanol yeast JAY291. The final alignment was performed against AWRI 796, whereby the alignment was to the 16 yeast chromosomes.

Single nucleotide polymorphisms (SNPs) within open reading frames between L2056 and L2056-C7 were then identified using CLC Genomics workbench software. Initially, the two wine sequences were compared with S288C and EC1118 to determine the SNPs, prior to identifying which SNPs were unique to L2056-C7. From past studies we believed that L2056-C7 was effectively homozygous whilst the

L2056 was heterozygous, so we hypothesised that we would find hetereozygosity in certain alleles. In this initial investigation this was only taken in to consideration at the final analysis of deleterious SNPs.

Final sequence contigs will soon be deposited at DDBJ/EMBL/GenBank.

Phenotype Microarrays

Phenotype microarrays were performed by BIOLOG (www.biolog.com, Hayward, CA, USA) as described by (Bochner *et al.*, 2001).

Results and Discussion:

Development of optimal methodology for the metabolomics analysis of AE (and other interesting) wine yeast strains

To further evaluate the basis of strains of interest (in this case adaptively evolved and specific gene mutants), and consequently their effect on an oenological fermentation we decided to develop methods that would enable us to measure as many metabolites as possible, this was a specific objective outlined in UA 05/01. The possibility of measurement of all metabolic outcomes (metabolomics) allows for the direct evaluation of cellular responses. The application of this type of analysis within the field of winemaking is very exciting since it has the potential to reveal the exact metabolic differences between wines, without any prior knowledge and thus a precise measure of experimental treatments. The scope within this project for the use of metabolomics as a rapid wine yeast phenotyping tool is also very valuable.

At the time these analyses were conducted, the exact and appropriate method of sampling and extraction was still in debate, leaving many choices for experimental design. Not only this, but in a microbiological context, strains can be analysed from different growth phases and environmental conditions. We wished to discover which metabolic differences were detectable when the metabolic profile of two closely related wine strains were compared in different media (notably one which is similar to an oenological fermentation), with different extractions and at different growth phases. Following on from this we wished to use these profiles as a tool to predict the physiological differences between an adaptively evolved strain (L2056-C7) and its parent.

We chose 3 key yeast strains of interest to evaluate; L2056-C7, W303 *ngr1*, W303 *gid7*, due to their efficient fermentation characteristics as previously described, UA 01/04, (Gardner *et al.*, 2005, McBryde *et al.*, 2006). However, this report only contains results pertaining to L2056-C7. Inclusion of Δ*ngr1* and Δ*gid7* strains contributed to the understanding of a broader range of fermentation efficient mutants, however, for the purpose of this research project we concentrated on L2056-C7 due to its potential for being directly applicable to industry (as it is a non-GM wine yeast).

Identification and measurement of each and every metabolite of an entire metabolome is a difficult task due to both their complex and unexplored nature. Advances in this field however are being made at an extremely rapid rate. At the time of these analyses, the best available option was the use of Gas Chromatography Electrospray Ionisation Time of Flight Mass Spectrometry (GC-ESI-TOF-MS) a system currently being used for yeast metabolomics at the University of Manchester. The robustness of peak data across this platform allows for a more straightforward method of metabolite identification.

For application of metabolomics to the wine industry, obviously the metabolome of the finished wine is of the highest priority, however, since we were interested in the metabolite changes induced by particular strains of yeast we also examined their intracellular metabolome. This is known as the endometabolome, whereas the medium that the yeast is/has been in, and has contributed metabolites to, would be referred to as the exometabolome. In the case of winemaking the exometabolome equates to the compositon of the must or finished wine. Unfortunately initial attempts to prepare samples of the exometabolome from Chemically Defined Grape Juice Medium for analysis by Gas Chromatography were unsuccessful since this analytical method requires samples to be dried down. The presence of high concentrations of salts (amongst other metabolites) did not allow this to occur successfully. Consequently the exometabolome was examined using Liquid Chromatography.

Due to the technical difficulties and requirement of expertise for chromatographic analyses, and subsequent complex data interpretations such as these, all of the work related to examination of the metabolome was done with either our collaborators at The University of Manchester (the groups of Steve Oliver, now located in Cambridge, and Douglas Kell) or through Metabolomics Australia. Since the bulk of analytical work was carried out by third parties, and indeed many of the methods utilised were new technologies (in the case of MA, this was the first type of analysis of this kind) we had little control over timelines and as such, many delays were encountered.

Three different media were used to examine their usefulness in evaluation of industrial strains, these being Footprinting medium (FM, Allen *et al.*, 2003), F1-limited nitrogen medium (F1, Baganz *et al.*, 1997, Castrillo *et al.*, 2007) and Chemically Defined Grape Juice Medium (CDGJM, McBryde *et al.*, 2006). Briefly, FM is a synthetically defined minimal medium supplemented with a cocktail of amino acids, bases and organic acids. It was specifically designed to minimise the contribution of small inorganic ions lest these interfere with the mass spectrometric analysis. F1 has been used to examine the effect of a number of nutrient limitations by metabolomics analysis. We chose a nitrogen limited version of F1 as this has specific relevance to wine making, with many musts found to be nitrogen deficient (Amerine *et al.*, 1980, Henschke & Jiranek, 1993). Thirdly, CDGJM is the commonly used synthetic medium for industrial wine making studies (Henschke & Jiranek, 1993, McBryde *et al.*, 2006).

Both exometabolic and endometabolic samples were collected from cultures grown in each medium from both exponential and stationary phases. However, as mentioned, a number of the exometabolic samples were not able to be processed using methods for gas chromatographic analysis, as the requirement for concentration and drying often resulted in formation of an insoluble gel. For instance drying of samples of the exometabolome from CDGJM or F1 was unsuccessful, most likely arising due to the residual salts and/or sugars present. Thus, the only exometabolic fraction examinable by gas chromatography was from cultures grown in FM. As described, the exometabolic fraction of cultures grown in CDGJM was instead analysed by LC-MS. Two different extraction methods were also trialled, hot ethanol extraction (HEE) and cold methanol extraction (CME).

Based on the significant ions selected from the GC-MS and LC-MS data, principal component analysis was used to determine which metabolites (including those of which the identity still remains unknown) were discriminating between conditions or strains.

Discrimination of a mutant strain from its parent was achievable by PCA of internal metabolites in all three media. An example of GC-MS data is shown in Figure H.1 and a summary of all LC-MS analysis in CDGJM is shown in Figure H.2. Separate clustering of each strain in the scores plots shows that each of these analytical methods can differentiate between these strains at a metabolic level. Put more simply, these analytical methods can detect differences in the metabolome of these yeast strains, when cultured in F1, FM or CDGJM, and sampled at either stationary or exponential growth phase.

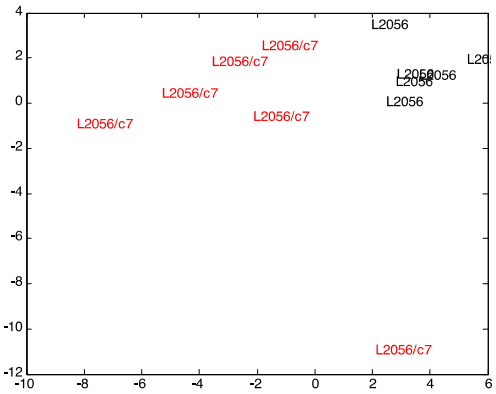


Figure H.1: The scores plot of a principle component analysis of endometabolites at stationary growth phase of L2056 and L2056-C7 when grown in footprinting media. The axes represent the two main principle components.

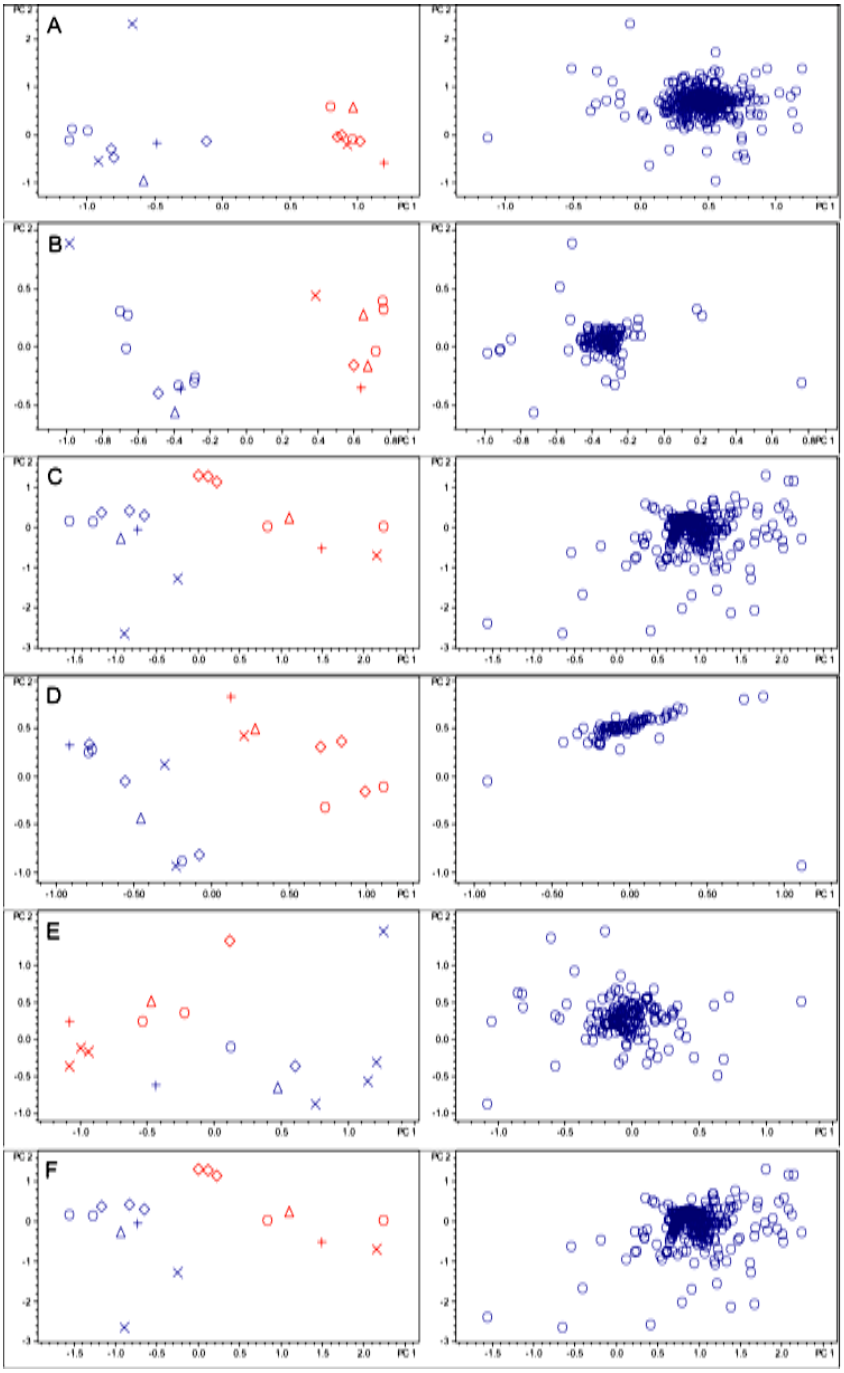


Figure H.2: Principal Component Analysis Scores and Loadings plots for all LC-MS analysis methods of spent chemically defined grape juice medium. Scores plot; L2056-C7 (mutant) is represented in red, whilst L2056 (parent) is in blue. Results from each LC-MS method is shown separately; A) Reverse Phase ESI +, B) Reverse Phase ESI -, C) Reverse Phase APCI +, D) Reverse Phase APCI -, E) HILIC ESI +, F) HILIC ESI-.

It is commonly known that single metabolomics methods only have the potential to probe a proportion of the measurable metabolome, and choices of methodology largely impact on these. For this reason, we decided to investigate a number of preparatory methods (then analysed by GC-MS), to not only analyse the outcomes, but to appreciate where limitations are evident and also to reveal as complete a metabolome as possible. Initial observation of raw chromatograms revealed many differences between mutants and parents, which was certainly promising, and the complexity of data generated is shown in an example chromatagram (Figure H.3). To gain some understanding of how different analytical choices might contribute to a metabolome, we undertook an analysis of detected metabolites for each condition tested. Unfortunately not all permutations of media x growth stage x extraction techniques could be completed as

there was limited access to analytical equipment in our collaborators' laboratories. However enough conditions were examined to allow us to make some recommendations for future studies. For example, the metabolome of yeast grown in CDGJM or F1 was more complex than that grown in FM as shown in Table H.2. Here, the average total and identified metabolites detected for each condition and analysed by GC-MS is displayed, with cultures grown in F1 or CDGJM clearly having approximately double the number of detectable metabolites (96 and 84 respectively *vs* 34 - 48). This is not surprising since these media are themselves more complex, thus able to probe a greater number of yeast metabolic pathways.

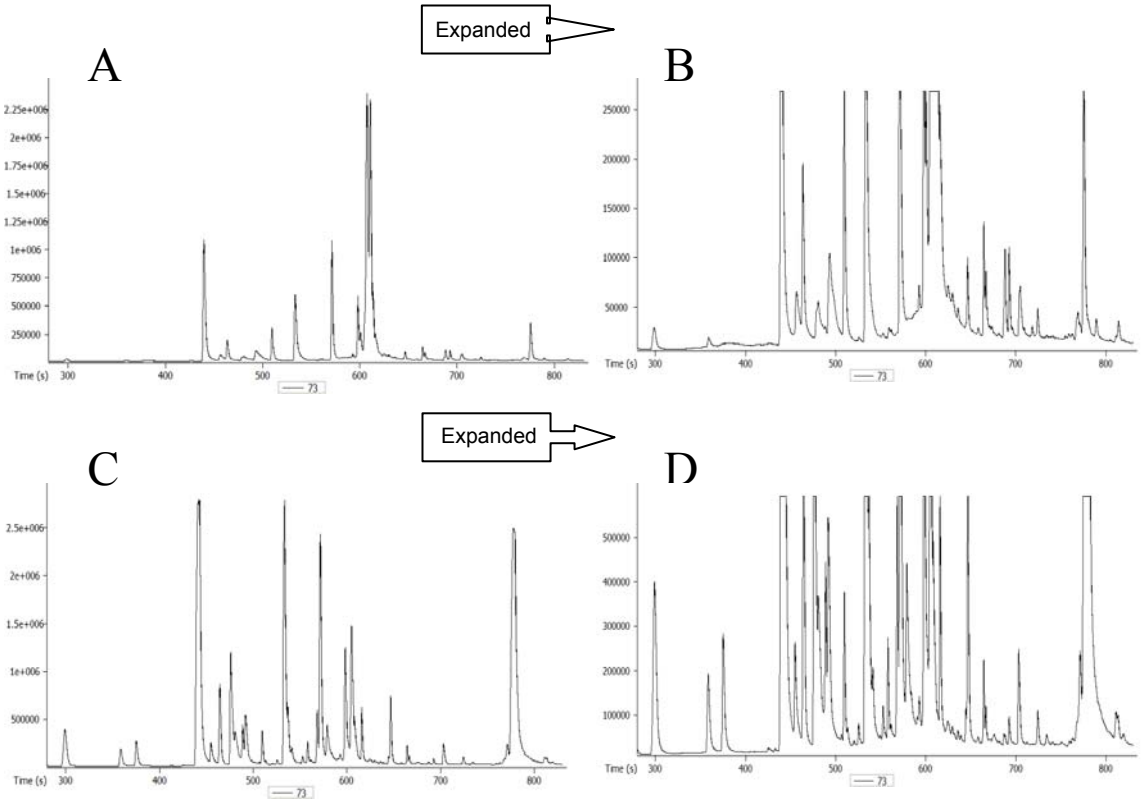


Figure H.3: Chromatograms of the endometabolome of yeast strain L2056, (A, B) and L2056-C7 (C, D) grown in CDGJM and sampled at stationary phase and extracted by HEE. (B, D) display expanded versions of chromatograms A and C.

Table H.2: The average total features detected and metabolites identified from either the endometabolome (ENDO) or exometabolome (EXO) of average (combined) results from L2056 and L2056-C7 cultures grown in various media, at either exponential (EXP), stationary (STAT) phase or from the end of fermentation (EOF), that is upon exhaustion of all sugars. Features chosen are those whose $p < 0.05$.

COMPARTMENT	ENDO	ENDO	ENDO	ENDO	ENDO	ENDO	EXO	ENDO
MEDIUM	F1	F1	FM	FM	FM	FM	FM	CDGJM
EXTRACTION METHOD	HEE	HEE	HEE	HEE	CME	CME	N/A	HEE
GROWTH PHASE	EXP	STAT	EXP	STAT	EXP	STAT	STAT	EOF
TOTAL FEATURES DETECTED	97	67	46	42	44	34	48	84
TOTAL METABOLITES IDENTIFIED	42	30	19	17	21	16	34	36
% METABOLITES IDENTIFIED	43%	45%	41%	40%	48%	47%	71%	42%

The actual percentage of metabolites identified from those detected was usually around 45%, except where the exometabolome was examined from FM. This is most likely a reflection of the knowledge of original media components and subsequent purposeful inclusion of them in spectral libraries. In general, more metabolites were identified from the exponential growth phase, which is not surprising since it is at this stage that cells are in the process of rapid growth and active metabolism. The hot ethanol extraction also tended to yield marginally more detectable metabolites, but as discussed below cold methanol extraction is definitely a useful inclusion.

As we were ultimately interested in determining the metabolite differences between parent and mutant strains, an analysis of just the discriminatory metabolites identified using GC-MS was also performed across the various methods, and is represented as Venn diagrams in Figure H.4. Overall it is evident that there is little overlap between detected metabolites between conditions, revealing that in fact only a proportion of the metabolome is detectable by a single condition, and highlighting the advantage of multiple analyses. Comparison of the endo- and exometabolomes also reveals few common discriminatory metabolites (Figure H.4C).

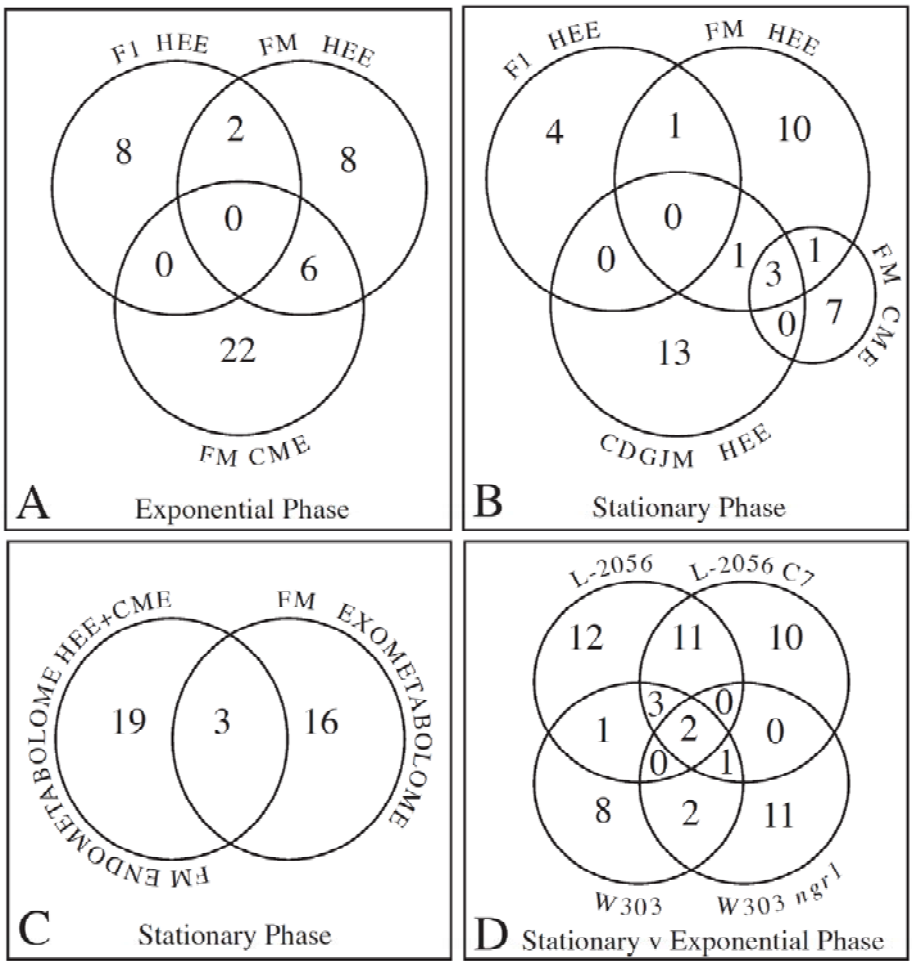


Figure H.4 A-C: Correlation of discriminatory metabolites of the L2056-C7 endometabolome, (except where highlighted as exometabolome in C), in comparison to L2056 at exponential phase (A), or stationary phase (B) when grown in F1-N limiting (F1), Footprinting (FM) or Chemically defined grape juice (CDGJM) medium. Either Hot ethanol extractions (HEE) or cold metahanol extraction (CME) were performed. D: Correlation of discriminatory metabolites of stationary and exponential phases identified from various yeast strains when grown in FM.

In some cases, the same discriminatory metabolites were identified from growth in different media. For instance, four discriminatory metabolites were identified between L2056-C7 and its parent, when grown in either FM or CDGJM at stationary phase (Figure H.4B), with three of these, namely fumaric acid, isopropylmalic acid or 2-hydroxy glutaric acid being extracted by either the hot ethanol or cold methanol methods. This is of particular interest as it introduces robustness into the evaluation of how to decipher how a particular genotype impacts the production of certain metabolites. Not only this, but the difference in yeast response to changes in media composition is also of interest and can potentially be informative when relating back to the original media composition.

Discriminatory metabolomics may also be used to reveal underlying processes of complex metabolism in a robust manner. For instance, we compared the endometabolome of cells in either stationary or exponential growth phase, identified discriminatory metabolites and then compared these results across four strains (Figure H.4D). Two of the 62 metabolites identified were found to be discriminatory in all strains and a further three in at least three of the four strains. This result uncovers the potential for the identification of "global metabolic responses", for instance, those metabolites that change irrespective of these particular strain differences, under these particular conditions. Potentially, this method could be expanded upon, to reveal specific metabolic biomarkers.

Analysis of the metabolome of yeast strain L2056-C7

Since the desirable fermentation phenotype of L2056-C7 is evident in CDGJM and our research priority is to elucidate effects on oenological fermentation, this was the medium of choice for a metabolomic evaluation. The use of a defined medium instead of gape juice here was necessary since a known initial medium composition would allow a more straightforward examination of the metabolite changes.

The endometabolome of cells cultured in CDGJM, sampled at the end of fermentation (after exhaustion of all available sugars) and extracted by HEE was evaluated by GC-ESI-TOF-MS and the exometabolome was analysed by all 6 LC-MS analytical protocols as detailed in the methods section of this Output. Note that the GC and LC analyses were performed in separate laboratories (Uni. of Manchester and Metabolomics Australia, respectively) and for this reason the statistical analyses was also performed separately. GC-MS analysis revealed that 84 features were detected with 36 of these being identified (Table H.2). At a statistical confidence of $p < 0.05$, the concentration of 16 of these features differed (data not shown) of which 9 were putatively identified by cross referencing to spectral libraries (Table H.3A and H.3B). Analysis of gross fermentation metabolites of spent fermentation media was also performed by HPLC, as is standard operation within this laboratory, but no significant differences were found (Table H.3C).

Table H.3A: Putatively annotated discriminatory metabolites of L2056-C7 identified by both GC-ESI-TOF-MS and a combination of LC-MS methods. Compounds with alike biological groups are consolidated and whether they have been reported in the literature as being found in yeast is noted. The fold change by comparison of the peak area (mutant/parent) or whether they are only detectable in one or other strain and the detected location of the metabolite (EXO: exometabolome, ENDO: endometabolome) is listed. Metabolites identified from the EXO by LC-MS methods are at a significance of $p < 0.02$ and from the ENDO by GC-MS at $p < 0.05$.

Biological Group	Dicriminatory Metabolite	Involved in	Known in Yeast?	Fold Change (mutant/parent)	Location
Steroid Hormones	Androst-4-ene-3, 17 dione	Steroid hormone biosynthesis	No	Parent only	EXO
	Urocortisone	Steroid hormone biosynthesis	No	Parent only	EXO
	Estradiol-17B (Beta)	Steroid hormone biosynthesis	No	4.57	EXO
	Estradiol-17A (Alpha)	Estrogen biosynthesis	No	4.88	EXO
Jadomycin intermediates	Prejadomycin	Jadomycin biosynthesis	No	1.12	EXO
	UWM6	Jadomycin biosynthesis	No	1.19	EXO
Terpenoids and Quinones	6-decylubiquinone	synthetic derivative of ubiquinone	No	Parent only	EXO
	2-methyl-6-farnesyl-1,4-benzoquinol	Plastoquinone-9 and tocochromanol biosynthesis	No	Parent only	EXO
	3-geranyl-4-hydroxybenzoate	Glutathione and shikonin biosynthesis	Yes	Parent only	EXO
	Menaquinone-2	Ubiquinone and other terpenoid-quinone biosynthesis	No	-3.07	EXO
	Gibberellin A15 (closed lactone from)	Gibberrellin biosynthesis	Yes	Mutant only	EXO
TCA cycle intermediates	Fumaric acid	TCA cycle, tyrosine, arginine and proline metabolism	Yes	9.57	ENDO
	Succinic acid	TCA cycle	Yes	11.86	ENDO
Amino acid metabolism	Alanine	Amino acid metabolism	Yes	1.67	ENDO
	2-isopropylmalic acid	Amino acid metabolism	Yes	5.35	ENDO
Unsaturated fatty acids	Oleic acid	Fatty acid biosynthesis	Yes	Parent only	ENDO
	Palmitin	Fatty acid biosynthesis	Yes	-1.67	ENDO
	(6Z,8E,10E,14Z)-(5S,12R)-5,12,20-Trihydroxyicosa-6,8,10,14- tetraenoate	Arachidonic acid synthesis	Yes	Mutant only	EXO

Table H.3B: Putatively annotated discriminatory metabolites of L2056-C7 identified by both GC-ESI-TOF-MS and a combination of LC-MS methods. Compounds do not have obvious similar biological groups and whether they have been reported in the literature as being found in yeast is noted. The fold change by comparison of the peak area (mutant/parent) or whether they are only detectable in one or other strain and the detected location of the metabolite (EXO: exometabolome, ENDO: endometabolome) is listed. Metabolites identified from the EXO by LC-MS methods are at a significance of $p < 0.02$ and from the ENDO by GC-MS at $p < 0.05$.

Biological Group	Dicriminatory Metabolite	Involved in	Known in Yeast?	Fold Change (mutant/parent)	Location
Ungrouped metabolites	Elymoclavine	An Ergoline	No	Parent only	EXO
	Indole-3-glycol	Tryptophan degradation	Yes	Parent only	EXO
	5'-amino-5'-deoxyuridine	Deoxynucleoside	Yes	Parent only	EXO
	Flavin	Mitochondrial enzymatic cofactor	Yes	Parent only	EXO
	5-pentadecatrienyl resorcinol-3-methyl ether	Sorgoleone biosynthesis in plants	No	Parent only	EXO
	4-hydroxy-5-methyl-2-propyl-3(2H)-furanone	Furaneol biosynthesis	No	Parent only	EXO
	2-hydroxyglutaric acid	Butanoate metabolism	Yes	-4.23	ENDO
	Bestatin	Statin biosynthesis	No	-3.07	EXO
	(-)-jasmonoyl-L-valine	Jasmonate pathway	No	-3.07	EXO
	1-acyl-sn-glycero-3-phosphoethanolamine (n-C12:0)	Glycerophospholipid metabolism	Yes	-2.38	EXO
	6,7-dihydropteridine	Folate biosynthesis	Yes	-1.47	EXO
	Me-IP	Cytokinin degradation	Yes	-1.41	EXO
	2-oxo-9-methylthiononanoate	Glucosinolate biosynthesis	Yes	3.13	EXO
	Myoinositol	Phosphatidylinositol biosynthesis	Yes	4.1	ENDO
	Lactic acid	Glycolysis	Yes	4.91	ENDO
	Valpromide	A Pentanoic acid	No	5.65	EXO
	Eserine	Melatonin pathway	No	Mutant only	EXO

Table H3.C: HPLC analysis of spent fermentation media from fermentations performed in CDGJM by L2056-C7 and L2056. These are the same biological samples used for exometabolic analysis by LCMS. The average and standard deviation of six biological replicates are shown as well as the percentage detected in L2056-C7 as compared to the parent.

Strain	Glucose	Fructose	Glycerol	Acetaldehyde	Ethanol	Citric	Tartaric	Malic	Succinic	Lactic	Acetic Acid
L2056 (average)	0	1.39	7.55	0.30	138.7	0.43	1.13	2.85	0.48	0.60	1.83
L2056 (st. deviation)	NA	0.20	0.23	0.01	0.53	0.07	0.40	0.07	0.02	0.02	0.16
L2056-C7 (average)	0	0.92	7.85	0.31	136.3	0.41	1.03	2.77	0.46	0.61	1.85
L2056-C7 (st. deviation)	NA	0.11	0.06	0.02	3.06	0.01	0.37	0.04	0.01	0.02	0.06
Pecentage of parent (%)	100	66.15	104.01	103.28	98.28	95.16	91.49	97.20	94.40	100.78	101.30
	NA	12.46	0.73	6.13	2.24	2.06	35.61	1.37	2.21	2.53	3.01

Statistical analysis of LC-MS data was immediately focussed on only the discriminatory features, thus a total feature count is currently unavailable. Nonetheless, many discriminatory features are detected with this method, for example at a confidence of $p < 0.05$, 197 peak groups (most likely to be unique metabolites) are detected (Table H.4). Since the process of putative annotation of these features is quite laboursome and we currently do not have the in-house expertise, we were limited to examination of only those peak groups with a p value of < 0.002 and those only detected in parent or mutant. Of these 59 peak groups, 26 metabolites were putatively annotated (Table H.3A and H.3B). As explained in the methods, these putative identifications were achieved by comparison to spectral libraries, which is not as robust a method of identification as in GC-MS. Thus for definite identification, standards of each metabolite must be run on the same machine as the data was obtained. This is currently underway for this set of 26 metabolites with Metabolomics Australia. Obviously further identifications of all discriminatory peak groups at a confidence of $p < 0.05$ would be extremely valuable.

Table H.4: Discriminatory features counted from the combination of all LC-MS analyses at different statistical relevance from the exometabolome of L2056-C7 cultures grown in CDGJM. A ranking of significant features was performed to select individual features with low p values. Some features were found to be related and assumed to be derived from the same metabolite, these were assigned to a peak group (most likely these groups represent unique metabolites). Any features that were present in a peak group with a ranked feature was given a promoted ranking to the lowest number rank score for that peak group. 4 (all and none) encompasses those features that are only detectable in one or other of L2056 or L2056-C7.

Marker priority score	Feature count	Promoted feature count	Peak group count
1 ($p < 0.002$)	30	50	23
2 ($p < 0.01$)	65	90	48
3 ($p < 0.05$)	114	171	90
4 (all and none)	48	53	36
Total	**257**	**364**	**197**

Of the discriminatory metabolites identified, none are identified in both endometabolome and exometabolome, possibly a reflection of the subset of metabolites examined and that not all compounds are transported outside of the cell. However upon examination of discriminatory metabolites from FM media in stationary phase (Table H.5), there are some commonly identified compounds both between the endo and exo-metabolome (glycerol, isomaltose and sucrose), and between FM and CDGJM media (fumaric acid, 2-isopropylmalic acid and 2-hydroxyglutaric acid). However, this may be a reflection of preparation and analytical methods, as each of these were identified in the GC-MS analysis.

Table H.5: Discriminatory metabolites of L2056-C7 identified by GC-MS from stationary phase cultures in FM medium from either the endometabolome or the exometabolome. (Fold change ratio not yet determined). The total discriminatory features from the endometabolome = 22 and exometabolome = 19).

ENDOMETABOLOME	EXOMETABOLOME
Glycerol	Glycerol
Isomaltose	Isomaltose
Sucrose	Sucrose
Fumaric acid	Phosphocreatine
2-isopropylmalic acid	Malic acid
2-hydroxyglutaric acid	Threiotol or erythritol
Uracil	Octadecanoic acid
Mannose	
Glucose	

Analysis of the metabolic consequence of fermentation with L2056-C7

Preliminary analysis of the putative discriminatory metabolites was undertaken using a number of programs designed to find biologically meaningful patterns, such as PAPi (Aggio *et al.*, 2010) and MetPA (Xia & Wishart, 2010). Unfortunately, using these tools, no patterns were revealed. Upon discussion with others in the field (discussions held at *The International Metabolomics Society Conference 2011*, funded by GWT 1015) we found that this was not an uncommon occurrence, most likely since only a subset of the metabolome had actually been identified and only a proportion of these annotated to a central reference library (Jewison *et al.*, 2011). Nonetheless we then began a manual investigation of each metabolite, its possible relationship with any other from the list, and how the change in its concentration may be a consequence or cause of the efficient fermentation phenotype of L2056-C7.

From this manual analysis we were able to class 18 of the 35 metabolites to 6 broad biological groups with 17 being seemingly unrelated (Tables H.3A-B). Many of these compounds have not yet been described in yeast. Whether they are in fact a misidentification, or that they actually are present is still under investigation. Since this analysis is on-going (Metabolomics Australia are in the process of refining the analysis with the inclusion of standards), and when considered in isolation, effects of such are only speculative, as such details of only a few metabolites are highlighted in this section. More detail, where meritorious is provided in following sections. Those metabolites not found in yeast or that do not have a known yeast derived precursor are, in general, not discussed.

Classical steroid hormones such as those highlighted in Table H.3A are thought to be absent from *S. cerevisiae*. In fact typical steroid biosynthetic pathway of yeast ends in the production of ergosterol (Brocard-Masson & Dumas, 2006) whereas in plants and mammals this is only the starting entity for steroid hormone biosynthesis. There are many conversions required for the production of steroid

hormones such as these from ergosterol, thus it is unlikely these are in fact present. Yeasts are known however to have steroid metabolizing enzymes, such as Atf2p, which can catalyse the conversion of pregnenolone to acetylpregnenolone (Cauet *et al.*, 1999) and interestingly Atf2 is an alcohol O-acetyl transferase responsible for the formation of volatile esters in yeast (Verstrepen *et al.*, 2003).

The yeast isoprenoid pathway results in production of many end products including precursors for steroid like metabolites including the essential and well studied ergosterol (as reviewed by Kuranda *et al.*, 2010). A number of other non-steroid like discriminatory metabolites of L2056-C7 are also associated with this pathway, notably 3-gernayl-4-hydroxybenzoate, menaquinone-2 and Gibberellin A15. Each of these compounds have gernayldiphosphate as a common (but not direct) precursor for their synthesis in various fungi, however as yet there is no definitive evidence for their synthesis in *S. cerevisiae*. Putative identification of these from this study would be their first report, and certainly interesting from the point of view of involvement of the isoprenoid/mevalonate pathway in fermentation. Additionally, gibberellins are well known as growth hormones in higher plants and have been recently examined in fungi (Tudzynski, 2005). Gibberellins may have a similar role to plants (perhaps in environemtal signalling) in yeast, and since Gibberellin A15 is only detectable in L2056-C7, its presence may modify cellular processes in response to the environment.

The citrate cycle (TCA cycle) is an important pathway during aerobic growth that provides reducing equivalents for the respiratory chain, which in turn produces energy in the form of ATP. However during fermentative growth, such as that studied here, the TCA cycle is repressed by glucose and lack of oxygen. Residual TCA pathway activity does however remain, in fact it is commonly thought to function in two separate branches, instead of a cycle, one oxidative, leading to formation of 2-oxoglutarate (Nunez de Castro *et al.*, 1970), the other reductive, leading to the formation of organic acids such as fumarate and succinate (Camarasa *et al.*, 2003). The increased abundance of fumarate (*ca.* 9.57-fold more) and succinic acid (*ca.* 11.86-fold more) detected intracellularly in L2056-C7 is expected to be a result of increased flux through the reductive arm of the TCA cycle. These organic acids are of interest in winemaking since they (succinic acid in particular) contribute to wine acidity. However, it is worth noting that no increase in extracellular succinic acid was found by HPLC (Table H.3C). Succinic acid can also be formed by the cytosolic located glyoxylate cycle, an off-shoot of the TCA cycle where isocitratelyase catalyses the formation of succinate from isocitrate (as reviewed in Strijbis & Distel, 2010). However, flux through this cycle is normally only activated by growth on non-fermentable carbon sources. Lin *et al.*(2011) found that deletion of genes such as *FUM1* (fumerase) led to accumulation of fumarate and succinate in respiring cells and is linked to the retrograde response pathway, and as such further studies should include checking the activity of Fum1p in L2056-C7.

Isopropylmalic acid, is an intermediate of leucine biosynthesis, generated from 2-oxoisovalarate or from pyruvate metabolism. It is also known to be secreted and then thought to chelate aluminium ions leading to aluminium tolerance (Suzuki *et al.*, 2007). The more than five fold increase in intracellular 2-isopropylmalic acid in L2056-C7 is expected to reflect an increase in flux of this pathway and may result in an increased tolerance to potentially damaging environmental ions.

Myo-inositol, the most prominent form of inositol in nature plays an important role as the structural basis for a number of secondary messengers in eukaryotic cells, including inositol phosphates, phosphatidylinositol and phosphatidylinositol phosphate lipids, also important structural components for cellular membranes. The four fold increase of intracellular myo-inositol in L2056-C7 may be a reflection of a modified membrane structure. Earlier work by Jiranek *et al.* (1998) has in fact demonstrated

increesed stress (salt) tolerance by laboratory strains that overproduce inositol. Whether a similar effect is being observed in the evolved wine strains is to be confirmed.

Fatty acids are also important membrane components, together with energy storage and protein modification their synthesis is essential since yeast rarely grow in the presence of utilisable fatty acids. The biosynthesis of the two unsaturated fatty acids highlighted by this study, oleic acid and palmitin, are generally considered the most common products of fatty acid biosynthesis (Tehlivets *et al.*, 2007). The third highlighted fatty acid, (6Z,8E,10E,14Z)-(5S,12R)-5,12,20-trihydroxyicosa-6,8,10,14- tetraenoate, also known as 20-OH-LTB4, is thought to be a by-product of arachidonic acid metabolism. Interestingly some very recent and preliminary results from a collaborative laboratory (David Block, UC Davis, CA) report that the ratios of phospholipids found in L2056-C7 membranes were in fact altered (data not shown) similar to what they have seen with a selection of wine yeast that have varying tolerance to ethanol (Henderson *et al.*, 2011).

Many more discriminatory metabolites have been found from experiments with L2056-C7, however, as highlighted earlier, without the integration with other related data sets, the mechanisms causing these changes can only be speculated upon. Future work ion these is planned however.

Genomic analysis of L2056-C7

The genomic sequences of the adaptively evolved strain L2056-C7 and its parent L2056 were obtained and compared. A total of 200 mutations, or single nucleotide polymorphisms (SNPs), which led to amino acid changes (non-synonymous) were identified in 176 open reading frames (ORFs, Table H.6). SNPs from non-coding regions are yet to be determined, however, this has recently become a priority to complete since recent discoveries reveal the extreme complexity and importance of cellular regulation of yeast, (as well as almost all other living organisms) by non-coding or thought to be non-coding, genetic material (Ito *et al.*, 2008, Granovskaia *et al.*, 2010, Mercer *et al.*, 2011).

Interestingly in 23 cases, 2 mutations are found in the same ORF, and a single gene YDR179W-A, currently of unknown function harbours 3. Perhaps this set of genes could be viewed as “hot spots” for mutations during this particular adaptive evolution, a possibility that certainly warrants separate detailed analysis.

A Gene Ontology (GO, Ashburner *et al.*, 2000; date performed: 10/09/11) analysis was then performed on this list of genes to determine if any biological processes were enriched for SNPs. Using either GO TERM FINDER (function) or GO STATS (both part of the suite of tools from GO TOOLBOX) only one GO term; Histone Binding, was found to be significantly enriched, with 7 genes from 23 of those annotated to this term existing within this set (Table H.7 and H.10). Analysis with GO TERM FINDER (process) revealed 8 more significant GO terms notably DNA packaging, regulation of various cellular processes and Response to Stimulus (Table H.8). It is worth noting that a number of genes associated with Histone Binding are also associated with DNA packaging (Table H.10). GO PROXY analysis of how the selected genes cluster resulted in 84 classes of GO terms (data not shown).

Table H.6: The distribution of mutations (SNPs) in the L2056-C7 genome compared to L2056

Chromosome	Open Reading Frames	Chromosome length	Total genes (mutated)	Total mutations	% mutated genes in chromosome	Chr as % of genome
16	511	948,066	27	28	5.3	7.9
15	598	1,091,291	6	7	1.0	9.0
14	435	784,333	8	10	1.8	6.5
13	505	924,431	12	12	2.4	7.7
12	578	1,078,177	23	28	4.0	8.9
11	348	666,816	2	2	0.6	5.5
10	398	745,751	0	0	0.0	6.2
8	321	562,643	8	11	2.5	4.7
7	583	1,090,940	14	14	2.4	9.0
6	141	270,161	4	4	2.8	2.2
5	324	576,874	4	4	1.2	4.8
9	241	439,888	0	0	0.0	3.6
4	836	1,531,933	61	72	7.3	12.7
3	183	316,620	5	6	2.7	2.6
2	456	813,184	1	1	0.2	6.7
1	117	230,218	1	1	0.9	1.9
TOTAL	**6575**	**12, 071,326**	**176**	**200**	**100**	**100**

Table H.7: GO (Function) Terms enriched in L2056-C7 SNP set, p < 0.05

GO Term	Cluster frequency	Background frequency	P-value	Genes annotated to the term
Histone Binding	7 out of 175 genes, 4.0%	40 out of 7168 background genes, 0.6%	0.00602	*RAD9/YDR217C: SIR4/YDR227W: HIF1/YLL022C: SIR3/YLR442C: FPR4/YLR449W: CAC2/YML102W: RTT106/YNL206C*

Table H.8: GO (Process) Terms enriched in L2056-C7 set, p < 0.05

GO Term	Cluster frequency	Background frequency	P-value
DNA packaging	8 out of 175 genes, 4.6%	49 out of 7168 background genes, 0.7%	0.01304
cellular process	145 out of 175 genes, 82.9%	4966 out of 7168 background genes, 69.3%	0.01616
regulation of cellular biosynthetic process	32 out of 175 genes, 18.3%	628 out of 7168 background genes, 8.8%	0.0271
regulation of biosynthetic process	32 out of 175 genes, 18.3%	628 out of 7168 background genes, 8.8%	0.0271
regulation of cellular metabolic process	36 out of 175 genes, 20.6%	751 out of 7168 background genes, 10.5%	0.03066
regulation of cellular macromolecule biosynthetic process	31 out of 175 genes, 17.7%	606 out of 7168 background genes, 8.5%	0.03384
regulation of macromolecule biosynthetic process	31 out of 175 genes, 17.7%	608 out of 7168 background genes, 8.5%	0.03603
response to stimulus	41 out of 175 genes, 23.4%	917 out of 7168 background genes, 12.8%	0.04132

Table H.9: Expanded GO (Process) Term DNA Packaging enriched in L2056-C7 set, p < 0.05

GO Term	Cluster frequency	Background frequency	P-value	Genes annotated to the term
DNA packaging	8 out of 175 genes, 4.6%	49 out of 7168 background genes, 0.7%	0.01304	*MCD1/YDL003W: SCC2/YDR180W: YCG1/YDR325W: HIF1/YLL022C: SIR3/YLR442C: FPR4/YLR449W: CAC2/YML102W: RTT106/YNL206C*

Further analysis used the Pathway Enrichment tool (part of YEAST MINE, http:// yeastgenome.org), which is designed to identify those curated pathways that are also enriched within the gene set, highlighted the isoprenoid/mevalonate pathway (p = 0.02). Only 2 genes however, *ERG10* and *HMG2* from this gene set are included, with the mutations in L2056-C7 predicted to be tolerated (Table H.10).

The GO term Histone Binding is defined as "Interacting selectively and non-covalently with a histone, any of a group of water-soluble proteins found in association with the DNA of plant and animal chromosomes". They are involved in the condensation and coiling of chromosomes during cell division and have also been implicated in nonspecific suppression of gene activity (EMBL-EBI, http://www.ebi.ac.uk/QuickGO). It is well known that access to transcriptional binding sites is a common method of transcriptional regulation, and a general modification such as this may be indicative of loosening of transcriptional suppression. This has recently become quite a hot topic with the discovery that nucleosomes are preferentially positioned over exons, another method of regulation and transcriptional feedback (Nahkuri *et al.*, 2009, Tilgner *et al.*, 2009). Perhaps with this gene complement the evolved mutant L2056-C7 has less restrictions placed upon genes contributing towards fermentation activity. Presumably a loss or modification of general transcriptional suppression at the level of chromosomal superstructure, in part controlled by histone binding, at key times when normally they would be down regulated due to environmental cues (such as an increase in environmental stress such as that in the latter parts of an oenological fermentation) results in the cell maintaining an efficient fermentation rate.

It is well known that for transcription of a gene, the necessary machinery must first assemble and then move along the body of the gene, but before this can occur DNA must be unravelled from its packaging in nucleosomes (Williams & Tyler, 2007). Precise regulation of transcription at the level of chromatin is possible since nucelosomes and indeed histones can be removed by the action of a number of factors, such as histone chaperones, in a coordinated fashion (Avvakumov *et al.*, 2011). Some of the genes found to have mutations in L2056-C7 appear to have precisely this role in gene silencing at the level of chromatin, for example *SIR3*, *SIR4, HIF1, FPR4* and *CAC2,* interestingly *SIR3* accommodating two separate amino acid changes (K305E and M755L). However only the E324G amino acid change of *HIF1* in L2056-C7 is predicted by SIFT analysis to result in one which affects protein function (Table H.10). Hif1p is an interacting and linking component between the yeast heterotrimeric HAT-B complex and chromatin assembly in the nucleus (Ai & Parthun, 2004, Poveda *et al.*, 2004). HAT-B, made up of the gene products of *HAT1* and *HAT2*, specifically acetylates lysine 12 and 5 of free non-chromatin-bound histone H4. Histone acetylation is known to be a dynamic process that correlates with modulation of gene expression (Roth *et al.*, 2001). Hif1p seems to be a non-essential subunit of the complex since *HIF1* mutants still have functional HAT-B. However, this deletion does cause a minor telomeric silencing defect, similar to that seen in Δ *hat1* or Δ *hat2* mutants, as observable in the presence of certain histone

H3 lysine-to-arginine mutations (Poveda *et al.*, 2004).This may indicate that again, L2056-C7 may well have a modified transcriptional regulation acting through more the mechanisms of DNA architecture.

Table H.10: SIFT predictions of selected gene mutations of L2056-C7. Scores predicted by SIFT are shown, an amino acid substitution is predicted damaging if the score is <= 0.05, highlighted in red, and tolerated if the score is > 0.05. Scores < 0.2 are highlighted in orange as these should be examined in more detail.

GENE	aaMUTATION	SIFT PREDICTION	SCORE
LIPID AND STEROL METABOLIC PROCESS			
ERG10	E311Q	TOLERATED	0.33
HMG2	D940A	TOLERATED	0.12
SUR2	V87I	TOLERATED	0.86
CRC1	S224R	AFFECT PROTEIN FUNCTION	0
IPT1	L90I	TOLERATED	1
ARE1	V57M	TOLERATED	0.21
CELLULAR AMINO ACID METABOLIC PROCESS			
ARO3	N54G	TOLERATED	0.32
AGX1	R239G	TOLERATED	0.1
ASP1	Q130stp	AFFECT PROTEIN FUNCTION	0
SPE4	R129H	AFFECT PROTEIN FUNCTION	0
BNA7	L53P	TOLERATED	0.15
HISTONE BINDING			
RAD9	T50A	TOLERATED	0.72
SIR4	S336L	TOLERATED	0.32
DNA PACKAGING			
MCD1	L176I	TOLERATED	0.86
SCC2	T104I	TOLERATED	0.18
YCG1	T913I	TOLERATED	0.21
HISTONE BINDING and DNA PACKAGING			
HIF1	E324G	AFFECT PROTEIN FUNCTION	0.02
SIR3	M755L	TOLERATED	0.65
SIR3	K305E	TOLERATED	1.0
FPR4	N58T	TOLERATED	0.38
CAC2	N243T	TOLERATED	0.35
RTT106	N439G	ANALYSIS NOT AVAILABLE	N/A
RTT106	N328G	ANALYSIS NOT AVAILABLE	N/A

Integration of genomic and metabolomic data from L2056-C7

Genomic and metabolomic data were manually inspected for commonalities. Initially GO terms generated by GO PROXY were sorted for those that have links to the discriminatory metabolites. Two broad classes were highlighted by this analysis, that is, lipid and sterol metabolic processes and amino acid metabolic processes. Mutations in genes belonging to these classes were checked by SIFT analysis (Table H.10),

among those of the lipid and sterol metabolic processes a S224R change in *CRC1* is predicted to be deleterious to protein function and a D940A change in *HMG2* is predicted to be tolerated, however, with low confidence (a SIFT score of 0.12) inferring that this change should be examined more closely. Similarly from the cellular amino acid processes mutations in *ASP1* and *SPE4* are predicted to affect protein function and *AGX1* and *BNA7* should be further examined.

Phospholipids in yeast are the major building blocks of plasma and organelle membranes. However a variety of other lipid types are also found, for example glycolipids, inositol phospholipids, free fatty acids and sterols. The composition of a membrane is extremely influential upon cell health since it modulates the fluidity, permeability, thickness and opportunity for signalling. Thus the involvement of the lipid and sterol metabolic processes in this mutant could be a direct reflection of a general improvement of cell health in a fermentation environment. SNPs in the genes *CRC1* and *HMG2* have been found to be likely effectors.

CRC1 encodes the mitochondrial inner membrane carnitine transporter, the orthologue of the human mitochondrial carnitine acyl carnitine translocase and is required for transport of acetyl CoA (a product of beta-oxidation of fatty acids in the peroxisome) into the mitochondrial inner membrane where it is used in the citric acid cycle (van Roermund *et al.*, 1999). Biosynthesis of fatty acids and in turn phospholipids is dependent upon the availability of an essential building block, acetyl CoA (as reviewed in Strijbis & Distel, 2010). Briefly, acetyl CoA is produced from pyruvate (ex glycolysis) and in yeast via the beta-oxidation of fatty acids in the peroxisome. Acetyl CoA alone can not transverse membranes, thus requires transport, for example by Crc1p. Δ*Crc1* strains have been reported to be viable, have increased competitive fitness (on minimal media) (Breslow *et al.*, 2008) and a putative increase in replicative lifespan (this mutation was short listed, however failed to pass a very strict verification criteria) (Kaeberlein *et al.*, 2005). Perhaps during fermentation, a mutation in Crc1p allows more readily available cytosolic acetyl CoA leading to a modification in the production of lipid components (produced in the cytosol) and in turn a robustness during fermentation.

HMG2 encodes one of two isozymes of HMG-CoA reductase that convert HMG-CoA to mevalonate, part of the mevalonate/isoprenoid (as reviewed in Kuranda *et al.*, 2010). Δ*Hmg2* strains exhibit abnormal lipid particle morphology in stationary phase cultures as well as low lipid droplet content (Bozaquel-Morais *et al.*, 2010). As discussed above, the D940A mutation found in L2056-C7 was predicted to be tolerated (at low confidence), however, it merits discussion here since there are multiple links with identified discriminatory metabolites. The early parts of the mevalonate pathway appear to be located in the cytosol (Farhi *et al.*, 2011) and begin with 2 acetyl CoA (possibly providing a link with a modified *CRC1* as explained previously). Subsequent reactions lead to the formation of farnesyldiphosphate, geranyldiphosphate and geranylgeranyldiphosphate, amongst others. These compounds are non-direct precursors for steroid biosynthesis, ubiquinone and other terpenoid biosynthesis, and the formation of gibberrellins, which are amongst some of the identified pathways of discriminatory metabolites of L2056-C7, as reported above (see Figure H.5 for a diagramatic representation). Also, a modification of *ERG10*, an acetyl-CoA C-acetyltransferase involved in the first step in mevalonate biosynthesis, is found in L2056-C7; E311Q which is again, predicted to be tolerated.

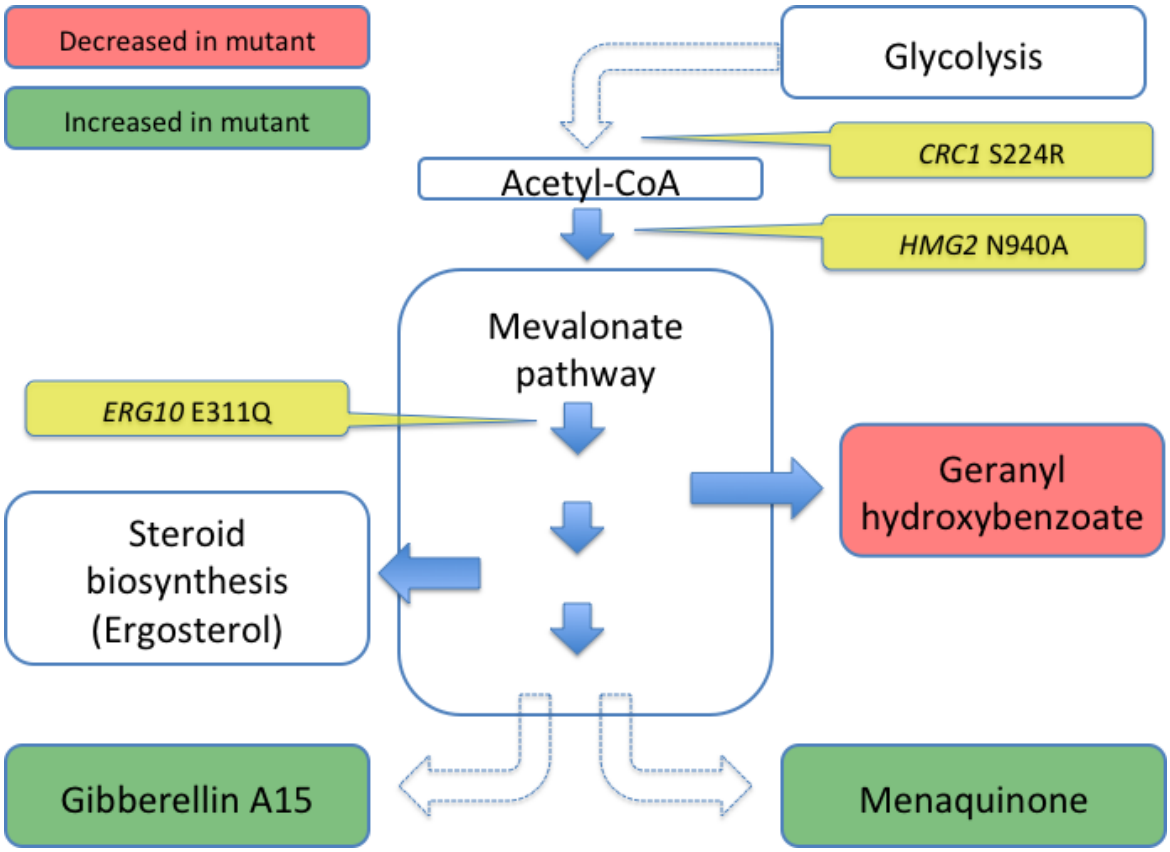


Figure H.5: Links between discriminatory metabolites of L2056-C7 and amino acid changes surrounding the mevalonate/Isoprenoid pathway of *S. cerevisiae*. Genes mutated in L2056-C7 and their associated amino acid changes are highlighted in yellow and discriminatory metabolites and their relative change in concentration are in red (decreased in L2056-C7 compared in L2056) or green (increased in L2056-C7).

The involvement of cellular amino acid processes directly links the changes of metabolism of this mutant to nitrogen metabolism. The importance of yeast assimilable nitrogen (YAN) in wine fermentation is well known, with deficiencies of such a common cause of fermentation faults. Amino acids are the main source of YAN in grape juice and their relative compositions are extremely variable (Henschke & Jiranek, 1993). Potentially deleterious SNPs found in *ASP1* and *SPE4* associates these genes.

ASP1 encodes the cytosolic L-asparaginase, involved in asparagine catabolism, it is constitutively expressed and responsible for the main flux of conversion of asparagine to aspartate and ammonia (Sinclair *et al.*, 1994). Another asparaginase does exists in yeast, Asp3p, of which there are 4 copies, it is activated in response to nitrogen starvation (Bon *et al.*, 1997). Asparagine however is only reported to be present at very low percentages worldwide, with a mean of less than 1% found across a range of Australian grape varieties (Henschke & Jiranek, 1993). Certainly since asparagine was not included in the selection medium for the evolution of L2056-C7 the loss of an asparaginase would appear not to have been a selective determinant, and in fact is most likely to have little effect. Asparagine is however rapidly transported and utilized by wine yeast (Jiranek *et al.*, 1995) and is described as a preferred nitrogen source (Cooper, 1982). Perhaps this is indicative that a modification in asparagine catabolism could influence central nitrogen catabolic pathways resulting in an increase in fermentation efficiency through an adjustment of central nitrogen metabolism. *ASP1* alleles have also recently been highlighted as important to industrial oenological fermentation since Marullo and colleagues (2007) identified *ASP1* as a

quantitative trait loci (QTL) responsible for variation in acetic acid production between two non commercial yeast strains directly isolated from grape must.

SPE4 encodes spermine synthase, required for the biosynthesis of spermine from spermidine (Hamasaki-Katagiri *et al.*, 1998). Spermine in yeast is a precursor for β-alanine (and subsequently pantothenic acid) intermediates of coenzyme A (CoA) biosynthesis, a cofactor required by a large number of metabolic enzymes. Spermine is ubiquitously produced as the final product of polyamine biosynthesis and is essential for growth when β-alanine and/or pantothenic acid are lacking from the medium (White *et al.*, 2001). Interestingly the metabolome analysis of L2056-C7 highlighted an increase in intracellular alanine which is strangely the reverse of that which is expected in an *SPE4* mutant, pending the relationship between alanine and β-alanine. This increase in alanine could alternatively be explained by the disruption of *AGX1* (R239G in L2056-C7, tolerated with a SIFT score of 0.1). *AGX1* encodes the alanine glyoxylate aminotransferase, for glycine production on non-fermentable carbon sources (Schlosser *et al.*, 2004). Perhaps the inactivity of *AGX1*, even in the presence of fermentation, leads to an accumulation of alanine.

As discussed above the methodologies for analysis of connections between genes and their products, as used here in the form of a GO analysis is extremely useful in this type of analysis. Unfortunately the current methods for discovery of connections with metabolites to each other and within biochemical pathways overlaid on the underlying enzymes involved are not as well advanced. We however used knowledge from the literature to link metabolites with GO terms and then looked for enrichment of those terms. The term Lipid Biosynthetic Process, which encompasses daughter terms such as Fatty Acid Biosynthesis, Isoprenoid Biosynthetic Process, Glycerophospholipid Biosynthetic Process, Steroid and Sterol Biosynthetic Process was highlighted with nine metabolites being linked. Notably whichever analysis is used to relate genomic and metabolomic data, commonalities between biochemical pathways have been uncovered.

Analysis of L2056-C7 by phenotype microarray

Phentoype microarrays comparing L2056-C7 and L2056 were performed by BIOLOG (Hayward, CA, USA). In brief this analysis can compare around 2000 phenotypes in a single experiment by use of cellular respiration as a universal reporter when exposed to different mediums (Bochner *et al.*, 2001). An actively respiring cell (for instance in a medium with a particular carbon source) reduces a tetrazolium dye, resulting in an easily measurable colorimetric reaction.

This analysis revealed that L2056-C7 was somewhat resistant to potassium chromate and was unable to utilise four compounds as sources of phosphate, these being the cyclic monophosphates, adenosine 2’3’ cyclic monophosphate, cytidine-2’3’ cyclic monophosphate and thymidine 3’5’ cyclic monophosphate as well as dithiophosphate.

Potassium chromate is used as a toxic analogue of sulphate, and as such resistance is due to a modification of the sulphate assimilation pathway. Mutations in genes such as the *SUL* family of sulphate transporters (Cherest *et al.*, 1997), or the *MET* (mainly *MET3*) family involved in the incorporation of sulphate in to methionine and cysteine (Breton & Surdin-Kerjan, 1977) have been shown, however the coding regions of these genes in L2056-C7 were identified as being the same as wildtype. As explained previously, it can not be ruled out that regulation of one or more of these genes is altered in L2056-C7, and analysis of non-coding regions will reveal this. There are many interacting factors known for both the *SUL* and *MET* genes, and although an exhaustive comparison of each of these has not yet been completed, 2 genes, *RAS2* and *APA1*, that are interacting factors of *MET3* are indeed altered in L2056-C7.

The availability of phosphate from the environment is a critical determinant for yeast growth. Environmental phosphate limitation leads to activation of the PHO response, where acquisition of phosphate from the environment is done so in a coordinated fashion. This includes transcriptional up-regulation of acid phosphatases which break down extracellular phosphate containing molecules, such as nucleotides. Of these acid phosphatases, the gene encoding Npp2p (described as a Nucleotide Pyrophosphatases/Phosphodiesterases (Kennedy *et al.*, 2005)), is mutated in L2056-C7. Furthermore this I474F amino acid change is predicted by SIFT to be deleterious with a score of 0. We expect this mutation is the cause of this strains auxotrophy for the above phosphate sources. Since it appears that Npp2p is only required for utilization of these specific phosphate sources, and that indeed many others are available in grape juices, it is not expected that this would cause a problem in industrial applications, however this phenotype certainly warrants further exploration.

Interestingly in humans, the NPP family regulate extracellular nucleotide metabolism and nucleotide-derived intercellular signaling. Intercellular signaling is also very important in yeast, including during sporulation (Jakubowski, 1986). For instance yeast cells are known to maintain high concentrations of extracellular purines and produce adenosine 5'tetraphosphate and adenosine 5'-pentaphosphate to mark ascospore formation during sporulation and intriguingly *NPP2* expression is strongly induced during sporulation (Chu *et al.*, 1998). Kennedy *et al.* (2005) infer that for these reasons *NPP2*, may be implicated in a novel intercellular signaling role by regulating extracellular nucleotide metabolism. This is further supported since *NPP2* deletions have been reported to have an increased resistance to hyperosmotic stress (1 M NaCl) and also in increased competitive fitness. These phenotypes certainly align well with the known fermentation phenotype of L2056-C7.

Conclusion:

Our approach to develop the use of metabolomics as a phenotyping tool for wine yeast involved many facets of experimental design, including choices such as medium, growth stage, extraction and analytical techniques. We found that both GC-MS and LC-MS analytical techniques were extremely sensitive, being affected by most parameters. This led us to choose, where possible, to include as many variables as was practical in each experiment and subsequently led us to successfully develop an analytical pipeline appropriate for the use of metabolomics in this context of wine yeast in oenological fermentation.

The use of a multi-platform approach to elucidate mechanisms behind the fermentation efficient phenotype of L2056-C7 has certainly proven fruitful. The major findings so far are stated below in brief. Metabolomic analysis at the end of fermentation revealed 35 putatively identified discriminatory metabolites of L2056-C7, of which some were from similar chemical and biological groups. Of these the mevalonate/isoprenoid pathway was shown to be significantly enriched for discriminatory metabolites. Whole genome sequencing of both L2056-C7 and L2056 revealed 200 single nucleotide polymorphisms which lead to amino acid changes. For the affected genes, enriched GO terms included Histone Binding (function) as well as the processes of DNA Packaging, Regulation of Biosynthetic Processes and Response to Stimuli. Phenotype array analysis found very few significant differences between L2056-C7 and its parent, but did include the inability to utilise four phosphate sources as well as resistance to potassium chromate.

Integration of each of these data sets has revealed a number of pathways that are likely to link the fermentation efficiency phenotype of L2056-C7 and those highlighted are more than worthy of further investigation. For example a number of SNPs and related discriminatory metabolites were found in the

mevalonate/isoprenoid pathway, as were in Fatty Acid/Phospholipid Biosynthesis. Extracellular Nucleotide Metabolism and involvement of Methionine/Cysteine Biosynthesis was also highlighted in phenotype microarrays with SNPs found in L2056-C7 that are the likely effectors.

We hypothesise that a complex combination of metabolic changes contribute to the phenotype of L2056-C7 during fermentation, but of particular interest are those that contribute to the ability to maintain fermentation in an ever increasing stressful environment. Based on these analyses this may involve alteration of lipids in the membrane, contributing to stress resistance, gene silencing (or an inability of such) through histone binding activities and DNA architecture, leading to continuation or slowing of the deactivation of fermentation, a change in cell signaling, perhaps due to the loss of Npp2p associated intercellular signaling or the change in production of signaling compounds, such as gibberellin. Further analysis will involve testing of key components highlighted from this study and identification of SNPs from the non-coding regions of L2056-C7. Also a comprehensive transcriptional profile of multiple time points during fermentation, employing RNA deep sequencing would be extremely useful to elucidate if there is differential regulation, RNA signaling, or even editing at the RNA level, as seen in higher eukaryotes (Mattick, 2010).

Ultimately these data will be useful in guiding strain construction, isolation or generation strategies. Specifically genes or processes deemed important to a given attribute of yeast will be targeted to develop superior trains. Alternatively, the conditions prevailing during wine making may be able to be modified in a determined manner to evoke a desired response in yeast in terms of fermentation kinetics or metabolite yields.

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Output I: New yeast/LAB strains constructed

14. 1-2 new strains with greater robustness and reliability (eg sugar and/or salt tolerance) constructed via methods and expertise derived from early part of project.

As detailed above, in previous progress reports and the application for project UA 11/01, much of this Output has been delayed given its dependence on the preceding parts of the project. The activities planned here will largely be undertaken in UA 11/01. Output I does warrant mention however that strain FM16 or its derivatives, whilst produced in UA 01/04, have been the subject of much research effort in the present project. Such strains are being explored further specifically because of interest in commercialising them and thereby making available to the wine industry. The data generated for these strains will help define the specific conditions under which they offer benefit to the winemaker. In addition, such data is forming part of a submission to secure IP protection over the strains.

Output J: Linkages with wineries and industry suppliers

15. Initiation of Phase 1 industry trials of yeast, bacteria and enzymes. Initiate commercial preparation.

The Jiranek group has ongoing working relationships with numerous industry suppliers and wineries. All have expressed an interest in extending existing collaborations to include evlaution of strains and enzymes derived from this project. These opportunities will be followed-up as part of UA 11/01.

Output K: Data from Phase 2 industry trials and commercialisation plan

16. Data (incl. sensory) for novel yeast, bacteria and enzyme treatments collected in '10 vintage. Wines set aside for medium-term (<2 yrs) storage trials of above.

17. Plan for commercialisation of beneficial strains or treatments developed.

Contributors:

Michelle Walker, Jennie Gardner, Joanna Sundstrom, Tommaso Liccioli, Assoc Prof Vladimir Jiranek and Charles "Chik" Brennan (Winemaker,UC Davis)

Methods:

Strains and growth conditions

Essentially all was performed as described in the Methods for Outputs A, F and H.

Laboratory scale micro-plate fermentations

Sacrificial micro-plate fermentations were performed essentially as described by Liccioli *et al.* (2011). Briefly, 600 µL YPD cultures were grown in deep well plates at 30°C overnight. Following this, 2 uL of each was used to inoculate 200 µL of CDGJM starter medium (100 g/L glucose:fructose, 450 mg/L of nitrogen as a complex mixture of ammonium and amino acids) in fresh deep well plates and grown again overnight. These plates were then diluted with 800 µL of fresh CDGJM and acted as the source plate to inoculate (10 µL) in nine replicates of fresh plates with 190 µL CDGJM. Plates were grown at 30°C. At each time point of interest during fermentation a replicate plate was placed at -20°C. Glucose and fructose was measured by enzymatic assay.

Industrial Scale fermentations - University of Adelaide

Small industrial-like red grape fermentations were performed in triplicate with 20 kg lots of randomly sorted handpicked grapes. Each lot was de-stemmed and crushed separately and then transferred immediately to 30 litre plastic drums, provided with a 7 mm hole for release of CO_2. Two different batches of Merlot grapes were used and the initial juice parameters are shown in Table K.1. Juices were ameliorated with tartaric acid (respectively 2.3 and 1 g/L), nitrogen as diammonium phosphate (200 and 40 mg/L at 50 and 80 hrs post inoculation, respectively) and potassium metabisulphite (50 mg/L to give 25 mg/L of molecular SO_2). Yeast was inoculated (from fresh YPD-Agar) into YPD broth and grown overnight at 30°C with agitation at 160 rpm on a shaker bench. Cells of each culture were inoculated into aliquots of pre-sterilized white grape (Chardonnay 2010) juice diluted 1:1 with sterile water to an inoculation density of 2.5×10^6 cells/mL and grown overnight at 20°C with agitation at 160 rpm on a shaker bench. Triplicate ferments were then inoculated at 5×10^6 cells/mL and fermentation conduced at room temperature (~25°C). Every 12 hours, caps were plunged for 1 minute and 1 mL samples were collected and stored at -20°C for later analyses (residual sugars, relevant organic acids and alcohols and colour measurements).

Table K.1: Key compositional features of 2010 Merlot grapes used to evaluate mutant fermentation performance. Grapes were harvested from the Waite Institute experimental vineyards (Adelaide, South Australia) or from Lobethal (South Australia).

	VALUES	
PARAMETER	Merlot "Waite"	Merlot "Lobethal"
Glucose + fructose (g/L)	250	265
pH	4.02	3.87
Total acidity (g/L)	3.3	5.0
Alpha Amino Nitrogen (mg/L)	96	93
Ammonia (mg/L)	34	22
Yeast Assimilable Nitrogen (mg/L)	124	111
ADDITIONS		
SO_2 (mg/L)	25	25
Tartaric acid (g/L)	2.3	1
Diammonium phosphate (mg/L)	200	40

Fermentations - University of California Davis

Small scale fermentations were conducted in triplicate at the UC Davis Winery, California using 2011 Cabernet Sauvignon must. Each fermentation tank (33.5 US gallons or ~127 L working volume) was equipped with sensors for on-line monitoring of temperature (°C) and sugar content (°Brix). Data was collected from fermentations at 96 timepoints per day. The resultant wines were bottled and stored at the Winery for later shipment to University of Adelaide for sensory and chemical analyses.

Microsatellite typing

This was undertaken essentially as described in Output F.

Evaluation of stability of clones of L2056-C7 through a 50-generation passage

10 mL of YPD was inoculated with a fresh streak of yeast (either L2056-C7 or L2056) grown on YPD-Agar and grown overnight with shaking at 28°C. Cell number was enumerated and fresh YPD was inoculated with 1 x 10^6 cells/mL. This was repeated seven times to total approximately 50 generations of cell growth. Cultures were then streak plated and 34 single isolates were selected at random.

Results and Discussion:

Molecular Typing of adaptively evolved strain L2056-C7

Microsatellite analysis of both mixed cultures and clonal cultures of evolved strains was performed at 10 loci (data not shown). There was no discernable difference between FM16, L2056-C7 and their original parent L2056, providing good evidence for direct inheritance but not a method to differentiate them.

Stability of the fermentation efficient phenotype of L2056-C7 through multiple generations

Since L2056-C7 is being developed for commercial release, it is important to check the stability of the genetic changes, or more specifically the desirable phenotype for which it was chosen. To examine this we passaged L2056-C7 (and its parent L2056) for 50 generations with no selection and tested 34 clonal isolates to see if they had retained the fermentation efficient phenotype. Additional to this we also

included, what we believe to be a heterozygous version of L2056-C7. Micro-fermentations were performed so as to examine many isolates in a single experiment. Nine timepoints throughout fermentation.

All of the passaged isolates were found to behave as their parents (an example of a single time point, 185 hrs is shown in Figure K.1), irrespective of being passaged through 50 cellular generations. Thus, we believe the fermentation efficient phenotype of L2056-C7 is suitably stable.

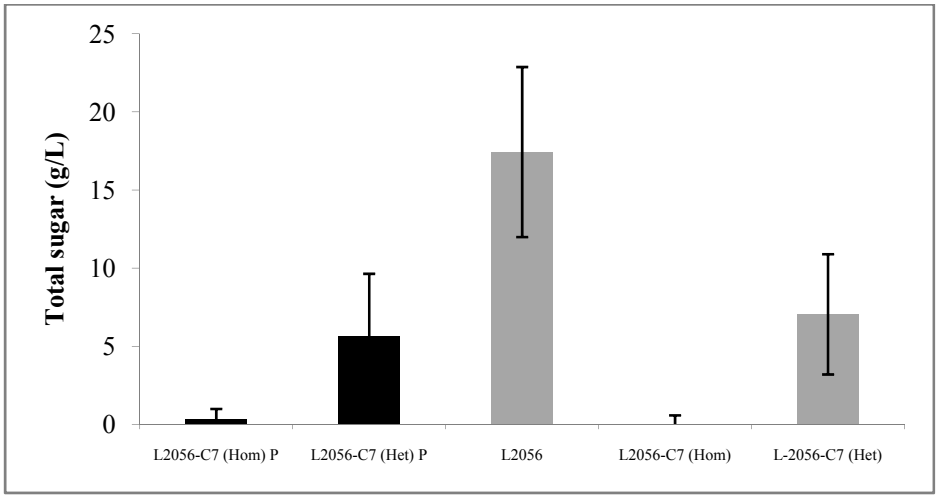


Figure K.1: The average total sugar (glucose + fructose) remaining in the medium after 185 hrs of fermentation by L2056-C7, homozygous or heterozygous version or L2056 (grey bars) or an average of 34 clonal isolates after being passaged for 50 cellular generations (black bars).

Small industrial scale fermentations utilising L2056-C7

The fermentation performance of L2056-C7 was evaluated in small industrial-scale grape juice fermentations from the 2010 vintage. A number of grape varieties were chosen, however due to the poor quality of some of the parcels, some failed to complete. However, in almost all cases, L2056-C7 performed either as well as, or better than the parent. As an example the fermentation profile of two separate parcels of Merlot (one from Waite and another from Lobethal) are shown in Figures K.2 and K.3. L2056-C7 finished fermentation in 1 or 2 days prior to L2056 (Waite Merlot: 157 hrs *vs* 180 hrs and Lobethal Merlot: 230 hrs *vs* 270 hrs). Major metabolites found in the final wine were very similar (Figures K.4-5). There was however a slight decrease in lactic acid from fermentations with L2056-C7 across both wines.

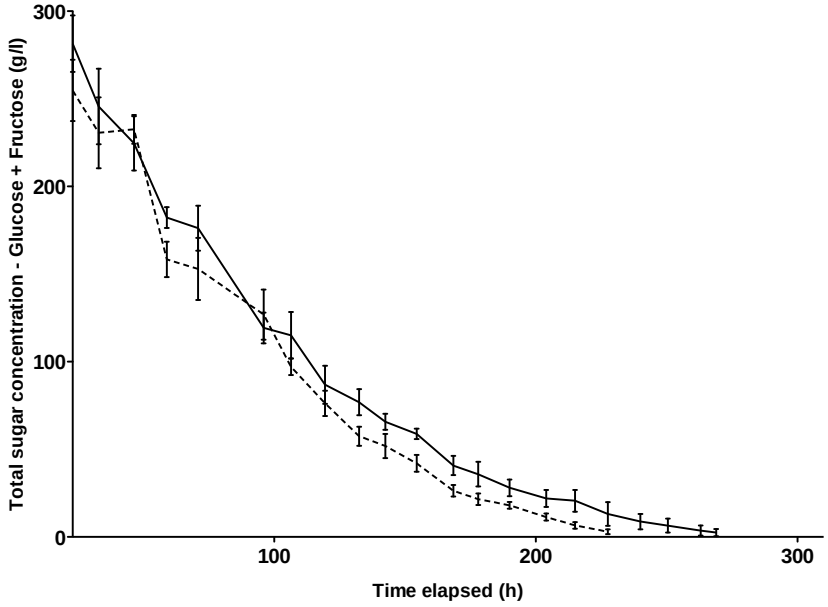


Figure K.2: Catabolism of sugars by L2056-C7 (dotted line) and L2056 (solid line) when grown in grape must (2010 Merlot from Lobethal) under industrial-like oenological conditions. Standard deviations are also included.

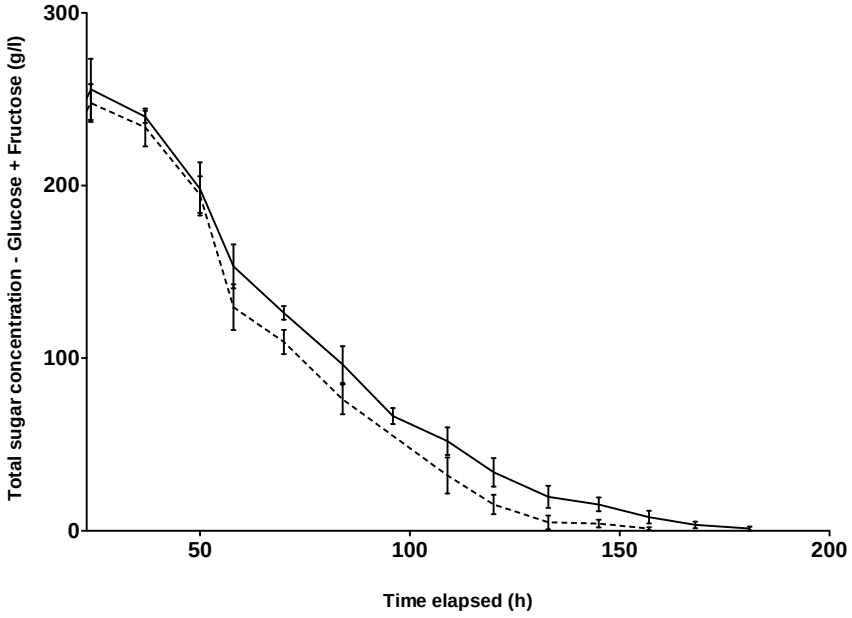


Figure K.3: Catabolism of sugars by L2056-C7 (dotted line) and L2056 (solid line) when grown in grape must (2010 Merlot from Waite) under industrial-like oenological conditions. Standard deviations are also include

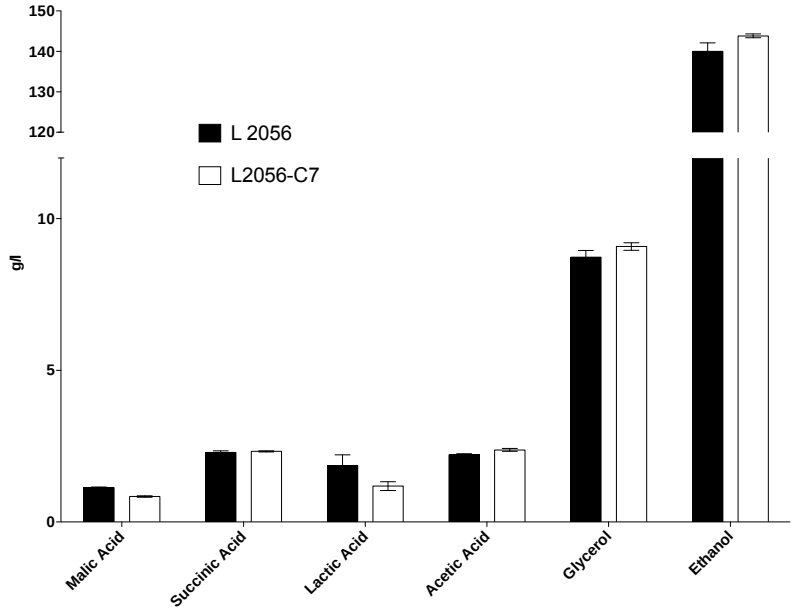


Figure K.4: Major metabolites, measured by HPLC of spent fermentation samples of 2010 Lobethal Merlot fermented by either L2056 (solid bars) or L2056-C7 (white bars). Standard deviations are also included.

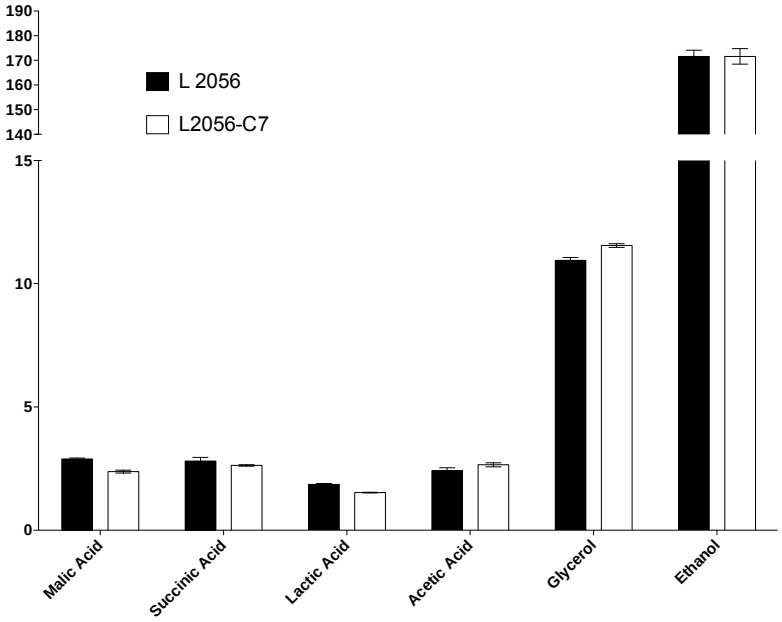


Figure K.5: Major metabolites, measured by HPLC of spent fermentation samples of 2010 Waite Merlot fermented by either L2056 (solid bars) or L2056-C7 (white bars). Standard deviations are also included.

Comparative fermentation trials were also conducted at the UC Davis winery by Charles Brenneman (resident winemaker) using 2011 Cabernet Sauvignon must. Fermentation kinetics of L2056-C7 was compared to L2056 and PDM. PDM being used here as our 'benchmark' strain, being regarded as the best commercial strain available in terms of fermentation vigour and performance. The initial juice parameters are still forthcoming and unfortunately not available at the time of the report. The evolved yeast strain, L2056-C7, performed similarly to the parental strain L2056 and was only 15 hours slower than PDM as shown in Figure K.6. Noteably the difference in fermentation times may be smaller given that the data is in °Brix and not residual sugar, whereby less than 2.5 g/L is considered dry. We are also in the process of

negotiating shipment of wines, and will use the bottled wine to define the metabolic and sensory impacts of the use of L2056-C7 on an American Cabernet must.

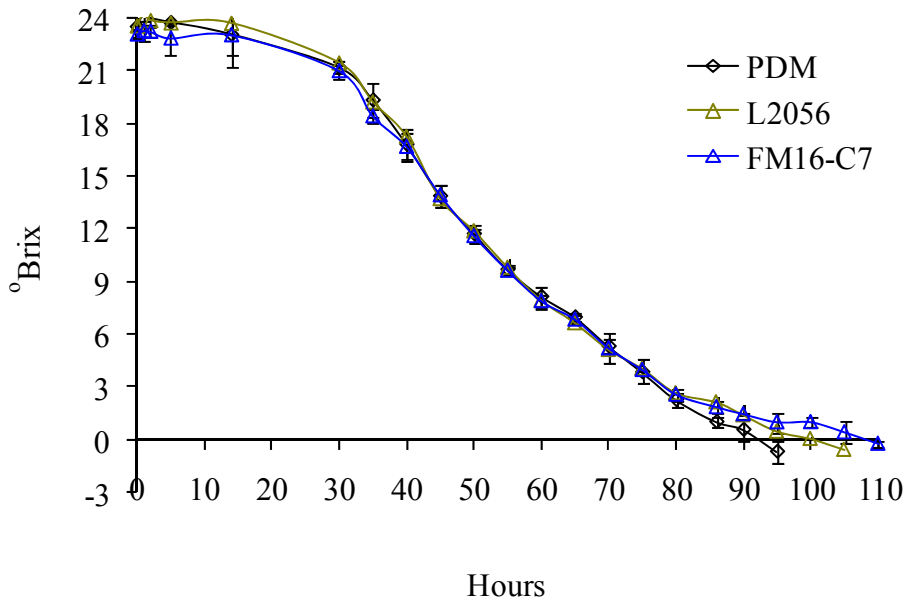


Figure K.6: Fermentation profiles of evolved yeast strain L2056-C7 with parental strain L2056, and PDM

Commercialisation plan

A key objective of this project was to deliver microbial tools which could be applied by winemakers to deal with fermentation problems and improve flavour complexity. Since the University of Adelaide and the Jiranek group are not in a position to directly market and distribute such tools to the industry, we are obliged to partner with an industry supplier. In securing such a partnership it is essential to develop a portfolio of information highlighting the attributes of the particular strain or derivative we wish to share. It is generally accepted that securing IP protection over these developments will also aid in this goal.

To that end much of the work in this project has been an attempt to define the precise genetic and physiological basis for the novel attributes of e.g. evolved yeast strains, determine the particular fermentation scenario they are most relevant for (e.g. high sugar, low nutrient juices, etc) demonstrate their benefits in at least a number of red and white juices.

This work is ongoing, but we have already begun to define the 'typical' behaviour of the evolved strains. Specifically they typically perform better than (i.e. ferment faster) or are equal to the lead commercial reference strains PDM or EC1118. We are taking the novel approach of seeking Plant Breeders Rights (covers plants and fungi – yeast is a micro fungi) to protect our strains, since PBR can be cheaper or easier to secure compared to a patent. To assist in the process, Dr Jiranek was formally registered with IP Australia in 2010 as a so-called "Qualified Person", who can aid in prepation of the necessary submission to IP Australia. In support of a PBR application we are collecting both fermentation data as well as distinguishing attributes, the latter not necessarily being related to fermentation, for example, colony and cell morphology. Sensory attributes of finished wines will also be important. For this reason obtaining the wines produced a UC Davis in early 2012 will be important.

Once PBR have been secured and the strain is licenced to a yeast manufacturer, it will ideally be produced as a active dried yeast preparation and sold to the industry.

Conclusion:

We have examined the fermentation dynamics of the adaptively evolved yeast strain, L2056-C7, in a number of different industrial scale fermentations, with grape must from different South Australian regions as well as one from Californina, and in each case it performed equally as well as its parent, or PDM (commonly regarded as a robust fermentation yeast) and in some juices the use of L2056-C7 actually shortened fermentation duration. Wines produced by L2056-C7 also had slightly lower lactic acid. It is also noteworthy to mention that this novel strain also performed well in the hands of winemakers other than those from this group.

It was also pleasing to find that the phenotype exhibited by this strain, under controlled conditions was also shown to be very stable. After 50 generations of growth on non-selective medium, the same reduction in fermentation was apparent. Genetic stability is central to commercialisation potential, considering the strain is required to be grown in large volumes prior to dehydration of the yeast for packaging.

We have accumulated extensive data regarding this strain's genetic background, the potential relationship between genotype and phenotype (fermentation traits) and metabolite profile of both the intracellular contents of the yeast and extracellular metabolites that would impact on the quality of a wine (see Output H). This data is in support of the application for the future commercialisation of L2056-C7.

Output L: Honours and PhD graduates and recruitment

18. 1-3 PhD candidates (currently part of UA01/04) to complete and submit their theses (2005-2006).

19. 1 Honours graduate from work undertaken in 2006-2007.

20. 1 Honours graduate from work undertaken in 2007-2008.

21. 1-2 PhD students recruited to work on aspects detailed within this project application (2005-2006).

22. 1-3 additional Hons/PhD students recruited to the project to support emerging areas (2008-2009).

The Jiranek group has recruited a number of PhD, Masters and Honours students, as well as a number of undergraduate students who completed projects over the summer vacation period, throughout the duration of project UA 05/01. These student recruitments and completions have seen completion of Output L 18-22 and are summarised below.

PhD Students

Name	Thesis Title	Year Completed	Output
Jennifer M. Gardner	Identification of genes affecting glucose catabolism in nitrogen-limited fermentation	2006	L 18
Colin McBryde	Development of novel wine yeast strains using adaptive evolution	2006	L 18
Angela H. Matthews	Biochemical and molecular characterisation of oenologically important enzymes identified in lactic acid bacteria	2007	L 18
Alana Capaldo	Genes and mechanisms responsible for the β-glucosidase metabolisim in the oenologically important lactic acid bacteria *Oenococcus Oeni*	2011 (submitted)	L 21
Name	**Thesis Title**	**Year Commenced**	**Output**
Krista Sumby	Identification and characterisation of esterases from wine associated lactic acid bacteria	2007	L 21
Jade Forrester	Characterisation of the wine meta-metabolome: Linking sensory attributes to yeast genotype	2010	L 22
Trung D. Nguyen	Generating robust yeast for successful fermentation in high sugar media	2010	L 22
Jin Zhang	Assimilable nitrogen catabolism by wine yeast	2010	L 22

Master of Oenology Students

Name	Thesis Title	Year Completed	Output
Scott Robinson^	The effect of sodium chloride on malolactic fermentation	2009	N/A
Leah Adint^	Limiting hydrogen sulfide production in wine yeast by knockout of sulfate transporters	2011	N/A

Honours Students

Name	Thesis Title	Year Completed	Output
Katherine Katalinic*	Genetic engineering of an *opi1* mutant of *Saccharomyces cerevisiae* to biosynthesise inositol and its derivatives	2005	N/A
Matthew Zadow*	Predicting fermentation outcome. What do measures of yeast assimilable nitrogen needs actually tell us?	2005	N/A
Alana Capaldo**	Characterisation of β-glucosidase gene from *Oenococcus oeni* for oenological use	2006	L 19
Robert Storey*	Use of a high throughput fermentation system to evaluate novel yeast strains	2007	L 19
Rauri Donkin*	Sodium chloride in Australian grape juice and its effect on fermentation	2008	L20
Paul Easton*	Identification of genes important to an oenological fermentation	2009	L22
Thomas Parker**	Identification of genes haploproficient for wine fermentation	2009	L22
Vic Xin Yang	Investigation of the role of Dmo1p, a novel cation transporter, in yeast fermentation	2010	N/A

*12 unit honours = One year, half-time research component and half-time coursework
**24 unit honours = One year, full-time research component

Summer Scholars

Name	Project Area	Year
Ruben Jacobs	Yeast gene deletion library replication	2006/2007
Damian DeSouza	Yeast gene deletion library replication	2006/2007
Christian Knott	Fermentation trials of adaptively evolved yeast strains	2007
May Yong	Yeast gene deletion library pilot screen	2008/2009
Phyllicia Toh	Yeast gene deletion library pilot screen	2008/2009
Nick Kalatzis	Yeast gene deletion library screening	2009/2010

Visiting Scholars/Staff

Name	Project Area	Year
Karen Block	Yeast overexpression library set up	2007/2008
Nga Lam	Yeast overexpression library plasmid amplification	2008/2009

Output M: Disseminated findings

23. 1-3 publications/seminar presentations detailing project findings (2005-2006).

24. 1-3 publications/seminar presentations detailing project findings (2006-2007).

25. 1-3 publications/seminar presentations detailing project findings (2007-2008).

26. 1 paper describing properties of initial AE strain with commentary on gross genetic differences in the strain (2008-2009).

27. Initial trials of above conducted during '09 vintage. Data disseminated via 1-3 papers, seminars or workshops (2009-2010).

28. Data from Phase 1 trials disseminated. 1-3 publications, seminar presentation or workshops. Benefits for industry promulgated (2010-2011).

29. Final report on project to date. 1-3 publications, seminars or workshops for researchers and winemakers (2010-2011).

The Jiranek group has disseminated project findings through a number of peer-reviewed journal articles, other forms of written media, invited seminar presentations and poster presentations and talks at a number of national and international conferences. These are summarised below and achieve Output M 23-25 and part of output M 29 (this report forming the final part of Output M 29).

Output M 26 was delayed up until now as the manuscript describing the properties of the initial AE strain L2056-C7 required a greater scope of experimental work and analysis to be suitable for publication. Having recently obtained the required metabolomic and genome sequence data, the manuscript is under revision for proposed submission in early 2012. Outputs M26-28 which related to data collection from several comparative fermentation trials were disseminated as part of this report and seminar presentations. The collective data (including that from work forming part of the continuation project UA 11/01) will be reported in industry journals/magazines and local seminars.

Refereed journal articles (Attached in Appendix 1)

Grimaldi A., Bartowsky E. & Jiranek V. (**2005**) A survey of glycosidase activities of commercial strains of *Oenococcus oeni.* International Journal of Food Microbiology **105**:233-244

Grimaldi A., Bartowsky E. & Jiranek V. (**2005**) Screening of *Lactobacillus* spp. and *Pediococcus* spp. for glycosidase activities that are important in oenology. Journal of Applied Microbiology **99**:1061-69

Gardner J. M., McBryde C., Vystavelova A., de Barros Lopes M. & Jiranek V. (**2005**) Identification of genes affecting glucose catabolism in nitrogen limited fermentation. FEMS Yeast Research **5**:791-800

Walker M. E., Vystavelova A., Pedler S., Eglinton J. & Jiranek V. (**2005**) PCR-based gene disruption and recombinatory marker excision to produce modified industrial *Saccharomyces cerevisiae* without added sequences. Journal of Microbiology Methods **63**:193-204

McBryde C., Gardner J. M., de Barros Lopes M. & Jiranek V. (**2006**) Generation of novel wine yeast strains by adaptive evolution. American Journal of Enology and Viticulture, **57**:423-430

Matthews A., Grbin P. R. & Jiranek V. (**2006**) A survey of lactic acid bacteria for enzymes of interest to oenology. Australian Journal of Grape and Wine Research **12**:235-244

Astorga A. M., Gardner J. M., McBryde C., Schmid F., Walker M. & Jiranek V. (**2007**) Optimisation of industrial strains of *Saccharomyces cerevisiae* using recombinant and non-recombinant methods. Microbiology Australia **25**:62-63

Matthews A., Grbin P. R. & Jiranek V. (**2007**) Biochemical characterisation of the esterase activities of wine lactic acid bacteria. Applied Microbiology and Biotechnology **77**:329-337

Poole K., Walker M. E., Warren T.,Gardner J. M., McBryde C., de Barros Lopes M. &. Jiranek V. (**2009**) Proline transport and stress tolerance of ammonia-insensitive mutants of *PUT4*-encoded proline-specific permease in yeast. Journal of General Applied Microbiology **55(6)**:427-39

Sumby K. M., Matthews A. H., Grbin P. R. & Jiranek V. (**2009**) Cloning and characterization of an intracellular esterase from the wine-associated lactic acid bacterium *Oenococcus oeni*. Applied and Environmental Microbiology **75(21)**: 6729–6735

Sumby K. M., Grbin P. R. & Jiranek V. (**2010**) Microbial modulation of aromatic esters in wine: Current knowledge and future prospects. Food Chemistry **121**:1-16

Capaldo A., Walker M. E., Ford C. M. & Jiranek V. (**2011a**) β-Glucoside metabolism in *Oenococcus oeni*: Cloning and characterisation of the phospho-β-glucosidase bglD. Food Chemistry **125**:476–482

Capaldo A., Walker M. E., Ford C. M. & Jiranek V. (**2011b**) β-Glucoside metabolism in *Oenococcus oeni*: Cloning and characterisation of the phospho-β-glucosidase CelD. Journal of Molecular Catalysis B: Enzymatic **69**:27–34

Other written articles

Gardner J. M., McBryde C., de Barros Lopes M. & Jiranek V. (**2005**) Recent efforts to optimise wine yeast and its fermentation performance. Australian and New Zealand Wine Industry Journal **20(6)**:43-45

McBryde C., Gardner J.M., de Barros Lopes M. & Jiranek V. (**2006**) Adaptive (directed) evolution yields superior, "ready-to-use" wine yeast strains. Australian and New Zealand Wine Industry Journal **22(2)**:44-48

V. Jiranek (**2007**) Adaptive evolution can produce hardy yeast. R&D at Work: GWRDC Newsletter, April 2007

McBryde C., Gardner J. M., de Barros Lopes M. & Jiranek V. (**2008**) Ready-to-Use wine yeasts: Generation of superior "ready-to-use" wine yeasts using adaptive (directed) evolution. Wine Business Monthly, April 2008

Conference and seminar presentations

2006

Jiranek, V. "Making yeast make more of nitrogen". *Invited oral presensentation at the International Wine Microbiology Conference, Yosemite, USA*

2007

Astorga, M. A., Gardner, J. M., Walker, M. E., de Barros Lopes, M. & Jiranek, V. "Screening of transposon mutation libraries in a wine yeast background to identify genes contributing to successful fermentation". *Poster presentation at the 23rd International Conference on Yeast Genetics and Molecular Biology*, *Melbourne, Australia*

Capaldo A., Walker M. E., Ford C. & Jiranek V. "Expression and characterisation of a β-glucosidase gene from *Oenococcus oeni* for oenological use". *Poster presentation at the 13th Australian Wine Industry Technical Conference, Adelaide, Australia*

Gardner, J. M., Astorga, A.M., Walker, M. E. & Jiranek, V. "Screening of a mutagenic transposon library in a wine yeast background to identify genes contributing to successful fermentation". *Poster presentation at the 23rd International Conference of Yeast Genetics and Molecular Biology, Melbourne, Australia*

Gardner, J., McBryde, C., Astorga, M., Walker, M. & Jiranek, V. "Tackling the impacts of climate change on winemaking through novel and improved wine yeasts". *Invited oral presentation discussion at the 8th International Symposium on Innovations in Enology*, *Stuttgart, Germany*

Jiranek V. "An update on the nutritional management of the primary fermentation". *Invited oral presentation at the 13th Australian Wine Industry Technical Conference, Adelaide, Australia*

Jiranek V. "Getting more out of winemaking microorganisms". *Invite oral presentation at the Australian Society for Microbiology Annual Conference*, *Adelaide, Australia*

Jiranek V. "Optimisation of winemaking strains: A range of approaches for a range of tastes". *An invited oral presentation at CSIRO, Plant Industry, Adelaide, Australia*

Lam N., Walker M. E. & Jiranek V. "Construction of wine yeast with increased tolerance to osmotic stress and their evaluation under wine fermentation". *Poster presentation at the 13th Australian Wine Industry Technical Conference, Adelaide, Australia*

Matthews A., Grbin P. R. & Jiranek V. "Esterases of *Oenococcus oeni*". *Poster presentation at the 13th Australian Wine Industry Technical Conference, Adelaide, Australia*

McBryde, C.M., Gardner, J.M. & Jiranek, V. "Using adaptive evolution to enhance wine yeast". *Poster presentation at the 23rd International Conference of Yeast Genetics and Molecular Biology, Melbourne, Australia*

McBryde, C.M., Gardner, J.M. & Jiranek, V. "Characterisation of adaptively evolved wine yeast under pilot conditions". *Poster presentation at the 23rd International Conference of Yeast Genetics and Molecular Biology, Melbourne, Australia*

Schmid F., Walker M. E. & Jiranek V. "Automated fermentation system for evaluation of large numbers of yeast strains in laboratory wine fermentations". *Poster presentation at the 23rd International Conference on Yeast Genetics and Molecular Biology, Melbourne, Australia*

Schmid F., Walker M. E. & Jiranek V. "An automated fermentation system for evaluation of large numbers of yeast strains in laboratory wine fermentations". *Poster presentation at the 13th Australian Wine Industries Technical Conference Adelaide, Australia*

Sumby K., Capaldo A., Walker M. E., Grbin P. R., Ford C. & Jiranek V. "Enzymatic activities of lactic acid bacteria: Distribution, cloning and characterisation of esterases and glycosidases". *Poster presentation at the 13th Australian Wine Industries Technical Conference Adelaide, Australia*

Walker M. E., Gardner J. M., McBryde C., Schmid F., Macintyre A., Bishop A. & Jiranek V. "Strategies for the generation of wine yeast strains with novel fermentation traits". *Poster presentation at the 23rd International Conference on Yeast Genetics and Molecular Biology, Melbourne, Australia*

Walker M. E., Gardner J. M., McBryde C., Schmid F., Macintyre A., Bishop A. & Jiranek V. "Strategies for the generation of wine yeast strains with novel fermentation traits". *Poster presentation at the 13th Australian Wine Industry Technical Conference, Adelaide, Australia*

2008

Jiranek V. "Yeast genomics: Medium and long term goals". *Invited oral presentation at the Wine 2030 Blue Sky 2008 Symposium: Research to Transform the Wine Industry, Adelaide, Australia*

2009

Gardner J. M., McBryde C., Walker M. E. & Jiranek V. "Progress towards characterisation of an adaptively evolved wine yeast strain". *Poster presentation at the 4th Australian Conference on Yeast: Products and Discovery, Adelaide, Australia*

Jiranek V. "Challenges of commercialisation (of microbial tools)". *Invited seminar at the Wine 2030 Blue Sky 2009 Symposium: Research to Transform the Wine Industry, Adelaide, Australia*

Jiranek V. "Characterisation and optimisation of wine microbes". *Invited seminar at the Department of Enology and Viticulture, UC-Davis, Davis, California, USA*

Jiranek V. "Inside the microbial 'black box' of winemaking". *Invited oral presentation at the Wine Innovation Cluster Research Day Symposium.*

McBryde C., Gardner J. M., Walker M. E. & Jiranek V. "Adaptive evolution strategies for the generation of novel wine yeast". *Poster presentation at the 24th International Conference on Yeast Genetics and Molecular Biology, Manchester, UK*

Walker M. E., Liccioli T., Warren T., Capaldo A., Schmid F. & Jiranek V. "The influence of wine yeast on colour extraction and stabilisation in red wine". *Poster presentation at the 24th International Conference on Yeast Genetics and Molecular Biology, Manchester, UK*

Walker M. E., Gardner J. M., Liccioli T., Schmid F., McBryde C. & Jiranek V. "Progress towards improvement of industrial yeast for the wine industry". *Oral presentation at the 4th Australian Conference on Yeast: Products and Discovery, Adelaide, Australia*

2010

Gardner J. M., McBryde C., Dunn W., Brown M., Oliver S. & Jiranek V. "Using metabolomics to probe the impact of an adaptively evolved wine yeast on oenological fermentation". *Poster presentation at the 14th Australian Wine Industry Technical Conference, Adelaide, Australia*

Jiranek V. "Development of new yeast strains for the wine industry". *Invited seminar at the Oregon Wine Industry Symposium, Eugene, Oregon, USA*

Jiranek, V. "Overview of the Jiranek group's research". *Invited seminar at the University of Rioja, Logrono, Spain*

Jiranek V. "Temperature distributions in fermentors during red winemaking: potential implications". *Invited oral presentation and panel discussion at the 9th International Symposium on Innovations in Enology*, *Stuttgart, Germany*

Jiranek V. "Wine microbiology for fermentation success and wine quality". *Invited seminar at the School of Biological Sciences, University of Auckland, Auckland, New Zealand*

Walker M.E., Liccioli T., Schmid F., Kalatzis N., Gardner J.M., Grbin P.R. & Jiranek, V. "The identification of genes with the potential to influence wine colour and fermentation duration". *Poster presentation at the 14th Australian Wine Industry Technical Conference, Adelaide, Australia*

Walker M.E., Liccioli T., Schmid F., Kalatzis N., Gardner J.M., Grbin P.R. & Jiranek V. "The identification of genes with the potential to influence wine colour and fermentation duration". *Poster presentation at the 14th Australian Wine Industry Technical Conference, Adelaide, Australia*

2011

Gardner J. M., McBryde C., Dunn W., Brown M., Oliver S. & Jiranek V. "Using metabolomics to probe the impact of an adaptively evolved wine yeast on oenological fermentation". *Poster presentation at the 7th International Conference of the Metabolomics Society, Cairns, Australia*

Gardner J. M., McBryde C., Dunn W., Brown M., Oliver S. & Jiranek V, "Using metabolomics to probe the impact of an adaptively evolved wine yeast on oenological fermentation". *Talk at Crush2011 - The Grape and Wine Science Symposium, Adelaide, Australia*

Jiranek V. "Superior wine microbes: Finding/making them, understanding them and making them better". *Invited seminar at the University of British Columbia, Okanagan, Canada*

Walker M. E., Liccioli T., Schmid F., Trung D. N., Kalatzis N., Sundstrom J. F. , Gardner J. M. & Jiranek V. "Identification and classification of *Saccharomyces cerevisiae* genes required for high-sugar fermentation using a genome-wide screening approach". *Talk at Crush2011 - The Grape and Wine Science Symposium, Adelaide, Australia*

Zhang J., Gardner J. M., Walker M. E., Sundstrom J. F., Grbin P. R. & Jiranek V. "Investigation of the genetic basis of high nitrogen efficiency (HNE) in wine yeast". *Talk at Crush2011 - The Grape and Wine Science Symposium, Adelaide, Australia*

Outcome/Conclusion

This project succeeded in providing greater insight into the role of microorganisms in the winemaking process. Specifically, we were able to generate superior strains of yeast (in terms of fermentation robustness) via a range of methods, and identify LAB and their enzymes which have potential as ways of modifying wine composition. Such yeast strains include those with increased nitrogen efficiency as well as general improvements in wine fermentation performance, whilst the LAB included strains with specific esterase and β-glucosidase activities. Enzyme preparations from LAB were also generated.

In addition to the above we have established a comprehensive set of methodologies and expertise for the generation and screening of candidate optimised yeast and bacteria, along with methodologies (bioinformatics, functional genomics, metabolomics, etc) to characterise such developments to a high degree.

The ambitious nature of this project combined with difficult vintages meant that some aspects were not advanced to the extent intended. However, on the whole, performance targets that were achieved were exceeded many-fold in terms of number of strains or enzymes examined.

There are undoubtedly significant potential benefits for the industry. We are in the process of developing a case to protect (in terms of IP) and commercialise some of the strains from this work. The same can be said of some of the LAB enzymes characterised herein. We would hope to see novel yeast strain from this work enter the marker in 2012/2013. At very least the winemaker could see immediate advantages from the selective use of LAB strains in the winemaking process.

Recommendations

Many of the recommendations that would have come from this project have already been taken up by virtue of the continuation of funding to support specific parts of this project though UA 11/01. That wine microbiology can influence wine processing and composition remains and undeniable fact. As such the industry would be well served to continue support of research in this area. We have developed a large number of promising strains, derivatives and leads. Not all will be accommodated within UA 11/01. As such we will seek to underwrite further work in this area by continuing to attract research students to work on relevant topics.

Appendix 1: Communication

Extensive communication and dissemination of findings from this research has been achieved. In addition to the written and oral presentations detailed in Output M, outlines of this work have been provided to wine industry groups visiting the Waite campus. In addition the work has been detailed as cutting-edge developments in wine microbiology through lectures to undergraduate and postgraduate oenology students as well as BSc (Biotechnology) students at the University of Adelaide.

The peer-reviewed publications largely arising from this work are attached here.

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A survey of glycosidase activities of commercial wine strains of *Oenococcus oeni*

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Abstract

Lactic acid bacteria play an important role in winemaking by undertaking the malolactic fermentation, yet little information is available on other aspects of their physiology, such as their profile of external enzymatic activities. In this study we sought evidence for the existence and action of glycosidase enzymes in wine isolates of *Oenococcus oeni*. This group of enzymes is of interest because of their potential for liberation of grape-derived aroma compounds from their natural glycosylated state. This comprehensive study reveals that these bacteria produce glycosidases that might be important in winemaking. Strains did not necessarily hydrolyse all substrates tested, but rather were grouped according to substrate specificity. Thus a subset comprising strains 2, 5 and 16 possessed high cumulative activities against β-D- and α-D-glucopyranoside substrates, while a group comprising strains 4, 21 and 22 was noted for superior hydrolysis of β-D-xylopyranoside, α-L-rhamnopyranoside and α-L-arabinofuranoside substrates. Key physico-chemical inhibitors of analogous systems from other microorganisms were seen to produce variable responses across the strains investigated here. Accordingly, several strains retained significant hydrolytic activity at typical wine pH values (~3.0–4.0), residual glucose and fructose contents (up to 20 g/L), and ethanol contents (up to 12%). These findings highlight the potential of *O. oeni* as a useful alternative source of glycosidase enzymes for use in winemaking.
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Keywords: Glycosidase; Lactic acid bacteria; Wine; MLF; Aroma; Flavour

1. Introduction

Many studies have been carried out to determine the impact of wine microorganisms on wine aroma and flavour (Antonelli et al., 1999; Kotseridis and Baumes, 2000; Lambrechts and Pretorius, 2000; Reynolds et al., 2001). Few of these works, however, have focused on Lactic Acid Bacteria (LAB) and in particular, *Oenococcus oeni*, the main bacterial species that conducts the malolactic fermentation (MLF) in wine. It is only recently that interest in this organism has shifted from the MLF to consider its impact on wine aroma, especially through elaboration of glycosidase enzymes. Many chemical compounds with the potential to contribute significantly to wine aroma, including monoterpenes, C_{13}-norisoprenoids, benzene derivatives and aliphatic alcohols, can occur in grapes in free form or as odourless non-volatile glycosides (Günata et al., 1988; Williams et al., 1982; Winterhalter and Skouroumounis, 1997). Included are aroma compounds occurring in either the α-L-arabinofuranoside, α-L-rhamnopyranoside, β-D-apiofuranoside, β-D-glucopyranoside, or β-D-xylopyranoside forms (Williams et al., 1982; Günata et al., 1985; Strauss et al., 1986; Voirin et al., 1990). These glycoconjugates can be either monoglucosides or disaccharide glycosides where the glucose bound to the aglycon is further substituted with an α-L-arabinofuranoside, α-L-rhamnopyranoside, β-D-xylopyranoside or β-apiofuranoside. The release of the aglycon from the disaccharide involves the sequential release of the sugar moities (Günata et al., 1988; D'Incecco et al., 2004). Specific glycosidases are now recognised as effective tools for the liberation of these aroma compounds (van Rensburg and Pretorius, 2000), with fewer of the negative impacts on wine structure and quality found with other treatments such as heating, acid hydrolysis or application of crude enzyme preparations (Francis et al., 1992; Spagna et al., 1998; Schneider et al., 2001).

Present knowledge about the glycosidases of *O. oeni* is modest compared to that of other industrially important LAB, particularly those applied in the manufacture of dairy products (De Vos and Gasson, 1989; Tzanetakis and Litopoulou-Tzanetakis, 1989; Antuna and Martinez-Anaya, 1993; Bianchi-Salvadori et al., 1995; Marasco et al., 1998, 2000). Most previous studies of the topic have examined the activity of these enzymes during the MLF of wine (Boido et al., 2002; Ugliano et al., 2003; Barbagallo et al., 2004; D'Incecco et al., 2004). In this study we have sought to define the nature and extent of glycosidase activity of 22 strains of *O. oeni* under optimised and reproducible conditions. As such, these organisms were grown in defined media, and presented with *p*-nitro-phenyl-linked glycosides of relevance to wine. The influence of critical wine parameters, such as the concentrations of residual sugars and ethanol, pH and temperature were investigated both individually and in combination to identify the most promising candidates for application to wine.

Abbreviations: *p*NP-βGlu, *p*-nitro-phenyl β-D-glucopyranoside; *p*NP-αGlu, *p*-nitro-phenyl α-D-glucopyranoside; *p*NP-βXyl, *p*-nitro-phenyl β-D-xylopyranoside; *p*NP-αRha, *p*-nitro-phenyl α-L-rhamnopyranoside; *p*NP-αAra, *p*-nitro-phenyl α-L-arabinofuranoside.

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2. Materials and methods

2.1. Bacterial strains and cultivation

The strains of *O. oeni* used in this study are listed in Table 1. Most were single colony isolates from commercial preparations (some formulated as mixtures of strains) of freeze-dried starter cultures used for the initiation of MLF during winemaking. Long term storage of the bacteria was achieved using vials containing treated beads in a cryopreservative fluid at −80 °C (Protect®, Technical Service Consultants Ltd.). Pre-cultures of each strain were prepared on an ongoing basis (each 2–4 days) by adding one bead into de Mann/Rogosa/Sharp broth medium supplemented with preservative free apple juice (20%),

Table 1
Strains and source of *Oenococcus oeni* used in this study[a]

Isolate	Origin
1	Lallemand O.S.U. (VI 77)
2	Lalvin 4× (VL 92)
3	Inobacter
4	Bitec Vino
5	Lalvin-Inobacter
6A	Lalvin MT01 Standard
7	Lallemand 3× (E218)
8	CHR Hansen Viniflora
9	CHR Hansen Viniflora
10	Lalvin EQ54 MBR
11	Lalvin MCW
12	Lalvin 3× 1Step
13	Lalvin IB Standard
14	Enoferm Alpha
16	Lalvin 3× Standard
17	Lalvin OSU MBR
20	Lallemand No. 3
21	Lallemand No. 4
22	Lallemand No. 5
23	Lallemand No. 8
28	Lallemand No. 2
29	Lallemand No. 9

[a] Strains 20–29 were derived from pre-commercial trails samples kindly provided by Lallemand.

adjusted to pH 5.0 (MRSA) (Kelly et al., 1989) and incubated at 25 °C.

Cultures used for the study of glycosidase activities were prepared by inoculating precultures into 5 mL of MRSA to an optical density measured at a wavelength of 600 nm (OD_{600}) of approximately 1 and incubating at 25 °C for 42 h (66 h for *O. oeni* strains 3 and 5). Cells were harvested and washed twice by centrifugation (20,000 × g for 5 min) with 0.85% w/v NaCl in distilled water. All strains were not necessarily analysed in a single experiment since occasionally one or more strains grow insufficiently.

2.2. Determination of glycosidase activity of culture biomass

Glycosidase activity was determined according to a method described previously (Grimaldi et al., 2000), with modifications to allow the use of a micro-plate spectrophotometer. Assays were reduced to a final volume of 80 µl and performed in standard 96-wells plates. For each reaction, 40 µl of 0.2 M McIlvane buffer (0.1 M citric acid and 0.2 M K_2HPO_4) was used (Dawson et al., 1986). When required McIlvane buffer was prepared to pH 4.0 and included the following at the final assay concentrations indicated: ethanol (0, 4, 8 and 12 v/v), glucose or fructose (0, 0.01, 0.1, 0.75 and 2% w/v). Each well was then dosed with 20 µl of a suspension of the appropriate *O. oeni* strain prepared in 0.85% NaCl and standardised to yield a final OD_{600} in the assay of 0.5. Substrate solutions (20 µl) were added to give the following final concentrations: *p*-nitrophenyl β-D-glucopyranoside (10 mM), *p*-nitro-phenyl α-D-glucopyranoside (10 mM), *p*-nitro-phenyl xylopyranoside (7.5 mM), *p*-nitro-phenyl α-L-rhamnopyranoside (7.5 mM), and *p*-nitro-phenyl α-L-arabinofuranoside (7.5 mM) (Sigma). Assays were incubated at 37 °C and enzymatic activity was stopped after 1 h by the addition of 160 µl of 0.5 M Na_2CO_3 and the 96-well plate centrifuged (2500 × *g* for 18 min) to remove the cells from the reaction. Supernatants (200 µl) were transferred into corresponding wells in a fresh 96-well plate and the absorbance of each at 400 nm determined with a multi-plate spectrophotometer (µQuant®, BIO-TEK Instruments Inc.) set to automatic path-length correction. Blanks were prepared without bacterial cells but otherwise treated in the same manner. All reactions were performed in at least duplicate with replicate values typically being within 5% of one another. Non-enzymic hydrolysis of *p*-nitro-phenyl α-L-arabinofuranoside under alkaline conditions occurred after the addition of the 0.5 M Na_2CO_3 solution, and resulted in a doubling of sample absorbance approximately every 20 min. A similar increase was observed, in both samples and blanks (data not shown) and thus samples were processed as quickly as possible. Accordingly, centrifugation was applied for only 12 min after which supernatants were immediately transferred and analysed in the multi-plate spectrophotometer. In all cases, one unit of activity was defined as mmols of *p*-nitro-phenol liberated per min per milligram of cell dry weight. Culture dry cell weight was determined from 10 mL cultures which had been grown for 42 h.

2.3. Temperature dependence of glycosidase activity

Where the influence of temperature on glycosidase activity was investigated the same method as described above was used with the exception that all volumes were increased by 25% to give a final assay volume of 100 µl. Assays were performed in 200 µl disposable PCR tubes thereby allowing the use of a PCR thermocycler (Mastercycler Gradient®, Eppendorf) for accurate temperature control. Tubes were incubated for 1 h at temperatures between 14.9 °C and 57.7 °C. At the end of the incubation period, 80 µl from each assay were transferred to a well of a 96-well plate to which had previously been added 160 µl of 0.5 M Na_2CO_3. The 96-well plate was then centrifuged as above to pellet cells and 200 µl of supernatant transferred to a fresh 96-well plate for quantitation of sample absorbance at 400 nm as described.

3. Results

With few exceptions, previous studies of glycosidases of wine LAB have been limited to investigations of β-glucosidase (β-D-glucopyranosidase) activity. Our earlier study (Grimaldi et al., 2000) appears to be the first to use other substrates to specifically identify enzymatic activities, albeit limited, beyond β-glucosidase. Regardless of the source

organism or the enzyme activities under investigation, an influence by parameters including pH, temperature and the presence of inhibitors such as sugars and ethanol has been a common observation (e.g. Aryan et al., 1987; Winterhalter and Skouroumounis, 1997; Grimaldi et al., 2000; Spagna et al., 2002; Barbagallo et al., 2004). In this study we sought to expand our earlier preliminary findings by more fully defining the nature and functional limitations of the glycosidases associated with up to 22 different *O. oeni* strains.

3.1. pH and substrate interactions

Strains of *O. oeni* were cultured in MRSA and evaluated for their ability to liberate nitrophenol from each of five glycosylated *p*-nitro-phenyl-substrates over a range of pH values (2.6 and 7.0). General observations from this survey (Fig. 1) include that *O. oeni* glycosidase activities were widely distributed, with most strains acting on several of the substrates tested. Also, in confirmation of previous findings, assay pH greatly altered the glycosidase activity for the majority of *O. oeni* strain and substrate combinations. This finding reinforces the necessity of examining these properties under a broad range of pH values in order to develop a fuller view of the potential of individual *O. oeni* strains.

3.1.1. β-D-Glucopyranosidase

All strains tested possessed a detectable β-glucosidase activity against *p*NP-βGlu. The magnitude of this activity ranged by up to 16-fold in a strain and pH dependent manner with strains 2, 5, 16, 21 and 22 typically showing highest activities, whilst strains 6A, 13, 14 and 28 were representative of the lower extreme (Fig. 1A). Across the 17 strains surveyed for this activity, it is evident that pH of the assay buffer markedly influenced β-glucosidase activity. Some strains in fact displayed two peaks of activity: one near pH 3.4 and a second at a pH>5.8, while in other strains maximal activity was centred around a single pH of 3.8.

3.1.2. α-D-Glucopyranosidase

As observed for β-D-glucopyranosidase activity, all isolates possessed an activity against *p*NP-αGlu (Fig. 1B). Values ranged by approximately 10-fold across the strains and pH values studied. The optimal pH for α-L-glucopyranosidase activity varied such that it occurred at around pH 3.8 for strain 22, pH 4.6 for strain 2 and pH 6.6 for strains 1, 5, 12, 16 and 17. Dual pH optima of the sort observed for β-D-glucopyranosidase activity in many strains, were atypical for α-D-glucopyranosidase activity, and were most obviously seen with strain 17 at pH 3.8 and 6.6.

3.1.3. β-D-Xylopyranosidase

Liberation of nitrophenol from *p*-nitro-phenyl β-D-xylopyranoside was most effective at lower pH values, with a peak in activity typically occurring at pH 3.0–3.4 (Fig. 1C). Maximum activities of approximately 0.9 units were seen for strain 21 and 22 at pH 3.0. Strain 16 demonstrated a duality of pH optima, being at 3.0 and 5.4.

3.1.4. α-L-Rhamnopyranosidase and α-L-arabinofuranosidase

The response of individual strains to the final pair of substrates studied were similar and thus only selected data are shown in Fig. 1D. Despite an oenological importance being placed on α-L-rhamnopyranosidase because of the frequent occurrence of rhamnose-linked aroma compounds in grapes (Bayonove et al., 1992; Razungles et al., 1993; Bureau et al., 1996), poor hydrolysis of the corresponding *p*-nitrophenyl substrate (*p*NP-αRha), with a maximum of only 0.2 units was observed. Again, lower pH values gave optimal hydrolysis of *p*NP-αRha, whereas trace or no activity was seen above pH values of 4.6–5.0. Given the scarcity of this activity any further discussion of it is limited. Hydrolysis of *p*-nitro-phenyl α-L-arabinopyranoside was readily evidenced by all but two of the 21 strains studied. Again, only the findings for a selection of strains is reported here (Fig. 1D). In several strains two pH optima seemed apparent: one at around 3.4–3.8 and a second above pH 6.6. Highest activities of the order of 0.4 units, as typified by strain 20, occurred near pH 3.4.

A ranking of strains according to their ability to hydrolyse a given substrate was made difficult by virtue of the fact that such rankings changed according to the pH at which activity was quantified. For this reason, 'total' glycosidase activity was determined for each strain by summing activity measurements made across all pH values for a given substrate (Table 2). In this way, the strains most active against a particular

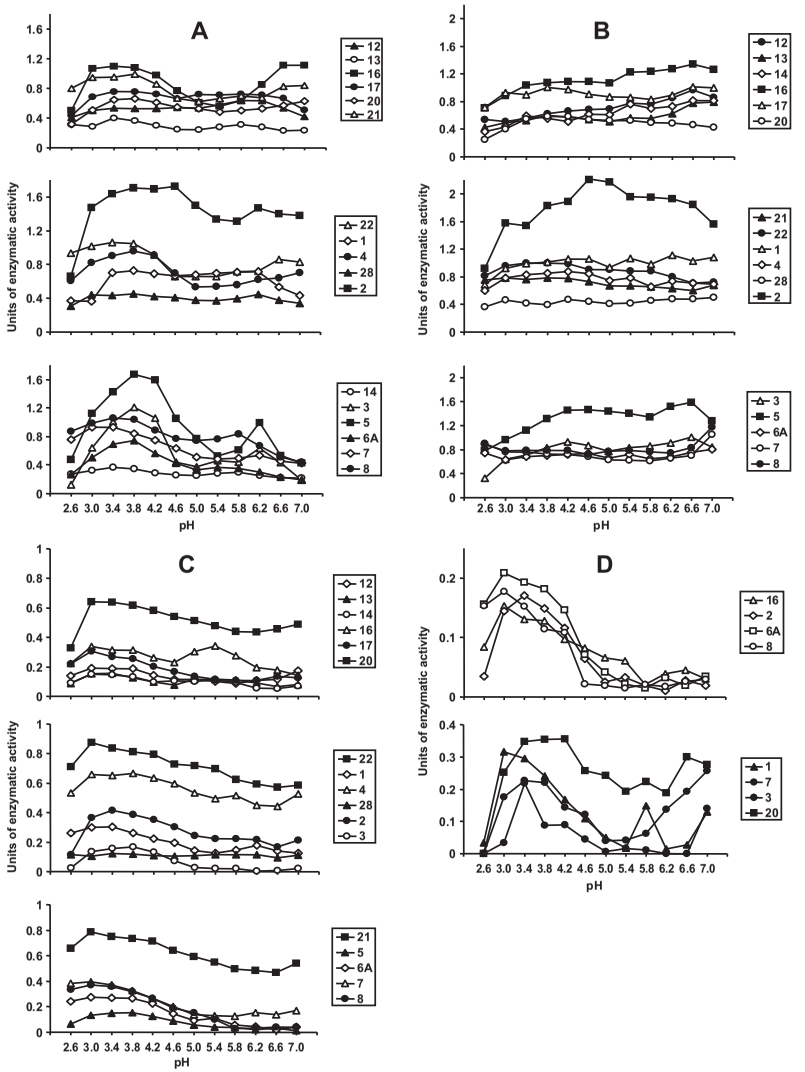


Fig. 1. Influence of pH on the glycosidase activity of *Oenococcus oeni* strains against *p*-nitro-phenyl β-D-glucopyranoside (A), *p*-nitro-phenyl α-D-glucopyranoside (B), *p*-nitro-phenyl β-D-xylopyranoside (C), *p*-nitro-phenyl α-L-rhamnopyranoside (D, open symbols) and *p*-nitro-phenyl α-L-arabinofuranoside (D, close symbols). Values are the mean of duplicate determinations.

Table 2
Total glycosidic activities for *Oenococcus oeni* strains against each of five substrates[a]

Strain	Substrate					Total[b]
	*p*NP-βGlu	*p*NP-αGlu	*p*NP-βXyl	*p*NP-αAra	*p*NP-αRha	
28	4.76	5.31	1.34	0.32	0.04	11.77
13	3.47	6.99	1.28	0.74	1.34	12.48
14	3.39	7.51	1.21	0.69	1.35	14.15
6A	4.96	8.48	1.78	1.12	0.80	17.14
12	6.34	8.50	1.66	0.46	1.16	18.12
3	7.00	9.54	0.79	0.57	0.65	18.55
7	7.74	8.84	2.80	0.91	1.62	21.91
20	6.51	5.84	6.15	0.80	2.99	21.96
1	7.28	11.92	2.40	0.77	1.55	23.22
17	8.04	10.89	2.16	0.86	1.64	23.59
8	9.57	9.84	2.23	0.85	1.28	23.77
4	8.51	9.12	6.70	1.05	1.67	27.05
21	9.52	8.48	7.41	1.10	1.66	28.17
16	10.35	13.27	3.11	0.94	0.63	28.30
5	11.19	15.66	0.91	0.57	ND[c]	28.33
22	10.06	10.57	8.55	1.15	1.65	31.98
2	17.29	21.37	3.23	0.81	0.96	43.66

[a] Values are a summation of activities determined at each of the examined pH values between 2.6 and 7.0 (see Fig. 1).
[b] Measures of total glycosidic activity are derived from the totals determined for each substrate.
[c] ND, not determined.

substrate could be identified while also revealing trends across the five substrates tested. Thus strains 16, 22, 2, 5, and 8 were amongst the most highly active group for both *p*NP-βGlu and *p*NP-αGlu, whereas strains 21, 22, and 4 were prominent amongst the remaining substrates. For practical reasons, all substrates were not applied at the same concentration in this survey and thus a summation of total activities for each strain across all substrates and pH values will not equate to an absolute measure of total glycosidic activity for that strain. Nevertheless, such summations provide a useful means of achieving an overall ranking of the strains studied. This cumulative value highlights *O. oeni* strains 2 and then 22, 5, 16 and 21 as possessing the greatest overall glycosidic activity (Table 2).

3.2. Temperature optima of glycosidase activities

The ability of observed glycosidase activities to operate over a broad range of temperatures was determined. While absolute values differed, general temperature trends were largely consistent for all strains. As such, only a selection of strains are reported here, and for clarity, typically those with highest activities (Fig. 2). In general terms, maximal hydrolysis of any of the substrates occurred at temperatures near 40 °C. Nitrophenol release was rapidly reduced as the temperature increased to 60 °C, while a more gradual decline was observed at temperatures below the optimum. Only low activities were observed at temperatures of 20 and 23 °C, which might be considered more relevant to winemaking conditions.

3.3. Influence of ethanol, glucose or fructose on glycosidase activity

Ethanol and sugars (glucose and fructose) have often been reported as the cause of inhibition of glycosidase enzymes (Aryan et al., 1987; Winterhalter and Skouroumounis, 1997; Sanchez-Torres et al., 1998; Grimaldi et al., 2000; Spagna et al., 2002; Barbagallo et al., 2004). The influence of these compounds was therefore investigated in this study. Examination of all 22 strains revealed a limited number of patterns of responses to the inclusion of either ethanol, glucose or fructose in the assay buffer. Accordingly, a representative selection only of the studied strains is reported.

Ethanol at 4% v/v resulted in a marked increase in β-D-glucopyranosidase activity by strains 2 and 4 (Fig. 3, A1), a trend repeated for 70% of all strains studied (data not shown). A reduced or no enhancement was seen for the remaining strains or at higher ethanol contents. In strains with low initial activities,

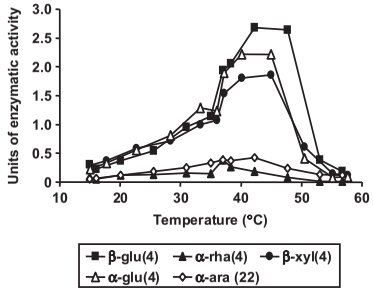


Fig. 2. Influence of temperature on glycosidic activity for a selection of *Oenococcus oeni* strains (in parentheses) and substrates. Values are the mean of duplicate determinations.

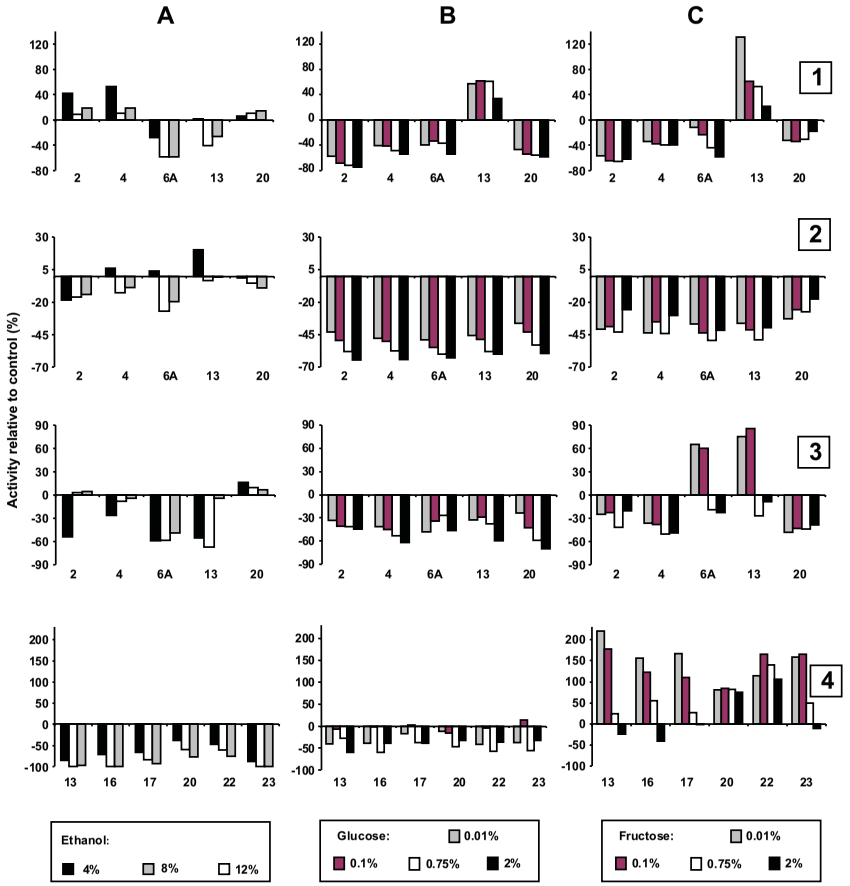


Fig. 3. Glycosidic activity of *Oenococcus oeni* strains observed in the presence of ethanol (column A), glucose (column B) or fructose (column C) against *p*-nitro-phenyl β-D-glucopyranoside (row 1), *p*-nitro-phenyl α-D-glucopyranoside (row 2), *p*-nitro-phenyl β-D-xylopyranoside (row 3) and *p*-nitro-phenyl α-L-arabinofuranoside (row 4). Values are relative (%) to the activity observed for the control performed under the same conditions in the absence of either ethanol or added sugars. Values are the mean of duplicate determinations.

as typified by 6A and 13, higher ethanol contents were typically inhibitory to β-glucopyranosidase. Inhibition of β-glucopyranosidase activity was also observed for glucose and fructose, even when present at only 0.01% w/v (Fig. 3, B1 and C1). Such reductions, typically of the order of 40%, were seen for approximately 80% of the 15 strains studied (data not shown). Interestingly, a 200-fold increase in the concentration of glucose or fructose did not greatly increase inhibition. Strain 13 stood out from others by displaying a marked enhancement of activity, particularly in the presence of fructose.

Ethanol enhancement was also observed for α-D-glucopyranosidase, again being most apparent at 4% v/v (Fig. 3, A2), albeit to a lesser degree and only occurring in 60% of all strains examined (data not shown). Glucose, still at only 0.01% w/v, was highly inhibitory to α-D-glucopyranosidase. Across all strains, glucose produced an average decline in activity of approximately 45% (Fig 3, B2). The response to fructose was lower (Fig 3, C2).

When considering β-D-xylopyranosidase activity, the impact of ethanol was modest and variable (Fig. 3, A3). At lower concentrations, glucose more so than fructose, produced a modest drop in nitrophenol liberation from *p*NP-Xyl (Fig. 3, B3 and C3). Determinations of the influence of ethanol, glucose and fructose on α-L-rhamnopyranosidase activity were performed, however, the minimal nature of this activity made some responses difficult to discern clearly. It is noteworthy however that several strains, that is 2, 7, 8, 9, 10 and 20, tended towards enhanced activity in the presence of concentrations of ethanol greater than 0.01%, as well as in the presence of sugars (data not shown).

Results for the α-L-arabinofuranoside substrate showed a strong inhibition by ethanol, so much so that at higher ethanol levels (8% and 12%) there was a total lack of enzymatic activity (Fig. 3, A4). Glucose was also inhibitory, though not as completely, particularly at lower concentrations. The most interesting result was obtained with the inclusion of fructose in the assay medium. Most strains exhibited a large increase in activity (>200% for strain 13) over that of the control when incubated with fructose (Fig. 3, C4). Only at the highest concentration (2%) was fructose inhibitory for some strains.

3.4. Influence of multiple parameters on glycosidase activity

In most settings outside the laboratory, bacteria are likely to face a combination of the factors investigated above. This is certainly true for winemaking. To study the interplay of these factors, glycosidase activity in representative strains was assayed at pH 3.5 with or without 10% v/v ethanol, 0.2% w/v glucose and/or 0.1% w/v fructose, for four substrates. Once again, *p*NP-αRha was omitted from this survey due to the low activity previously seen for this substrate. For most of the strains examined, the inclusion of potential inhibitors, ethanol and glucose had little affect on glycosidase activities against *p*NP-βGlu, *p*NP-αGlu and *p*NP-βXyl substrates (Fig. 4). Inhibition was most apparent for the action of strain 4 against *p*NP-βXyl. For several other strain and substrate combinations, an enhancement of activity was observed, particularly for strains 21 and 22 and *p*NP-βGlu. Given the stimula-

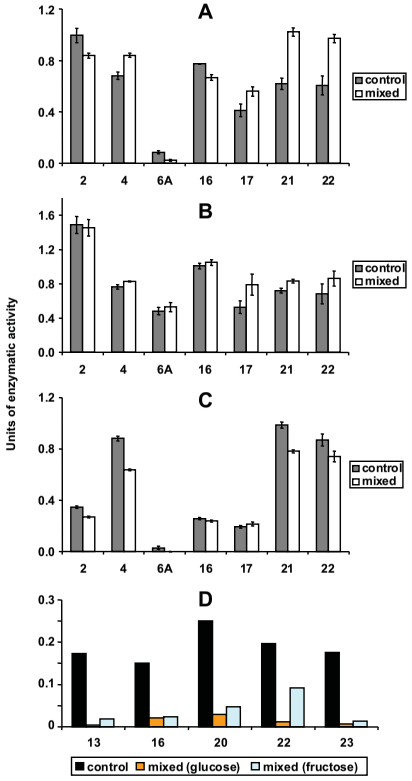


Fig. 4. Influence of ethanol and glucose (A–C) or ethanol and fructose (D) on the glycosidic activities of selected *Oenococcus oeni* strains against (A) *p*-nitro-phenyl β-D-glucopyranoside, (B) *p*-nitro-phenyl α-D-glucopyranoside, (C) *p*-nitro-phenyl β-D-xylopyranoside and (D) *p*-nitro-phenyl α-L-arabinofuranoside. Values are the mean of triplicate determinations.

tory affect of fructose on α-arabinofuranosidase activity (Fig. 3), an additional assay condition incorporating fructose instead of glucose was tested. For all five strains studied, the inclusion of ethanol and glucose or fructose was highly inhibitory on α-arabinofuranosidase activity (Fig. 4). Only in the case of strain 22 incubated with ethanol and fructose was there any clear suggestion that fructose was at least less inhibitory than glucose.

4. Discussion

Previous studies have either indirectly or specifically sought to demonstrate the presence of glycosidic, typically β-glucosidase (β-glucopyranosidase), activities amongst *O. oeni* associated with wine (McMahon et al., 1999; Grimaldi et al., 2000; Mansfield et al., 2002). To the best of our knowledge, the present work represents the most comprehensive investigation of this type in terms of numbers of strains and particularly, substrates and impacting parameters. This work has succeeded in demonstrating that the possession of glycosidic activities is both widespread amongst strains of *O. oeni* and is not limited to only β-glucosidase. We have also examined the influence of potentially inhibitory parameters such as pH, temperature and selected wine components (ethanol, glucose and fructose), both singularly and in combination on the activity of five glycosidases.

At the commencement of this study, we modified the methodology used in our preliminary report (Grimaldi et al., 2000) and made several improvements in order to accommodate the range of compositional parameters used and the variability in growth seen for the expanded strain collection under investigation. Specifically, a 0.5 M Na_2CO_3 solution was used prior to spectrophotometric quantitation of *p*-nitrophenol because of its greater ability to alkalise assay samples of low pH. Also, the addition of bacterial biomass was standardised to a final OD_{600} of 0.5 in the assay. With these modifications it has been possible to more precisely assess the influence of pH on glycosidic activities. In keeping with their anticipated appropriateness for use in the liberation of glycosides under wine conditions, most of the *O. oeni* strains studied had relatively high glycosidase activity at pH values between 3.0 and 4.0 (i.e. wine pH). The fact that this pH optimum does not correspond with the optimal pH for the growth of these organisms (~pH 4.2–4.8; van Vuuren and Dicks, 1993) argues against the influence of pH merely being a consequence of differential growth of the biomass during the assay period.

For the three glycosidases, β-D-glucopyranosidase, α-D-glucopyranosidase and β-D-xylopyranosidase, marked hydrolytic activity was retained above wine pH values, up to neutral, suggesting the possible use of these enzymes at such pH values. In contrast, hydrolysis of *p*NP-αRha and *p*NP-αAra quickly diminished with pH values above 4.0. This latter finding also explains our previous inability to detect significant α-L-rhamnopyranosidase activity in 13 *O. oeni* isolates, where measurements were conducted at pH 5.0 (Grimaldi et al., 2000). Also of note is the existence of two peaks of β-D-glucopyranosidase activity at quite distinct pH values (Fig. 1). This result might be indicative of the involvement of multiple enzymes, each with their own pH optimum. Alternatively, it is possible this study has actually revealed another example of that uncommon group of enzymes that have dual pH optima (e.g. Levin and Bodansky, 1966; Gee et al., 1988; Nagashima et al., 1999). Opposing the latter notion are reports of multiple β-D-glucopyranosidases in *Lactobacillus plantarum* (Marasco et al., 1998, 2000) and other lactic acid bacteria. A preliminary search of the *Oenococcus* genome sequence (Coutinho and Henrissat, 1999) also reveals the presence of multiple putative open reading frames with high homology to a consensus sequence derived from more fully characterised β-D-glucopyranosidases from other organisms (data not shown). Detailed characterisation of purified forms of the enzyme(s) in question will resolve this issue.

The finding that few *O. oeni* strains possessed consistently high activities for all substrates used (Table 2), reiterates the importance of strain selection when considering the application of *O. oeni* in the modification of the glycoside profile of wine. Such variability might also be considered indicative of the involvement of multiple enzymes with limited substrate specificity, rather than a single enzyme able to act on all the substrates tested. The presence of minimal α-L-rhamnopyranosidase and α-L-arabinofuranosidase activities in particular agrees with recent findings that *O. oeni* has low specificity for this important group of disaccharide aroma-related compounds in wine (D'Incecco et al., 2004). The industrial significance of these activities, which is nonetheless detectable in these strains, does still warrant further investigation.

In investigating the influence of specific physiochemical parameters, some light has been shed on the scope of the applicability of the enzymes examined in this study. Retention of some glycosidic activity at temperatures below 20 °C (Fig. 2) is of importance for the wine industry, given that wines are usually stored in this range during the MLF. The greatest activity was observed above 35 °C. Whilst this is outside the typical operating range for winemaking, it becomes more important for alternate processing of small batches of juice or wine or else other industrial applications (e.g. Mourgues and Bénard, 1982; Girard et al., 1997).

The enhancing effect of ethanol, occurring most often at lower concentrations (e.g. 4% v/v; Fig 3), mirrors previous findings for *O. oeni* (Grimaldi et al., 2000) and yeast biomass (Blondin et al., 1983; Gondé et al., 1985), though expression of a β-glucosidase gene from *L. plantarum* has been shown to be repressed by 12% (v/v) ethanol (Spano et al., 2005). Given the duration of the assay used in this study it is more likely that observed influences of ethanol related to existing enzyme rather than *de novo* synthesis. Here, ethanol partially inhibited glycosidase activities, with complete inhibition being most often seen for α-arabinofuranosidase activity. The retention of glycosidase activity in the presence of up to 10% ethanol supports the potential use of such enzymes in the processing of alcoholic beverages, such as wine and beer, with ethanol concentrations within this range. A characterisation of purified enzymes is necessary to determine the precise manner in which ethanol influences their activity.

Non-distilled alcoholic beverages often contain residual sugars, which in the case of wine are mainly glucose and fructose (Boulton et al., 1996). In keeping with numerous reports for glycosidase enzymes from various sources (Aryan et al., 1987; Cordonnier et al., 1989), the presence of such sugars was found to reduce activity (Fig 3). The key exception being a marked enhancement of some activities by fructose even when present at concentrations as high as 0.75% v/v, the specified maximum residual sugar content for dry table wines (Iland and Gago, 2002). As a general trend, although the threshold concentration for appearance of fructose inhibition was as at least as low as 0.01%, a 75-fold increase in fructose concentration did not produce a proportional increase in inhibition. This represents an important distinction between *O. oeni* and other sources of glycosidases wherein the degree of inhibition is much more closely linked to the concentration of sugar present (Aryan et al., 1987; Cordonnier et al., 1989).

In summary, in the single parameter experiments, ethanol, sugars (glucose and fructose) and pH, are able to impact upon the glycosidic ability of *O. oeni* cells in a manner that ranges between highly inhibitory to highly stimulatory. By additionally examining the influence of these parameters when applied in a multifactorial fashion, it has been possible to gain an insight into the potential of these activities under conditions more analogous to an industrial setting. Most notably, combination of these parameters generally resulted in a more moderate influence on detectable glycosidase activity, especially that against *p*NP-βGlu, *p*NP-αGlu and *p*NP-βXyl (Fig. 4). The ability to hydrolyse *p*NP-αAra (and *p*NP-αRha; data not shown) was again found to be weak and, in this multifactorial experiment, one sensitive to sugars. Thus, whereas fructose on its own was at times seen to be stimulatory, when supplied with ethanol at pH 3.5 a marked inhibition was apparent. This observation is a likely explanation for the inability of D'Incecco et al. (2004) to detect any glycosidase activity in wine (containing ethanol) other than β-glucosidase. There are no obvious links between the findings for the multifactorial experiments compared to those in which single parameters were studied. These observations serve only to highlight the complexity of the interactions involved and reiterates the importance of further work with purified glycoside enzymes. Nevertheless, several strains stand out as possessing high levels of activity against the various substrates studied. Consideration of such findings along with the relative sensitivity of each activity to the wine-related inhibitors studied allows for the selection of strains for further evaluation: either during MLF of wine or else upon incubation with purified grape glycosides. In addition, work is currently underway in this laboratory to purify the probably numerous glycosidase enzymes found in these bacteria, ahead of their more precise characterisation.

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Screening of *Lactobacillus* spp. and *Pediococcus* spp. for glycosidase activities that are important in oenology

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ABSTRACT

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Aims: To assess glycosidase activities from a range of *Lactobacillus* and *Pediococcus* species and characterize these activities under conditions pertinent to the wine industry.

Methods and Results: Lactic acid bacteria were cultured in MRS broth supplemented with apple juice before being harvested, washed and assayed for glycosidase activity using *p*-nitrophenol-linked substrates. All strains exhibited a detectable capacity for the hydrolysis of the β- and α-D-glucopyranosides. The magnitude of these activities and their response to the physico-chemical parameters investigated varied in a strain-dependent manner. The use of an assay buffer with a pH below 4 generally resulted in a reduced hydrolysis of both substrates while temperature optima ranged between 35 and 45°C. The effect of the inclusion of ethanol in the assay buffer (up to 12%, v/v) ranged from near complete inhibition to increases in activity approaching 80%. With the clear exception of a single strain, glucose and fructose (0·1–20 g l^{-1}) acted as inhibitors. An assessment of glycosidase activity during simultaneous exposure to glucose and ethanol at a pH of 3·5 suggested that ethanol decreased loss of activity under these wine-like conditions.

Conclusions: *Lactobacillus* spp. and *Pediococcus* spp. possess varying degrees of β- and α-D-glucopyranosidase activities, which in turn are influenced differently by exposure to ethanol and/or sugars, temperature and pH. Several strains appeared suited for further evaluation under winemaking conditions.

Significance and Impact of the Study: This work highlights the fact that strains of *Lactobacillus* and *Pediococcus* have the potential to influence the glycoside composition of wine. Tailoring of wine may therefore be possible through selective application of strains or enzymatic extracts thereof.

Keywords: aroma, flavour, glycosidase, lactic acid bacteria, malolactic fermentation, wine.

INTRODUCTION

Three genera of lactic acid bacteria (LAB) are commonly associated with the winemaking process: *Oenococcus*, *Pediococcus* and *Lactobacillus* (Wibowo *et al.* 1985; Fugelsang 1997). Of these, often only *Oenococcus* is able to survive in the later stages of the process when ethanol concentrations are high (Wibowo *et al.* 1985; Lonvaud-Funel 1999; Liu 2002), up to 15% (w/v) (Iland and Gago 2002). This makes *Oenococcus oeni* the main bacterium of the malolactic fermentation (MLF) in wine. By this process, L-malic acid, an astringent acid conferring a flavour of green apples (unripe fruit), is transformed to lactic acid, which, thanks to its monocarboxylic nature, imparts a more elegant and round taste to the wine. While some species of *Lactobacillus* are thought to strongly contribute to MLF (Sieiro *et al.* 1990), the main influence on wine by *Pediococcus* and *Lactobacillus* is likely to originate from their presence outside alcoholic fermentation. That is, in the earlier stages of a normal or delayed fermentation (prior to accumulation of high concentrations of ethanol) or in a spoilage context in wines with low sulfur dioxide and/or higher pH values (>3·5) (Costello *et al.* 1983; Fugelsang 1997). Given their natural association with the winemaking process and the fact that *O. oeni* growth performance in wines is at times poor, *Pediococcus* and particularly *Lactobacillus* are attracting greater interest as MLF starters (Pilatte and Prahl 1997).

Whereas *Lactobacillus* and *Pediococcus* have been extensively studied in the dairy industry where they play a pivotal role in determining the flavour and textural properties of several dairy products (e.g. see reviews by Torriani *et al.* 1994; Matthews *et al.* 2004), comparable data obtained under oenological conditions or for strains from wine are distinctly lacking. One mechanism by which these organisms may influence wine sensory properties is as a consequence of the production of glycosidase activities, which can act on compounds with potential sensory significance. Grape aglycones include monoterpenes, C_{13}-norisoprenoids, benzene derivatives and aliphatic alcohols, which can occur in free form or as odourless nonvolatile glycosides (Williams *et al.* 1982; Günata *et al.* 1988; Winterhalter and Skouroumounis 1997). Release of these bound volatile aglycones is either by acid hydrolysis, which usually occurs slowly during wine storage, or by the action of glycosidase enzymes. Typically, the target glycosides are β-D-glucose and diglycoside conjugates, with the latter comprising glucose and a second sugar unit being α-L-arabinofuranose, α-L-rhamnopyranose or β-D-apiofuranose. Volatile aglycones present as monoglucosides are liberated via β-D-glucosidase (glucopyranosidase), whereas, diglycoside-bound algycones are liberated through a sequential release by α-L-arabinofuranosidase, α-L-rhamnopyranosidase or β-D-apiofuranosidase followed by β-D-glucosidase (Günata *et al.* 1988; D'Incecco *et al.* 2004). α-D-glucosidases have been suggested to be of importance in the degradation of yeast-derived macromolecules thereby providing nutrients for the growth of LAB (Guilloux-Benatier *et al.* 1993).

Numerous studies have investigated the glycosidase activity of *O. oeni* strains demonstrating a range of capabilities (McMahon *et al.* 1999; Grimaldi *et al.* 2000, 2005; Boido *et al.* 2002; Mansfield *et al.* 2002; Ugliano *et al.* 2003; D'Incecco *et al.* 2004); however, there has been minimal characterization of glycosidases of *Lactobacillus* and *Pediococcus*. In this paper we specifically evaluate such activities. These findings have implications for the selection and, ultimately, the application of these organisms in winemaking to effect predictable alterations in the aroma properties of wine.

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MATERIALS AND METHODS

Bacterial strains and cultivation

The strains of *Lactobacillus* and *Pediococcus* used in this study are listed in Table 1. Most were single-colony isolates from commercial preparations of freeze-dried starter cultures (containing *O. oeni* strains) used in initiation of the MLF in winemaking. *Pediococcus* strain Ped18 and *Lactobacillus* strain Lac19 were isolated from commercial olive products and identified to the genus level (as were all other unidentified isolates) using physiological test strips (API 50 CHL, bioMérieux, Marcy l'Etoile, France). Long-term storage of all strains was achieved using vials containing treated beads in a cryopreservative fluid at −80°C (Protect®, Technical Service Consultants Ltd, Heywood, UK). Fresh cultures of each strain were prepared as required by adding one bead into MRS broth medium supplemented with preservative free apple juice (20%) and adjusted to pH 5·0 (MRSA) (Kelly *et al.* 1989) and incubated at 25°C for 2–4 days. Cultures used for the study of glycosidase activities were prepared by inoculating 5 ml of MRSA to an optical density measured at a wavelength of 600 nm (OD_{600}) of approx. 0·5 for *Pediococcus* and 0·3–0·4 for *Lactobacillus*, and incubating at 25°C for 42 h.

Determination of glycosidase activity

Culture biomass was assessed for type and degree of glycosidase activity as described in previous work (Grimaldi *et al.* 2005) with some modifications. Briefly, cells were harvested and washed twice by centrifugation (20 000 *g*,

Table 1 Bacterial strains used in this study

Strain	Genus/species	Origin
Lac6A	*Lactobacillus*	Lalvin MT01 Standard
Lac12	*Lactobacillus*	Lalvin 3X 1 Step
Lac13	*Lactobacillus*	Lalvin IB Standard
Lac15	*Lactobacillus*	Lavin 31 MBR
Lac16	*Lactobacillus*	Lalvin 3X Standard
Lac24	*Lactobacillus*	Experimental starter culture*
Lac25	*Lactobacillus*	Experimental starter culture*
Lac19	*Lactobacillus*	Commercial olive product
Lac26	*Lactobacillus plantarum*	Experimental starter culture*
Ped3	*Pediococcus*	Inobacter
Ped10	*Pediococcus*	Lalvin EQ54 MBR
Ped11	*Pediococcus*	Lavin MCW
Ped15	*Pediococcus*	Lalvin 31 MBR
Ped16	*Pediococcus*	Lalvin 3X Standard
Ped17	*Pediococcus*	Lalvin OSU MBR
Ped24	*Pediococcus*	Experimental starter culture*
Ped27	*Pediococcus*	Experimental starter culture*
Ped18	*Pediococcus*	Commercial olive product

*Strains derived from precommercial trails samples kindly provided by Lallemand, Montpellier, France.

5 min) with 145 mmol l^{-1} NaCl in distilled water. Assays (80 μl) were performed in flat-bottomed 96-well plates and comprised 40 μl of 0·2 mol l^{-1} McIlvane buffer (0·1 mol l^{-1} citric acid and 0·2 mol l^{-1} K_2HPO_4, pH 5·0, unless otherwise stated) and 20 μl of a suspension of the appropriate bacterial strain prepared in 145 mmol l^{-1} NaCl and standardized to yield a final OD_{600} in the assay of 0·5. Substrate solutions (20 μl) were then added to give a final concentration of 10 mmol l^{-1} for either *p*-nitrophenyl-β-D-glucopyranoside (*p*NP-βGlu) and *p*-nitrophenyl-α-D-glucopyranoside (*p*NP-αGlu) (Sigma). Assays were incubated at 37°C (unless otherwise stated) for 1 h, stopped by the addition of 160 μl of 0·5 mol l^{-1} Na_2CO_3 and clarified (96-well plate rotor, 2500 *g*, 18 min). Supernatants (200 μl) were transferred to a fresh 96-well plate and the absorbance (400 nm) determined using a micro-plate spectrophotometer (μQuant®, Bio-Tek Instruments Inc., Winooski, VT, USA) set to automatic path-length correction. Blanks were prepared without bacterial cells but otherwise treated in the same manner. All reactions were performed in at least duplicate or, for the combined parameter test, in triplicate. Values derived from replicates were within 6% of one another.

Influence of inhibitors

The influence of assay buffer pH was examined by preparing McIlvane buffer at pH values between 2·6 and 7·0. In separate trials the influence of the inclusion of ethanol (4, 8 and 12%, v/v) or glucose or fructose (0. 1, 1, 7·5 and 20 g l^{-1}) was also examined.

Influence of temperature on glycosidase activity

Glycosidase activities were determined as above with the exception that all volumes were increased by 25% to give a final assay volume of 100 μl. Assays were performed in 200 μl disposable PCR tubes thereby allowing the use of a PCR thermocycler (Mastercycler Gradient®, Eppendorf, Hamburg, Germany) for accurate temperature control. Tubes were incubated for 1 h at temperatures between 14·9 and 57·7°C. At the end of the incubation period, 80 μl was transferred from each assay to a 96-well plate to which had been added 160 μl of 0·5 mol l^{-1} Na_2CO_3. The plate was then centrifuged as above to pellet cells and 200 μl of supernatant transferred to a fresh 96-well plate for quantitation of sample absorbance at 400 nm as described. Controls without cells were treated in the same way as samples and included in the same 96-well plate. A unit of activity was defined as mmols of *p*-nitrophenol liberated per minute per milligram of cell dry weight.

RESULTS

Lactobacillus and *Pediococcus* strains were tested with all the *p*-nitrophenyl forms of the key glycosides of importance in winemaking. Unlike earlier work which showed detectable and variable activity by *O. oeni* against five substrates (Grimaldi *et al.* 2000, 2005), preliminary work showed *Lactobacillus* spp. and *Pediococcus* spp. only acted against β- and α-D-glucopyranosides (data not shown). Accordingly, only these substrates were included in the more detailed investigation described herein. In the following sections we present results concerning β- and α-D-glucopyranosidase activities and the manner in which they are influenced by various wine parameters.

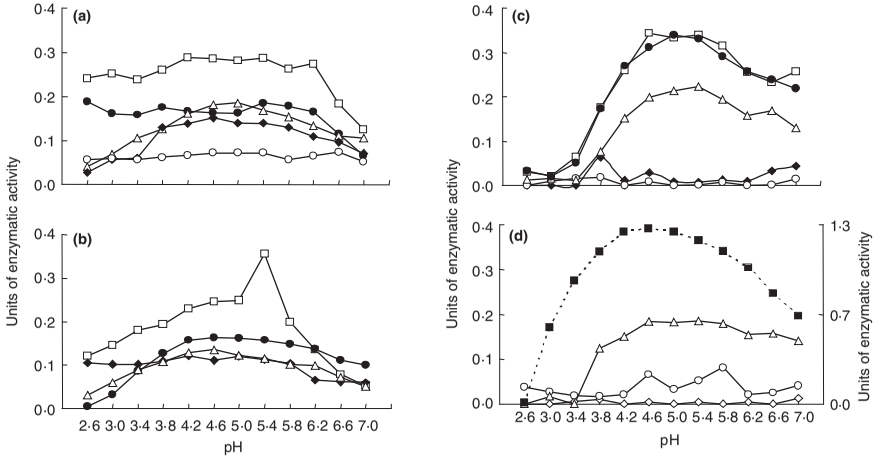


Fig. 1 Influence of pH on the enzymatic activities of *Lactobacillus* spp. (panel a: □ Lac19, ● Lac16, △ Lac12; ◆ Lac6A, ○ Lac25; panel b: □ Lac24, ● Lac26, ◆ Lac15, △ Lac13) and *Pediococcus* spp. (panel c: ◆ Ped3, ○ Ped17, △ Ped10, □ Ped15, ● Ped27; panel d: ◇ Ped24, ○ Ped11, △ Ped16, ■; Ped18) against *p*NP-βGlu. Values are the mean values of duplicate determinations. NB: values for Ped18 are plotted against the right-hand axis

Effect of pH

The effect of pH on the hydrolysis of β-D-glucopyranoside is shown in Fig. 1. For both *Lactobacillus* spp. (Fig. 1a,b) and *Pediococcus* spp. (Fig. 1c,d) maximum activities ranged between 0·1 and 0·4 units, with only strain Ped18 showing a higher activity (1·2 units). Several strains (Ped17, Ped3, Ped11, Ped24 and Lac25) possessed either low activity or else appeared unable to hydrolyse *p*NP-βGlu under the conditions used. In the case of *Pediococcus* strains with marked activity showed a conventional bell curve response to pH whereas, for most *Lactobacillus* strains, activities were less influenced by pH. Importantly, at wine pH values (*c.* pH 3–4) *Lactobacillus* spp. retained most of its activity, whereas *Pediococcus* spp. most typically did not effect marked hydrolysis until above pH 3·4–3·8. Again, the activity of strain Ped18 stood out, in this case being able to act below this pH range. All *Lactobacillus* strains acted against *p*NP-αGlu as substrate (Fig. 2a,b), but strain Ped18 was the only *Pediococcus* spp. able to do so (Fig. 2c). The range of maximum activities was similar to that seen for *p*NP-βGlu with strong activity being retained at wine pH values.

Effect of ethanol

Ethanol can occur in wine at concentrations above 15% (v/v) and has been repeatedly shown to influence glycosidase activities both positively and negatively in a highly strain- and concentration-dependent manner (Williams 1993; Grimaldi *et al.* 2000, 2005). Spano *et al.* (2005) also showed a β-glucosidase gene from *Lactobacillus plantarum* to be repressed by 12% (v/v) ethanol. When ethanol was present in the assay medium used in this study, responses were again highly strain dependent (Fig. 3). Nevertheless, β-D-glucopyranosidase activities were typically enhanced amongst *Lactobacillus* spp. (Fig. 3a), whereas most often for *Pediococcus* spp., little change was seen (Fig. 3c). The greatest enhancement of β-D-glucopyranosidase activity occurred at 4 or 8% (v/v) ethanol. Strains Lac15 and Lac16 showed increases of 50% or more at 4% ethanol. For all *Lactobacillus* strains except Lac12, lowest activities were seen at 12% ethanol (v/v). Hydrolysis of *p*NP-βGlu was strongly inhibited for *Pediococcus* strain Ped3 at all ethanol concentrations. When considering α-D-glucopyranosidase in *Lactobacillus* spp., the response tended towards an enhanced activity with increasing amounts of ethanol (Fig. 3b), and only strain Lac12 showing marked inhibition at all ethanol concentrations. The response of *Pediococcus* strains was opposite: all but strain Ped18 being strongly inhibited (Fig. 3d).

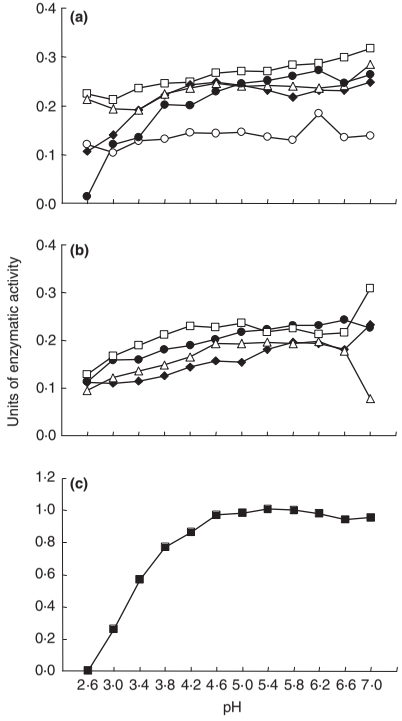


Fig. 2 Influence of pH on the enzymatic activities of *Lactobacillus* spp. (panel a: □ Lac6A, ◆ Lac12, △ Lac13, ○ Lac25, ● Lac26; panel b: ● Lac15, ◆ Lac16, □ Lac19, △ Lac24) and *Pediococcus* spp. (panel c: ■ Ped18) against *p*NP-αGlu. Values are the mean values of duplicate determinations. NB: a different scale is used in panel c

Effect of glucose and fructose

The natural grape sugars glucose and fructose have been shown to be inhibitory to glycosidase enzymes even at the residual concentrations found in wines (Williams 1993;

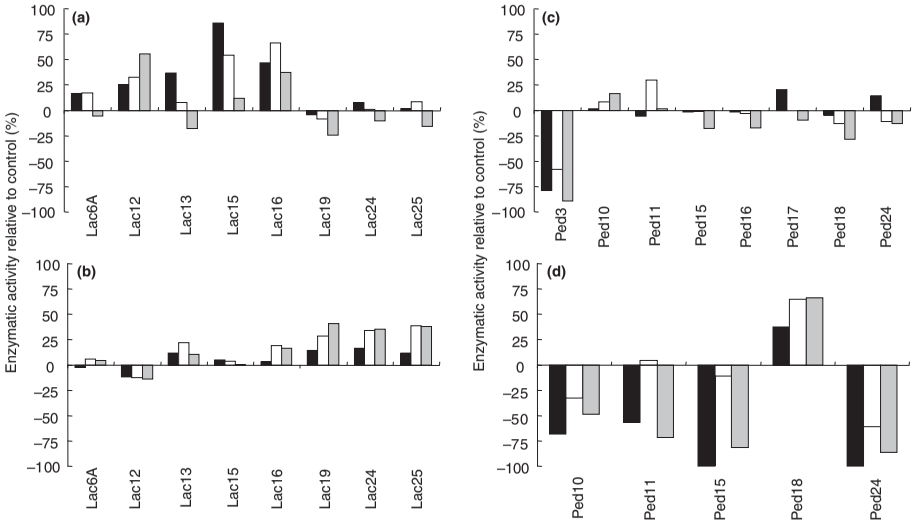


Fig. 3 Influence of ethanol on the enzymatic activities of *Lactobacillus* spp. against *p*NP-*β*Glu (a) or *p*NP-*α*Glu (b) and *Pediococcus* spp. against *p*NP-*β*Glu (c) or *p*NP-*α*Glu (d). Values are shown relative (%) to the activity observed for the control performed under the same conditions in the absence of ethanol and are the mean values of duplicate determinations. Ethanol: ■ 4% v/v; □ 8% v/v; ■ 12% v/v

Winterhalter and Skouroumounis 1997; Grimaldi *et al.* 2000, 2005). Inclusion of glucose at one of the four concentrations examined in this study (0·1–20 g l^{-1}) produced a similar pattern of inhibition for both *β*-D-glucopyranosidase and *α*-D-glucopyranosidase activities in *Lactobacillus* spp. (Fig. 4a,b). While glucose inhibition increased with sugar concentration, the response was not linear and strong inhibition was apparent even at 0·01% glucose. In *Pediococcus* strains Ped10 and Ped15, *β*-D-glucopyranosidase was completely inhibited at all glucose concentrations (Fig. 4c). In strain Ped24 inhibition was minimal while in strain Ped17 the response was one of enhancement with 1 g l^{-1} glucose mediating a 125% increase of activity against *p*NP-*β*Glu compared with the glucose-free control (Fig. 4c).

The typical response to fructose (Fig. 5) was also an inhibition and in fact was similar to that seen in the presence of glucose. Again *Pediococcus* strain Ped17 defied the trend by possessing a *β*-D-glucopyranosidase that was enhanced in the presence of fructose.

Effect of temperature

Given that much of the winemaking process occurs at temperatures between approx. 10 and 30°C, the impact of incubation temperature on the glycosidic activities of a selection of the *Lactobacillus* and *Pediococcus* strains under investigation was examined. In all cases temperature optima occurred at or above 35°C for both enzymatic activities (Fig. 6). For *Lactobacillus* strains Lac16 and Lac19, maximal hydrolysis of *p*NP-*α*Glu occurred around 45°C. In all cases, as temperatures approached 50°C both activities were rapidly reduced to zero at 60°C. At temperatures used in winemaking, significant activities were retained.

Effect of combined parameters

Evaluation of strains for hydrolysis of *p*NP-*β*Glu and *p*NP-*α*Glu in the presence of a combination of the parameters tested above revealed similar response patterns for many. Accordingly, only findings for the most representative strains are reported in Fig. 7. Both activities displayed by *Lactobacillus* strains Lac12 and Lac19 showed a small increase when assayed in the presence of 10% ethanol and 2 g l^{-1} glucose (Fig. 7a,b). Meanwhile, *β*-D-glucopyranosidase activity of *Pediococcus* strain Ped18 was about 30% of that observed for the control (Fig. 7a) whereas *α*-D-glucopyranosidase activity was increased by approx. 100% relative to the control (Fig. 7b). Strain Ped15 displayed minimal activity.

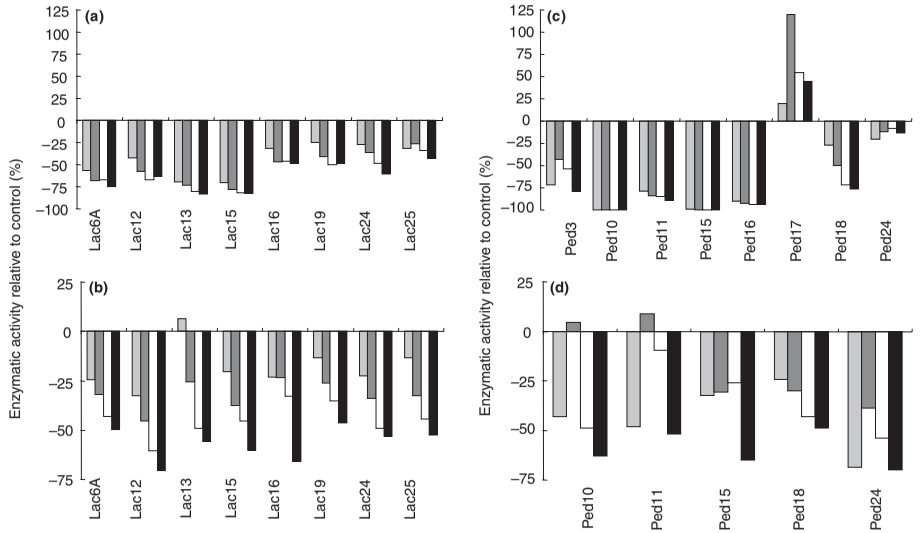


Fig. 4 Influence of glucose on the enzymatic activities of *Lactobacillus* spp. against *p*NP-*β*Glu (a) or *p*NP-*α*Glu (b) and *Pediococcus* spp. against *p*NP-*β*Glu (c) or *p*NP-*α*Glu (d). Values are shown relative (%) to the activity observed for the control performed under the same conditions in the absence of ethanol and are the mean values of duplicate determinations. Glucose: ■ 0·1 g l^{-1}; ■ 1 g l^{-1}; □ 7·5 g l^{-1}; ■ 20 g l^{-1}

DISCUSSION

Many studies have been carried out to investigate the ability of *Lactobacillus* and *Pediococcus* species to produce glycosidase enzymes (De Vos and Gasson 1989; Tzanetakis and Litopoulou-Tzanetakis 1989; Antuna and Martinez-Anaya 1993; Bianchi-Salvadori *et al.* 1995; Marasco *et al.* 1998, 2000). The majority of such works, however, have concentrated on dairy isolates. Little attention, if any, has been dedicated to the characterization of the glycosidases of wine *Lactobacillus* and *Pediococcus* species. Nevertheless, given that these organisms form part of the natural microflora of the winemaking process (Costello *et al.* 1983; Boulton *et al.* 1996; Fugelsang 1997) and have become commercially available as alternate MLF starters (Pilatte and Prahl 1997), a more detailed characterization is justified.

Our investigations have shown that the *Lactobacillus* and *Pediococcus* strains studied here produce little beyond *α*- and *β*-D-glucopyranosidase activities. The former activity may be of importance in the liberation of nutrients from yeast-derived macromolecules which can enhance the growth of LAB (Guilloux-Benatier *et al.* 1993). While *β*-D-glucopyranosidases can play a role in liberation of sensorially detectable grape aglycones from their glycosylated forms, this process may require the removal of multiple glycoside species, potentially in a sequential manner (Günata *et al.* 1988). Accordingly, the limited number of additional glycosidase activities from these organisms reduces the potential impact of *Lactobacillus* and *Pediococcus* strains on the typically mixed population of flavour precursors found in grapes. Furthermore these activities were approximately one order of magnitude less than those seen for *O. oeni* in previous work (Grimaldi *et al.* 2000, 2005). Even so, the activities studied here might still have marked impact on the flavour profile of wine. To determine their relative importance in a wine-like environment, several key parameters were examined individually and in combination.

Mirroring recent findings for *O. oeni* (Grimaldi *et al.* 2000, 2005; Ugliano *et al.* 2003; Barbagallo *et al.* 2004), both *β*- and *α*-D-glucopyranosidase activities were influenced by pH, though to varying degrees (Fig. 1). The relative stability of *Lactobacillus β*- and *α*-D-glucopyranosidase activities to pH changes and low pH values suggests that these isolates rather than the wine-related *Pediococcus* would be of greater importance in wines with typical pH values between 3·0 and 4·0. The sole exception, *Pediococcus* strain Ped18, which was not of wine origin, might, in fact, enjoy oenological applications. The high overall activity of Ped18 was such that even

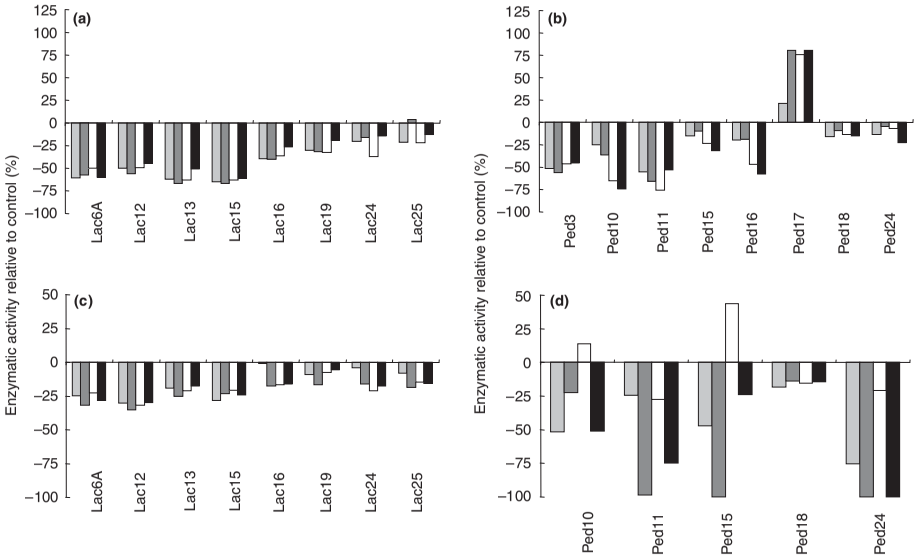


Fig. 5 Influence of fructose on the enzymatic activities of *Lactobacillus* spp. against *p*NP-*β*Glu (a) or *p*NP-αGlu (b) and *Pediococcus* spp. against *p*NP-*β*Glu (c) or *p*NP-αGlu (d). Values are shown relative (%) to the activity observed for the control performed under the same conditions in the absence of ethanol and are the mean values of duplicate determinations. Fructose: ▩ 0·1 g l^{-1}; ■ 1 g l^{-1}; □ 7·5 g l^{-1}; ■ 20 g l^{-1}

after being diminished by wine pH values, marked residual activities, comparable to *Lactobacillus* strains, remained.

The variability of the response of the studied glycosidase activities to ethanol, glucose and fructose is not without precedent in other organisms, or *O. oeni* (Williams 1993; Winterhalter and Skouroumounis 1997; Grimaldi *et al.* 2000, 2005). While still showing strong strain dependence, our findings reveal ethanol to induce a trend towards enhancement of hydrolysis of both substrates amongst *Lactobacillus* strains compared with a reduction or unaltered hydrolysis by *Pediococcus* strains (Fig. 3). While this data offer no further clues as to the basis for these responses, the availability of considerable strain variation introduces the possibility of selecting and applying isolates according the ethanol concentration of the fermenting juice or wine. Contrasting the variability of the response to ethanol, glucose (a possible end-product of glycosidase activity) was seen to be inhibitory to hydrolysis of both *p*NP-*β*Glu and *p*NP-αGlu with few exceptions (Figs 4 and 5). Again the existence of an isolate (Ped17) whose *β*-D-glucopyranosidase activity is strongly enhanced by both glucose and fructose, offers a potentially valuable source from which a superior enzyme might be purified. Such a preparation or strain is an excellent candidate for overcoming the marked sensitivity seen amongst existing enzyme preparations to these sugars (reviewed by Winterhalter and Skouroumounis 1997).

The final parameter considered in this study was included out of recognition of the fact that it varies widely during winemaking. Optimal temperatures for *p*NP-*β*Glu and *p*NP-αGlu hydrolysis (35–45°C) were higher than the typical working range seen during fruit processing, fermentation and maturation (Boulton *et al.* 1996). Compared with *O. oeni* (Grimaldi *et al.* 2005), *Lactobacillus* and *Pediococcus* strains showed a reduced sensitivity to temperatures outside the optima. Again this fact suggests that these genera may be a better source of enzymes for application to low temperature juices or wines.

An analysis of the impact of the simultaneous exposure of bacterial biomass to multiple parameters proved informative. Both *Lactobacillus* strains Lac12 and Lac19 tended towards enhanced activities, suggesting that the response to ethanol was the dominant effect. The overriding influence on activity by *Pediococcus* strain Ped15 was the low pH of the medium (i.e. 3·5) resulting in minimal activity upon which the influences of ethanol and glucose were minor. The nature of the response to ethanol and glucose was more

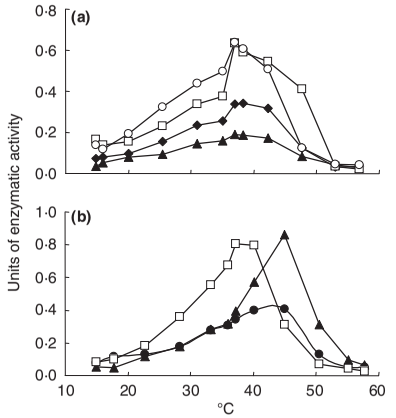


Fig. 6 Influence of temperature on enzymatic activities of *Lactobacillus* spp. and *Pediococcus* spp. against *p*NP-*β*Glu (panel a: ◆ Lac16, ▲ Lac19, □ Ped27, ○ Ped28) and *p*NP-αGlu (panel b: ● Lac16; ▲ Lac19; □ Ped18). Values are the mean of duplicate determinations

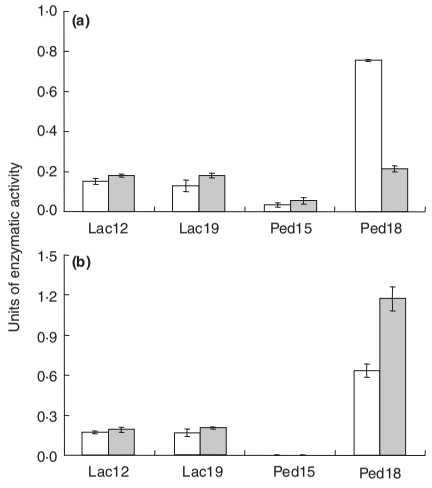


Fig. 7 Influence of ethanol and glucose on *Lactobacillus* spp. and *Pediococcus* spp. against *p*NP-*β*Glu (a) and *p*NP-αGlu (b). Assays were conducted at pH 3·5, in the presence or absence of ethanol (10%, v/v) and glucose (2 g l^{-1}). Values are the mean of triplicate determinations ± standard deviation. □ Control; ▩ Mixed

clearly seen against the background of the higher activity of strain Ped18. In this case, *β*-D-glucopyranosidase activity was reduced, replicating the pattern seen in the presence of ethanol alone (Fig. 3c). Similarly, α-D-glucopyranosidase activity was increased, again as seen previously (Fig. 3d) when this strains was exposed to ethanol only.

In summary, this study completes the survey performed on wine LAB strains in our collection (Grimaldi *et al.* 2000, 2005). Having demonstrated that all tested strains have at least one clear detectable glycosidic activity, some points can be made regarding differences between the genera, *Lactobacillus*, *Pediococcus* and *Oenococcus*. While the latter have a very wide range of activities, the former effect hydrolysis of only a limited number of substrates, specifically the *β*- and α-D-glucopyranosides. The ability of *Lactobacillus* and *Pediococcus* to hydrolyse the *p*NP-*β*Glu and *p*NP-αGlu substrates was also lower than *Oenococcus*. Importantly, the presence of sugars in the assay medium was highly inhibitory to both enzymatic activities. At face value this might imply that use of such organisms for flavour liberation is limited to the latter stages of fermentation when juice sugar contents are low. Interestingly, sugar concentrations as low as 0·1 g l^{-1} were inhibitory to glycosidic activities, and concentrations up to 20 g l^{-1} were only marginally more inhibitory. In addition, enhancement of hydrolysis by ethanol appeared to override inhibition by sugar. Thus the window of sugar concentrations within which such strains might be applied during the fermentation may well be wider than that examined here. It is also worth reiterating that glycosidic activities by one strain in this survey, Ped17, were in fact enhanced by sugars.

The findings reported here form the basis for the selection of isolates for the purification and further characterization of potentially useful glycosidase enzymes. In addition, this study is being extended to consider the ability of selected isolates to act on natural grape glycosides under wine-like conditions and during winemaking, with the ultimate aim of tailoring wine composition.

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Identification of genes affecting glucose catabolism in nitrogen-limited fermentation

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Abstract

In recognition of the importance of assimilable nitrogen in the successful completion of several fermentation processes, we have sought to develop yeast strains that utilise this typically limited nutrient group more efficiently. With the aid of transposon mutagenesis together with a high-throughput method for analysis of multiple fermentations, we have identified 'nitrogen-efficient' mutants that catabolise more sugar for a given amount of nitrogen utilised. In this way we have identified two genes, *NGR1* and *GID7*, whose disruption leads to an enhanced catabolism of sugar in an industrial strain and/or a laboratory strain, during growth in a chemically defined grape juice medium with limiting nitrogen. Deletion of *NGR1* or *GID7* also resulted in minor changes in metabolites produced, and biomass yield, measured as dry weight, was also decreased in *NGR1* mutant strains.
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1. Introduction

The natural substrates for many fermented beverages, including wine, are frequently deficient in assimilable nitrogen. This fact is recognised to be a common cause of fermentation difficulties [1–5], and in turn the reason for the widespread and routine use of nitrogen supplements. Nevertheless, supplementation is not always successful. The timing of additions can be critical [2] and over-addition may increase the risk of microbial instability, that is increase the risk of contaminating microbial growth post-fermentation, of dramatic changes in the sensory profile, and of formation of carcinogens and biogenic amines [6–9]. Moreover consumers are calling for fewer additives to foods and beverages. An ideal solution therefore is to enable the inoculated yeast to more efficiently exploit the naturally occurring assimilable nitrogen [10]. This approach would both facilitate reliable completion of fermentation and eliminate or minimise the need for additives.

Wine yeast strains are known to differ in the efficiency with which they exploit nitrogen sources [11,12]. As a consequence, so called 'nitrogen-efficient' strains may offer greater fermentation reliability [12–14]. We sought to identify genes that could be deleted to improve nitrogen efficiency as a prelude to improving this attribute in industrial strains with otherwise desirable properties. A transposon mutagenesis system was used together

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with a high-throughput, micro-fermentation method for mutant evaluation. Mutants displaying improved nitrogen efficiency were further characterised in shake-flask fermentations and their affected genes identified by PCR amplification and determination of sequences adjacent to the transposon insert. Subsequent investigations were undertaken with disruptants in both laboratory and wine yeast backgrounds.

2. Materials and methods

2.1. Yeast strains and culture conditions

The *Saccharomyces cerevisiae* strains W303, C9 and their derivatives (Table 1) were used for this study. The laboratory strain W303 was selected as it is able to ferment chemically defined grape juice medium (CDGJM) [15] containing sugar concentrations alike to those found in typical grape must (approximately 200 g l^{-1}). Except where indicated in Table 1, all strains were derived from the prototrophic strains KP2 and KP3, which were produced by mating W303 with P49 [16] followed by five back-crosses with W303. Strain C9 was produced in this laboratory [17] and is a haploid derivative of the common industrial wine yeast strain, L-2056 (Lallemand). Yeast deletion strains 3352 (BY4741 *ngr1*::*KANMX4*) and 3446 (BY4741 *gid7*::*KANMX4*), were acquired from the *S. cerevisiae* Deletion Project, http://www-sequence.stanford.edu/group/yeast_deletion_project/deletions3.html (Invitrogen, Carlsbad, CA).

Yeast were routinely grown in complete (YPD: 1% yeast extract, 2% Bactopeptone and 2% glucose) or minimal [18] medium, with an addition of 2% Bacto-agar where necessary. Experimental cultures were grown in CDGJM [15]. Where appropriate, low to moderate concentrations of total nitrogen (typically 75–100 mg FAN l^{-1} as either amino acids and ammonium, or ammonium alone) were present in CDGJM to simulate a limiting-nitrogen environment. Potassium acetate medium (1% potassium acetate, 0.1% yeast extract, 0.05% glucose, 2% Bacto-agar) was utilised for yeast sporulation before spore dissection and mating, carried out as described [19]. Yeast transformation was performed following lithium acetate treatment [20], and positive transformants were selected on YPD-geneticin (400 μg ml^{-1}).

2.2. Construction of JMG2 and JMG3

JMG2 was constructed by deletion of *URA3* from KP2 via the *KANMX* gene replacement strategy [21]. A PCR product corresponding to the *KANMX4* module flanked with 59 and 60 bp of *URA3* sequences (+653 → + 712 and +102 → + 160) was amplified from pBS418 with primers ura3KANF and ura3KANR (Table 2). PCR was performed in 1X Dynazyme EXT buffer with 0.5 mM dNTPs, 0.5 mM $MgCl_2$ and 1 U of Taq (Sigma, St. Louis, MO). Cycling conditions were as follows: 30 cycles of 94 °C, 30 s; 52 °C, 1 min; 72 °C, 2 min. KP2 was transformed with this PCR product and positive transformants were selected on YPD-geneticin (400 μg ml^{-1}). Deletion of *URA3* in JMG2 was indicated by non-growth on minimal medium and confirmed by PCR with primers URA3F1 and URA3R. PCR was performed in 1X Dynazyme EXT buffer with 0.5 mM dNTPs, 1 mM $MgCl_2$ and 1 U of Dynazyme EXT. Cycling conditions were as follows: 30 cycles of 94 °C, 30 s; 62 °C, 1 min; 72 °C, 3 min. Strain JMG2 was crossed with KP3, diploids were sporulated, dissected and JMG3 was identified by PCR.

Table 1
Yeast strains used in this study

Yeast strain	Relevant genotype	Source
KP2	Prototrophic derivative of W303 *MAT*α	This laboratory
KP3	Prototrophic derivative of W303 *MAT*a	This laboratory
JMG2	*ura3*::*KANMX3 MAT*α	This study
JMG3	*ura3*::*KANMX3 MAT*a	This study
JMG2.113	*ura3*::*KANMX3 ngr1*::mTn-3xHA/GFP	This study
JMG2.120	*ura3*::*KANMX3 gid7*::mTn-3xHA/GFP	This study
3352	BY4741 *ngr1*::*KANMX4*	SGD[a]
3446	BY4741 *gid7*::*KANMX4*	SGD[a]
KP2 *ngr1*	*ngr1*::*KANMX4 MAT*α	This study
KP2 *gid7*	*gid7*::*KANMX4 MAT*α	This study
KP3 *ngr1* B	*ngr1*::*KANMX4 MAT*a	This study
KP2/3 *ngr1 gid7*	*MAT*a *ngr1 gid7*	This study
C9	*MAT*a *ho*	[17]
C9 *ngr1*	*MAT*a *ho ngr1*::*KANMX4*	This study
C9 *gid7*	*MAT*a *ho gid7*::*KANMX4*	This study

[a] SGD, *Saccharomyces cerevisiae* Deletion Project, http://www-sequence.stanford.edu/group/yeast_deletion_project/deletions3.html.

Table 2
Oligonucleotides used for PCR

Primer	Sequence (5′–3′)
ura3KANF	TTCATTGGATGTTCGTACCACCAAGGAATT ACTGGAGTTAGTTGAAGCATTAGGTCCCAG ACATGGAGG CCCAGAATAC
ura3KANR	ATAGTCCTCTTCCAACAATAATAATGTCAG ATCCTGTAGAGACCACATCATCCACGGTTC AGTATAGCGACCAGCATT C
URA3F1	AGGGAAGACAAGCAACGAAA
URA3R	TTGGTTCTGGCGAGGTATTG
IPCR3	GTTTTCGTTCCACTGAGCGT
IPCR4	ACAAGACACGTGCTGAAGTCA
GFPprobeF	ACGCTCAGTGGAACGAAAAC
GFPprobeR	TGACTTCAGCACGTGTCTTG
NGR1A	AAAACCATCTGCGTGTTATATCCTA
NGR1D	GACTTTGTAGCGATTTTCACATTTT
GID7A	GTTTCAGATCTATGCTGAGACACG
GID7D	CCATTTGGTATGGATTATCACTAGG
G418F	AAAAGACTCACGTTTCGAGGC
G418R	CGAGCATCAAATGAAACTGC

2.3. Generation of yeast mutants using a transposon insertion genomic library

A yeast genomic library containing random transposon insertions was obtained from Dr Michael Snyder, Yale University [22]. A library of mutants was generated by transformation of JMG2 or JMG3 with genomic DNA fragments containing mTn-3xHA/*GFP URA3* insertions. Briefly, 5×10^8 cells were transformed [20] with 1 μg of *Not*I digested and purified mutagenised DNA. Transformants were selected on minimal medium (to eliminate auxotrophic mutants).

2.4. Selection of nitrogen-efficient strains

During the primary screen, 1 ml YPD starter cultures of 5000 randomly selected transposon mutants were grown overnight at 30 °C in 48-well plates (#3548, Corning Costar, Rochester, NY) with shaking (~150 rpm). A 48-pinned replicator was then used to inoculate screening fermentations comprising 1 ml of CDGJM (75–100 mg FAN l^{-1} as ammonium) in fresh 48-well microtitre plates, before aerobic incubation at 30 °C with shaking (100 rpm). Fermentation progress was determined after 10–14 days from the residual sugar content of each culture as estimated using a handheld refractometer. Mutants that catabolised the greatest amount of sugar were selected for further evaluation in the form of a secondary screen, performed in triplicate 5–20 ml cultures of CDGJM containing 75–750 mg FAN l^{-1} as ammonium or as a mix of nitrogen compounds (a combination of amino acids and ammonium, as described in [15]). Fermentation progress was again monitored by refractive index. Mutants achieving the greatest degree of sugar catabolism after incubation for 10–14 days were selected for further investigation.

2.5. Identification of *mTn3 × HA*/GFP/URA3 insertion points in nitrogen-efficient strains

The location of the transposon and therefore the identity of the affected gene of each strain of interest was determined from sequence homology of the insertion site to the *Saccharomyces* Genome Database (http://www.yeastgenome.org/). Regions adjacent to and including the inserted transposon were amplified by inverse PCR [23,24] and sequenced. To achieve this, a *Dra*I genomic digestion of the transposon mutant was ligated at a low DNA concentration (approximately 1 μg ml^{-1}) in the presence of T4 DNA ligase (5 U ml^{-1}) to obtain a high proportion of self-annealed products. Ligation reactions were purified with a *Perfect Prep* kit (Eppendorf) to a final volume of 30 μl and PCR was then performed with primers IPCR3 and IPCR4. The PCR reaction was performed in 1 × Dynazyme EXT (Finnzymes, Espoo, Finland) reaction buffer with 100 pmol of each primer, 1 μl of purified ligation reaction, 2 mM $MgCl_2$, 100 μM dNTPs and 1 U of Dynazyme EXT. The PCR amplification conditions were as follows: 10 min at 94 °C and then 30 cycles of 94 °C, 30 s; 56 °C, 1 min; 72 °C, 4 min. The size of the generated IPCR fragment was confirmed by comparison to a Southern blot [25,26] of *Dra*I digested mutant DNA hybridised with a probe specific to *GFP* (data not shown). The latter was generated by PCR using transposon mutant genomic DNA (200 ng), primers GFPprobeF and GFPprobeR (100 pmol each), 1 mM $MgCl_2$, 50 μM dNTPs, 50 μM DIG labelled dNTPs (Roche, Rotkreuz, Switzerland) and 1 U of Dynazyme EXT DNA polymerase (Finnzymes). The PCR reaction was performed in 1 × Taq reaction buffer (Sigma) over 30 cycles at 94 °C, 30 s; 56 °C, 1 min; 72 °C, 1 min. Sequencing of the amplified genomic fragments (automated sequencing by IMVS Adelaide with primers IPCR3 and IPCR4) and comparison to the yeast genome utilising the BLAST homology search engine (http://seq.yeastgenome.org/cgi-bin/nph-blast2sgd) revealed the identity of the affected gene.

2.6. Construction of NGR1 and GID7 deletants in KP2 and C9

NGR1 and *GID7* were disrupted in strains KP2 and C9 [17] using the *KANMX* gene replacement strategy. We took advantage of the readily available yeast

deletion strains from the Yeast Deletion Project by amplifying a PCR product corresponding to the *KANMX4* module flanked with *NGR1* or *GID7* sequences (−260 → −1, +2020 → +2329 and −445 → −1, +2239 → + 2613, respectively) from genomic DNA purified from the corresponding yeast deletion mutants 3352 and 3446, using the primer pairs NGR1A, NGR1D and GID7A and GID7D. Strains KP2 and C9 were then transformed with these PCR products and transformants were selected on YPD-geneticin. Deletion of each gene was confirmed by Southern blot hybridisation with a probe specific for *KANMX4*. The *KANMX4* probe was PCR-amplified from pFA6-lacKanMX4 [21] using primers G418F and G418R. PCR conditions employed were the same as for the GFP probe. KP2/3 *ngr1 gid7* was generated by back-crossing KP2 *ngr1* with KP3 followed by sporulation and dissection to generate KP3 *ngr1*B. Subsequently KP3 *ngr1*B was mated with KP2 *gid7*, sporulated and dissected to generate the double mutant KP2/3 *ngr1 gid7*. Mating type was determined by PCR (as described by [27]), and gene disruptions by Southern-blot hybridisation, as above.

2.7. Small-scale fermentations

Fermentations were carried out (according to [17]) under anaerobic conditions, in triplicate cultures of 150 ml of CDGJM containing 200 g l^{-1} of glucose, where the concentration of nitrogen varied from low (75 mg FAN l^{-1}) to high (750 mg FAN l^{-1}) and was supplied either as ammonium only or as a mixture of amino acids and ammonium [15]. Samples were regularly collected, clarified and fermentation progress was evaluated by enzymatic determination (Roche kit #0139106) of residual glucose. Residual ammonium was also determined enzymatically (Roche kit #1112732). Ethanol, glycerol, acetic acid, succinic acid and acetaldehyde were quantified by HPLC analysis in undiluted and diluted (1:20) terminal samples, clarified through syringe filters (0.45 μm PVDF, Millipore, Bedford, MA). HPLC analysis utilized an Aminex HPX-87H column (300 mm × 7.8 mm; BioRad) and was performed at 60 °C with 2.5 mM H_2SO_4 at a flow rate of 0.5 ml min^{-1}. Peaks were detected with a RID-10A refractive index detector (Shimadzu, Kyoto, Japan) and quantified by comparison with prepared standards in CDGJM using Delta integration software (DeltaWare Dataworks, Brisbane, Australia).

Optical density (600 nm) of appropriately diluted culture samples was used as an estimate of yeast cell growth. Biomass yield was determined from the weight of washed (10 ml deionised water) and dried cells (600 W microwave oven, medium setting, 10 min) collected from 5 to 10 ml culture samples on 0.22 μm filter disks (pre-dried and pre-weighed).

3. Results

3.1. Screening for nitrogen-efficient yeast mutants

Transposon insertion mutants were generated in the genome of strains JMG2 and JMG3. Selection on minimal medium eliminated auxotrophic mutations. Preliminary work (data not shown) suggested that nitrogen-efficient strains could be identified either from their reduced nitrogen consumption in nitrogen-excess:carbon-limited media (e.g. 750 mg FAN l^{-1}, 200 g glucose l^{-1}) or else from their greater glucose catabolism in nitrogen-limited:carbon-excess media (~75 mg FAN l^{-1}, 200 g glucose l^{-1}). The later formulation was adopted for the medium in which large numbers of mutants were evaluated in the primary screen, since the extent of glucose catabolism was easily estimated using a refractometer. In this way, from the 5000 mutants assessed, 110 (2%) preliminary isolates were selected as the most nitrogen-efficient and retained for further investigation (Fig. 1). These mutants catabolised at least 10% more glucose than the parental strain. In a secondary screen using the same medium but performed in triplicate 5 ml fermentations, 40 of these mutants (36%) were again shown to catabolise at least 10% more glucose than the parental strain (data not shown). This 16.5-fold enrichment for nitrogen-efficient mutants supported the suitability of the method.

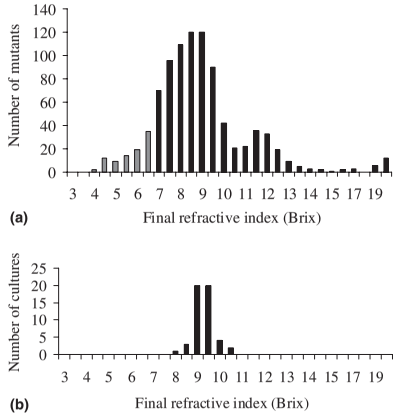


Fig. 1. Histograms showing the distribution of final refractive index values for individual micro-fermentations performed by approximately 1000 transposon mutants (a) or 50 separate cultures of the wild type KP2 (b). All cultures were grown for 14 days in CDGJM containing 100 mg FAN l^{-1} as ammonium. Values are derived from a typical experiment for a subset of the total 5000 mutants examined. The grey bars (Panel a) correspond to the mutants selected for further investigation.

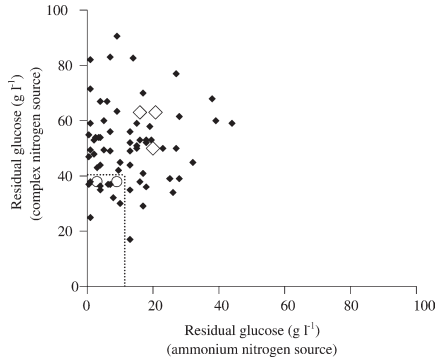


Fig. 2. Comparison of the residual glucose concentrations after 14 days of fermentation by selected transposon mutants (◆), in particular JMG2.113 and JMG2.120 (○), and the wild type, KP2 (◇), when grown in 5 ml of CDGJM containing 75 mg FAN l^{-1} supplied as ammonium or as an ammonium and amino acid (complex) nitrogen source. Mutant strains that catabolised the most glucose, that is those fermentations with less residual glucose than the selection threshold (dotted lines) were chosen for further analysis.

Grape juices typically contain assimilable nitrogen as a complex mixture of compounds, primarily amino acids and ammonium [15,28]. All screening of mutants up to this point, however, had been conducted using ammonium alone. The relative nitrogen efficiency of the selected mutants was therefore examined during growth in a limited-nitrogen medium (75 mg FAN l^{-1}), instead comprised of a mix of nitrogen compounds. In general the extent of glucose catabolism was decreased when a mixed, limiting source of nitrogen was provided, yet the majority of mutants still proved superior to the parental strain (Fig. 2). For a small number of mutants there was little change in efficiency across the two nitrogen sources.

3.2. Identification and characterisation of genes influencing nitrogen efficiency

Ten mutants with the most enhanced performance, that is where significantly more glucose is catabolised when nitrogen is supplied as a complex mix or as solely ammonium, were selected from the above screens and assessed in 150 ml fermentations of CDGJM containing 75 mg FAN l^{-1} as amino acids and ammonium. Such volumes permitted a more comprehensive sampling regime and hence more accurate determination of fermentation kinetics, yield of biomass and key metabolites. Under these conditions mutants JMG2.113 and JMG2.120 were chosen for genetic analysis because in comparison to the parental strain, after glucose fermentation had become protracted they had catabolised 7.3% and 22.2% more glucose, respectively, under the nitrogen-limited conditions (data not shown). The genomic location of the transposon was therefore determined for both strains by IPCR amplification and sequencing of genomic fragments containing the insert. JMG2.113 bore an insertion at +1533 bp of *NGR1*/*YBR212W* (Negative Growth Regulatory protein) [29], and JMG2.120 at +956 bp of *GID7*/*YCL039W* (glucose-induced degradation process of FBPase) [30].

The entire open reading frame of each gene was in turn deleted in strain KP2 and the wine yeast derivative, C9, and the resultant deletants cultured in media of various nitrogen contents. Deletion of *NGR1* or *GID7* from the laboratory strain KP2 increased the amount of glucose catabolised under most conditions examined. In media containing limiting ammonium these strains failed to support completion of fermentation, that is

Table 3
Duration of glucose fermentation and the yield of biomass for wild type and mutants during growth at two nitrogen concentrations, supplied as either ammonium or ammonium and amino acids

	Ammonium				Complex N			
	75 mg FAN l^{-1}		300 mg FAN l^{-1}		75 mg FAN l^{-1}		300 mg FAN l^{-1}	
	Duration (h)	Biomass (g l^{-1})	Duration (h)	Biomass (g l^{-1})	Duration (h)	Biomass (g l^{-1})	Duration (h)	Biomass (g l^{-1})
KP2	–[a]	2.2 ± 0.1	105	5.6 ± 0.1	326	3.4 ± 0.1	74	6.1 ± 0.2
C9	190	3.1 ± 0.1	70	6.9 ± 0.4	190	2.9 ± 0.1	78	6.9 ± 0.4
	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)
KP2 *ngr1*	4.9[a]	104 ± 3.9	100	92 ± 5.1	100	101 ± 5.2	95	107 ± 7.2
KP2 *gid7*	17.3[a]	100 ± 3.1	90	98 ± 2.1	92	82 ± 4.4	95	110 ± 2.0
C9 *ngr1*	85	85 ± 2.0	100	77 ± 7.2	73	78 ± 2.1	85	87 ± 1.3
C9 *gid7*	100	100 ± 2.0	100	98 ± 1.7	91	97 ± 1.5	87	108 ± 9.7

Values for the mutant strains are expressed as a percentage of the parental strain grown under the same conditions. Biomass is expressed as ± SD. Fermentation durations are the average of triplicate fermentations and were within 3% of the reported figure.

[a] Complete catabolism of glucose did not occur, thus values reported are the percentage increase in glucose catabolism compared to that of the parental strain, after 450 h.

they became "stuck" (Table 3). Nevertheless strains KP2 *ngr1* and KP2 *gid7*, grown in the media used for the primary and secondary screen (i.e. 75 mg FAN l^{-1}, supplied as ammonium), still consumed more glucose than the wild type, KP2, before fermentation arrested. In media of higher ammonium content or else complex nitrogen-limited media, KP2 *gid7* completed fermentation sooner, that is in approximately 90% of the time of the wild type. In complex media of 300 mg FAN l^{-1} both deletants in KP2 displayed a fermentation duration similar to that of the parent strain.

Where deletions were introduced into the C9 background and the strains cultured in media containing ammonium as sole nitrogen source, improvements in fermentation performance were seen for C9 *ngr1* cultured in nitrogen-limited media (Table 3). In this case, fermentation duration was reduced to 85% of the wild type. By comparison, deletion of either *NGR1* or *GID7* in C9 reduced the duration of fermentation in all cases where media comprising mixed nitrogen sources were used. Most notably, C9 *ngr1* grown in complex nitrogen-limited media was able to reduce fermentation duration by 27% compared with the wild type grown under the same conditions.

Biomass yields by all cultures were determined to establish whether any improvements in nitrogen efficiency were attributable to a reduced formation of biomass. There was, however, no correlation between biomass yield and fermentation duration.

3.3. Glucose utilisation by KP2/3 ngr1 gid7

The double-deletion strain KP2/3 *ngr1 gid7* was constructed as described in Section 2. Deletion of both genes was confirmed by Southern-blot hybridization (data not shown). In fermentation trials utilising nitrogen-limited media containing ammonium as sole nitrogen source, the rate of glucose catabolism was markedly increased in KP2/3 *ngr1 gid7* such that only this strain completed the fermentation of all the glucose (200 g l^{-1}) within the duration of the experiment (Fig. 3). In so doing the double mutant catabolised 71% more glucose than the wild-type strain (KP2, 116.5 g l^{-1}), 44% more glucose than KP2 *ngr1* (139.3 g l^{-1}) and 30% more than KP2 *gid7* (154.3 g l^{-1}). Attempts were made to construct an *NGR1* and *GID7* double mutant in C9, however, the combination of both mutations in this background appeared to be lethal.

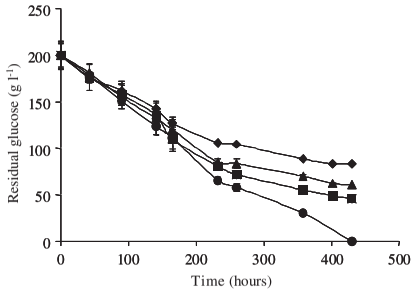


Fig. 3. Catabolism of glucose by wild-type strain KP2 (◆) and deletion strains KP2 *ngr1* (▲), KP2 *gid7* (■) and KP2/3 *ngr1 gid7* (●). Fermentations were performed in CDGJM with 75 mg FAN l^{-1} as ammonium. Data points are the mean from triplicate fermentations ±SD. NB: smaller error bars are concealed behind the data symbols.

3.4. Nitrogen utilisation, biomass and glycerol yields by deletants

Differences in nitrogen efficiency have previously been suggested through determinations of the maximum amount of nitrogen that is utilised by a strain in conditions of nitrogen excess [11,12]. Therefore the maximum nitrogen utilisation of the deletants was determined using media containing ammonium as sole nitrogen source and supplied in excess to the requirements of these strains (750 mg FAN l^{-1}). Interestingly, nitrogen utilisation was not generally seen to be reduced for these deletants compared with the parental strains (276 ± 18 mg l^{-1} for KP2 and 218 ± 14 mg l^{-1} for C9). In fact, strain C9 *gid7* tended toward a higher consumption of ammonium (246 ± 9 mg l^{-1}).

Biomass yields and cell numbers were also determined. No differences were seen in the latter between the mutants and corresponding wild type (data not shown). However, just as biomass yield had not correlated with fermentation duration (Table 3), there was no clear link between biomass yield and degree of nitrogen consumption determined under the conditions of this experiment (data not shown). Strain C9 *ngr1* was perhaps the sole exception, showing comparable reductions in biomass yield and nitrogen utilisation. Reduced biomass yields were a recurring feature of C9 *ngr1*, such that across all experiments biomass yields ranged between 77% and 87% of that of the wild type, independent of the amount or type of nitrogen supplied. Reduced biomass yields combined with unchanged cell numbers suggest that C9 *ngr1* produce cells that were smaller than the wild type.

The final parameter determined at the conclusion of fermentation was the yield of gross metabolites, namely, ethanol, glycerol, acetic acid, succinic acid, citric acid and acetaldehyde. Most analytes were unchanged in their concentration across the strains examined (data not shown). Glycerol yields appeared to increase in most cases, but significant increases of up to ~17% were limited to strain C9 *ngr1* in both media (Fig. 4).

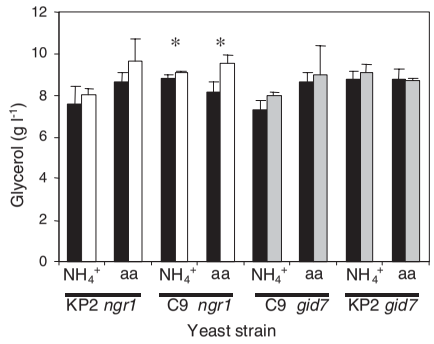


Fig. 4. The concentration of glycerol at fermentation completion or arrest (in the case of KP2, KP2 *ngr1* and KP2 *gid7* supplied with ammonium), when conducted by wild type (■) KP2 and C9 or their derivatives bearing deletions of *NGR1* (□) or *GID7* (▨). Triplicate fermentations were performed in CDGJM containing low nitrogen (75 mg FAN l^{-1}) as either ammonium or as a mixture of complete amino acids and ammonium (aa) as indicated. Values shown in the first eight bars were derived from a separate experiment to that which yielded the values shown in the last eight bars. Glycerol yields by mutants which were significantly different ($p < 0.05$, Student's *t*-test) from the corresponding wild type are indicated (*).

4. Discussion

The catabolism of essentially all sugars from an industrial wine fermentation is vital to the successful production of a 'dry' wine within stylistic specifications [31]. Problem fermentations, which often arise due to an insufficient supply of yeast-assimilable nitrogen [1–5] are typically addressed through vineyard or winery practices that increase the amount of available nitrogen. The exploitation of yeast strains that are more nitrogen-efficient [10,13,14] is becoming a more common solution. Conceptually, increased nitrogen efficiency might come about either with or without a nett reduction in the amount of nitrogen assimilated. Such improvements could presumably arise through one or a combination of (i) maintenance of sugar transport systems, catabolic and associated enzymology and processes or (ii) a reduced expenditure of nitrogen on non-essential processes, thereby disengaging nitrogen to support core fermentative activities. Highly nitrogen-efficient strains that are a manifestation of either scenario would be useful to industry.

We sought to increase the understanding of the basis for differences in nitrogen efficiency, as a prelude to manipulating this attribute in industrial strains for greater fermentation reliability. A transposon-based system and multi-well plate format fermentations were successfully used to generate and identify mutants whose capacity to catabolise sugar had been altered (see Fig. 2). Mutants with increases or decreases in nitrogen efficiency were both considered potentially valuable to this study. Nevertheless, given that most mutations probably resulted from a loss of function in the target gene [32,33], those yielding an increase in nitrogen efficiency were considered more likely to be linked with changes in nitrogen metabolism. Whereas mutants with reduced nitrogen efficiency might arise from any mutation that debilitates growth and/or any stage of sugar catabolism, mutants with increased nitrogen efficiency were hypothesised to come about through disruption of a gene or metabolic outcome normally antagonistic to efficient exploitation of nitrogen.

Up to 40 transposon mutants as well as deletants of *NGR1* and *GID7* in a laboratory and a wine strain derivative were evaluated through several fermentations. The transposon mutants, shown to have increased nitrogen efficiency in aerobic fermentations of media with limited nitrogen supplied as ammonium, typically catabolised less glucose when grown on a complex nitrogen source (Fig. 2). Subsequent trials with the deletants conducted under semi-anaerobic conditions generally yielded superior performance in media with mixed nitrogen sources (Table 3). These apparently discrepant results in fact agree with previous reports and may be attributed to the different availability of oxygen in each case. Ammonium is a more efficient nitrogen source than most amino acids in aerobic cultures of laboratory strains [34] whereas, under self-anaerobic oenological conditions, supplementation of grape juice fermentations yields higher fermentation rates from mixed nitrogen sources rather than ammonium [35,36].

Quantitation of the nitrogen requirement of deletants revealed that unlike previously characterised nitrogen-efficient wine yeasts [10,11], deletion of *GID7* or *NGR1* typically did not reduce the amount of nitrogen removed from the medium. Fermentations of nitrogen-deficient media by such strains might therefore be expected to become nitrogen-depleted, even though fermentation should progress and complete ahead of the wild type. The usefulness of these strains as a means of effecting fermentation of nitrogen-deficient juices remains high, however, the potential for development of other problems, such as liberation of hydrogen sulfide [37], would need to be assessed under such conditions.

As already described, two genes were isolated and deleted from a laboratory and a wine strain derivative to allow more detailed characterisation. That deletion of *NGR1* or *GID7* has benefits for nitrogen efficiency was made clear by the fact that out of the 16 deletant:media combinations trailled, eleven resulted in more glucose catabolised than by the parent strain. For the remaining five combinations, deletants performed equivalently to the wild type (Table 3). The most rapid fermentation of a single-gene deletion was observed for C9 *ngr1* grown in a medium of limited, mixed nitrogen, wherein

total fermentation duration was reduced by up to 27% (approx. 50 h). In the context of an industrial fermentation process like winemaking, such a reduction in the residence time in expensive fermenter infrastructure can have significant benefits for plant throughput and productivity. The deletion of both *NGR1* and *GID7* from KP2 seemed also to significantly enhance fermentation, increasing the total glucose catabolised by up to 71% of the parent strain. Unfortunately, this combination of deletions appears lethal in the wine yeast strain C9. Further work with other industrial strains will help determine whether lethality of the *ngr1*/*gid7* deletions was specifically related to these modifications or else arose through unrelated effects such as a randomization of chromosomes.

A second criterion for evaluation of deletants was their pattern of metabolite production. It is noteworthy that not only did deletion of *NGR1* or *GID7* fail to produce detrimental consequence for the parameters examined, in some cases potential improvements were noted. Production of glycerol, a metabolite conferring desirable 'mouth-feel' properties to wine when in concentrations above its taste threshold level of 5.2 g l^{-1} [38], was in fact produced in increased amounts (by up to ~17%) by some deletants (Fig. 4). Apart from any sensory contributions, increased production of glycerol may indicate an alteration in the redox balance of deletants. Glycerol is produced during the regeneration of NAD^+, a requirement for anabolic metabolism, particularly during anaerobiosis. It is also known to be important in the balance of osmotic pressure [39]. Previously, a glycerol-overproducing strain, generated by overexpressing *GPD1*, was shown to have a higher fermentation rate at stationary phase [40]. Researchers hypothesise that this could be due to an enhanced release of inorganic phosphate or a reduced production of ATP [41]. It is therefore possible that any benefit for fermentation seen in *NGR1*/*GID7* deletants is a secondary effect of elevated glycerol production or fluxes of inorganic phosphate or ATP.

Determining precisely how deletion of *NGR1* or *GID7* increases nitrogen efficiency will require further investigation. No clear explanations can be derived from reports to date on the characterisation of these genes. Nonetheless, the insights offered by this study do allow for some speculation as to the basis for the influence of *NGR1* and *GID7* on nitrogen efficiency. A laboratory strain bearing a deletion of both genes was capable of catabolising a greater amount of glucose during fermentation than either single deletant under equivalent nitrogen-limited conditions (Fig. 3). It therefore seems likely that in KP2 the genes function in separate pathways in order to produce an additive effect. However, this combination of deletions in C9 appears to confer lethality suggesting a functional link between these two genes.

The precise biological function(s) of *NGR1* is unknown but the gene is glucose-repressible [29], is linked to the response to high-sugar stress [42] and, most recently, *NGR1* (*RBP1*) has been shown to promote degradation of mitochondrial porin mRNA [43], possibly by binding to its 3′UTR via its RNA recognition motifs [29]. Of relevance to the present study is the fact that *NGR1* (negative growth regulator (1) was originally isolated based on its influence on growth rate [29,44,45]. Deletion of the open reading frame increased early log-phase growth rate by 30% during growth on glucose, or by 60% or 75% during growth on galactose or glycerol, respectively. Conversely, over-expression of *NGR1* (*RBP1*) resulted in reductions in growth rate [45]. We have not detected any difference in growth rate of *ngr1* strains in this study (data not shown). The only impact on a growth attribute is that deletants consistently yield a reduced biomass with little change to cell number. Cells of *ngr1* strains are therefore smaller and/or lighter. The proposed reduction in cell size did not alter the uptake of ammonium (data not shown), as suggested for other oenological strains of reduced size [46]. However, it is possible that the basis for the increased nitrogen efficiency seen in such mutants is related to a reduction in nitrogen-demanding syntheses, perhaps those involved in formation of cellular reserve compounds or cell enlargement, thereby setting free nitrogen to support faster or extended fermentative activity.

GID7 is one of several *GID* genes, known to be involved in catabolite degradation of fructose-1,6-phosphatase, since this activity is still present in *gid7* strains that have been shifted to a glucose-containing medium [30]. The putative *GID7* protein is predicted to contain five WD40 repeats (SMART, http://smart.embl-heidelberg.de/), which are thought to mediate protein–protein interactions and are found in many regulatory proteins such as the β-subunit of the heterotrimeric G-protein GTPase [47]. Systematic searches for protein interactions have highlighted putative interactions of Gid7p with other GID proteins [48], but also with the high-affinity hexose transporter encoded by *HXT7*. This transporter is important at the end of fermentation for maintenance of hexose transport [49] and known to be rapidly degraded upon the depletion of nitrogen [50]. Perhaps, therefore, the absence of Gid7p prevents deactivation of Hxt7p, alike to FBPase, enhancing the mutants ability to maintain its glucose-catabolic activity under conditions of nitrogen starvation. An alternative possibility for the impact on nitrogen efficiency of deletion of *GID7* comes from work on another *GID* gene. *VID30* (*GID1*), is up-regulated on low ammonium [51], resulting in a channeling of nitrogen metabolism towards glutamate formation. It might therefore be speculated that *GID7* functions in a similar manner to *VID30* such that disruption of *GID7* brings about a re-direction of nitrogen which ultimately benefits maintenance of fermentation.

Regardless of how deletion of *NGR1* and *GID7* enhances nitrogen efficiency during fermentation, these findings provide new strategies for the construction or selection of optimised strains with greater fermentation reliability. While the superior nitrogen efficiency of the double-deletion strains is attractive, it currently seems inapplicable for producing improved wine yeasts, at least in the C9 derivatives of the wine strain, L-2056. Also, a comprehensive check for the introduction of any detrimental changes, such as over-production of undesirable metabolites [52], is yet to be carried out. Further work is therefore required not only to determine if this strategy can be applied to other wine yeast strains, but also to define the metabolic basis for the enhancement in these strains as well as any further mutants isolated using the method set-out in this report.

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PCR-based gene disruption and recombinatory marker excision to produce modified industrial *Saccharomyces cerevisiae* without added sequences

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Abstract

The dominant selectable *Kan*^{r} marker, which confers geneticin resistance in yeast, is extensively used for PCR based disruption of genes in functional analysis studies in laboratory strains of *Saccharomyces cerevisiae*. We have developed a gene disruption cassette, which incorporates the *Kan*^{r} marker, and direct repeat sequences designed from the target gene to enable the deletion of the gene without the introduction of added DNA sequences. We report on the disruption of the *HO* gene as a test case, using the *hodr–Kan*^{r}*–hodr* cassette. The cassette was shown to integrate at the *HO* locus and the *Kan*^{r} marker excised by recombination between the two direct repeat sequences. The disruption/excision event resulted in the removal of one direct repeat and the coding sequence of the gene, and hence in this case loss of *HO* function, with the introduction of no foreign or additional sequences, including the *Kan*^{r} marker. Having been derived from the target site, the remaining direct repeat sequence is native sequence in its native location. This design template has the potential to be adapted to other genes, and as such will be of advantage in instances such as the optimization of strains by recombinant DNA technology where the retention of minimal or no foreign sequences is desired.
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Keywords: G418; PCR-based gene disruption; Homologous recombination; Wine yeast; Haploid

1. Introduction

The demand for improved industrial strains of *Saccharomyces cerevisiae* remains strong. However, where improvement strategies utilise recombinant techniques, the prototrophic and higher-ploidy nature of industrial strains precludes the use of traditional

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selection systems based upon complementation of recessive markers. The complication imposed by ploidy has been tackled by the generation of stable haploid derivatives through neutralisation of the *HO* gene, which is responsible for spontaneous mating-type switching and hence re-diploidisation of haploids. This has been achieved by either non-recombinant approaches (Bakalinsky and Snow, 1990; Michnick et al., 1997) or else gene-disruption technology whereby an integration event directed by sequence homology results in replacement of the target gene, *HO*, with a dominant selectable marker, such as the geneticin resistance gene, *KanMX* (Rothstein, 1983; Wach et al., 1994). We reported (Walker et al., 2003) on the development of such a construct, pGEMTho::KanMX, which in addition to achieving *HO* disruption described by others (Tamai et al., 2001), also allowed subsequent removal of much of the disruption construct. An analogous, removable-marker system has been shown to be effective for disruption of at least one other gene, *URA3*, in a wine strain (Puig et al., 1998).

Where industrial yeast are the research subject, the criteria for gene disruption are somewhat different to, for example, gene function studies in laboratory yeast, wherein a large number of genes are knocked out by PCR-mediated gene disruption cassettes (Wach et al., 1994; Entian et al., 1999; Giaever et al., 2002), and excision of the selectable marker is not always deemed necessary. The ability to remove and hence reuse the selectable marker has obvious benefits for the industrial yeast researcher. However, this outcome is even more appealing to regulatory and consumer bodies who advocate minimal foreign sequences in strains destined for commercial use (e.g. see review by Dequin, 2001). Accordingly, an important goal of many recombinant programs aimed at improvement of industrial strains is the minimisation of extraneous vector/construct sequences. Gene disruption protocols that offer specific and complete removal of the target gene and also allow for removal of the selectable marker are therefore attractive. Marker removal is possible in constructs that incorporate direct repeat (DR) sequences flanking the marker. Homologous recombination between the DR sequences results in excision of all but a single DR. In earlier constructs, the DR retained at the target site comprised several hundred bases of sequence from a filamentous fungus, *Ashbya gossypii* (Wach et al., 1994). A more recent version, which utilises *S. cerevisiae* DRs, does achieve the goal of self-cloning (i.e. uses *S. cerevisiae* sequences), however these sequences are left near the target site and not in their native location (Estruch and Prieto, 2003).

Another system for removal of a selectable marker after integration of a gene disruption construct utilises the cre–*lox*P system (Güldner et al., 1999) and has the marker flanked by two *lox*P sites. A second transformation with a plasmid bearing the cre recombinase is required to bring about excision of the marker. The utility of this system is enhanced through the development of several constructs, each bearing different selectable markers, by which multiple disruptions can be carried out ahead of a single excision step that simultaneously removes all markers (Delneri et al., 2000). While such systems are effective and have been successfully applied in industrial strains (Panoutsopoulou et al., 2001) the fact remains that excision leaves behind foreign sequences (a single *lox*P site) and may increase the likelihood of chromosomal rearrangements between these and cryptic *lox*P sites (Delneri et al., 2000).

A system for in vivo site-directed mutagenesis (Storici et al., 2001) utilises an initial gene replacement step with a construct bearing a counter-selectable reporter cassette (CORE) to mark the target site. A subsequent transformation with integrative recombinant oligonucleotides (IROs) is used to bring about deletion or introduce specific mutations as well as removal of the CORE. This so called *delitto perfetto* system is effective but requires the generation of multiple PCR products (i.e. target-specific CORE and IROs) and two transformations.

In this study we sought to further the refinement of gene disruption constructs to yield a system that i) offers targeting of the desired gene, ii) utilises a selectable marker that is both dominant and removable and iii) results in only the retention of sequences that are not foreign to the organism per se nor their genomic location. This goal of 'seamless' gene disruption/deletion was achieved for the *HO* gene as a case study, using the *Kan*^{r} marker flanked by DRs derived from sequences 5′ of the target ORF, *HO* itself. We sought to apply design principles aimed at the ideal of generating such a disruption construct via a single PCR, differential restriction digestion and ligation steps. The resulting paradigm for design of gene disruption cassettes has potential applications in laboratory and particularly industrial strains, whether used as research tools, or as intermediates in the construction of optimised industrial strains.

2. Materials and methods

2.1. Strains and media

Escherichia coli strain DH5α was used for plasmid propagation. *E. coli* transformation was according to Mandel and Higa (1970). Ampicillin (100 mg/l) was used for plasmid selection in *E. coli*.

The *S. cerevisiae* strains used were wine yeast Lalvin L2056 and CY3079 and two laboratory mating-type tester strains, KP2 (*MAT*α) and KP3 (*MAT*a). Yeast strains were grown in YPD media (1% yeast extract, 2% bactopeptone, 2% D-glucose), which was solidified with 2% bacto-agar when necessary. For selective growth, geneticin (Astral Scientific) was added at 300 mg/l after autoclaving of the media. Yeast were sporulated on potassium acetate (KOAc) medium (1% potassium acetate, 0.1% yeast extract, 0.05% glucose, 2% bacteriological agar) for 8 days at 30° C. When required, asci were digested, spores dissected and mating carried out using standard classical genetic techniques (Guthrie and Fink, 1991).

2.2. Nucleic acid manipulation

Genomic DNA was isolated from yeast according to Adams et al. (1997). Plasmid DNA extraction was accomplished using commercial DNA purification kits (*Wizard® Plus Mini-preps*, Promega). DNA manipulations were performed according to Maniatis et al. (1982), using commercially available restriction enzymes, and T4 DNA ligase (Geneworks). *Dynazyme EXT* DNA polymerase (Finnzymes) was used in PCR reactions and associated primers are reported in Table 1. Plasmid constructs and PCR products were verified by DNA sequencing (IMVS, Adelaide, Australia).

2.3. Construction of plasmid pBS418

The gene encoding bacterial aminoglycoside-3′ phosphotransferase (*Kan*^{r}), which confers resistance to the aminoglycoside G418 (geneticin) when expressed in yeast, was amplified, together with the TEF^p and TEF^t flanking regions (from *A. gossypii*), by PCR from the plasmid pFA6a (Wach et al., 1994) using Pfu DNA polymerase (Promega). The primers were TEFfwd and TEFrev (Table 1). The plasmid, pBluescript SK-(Stratagene) and the PCR product from pFA6a were digested with *Sma*I and *Eco*RI and the fragments ligated together to produce pBS418. Correct function of the cloned *Kan*^{r} gene was confirmed by growing *E. coli* XL10-gold (Stratagene) containing pBS418 on agar plates supplemented with kanomycin (25 mg/l).

2.4. Construction of plasmid pBS418HODR4 for integration at HO and excision of Kan^r marker

Primers HO1 and HO2 (Table 1) designed according to the S288C genomic sequence from the *Saccharomyces* Genome Database (SGD, Accession number 9169867) (Jacq et al., 1997), were used to amplify genomic sequences from strain L2056. This 425 bp sequence contained the 5′ untranslated region of the *HO* gene (−724 to −368) and, as a 3′ targeting sequence, 65 bp of nucleotide sequence homologous to the 3′ untranslated region (+1965 to +2030). The direct repeat sequences in the disruption cassette were derived from a 272 bp nucleotide sequence (−670 to −399) within the PCR product. Unique restriction sites were engineered to allow the directional cloning of the direct repeat sequences and 3′ targeting sequence, as two fragments into plasmid pBS418.

PCR amplification was performed in a 50 μl reaction mixture using Taq DNA polymerase (Sigma), according to manufacturers instructions, and contained 50 pmoles of each primer (HO1 and HO2), and 200 ng of genomic DNA (from L2056) as template. The PCR cycle was initiated at 94 °C for 2 min, followed by 30 amplification cycles (94 °C, 30 s; 56.2 °C, 45 s; 72 °C, 1 min) and terminated with a 10 min extension at 72 °C. The product was purified using *UltraClean* kits (MioBio Laboratories).

The 425 bp PCR product and plasmid pBS418 were digested with *Kpn*I and *Asc*I, and ligated together, yielding plasmid pBS418HODR1. The latter contains a direct repeat sequence and 3′ targeting sequence, within a 394 bp sequence adjacent to the

Table 1
Sequences of the primers used in this study

Primer	Sequence (5′→3′)
HO1[a,b]	TCATgagctcTGtACAACATGGTAggcggccTGTCTCATGGAAATTGATGCAG
HO2[a,b]	TTGGggtaccACAATTAAAATATTTGTTTTTCTTTCGCGTAAATATTTAGCTTGATAACTGTTACTGATAgttaacGTGGACATGACATGAATTTTCGC
HOPIIrev[a,b]	TATTTACATCAACATATCGCGAAAATTCATGTCATGTCCACgttaacATATCAGTAACAGTTATCAAGCTA
HOPCRrev[b,c]	TCAGCAGATGGGTCTTCGAT
HOPIV[a,b]	GTGAACGTggtaccTGTAAAGAAAACACTTTGTTGGGTG
HOPV[a,b]	AGATGgtcgacCCAAGCTGAAGAGTTCAAGGC
HOPVI[b]	TGAAGAGTTCAAGGCTGCCGATG
HOPVIII[b]	GCTGGCATCATTGGGATGTTC
HORga[a,b]	CAAACCTAATGTGACCGTCGCT
HOFga[a,b,c]	TGAAGCTGTTGCTTACGGTGC
HOFwd[b]	GTCACGGCTAACTCTTACGTTATGTGCGCA
HOcRev[b]	GCTTCACGAACTTCTATATGCTCGCCGTAC
G418Fwd[c,d]	AAAAGACTCACGTTTCGAGGC
G418Rev[c,d]	CGAGCATCAAATGAAACTGC
TEFfwd[d]	CGATTGGACCCGGGGACATGGAGGCCCAGAA
TEFrev[d]	CAAGTCTAGAATTCGCATGCGGCGCGCCCGGCGTTAGTATCGA

Lower case sequences show novel restriction sites designed for cloning of the PCR products. Underlined sequence marks the part of the primer, representing overhangs not involved in annealing of primer during amplification of *HO* specific PCR product.
[a] Primers used in PCR amplification of *HO* gene fragments.
[b] Primers used in DNA sequencing of *HO* gene.
[c] Primers used as probes in Southern blot analysis.
[d] Primers used in PCR amplification of *Kan*^{r} sequences.

Kan^r gene. Digestion of the 425 bp PCR product with *Sac*I and *Hpa*I generated a 336 bp fragment (containing a single direct repeat) which was subcloned into *Sac*I-*Sma*I cleaved pBS418HODR1 to form pBS418HODR2 (Fig. 1).

The 3′ targeting sequence was extended from 65 bp to 619 bp (+2584 to +1965), to enhance gene disruption of *HO* by homologous recombination. A 687 bp PCR fragment was amplified from plasmid pGEMT-HO (Walker et al., 2003) as template and primers HOPIV and HOPIIrev, using *Dynazyme EXT* DNA polymerase (Finnzymes). The fragment was purified and used as template for amplification of the 687 bp fragment using the above primers and primer HOPV (converting the *Hpa*I site to *Sal*I at +2395 for ease of cloning.) Digestion with *Kpn*I and *Hpa*I yielded a 644 bp fragment, which was subcloned into *Kpn*I–*Hpa*I cleaved pBS418HODR2 to form pBS418HODR4 (Fig. 1).

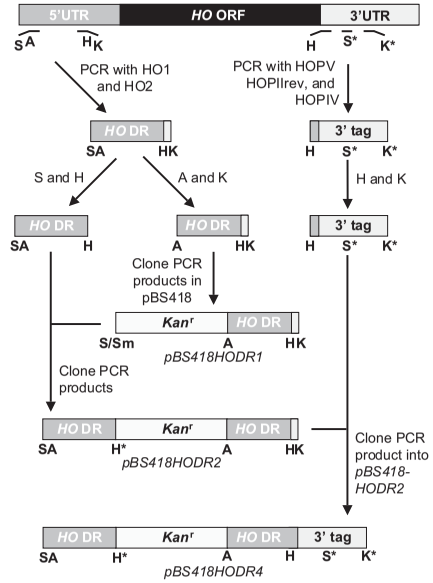


Fig. 1. Construction of *HO*-disruption cassette (*hodr–Kan*^{r}*–hodr*) in plasmid pBS418HODR4. *HO* ORF is indicated in black, the direct repeat sequences (−670 to −399) amplified from the 5′ untranslated region (−724 to −368) are in mid-grey, whilst the 3′ tag or targeting sequences of 65 bp (+1965 to +2030) or 619 bp (+2584 to +1965) are in light-grey. Primers used in the amplification are indicated as solid lines (with letters, depicting engineered restriction sites in the sequence: S (*Sac*I), H (*Hpa*I), A (*Asc*I), K (*Kpn*I)). H* indicates destroyed *Hpa*I site (H) via cloning in *Sma*I (Sm) site of plasmid pBS418HODR2. *Hpa*I sites in *HO* gene sequence modified in construction of plasmid pBS418HODR4 are: K* (*Hpa*I [GTT↓AAC] to *Kpn*I [GGTAC↓C]), S* (*Hpa*I [GTT↓AAC] to *Sal*I [G↓TCGAC]).

2.5. Construction of plasmid pBS418HODR6 for integration at HO

Plasmid pBS418HODR6 was constructed as a derivative of pBS418HODR4 which was devoid of the 3′ direct repeat and hence lacking the ability to excise the *Kan*^{r} marker following homologous recombination at the target locus. Therefore a 644 bp *Kpn*I–*Hpa*I fragment containing the 619 bp 3′ targeting sequence was subcloned into *Kpn*I–*Eco*RV cleaved pBS418, resulting in plasmid pBS418HODR5. The 336 bp *Sac*I–*Hpa*I fragment containing the repeat sequence found in pBS418HODR4 was subcloned into *Sac*I–*Sma*I cleaved pBS418HODR5 to form pBS418HODR6. In the latter, the single repeat sequence functions as a 5′ targeting sequence.

2.6. HO disruption and identification of ho::kan^r (geneticin resistant) haploid derivatives

HO was disrupted by the one-step gene disruption method (Rothstein, 1983). Wine yeast strains were transformed (Gietz et al., 1992) with either a 2.6 kb (*hodr–Kan*^{r}*–hodr*) *Kpn*I–*Sac*I fragment from pBS418HODR4 or a 2.3kb (*ho*::*Kan*^{r}) *Kpn*I–*Sac*I fragment from pBS418HODR6. The disruption of the chromosomal *HO* gene in the geneticin resistant transformants and their sporulation products was confirmed by Southern blot hybridisation (Maniatis et al., 1982) with chemiluminescent detection (Van Miltenburg et al., 1995), using a PCR-generated DIG-labelled 671 bp *HO* probe (primers: HOFga and HOPCRrev) and a 786 bp *Kan*^{r} probe (primers: G418Fwd and G418Rev). The haploid state of spores possessing a dysfunctional *HO* gene, was verified by PCR determination of mating type, the failure of the germinated spores to rediploidise and their appropriate behaviour with mating type tester strains (Walker et al., 2003).

2.7. Kan^r marker loss and deletion of the HO gene

Haploid (Δ*ho*) derivatives were identified, following selection of geneticin sensitive sporulation products, and subsequent determination of mating type (Huxley et al., 1990). Confirmation of the nature of the sequences retained at the disruption/excision site was achieved via sequencing of the region. The *HO* genes from the parental wine strains and haploid (Δ*ho*) derivatives were amplified using primers HOFga and HORga. DNA sequencing was carried out on the purified PCR products using primers spanning the *HO* gene (Table 1). Sequence analysis was performed using the BLAST program, the sequences being compared with laboratory strain S288C (http://www.yeastgenome.org/).

3. Results

3.1. Gene disruption of industrial yeast using hodr–Kan^r–hodr cassette

The original concept was to design gene-specific disruption cassettes, which possess a dominant selectable marker flanked by direct repeats of non-coding sequences from the target gene, as well as a short sequence (50–70 bp) to target insertion by homologous recombination. A single PCR product would provide the necessary sequences (direct repeats and targeting sequence), which could be selectively cleaved at unique sites and cloned into a plasmid in two steps (Fig. 1, pBS418HODR2). Transformation efficiency of this construct however proved impractically low and all transformants were determined to have mis-targeted integration (data not shown). The 65 bp 3′ targeting sequence in pBS418HODR2 was therefore replaced with a longer one of 619 bp (Fig. 1, pBS418HODR4). Transformation with the resulting *Kpn*I–*Sac*I (*hodr–Kan*^{r}*–hodr*) fragment was variable, yielding the equivalent of ~10 transformants per μg of *Kpn*I–*Sac*I fragment, which was ~4–6 fold less than with the *KanMX* disruption cassette previously reported by our laboratory (Walker et al., 2003) (data not shown).

3.2. Characterisation of HO disruption in haploid wine yeast

Southern blot analysis confirmed the correct localisation of the disruption cassette to the *HO* locus in two out of five transformants derived from L2056, and a single transformant from CY3079 (data not shown). Haploid (*ho*::*Kan*^{r}) derivatives of the geneticin resistant *HO*/*ho*::*Kan*^{r} transformants were obtained after sporulation and tetrad dissection. During dissection, it was observed that for most tetrads (84%) derived from L2056, only two of the four spores were viable. Variation in spore viability was also noted in tetrads derived from the transformant of CY3079, cyDR4, and ranged from 17% with four viable spores, 63% with three viable spores and 20% with two viable spores (Table 2). These findings are in contrast to our previous observations with *HO*-disruption using the *KanMX* construct in the same wine strains (Walker et al., 2003) where all spores were found to be viable. The expected pattern of segregation of *HO* (geneticin sensitive) and *ho*::*Kan*^{r} genes (geneticin resistance), that is 1:1 or 2:2, was observed in only 40 out of 73 (i.e. 55%, L2056) and 13 out of 54 (i.e. 25%, CY3079) of dissected tetrads.

A further construct was made to determine whether the DNA configuration (*hodr–Kan*^{r}*–hodr*) in pBS418HODR4 was responsible for the unexpected segregation of the *HO* and *ho*::*Kan*^{r} genes

Table 2
Tetrad analysis of the (*HO*/*ho*::*kan*^{r}) transformants

Transformant	Tetrads with the indicated number of viable spores					Number of tetrads yielding the indicated segregation pattern of G418^R:G418^S spores													Number of Δ*ho* progeny[a]
	0	1	2	3	4	1:0	0:1	1:1	2:0	2:1	2:2	1:2	0:2	3:0	3:1	1:3	0:3	4:0	
DR4-1	0	0	15	1	1	0	0	10	3	0	0	0	2	1	0	0	0	1	1
DR4-2	0	5	46	4	1	3	2	29	9	1	1	1	8	0	0	0	2	0	0
cyDR4	0	0	11	34	9	0	0	8	1	19	5	16	1	0	1	2	1	0	1
DR6-2	0	0	0	0	11	0	0	0	0	0	11	0	0	0	0	0	0	0	ND[b]

[a] Number of G418^S spores determined to be haploid and carrying the deleted *HO* gene.
[b] ND. Not determined.

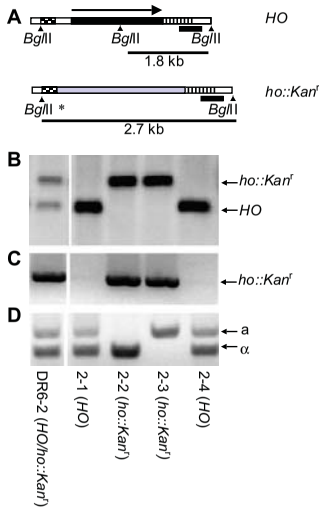


Fig. 2. Characterisation of *HO*-disruptant strains by Southern blot and PCR analysis. Block diagram (panel A) depicts the main features of the *HO* and *ho*::*Kan*r alleles on the chromosome locus. The 5′ and 3′ *HO* untranslated sequences are indicated by open bars, *HO* open-reading frame in black, and *Kan*^{r} sequence in grey. The 5′ targeting sequence within the disruption cassette consists of a single direct repeat generated from the 5′ untranslated sequence, shown as a stippled region, whilst the 3′ tag or targeting sequence is shown by a striped region. The direction of *HO* gene transcription is shown with an arrow, and solid bars indicate the 671-bp *HO* probe. Labelled (1.8 and 2.7 kb) lines indicate the fragments liberated from the *HO* and *ho*::*Kan*^{r} alleles upon *Bgl*II digestion and contributing to the banding pattern seen in the Southern blot analysis (panel B) of genomic DNA from *ho*-disruptant strains when hybridised with the 671-bp *HO* probe. The engineered *Sac*I site in the disruption construct is restored during homologous recombination to the *Bgl*II site (*Bgl*II*) originating from the native *HO* gene. The presence of the *Kan*^{r} marker was shown by the presence of a 786-bp PCR product using *Kan*^{r} specific primers G418Fwd and G418Rev (panel C). Mating type of the strains was shown by the presence or absence of the *MAT*a (554-bp) or *MAT*α (404-bp) PCR amplification products (panel D).

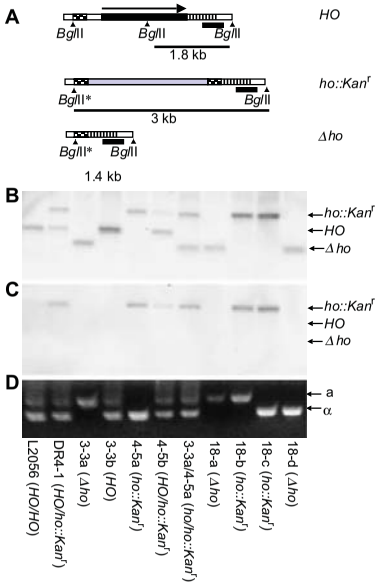


Fig. 3. Characterisation of haploid *ho*::*Kan*^{r} and Δ*ho* strains by Southern blot analysis and PCR analysis. Block diagram (panel A) depicts the main features of the *HO*, *ho*::*Kan*^{r} and Δ*ho* alleles on the chromosome locus. The 5′ and 3′ *HO* untranslated sequences are indicated by open bars, *HO* open reading frame in black, and *Kan*^{r} sequence in grey. Direct repeat sequences generated from within the 5′ untranslated sequence, are shown as a stippled region, and 3′ tag or targeting sequence by a striped region. The direction of *HO* gene transcription is shown with an arrow, and solid bars indicate the 671-bp *HO* probe. Labeled (1.8, 3 and 1.4 kb) lines indicate the fragments liberated from the *HO*, *ho*::*Kan*^{r} and Δ*ho* alleles upon *Bgl*II digestion and contributing to the banding pattern seen in the Southern blot analysis (panels B and C) of genomic DNA from *HO*-disruptant strains. The engineered *Sac*I site in the disruption construct is restored during homologous recombination to the *Bgl*II site (*Bgl*II*) originating from the native *HO* gene. The 671-bp *HO* probe (primers: HOFga and HOPCRrev) was used in panel B, and the 786-bp *Kan*^{r} probe (primers: G418Fwd and G418Rev) was used in panel C. Mating type of strains was shown by the presence or absence of the *MAT*a (554-bp) or *MAT*α (404-bp) PCR amplification products (panel D). Strains shown include the parental L2056 diploid, transformant DR4-1, two pairs of surviving tetrads (3-3a, 3-3b and 4-5a, 4-5b), the diploid 3-3a/4-5a derived from these, and a complete tetrad (18-a–18-d) from the latter diploid.

during meiosis. In the new construct, pBS418HODR6, the 336 bp *Sac*I–*Hpa*I fragment containing a single direct repeat sequence (identical to those sequences found in pBS418HODR4) and the 644 bp *Kpn*I–*Hpa*I fragment containing the 3′ targeting sequence were cloned either side of *Kan*^{r}. Transformation of L2056 with the cassette resulted in two transformants. Eleven tetrads were dissected from a single transformant (DR6-2) and were shown to exhibit four spore viability and the expected 2:2 segregation of *HO* and *ho*::*Kan*^{r} genes (Table 2).

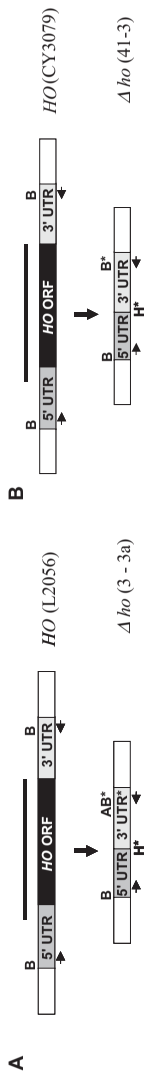
Transformant DR6-2 (*HO*/*ho*::*Kan*^{r}) and its sporulation products were further characterised using Southern blot analysis (Fig. 2). A 1.8 kb band detected by the *HO*-probe in the parental wine strain L2056 and DR6-2 was consistent with the wild-type *HO* gene predicted from sequence published on the *Saccharomyces* Genome Database (Jacq et al., 1997). A second larger band (2.7 kb) corresponding to the disrupted *ho* gene (*ho*::*Kan*^{r}), was detectable in DR6-2. The classical 2:2 segregation of *HO* and *ho*::*Kan*^{r} genes was observed in the dissected spores (Fig. 2B). The presence of the *ho*::*Kan*^{r} gene in DR6-2 and two of the spore progeny (2-2 and 2-3) was indicated by the presence of a 786 bp band after PCR amplification with *Kan*^{r} specific primers (Fig. 2C). These spores were further identified as either *MAT*a or *MAT*α by PCR (Fig. 2D), whereas the remaining two spores in the tetrad possessed both *MAT* bands, thereby indicating they possessed a functional *HO* gene (corresponding to the 1.8 kb band) which had caused mating type switching and rediploidisation (Herkowitz and Oshima, 1983).

3.3. Identification of excision of Kan^r marker in haploid (Δho) wine yeast

Loop-out of the *Kan*^{r} marker was shown to occur during sporulation of the geneticin resistant (*HO*/*ho*::*Kan*^{r}) transformants of L2056 and CY3079, namely DR4-1 and cyDR4. The dissected tetrads (each with two viable spores) were initially examined on plates and assayed for geneticin sensitivity. The mating type of the geneticin sensitive colonies was determined by PCR prior to Southern blot analysis. A single geneticin sensitive haploid colony was identified for each of L2056 (3-3a; *MAT*a) and CY3079 (41-3; *MAT*α) and both were characterised in parallel. Findings for derivatives of each haploid were comparable, therefore only data for derivatives of L2056 are reported here. As shown in Fig. 3, strain 3-3a, the haploid derivative of L2056, carried a deletion of the *ho* gene: indicated by a 1.4 kb band during Southern blot analysis (Fig. 3B), or a similar sized band following PCR amplification using HORga and HOFga primers (data not shown). These fragment sizes were consistent with that predicted for removal of the *Kan*^{r} gene and a single direct repeat (DR) sequence. Loss of the selectable marker was also confirmed by the absence of fragments with homology to the *Kan*^{r} probe (Fig. 3C). In comparison, most haploid *ho*-disruptants in which loop out had not occurred possessed a 3 kb band that hybridised with both the *HO* and *Kan*^{r} probes (Fig. 3B and C).

Classical genetics was used to obtain haploid Δ*ho* derivative strains of L2056 of both mating types. Strains 3-3a (*MAT*a, Δ*ho*) and a second haploid derivative of L2056, namely 4-5a (*MAT*α, *ho*::*Kan*^{r}) were crossed, diploids isolated by micro-dissection

Fig. 4. DNA sequence analysis of the *HO* gene after loop out of *Kan*^{r} and direct repeat (DR) sequences. Block diagrams showing the DNA sequences retained at the *HO* locus following gene disruption and marker excision by homologous recombination in the commercial strains L2056 (panel A) and CY3079 (panel B). Deleted *HO* sequence is indicated by the solid line above the native *HO* gene. *HO* ORF is indicated in black, the 5′ untranslated region in mid-grey, the 3′ untranslated region in light grey whilst flanking sequences are in white. The *Bgl*II sites recognized in Southern blot analysis (Fig. 3) are indicated as B and B* (*Sac*I→*Bgl*II); the latter site is shown as *agatct* in the DNA sequences derived from the marker-free haploids, 3-3a and 41-3 (panel C, see text for provenance details). The introduced *Hpa*I (H*) and *Asc*I (A) sites and positions of the primers HORga and HOFga (—▶) are also shown. Panel C: DNA sequence of *HO* gene amplified using HORga and HOFga primers. The amplified sequence includes flanking regions (standard font) and the modified sequence comprising the 5′ untranslated region (underlined standard font) and the residual direct repeat (underlined bold italics) and the 3′ untranslated targeting sequence (bold). Restriction sites indicated in bold and underlined font are the *Asc*I site (GgCGcGcc) engineered in the *HO* sequence through Primer HO1, and native *Hpa*I sites (GTTAAC) and the introduced *Hpa*I site at the juxtaposition of the 5′ and 3′ sequence (GTTAAc). The *Bgl*II and *Hpa*I restriction sites restored during homologous recombination are indicated in underlined lower case. Nucleotide changes relative to the parental strain are noted in lower case.



C

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3-3a CAAACCTAAT GTGACCGTCG CTGATTAGCG GATCACGAAA AGTGATCTCG ATACAATTAG AGGATCCACG AAAATGATGT GAATGAATAC ATGAAAGATT 100
41-3 CAAACCTAAT GTGACCGTCG CTGATTAGCG GATCACGAAA AGTGATCTAG ATACAATTAG AGGATCCACG AAAATGATGT GAATGAATAC ATGAAAGATT 100

3-3a CATGagatct GACAACATGG TAGgCGcGcc TGTCTCATGG AAATTGATGC AGTTGAAGAC ATGTGCGTCA CGAAAAAAGA AATCAATCCT ACACAGGGCT 200
41-3 CATGagatct GACAACACGG TAGACGTGTG TGTCTCATGG AAATTGATGC AGTTGAAGAC ATGTGCGTCA CGAAAAAAGA AATCAATCCT ACACAGGGCT 200

3-3a TAAGGGCAAA TGTATTCATG AAAGTGAGGT AACTAAATAC ACGATACCA TGGAAATTAA CGTACCTTTT TTGTGCGTGT ATTGAAATAC 300
41-3 TAAGGGCAAA TGTATTCATG TGTGTCACGA AAAGTGAGGT AACTAAATAC ACGATACCA TGGAAATTAA CGTGCCTTTT TTGTGCGTGT ATTGAAATAC 300

3-3a TATGACATAT TACAGAAAGG GTTCACAAGT CCTGTTTCTA TGCCTTTCTC TTAGTAATTC ACGAAATAAA CCTATGGTTT ACGAAATGAT CCACGAAAAT 400
41-3 TATGACATAT TACAGAAAGG GTTCACAAGT CCTGTTTCTA TGCCTTTCTC TTAGTAATTC ACGAAATAAA CCTATGGTTT ACGAAATGAT CCACGAAAAT 400

3-3a CATGTTATTA TTTACATCAA CATATCGCGA AAATTCATGT CATGTCCACG TTAAcATATC AGTAACAGTT ATCAAGCTAA ATATTTACGC GAAAGAAAAA 500
41-3 CATGTTATTA TTTACATCAA CATATCGCGA AAATTCATGT CATGTCCACG TTAAcATATC AGTAACAGTT ATCAAGCTAA ATATTTACGC GAAAGAAAAA 500

3-3a CAAATATTTT AATTGTGATA CTTGTGAATT TTATTTTATT AAGGATACAA AGTTAAGAGA AAACAAAATT TATATACAAT ATAAGTAATA TTCATATATA 600
41-3 CAAATATTTT AATTGTGATA CTTGTGAATT TTATTTTATT AAGGATACAA AGTTAAGAGA AAACAAAATT TATATACAAT ATAAGTAATA TTCATATATA 600

3-3a TGTGATGAAT GCAGTCTTAA CGAGAAGACA TGGCCTTGGT GACACACTCTC TTCAAACCAA CTTCAGCCTT TCTCAATTCA TCAGCAGATG GGTCTTCGAT 700
41-3 TGTGATGAAT GCAGTCTTAA CGAGAAGACA TGGCCTTGGT GACAACTCTC TTCAAACCAA CTTCAGCCTT TCTCAATTCA TCAGCAGATG GGTCTTCGAT 700

3-3a TTGCAAAGCA GCCAAAGCAT CGGACAAAGC AGCTTCAATC TTGGACTTGG AACCTCTCTT CAATTTAGAA GACAAGACTG GGTCAGTGAC AGTTTGTTCG 800
41-3 TTGCAAAGCA GCCAAAGCAT CGGACAAAGC AGCTTCAATC TTGGACTTGG AACCTCTCTT CAATTTAGAA GACAAGACTG GGTCAGTGAC AGTTTGTTCG 800

3-3a ATGGAGGCAA CGTAGGATTC CAATCTTTGT CTAGCTTCGT GCTTCTTGGC AAAAGCTTCA TCGGCAGCCT TGAACTCTTC AGCTTGGTTA ACCATCTTTT 900
41-3 ATGGAGGCAA CGTAGGATTC CAATCTTTGT CTAGCTTCGT GCTTCTTGGC AAAAGCTTCA TCGGCAGCCT TGAACTCTTC AGCTTGGTTA ACCATCTTTT 900

3-3a CAATTTCTTC AGAAGACAAT CTACCACAG CGTTAGAGAT AGTGATGTTA GAAGACTTAC CGGTAGACTT TTCGACGGCA GTAACCTTCA AGATACCGTT 1000
41-3 CAATTTCTTC AGAAGACAAT CTACCACAG CGTTAGAGAT AGTGATGTTA GAAGACTTAC CGGTAGACTT TTCGACGGCA GTAACCTTCA AGATACCGTT 1000

3-3a AGCATCAACT TCGAAGATAG CTTCCAAGAC TGGTTCACCA GCTGGCATCA TTGGGATGTT CTTCAAGTCG AATTCACCCA ACAAAGTGTT TTCTTTACAg 1100
41-3 AGCATCAACT TCGAAGATAG CTTCCAAGAC TGGTTCACCA GCTGGCATCA TTGGGATGTT CTTCAAGTCG AATTCACCCA ACAAAGTGTT TTCTTTACAg 1100

3-3a ttaacACGTT CACCTTGGTA GACTGGGAAT TGAACGGcGG TTTGGTTGTC AGCACATGTA GTAAAGGTTC TTCTCTTGAT GGTTGGAACA GTAGTGTTTC 1200
41-3 ttaacACGTT CACCTTGGTA GACTGGGAAT TGAACGGTGG TTTGGTTGTC AGCACATGTG GTAAAGGTTC TTCTCTTGAT GGTTGGAACA GTAGTGTTTC 1200

3-3a TTGGAACAAC GATACCGAAC ATGTCACCTT GCATACCAAC ACCTAGAGAT AATGGAGCAA CATCTAACAA CAACAAGTCC TTGGTTTCGT CAGATGTGGA 1300
41-3 TTGGAACAAC GATACCGAAC ATGTCACCTT GCATACCAAC ACCTAGAGAT AATGGAGCAA CATCTAACAA CAACAAGTCC TTGGTTTCGT CAGATGTGGA 1300

3-3a TTGGCCGGTC AAGATAGCAC CTTGAACAGC AGCACCGTAA GCAACAGCTT CA
41-3 TTGGCCGGTC AAGATAGCAC CTTGAACAGC AGCACCGTAA GCAACAGCTT CA
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and sporulated. The resulting tetrads were dissected and the spores grown on YPD prior to replica streaking onto YPD in the presence and absence of geneticin. Interestingly, spore viability was restored, with 85% of dissected tetrads bearing four viable spores and the remaining 15% of tetrads having three viable spores. The expected 2:2 pattern of segregation of Δ*ho* (geneticin sensitive) and *ho*::*Kan*^{r} (geneticin resistant) alleles was observed in the dissected spores. Characterisation of a typical tetrad (18-a–18-d) originating from this 3-3a/4-5a (Δ*ho*/*ho*::*Kan*^{r}) diploid is shown in Fig. 3. Subsequent fermentation trials of both Δ*ho* and *ho*::*Kan*^{r} disruptants revealed fermentation profiles similar to the parental wine strain L2056, while spore viability was seen to be restored to 100% upon successive rounds of mating and sporulation (data not shown).

3.4. Characterisation of HO gene after excision of Kan^r marker

The *HO* gene was sequenced in Δ*ho* strains derived from L2056 (3-3a, *MAT*a) and CY3079 (41-3, *MAT*α) to verify the loss of the *Kan*^{r} gene and a single DR, and to determine the precise nature of any residual sequences (Fig. 4). The region was amplified using the HOFga and HORga primers, which flank the manipulated region in the disruption construct, i.e. the sequence between the *Bgl*II (*agatct*) and *Hpa*I (*gttaac*) sites. These sites were regenerated at the *HO* locus during gene disruption via homologous recombination; the sequence of the *Hpa*I (GTT↓AAC) site was modified to a *Kpn*I (GGTAC↓C) recognition site whilst the *Bgl*II (A↓GATCT) site was modified to a *Sac*I (GAGCT↓C) recognition site in the plasmid pBS418HODR4 (Fig. 1). The engineered *Sac*I and *Kpn*I sites were at the 5′ and 3′ ends of the 2.6 kb (*hodr*–*Kan*^{r}–*hodr*) fragment used for the gene disruption of L2056. The restoration of the *Bgl*II site was confirmed by Southern blot analysis of the *ho*::*Kan*^{r} and Δ*ho* strains (Figs. 2 and 3). The size of the hybridising band observed in strain 3-3a (Δ*ho*) corresponded to that predicted from the DNA sequence originating in S288c. The other engineered sites; *Asc*I (GG↓CGCGCC) in Primer HO1, *Hpa*I (GTT↓AAC) in Primer HO2 were unaltered, whilst the *Sal*I site (G↓TCGAC) in Primer HOPV was restored to *Hpa*I (GTT↓AAC). A single transition (T–C at 455) was also observed in the DNA sequence of *HO* originating from 3-3a when compared to the parental strain L2056. These six nucleotide changes, (five of which were as a consequence of the engineered restriction sites), are minor modifications, when compared to the residual direct repeat sequences (~400 bases) left at the target locus in gene disruptions using *KanMX* (Puig et al., 1998; Walker et al., 2003). In the case of strain 41-3, DNA repair during recombinatory marker excision and subsequent meiosis, resulted in the restoration of the sequences native to *HO*, with the exception of a single cytosine creating the *Hpa*I site (GTT↓AAC) juxtaposing the 5′ and 3′ untranslated sequences (Fig. 4).

4. Discussion

We have undertaken to develop a construct that allows a seamless disruption, in this case of the *HO* gene, in both industrial and laboratory strains. Importantly, direct repeat (DR) sequences sourced from the target gene have been incorporated with the goal of enabling the removal of the dominant selectable marker by homologous recombination, leaving only self-DNA in its native location. Several haploid and diploid Δ*ho* strains have been constructed (the latter through mating), which can be considered as 'self-cloned' yeast. Sequence analysis of the target region has revealed the precise nature of the sequences left after loop-out of the marker. The configuration of the disruption construct did in fact enable effective removal of the intervening selectable marker and one of the DR sequences. Further, the residual DR and 3′ tag (or targeting sequence) were found in their correct position with only the ORF that ordinarily separates them now being absent. Only a single base pair change was introduced through the process, with all other deviations arising from the deliberate introduction of restriction sites to aid construction. The latter might be avoided altogether, where appropriate restriction sites unique to the target gene and not found in the dominant selectable marker occur naturally at the target site in a configuration appropriate for cloning purposes. Alternatively, deliberate introduction of single base modifications would allow for site-directed mutagenesis of the sequence incorporated in the DR.

In what may be a feature of the construct used for this gene only, the heterozygous transformants displayed reduced spore viability upon sporulation. These findings contrast those obtained previously with a forerunner construct also used to disrupt the *HO* gene (Walker et al., 2003). Investigations into the basis for this observation using a second construct, pBS418HODR6, which lacked the DRs but spanned the same region of the *HO* gene and flanking sequences were informative. Spore viability and segregation patterns were as expected using pBS418HODR6, thereby suggesting that for pBS418HODR4, the loop-out process or the direct repeats themselves were the source of the reduced spore viability. The fact that spore non-viability did not co-segregate with the *ho*::*Kan*^{r} allele in the present work is taken to indicate the involvement of a post-meiotic event. Given the range of responses seen for derivatives of L2056 compared to CY3079, this phenomenon is apparently also manifested in a strain dependant manner. It is proposed that this event is caused by the proximity of the direct repeats to each other, affecting separation of the chromatids during metaphase (Davis and Smith, 2001). Interestingly, viable spores carrying the *ho*::*Kan*^{r} allele were subsequently shown to behave predictably during the classical genetic breeding used to generate both mating types of the haploid Δ*ho* strains.

Since the demand for enhanced strains for industrial use remains high, tailoring of this disruption system to other genes of interest represents one approach to targeted modification of industrial strains. A key advantage of such constructs is that they enable production of recombinant strains free of extraneous sequences, thereby heightening the regulatory and consumer acceptability of resulting strains. The utility of this system also extends to research conducted with laboratory strains. The generation of isogenic pairs of deletion and wild type strains by gene replacement (Rothstein, 1983; Wach et al., 1994), typically exploits auxotrophic markers or 'gain-of-function' selectable markers (i.e. antibiotic resistance genes) for identification of transformants and hence disruptants. However, recent reports put the assumption of isogenicity of such strains into doubt. Genetic complementation and supplementation of the growth medium do not produce equal responses (Çakar et al., 1999; Pronk, 2002) and the metabolic load associated with continued expression of an antibiotic resistance gene might make such strain sets inappropriate for use in studies of the metabolic consequences of deletion of the target gene. Accordingly, systems that bring about seamless gene deletion are important tools for modification of laboratory strains and are vital for manipulation of industrial strains.

To our knowledge, only the present system and the *delitto perfetto* system (Storici et al., 2001) satisfy the criteria set out at the commencement of this study. That is, target specificity, use of a dominant selectable marker that is removable and does not leave sequences foreign to the organism or outside their native location. It is clear no gene disruption system will be applicable in all situations. Limitations may be encountered in the generation of the disruption construct or the efficiency of transformation or marker removal. As such the availability of alternate gene disruption systems is an advantage. However the present system has advantages beyond simply being an alternative. Unlike the *delitto perfetto* system (Storici et al., 2001), our system does not require two constructs and involves only a single transformation. Further enhancement of our disruption cassette might include introduction of a second counter selection marker, such as the growth inhibitory *GIN11* sequence of the *GAL10* promoter (Akada et al., 2002) or general amino acid permease (*GAP1*) gene (Regenberg and Hansen, 2000), to improve selection of marker gene(s) excision by homologous recombination.

To conclude, we have described a viable system for seamless gene deletion in both laboratory and industrial yeast. The system utilises a DR–*Kan*^{r}–DR construct whereby integrants are selected by virtue of their acquisition of resistance to geneticin. A key feature of the system is that the DR sequences are derived from the target gene. As a result the integration event is targeted, and following marker removal by loop-out, the single remaining DR is correctly located within the native sequence. Appropriate design of one of the DR sequences would also allow site-directed mutagenesis. This system is therefore useful in the introduction of specific gene deletions in industrial strains and may additionally offer some advantages to construction of laboratory strains in certain circumstances.

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Generation of Novel Wine Yeast Strains by Adaptive Evolution

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Abstract: As a non-recombinant means of strain improvement, adaptive evolution is a technique with great potential. In this first report of the use of adaptive evolution in the improvement of a commercial wine yeast strain, a sequential batch fermentation system was used to adaptively evolve the wine strain L-2056 and its haploid derivative, C9. Mutants were isolated under the selective pressures of a winelike fermentation after approximately 350 generations (from L-2056) and 250 generations (from C9) and were demonstrated to have altered production of metabolites, including ethanol, glycerol, and succinic and acetic acid. Additionally, the evolved isolate of the commercial wine yeast was able to more rapidly catabolize all available sugars under these conditions. These results endorse the potential of adaptive evolution as a tool for the non-recombinant modification and optimization of industrial yeast strains.

Key words: adaptive evolution, *Saccharomyces cerevisiae*, wine, strain optimization

In the production of fermented beverages such as wine, beer, and cider, yeast facilitate the biochemical conversion of sugars to ethanol and carbon dioxide and produce a number of sensorily important metabolites (e.g., higher alcohols, organic acids, and esters), consequently influencing product quality (Romano et al. 1998, Lambrechts and Pretorius 2000). Selected strains of *Saccharomyces cerevisiae* are widely used in these industries because of their generally greater fermentation reliability and their preferred sensory contribution to the product. Nevertheless, their application does not guarantee fermentation will proceed without difficulties. For example, a common problem affecting winemaking, often despite inoculation with selected strains, is the occurrence of stuck or sluggish fermentations, which result from a attenuation of yeast sugar catabolic capacity and/or restricted biomass formation (Salmon 1996, Bisson and Butzke 2000). The problem can arise for many reasons (Alexandre and Charpentier 1998), but common causes are nutrient deficiencies or the inability of the yeast to function effectively in the hostile environment prevailing in grape juice: low pH values (~3.5), high osmolarity (sugar content, up to 300 g L^{-1} for dry table wines), temperature extremes during processing (0 to 35°C), low oxygen availability or oxidative stress through aerobic handling, among others (Boulton 1980, Bisson 1999). Incompletely fermented wines not only fall outside specifications for some wine styles but also are at increased risk of microbial spoilage and oxidation, and hence loss of commercial value. As a consequence, there is a strong demand for strains that are better suited to these conditions and that can make a positive sensory contribution to the wine.

Optimized strains can be developed by several methods including hybridization or genetic engineering. However, hybridization strategies may be difficult to apply since wine yeasts are generally homothallic and frequently exhibit poor sporulation and spore viability (Snow 1983, Walker et al. 2003). Additionally, screening for hybrids can be complicated since industrial strains do not necessarily have auxotrophic markers and dominant markers (arising through spontaneous mutation) must be specifically isolated and checked for retention of original fermentative properties (Pérez et al. 2000, Ambrona et al. 2005). As an alternative, recombinant techniques are precise but the use of this technology to optimize strains intended for fermented beverage production is not favored by some consumer groups (Pretorius and Bauer 2002). Adaptive evolution is an approach that has yet to be widely used in the development of optimized industrial yeast.

Adaptive evolution defines a set of mutations that occur in response to a specific challenge and that are advantageous to the cell under these conditions (Foster

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1999). By this technique an organism is subjected to serial or continuous cultivation for many generations under conditions to which it is not optimally adapted (Brown et al. 1998, Ferea et al. 1999). In the presence of such an environmental stress, an increase in fitter variants occurs because of natural selection. The mechanism of adaptive evolution is still debated, but three models for this process are postulated: mutations are targeted toward genes in which a mutation directly relieves the stress (directed mutation model), genome wide mutation rates increase such that both adaptive and nonadaptive mutations are stimulated (hypermutation model), or mutations arise infrequently but DNA replications acting on multiple DNA copies making normal mutation appear enhanced or gene specific (cryptic growth model) (Hersh et al. 2004).

As a strain-optimization technique, adaptive evolution has the advantage of not necessarily requiring previous genetic modification of the strain of choice or application of a complicated method to identify desirable derivatives or knowledge of the genes involved in the attribute. Furthermore, the increased ploidy (that is, aneuploid and polyploid) of wine strains could be well suited to adaptation through this technique (Bakalinsky and Snow 1990, Querol et al. 2003). Consequently, adaptive evolution may offer a versatile non-recombinant means for the development of optimized wine yeast.

The aim of this study was to investigate the merits of the use of adaptive evolution to derive novel wine yeast strains, especially those suited to the stresses of fermentation. Given the complex nature of industrial grape juice/must fermentations, we used a system that would emphasize a specific subset of stresses prevalent under such conditions. These stresses were chosen to reflect typical practices in New World winemaking regions: deliberate inoculation of each batch of grape juice with an aerobically propagated starter culture. Accordingly, apart from the high sugar and low pH environment of the growth medium, we used a sequential batch fermentation system with each batch subjected to an aerobic propagation step followed by anaerobic fermentation. It was hypothesized that this multistressed approach would result in adaptation of the yeast culture to one or more of these parameters, yielding derivatives that were better able to grow under the imposed stress or yield a different profile of metabolites.

Materials and Methods

Yeast strains. *Saccharomyces cerevisiae* strains L-2056 (Lallemand, Adelaide, Australia, selected from vineyards of the Cotes du Rhone) and C9 (L-2056 *ho MAT*a), a stable haploid derivate thereof, were used. The construction of C9 by disruption of *HO* using a removable *KanMX*-based disruption construct is described elsewhere (Walker et al. 2003).

Sequential batch fermentation. A starter culture was grown aerobically for 24 hr at 30°C in a 250-mL shake flask containing 50 mL of YPD medium (1% w/v yeast extract, 2% w/v bactopeptone, 2% w/v D-glucose) and shaken at 160 rpm on a rotary platform. Adaptation of strains to high-stress conditions was achieved by growth in 1 L of a chemically defined grape juice medium (CDGJM) in sequential batch fermentations in bench-top fermentors (2 L, B Braun Biotech, Melsungen, Germany). The formulation of CDGJM used here differed from that originally reported (Henschke and Jiranek 1993) in that it contained glucose and fructose (each at 100 g L^{-1}) and ammonium sulfate (580 mg free amino nitrogen L^{-1}) as the sole nitrogen source (to eliminate mutants with amino acid auxotrophies, which would be impractical in industry) (Table 1). The sterols and unsaturated fatty acids, otherwise known as oxygen substitutes or survival factors (Larue et al. 1980), were deliberately omitted from the medium. Fermentations were initiated by introduction of the yeast starter culture to 5 x 10^6 cells mL^{-1} and the inoculated medium aerated

Table 1 Chemically defined grape juice medium (CDGJM) adapted from Henschke and Jiranek (1993). The medium was adjusted to pH 3.5 before dilution to the final volume and sterilization by filtration (0.45 µm).

Component	Amount (/L)
Carbon	
Glucose	100 g
Fructose	100 g
Salts and organic acids	
L-Malic acid	3.0 g
Potassium sodium tartrate	2.5 g
Citric acid	1.5 g
K_2HPO_4	1.14 g
$MgSO_4.7H_2O$	1.23 g
$CaCl_2.2H_2O$	0.44 g
Trace minerals	
$MnCl_2.2H_2O$	198.2 µg
$ZnCl_2$	135.5 µg
$FeCl_2$	32.0 µg
$CuCl_2$	13.6 µg
H_3BO_3	5.7 µg
$Co(NO_3)_2.6H_2O$	29.1 µg
$NaMoO_4.2H_2O$	24.2 µg
KIO_3 (10.8 µg)	
Vitamins	
Myo-inositol	100 mg
Pyridoxine HCl	2.0 mg
Nicotinic acid	2.0 mg
Calcium pantothenate	1.0 mg
Thiamine HCl	0.5 mg
p-Amino benzoic acid	0.2 mg
Riboflavin	0.2 mg
Biotin	0.125 mg
Folic acid	0.2 mg
Nitrogen	
Ammonium sulfate	2.78 g

using sterile compressed air (0.45 µm, 2 VVM) for 4 hr with agitation at 350 rpm. Aeration sought to replicate typical industry practices for generation of an inoculum by aerobic cultivation, during which a theoretical preaccumulation of sterols and unsaturated fatty acids could occur. Such an extensive aeration was considered to lead to an oxidative stress. Culture vessels were then fitted with a fermentation lock to allow them to become self-anaerobic and were maintained at 30°C with agitation at 150 rpm. Fermentations were complete when less than 2.5 g L^{-1} of residual sugars remained, at which point the fermentor was drained. Sufficient culture remained in the fermentor to inoculate a fresh batch of media to ~1 x 10^5 cells mL^{-1}. The refilled fermentor was sparged with compressed air as before, the fermentation lock refitted, and the culture was again monitored for fermentation completion before the entire cycle (draining, refilling, sparging, fermentation) was repeated.

Analysis of culture isolates. Given each batch fermentation was inoculated to ~1 x 10^5 cells mL^{-1} and resulted in ~2 x 10^8 cells mL^{-1}, there were of the order of 11 generations per fermentation cycle. Culture samples were collected at 50-generation (culture doubling) interims, checked for purity by plating, and designated FM1 to FM5 (for C9) and FM10 to FM16 (for L-2056). The fermentation performance of cultures from each sample was compared to the parental strains in separate shake-flask cultures. Upon identification of novel attributes, after some 250 and 350 generations for strains C9 and L-2056, respectively, the fermentation performance of both the mixed cultures and the 10 single-colony clones of each evolved culture was evaluated. Clones were chosen randomly through colony isolation after streak-plating and incubation on YPD. Batch fermentations where conducted by inoculating yeast from plates into 10 mL YPD and grown overnight (to ~2 x 10^8 cells mL^{-1}) before inoculation at 1 x 10^5 cells mL^{-1} into 100 mL CDGJM. Triplicate fermentations of parental strains and mixed culture isolates were conducted. Single-colony isolates were conducted as single fermentations. Fermentations were performed as described previously (Walker et al. 2003) in 250-mL conical flasks fitted with a fermentation lock. Near-anaerobic conditions of industrial fermentations were mimicked by flushing the headspace of each flask after inoculation with filtered (0.45 µm), high-purity nitrogen (~20 VVM, 5 min). Flasks were incubated at 30°C with shaking at 160 rpm.

Analytical methods. Culture development was monitored during logarithmic phase by determining the optical density at 600 nm (OD_{600}). Culture dry weight was determined by collecting biomass from 5-mL culture aliquots on a predried and preweighed 0.22-µm membrane filter, followed by washing, drying, and reweighing. Commercially available kits were used for enzymatic quantitation of glucose and fructose (Boehringer Mannheim, Mannheim, Germany). Succinic acid, acetic acid, acetaldehyde, glycerol, and ethanol were determined by HPLC analysis as described previously (Walker et al. 2003).

Results

Chemostat selection and analysis of candidate variants. In order to isolate yeast strains that displayed phenotypes of greater fermentation performance under stressful winelike conditions, *S. cerevisiae* strains L-2056 and C9 were subjected to adaptive evolution through sequential batch fermentation. Experimental design was based on the notion that the condition of excess oxygen before an extended anaerobic fermentation of a medium devoid of sterols and unsaturated fatty acids, high in sugar, and low in pH would provide a selective pressure(s). Low inoculum density further exacerbated the influence of these stresses by encouraging greater cell division and therefore a reduction in the cellular concentrations of sterols and unsaturated fatty acids at the end of fermentation. It was hypothesized that evolved strains dominating the culture after extended exposure to these stresses would be better able to complete fermentation during exposure to one or more of these parameters.

Each chemostat was sampled every 50 generations, and a number of fermentation parameters were analyzed. Comparative small-scale batch fermentations of mixed cultures were conducted to allow monitoring of fermentation duration, glucose, and fructose utilization and the formation of biomass, acetic acid, succinic acid, glycerol, and ethanol. Fermentation duration by each 50-generational sample was seen to match the parent strains until a point where an adaptive mutation(s) was proposed to have occurred: after 250 generations under selective pressure for C9 and after 350 generations for L-2056. The mixed culture (FM16-M; M denotes the mixed, unpurified culture) derived from L-2056 displayed superior fermentative abilities (Figure 1) as well as altered metabolite yields (data not shown). While that was initially also the case for cultures derived from C9 (FM5), subsequent analysis revealed that significant differences were limited to parameters other than fermentation kinetics (Table 2). Thus, FM5-M produced 8.6% more biomass at the end of fermentation than the parent strain. Also, ethanol yield was decreased, with a concurrent increase in glycerol, acetic acid, and acetaldehyde.

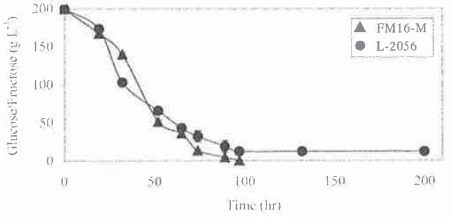


Figure 1 Fermentation kinetics of the mixed evolved culture FM16-M compared to the parental strain L-2056. Fermentations were performed in CDGJM. Data points are means of triplicate fermentations ± standard deviation.

For FM16-M, the differences between the evolved cultures and the parent were typically greater in magnitude (Table 3). FM16-M was able to catabolize all available sugar after 96.5 hr, which is ~69% of the time required by L-2056 from which FM16-M was derived. No deviation in biomass formation was apparent between FM16-M and the parent strain; however, the production of most metabolites by FM16-M did deviate. For instance, there was a marked increase (12%) in glycerol production in the mixed evolved culture FM16-M and less succinic acid produced compared with L-2056 (Table 3). Unlike the FM5-M and C9 comparison, there was little deviation between FM16-M and L-2056 in the production of acetic acid. Culture growth, estimated by absorbance during exponential growth and as a function of cell numbers at the end of fermentation, did not differ markedly between evolved cultures and their respective parental strain (data not shown).

Table 2 Metabolites and fermentation kinetics of adaptively evolved cultures derived from the parental population of C9.

Parameter	C9[a]	FM5-M[b]	Mean of clones (range)
Dry weight (mg mL^{-1})	6.236 ± 0.241	108.6	105.4 (92.5–155.6)
Fermentation duration (hr)	62	100.0	100 (100)
Succinic acid (g L^{-1})	0.22 ± 0.01	97.4	109.4 (89.7–125.1)
Glycerol (g L^{-1})	7.09 ± 0.07	110.3	108.2 (102.2–115.2)
Acetic acid (g L^{-1})	1.39 ± 0.03	109.1	113.9 (97.8–127.6)
Acetaldehyde (g L^{-1})	0.003	120.0	110.0 (90.0–150.0)
Ethanol (g L^{-1})	102.36 ± 1.17	86.5	89.3 (87.1–100.3)

[a]Absolute values ± standard deviation are shown for C9.
[b]Data for FM5-M (mixed culture) and the mean of the nine single-isolate clones (C mean) are expressed as a percentage of C9.

Table 3 Metabolites and fermentation kinetics of adaptively evolved cultures derived from the parental population of L-2056.

Parameter	L-2056[a]	FM16-M[b]	Mean of clones (range)
Dry weight (mg mL^{-1})	4.674 ± 0.071	98.7	95.7 (85.1–101.6)
Fermentation duration (hr)	140	68.9	70.3 (55.4–85.0)
Succinic acid (g L^{-1})	0.35 ± 0.05	92.9	79.0 (50.4–109.6)
Glycerol (g L^{-1})	7.54 ± 0.11	112.0	109.2 (98.9–131.2)
Acetic acid (g L^{-1})	1.67 ± 0.02	105.8	98.2 (92.3–105.3)
Acetaldehyde (g L^{-1})	0.007	90.5	106.4 (85.7–128.6)
Ethanol (g L^{-1})	106.28 ± 1.21	96.5	98.8 (94.4–101.1)

[a]Absolute values ± standard deviation are shown for L-2056.
[b]Data for FM16-M (mixed culture) and the mean of nine single-isolate clones (C mean) are expressed as a percentage of L-2056.

To further elucidate the phenotypic differences between the two mixed-culture evolved cultures and their respective parents, 10 random single-colony representatives from the FM5-M and FM16-M populations were selected to undergo preliminary fermentation analysis (metabolite and fermentation data are shown in Tables 2 and 3, respectively). In both instances, one of the 10 analyzed clones failed to complete fermentation within the time frame of the experiment; therefore, the data for this strain was omitted from mean data calculations. For the most part, the mean for the clones from each culture modeled the trends of the corresponding mixed culture. However, there was occasional large variation in the production of metabolites and the measured fermentation dynamics for the single clones. For example, the mean succinic acid yields by the clonal cultures were seen to increase by ~10% rather than remaining largely unchanged in the mixed culture derived from FM5. Interclonal variation was also highlighted by the measures of dry cell weight; however, again the mean resembled that of the parent, C9. Metabolite differences between C9 and FM5, which are of greatest industrial importance, are highlighted in Figures 2 and 3. Most notably, FM5-M clearly produces more glycerol and less ethanol, with almost all FM5-C strains mimicking this result. Preliminary results for FM5-C3 suggest that this strain may demonstrate the most favorable metabolite production with regard to enological significance; that is, an increase in glycerol production and a reduction in ethanol production. Further characterization of this strain will better define the scale of these highlighted changes.

Similar patterns were seen in the FM16 single isolates, with the mean values for metabolite yields by these isolates broadly agreeing with those established for the mixed culture. Succinic acid values did vary widely, however (Table 3). Fermentation duration was dramatically reduced compared to the parent strain, L-2056 (Figure 4), although the variation between clonal cultures was wide (77.5 to 119 hr). Glycerol production by the same evolved isolates also consistently increased over the parent strain (Figure 5), and the mean of the single isolates was similar to that of the mixed culture. As with the analysis of the clonal derivatives from C9, examples can be found among the set of derivative of L-2056 which indicate marked changes in metabolite yields and fermentation duration. Because of the potential sensory and processing significance of these changes, such examples (FM16-C7) warrant further investigation under a broader set of conditions.

Discussion

The sluggish catabolism of sugars displayed by L-2056, as correlated by the protracted fermentation compared to the evolved cultures (Figure 1), is considered highly undesirable under industrial enological conditions. When sugar catabolism subsides toward the end of fermentation, it has a high likelihood of becoming stuck, which is problematic because residual sugar of even 5 to 10 g L^{-1} can dramatically change the style of a wine, especially when dry styles are the intended product (Iland and Gago 2002). In addition, restarting a fermentation in order to bring a wine within specification can be extremely difficult where arrest has occurred in the later stages when ethanol concentrations are high.

Numerous stresses are typically imposed on yeast under enological conditions, and such stresses may have contributed to the unintentional adaptive evolution of contemporary strains from less well-suited progenitors (Querol et al. 2003). It was therefore hypothesized that *deliberate* application of a selective pressure(s) through a laboratory-based adaptive evolution experiment could be used to yield yeast more adept at completing fermentation under winelike conditions. We applied an environment reminiscent of winemaking that could be considered highly stressful: oxidative stress from prefermentation propagation and aerobic handling of grape must/juice, extended fermentation induced by high concentrations of glucose and fructose, anaerobic growth in the absence of oxygen substitutes (sterols and unsaturated fatty acids), low pH, and a low rate of inoculation. The work was performed with a wine strain (L-2056) and, to facilitate later genetic characterization, a haploid derivate (C9) (Walker et al. 2003).

The measured fermentation duration for generational isolates up to and including FM4 (200 generations) and FM15 (300 generations) were similar to the parental strain in each case. However, adaptation to the selective pressure occurred in the haploid C9 culture after approximately 250 generations and the commercial L-2056 culture after approximately 350 generations. The evolved cultures were able to ferment more rapidly and/or produce altered amounts of metabolites with sensorial or

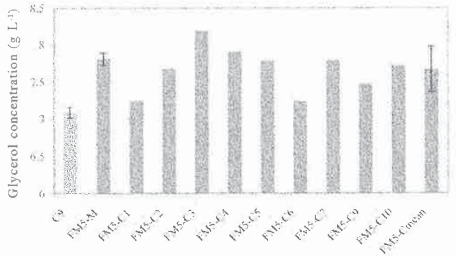


Figure 2 Concentration of glycerol produced by C9, the mixed evolved culture (FM5-M), and clonal isolates thereof (FM5-C1 to C10). Fermentations were performed in CDGJM. Values for C9 and FM5-M are the mean of triplicate fermentations ± standard deviation. Data for isolate FM5-C8 has been excluded as this culture failed to complete fermentation.

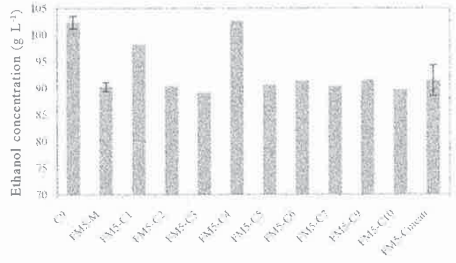


Figure 3 Concentration of ethanol produced by C9, the mixed evolved culture (FM5-M) and clonal isolates thereof (FM5-C1 to C10). Fermentations were performed in CDGJM. Values for C9 and FM5-M are the mean of triplicate fermentations ± standard deviation. Data for isolate FM5-C8 has been excluded as this culture failed to complete fermentation.

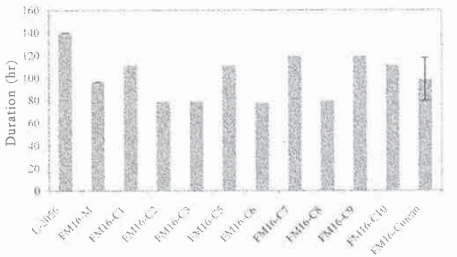


Figure 4 Fermentation duration of L-2056, the mixed evolved culture (FM16-M), and clonal isolates thereof (FM16-C1 to C10). Fermentations were performed in CDGJM. Values for C9 and FM16-M are the mean of triplicate fermentations ± standard deviation. Data for isolate FM16-C4 has been excluded as this culture failed to complete fermentation.

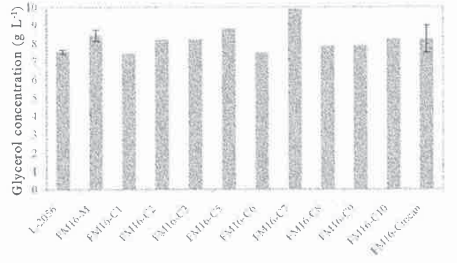


Figure 5 Concentration of glycerol produced by L-2056, the mixed evolved culture (FM16-M), and clonal isolates thereof (FM16-C1 to C10). Fermentations were performed in CDGJM. Values for C9 and FM16-M are the mean of triplicate fermentations ± standard deviation. Data for isolate FM16-C4 has been excluded as this culture failed to complete fermentation.

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quality importance for wine. Consequently, it is proposed that a defining mutation or mutations occurred after the points where FM4 and FM15 had been collected.

While the rapid fermentation seen by FM16-M and clonal isolates may be considered undesirable under optimal conditions, under suboptimal conditions such as during the nutrient limitations that commonly occur during grape juice fermentations (Henschke and Jiranek 1993, Alexandre and Charpentier 1998, Bisson 1999), this property may help overcome protracted fermentation. For the wine industry, fermentation reliability is a key yeast attribute (Pretorius 2000, Pretorius and Bauer 2002); thus a strain with such an improvement would be highly prized by winemakers. Perhaps of equal importance to rapid fermentation is completion of fermentation, again a property displayed by the majority of the clonal isolates from FM16-M. Together the attributes of reduced fermentation duration and complete catabolism of sugars can go a long way to achieving a final product without off-flavors and poor palate structure, thereby enhancing (or preserving) the commercial value of the wine.

Without large-scale gene or genome analysis techniques, the existence and precise nature, number, or origin of the mutations proposed to have occurred in this study cannot be defined. Nevertheless some comments about the adaptive evolution process are warranted. First, yeast in this experiment spent most time in a resting state. Thus of the three mechanisms by which mutations may arise (directed mutation model, hypermutation model, or the cryptic growth model) (Hersh et al. 2004), any mutations that occurred were likely to have arisen from a replication independent process (Heidenreich et al. 2003). It is also noteworthy that adaptive evolution of the haploid strain occurred some 100 generations sooner than it did for the commercial strain (presumed to be a diploid) (Walker et al. 2003). Ploidy may be important in this outcome (Orr and Otto 1994, Zeyl et al. 2003), although this disparity may not be the same in other circumstances. By definition, a new allele requires a molecular mutation event followed by a fixation process, whereby the new allele replaces all others in the population (Zeyl 2004). While higher ploidy reduces the waiting period by providing more alleles from which the next adaptive mutation can arise (Paquin and Adams 1983), nondominant mutations may take longer to fix because they confer smaller selective advantages on heterozygotes (Zeyl et al. 2003). Thus in a larger population, as in this experiment, fixing time rather than the waiting time takes precedence, and haploids should evolve more rapidly (Orr and Otto 1994). Further experiments with strains of different ploidy would help explain the basis for the different evolution rates.

Given that such an extended incubation was required to yield a culture with enhanced enological properties, a clearly predominant, single clone might have been expected (Sonderegger and Sauer 2003). That was not the case, however. Characterization of clonal isolates from the mixed cultures, FM5-M and FM16-M, revealed clear heterogeneity. The single-clone fermentations derived from both FM5-M and FM16-M generally yielded a mean response that was similar to that of the mixed-culture populations (Tables 2 and 3). One advantage of considering individual clones is that the performance of some appeared better than that of the mixed cultures. More detailed characterization of these and additional isolates might uncover even better clones.

Numerous groups have isolated evolved strains using continuous culture with defined working volumes and specified dilution rates (Ferea et al. 1999, Sonderegger and Sauer 2003). Here, use of a sequential batch cultivation system ensured that any evolved strains selected would be suited to fermentation under enological conditions and seemed to eliminate selection of alterations that are disadvantageous to general fermentation performance. This system, although useful in this application, extended the duration of the selection process considerably. In a similar work on the adaptation of a haploid recombinant strain to anaerobic xylose utilization, Sonderegger and Sauer (2003) isolated an evolved strain after 460 generations over 266 days, whereas isolation of FM5-M after some 250 generations required 198 days. The difference in total duration between our batch-fed continuous fermentation and a continuous culture is mainly due to the prevalence of stationary phase during high sugar fermentations.

The increased biomass production (dry weight) demonstrated by evolved cultures at the end of fermentation might have arisen through a divergence of carbon during glycolysis. With FM5-M, increased biomass yield could be partly related to a reduced production of ethanol and/or a response to the toxicity of these metabolites (Aranda and del Olmo 2003). In a winemaking context, reduced ethanol yield can be a very positive outcome. Ethanol increases the perception of "heat" on the back palate and masks many flavor and aroma compounds of wine (Iland and Gago 2002). Thus the avoidance of higher ethanol concentrations, particularly those resulting from the use of overripened fruit, can be most beneficial. So-called low-ethanol yeasts are attracting interest (Pretorius 2000, Pretorius and Bauer 2002), particularly in New World wine-producing countries where flavor-ripe grapes are readily achieved and the resulting flavor-intense wines are valued by consumers. A reduced ethanol yield would presumably also benefit the yeast itself by reducing the toxic effects of this metabolite during fermentation (Ansanay-Galeote et al. 2001). The superior performance of FM5 may in part be a result of the lower ethanol concentration of these fermentations.

Glycerol yields were increased by approximately 10% in both mixed evolved cultures. Recombinant strains that overproduce glycerol have previously been shown to also yield elevated amounts of acetic acid because of an oxidation of acetaldehyde by aldehyde dehydrogenases (Eglinton et al. 2002) as a mechanism for maintaining redox balance. While increases in acetic acid production were seen by the strains investigated here, the extent to which

such a compensatory process might explain these yields is uncertain. Similarly, the basis for the glycerol response of the evolved strains investigated here is yet to be determined, but mechanism aside, increased diversion of carbon to glycerol is likely to be viewed favorably by winemakers, as glycerol has the potential to enhance the mouthfeel of wine (Nieuwoudt et al. 2002).

Succinate production was increased in the haploid-evolved strains; however, a reduction was generally observed in the diploid. There may be several reasons for this deviation. Diversion of more carbon through the tricarboxylic acid pathway is one possibility. During fermentation, the tricarboxylic acid pathway operates in a branched fashion, and it is thought that most succinic acid is formed by the reductive pathway (Camarasa et al. 2003). Alternate routes rely on glutamate being present and therefore are probably unimportant for the ammonium-grown cultures investigated (Albers et al. 1998, Coleman et al. 2001, Tate and Cooper 2003). However, the genes involved in these pathways will be targeted in further work to help determine the extent of their involvement in the modified succinic acid yield of FM5-M, FM16-M, and clonal isolates.

Conclusion

Although metabolic engineering has become a standard practice for strain improvement in other industries, issues concerning consumer acceptance currently impede such use of strains in the wine industry. Adaptive evolution offers a non-recombinant technique to isolate commercial wine yeast strains that are highly tailored to the stressful conditions of a typical wine fermentation. To our knowledge, this report is the first to detail application of this technique to a commercial wine yeast strain. Our findings indicate the feasibility of this technology in this field even if some selection strategies, such as the sequential batch approach used here, require extended culture times.

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A survey of lactic acid bacteria for enzymes of interest to oenology

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Abstract

Isolates from wine-associated genera of lactic acid bacteria (LAB), including *Lactobacillus*, *Oenococcus* and *Pediococcus*, were examined for enzymatic properties that could be of value in the production and processing of wine. Up to 438 combinations of bacterial isolates and substrates were examined. Across the 50 isolates investigated, all were found to hydrolyse esters, with the greatest activity being seen amongst the oenococci followed by the lactobacilli and pediococci. Lipase activity was restricted to three *Lactobacillus* isolates, while all tested isolates displayed tannase activity. Cellulase, β-glucanase and lichenase activities were observed for almost all *Pediococcus* and *Lactobacillus* isolates, while such activities were seen for only a minority of oenococci. No degradation of the xylan substrate was detected. Once confirmed under wine-like conditions, our findings will help define potentially interesting genera of LAB for winemaking.

Abbreviations

LAB lactic acid bacteria; **MLF** malolactic fermentation; ***p*NP** *p*-nitrophenyl

Key words: *Lactic acid bacteria, hydrolytic enzymes, vinification*, Lactobacillus, Oenococcus, Pediococcus

Introduction

Wine is produced from grape juice largely through an array of microbial and non-microbial enzyme-driven reactions. Traditionally, the sources of such enzymes included grape berry tissues, external (surface) microorganisms associated with the fruit (in particular, *Botrytis cinerea*), yeasts mediating the primary fermentation, lactic acid bacteria (LAB) during the malolactic fermentation (MLF) and spoilage organisms.

The primary role of LAB in wine production is to perform a secondary or malolactic fermentation. MLF involves the decarboxylation of malic acid to produce lactic acid and carbon dioxide by the malolactic enzyme, and is commonly conducted in wine after the yeast have completed the alcoholic fermentation (Liu 2002). *Oenococcus oeni* has become the preferred LAB species for conducting MLF, owing to its ability to tolerate the harsh conditions posed by wine (Liu 2002). The malolactic enzymes aside, the current understanding of the enzymes produced by LAB in wine remains limited.

However, a review of relevant literature (Matthews et al. 2004), including publications from the dairy industry, makes it clear there exists great potential for the identification and exploitation of enzymes from wine related LAB in the winemaking process. For example, proteolytic enzymes have been reported in wine *Lactobacillus* strains (Dicks and Van Vuuren 1988) and more recently, characterised in *O. oeni* (Manca de Nadra et al. 1999, Farías and Manca de Nadra 2000, Manca de Nadra et al. 2004). Davis and coworkers (1988) conducted a brief study and found some wine LAB produced lipase and esterase enzymes. Tannases were also recently observed in a number of wine *Lactobacillus plantarum* strains (Vaquero et al. 2004). A final group of enzymes, the glycosidases, would be highly valuable in liberating desirable aroma compounds from their non-volatile glycosidic forms in wine and have been reported in all three wine LAB genera and particularly *O. oeni* (Grimaldi et al. 2000, 2005a, 2005b, Boido et al. 2002, Barbagallo et al. 2004, D'Incecco et al. 2004, Ugliano et al. 2003).

Against that background, we sought a clearer definition of enzymic capabilities in LAB with special emphasis on enzymes that could be of value in winemaking. Accordingly, we screened 50 LAB isolates for production of esterases, lipases, polysaccharide-hydrolysing enzymes and tannases. Those assessments are reported below.

Materials and methods

LAB strains

The LAB strains used in this study originated from a number of sources, the details of which are presented in Table 1. The collection consisted of 11 pediococci, 16 lactobacilli and 23 *O. oeni*. Most of the bacteria were isolated from freeze-dried LAB starter cultures for use in commercial winemaking. *Lactobacillus* strains 31 to 40 inclusive and *Pediococcus* strains 41 and 42 were purchased from the Australian Starter Culture Research Centre, while *Pediococcus* strain18 and *Lactobacillus* strain19 were isolated from a commercial olive product.

Table 1. Details of the lactic acid bacteria strains used in this study.[a]

Lactobacilli

Strain	Genus/species[b]	Origin
Lac6	*Lactobacillus* spp	Lalvin MT01 Standard
Lac12	*Lactobacillus* spp	Lalvin 3X Step 1
Lac13	*Lactobacillus* spp	Lalvin 1B Standard
Lac15	*Lactobacillus* spp	Lalvin 31 MBR
Lac16	*Lactobacillus* spp	Lalvin 3X Standard
Lac19	*Lactobacillus* spp	Olives
Lac24	*Lactobacillus* spp	Lallemand nuovi Ceppi Oo7
Lac25	*Lactobacillus plantarum*	Lallemand nuovi Ceppi Lp1
Lac26	*Lactobacillus* spp	Lallemand nuovi Ceppi Oo1
Lac31	*Lactobacillus fermentum* CSCC-5217	SCRC
Lac32	*Lactobacillus plantarum* CSCC-5275	SCRC
Lac33	*Lactobacillus casei* CSCC-2600	SCRC
Lac34	*Lactobacillus hilgardii* CSCC-5489	SCRC
Lac35	*Lactobacillus buchneri* CSCC-5400	SCRC
Lac39	*Lactobacillus brevis* ASCC-1300	SCRC
Lac40	*Lactobacillus helveticus* ASCC-905	SCRC

Oenococcus oeni

Strain	Species	Origin
O.oeni1	*Oenococcus oeni*	Lallemand OSU (VI77)
O.oeni2	*Oenococcus oeni*	Lalvin 4X (VL92)
O.oeni3	*Oenococcus oeni*	Inobacter
O.oeni4	*Oenococcus oeni*	Bitec Vito
O.oeni5	*Oenococcus oeni*	Lalvin-Inobacter
O.oeni6	*Oenococcus oeni*	Lalvin MT01 Standard
O.oeni7	*Oenococcus oeni*	Lallemand 3X (E218)
O.oeni8	*Oenococcus oeni*	CHR Hansen Viniflora
O.oeni9	*Oenococcus oeni*	CHR Hansen Viniflora
O.oeni10	*Oenococcus oeni*	Lalvin EQ54 MBR
O.oeni11	*Oenococcus oeni*	Lalvin MCW
O.oeni12	*Oenococcus oeni*	Lalvin 3X 1Step
O.oeni13	*Oenococcus oeni*	Lalvin 1B Standard
O.oeni14	*Oenococcus oeni*	Enoferm Alpha
O.oeni16	*Oenococcus oeni*	Lalvin 3X Standard
O.oeni17	*Oenococcus oeni*	Lalvin OSU MBR
O.oeni20	*Oenococcus oeni*	Lallemand nuovi Ceppi Oo3
O.oeni21	*Oenococcus oeni*	Lallemand nuovi Ceppi Oo4
O.oeni22	*Oenococcus oeni*	Lallemand nuovi Ceppi Oo5
O.oeni23	*Oenococcus oeni*	Lallemand nuovi Ceppi Oo8
O.oeni28	*Oenococcus oeni*	Lallemand nuovi Ceppi Oo2
O.oeni29	*Oenococcus oeni*	Lallemand nuovi Ceppi Oo9
O.oeni30	*Oenococcus oeni*	BioStart oenos SK1

Pediococci

Strain	Genus/species	Origin
Ped3	*Pediococcus* spp	Inobacter
Ped10	*Pediococcus* spp	Lalvin EQ54 MBR
Ped11	*Pediococcus* spp	Lalvin MCW
Ped15	*Pediococcus* spp	Lalvin 31 MBR
Ped16	*Pediococcus* spp	Lalvin 3X Standard
Ped17	*Pediococcus* spp	Lalvin OSU MBR
Ped18	*Pediococcus* spp	Olives
Ped24	*Pediococcus* spp	Lallemand nuovi Ceppi Oo7
Ped27	*Pediococcus* spp	Lallemand nuovi Ceppi Oo9
Ped41	*Pediococcus acidilacti* CSCC-2302	SCRC
Ped42	*Pediococcus pentosaceus* CSCC-2304	SCRC

[a] SCRC: Starter Culture Research Centre.
[b] Species listed where it is known. The provenance and identification of strains from sources other than designated culture collections is described in earlier reports (Grimaldi et al. 2000, 2005a, 2005b), and in some cases included minor, non-oenococci contaminants.

Preculturing conditions
All bacteria were stored in cryopreservative-treated beads (Protect, Technical Service Consultants Ltd.) at –80ºC until required. In preparation for experiments, bacteria were cultured from the stock beads in de Mann Rogosa Sharp medium (MRS) (Amyl Media) which was supplemented with 20% (v/v) preservative-free apple juice and adjusted to pH 5.0 prior to autoclaving (MRS+AJ). When required for screening, these cultures were used to inoculate 5 mL volumes of fresh MRS+AJ to yield an optical density at 600 nm (OD_{600}) of approximately 1.0 for *Oenococcus* strains and 0.3 for both *Lactobacillus* and *Pediococcus* strains. All bacteria were then incubated at 25ºC for 24 hours, except *O. oeni*, which was incubated for 40 hours (O.oeni 3 and 5 were incubated for 64 hours).

Biomass preparation
Several of the enzyme assays were conducted using liquid suspensions of bacteria rather than on solid media. In such cases, the concentration of bacterial cells required to produce an optical density at 600nm (OD_{600}) of 0.5 in the final assay was determined spectrophotometrically. The cells were harvested and washed once in 0.85% (w/v) NaCl solution by centrifugation (5,000 × *g*, 5 minutes). Cells were then resuspended in the appropriate reaction mixtures as described below. Dry weights were determined using 10 mL culture samples adjusted to an OD_{600} of 0.5.

Replication
All assays were, as a minimum, performed in duplicate and assays for which replicates disagreed by more than 5% were repeated.

Esterase activity
Esterase activity was assessed using three *p*-nitrophenol-linked ester substrates (Sigma), namely *p*NP-acetate, *p*NP-butyrate and *p*NP-octanoate. Briefly, a 25 mM stock substrate solution was prepared in dimethyl sulphoxide or propan-2-ol and stored at –20ºC until required. The final 1 mL reaction mixture contained McIlvaine buffer (pH 5.0, Dawson et al. 1986) and washed cells to give a final OD_{600} of 0.5 and 40 µL stock substrate solution to yield a final substrate concentration of 1 mM. Control assays consisted of the same reaction mixture to which no cells were added. The samples and controls (performed in duplicate) were incubated for 2 hours at 37ºC. Following incubation, cells were pelleted by centrifugation (13,000 × *g*, 7 minutes), 900 µL of the supernatant was then transferred to a 1.5 mL disposable cuvette and 100 µL of 0.5 M sodium hydroxide was added for alkalinisation. The absorbance at 410 nm was immediately determined spectrophotometrically and the activity was quantified by comparing with *p*-nitrophenol standards.

Lipase activity
Lipase activity was detected by observing the degradation of tributyrin in agar plates based on the method of Oterholm and Ordal (1966). This method was adapted for LAB by using MRS+AJ medium solidified with 20 g/L agar and adjusted to pH 5.0 prior to autoclaving. Tributyrin at 0.3% (v/v) was dispersed into the medium by sonicating the autoclaved agar with an ethanol sterilised sonotrobe for 90 seconds at 70 watts using a Branson B-12 Sonifier immediately prior to pouring. Duplicate plates were inoculated with 30 µL drops of a pre-culture of the appropriate strain (described above), and then incubated at 28ºC and observed daily for one week. Producer colonies were identified by the formation of a clear halo in the opaque medium. As a positive control the lipolytic yeast *Yarrowia lipolytica* (Charles Sturt University culture collection), which had been grown overnight at 28ºC on YPD (1% w/v yeast extract, 2% w/v bactopeptone, 2% w/v D-glucose, 2% w/v agar), was bar streaked concurrently onto the same medium.

Tannase activity
Tannases were detected using methyl gallate substrate based on methods reported by Osawa and Walsh (1993) and Vaquero et al. (2004). Briefly, a stock solution of 20 mM methyl gallate was prepared in 0.2 M McIlvaine buffer at pH 5.0 and stored at 4ºC until required. The assay contained 600 µL of substrate solution and washed cells (harvested using method described above) to a final OD_{600} of 0.5. Control assays were prepared without cells. All assays were incubated at 37ºC for 6 hours. Following incubation, the samples were centrifuged (13,000 x *g*, 5 minutes) and 500 µL of supernatant was removed and added to an equal volume of a saturated solution of $NaHCO_3$ in a 48-well plate (Costar). The plates were then covered and held at room temperature for 1 hour. The absorbance of the resulting green colour was measured using a microplate spectrophotometer (Bio-Tek Instruments Inc. µQuant) at 440 nm. All assays were conducted in duplicate and the results were then corrected for non-enzymatic degradation of methyl gallate using a cell-free control.

Table 2. Substrates used to detect polysaccharide-degrading enzymes.

Enzyme	Substrate	Substrate concentration	Reference
Cellulase	carboxymethyl-cellulose, sodium salt	1% (w/v)	Strauss et al. (2001)
β-glucanase	barley β-glucan	0.02% (w/v)	Strauss et al. (2001)
Lichenase	lichenan	0.02% (w/v)	Strauss et al. (2001)
Xylanase	Remazol Brilliant Blue Xylan	0.1% (w/v)	Farkaš et al. (1985)

Activity of polysaccharide-degrading enzymes
The substrates and their concentration as used to quantify the degradation of various polysaccharides are listed in Table 2. Details for individual substrates are shown below.

Cellulases
Bacteria were screened for cellulase enzymes using a method derived from Strauss et al. (2001). Bacteria were grown on agar medium of pH 5.0 containing 5 g tryptone (BD), 2.5 g yeast extract (Amyl Media), 1 g glucose, 10 g carboxymethylcellulose sodium salt (Fluka) and 20 g bacteriological agar per litre. 30 µL of bacterial pre-culture were pipetted onto the plate surface in duplicate and plates incubated at 28ºC for three days in the case of *Lactobacillus* and *Pediococcus* and five days in the case of *O. oeni*. Following incubation, the colonies were removed by washing with sterile MQ water before the plate was stained with 0.1% (w/v) Congo Red for 15 minutes and destained with 1 M NaCl as per Teather and Wood (1982). Cellulolytic colonies were identified by the presence of an unstained halo around the colony site against an orange-pink background. Purified cellulase enzyme from *Aspergillus niger* (Sigma) was used as a positive control.

β-glucanases
The activity of β-glucanases was detected using barley β-glucan and lichenan substrates using a method based on that described by Strauss et al. (2001). The modified method used a medium consisting of 5 g tryptone, 2.5 g yeast extract, 1 g glucose, 20 g bacteriological agar and either 0.2 g lichenan (Sigma) or 0.2 g barley β-glucan (Sigma) per litre. Plates were incubated under the conditions described for the cellulase assay. Following incubation, the plates were stained with Congo Red (Teather and Wood 1982) and producer colonies were evident by the presence of an unstained halo around the colony site against a stained orange-pink background.

Xylanases

Remazol Brilliant Blue Xylan (Sigma) substrate was used in a plate method based on that described by Farkaš et al. (1985) in order to detect xylanase activity. The medium contained 5 g tryptone, 2.5 g yeast extract, 1 g Remazol Brilliant Blue Xylan and 20 g agar per litre and 30 µL of LAB pre-cultures were pipetted in duplicate onto the plates and incubated at 25°C for one month and observed every two days for producer colonies. The effectiveness of the method at detecting xylanase activity was confirmed using a concentrated xylanase product (Sigma).

Results

Esterases

The results for the esterase screen using the *p*-nitrophenol-linked substrates are presented in Figure 1. All of the LAB had some activity towards the ester substrates, but there was great variation between the three genera. The *Pediococcus* strains, except the olive isolate Ped18, showed very weak esterase activity towards the three substrates. Most of the lactobacilli and oenococci had similar esterase activity towards *p*NP-acetate (average of 0.013 and 0.019 units of activity, respectively), with the exception of *O. oeni* strains 8, 9, and 12, which were strongly esterolytic towards this substrate (approx. 0.040 to 0.050 units). Esterase activity towards *p*NP-butyrate (Ave. = 0.031 units) and *p*NP-octanoate (Ave. = 0.036 units) were similar within the *Oenococcus* genus, but within the *Lactobacillus* genus activity was minimal towards both substrates (Ave. = 0.004 and Ave. = 0.003 units, respectively), with the exception of Lac31 and Lac39 which showed slightly higher activity towards *p*NP-butyrate. Overall, *O. oeni* strains 8, 9, 12 and 28 showed the strongest esterase activity.

Lipases

Only three of the *Lactobacillus* strains, Lac25, Lac16 and Lac15, were found to be able to hydrolyse tributyrin, as seen by a clearing of the opaque medium surrounding the cell growth in the plate assay (data not shown). None of the *Pediococcus* or *Oenococcus* strains possessed lipase activity as determined using this method.

Tannases

The findings from the screen for tannase activity are shown in Figure 2. The green colouration observed during the assay is due to tannase-liberated gallic acid undergoing a non-enzymatic oxidation and polymerisation (Osawa and Walsh 1993). All of the LAB tested displayed some tannase activity as determined by this assay. Generally, the pediococci strains had lower activity (Overall average OD of 0.211) than both the lactobacilli (Ave. OD of 0.328) and oenococci (Ave. OD of 0.395). However, there were some exceptions amongst the pediococci, namely Ped17 and Ped18, whose activities (OD of 0.308 and 0.350, respectively) approached that of the

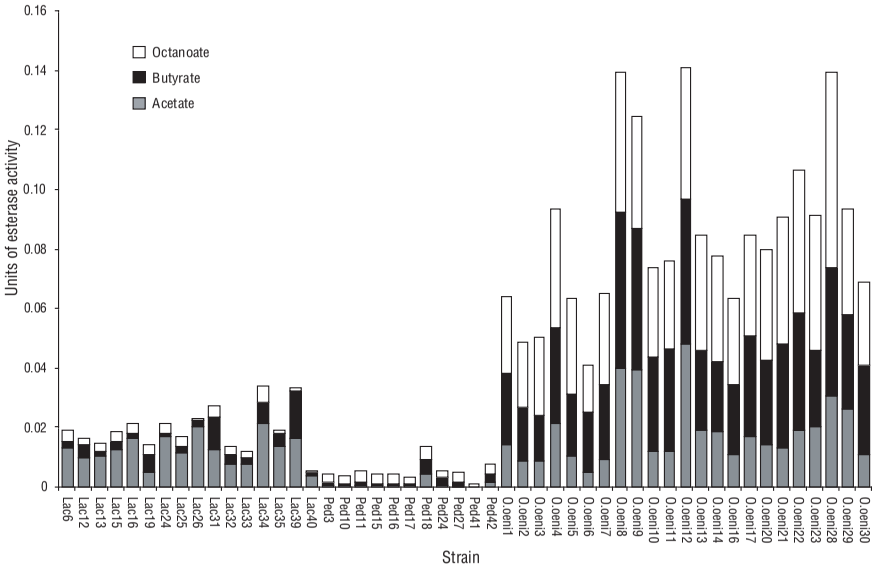


Figure 1. Esterase activity of strains of lactic acid bacteria towards *p*-nitrophenol-linked substrates, expressed as µmoles of *p*-nitrophenol liberated per minute per mg of dry cell weight. Values are the mean of determinations from duplicate assays, which in each case were within 5% of one another. Strains are listed in Table 1.

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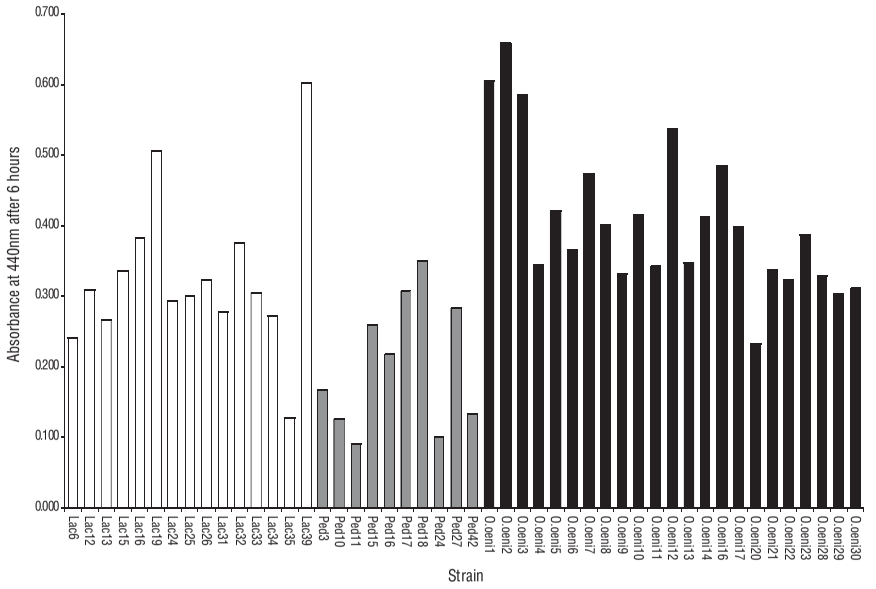


Figure 2. Tannase activity of LAB toward methyl gallate substrate, expressed as absorbance at 440 nm. Values for each strain are the mean of duplicate determinations, which in each case were within 5% of one another. Strains are listed in Table 1.

lactobacilli and oenococci. Within each genus, there was great variation in activity, suggesting tannase activity is not only genus, but also strain dependent. Of the lactobacilli, *Lb. brevis* strain Lac39 had the greatest tannase activity (i.e. OD of 0.602) while *Lb. buchneri* strain Lac35 showed the lowest activity (OD of 0.127).

Polysaccharide degrading activities

Cellulase activity in LAB was evidenced by their ability to break down carboxymethylcellulose, which in turn yielded an unstained halo around the colony site following staining of plates with Congo Red. All of the *Lactobacillus* and *Pediococcus* strains were able to break down this substrate, with the exception of the *Lb. fermentum* strain Lac31 (Table 3). By comparison, cellulolytic activity was less frequent amongst the *O. oeni*, with fewer than half (9 out of the 22 strains studied) giving a positive response.

Using barley β-glucan as substrate, LAB which produced β-glucanase activity were again identified by the formation of an unstained halo upon treatment of growth plates with Congo Red. Accordingly, all of the lactobacilli were shown to produce a β-glucanase activity with the exception of *Lb. helveticus* strain Lac40. Similarly, all of the pediococci gave a positive response with the exception of the olive isolate, Ped18, which did not. However, two of these strains (Ped10 and Ped11) produced small halos, suggesting weak glucanase activity. Of the 22 strains of *O. oeni* tested only 6 exhibited a β-glucanase activity. A second β-glucan, lichenan, was also used in order to further differentiate the strains. All of the *Lactobacillus* strains studied were able to hydrolyse lichenan with the exception of *Lb. fermentum* strain Lac31, which previously had been identified as the sole *Lactobacillus* strain that lacked cellulase activity. Of the 10 *Pediococcus* strains, seven possessed lichenase activity. Similar to the cellulase findings, lichenase activity was less frequent within the *Oenococcus* genus with only 3 out of 22 strains producing a detectable activity.

The final polysaccharide substrate to be examined was Remazol Brilliant Blue xylan, however none of the LAB proved positive for xylanase using this substrate.

Discussion

We have described a survey of wine-related lactic acid bacteria for potentially useful enzymic activities, which, to our knowledge, is the most comprehensive survey of its type to date. Our findings indicate a broad range in the specificity and magnitude of these activities, thereby reiterating the notion that LAB have the potential to influence wine composition in a manner which extends well beyond malate decarboxylation. The significance of the individual activities detected is discussed in further detail below.

Table 3. Production of polysaccharide-degrading enzymes by wine lactic acid bacteria.[a]

Strain	Cellulase	β–glucanase	Lichenase	Xylanase
Lac6	+	+	+	–
Lac12	+	+	+	–
Lac13	+	+	+	–
Lac15	+	+	+	–
Lac16	+	+	+	–
Lac19	+	+	+	–
Lac24	+	+	+	–
Lac25	+	+	+	–
Lac26	+	+	+	–
Lac31	–	+	–	–
Lac32	+	+	+	–
Lac33	+	+	+	–
Lac34	+	+	+	–
Lac35	+	+	+	–
Lac39	+	+	+	–
Lac40	+	–	+	–
Ped3	+	+	+	–
Ped10	+	weak	+	–
Ped11	+	weak	+	–
Ped15	+	+	+	–
Ped16	+	+	–	–
Ped17	+	+	+	–
Ped18	+	–	+	–
Ped24	+	+	–	–
Ped27	+	+	–	–
Ped42	+	+	+	–
O.oeni1	–	–	–	–
O.oeni2	+	+	–	–
O.oeni3	–	nd	–	–
O.oeni4	–	–	–	–
O.oeni5	–	nd	–	–
O.oeni6	+	+	–	–
O.oeni7	–	–	–	–
O.oeni8	–	–	–	–
O.oeni9	–	–	–	–
O.oeni10	–	–	–	–
O.oeni11	+	+	–	–
O.oeni12	–	–	–	–
O.oeni13	+	–	+	–
O.oeni14	+	–	–	–
O.oeni16	–	+	–	–
O.oeni17	–	–	–	–
O.oeni20	+	+	+	–
O.oeni21	+	+	–	–
O.oeni22	–	–	–	–
O.oeni23	+	–	–	–
O.oeni28	–	–	–	–
O.oeni29	+	–	+	–

[a] + = activity detected, – = no activity detected, nd = not determined

Esters are qualitatively one of the most important groups of aroma compounds found in wine, and are generally acknowledged to contribute a desirable, fruity aroma (Etievant 1991). They are derived from the grape berry (Rapp and Mandery 1986) and, perhaps more significantly, they are produced by yeasts during fermentation (Younis and Stewart 1998, Lambrechts and Pretorius 2000). Some esters (and their descriptors) commonly found above their sensory threshold in wine include ethyl acetate ('fruity' at low concentrations, but changing to 'nail polish remover' at higher concentrations), isoamyl acetate (banana), ethyl hexanoate (fruity, violets), ethyl octanoate (pineapple, pear) and ethyl decanoate (floral) (Margalit 1997, Lambrechts and Pretorius 2000).

Until now, perhaps the most important evidence that wine LAB possess esterase activity has been from wine-volatile profiling studies which have investigated the changes in concentrations of individual esters during MLF. For example, increases in ethyl acetate (Maicas et al. 1999, Delaquis et al. 2000), isoamyl acetate (Laurent et al. 1994, Maicas et al. 1999, Ugliano and Moio 2005) and ethyl lactate (Delaquis et al. 2000, Gambaro et al. 2001, Ugliano and Moio 2005) have been observed, while Zeeman et al. (1982) reported a decrease in some esters following MLF. In this study, LAB were screened for the production of esterases capable of hydrolysing an ester substrate. The strains found to have the greatest hydrolytic esterase activity belonged to *O. oeni*, the organism most commonly responsible for conducting MLF in commercial wineries. Davis et al. (1988) reported similar results in their study, finding 23 strains of wine LAB to have esterase activity, including 15 strains of *Leuconostoc oenos* (now classified as *O. oeni*). These results further confirm that wine LAB produce hydrolytic esterases and suggest LAB could have an impact on wine aroma, and by extension wine quality. Whether or not these activities are indicative of potential ester synthetic capability is yet to be determined.

Wine lipids can originate from a number of sources. Initially, lipids are derived from the grape berry (including the skin, seeds and pulp), with the concentrations and types of lipids varying depending upon grape variety (Gallander and Peng 1980), fruit maturity at harvest (Bauman et al. 1977), and climate (Izzo and Muratore 1993) and presumably via methods of processing of the grapes. Subsequently, lipids are liberated from yeast during autolysis, including tri-, di- and mono-acylglycerols and sterols (Pueyo et al. 2000).

The action of lipases on wine lipids could result in the formation of a range of volatile compounds, but perhaps the most important would be liberation of fatty acids. Many fatty acids have low aroma thresholds and can contribute to wine aroma, although with their odours being described as 'vinegar', 'cheesy' and 'sweaty', they could have a detrimental effect on wine aroma (Etievant 1991). However, a positive contribution to the aroma of wine could then be made by some of the volatile compounds which are in turn derived from fatty acids, including esters, ketones and aldehydes (Charpentier and Feuillat 1993).

The lipolytic activity of wine LAB has not previously been thoroughly investigated. In this present study, lipase activity was observed in three *Lactobacillus* strains, but not in any of the pediococci or *O. oeni*. Similar results were reported by Herrero et al. (1996), who failed to find lipolytic activity amongst wine isolates comprising 2 *Leuconostoc* and 3 *Lactococcus* strains or any of the 32 *Lactobacillus* strains they also investigated. By contrast, in a study of LAB isolated from wines, Davis et al. (1988) observed lipase activity towards the tributyrin substrate in several strains of *Leuconostoc oenos* and one species of *Lactobacillus*.

As noted above, the lipolytic activity of LAB has been more extensively researched in other areas of food production. In the dairy industry *Lactobacillus* lipases are known to contribute important flavour compounds, particularly to cheeses (Collins et al. 2003), while *Pediococcus* species can show lipase activity (e.g. El Soda et al. 1986, Andersen et al. 1995, Katz et al. 2002) and are also found in wine. None of those published studies were focussed on wine strains specifically.

Tannases were explored in our study because of the importance of tannins to the quality of red wines. Tannases were observed in all of the LAB investigated and several of the *O. oeni* strains were found to have high tannase activity. These findings conflict with those reported by Vaquero et al. (2004) who screened a total of 78 grape and wine LAB isolates from the genera *Lactobacillus*, *Pediococcus*, *Leuconostoc* and *Oenococcus* and found no tannase activity in any of the variants of *O. oeni*. One strain of *Lb. plantarum* was the sole tannase producer. Previous studies suggesting tannases may be produced by LAB were focused on isolates of *Lb. plantarum*, *Lb. paraplantarum* and *Lb. pentosus* from human faeces and fermented foods (Osawa et al. 2000, Nishitani et al. 2004), and isolates of *Lb. plantarum* from olive waste (Lamia and Moktar 2003). Additional work on tannase occurrence included LAB genera not endogenous to wine, including *Streptococcus* species (Osawa 1990, Osawa et al. 1995).

The possession of tannase activity by LAB could confer nutritional benefits on the bacteria. For example, gallic acid stimulates the early growth stages of *O. oeni* in wine, together with malate degradation (Vivas et al. 1997), whereas the ellagitannin, vescalagin, improves the viability of non-growing *O. oeni* (Vivas et al. 2000). Other workers have shown that the growth of *Lb. hilgardii* was stimulated by phenolic acids, including gallic, vanillic, *p*-coumaric, caffeic, ferulic, *p*-hydroxybenzoic, protocatechuic, and syringic acids (Campos et al. 2003). Under those same conditions, the growth of *O. oeni* was however inhibited. Clearly, further work is required to determine the precise nature and significance of the interaction of LAB with tannins.

Polysaccharides were the final group of substrates considered in this study. The principal polysaccharides of higher plant cell walls and middle lamellae, which are important to winemaking, include cellulose (primarily β-glucans), hemicellulose (primarily xylans) and pectic substances (Whitaker 1990). These macromolecules are released into grape juice either by the disruption of berries during crushing, or they are liberated through the action of grape enzymes. Enzymes which are capable of hydrolysing polysaccharides could (i) increase juice yields (Berg 1959, Haight and Gump 1994), (ii) improve wine quality by degrading grape cell walls to liberate colour and aroma-precursors (Wightman et al. 1997, Bakker et al. 1999, Pardo et al. 1999), (iii) aid in processing by avoiding difficulties caused by polysaccharides (including filter fouling and protracted juice sedimentation times), and (iv) alter flavour perception (Langourieux and Crouzet 1994). Glucans represent one group of polysaccharides of particular interest in this context.

Glucans are found in both grape juice and wine, and in addition to being grape-derived, they can originate from yeast, including *Saccharomyces cerevisiae*, during primary fermentation (Humbert-Goffard et al. 2004). Spoilage microorganisms associated with winemaking can also produce glucans. In particular, the spoilage LAB *Pediococcus damnosus* (formerly *Pediococcus cerevisiae*) secretes a trisaccharide, causing 'ropiness' in wines. Furthermore, the grape fungus *Botrytis cinerea* produces a high molecular weight glucose-based glucan which persists after fermentation (Dubourdieu et al. 1980, Canal-Llauberes 1993).

Regardless of origin, glucans can impair wine clarification and filtration. Early in vinification, glucans can prevent sedimentation of solids and reduce juice yields. Often such problems are overcome by centrifugation or bentonite fining. Although both these methods accelerate sedimentation, neither removes the causative glucan, and filtration problems persist (Humbert-Goffard et al. 2004), particularly post-fermentation when the problem is exacerbated by alcohol-induced polymerisation of the glucan molecules (Villettaz et al. 1984, van Rensburg and Pretorius 2000). β-glucanases hydrolyse the glycosidic linkages of β-glucan chains, yielding glucose and oligosaccharides (Humbert-Goffard et al. 2004), and have previously been shown to be effective at removing problem β-glucans from wine, including those from botrytised wines (Villettaz et al. 1984). LAB represent a potentially useful source of β-glucanases for overcoming some of the processing problems posed by glucans in winemaking. In this present study, β-glucanases were detected using β-glucan from barley. Of the 16 lactobacilli used in our study, 15 were found to be able to hydrolyse β-glucan. Similarly, 9 of the 10 pediococci showed β-glucanase activity, but activity was less common among the *O. oeni* with only 6 strains showing a positive result. Earlier reports have demonstrated *O. oeni* produces an extracellular glucanase early in the stationary phase of cell growth (Guilloux-Benatier et al. 2000). In that study, the enzyme was found to be β-1,3 in nature and capable of hydrolysing yeast cell-wall macromolecules, suggesting it could contribute to yeast cell autolysis after alcoholic fermentation. Further work will be required to confirm if *O. oeni* can aid in removing problem glucans, including those secreted by *Botrytis cinerea*.

Hemicellulose is a heteropolysaccharide and along with cellulose is a major component of grape berry cell walls. The primary type of hemicellulose is β-1,4-xylan (β-1,4-linked D-xylopyranosyl), which is both highly branched and highly polymerised (van Rensburg and

Pretorius 2000). Attached to the xylopyranosyl polymer backbone are a number of possible entities, including acetyl, arabinosyl and glucanosyl residues (van Rensburg and Pretorius 2000). Owing to the different side constituents present, a number of different and specific types of enzymes are required to completely degrade hemicellulose and these include galactanases, mannases and xylanases. In this present study, none of the LAB were capable of degrading xylan.

Lichenan is a linear 1,3-1,4-β-D-glucan, and having 1,3- linkages, it is structurally similar to the glucan produced by *Botrytis cinerea* during grape berry infection (Strauss et al. 2001). Lichenase (endo-β-1,3-1,4-glucanase) activity in LAB could aid in removing problem glucans, in particular those found in botrytised wines. Most of the *Lactobacillus* and *Pediococcus* strains in this study were capable of breaking down lichenan and while the lichenase activity was less frequent among the *O. oeni*, LAB represent a potential source of enzymes for targeting glucans in botrytised wine production. While the genetics and activity of lichenases has been characterised in other bacteria, including *Streptococcus bovis* (Ekinci et al. 1997), *Bacillus circulans* (Kim 2003), and *Clostridium thermocellum* (Piruzian et al. 2002), to our knowledge, this is the first report of lichenase activity among wine LAB.

O. oeni is the preferred organism for instigating MLF in commercial winemaking, principally because it is suited to the harsh wine environment and is not usually associated with the production of spoilage characteristics. In many Australian wineries, MLF is initiated by inoculating a must or wine with a commercial preparation of *O. oeni*. In this present study, *O. oeni* was shown to produce a number of enzymes of oenological significance. In general, *O. oeni* was found to have stronger esterase and tannase activity than any of the lactobacilli or pediococci. These findings imply that a range of LAB-derived enzymes could act on wine substrates during MLF. Interestingly, the enzymes capable of degrading polysaccharides were more frequently observed among the *Lactobacillus* and *Pediococcus* strains – an observation that accords well with the stage in winemaking when ethanol concentrations are lowest and these bacteria grow best. Moreover, their enzymes would then be most valuable, that is prior to primary fermentation, and at a stage when they could increase juice yields, as well as aid in juice settling and clarification. Further work is now required to examine both the production and the activity of the observed enzymes under wine conditions. In particular, their activities need to be assessed at the low pH and temperatures commonly encountered in commercial wine production and in the presence of wine inhibitors such as ethanol and SO_2. The activity of these enzymes also needs to be assessed using wine-specific substrates. Future research might also reveal any effects their actions may have on the organoleptic properties and overall quality of wines.

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Optimisation of industrial strains of *Saccharomyces cerevisiae* using recombinant and non-recombinant methods



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The production of fermented beverages such as beer and wine is typically achieved using strains of *Saccharomyces cerevisiae*, which have been selected for their reliable fermentative properties and desirable sensory contribution to the end product. Even so, these strains are not without shortcomings and new strains are constantly being sought. In aiming to provide such strains, working with industrial isolates has its own set of challenges. Wine strains of *S. cerevisiae* are typically of higher ploidy or aneuploid, frequently lack convenient selectable markers and are homothallic. Laboratory strains, along with their comprehensive set of genetic tools, might be a more convenient research vehicle, but the fact remains that such strains fare poorly under the highly stressful conditions of industrial fermentations: sugar contents of over 200 g/L; low nutrient availability and pH; and high final ethanol concentrations. More importantly, the wines produced by laboratory strains of yeast frequently display poor sensory properties. Put simply, laboratory yeast lack the desirable phenotypic characteristics needed to make wine of quality to enable industrially relevant studies.

Some progress has been made recently toward the generation of appropriate wine yeast resources through the development of heterothallic wine yeast derivatives [1]. Consequently a combined approach to the identification of genes important to wine fermentations has been possible: thus both laboratory and wine yeast backgrounds have been used to screen gene modifications (e.g. transposon mutant libraries [2]) for phenotypes of interest. Where mutations of interest have been highlighted in the former, the affected genes were identified by inverse PCR [3,4] before the phenotype associated with their disruption was confirmed in the wine yeast derivative. In this way several genes, including *NGR1* and *GID7*, have been found during searches for loci whose disruption proves beneficial to fermentation reliability under conditions of nitrogen limitation [5]. Such genes are considered important since a deficiency of assimilable nitrogen in grape juice is a common cause of poor biomass yield, reduced fermentative vigour and hence, slow or prematurely terminated (so-called 'stuck') fermentations by the inoculated yeast [6].

Ongoing attempts to develop commercial wine yeast strains that are more 'nitrogen efficient' and better able to function in nitrogen-deficient juices [7,8], may well incorporate modifications to the genes identified to date. Further genes of importance to fermentation success and wine sensory properties are being sought through screening of the standard commercially available laboratory yeast deletion and over-expression libraries. The latter will be especially important in revealing genes acting through mechanisms other than loss of function.

As well as identifying targets for modification by recombinant methods to attain optimised strains, work by this group also aims to achieve this goal by non-recombinant methods. Therefore, hybridisation strategies are being used as well as adaptive (directed) evolution approaches [9,10]. To date, strains have been produced which catabolise all available sugar in a stressful oenological fermentation (low inoculation rate, high sugar content, low pH and oxygenation) in about half the time required by the parent strain (Figure 1). Other potentially useful changes, such as altered yields of glycerol and ethanol, have also been observed. Importantly, having been derived by non-recombinant means, such optimised strains could conceivably be immediately adopted by industry. To our knowledge, the report arising from this work is the first to describe the use of adaptive evolution to optimise industrial yeast, in this case a wine yeast [11].

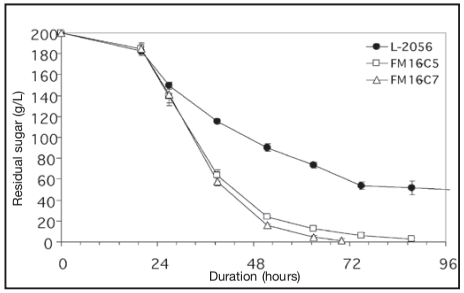


Figure 1. Relative fermentation performance of adaptively evolved strains FM16-C5 and FM16-C7 compared with the parental strain L2056. Fermentations were conducted in a chemically defined grape juice medium [11].

Given the enormous promise of the initial outcomes from the use of adaptive evolution, such work is being expanded to include further strains and other attributes. Through this work, many hundreds of candidate strains or mutants that require more intensive evaluation in laboratory model wine fermentations are being produced. To better accommodate such numbers an automated fermentation phenotyping system has been developed (Figure 2). Based on a system designed by researchers at INRA in Montpellier [12], the present system logs weight loss due to fermentative production of carbon dioxide from sugars. In this way the need for sampling and analysis of individual fermentation vessels in order to monitor fermentation progress is largely eliminated. Once deemed suitable in terms of basic fermentation properties and appropriate production of major metabolites, such novel strains will then undergo further assessment in pilot scale grape juice fermentations.

Conclusion

The production of optimised strains has been undertaken via a multifaceted approach. Recombinant strategies have utilised both existing laboratory yeast tools as well as recently developed derivatives of a commercial wine yeast strain. Having been identified through screens of various libraries of mutants, gene modifications influencing phenotypes of importance to winemaking are undergoing further evaluation and may in turn be incorporated into future strain improvement activities. Parallel non-recombinant strategies, particularly adaptive evolution, have also yielded highly promising, candidate optimised strains. Regardless of the method by which they were formed, large numbers of both recombinant and non-recombinant strains are able to be assessed using an automated system for conduct of monitored fermentation trials. Through this work we hope to achieve both the accelerated production of superior strains for the fermented beverage industries as well as a better understanding of the basis for efficient performance by yeast in high stress environments.

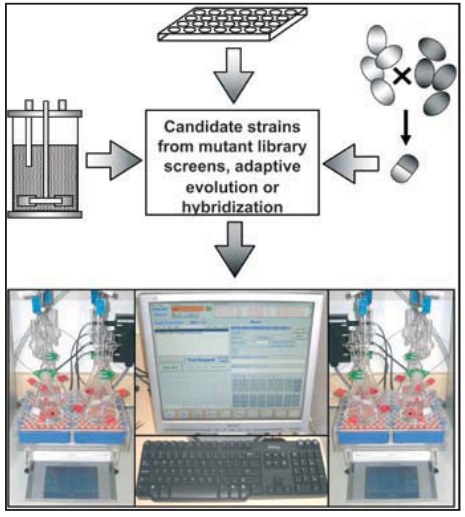


Figure 2. Strains of interest, whether derived through screening of deletion libraries, pools of hybrids or else isolated from adaptive evolution experiments, are assessed via a high throughput fermentation phenotyping system. Fermentations are typically conducted on a 100 ml scale in individually temperature controlled customised shake flasks. Four flasks are monitored simultaneously with the aid of custom control software and a pneumatic vessel lifting system. Chemical analysis of the culture medium is conducted off-line.

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Biochemical characterisation of the esterase activities of wine lactic acid bacteria

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Abstract Esters are an important group of volatile compounds that can contribute to wine flavour. Wine lactic acid bacteria (LAB) have been shown to produce esterases capable of hydrolysing ester substrates. This study aims to characterise the esterase activities of nine LAB strains under important wine conditions, namely, acidic conditions, low temperature (to 10°C) and in the presence of ethanol (2–18% v/v). Esterase substrate specificity was also examined using seven different ester substrates. The bacteria were generally found to have a broad pH activity range, with the majority of strains showing maximum activity close to pH 6.0. Exceptions included an *Oenococcus oeni* strain that retained most activity even down to a pH of 4.0. Most strains exhibited highest activity across the range 30–40°C. Increasing ethanol concentration stimulated activity in some of the strains. In particular, *O. oeni* showed an increase in activity up to a maximum ethanol concentration of around 16%. Generally, strains were found to have greater activity towards short-chained esters (C2–C8) compared to long-chained esters (C10–C18). Even though the optimal physicochemical conditions for enzyme activity differed from those found in wine, these findings are of potential importance to oenology because significant activities remained under wine-like conditions.

Keywords Esters · *Oenococcus* · *Lactobacillus* · *Pediococcus* · Hydrolysis

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Introduction

Wine is produced by the enzyme-driven fermentation of grape juice. In addition to those enzymes produced by yeast during alcoholic fermentation, a range of additional enzyme activities of various sources can influence both the quality and the style of the final wine. Lactic acid bacteria (LAB) represent one source of such enzymes. Three genera of LAB are associated with grape juice and wine, namely, *Lactobacillus*, *Pediococcus* and, perhaps most importantly, *Oenococcus*, of which there is only one species, *Oenococcus oeni* (Fugelsang and Edwards 2007). The primary function of LAB in winemaking is to conduct the malolactic fermentation (MLF), which involves the decarboxylation of L-malic acid to L-lactic acid. In addition, LAB have further impacts on wine composition (Liu 2002), yet these activities are far from being characterised to the extent of the malolactic enzyme.

LAB associated with grape juice and wine have been shown to produce a range of enzymatic activities that could potentially be important in wine production, including those capable of hydrolysing ester substrates (e.g. Davis et al. 1988; Matthews et al. 2004, 2006). All strains of LAB examined were found to have activity towards three different ester substrates, although a genus and strain dependence was observed, with *O. oeni* generally having higher esterase activity than the lactobacilli and pediococci (Matthews et al. 2006). Potential substrates for such esterases in the wine environment are qualitatively one of the most important groups of volatile compounds in determining wine flavour (Ferreira et al. 1998; Lilly et al. 2006). Such esters can be generally categorised as either ethyl esters of fatty acids, acetate esters of higher alcohols or esters of organic acids. Esters of organic acids are the predominant group in wine, followed by the acetate esters

and ethyl esters of fatty acids (Etievant 1991). However, some individual ethyl esters of fatty acids and acetate esters are also important given their low sensory threshold (Simpson and Miller 1984; Aznar et al. 2001; Ferreira et al. 2002).

Minor amounts of some esters are derived from the grape berry, but the principle source of esters in wine is from their production by yeast during alcoholic fermentation. Ethanol is a key substrate in the formation of many esters thus their biosynthesis is, at least to some extent, concurrent with that of ethanol during alcoholic fermentation (Rapp and Mandery 1986; Herraiz and Ough 1993; Plata et al. 2003). The quantities and types of esters produced during fermentation are dependent upon the yeast strain conducting the fermentation (Soles et al. 1982; Antonelli et al. 1999; Rojas et al. 2003), as well as the physiochemical conditions including temperature, pH and nitrogen levels of the juice or must (Lambrechts and Pretorius 2000). Ethyl acetate is the most important ester found in wine, contributing a desirable fruity aroma at low concentrations. At higher concentrations (>200 mg/L), an undesirable 'solvent' character arises, which is detrimental to both wine aroma and quality (Dittrich 1983; Margalit 1997). Ethyl acetate along with other esters, such as methylbutyl acetate, can therefore contribute to 'ester taint' (Sponholz et al. 1982; Sponholz 1993). Therefore, selective production or degradation of esters could be valuable in enhancing wine composition and overcoming taints in wines. To achieve such selectivity, a greater understanding of the esterase profile of LAB is required.

Biochemical characterisations of LAB esterases, including those of *Lactobacillus* (El Soda et al. 1986a; Khalid et al. 1990; Castillo et al. 1999) and *Pediococcus* (Østdal et al. 1996) strains, have been published. Much of this work, however, has been carried out in the dairy industry, where esterases contribute to the flavour development of some foods, in particular, cheeses (Holland et al. 2005). While esters are generally accepted as being important volatile compounds in wine, parallel work has not been conducted in the wine context, despite the fact that physicochemical conditions are generally harsher. For example, wine pH ranges from 2.8 to 4.0, with low pH being important for reducing the risk of microbial spoilage and promoting red wine colour. Temperatures range from less than 10°C (for example, during juice settling) to 25°C (for example, during alcoholic fermentation). Also, the concentration of ethanol, a potential inhibitor of enzyme function, is particularly important after alcoholic fermentation, which coincides with the time when many wines undergo MLF (Liu 2002). LAB esterases must therefore be able to function under these conditions if they are going to impact on wine esters. In this study, we investigated the influence of the above critical oenological parameters on the esterase activity of nine LAB strains.

Materials and methods

Bacterial strains, preculturing conditions and preparation of biomass

In total, nine strains of LAB, three lactobacilli, three pediococci and three oenococci, were used in this study (Table 1). Some of the bacteria were isolated from freeze-dried LAB starter cultures for use in commercial wine-making. Two *Lactobacillus* strains, Lac34 and Lac35, and *Pediococcus* strain Ped42 were purchased from the Australian Starter Culture Research Centre, while *Pediococcus* strain Ped18 was isolated from a commercial olive product. Bacteria were stored and cultured as described previously (Matthews et al. 2006). Briefly, all isolates were held on cryopreservative-treated beads (Protect, Technical Service Consultants) at −80°C until required. In preparation for experiments, bacteria were precultured in de Mann Rogosa Sharp medium, which was supplemented with 20% (v/v) preservative-free apple juice and adjusted to pH 5.0 prior to autoclaving (MRS + AJ). When required for experiments, the precultures of bacteria were used to inoculate 5 mL volumes of fresh MRS + AJ broth to yield an optical density at 600 nm (OD_{600}) of approximately 1.0 (*O. oeni*) or 0.3 (lactobacilli and pediococci) and incubated at 26°C for 24 h (lactobacilli and pediococci) or 40 h (*O. oeni*).

All of the esterase assays conducted in this study consisted of liquid suspensions of whole bacterial cells. The number of bacterial cells required to produce an OD_{600} of 0.5 in the final assay was determined spectrophotometrically. The required volume of cells was harvested and washed once in 0.85% (w/v) NaCl solution by centrifugation (5,000×*g*, 5 min) to remove the culturing media before being resuspended in an appropriate volume of 0.1 M McIlvaine buffer (Dawson et al. 1986) and aliquoted into the reaction tubes. Dry cell weights were determined using 10-mL culture samples adjusted to an OD_{600} of 0.5.

Table 1 Details of the LAB strains used in the biochemical characterisation study

Strain	Genus/species[a]	Origin
Lac26	*Lactobacillus* sp.	Lallemand nuovi Ceppi Oo1
Lac34	*Lactobacillus hilgardii* CSCC-5489	SCRC
Lac35	*Lactobacillus buchneri* CSCC-5400	SCRC
Ped11	*Pediococcus* sp.	Lalvin MCW
Ped18	*Pediococcus* sp.	Olives
Ped42	*Pediococcus pentosaceus* CSCC-2304	SCRC
O.oeni8	*Oenococcus oeni*	CHR Hansen Viniflora oenos
O.oeni9	*Oenococcus oeni*	CHR Hansen Viniflora oenos
O.oeni12	*Oenococcus oeni*	Lalvin 3X 1Step

SCRC purchased from the Australian Starter Culture Research Centre collection

[a] Species listed where it is known

Determination of esterase activity

Esterase assays were conducted using *p*-nitrophenyl (*p*NP) octanoate substrate with the exception of the substrate-specificity trials, in which seven different *p*NP-linked ester substrates were used, as described below. Reaction mixtures (final volume 1 mL) were prepared as detailed in each section below and contained bacterial biomass to yield an optical density at OD_{600} of 0.5. Reactions were incubated at 37°C for 2 h, with the exception of the temperature optima experiments (see below). Following incubation, cells were pelleted by centrifugation (13,000×*g*, 7 min), the supernatant (900 μL) was transferred to a 48-well microplate (Costar No 3548, Corning) and the liberated *p*-nitrophenol was quantified immediately by measuring the absorbance at 410 nm using a microplate spectrophotometer (μQuant, Bio-Tek Instruments). The results were then corrected for non-enzymatic degradation of the ester substrate using a cell-free control of the same reaction mixture that was treated in the same manner. All assays and controls were performed in triplicate and activities were quantified by comparing with *p*-nitrophenol standards.

Influence of pH, temperature or ethanol on esterase activity

The influence of pH on esterase activity was determined by varying the pH of the reaction mixture across the range pH 3.0 to 8.0. The buffers used were 0.1 M McIlvaine buffer (pH 3.0 to 7.5) and Na_2HPO_4/NaH_2PO_4 buffer (pH 8.0) (Dawson et al. 1986). Briefly, 1-mL reactions were prepared by combining 860 μL of the appropriate buffer, 100 μL of the required cell suspension in the same buffer and 40 μL of a 25-mM stock *p*NP-octanoate solution in ethanol, to yield a final substrate concentration of 1 mM. Following incubation and centrifugation (13,000×*g*, 7 min), 900 μL of each supernatant was transferred to a fresh 48-well microplate. For alkalinisation, 300 μL of 0.5 M NaOH was then added to the pH-3.0 to -4.0 samples and 100 μL of 0.5 M NaOH was added to the pH-4.5 and -5.5 samples prior to absorbance measurements being taken as described above. Results were quantified by comparison with standard *p*-nitrophenol solutions prepared under the same conditions.

Esterase activity was studied at six temperatures across the range of 10 to 60°C in dry heating blocks or a PCR thermocycler. Accuracy of temperature settings was confirmed with a thermometer immediately prior to initiation of the experiment. Assays were carried out as described for the pH optima study, but for all samples, 0.1 M McIlvaine buffer of pH 5.0 was used. Reaction mixtures containing only buffer and substrate were equilibrated at the appropriate temperature for 10 min before the bacterial cell suspension was added to initiate the reaction. Following incubation, all samples were centrifuged, transferred into 48-well microplates and alkalinised as above before absorbances were measured.

The effect of the presence of ethanol on esterase activity was studied at nine ethanol concentrations ranging from 2 to 18% (v/v). Reaction mixtures were prepared as described for the temperature optima experiments, but different volumes of ethanol were added from 20 μL (2% v/v) up to 180 μL (18% v/v) and the volume of buffer was adjusted accordingly to maintain a final reaction volume of 1 mL. Reactions were incubated for 2 h at 37°C and treated post-incubation as described for the temperature optima samples.

Determination of substrate specificity

To determine the specificity of individual LAB strains for different ester substrates, seven *p*NP-linked esters of varying carbon-chain length were used: *p*NP-acetate (Sigma), -butyrate (Sigma), -octanoate (Jomar), -decanoate (Sigma), -dodecanoate (Fluka), -tetradecanoate (Fluka) and -octadecanoate (Fluka). The method of Pencreac'h and Baratti (1996) was used with some modifications. Stock solutions (25 mM) of each substrate were prepared in ethanol (*p*NP-acetate, -butyrate, -octanoate) or propan-2-ol (*p*NP-decanoate, -dodecanoate, -tetradecanoate, -octadecanoate) and stored at −20°C until required. Reaction mixtures (final volume 1 mL) contained the following: 755 μL 0.1 M McIlvaine buffer (pH 5.0), 5 μL Triton X-100, 40 μL of stock substrate solution (to yield a final substrate concentration of 1 mM), 100 μL of 1% (w/v) gum arabic (Sigma) solution, and 100 μL of cell suspension in buffer. All samples were vortexed vigorously for 1 min and incubated at 37°C for 2 h with agitation (100 rpm) on a platform shaker. Following incubation, all samples were centrifuged and treated as described for the temperature optima experiments.

Statistical analysis

Analysis of variance of the data was conducted using Microsoft Excel. Significant differences between the means ($p=0.05$) were determined using the Multiple Range Test and Studentised Range Q values.

Results

Influence of pH on esterase activity

The effect of pH on the esterase activity of the nine LAB strains towards *p*NP-octanoate is shown in Fig. 1. Overall,

the results suggest that the influence of pH varies between LAB strains, with different patterns of activity being observed for each of the strains studied. Compared to the other strains, the *O. oeni* appeared least influenced by pH, particularly across the range of pH 4.0 to 6.5. For O.oeni12, esterase activity was highest between pH 6.0 and 6.5 (average of 48 units of activity) and there was no significant difference in activity at pH 4.5 compared to pH 6.5. More than 40% of maximal activity (Ave.=21 units) was retained at pH 3.0 for this strain. Similarly, O.oeni9 showed no significant difference in activity at pH 6.5 compared to that seen at pH 4.0. Of the three *O. oeni* strains examined, O.oeni8 had the greatest activity of all strains (Ave.=62 units); however, this activity was also most strongly inhibited by low pH.

All of the lactobacilli showed maximum esterase activity close to pH 6.5, and all three strains showed a rapid decrease in activity at pH above optimum. Thus, for Lac34, of the order of 80% of the activity seen at pH 7.0 was lost at pH 7.5. Lac34 also showed a marked decrease in esterase activity at pH values below 5.0, and minimal activity was observed at pH 3.0. Of the pediococci, Ped42 had the maximum esterase activity at pH 4.5 to 5.0, values which were lower than the optimum pH values for Ped18 (6.0 to 6.5) and Ped11 (6.0). Ped18 was the only strain of the pediococci examined to retain at least some esterase activity at pH 3.0.

Influence of temperature optima on esterase activity

Maximum esterase activity was observed between 30 and 40°C for all three of the *O. oeni* strains (Fig. 2). For both O.oeni8 and O.oeni9, no significant difference in esterase activity was observed between 30 and 40°C, but O.oeni12 had significantly higher activity at 40°C. Similar trends were obtained for the lactobacilli, albeit at lower activities. Significantly higher activity was observed at 40°C than at 30°C for both Lac26 and Lac34. Interestingly, esterase activity diminished quickly above 40°C for all three lactobacilli strains and was similarly reduced at 10°C. By comparison, activities associated with two of the pediococci (Ped11 and Ped18) had lower temperature optima than other strains, with significantly higher activities at 20°C than at 40°C. At 10°C, Ped18 maintained 64% of the maximum activity observed at 30°C, a fact that could be important in wine production where such lower temperatures are common.

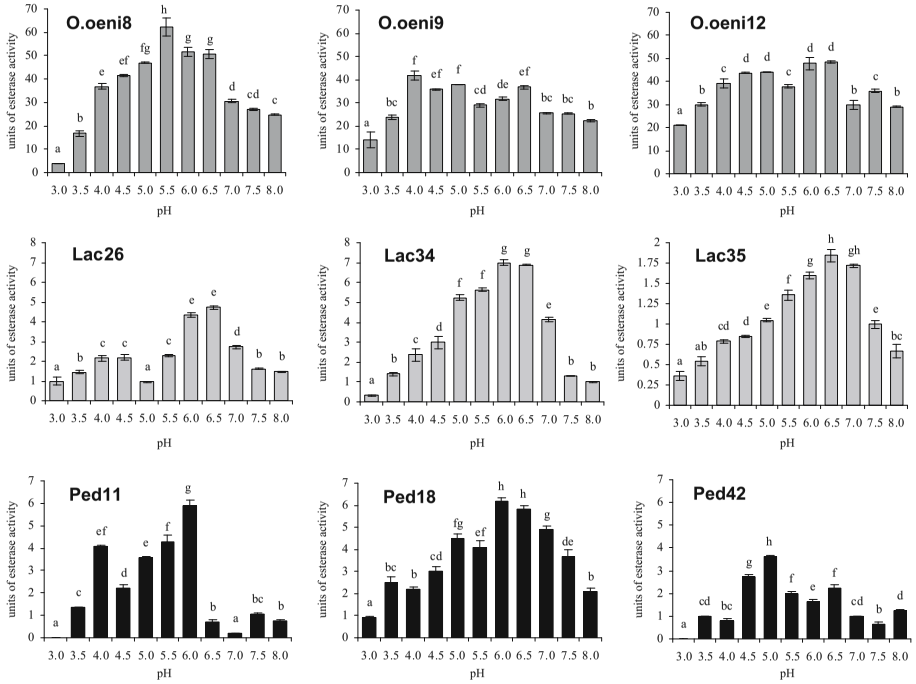


Fig. 1 Influence of pH on esterase activity of LAB strains. Units of esterase activity are defined as micromoles of *p*-nitrophenol liberated per minute per microgram of dry cell weight. Values are the mean of triplicates ± standard deviation. Within each panel columns with different letters are significant at the 5% level according to the multiple range test. NB: The scales differ across the genera and Lac35 has a different scale to Lac26 and Lac34

The influence of ethanol on esterase activity

Ethanol concentration is an important consideration during winemaking because MLF is often conducted after the completion of alcoholic fermentation, when ethanol concentrations of wine can exceed 13% (v/v). For this reason, the influence of ethanol on the ability of LAB strains to hydrolyse esters was investigated. Ethanol was not inhibitory to the esterase activity of all of the strains (Fig. 3). In fact, for all oenococci, ethanol was found to progressively increase the detected activity up to concentrations of the order of 16% (v/v). The influence of ethanol on the lactobacilli was generally inhibitory, with Lac26 showing the most dramatic reductions commencing from ethanol concentrations as low as 4%. Lac35 showed similar results, retaining as little as 30% of the activity found at 2% ethanol when exposed to an ethanol concentration of 18%. For Ped18, there was no significant difference in esterase activity at 2% compared to 16%, with intermediate concentrations yielding activities of up to approximately 30% higher.

Substrate specificity of esterases

Overall, the strains studied tended to show greater hydrolytic activity against the short-chained ester substrates (i.e. ≤C8) (Fig. 4). An exception was Ped18, which had equal or higher activity towards some of the longer esters. The remaining pediococci failed to hydrolyse any substrates from C12 and longer. All three *O. oeni* strains showed similar substrate specificity profiles with highest activity

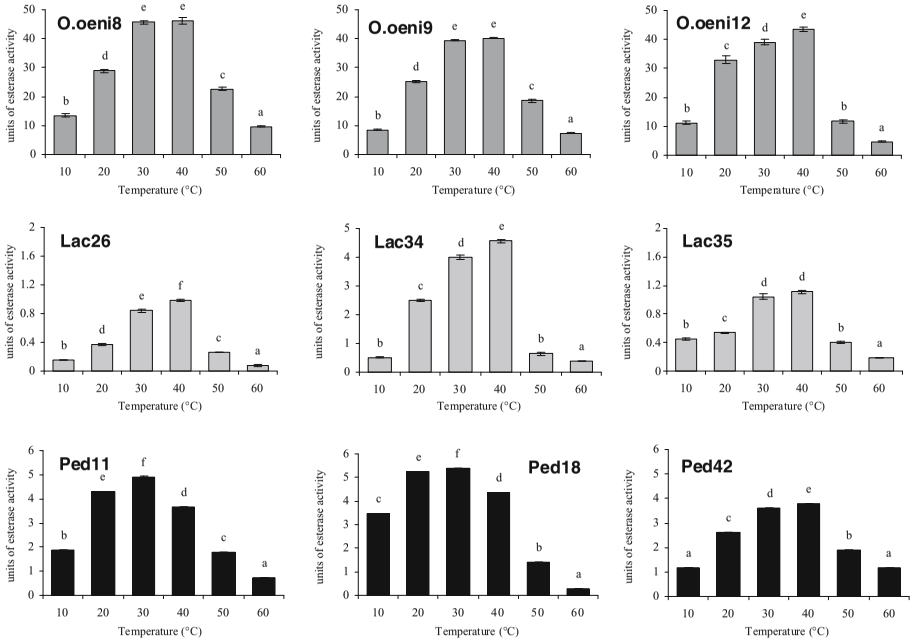


Fig. 2 Influence of temperature on esterase activity of LAB strains. Units of esterase activity are defined as micromoles of *p*-nitrophenol liberated per minute per microgram of dry cell weight. Values are the mean of triplicates ± standard deviation. Within each panel columns with different letters are significant at the 5% level according to the multiple range test. NB: The scales differ across the genera and Lac34 has a different scale to Lac26 and Lac35

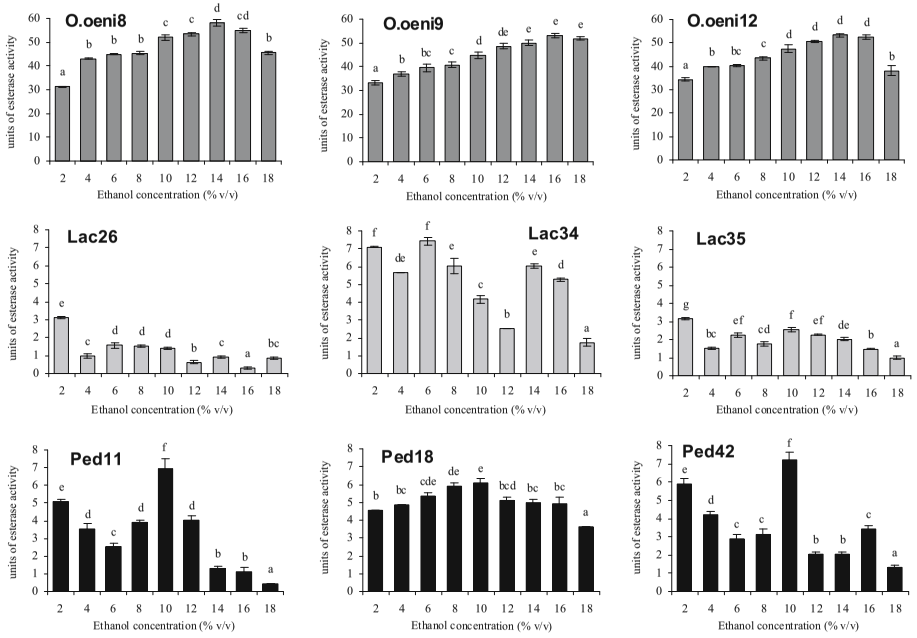


Fig. 3 Influence of ethanol on esterase activity of LAB strains. Units of esterase activity are defined as micromoles of *p*-nitrophenol liberated per minute per microgram of dry cell weight. Values are the mean of triplicates ± standard deviation. Within each panel columns with different letters are significant at the 5% level according to the multiple range test. NB: The scales differ across the genera

towards the four-carbon *p*NP-butyrate, but with high activity also towards *p*NP-acetate and *p*NP-octanoate. Similarly, all three strains had minimal activity towards the 14- and 18-carbon substrates. Amongst the lactobacilli, strong specificity towards the shortest substrate (*p*NP-acetate) was observed and all three strains showed minimal or no hydrolysis of the longer chained ester substrates.

Discussion

Esters are important contributors to the aroma profile of wine, and changes in their concentration have the potential to influence wine quality. The exploitation of esterases to modulate the ester composition of wine could either utilise purified enzyme preparations or selected microbial cultures bearing specific esterase activities. LAB and *O. oeni* in particular make good candidates for the latter approach because these organisms are routinely used to effect the MLF. Before such a notion can be tested, a fuller characterisation of the esterolytic capabilities of LAB is necessary. The work that is reported in this paper provides some of these data. In this study, the activities of nine LAB strains from the genera of *Lactobacillus*, *Pediococcus* and *Oenococcus* were characterised in relation to parameters of importance in the winemaking environment. The results of this investigation indicate that LAB possess esterase activities that could potentially alter the ester profile of wine.

The first of the physicochemical parameters examined, pH, is monitored throughout the entire winemaking process. Low pH, commonly within the range 3.0 to 3.6, is sought in both grape juice and wine for a number of reasons; to optimise the colour of red wines (Somers 1971), to reduce the likelihood of growth by spoilage micro-organisms (du Toit and Pretorius 2000), and to increase the concentration of molecular sulphur dioxide (Margalit 1997). In this study, esterase activity was examined across the pH range 3.0 to 8.0. Under these conditions, most of the LAB strains were found to have the highest esterase activity when the pH was close to 6.0 (Fig. 1). Esterases with pH optima close to neutral have previously been reported in LAB, including *Lactococcus lactis* (Holland and Coolbear

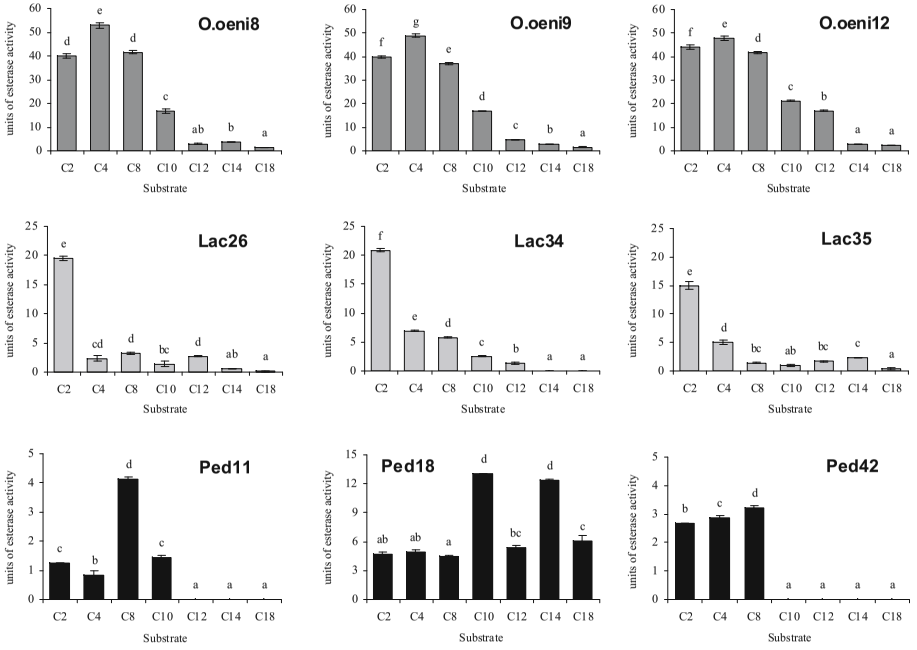


Fig. 4 Esterase activity of LAB strains towards substrates of different chain length. Substrates used were *p*NP-acetate (C2), -butyrate (C4), -octanoate (C8), -decanoate (C10), -dodecanoate (C12), -tetradecanoate (C14) and -octadecanoate (C18). Units of esterase activity are defined as micromoles of *p*-nitrophenol liberated per minute per microgram of dry cell weight. Values are the mean of triplicates ± standard deviation. Within each panel columns with different letters are significant at the 5% level according to the multiple range test. NB: The scales differ across the genera and the scale of Ped18 differs from Ped11 and Ped42

1996; Chich et al. 1997), *Lactobacillus casei* (Fenster et al. 2003) and *Lactobacillus helveticus* (Fenster et al. 2000). However, all of these results were obtained using purified enzyme preparations, rather than whole cell biomass, which was the case in this study. Despite having maximum activity close to pH 6.0, some of the strains, O.oeni9 in particular, showed activity over a broad pH range. Reports from studies conducted using dairy isolates of LAB have also described such ranges in pH optima (Chich et al. 1997; Castillo et al. 1999). In this report, some strains maintained significant esterase activity in the wine pH range. For example, both O.oeni12 and O.oeni9 retained some 50–60% of their maximum esterase activity at pH 3.5 compared to their optimum pH values.

As stated earlier, MLF is often conducted following alcoholic fermentation (Liu 2002). At that time, wine storage temperatures are preferably kept below 20°C, which coincides with the optimum growth temperature of LAB in wine (Asmundson and Kelly 1989). As with pH, some LAB have previously been reported to have esterases with broad temperature optima (Castillo et al. 1999). In this study, maximum esterase activity was generally found at temperatures that were higher than those considered suitable for wine production and storage (Fig. 2). Activity was, however, observed across a wide temperature range and some strains retained significant activity at lower temperatures. All strains had more than 40% of their maximum activity at 20°C, and *O. oeni* retained more than 60% of maximal activity at 20°C. These results therefore again suggest that esterases of wine LAB could be active at the temperatures applied during MLF and wine storage.

A further consideration of the inoculation of wine with LAB post-alcoholic fermentation for initiation of the MLF is that ethanol will be present at a high concentration. The growth of some LAB is inhibited by ethanol concentrations as low as 4% in wine (Capucho and San Romão 1994). By contrast, the malolactic activity of some strains of *O. oeni* is unaffected by ethanol concentrations of up to 12%, and

partial activity is retained at 16% (Capucho and San Romão 1994). The results reported for some strains are comparable with observations for the malolactic enzyme, that is that esterases are not inhibited by ethanol at concentrations commonly found in wine (Fig. 3). In fact, esterolytic capabilities of the *O. oeni* strains were stimulated by increasing ethanol concentration, up to about 14%. Other reports from our group on the β-glucosidase activity of LAB isolates describe a similar enhancement of activity by ethanol (Grimaldi et al. 2000, 2005a, b). A possible explanation for this trend is related to influence of ethanol on the permeability of the cell membrane, and hence, the degree of substrate and enzyme interaction.

Based on the findings reported for the artificial substrate used here, it appears probable that LAB esterases will retain at least partial activity under wine-like conditions of pH, temperature and ethanol concentration. While further work with natural substrates is necessary, a preliminary investigation of substrate specificity was also conducted to more fully define the potential applications of specific strains. As shown in Fig. 4, a distinct specificity for short- and medium-chained esters was found, with the exception of Ped18. Similar observations have been reported in dairy LAB, including some species also associated with wine, such as *L. helveticus* (Fenster et al. 2000), *L. casei* (El Soda et al. 1986b; Castillo et al. 1999; Fenster et al. 2003; Choi et al. 2004), *Lactobacillus plantarum* (El Soda et al. 1986b; Macedo et al. 2003), *Lactobacillus fermentum* (El Soda et al. 1986b) and *Lactobacillus brevis* (El Soda et al. 1986b). Examples of such esters in wine include ethyl esters, namely, ethyl acetate and ethyl butyrate. Such esters can contribute a desirable, fruity aroma to wine at low concentrations (Lilly et al. 2000); thus, degradation of these aromatic compounds would not necessarily be advantageous to wine quality. However, a beneficial role of these esterases might arise out of their deliberate application to treat wines tainted with unacceptably high concentrations of these esters. Such concentrations may arise through the action of the inoculated yeast or spoilage organisms including *Pichia anomala*, *Kloeckera apiculata* and *Hanseniaspora uvarum* in grape juice or wine (du Toit and Pretorius 2000) or species of *Acetobacter* growing in wine (Drysdale and Fleet 1989). The removal of excess ethyl acetate, which, at higher concentrations (>200 mg/L; Peynaud 1984), confers an acescent character to wine, would be an advantage. Strain Lac26, shown in our study to have high activity toward the C2 ester substrate and minor activity toward the larger substrates might therefore be used to specifically target this problem compound in wine.

Conversely, a number of the LAB had significant activity towards some of the longer chained esters. Both ethyl dodecanoate and ethyl tetradecanoate are examples that have been identified in wine (Francioli et al. 1999). The aroma contribution of these larger compounds becomes less desirable, and descriptors such as ‘soaplike’ and ‘stearic’ predominate (Rapp and Mandery 1986). Thus, amelioration of wines higher in undesirable long-chained ethyl esters could also be achieved through treatment with selected LAB strains.

While the work presented here goes some way to characterising the activity of LAB esterases, their action in wine is still not fully elucidated. Wine is a complex combination of all of the factors discussed here, as well as others, such as sulphur dioxide, which has previously been shown to inhibit the malolactic activity of LAB (Carreté et al. 2002). The consequences of the combined influences of such individual parameters remains to be studied, particularly because outcomes from such challenges are not necessarily predicted by the response to the single parameters (Grimaldi et al. 2000, 2005a, b). The effectiveness of the detected esterases against natural substrates is currently under investigation, as is the question of whether esterase activities of wine LAB are capable of making significant and detectable changes to the flavour of wines. The sensory contribution of ester-degradation products including fatty acids, higher alcohols and organic acids also requires exploration. Nevertheless, the results reported here show great promise for the deliberate use of LAB to selectively modify wine ester composition and thereby help justify further investigation.

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Full Paper

Proline transport and stress tolerance of ammonia-insensitive mutants of the *PUT4*-encoded proline-specific permease in yeast

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The imino amino acid, proline, has roles in both cellular nutrition and response to stress. Proline uptake in *Saccharomyces cerevisiae* is largely mediated by a high affinity, specific permease, Put4p, and a low affinity general amino acid permease, Gap1p. Both are subject to nitrogen catabolite repression (NCR) and nitrogen catabolite inactivation (NCI). In order for proline to be fully exploited, its transport must be derepressed, as occurs upon depletion of preferred nitrogen sources, and molecular oxygen must be present to allow the first step of catabolism via proline oxidase. This study focuses on the isolation of variants of Put4p, which are insensitive to repression by a preferred nitrogen source (ammonia) and their subsequent effect on proline transport and stress tolerance. Specific amino acid residues in the carboxy-terminal region of Put4p were targeted by site-directed mutagenesis. Substitution at Serine^{605}, a potential phosphorylation target, led to the amelioration of ammonia-induced down-regulation of Put4p. When combined with a promoter mutation (−160), the S^{605}A mutation resulted in increased proline uptake and accumulation. This increase in proline accumulation was associated with increased cell viability in conditions of high temperature and osmotic stress raising possible benefits in industrial fermentation applications.

Key Words——proline uptake; proline-specific permease; *PUT4*; *Saccharomyces cerevisiae*; site-directed mutagenesis; stress tolerance

Introduction

Beverage fermentations often involve the growth of yeast in a nutritionally complex and harsh medium. In grape must, the nitrogen compounds present include proteins, peptides, amino acids, ammonium ions and trace levels of vitamins and nucleotides (Henschke and Jiranek, 1993). Of these, yeast can utilize most α-amino acids, small peptides and ammonium under typical enological conditions. Assimilable nitrogen in grape must varies from 60 to 2,400 mg L^{-1} (Ough and Amerine, 1988) with low levels limiting yeast growth rate and total biomass formation resulting in a sluggish fermentation (Bely et al., 1990). Of the sources of nitrogen available to yeast in grape juice, proline often predominates (Ough and Amerine, 1988); however, unlike other amino acids, only small amounts of proline are utilized throughout enological fermentations

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(Ingledew et al., 1987; Ough and Stashak, 1974). This is due to the prevailing conditions, which effectively prevent the utilization of significant amounts of this amino acid.

Before it can be catabolized and utilized as a nitrogen source, proline must be transported into the cell. This occurs by a non-specific low affinity (K_m = 13 mM; Lasko and Brandriss, 1981) transport system encoded by the *GAP1* (General Amino-acid Permease) gene, and a high affinity (K_m = 0.025 mM; Magana-Schwencke and Schwencke, 1969) proline specific permease encoded by the *PUT4* (Proline UTilization) gene (Jauniaux et al., 1987). In addition, two SPS sensor regulated amino acids permeases, Agp1p and Gnp1p, with broad substrate specificities (Regenberg et al., 1999) have recently been shown to facilitate proline uptake (Andréasson et al., 2004). In contrast to *AGP1* and *GNP1*, both *GAP1* and *PUT4* are subject to nitrogen catabolite repression (NCR) and their protein products to nitrogen catabolite inactivation (NCI). Proline is catabolized within the mitochondrion in a two-step conversion to glutamate (Krzywicki and Brandriss, 1984; Wang and Brandriss, 1987), in which the first enzyme, proline oxidase (*PUT1*) is oxygen dependent (see Fig. 1). During the early stages of a wine fermentation, when oxygen may be present (depending on the extent of reductive handling of the juice), the high levels of preferred nitrogen sources, such as ammonia and glutamine, result in repression of transporter synthesis and inactivation of existing Gap1p and Put4p (Courchesne and Magasanik, 1988; Roberg et al., 1997; Soetens et al., 2001; Stanbrough and Magasanik, 1995). In subsequent stages of fermentation when levels of preferred nitrogen compounds decline and proline transport is derepressed, oxygen instead is no longer available (Poole, 2002).

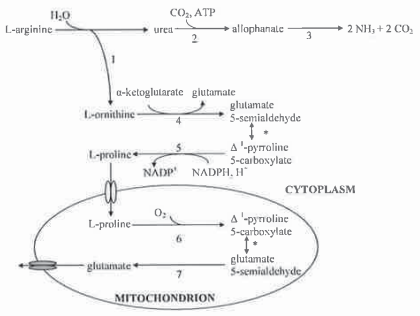


Fig. 1. Arginine and proline degradation in *Saccharomyces cerevisiae* adapted from Henschke and Jiranek (1993).

Enzymes: 1, arginase; 2, urea carboxylase; 3, allophanate hydrolase; 4, ornithine 5-aminotransferase; 5, Δ-pyrroline-5-carboxylate reductase; 6, proline oxidase; 7, Δ-pyrroline-5-carboxylate dehydrogenase; *, spontaneous.

The potential usefulness of proline as a nitrogen source is highlighted through work on the degradation of arginine, the third most prevalent amino acid in grape must (Ough and Amerine, 1988). Under anaerobic conditions, yeast is able to assimilate only three of the four nitrogen atoms of arginine with the fourth, as proline, being unassimilated. Martin et al. (2003) were however able to modify the anaerobic degradation of arginine, to allow for the assimilation of all four nitrogen atoms. That is, the NADPH-dependent P5C reductase (*PRO3*) and the *URE2* negative regulator were deleted, while a *PUT2* (encoding NAD(P) linked Δ^1-pyrroline-5-carboxylate dehydrogenase) devoid of a mitochondrial targeting sequence was strongly expressed (see Fig.1). In being able to utilize the fourth (proline) nitrogen in arginine, the recombinant strain was able to produce twice the biomass of the unmodified strain.

Given the typical predominance of proline in grape must and yet the frequent need for nitrogen supplementation of fermentations (Ough and Amerine, 1988), there is an obvious interest in yeast which can more efficiently utilize proline as a nitrogen source under enological conditions. Exploitation of this largely unassimilable but abundant nitrogen source could allow ferments to proceed more reliably and without supplementation. Problem fermentations may so be eliminated, thereby increasing production efficiency and wine quality.

Beyond offering a nutritional advantage, enhanced proline uptake may also convey a broad protective effect. Proline confers survival and growth advantages in the presence of herbicides (Chen and Dickman, 2005) and hydrogen peroxide (Terao et al., 2003), and acts as a cryoprotectant (Morita et al., 2002) in yeast, as well as compatible solute in plants (Delauney and Verma, 1993) and bacteria (Csonka, 1981). As such the ability of yeast to accumulate higher intracellular concentrations of proline, even without significant catabolism, may offer benefits in the high osmolarity environment of the grape juice fermentation.

This study aims to complement other studies by

overcoming the metabolic restrictions imposed by ammonia on proline utilization, through the constitutive expression of Put4p permease in yeast. Site-directed mutagenesis targeted amino acid residues in the C-terminal region of Put4p that are potentially involved in the ammonia-induced down-regulation of the permease. In addition to considering proline uptake and accumulation, tolerance of such strains to osmotic and thermal stress was also considered in light of the potential role of this amino acid in such attributes (Morita et al., 2002; Takagi et al., 2000).

Materials and Methods

Strains and media. The wild-type strain used in this study, KP2 (a haploid prototroph of W303**a**), was derived by mating W303**a** with a lys-2 marker yeast by classical genetics (Guthrie and Fink, 1991). The prototrophic haploid strain was backcrossed several times with VJ23, (a strain isogenic to W303 except for the *his3* and *leu2* mutations) in order to reconstitute the W303 genetic background of KP2. Deletion strains derived from KP2, including those harboring plasmid borne copies of *PUT4*, are listed in Table 1. Rich media (YPD) was used for routine culture maintenance. Yeast nitrogen base (YNB) media lacking ammonium sulfate and amino acids, was supplemented with the required amino acids and glucose at 20 g L^{-1}. The nitrogen source was 5 g L^{-1} ammonium sulfate or proline (Sherman, 1991). *Escherichia coli* were grown on Luria Bertani (LB) broth supplemented with ampicillin at 100 mg L^{-1} for plasmid selection, unless otherwise stated.

Transformations. Yeast cells were transformed by treatment with lithium acetate (Gietz et al., 1992). Geneticin (Astral Scientific) was used at 400 mg L^{-1} where indicated for selection of *kanMX* based gene disruptions (Walker et al., 2003). *E. coli* transformation was according to Ausubel et al. (1994).

Nucleic acid techniques. Plasmid DNA preparations from *E. coli* were made using the Promega Wizard *Plus* SV plasmid miniprep kit according to the manufacturer's instructions. Yeast genomic DNA and RNA was isolated as described by Adams et al. (1997) and Schmitt et al. (1990) respectively. DNA manipulations were performed using standard procedures (Maniatis et al., 1982). DNA was isolated and purified with an UltraClean 15 DNA purification kit (MO BIO Laboratories).

PCR amplification of DNA. PCR amplification was performed in 50 µl reactions containing 1 U DyNAzyme EXT DNA polymerase (Finnzymes), DyNAzyme EXT PCR buffer, 100 pmol of each primer, 50 ng of plasmid DNA or 200 ng of genomic DNA and 0.5 mM dNTPs. $MgCl_2$ concentrations and cycling parameters were optimized for each PCR reaction. Primers used for PCR amplification are listed in Table 2.

Table 1. Microbial strains used in this study.

Strain	Genotype	Source
Saccharomyces cerevisiae		
W303a	*ade2-1*, *can1-100*, *his3-11*, *-15*, *leu2-3*, *-112*, *trp1-1*, *ura2-1*, *MAT***a**	Susan Henry
Lys2 marker	*lys2*, *MATα*	Susan Henry
VJ23	*his3-11*, *-15*, *leu2-3*, *-112*, *MATα*	This laboratory
KP2	W303 (prototrophic), *MATα*	This study
KP30	W303, *MATα*, *npr2Δ::kanMX4*	This study
KP31	W303, *MATα*, *rcy1Δ::kanMX4*	This study
KP10	W303, *MATα*, *put4Δ::kanMX4*	This study
KP12	W303, *MATα*, *put4Δ::kanMX4*, *ura3Δ::kanMX4*	This study
KP21	KP12 harboring pTEF-PUT4	This study
KP13	KP12 harboring pFL38-PUT4	This study
KP52	KP12 harboring pFL38-PUT4(−160)	This study
KP61	KP12 harboring pFL38-PUT4($K^{604}R$)	This study
KP62	KP12 harboring pFL38-PUT4(LVAA)	This study
KP63	KP12 harboring pFL38-PUT4(LLAA)	This study
KP64	KP12 harboring pFL38-PUT4($S^{605}A$)	This study
KP65	KP12 harboring pFL38-PUT4($S^{605}E$)	This study
TW3	KP12 harboring pFL38-PUT4(−160, $S^{605}A$)	This study
Escherichia coli		
DH5α		Tony Focareta
Epicurian Coli XL1-Blue		Stratagene

Site-directed mutagenesis. Mutations in specific codons of Put4p and nucleotides in the *PUT4* promoter were introduced with the aid of the QuikChange Site-Directed Mutagenesis kit (Stratagene). The PCR reaction was according to the supplier's instructions, except that the extension time was 3 min/kb (24 min). Individual reactions contained pFL38-PUT4 (10 ng) as template and specific sets of primers as listed in Table 2. PCR products of the correct size were digested with *Dpn*I prior to transformation of Epicurian Coli XL1-Blue competent cells. DNA sequencing (in triplicate) of the isolated plasmids using primers SDMSEQ and MutSEQ1, confirmed the presence of specific point mutations. Plasmids pFL38 and p416TEF (Bonneaud et al., 1991; Mumberg et al., 1995) were the source material for plasmids described in Table 1, which (with the exception of pFL38-PUT4 and those containing single point mutations) were generated in vivo using the gap-repair method (Ma et al., 1987). A 1 : 5 ratio of linearized plasmid and amplified DNA (*PUT4* gene or *TEF2* promoter sequences), was used to transform yeast KP12. Plasmid constructs were verified by PCR analysis and DNA sequencing (IMVS, Adelaide, Australia).

PCR labeling of probes for Northern slot blot analysis. DNA probes for the detection of RNA were labeled with Digoxigenin-11-dUTP (DIG) by incorporation into PCR products. The PCR DIG Probe Synthesis Kit (Roche) was used as per the manufacturer's instructions. The DIG labeled products were gel purified before use. Primers used for the synthesis of DIG labeled probes are listed in Table 2.

Northern slot blot analysis. RNA slot blotting: Aliquots containing 5 µg of RNA were diluted in 100 µl of RNA dilution buffer (5 : 3 : 2 ratio of MQ water : 20×

Table 2. Sequences of the primers used in this study.

Primer	Sequence (5′→3′)
TEFPUTf[a]	GGAGTACTTGTTTTTAGAATATACGGTCAACGAAGTATAATTAACTAAACCCATCTAGAAATAAATCATGG
Put4r[a]	TTGCTGCAGTGGTAAAGATCA
Put4f[a]	AAGATTGTAGAAAGGATCCGC
P4UTRr[a,c]	ATTGTTCTTGTGGAAGGGCA
P4probeF[a,c,d]	TGCCCTTCCACAAGAACAAT
P4probeR[a,c,d]	AACAAGGCGTCCAAGAACTT
M1-160f[b]	CAGACGCATAAACATATATGCATATACATACTTATACACTCG
M1-160r[b]	CGAGTGTATAAGTATGTATATGCATATATGTTTATGCGTCTG
K604Rf[b]	GTCGAGATCGAGGAGAGATCAAGAGAAATTGAGGAGATGAG
K604Rr[b]	CTCATCTCCTCAATTTCTCTTGATCTCTCCTCGATCTCGAC
LV-AAf[b]	GATCGATGTTACTACAGGGGCAGCCGAGATCGAGGAGAAATC
LV-AAr[b]	GATTTCTCCTCGATCTCGGCTGCCCCTGTAGTAACATCGATC
Dileuf[b]	CAAAGACAAGTTCTTGGACGCCGCGGCGTAACGCTTTATGAAC
Dileur[b]	GTTCATAAAGCGTTACGCCGCGGCGTCCAAGAACTTGTCTTTG
S605Af[b]	GTCGAGATCGAGGAGAAAGCAAGAGAAATTGAGGAGATGAG
S605Ar[b]	CTCATCTCCTCAATTTCTCTTGCTTTCTCCTCGATCTCGAC
S605Ef[b]	GGCTAGTCGAGATCGAAGAGAAAGAAAGAGAAATTGAGGAG
S605Er[b]	CTCCTCAATTTCTCTTTCTTTCTCTTCGATCTCGACTAGCC
MutSEQ1[c]	AGGGTGTAAAGTGCGTGTG
MutSEQ2[c]	TACTGCAATGTGAATTCCC
MutSEQ3[c]	TATGACGTTTGGGTGGCCT
MutSEQ4[c]	TTATGTACATGCCCCAATC
SDMSEQ[c]	GTTCGGCCATAAGCTGTA
PDAf[d]	TCGCACCTGTATCTTCACAAA
PDAr[d]	AATCCCTAGAGGCAAAACCTT

[a] Primers used in PCR amplification of *PUT4* gene. [b] Primers used in site-directed mutagenesis of *PUT4* gene. [c] Primers used in DNA sequencing of *PUT4* gene and its variants. [d] Primers used for PCR synthesis of DIG-labeled probes.

SSC : formaldehyde) before being transferred to Hybond N+ nylon membrane (Amersham Life Scientific) using a slot blot apparatus (Hoeffer 648, Amersham Pharmacia Biotech). RNA was fixed to the membrane by UV crosslinking using the Amersham Life Sciences UV crosslinker apparatus (254nm @ 70,000 μJ/cm^2).

Hybridization and detection of DIG-labeled DNA probe bound to specific RNA: Prehybridization using DIG EASy Hyb buffer (Roche) was conducted at 50°C for 2 h. Hybridizations were carried out at 50°C overnight using 25 ng/ml of denatured DIG-labeled probe and washed to a stringency of 0.5× SSC/0.1% SDS at 68°C. Chemiluminescent detection of RNA was conducted according to Van Miltenburg et al. (1995). Briefly, the membrane was washed at room temperature, followed by incubation in 1× blocking solution (Roche) prior to incubation with anti-Digoxigenin-AP antisera (1 : 100,000 in blocking solution; Roche). The membrane was washed before equilibration in detection buffer and then incubated in the dark with 0.5 ml ECF substrate (Amersham Life Sciences) per 10 cm^2 of membrane. Fluorescent bands were detected using a Storm 860 phosphoimager (Molecular Dynamics).

Proline assays. Proline uptake: Measurement was as described by Lasko and Brandriss (1981) with minor variations. Cells (5–20 ml) were harvested by filtration, washed in YNB (lacking carbon and nitrogen sources), and incubated for 5 min in pre-warmed aerated YNB lacking a nitrogen source. [^{14}C]-proline (0.5 μCi, specific activity 260 mCi/mmol, Amersham Pharmacia, CFB71) was added to a final concentration of 0.2 mM and 500 μl samples were taken at 15, 60, 105 and 150 s after addition. The sampled cells were filtered, washed with YNB lacking a carbon source and containing 0.1% proline, and dried (microwave at 300 W, 2 min), before addition of 4 ml scintillation fluid (Starscint, 6013248). [^{14}C]-proline incorporation was determined as counts per min (CPM) using a Packard scintillation counter. Values were normalized (nmol proline min^{-1} mg^{-1} cells) by dry weight determination (in duplicate) of an identical sample at the time of sampling.

Proline accumulation: YNB media containing 150 g L^{-1} glucose, 1 g L^{-1} proline, and 0.427 mM [^{14}C]-proline (Amersham Pharmacia, CFB71) was inoculated with yeast cells at 1 × 10^6 cells ml^{-1}. Samples (500 μl) taken at 0, 3, 6, 9, 12 and 24 h intervals were harvested on 0.22 μm nitrocellulose filters, and washed twice with sterile MQ water before addition of 4 ml of scintillation fluid. [^{14}C]-proline incorporation was determined as described above. Values for the mean (including standard deviation) were from duplicate determinations of triplicate cultures.

Stress treatments. Osmotolerance: Strains precultured in YNB media containing 1 g L^{-1} proline as nitrogen source, were transferred (at 5 × 10^6 cells ml^{-1}) to YNB media of high osmolarity (1.1 M NaCl, 1.1 M glucose or 1.1 M sorbitol) and grown for 24 h. Conical flasks (250 ml) fitted with a septum sealed side port for sampling and side arm (to allow direct measurement of growth) were used in the experiment. Growth was monitored over 24 h, and measured directly in the culture flasks by inserting the side-arm (filled with culture) into a Hach 2100AN Turbidimeter fitted with a 610 nm filter. Culture density was expressed as nephelometric turbidity units (NTU). The percentage of viable cells present at the end of fermentation was measured by comparison of the number of colony forming units after 2–3 days of growth on YPD from appropriately diluted cultures (in phosphate buffered saline) to a total cell count of the same time point, using a hemocytometer.

Thermotolerance: Precultured cells were washed in fresh media and subjected to heat (20 min, 45°C). Viability measurements were performed prior to cells being transferred to YNB plus 1 g L^{-1} proline (5 × 10^6 cells ml^{-1}), and growth monitored as turbidity units (NTU). Values for the mean (including standard deviation) were from duplicate determinations of triplicate cultures.

Results

Generation of strains non-responsive to nitrogen catabolite repression

Strain KP2 (W303 prototroph, *MAT*α), in which *PUT4* expression is subject to NCR, was chosen as the wild type strain for this study (Poole, 2002). Gene-replacement of *PUT4* with the *kanMX4* selection marker was achieved by homologous recombination with a PCR-generated DNA fragment consisting of the *kanMX4* cassette with flanking *PUT4* sequences (Wach et al., 1994) and resulted in the construction of strain KP10 (*put4Δ::kanMX4*). This strain served as background for introduction through gap-repair and homologous recombination (Ma et al., 1987; Mumberg et al., 1995) of *PUT4* under the control of the constitutive *TEF2* promoter (i.e. *pTEF-PUT4*) to yield strain, KP21. A strain

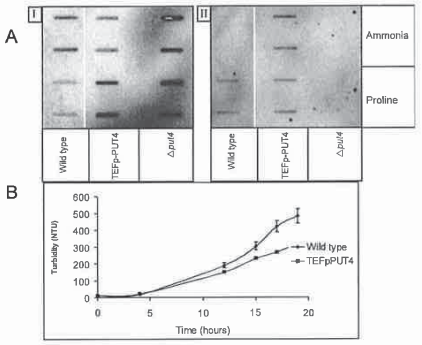


Fig. 2. Northern slot blot analysis of *PUT4* expression.
A: Strains KP2 (wild type) and KP21 (*TEFp-PUT4*) were grown in minimal media containing either proline or ammonium as the sole source of nitrogen. Cells were harvested at 5 × 10^7 cells/ml and cellular RNA was extracted and analyzed in duplicate by Northern slot-blot analysis as described in MATERIALS AND METHODS. Panel I: detection of *PDA* mRNA with DIG-labeled DNA probe corresponding to the *PDA* gene was the loading control of RNA samples. Panel II: *PUT4* mRNA detected with a DIG-labeled DNA probe corresponding to the *PUT4* gene. B: Strains KP2 (wild type) and KP21 (*TEFp-PUT4*) were grown (in triplicate) in minimal media containing ammonium as the sole source of nitrogen. Growth of the culture was measured directly using a Turbidimeter, and expressed as nephelometric turbidity units (NTUs).

with constitutive *PUT4* expression was constructed in order to study the effects of a nitrogen source on this proline-specific permease. This strain gave a constitutive pattern of *PUT4* expression (defined as presence of *PUT4* mRNA under ammonia-repressive conditions); however, its growth was typically of the order of 65% of the wild type (Fig. 2), presumably due to the metabolic load imposed on this strain by the relatively high strength of the *TEF2* promoter (Piruat et al., 1997).

Post-translational down-regulation of the proline-specific permease, Put4p

The presence of preferred nitrogen sources not only leads to the repression of *PUT4*, but also to the inactivation of the Put4p permease (Jauniaux et al., 1987). Therefore, to determine whether *PUT4* expression equates to Put4p activity, direct determination of Put4p transport activity by one of the constitutive strains (KP21, *pTEF-PUT4*) was carried out by isotopic assay (Lasko and Brandriss, 1981). To ensure transport occurred via Put4p and not Gap1p, radiolabeled [^{14}C]-proline was included in the assay at a final concentration of 0.2 mM, to exceed the K_m of Put4p (0.025 mM; Magana-Schwencke and Schwencke, 1969), whilst falling well below that of Gap1p (13 mM; Lasko and Brandriss, 1981). The contribution of the two SPS-regulated amino acid permeases, Agp1 and Gnp1, was considered negligible based on previous studies. Thus Agp1 has low affinity and broad specificity for various amino acid substrates, is not significantly expressed in cells grown in urea or proline alone whilst no detectable uptake of [^{14}C]-proline is observed at concentrations of 0.1 mM (Andréasson et al., 2004; Iraqui et al., 1999). Similarly *GNP1* is not functionally expressed when cells are grown in proline alone, with over-expression of *GNP1* failing to increase proline uptake relative to the parental strain (Regenberg et al., 1999; Schreve et al., 1998).

Compared to KP2 (wild type), Put4p activity was higher in the constitutive *PUT4* strain KP21, both in the absence and presence of ammonium (Fig. 3). Proline uptake was, however, lower in the presence of ammonium than when grown with proline alone, an expected consequence of the post-translational inactivation of Put4p, occurring with preferred nitrogen sources (Grenson, 1983). In keeping with early reports that Put4p activity decreases with progression through the growth phase (Horak and Rihova, 1982), we also found proline uptake to decline in all strains as the culture moved through the phases of growth.

In the shorter term, inactivation of Put4p was partial and transient. Thus, proline uptake activity was shown to decrease rapidly over the first 30 min after ammonium addition, declining from 35 ± 4 nmol proline min^{-1} mg^{-1} cells to 2 ± 2 nmol proline min^{-1} mg^{-1} cells (Fig. 4). At 60 and 120 min after the addition of ammonium, Put4p activity recovered to approximately 40% of that measured at the zero time point. No recovery of this sort is evident in similar studies of the Gap1p response to the addition of ammonium (Hein and André, 1997; Springael and André, 1998). Such post-translational regulation of Put4p is likely to involve several genes and factors. Two of these are *NPR2* and *RCY1*. Suggested to be a protein kinase that stabilizes amino acid transporters by antagonizing their ubiquitin-mediated degradation (De Craene et al., 2001) when deleted, *NPR2*, was expected to increase the loss of proline uptake activity upon exposure to am-

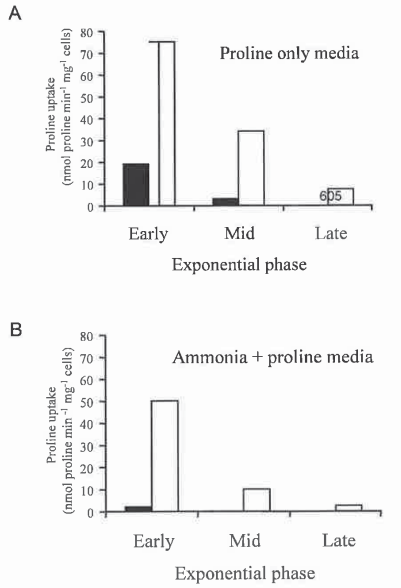


Fig. 3. The effect of growth phase on Put4p activity.

Cultures of KP2 (wild type, ■) and KP21 (*TEFp-PUT4*, □) were grown in media containing either (A) proline alone or (B) ammonia and proline as nitrogen sources. Cells were harvested at three points during growth: early (1×10^7 cells ml^{-1}), mid (5×10^7 cells ml^{-1}) and late (1×10^8 cells ml^{-1}) exponential phase, and assayed for Put4p activity using [^{14}C]-proline. Values are the mean of duplicate determinations from duplicate cultures. SD values for such experiments ranged from 2% to 12%.

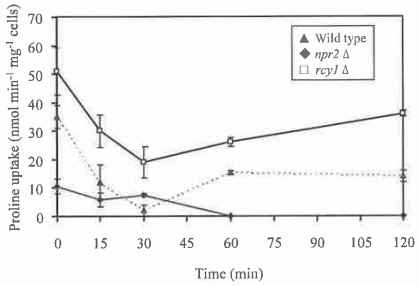


Fig. 4. The effect of ammonium addition on Put4p activity in wild type strain KP2, *npr2*Δ strain KP30 and *rcy1*Δ strain KP31.

YNB medium containing proline (as the nitrogen source), was inoculated with the yeast strains (at 5×10^6 cells ml^{-1} from a stationary phase culture) and grown until early exponential phase (1×10^7 cells ml^{-1}). Ammonium sulfate (5 g L^{-1}) was added and Put4p activity was measured using [^{14}C]-proline at 0, 15, 30, 60, 120 min post addition. Wild type strain KP2 (▲); *npr2*Δ strain KP30 (◆) and *rcy1*Δ strain KP31 (□). All values derived from the mean of duplicate determinations from triplicate cultures ± SD.

monium or else reduce the extent of recovery of lost activity. Both outcomes were evident (Fig. 4). In accordance with previous findings (Rousselet et al., 1995) the initial activity of Put4p was ca. 30% of the activity of the wild-type strain and by the 60 min time point no activity was detected and no recovery was observed.

The observations of Wiederkehr et al. (2000) suggest the *RCY1* gene product is involved in endocytic membrane traffic and recycling of early endosomes to the membrane in a process involving ubiquitin. Although not deficient in internalization of membrane fractions, the *rcy1* mutation blocks the intersection of the endocytic pathway with the vacuolar protein sorting pathway, thereby reducing degradation of internalized proteins. Initial Put4p activity was higher in the *rcy1*Δ strain than in the wild-type strain, yet followed a similar pattern of down-regulation and partial recovery of Put4p activity. As anticipated, however, a greater proportion (i.e. approx. 70% vs. 40%) of proline uptake activity was recovered by the 120 min time point. To further explore the role of Put4p inactivation, we sought to determine the consequences of key sequence changes to the ORF of *PUT4* through determination of short-term uptake activities and in longer term proline accumulation assays.

Characterization of PUT4 mutants resistant to nitrogen catabolite inhibition of Put4p

Ubiquitination of a number of plasma membrane proteins results in their endocytosis followed by vacuolar degradation (Galan et al., 1996; Hicke and Riezman, 1996; Omura et al., 2001; Springael and André, 1998). Previous reports have demonstrated specific lysine residues within the N-terminal domains of Gap1p (Soetens et al., 2001) and Put4p (Omura et al., 2005) to be ubiquitinated, and essential for down-regulation and direct sorting to the vacuole. Omura and coworkers (2005) further speculate that additional lysine resi-

dues, besides the N-terminal nine investigated, may be responsible for ubiquitination and subsequent degradation of the Put4p permease. Interestingly, a Lys to Arg substitution in the DXKSS sequence within the C-terminal domain of Ste2p resulted in the elimination of both ubiquitination and internalization of the variant (Hicke and Riezman, 1996). Further, replacement of a lysine residue within a similar motif in Gap1p (EEKAI) with glutamate, rendered the permease resistant to ammonium-induced inactivation (Hein and André, 1997).

Based on such studies, we targeted by site-directed mutagenesis specific amino acid residues in the Put4p protein identified as being analogous to those involved in the nitrogen catabolite inactivation of Gap1p (Hein and André, 1997; Springael and André, 1998). Put4p variants, with specific amino acid changes in the C-terminal domain, were analyzed for Put4p activity. A lysine residue within the EEKSR motif of Put4p was mutagenized (K^{604}R) using primers K604Rf/r (Table 2) to yield strain KP61. The resulting substitution did not significantly alter the uptake of proline, other than delaying recovery of uptake activity from the 30 min point after ammonium addition (Fig. 5). Hein and André (1997) demonstrated by site-directed mutagenesis that the replacement of a di-leucine motif (L^{575}, L^{576}) within a predicted α helix in the C-terminus of Gap1p

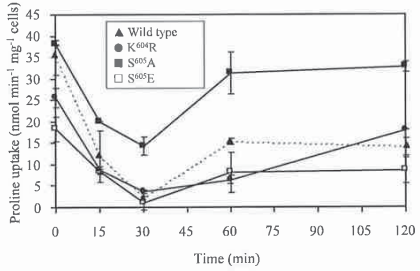


Fig. 5. The effect of ammonium addition on the activity of endogenous Put4p and variant Put4p permeases.

Strains were grown in media containing proline as the sole source of nitrogen. At time 0, ammonium sulfate (5 g L^{-1}) was added to the growth medium, and uptake of [^{14}C]-proline was assayed at the intervals shown, over a 120 min period. Values are the mean of duplicate determinations from triplicate cultures. Endogenous Put4p (▲) and Put4p K^{604}R (●), Put4p S^{605}A (■), Put4p S^{605}E (□) activities found in KP2, KP61, KP64, and KP65 respectively.

with two alanines protected the permease against ammonium-induced inactivation. Two similar motifs in the C-terminal region of Put4p, a leucine-valine motif at residues 598 and 599 and a di-leucine motif at residues 626 and 627, were replaced with two alanines (using primers LV-AAf/r and Dileuf/r, respectively; Table 2), in the strains KP62 (Put4p L^{598}A, V^{599}A) and KP63 (Put4p L^{626}A, L^{627}A). Both substitutions resulted in no detectable Put4p activity (data not shown), possibly attributed to either misfolding or incorrect sorting of the Put4p permease.

Interestingly, two types of di-leucine motifs are found in mammalian cells; one is dependent on the phosphorylation of a proximal residue, while the other is constitutively active and includes an acidic amino acid (Giesler et al., 1998). A single serine residue, S^{605}, the only predicted phosphorylation site in the C-terminal region of Put4p (Blom et al., 1999), was replaced with alanine (S^{605}A), using primers S605Af/r (Table 2) to yield strain KP64. Based on the supposition that the negative charge of glutamate could mimic the negative charge of a phosphorylated site (Stark, 1998), another substitution, serine to glutamate (S^{605}E), was created using primers S605Ef/r (Table 2) to produce strain KP65.

Proline grown cells expressing the Put4p S^{605}A variant (KP64) displayed similar rates of proline uptake to the wild type (38 ± 0.1 nmol proline min^{-1} mg^{-1} cells vs. 35 ± 4 nmol proline min^{-1} mg^{-1} cells; Fig. 5). When ammonium was added, the Put4p S^{605}A permease appeared resistant to inactivation, displaying higher activity (14 ± 0.3 nmol proline min^{-1} mg^{-1} cells) compared with wild type (2 ± 2 nmol proline min^{-1} mg^{-1} cells). The rate of proline uptake by Put4p S^{605}A not only recovered between the 30 and 120 min time points, but in fact achieved a value of 32 ± 0.1 nmol proline min^{-1} mg^{-1} cells or 86% of the activity found in the ammonium-free condition. In comparison, the S^{605}E modification led to a lower rate of proline uptake after growth in proline (18.3 ± 2.9 nmol proline min^{-1} mg^{-1} cells), but one which declined more slowly after ammonium addition. The proportion recovered by the 120 min time point was similar to that of the wild type (ca. 40%).

Proline accumulation in strains constitutively expressing Put4p S^{605}A

The longer term consequences of modifications intended to increase the half-life of the permease were

examined by combining these with those which resulted in ammonia insensitive over-expression. The *PUT4* gene with and without the $S^{605}A$ mutation, was driven by the *PUT4* promoter mutation (T→C at −160). The −160 mutation represents an example of the minimum modification needed in order for over-expression of *PUT4* under repressive conditions as occurs during growth in minimal media containing ammonia as the nitrogen source (data not shown). Proline accumulation was measured by monitoring the incorporation of the radiolabeled amino acid over a 24 h incubation. The two constitutive strains, KP52 (T→C at −160) and TW3 (T→C at −160, $S^{605}A$), were compared with KP13, a *put4*Δ strain harboring plasmid-borne *PUT4* of unmodified sequence and therefore subject to NCR.

The wild-type strain showed low accumulation with all over-expression strains accumulating more proline throughout the experiment (Fig. 6A). The $S^{605}A$ mutation resulted in a marked increase (up to 50-fold) in accumulation of proline within 3 h by TW3 (T→C at −160, $S^{605}A$) compared with KP52 (T→C at −160). These results indicate a stabilized Put4p permease which is insensitive to NCI, perhaps through disruption of phosphorylation and ubiquitination (Springael and André, 1998). The comparable growth of all three strains in YNB media (Fig. 6B) suggests that increased accumulation of proline was not detrimental in the time frame of this experiment. Further, since the first step in proline catabolism, mediated by the product of *PUT1*, proline oxidase, is repressed by ammonium (Xu et al., 1995), most proline that was accumulated would be expected to be present as the free amino acid, with minor amounts having been directly incorporated into new proteins.

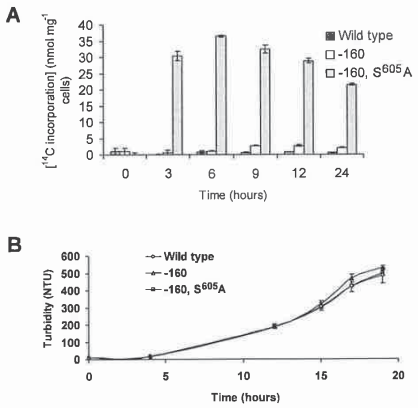


Fig. 6. Accumulation of proline from media by wild type and *PUT4* variant strains.

A: Proline accumulation assay: YNB media containing ammonium sulfate (5 g L^{-1}), proline (1 g L^{-1}) and 0.427 mM [^{14}C]-proline was inoculated in duplicate with 5 × 10^6 cells ml^{-1} and incubated at 30°C over a 24 h period. Proline incorporation (as [^{14}C] radioactivity) was measured in duplicate for each strain at three hourly intervals for 12 h and at 24 h. B: Comparative growth of strains to ascertain any pleiotrophic growth effects of the introduced plasmid-borne mutation(s). Parallel cultures were grown in media as described above (without [^{14}C]-proline). Growth was measured as described in MATERIALS AND METHODS. Data points represent means of triplicate cultures, standard error bars are shown. KP13 (Wild type), KP52 (−160), TW3 (−160, $S^{605}A$).

Consequences of enhanced proline accumulation

Proline is an osmoprotectant in plants and bacteria (Le Rudulier et al., 1984) and increased intracellular proline content confers increased viability after freezing and desiccation (Takagi et al., 2000). Therefore, the overexpression strains KP52 (T→C at −160) and TW3 (T→C at −160, $S^{605}A$), which are capable of increased proline accumulation, were evaluated for increased tolerance to stress. Strain TW3 (*PUT4* T→C −160, $S^{605}A$) exhibited significantly greater final viability following heat shock, and during growth in a medium containing 1.1 M NaCl but not 1.1 M sorbitol (Table 3). No significant differences were found in growth behavior between the strains when grown on medium containing 1.1 M glucose.

Discussion

In the presence of oxygen, *S. cerevisiae* is capable of growth on proline as the sole nitrogen source. However, proline is a poor nitrogen source; thus proline transport and catabolism are repressed and inhibited in the presence of superior nitrogen sources such as ammonium and glutamine (Courchesne and Magasanik, 1988; Lasko and Brandriss, 1981; Xu et al., 1995). The aim of this study was to isolate mutants of the *PUT4*-encoded proline-specific permease insensitive to ammonia repression, and investigate the effects on proline transport and stress tolerance in *Saccharomyces cerevisiae*.

Table 3. Percentage viability of wild type and constitutive *PUT4* strains exposed to heat shock or high osmolarity.

	KP13 (PUT4 wild type)	KP52 (−160)	TW3 (−160, $S^{605}A$)
Glucose	56.00 ± 7.00	51.00 ± 4.36	50.67 ± 9.70
Sorbitol	38.67 ± 6.23	47.00 ± 14.57	33.67 ± 3.84
NaCl	32.67 ± 6.74	40.00 ± 1.53	**63.67** ± 5.36
Heat	40.00 ± 3.61	38.67 ± 7.13	**63.67** ± 10.75

The average mean values (±SD) of triplicate determinations of cell viability reported. Values of significance highlighted in bold.

While over-expression of *PUT4* using the strong ammonia insensitive promoter *TEF* (considered constitutive) does lead to an increased rate of proline uptake, inactivation of the permease is apparent over the longer term (e.g. Fig. 3). Examination of the kinetics of inactivation revealed rapid loss of uptake activity over the initial 30 min after a shift into an ammonium-containing medium, followed by a partial recovery of activity in the subsequent 90 min. By comparison, no such recovery was observed during the ammonium-induced down-regulation of Gap1p (Hein and André, 1997). Deletion of the *RCY1* and *NPR2* genes confirmed the involvement of these genes in the inactivation of Put4p. In *rcy1* strains, degradation of internalized proteins has been shown to be reduced (Wiederkehr et al., 2000), an observation which might explain the higher initial activity and greater recovery of lost Put4p activity in an *rcy1* mutant (KP31) in this study (Fig. 4). Conversely, the fact that elimination of Npr2p, an antagonist of ubiquitin-based permease degradation, results in low and short-lived Put4p activity highlights the importance of this gene to maintenance of proline uptake activity. This latter observation also provides justification for investigation of ubiquitination targets in Put4p that are analogous to those found in Gap1p (Soetens et al., 2001).

The down-regulation (and inactivation) of Gap1p is dependent on the localization of the permease (De Craene et al., 2001; Roberg et al., 1997; Soetens et al., 2001; Springael and André, 1998). Ammonium-induced loss of measurable Gap1p activity thus reflects the progressive removal of Gap1p from the plasma membrane. During growth on proline Gap1p is ubiquitinated at two lysine residues upon the addition of a preferred nitrogen source, which leads to poly-ubiquitination and subsequent down-regulation (Springael et al., 1999b). This ubiquitination of Gap1p is dependent on the function of Npi1p and Npi2p (Springael et al., 1999a, b). The insensitivity of the $Gap1p^{LL\rightarrow AA}$ mutant to ammonium-induced down-regulation, despite the fact that it is ubiquitinated (Hein and André, 1997), suggests that ubiquitination is not solely responsible for Gap1p inactivation, and that multiple motifs are involved in Gap1p regulation.

The lysine residue within the EEKSR motif in the C-terminal region of Put4p was modified to arginine in an attempt to disrupt ubiquitination of Put4p. The $K^{604}R$ mutation did not ameliorate the ammonium-induced down-regulation of Put4p in comparison to the wild type, which indicates that this lysine residue is either not a ubiquitination site, or its ubiquitination has little effect on Put4p down-regulation. Interestingly, sequence identity does not necessarily correlate with function, as in the case of ubiquitination of the Gap1p and Put4p permeases. Recent studies have identified specific lysine residues, namely K^9 and K^{16} in Gap1p (Soetens et al., 2001) and K^9, K^{34}, K^{35}, K^{60}, K^{68}, K^{71}, K^{93}, K^{105}, and K^{107} in Put4p (Omura et al., 2005), as ubiquitination sites within the N-terminal sequences, in which there is only limited homology.

The phosphorylation state of Gap1p has been implicated in the ubiquitination and down-regulation of Gap1p. The kinetics of Gap1p down-regulation and dephosphorylation mirror each other (Stanbrough and Magasanik, 1995), implying that dephosphorylation of Gap1p is required for internalization. Recently, it was shown that Gap1p phosphorylation is decreased on inactivation of Npr1p (De Craene et al., 2001). On addition of ammonium to a proline-grown culture or the inactivation of Npr1p, neosynthesized Gap1p is targeted from the Golgi to the vacuole and Gap1p is internalized at the membrane (De Craene et al., 2001). It is interesting to note that the N-terminal region of Put4p contains 14 predicted phosphorylation sites (Blom et al., 1999), compared with the single phosphorylation site predicted in the C-terminal region (S^{605}). This prevalence of potential phosphorylation sites is consistent with evidence that the N-terminal region of Put4p is involved in ammonium-induced down-regulation, and ubiquitination (Omura et al., 2005).

It is also noteworthy that other determinants are likely to play a role in post-translational control, as in the case of Gap1p, where a di-leucine motif in the C-terminal region functions as an internalization signal (Hein and André, 1997). Modification of two potential internalization signals, $L^{558}V^{559}$ and $L^{626}L^{627}$ motif in the C-terminal region of Put4p, rendered the permease inactive. This might result either from mis-localization of Put4p or inactivation of the permease activity.

Our findings and others indicate that the intracellular levels of proline and arginine are correlated to the stress resistance of yeast (Morita et al., 2002; Takagi et al., 2000). The disruption of *PUT1* caused proline accumulation and led to increased viability in comparison to the control strain following exposure to freezing and desiccation (Takagi et al., 2000). Further, the survival rate of both wild type and *put1* strains increased in proportion to their arginine contents (Morita et al., 2002). Proline as a highly soluble compound functions as an osmoprotectant, and is accumulated in the cytoplasm and transported to the vacuole when in excess (Morita et al., 2002). The specific role of proline in osmoprotection and cryoprotection is, however, unclear.

We report on the isolation of variants of proline-specific permease, Put4p, which are able to accumulate proline, even when cells are grown in the presence of ammonia in the media. Furthermore, some of these mutants are able to withstand both osmotic and thermal stress. These findings raise the possibility that through an increased uptake of proline, which is potentially of nutritional as well as stress-tolerance significance, such strains may find use in high gravity, low nitrogen fermentations. Further work is now required to test this notion.

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Cloning and Characterization of an Intracellular Esterase from the Wine-Associated Lactic Acid Bacterium *Oenococcus oeni*▽†

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We report the cloning and characterization of EstB28, the first esterase to be so characterized from the wine-associated lactic acid bacterium, *Oenococcus oeni*. The published sequence for *O. oeni* strain PSU-1 was used to identify putative esterase genes and design PCR primers in order to amplify the corresponding region from strain Ooeni28, an isolate intended for inoculation of wines. In this way a 912-bp open reading frame (ORF) encoding a putative esterase of 34.5 kDa was obtained. The amino acid sequence indicated that EstB28 is a member of family IV of lipolytic enzymes and contains the GDSAG motif common to other lactic acid bacteria. This ORF was cloned into *Escherichia coli* using an appropriate expression system, and the recombinant esterase was purified. Characterization of EstB28 revealed that the optimum temperature, pH, and ethanol concentration were 40°C, pH 5.0, and 28% (vol/vol), respectively. EstB28 also retained marked activity under conditions relevant to winemaking (10 to 20°C, pH 3.5, 14% [vol/vol] ethanol). Kinetic constants were determined for EstB28 with *p*-nitrophenyl (*p*NP)-linked substrates ranging in chain length from C_2 to C_{18}. EstB28 exhibited greatest specificity for C_2 to C_4 *p*NP-linked substrates.

The quality of fermented foods and beverages is affected in part by their composition of aroma compounds. In winemaking, the malolactic fermentation is used to deacidify wine and is typically carried out by *Lactobacillus* spp., *Pediococcus* spp., and particularly *Oenococcus oeni* (10, 7). Numerous reports clearly show that outside of this core function, lactic acid bacteria (LAB) can also bring about significant changes of sensorial importance (2, 5, 12, 32, 40, 49, 55). Such studies typically examined the action of active cultures or whole cells; however, the promise of LAB as a source of purified enzymes for use as additives in winemaking has recently been highlighted (43).

Oenococcus oeni is acidophilic and indigenous to wine and similar environments. While the genome of the commercial PSU-1 strain has been sequenced and analyzed (44), there is limited information on the genes or their potential contribution to food and beverage aroma. The only such genes which have been cloned and partially characterized are *alsS* and *alsD* (24), which are thought to be responsible for the production of diacetyl, the principle compound conferring "buttery" aroma and flavor in wine (reviewed in reference 4). Analogous characterization of other flavor-related genes and enzymes not only may have practical implications for processes using LAB but also may be of fundamental interest.

As a group, esters are a quantitatively significant constituent of beverages such as wine (total of >100 mg · liter^{-1}) (15). Included in this group are the C_4 to C_{10} ethyl esters of organic acids, ethyl esters of straight-chain fatty acids (and branched-chain fatty acids to a lesser degree), and acetates of higher alcohols which are largely, if not exclusively, responsible for the fruity aroma of wine (13, 14). Some volatile esters are frequently found in fermented beverages in only trace amounts, often below threshold concentrations (3, 21, 25, 29, 50). However, they are extremely important for the flavor profile of these products, with different esters often having a synergistic effect to collectively affect aroma when their individual threshold concentrations are not exceeded. The fact that most esters are present in wine at concentrations around the threshold value implies that minor concentration changes might have a dramatic effect on the wine's flavor (3, 21, 25, 29, 50). For this reason, an understanding of the hydrolysis and synthesis of esters in winemaking and how these may be manipulated is essential.

A large amount of esters is formed during the primary fermentation by yeast; after this, LAB can contribute by increasing and decreasing the ester concentration (2, 5, 12, 40, 49, 55). Ester hydrolysis and synthesis can be catalyzed by esterases (6, 35, 38, 54). These enzymes commonly contain a catalytic triad composed of Ser, His, and Asp/Glu residues and a nucleophilic elbow structural motif (GXSXG), which contains the active-site serine residue (1, 31, 36, 48). They also contain an oxyanion hole, of which two residues donate their backbone amide protons to stabilize the substrate in the transition state. The oxyanion hole residues (in bold) have been divided into two groups termed G**X** and GG**G**X, with the glycine and a hydrophobic residue (X) being highly conserved (48).

While extensive research has been carried out on the enzymes responsible for ester formation by wine strains of *Saccharomyces cerevisiae* (22, 23, 45, 51), esterase activity for wine-related LAB is not well documented. Most characterization of esterases in LAB has focused on dairy isolates (9, 16–18, 20). Parallel work in a wine context is limited despite general acceptance of the importance of esters in wine. Until recently, most evidence that wine LAB possess esterase activity came from wine volatile profiling studies which investigated the

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changes in concentration of individual esters during malolactic fermentation (12, 40, 55). Such changes in ester concentration were strain specific and had the potential to greatly affect the final aroma of wine.

Our survey of the esterase activities of whole LAB cells found variations within species and even greater variation between the genera (42), with *O. oeni* showing greatest activity toward the *p*-nitrophenyl (*p*NP)-linked substrates tested. More recently (41), the esterase activities of whole *O. oeni*, lactobacillus, and pediococcus cells was determined under conditions with some relevance to wine. At least partial resistance to the harsh conditions used was observed, thereby demonstrating a necessary requirement of any enzyme intended for application in analogous environments. To more completely characterize esterases of LAB, the enzymes and their structural genes must be fully investigated. This study represents an effort to dissect the complex array of ester synthesis and hydrolysis activities in whole cells by cloning, heterologous expression, partial purification, and biochemical characterization of a single esterase from *O. oeni*. With a view to applying such an esterase under conditions found in wine and perhaps other industrial settings, enzyme function under the harsh physicochemical conditions frequently encountered in wine was examined.

MATERIALS AND METHODS

Strains, media, and plasmids. *Oenococcus oeni* strain Ooeni28 was isolated from freeze-dried LAB starter cultures for use in commercial winemaking and originates from Lallemand nuovi Ceppi Oo2 (42). In preparation for experiments, Ooeni28 was cultured from the stocks in de Man-Rogosa-Sharp (MRS) medium (Amyl Media) supplemented with 20% (vol/vol) tomato juice. *Escherichia coli* DH5α was used as a host strain for cloning and maintenance of plasmids. *E. coli* BL21(λDE3) was used as a host for expression of the *estB* gene under the control of the T7 promoter. *E. coli* transformants were grown at 37°C in either Luria-Bertani broth (LB) or Terrific broth (for expression only), which contained 100 μg/ml of ampicillin when required. All bacteria were stored at −80°C in 30% (vol/vol) glycerol.

DNA isolation and manipulations. Isolation of DNA from Ooeni28 was carried out using the UltraClean microbial DNA isolation kit (MoBio). Primers targeting the open reading frame (ORF) of the putative esterase gene were designed based on the PSU-1 *O. oeni* genome sequence (CP000411) (44). PCR was performed with pfuUltra II Fusion HS DNA polymerase (Stratagene) in an Eppendorf Mastercycler gradient according to the parameters accompanying the polymerase. Plasmids from *E. coli* were prepared with the Wizard SV miniprep system (Promega) according to the protocol provided by the manufacturer. Agarose gel electrophoresis was performed according to the method of Sambrook and Russell (52), and DNA fragments were isolated using the Wizard SV gel and PCR cleanup system (Promega). T4 DNA ligase and other DNA-modifying enzymes were purchased from New England Biolabs Inc. and were used as recommended by the manufacturer. Bacterial transformation was performed using chemically competent *E. coli* cells and heat shock at 42°C for 2 min.

Cloning of the *Oenococcus oeni* esterase gene into *E. coli*. Plasmid pET14.b//*estB28* was constructed by inserting the amplified *estB28* esterase gene from Ooeni28 into the NdeI and BamHI sites of pET14.b (Novagen). The forward primer 5′-GGC GGC CAT **ATG** TCA GAA ATA GTT TTA AAA-3′ was designed to contain an NdeI site (underlined) and the esterase gene start codon (bold). The reverse primer 5′-CGG CCC TCG AG**T** CAT TCT ACT GAT CTG TTA GAA C-3′ was designed to contain an XhoI site (underlined) and the esterase gene stop codon (bold) immediately after the final glutamic acid (GAA) codon. The ligation mixture was used to transform *E. coli* DH5α cells. A positive clone was selected on LBA plates (LB broth with 2% bacteriological agar) containing ampicillin, and the presence of the insert was initially confirmed by whole-cell PCR using the T7 promoter and terminator primers (Novagen). Positive clones were then sequenced, and the sequenced plasmid was then transformed into *E. coli* BL21(λDE3). Target proteins were produced using this clone and contained a polyhistidine tag and a thrombin cleavage site attached to the N-terminal region for purification purposes.

DNA sequencing and sequence analysis. The sequences of both strands of the plasmid construct were confirmed by nucleotide sequencing, with labeling and capillary separation on the AB 3730*xl* performed by the Australian Genome Research Facility (Brisbane). The T7 promoter and terminator primers were used for this purpose. Nucleotide sequencing results were analyzed using Chromas Lite version 2.01 (Technelysium Pty. Ltd., Australia), BioEdit (30), and NCBI BLAST (www.ncbi.nlm.nih.gov/BLAST).

Purification of the recombinant esterase. *E. coli* BL21(λDE3) harboring the pET14.b//*estB28* plasmid was used as the source of the recombinant enzyme. Cultivated cells were induced at an optical density at 600 nm of 0.6 with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma) and were then incubated at 14°C with shaking at 180 rpm for 48 h before harvest. The cell pellet was disrupted using EasyLyse bacterial protein extraction solution according to the manufacturer's instructions (Epicentre Biotechnologies). The crude extract was separated from cell debris by centrifugation at 11,000 × *g* for 30 min at 4°C. The recombinant esterase with a His tag was purified by using affinity chromatography and the BioLogic LP low-pressure chromatography system (Bio-Rad). The cell lysate was loaded onto a column containing Talon metal affinity resin (Clontech) and washed for 1 h at 1 ml · min^{-1} with wash buffer (50 mM sodium phosphate, 300 mM sodium chloride, 10 mM imidazole, pH 7.0). The recombinant enzyme was eluted at 0.5 ml · min^{-1} using McIlvaine buffer (pH 5.0). Chromatography data were recorded using LP Data View software (Bio-Rad). The molecular mass of the purified recombinant esterase was determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis with a 5% (wt/vol) stacking gel and either a 12% or 18% (wt/vol) separating gel, which were made and run according to the manufacturer's protocols (Bio-Rad). The His tag was cleaved using a thrombin CleanCleave kit (Sigma). Determination of enzyme activity (see below) revealed little difference between cleaved and uncleaved enzymes (data not shown). For this reason and because thrombin was lost to the enzyme preparation, we elected to conduct all characterization work with the uncleaved purified recombinant enzyme.

Determination and characterization of esterase activity. Esterase assays were conducted using *p*NP butanoate substrate, with the exception of the substrate specificity trials, in which eight different *p*NP-linked ester substrates were used, as described below. Reaction mixtures (final volume, 200 μl) were prepared in 96-well microplates (Sarstedt) as detailed in each section below and incubated at 37°C (for convenience) for 1 h, with the exception of the temperature optimum experiments. The liberated *p*-nitrophenol was quantified immediately by measuring the absorbance at 410 nm using a microplate spectrophotometer (μQuant; Bio-Tek Instruments). The data were then corrected for nonenzymatic degradation of the ester substrate using an enzyme-free control of the same reaction mixture that was treated in the same manner. All assays and controls were performed in triplicate, and activities were quantified by comparison with *p*-nitrophenol standards.

Determination of substrate specificity. To determine the specificity of EstB28 for different ester substrates, eight *p*NP-linked esters with different carbon chain lengths were used: *p*NP-acetate, -butanoate, -octanoate, and -decanoate (Sigma); *p*NP-hexanoate (TCI); and *p*NP-dodecanoate, -tetradecanoate, and -octadecanoate (Fluka). The method of Pencreac'h and Baratti (47) was used with some modifications. Stock solutions (25 mM) of each substrate were prepared in ethanol (*p*NP-acetate, -butyrate, -hexanoate, and -octanoate) or propan-2-ol (*p*NP-decanoate, -dodecanoate, -tetradecanoate, and -octadecanoate) and stored at −20°C until required. Assays were carried out as described for the pH optimum study, but for all samples the reaction mixtures (final volume, 200 μl) contained a modified 0.1 M McIlvaine buffer (pH 5.0, with the addition of 5 μl Triton X-100 and 100 μl of 1% [wt/vol] gum arabic [Sigma] solution per ml). Following incubation for 1 h at 37°C, the absorbance was measured at 410 nm.

To determine the constants K_m and k_{cat}, the enzyme was incubated with increasing amounts of substrate and the measured activities from duplicate experiments were analyzed using nonlinear regression and GraphPad Prism, version 5.01.

Influence of pH, temperature, or ethanol on esterase activity. The influence of pH on esterase activity was determined by varying the pH of the reaction mixture across the range from 3.0 to 8.0. The buffers used were 0.1 M McIlvaine buffer (pH 3.0 to 7.5) and Na_2HPO_4-NaH_2PO_4 buffer (pH 8.0) (11). Briefly, 200-μl reaction mixtures were prepared by combining 187 μl of the appropriate buffer, 5 μl of diluted enzyme (final amount in the reaction, 1 μg), and 8 μl of a 25 mM stock *p*NP-butanoate solution in ethanol, to yield a final substrate concentration of 1 mM. Following incubation, 40 μl of 0.5 M NaOH was then added to the pH 3.0 to 3.5 samples and 10 μl of 0.5 M NaOH was added to the pH 4.0 and 4.5 samples for alkalinization of samples prior to absorbance measurements being taken as described above. Results were quantified by comparison with standard *p*-nitrophenol solutions prepared under the same conditions.

Esterase activity was studied at six temperatures across the range of 10 to 60°C in dry heating blocks or incubators. The accuracy of temperature settings was confirmed with a thermometer immediately prior to initiation of the experiment. Assays were carried out as described for the pH optimum study, but for all samples, 0.1 M McIlvaine buffer at pH 5.0 was used. Reaction mixtures containing only buffer and substrate were equilibrated at the appropriate temperature for 10 min before the enzyme was added to initiate the reaction. Following incubation, all samples were transferred to a 96-well microplate and absorbances were measured.

The effect of the presence of ethanol on esterase activity was studied at 17 ethanol concentrations ranging from 2 to 50% (vol/vol). Reaction mixtures were prepared as described for the temperature optimum experiments, but different volumes of ethanol were added, from 4 μl (2%, vol/vol) up to 100 μl (50%, vol/vol), and the volume of buffer was adjusted accordingly to maintain a final reaction volume of 200 μl in a 96-well microplate. Reaction mixtures were incubated for 1 h at 37°C, and absorbance was measured.

Inhibition studies. The effect of sulfite was determined by adding sodium metabisulfite at concentrations ranging from 0 to 1.5 g · $liter^{-1}$ and at an extreme concentration of up to 20 g · $liter^{-1}$. Reaction mixtures were prepared as described for the temperature optimum experiments, but 0.1 M McIlvaine buffer at pH 3.5 was used and different volumes of a 100 g · $liter^{-1}$ stock solution of sodium metabisulfite were added. The volume of buffer was adjusted accordingly to maintain a final reaction volume of 200 μl in a 96-well microplate. Reaction mixtures were incubated for 1 h at 37°C, and absorbance was measured.

The effects of diethyl pyrocarbonate (DEPC), phenylmethylsulfonyl fluoride (PMSF), phenylarsine oxide (PAO), phenylglyoxal (PGO), and β-mercaptoethanol (β-ME) were also tested using 5 mM of each inhibitor. Reaction mixtures were prepared as described for the temperature optimum experiments, but inhibitors were added and the volume of buffer was adjusted accordingly to maintain a final reaction volume of 200 μl in a 96-well microplate. Reaction mixtures were incubated for 60 min at 37°C, and enzyme activity was measured at 15-min intervals and compared to that of controls without addition of inhibitors.

Statistical analysis. Analysis of variance of the data was conducted using Microsoft Excel and GraphPad Prism, version 5.01. K_m and k_{cat} values were calculated using nonlinear regression and GraphPad Prism, version 5.01.

RESULTS

DNA sequencing and sequence analysis. Based on the published sequences of LAB lipolytic enzymes, an ORF encoding a putative esterase gene in *O. oeni* was identified and successfully amplified from strain Ooeni28. Nucleotide sequencing of the putative esterase ORF was conducted in triplicate. The ORF consisted of 912 nucleotides encoding a deduced protein of 303 amino acids and was designated *estB28* (Fig. 1). The sequence was aligned with the *O. oeni* strain PSU-1 genome sequence using NCBI BLAST. The results indicate differences at six nucleotides in Ooeni28 compared to PSU-1, resulting in three changes when translated to the amino acid sequence (Fig. 1). The G+C content was 41.12%. No potential signal sequence was found in EstB28, as revealed by the PSORT program (46).

The deduced amino acid sequence of the putative esterase gene was compared with other known and predicted esterase sequences available from GenBank (NCBI database). The published esterase to which EstB28 showed highest homology was EstB from *Lactobacillus casei* LILA (21% identity) (18). Based on these alignments, putative lipolytic enzyme motifs were identified, showing that EstB28 does not show sequence similarity with other putative esterases along the entire length of the protein. Homology was localized to the region between the GGGX (N-terminal oxyanion hole residue) and GXSXG (nucleophilic elbow) motifs (Fig. 1), with low sequence similarity away from these regions. EstB28 belongs to family IV of bacterial lipolytic proteins (1) with a putative nucleophilic elbow sequence identified as GDSAG (Fig. 1) starting at residue 141. This also fits the GXSXG motif found in most esterases (see the supplemental material). The sequence GDSAG in the Ooeni28 esterase is also found in the characterized esterase (EstB) of *Lactobacillus casei* LILA (18). Based on alignment with other esterase sequences (data not shown) His-269 is most likely the catalytic histidine and the catalytic aspartic acid is tentatively identified as Asp-235 (Fig. 1).



FIG. 1. Nucleotide sequence of *estB28* from *Oenococcus oeni* strain Ooeni28. The predicted amino acid sequence is given below the nucleotide sequence in single-letter code. An asterisk indicates the stop codon. The GGGX motif containing the N-terminal oxyanion hole residue and the putative active-site serine consensus sequence are underlined. The putative residues of the catalytic triad are marked by a box. Sequence changes from strain PSU-1 are highlighted; in order they are C→T, T→C, C→T, A→G, T→C, and C→T. Amino acid changes are also highlighted; in order they are P→S, H→R, and V→A.

Cloning of the *Oenococcus oeni* esterase gene into *E. coli*. The putative esterase gene was cloned into pET14.b, and once positive clones had been sequenced, one was chosen for further experiments. The *E. coli* [BL21(λDE3)] cells containing the plasmid pET14.b//*estB28* were induced and cell extracts obtained for analysis by SDS-polyacrylamide gel electrophoresis. The induced BL21(λDE3) cells containing pET14.b//*estB28* overexpressed a protein of approximately 34.3 kDa (Fig. 2). A faint band at 34.4 kDa was observed in the uninduced sample but not in either of the samples containing induced BL21 (λDE3) cells with only the pET14.b plasmid (Fig. 2). Crude cell extracts were assayed for activity (data not shown), and BL21(λDE3) containing pET14.b//*estB28* had 10-fold-greater activity than BL21(λDE3) containing pET14.b. EstB28 was purified from the whole-cell lysate to electrophoretic homoge-

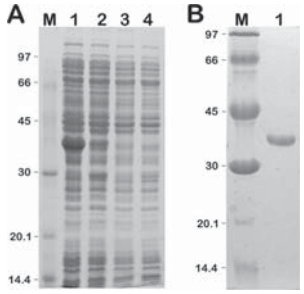


FIG. 2. (A) Overexpression of esterase from Ooeni28 in *E. coli* BL21(λDE3). Lanes: M, molecular mass standards (kDa); 1, *E. coli* BL21(λDE3) harboring plasmid pET14.b//*estB28* induced with IPTG; 2, *E. coli* BL21(λDE3) harboring plasmid pET14.b//*estB28* uninduced; 3, *E. coli* BL21(λDE3) harboring plasmid pET14.b induced with IPTG; 4, *E. coli* BL21(λDE3) harboring plasmid pET14.b uninduced. (B) Purified recombinant esterase from Ooeni28. Lanes: M, molecular mass standards (kDa); 1, purified EstB28 from Ooeni28. Protein samples were separated on an SDS-polyacrylamide gel and stained with Coomassie brilliant blue.

neity using affinity chromatography (Fig. 2). The purified enzyme could be stored without significant loss of activity for several weeks at −80°C in 0.1 M McIlvaine buffer (pH 5.0) supplemented with glycerol (10%, vol/vol).

Determination and characterization of esterase activity. Having established that the product of the cloned *estB28* gene possessed esterase activity, further characterization of this product commenced. Substrate specificity was determined using *p*NP-linked esters of various acyl chain lengths (C_2 to C_{18}) at 37°C. The enzyme was most active on substrates with a chain length of up to C_{10}, with minimal activity detected with the longer chain lengths. The highest activity was observed with *p*NP-acetate (C_2) and *p*NP-butanoate (C_4). The K_m increased with increasing chain length (Table 1), confirming that the enzyme is more specific for short-chain fatty acids than medium-chain fatty acids. The influence of pH, in the range of 3.0 to 8.0, on esterase activity was tested at 37°C. Although the enzyme was active at all of the pHs tested, the highest activity was observed at pH 5.0 (Fig. 3A). The esterase was also active at temperatures of between 10 and 60°C, although the highest activity was observed at 40°C (Fig. 3B). The influence of ethanol on esterase activity was tested at 37°C. Initial experiments were conducted with the range found in wine (up to 16% [vol/vol]); however, given that activity continued to increase with increasing ethanol concentration, higher concentrations (up to 50%) were examined. The highest esterase activity was observed at an ethanol concentration of 28% (Fig. 3C).

Assays to determine the effect of inhibitors on esterase activity using *p*NP-butanoate as the substrate showed that sodium metabisulfite, a potential inhibitor found in wine, did not inhibit the enzyme when present at concentrations commonly found in wine (Fig. 3D). The enzyme was only mildly (less than 50%) inhibited until high concentrations (i.e., 10 g · liter^{-1}) of sodium metabisulfite were reached (Fig. 3D). Assays with other inhibitors (Fig. 4) showed that the enzyme was strongly inhibited by DEPC, a histidine modifier, and PMSF, a serine protease inhibitor. PGO, which targets arginine residues, decreased activity by 82%. Inhibition by β-ME or PAO, a cysteine modifier, caused activity to decrease by 71% in each case.

DISCUSSION

This research focused on the cloning and biochemical characterization of an esterase identified by sequence homology using the published nucleotide sequence for *O. oeni* PSU-1. Nucleotide sequencing of the esterase from *O. oeni* Ooeni28, designated *estB28*, revealed a 912-bp ORF which could encode a protein of 34.5 kDa. This is differentiated from the ORFs determined for *Lactobacillus casei* LILA *estB* and *estC* (17, 18), which were 777 bp and 954 bp and encoded proteins of 35.7 kDa and 28.8 kDa, respectively. According to the system proposed by Arpigny and Jaeger (1), the amino acid sequence of EstB28 reveals it to be a member of family IV of lipolytic enzymes. This group of enzymes shows sequence similarity to the mammalian hormone-sensitive lipase. Holm et al. conduced site-directed mutagenesis of the serine at the center of the GXSXG nucleophilic elbow of mammalian hormone-sensitive lipase, resulting in complete absence of enzymatic activity (34). The deduced amino acid sequence of EstB28 was analyzed for key lipolytic enzyme motifs. Possible oxyanion hole residues, nucleophilic elbow pentapeptides, and catalytic triad resides (48) were identified. EstB28 showed no potential signal sequence (46), suggesting that this enzyme is located in the cytoplasm. This observation concurs with those made for *Lactobacillus helveticus* CNRZ32 EstA (16), *L. lactis* B1014 EstA (20), *Lactobacillus casei* LILA EstB (18), and *Lactobacillus casei* LILA EstC (17).

The esterase of *O. oeni* Ooeni28 (EstB28) was overexpressed in *E. coli* and purified using affinity chromatography. The purified recombinant enzyme was then characterized under conditions relevant to winemaking. The potential role of EstB28, when used as an additive in wine, in aroma development is dependent on the sensitivity of the enzyme to environmental conditions encountered therein. Low pH values, in the range 3.0 to 3.6, are sought for both grape juice and wine and are monitored throughout the winemaking process. At these values EstB28 was stable, and it retained 30 to 40% of the activity

TABLE 1. Hydrolysis of *p*NP esters by purified heterologously expressed esterase

Substrate	Chain length	Relative activity (%)[a]	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} \cdot \mu M^{-1}$)
*p*NP-acetate	C_2	100	11.54	942	81.6
*p*NP-butanoate	C_4	92	14.44	890	61.8
*p*NP-hexanoate	C_6	56	36.05	901	25.0
*p*NP-octanoate	C_8	50	47.67	527	11.0
*p*NP-decanoate	C_{10}	24	70.10	322	4.6
*p*NP-dodecanoate	C_{12}	2	168.73	153	0.9
*p*NP-tetradecanoate	C_{14}	—[b]	—	—	—
*p*NP-octadecanoate	C_{18}	—	—	—	—

[a] Enzyme activity, determined at 37°C in 0.1 M McIlvaine buffer (pH 5.5) containing Triton X-100 and gum arabic, is the mean from triplicate experiments with a standard deviation of less than 8%. Values are shown relative to the highest observed activity, which was arbitrarily designated 100%.

[b] —, not detected.

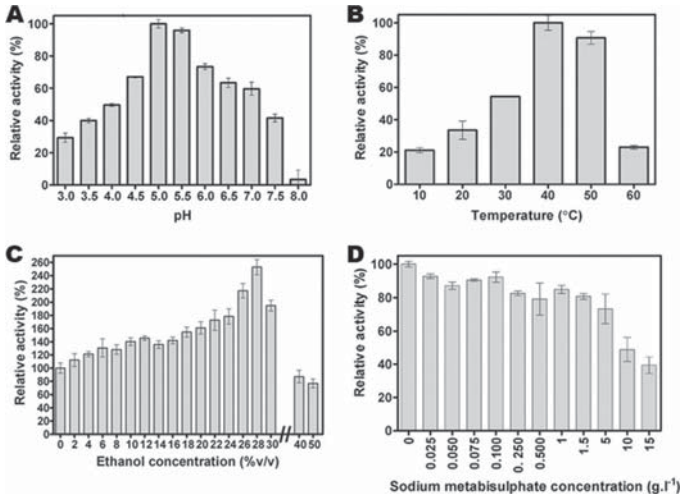


FIG. 3. Characterization of EstB28. Values are the means of triplicates ± standard deviations. Values are shown relative to the highest observed activity, which was arbitrarily designated 100%, except for panel C, where values are shown relative to the activity observed in the absence of ethanol, which was arbitrarily designated 100%. (A) Influence of pH on esterase activity of EstB28. (B) Influence of temperature on esterase activity of EstB28. (C) Influence of ethanol on esterase activity of EstB28. (D) Influence of sulfite on esterase activity of EstB28. Free SO_2 includes the concentrations of sulfur dioxide forms which are in equilibrium and are not bound to any compound in solution. This is defined as $[SO_2] + [HSO_3^-]$ ($[SO_3^{2-}]$ is minimal, i.e., 0.02%) at pH 3.5, where 98% of the sulfur dioxide exists as bisulfite ion (HSO_3^-).

under optimal conditions at pH 5.0 to 5.5 (Fig. 3A). Based on other esterases that have been purified from LAB and characterized, only EstC from *Lactobacillus casei* LILA (17) had an optimum activity in the same pH range. EstB28 and EstC show only 11% homology. Because EstC was from a dairy isolate, Fenster et al. characterized it under conditions relevant to cheese ripening, and no other aspects relevant to wine were assessed (17).

Maximum esterase activity of EstB28 was observed at 40°C, which is a temperature higher than those considered suitable for wine production and storage. Wine is usually stored at temperatures below 20°C. EstB28 retained 33% of its activity at 20°C and 21% at 10°C relative to that observed under optimal conditions. This suggests that the enzyme has the potential to affect the ester profile of wine even at these low temperatures. EstB28 might also be of value as an addition to ripening cheese, given that the cheese is stored at 10°C and its pH (5.0 to 5.5) (18, 19) corresponds to the optimum for EstB28. While the effects of NaCl levels, usually 4% in ripening cheese, were not considered in this study, it can be suggested that EstB28 will retain at least 20% activity if used at temperatures at which cheese is ripened. As with wine, high concentrations of some esters can produce undesirable fruity off-flavors in some cheeses (39), and investigation of the effect that addition of EstB28 has in ripening cheese could prove interesting.

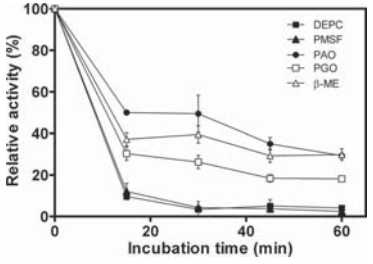


FIG. 4. Effects of inhibitors on the activity of recombinant EstB28 esterase. Inhibitors at 5 mM were incubated in triplicate with the enzyme for 60 min at 37°C. Readings were taken every 15 min using a microplate reader. Error bars indicate standard deviations.

A further potentially inhibitory enological parameter to be considered was ethanol. This alcohol in fact stimulated esterase activity with 14% (vol/vol) ethanol, producing 149% activity relative to the 0% ethanol control. EstB28 retained 79% of its activity at 50% (vol/vol) ethanol. Other reports from our group on the esterase activity (41) and β-glucosidase activity (26–28) of LAB isolates describe a similar enhancement of activity by ethanol when assaying whole cells. While the need for ethanol to enhance ester formation in cheese has been reported, only levels of 6% (vol/vol) have been assessed (33). There are no reports in the literature of LAB esterases retaining activity at 50% ethanol, although an esterase from *Bacillus*

licheniformis retained activity in various solvents, including 13% activity in 50% ethanol (54). While a likely application of this characterized esterase might be in floral, white wines, which rarely exceed 13.5% ethanol, the robustness of this enzyme suggests that it might also find applications in beverages of higher ethanol content (i.e., <15%, vol/vol). Outside of wine, the stability and high activity of EstB28 in ethanol suggest a possible use as an industrial biocatalyst. The use of EstB28 in organic synthesis and further characterization in other solvents could be of interest.

Sodium metabisulfite, the final wine-associated inhibitor to be considered here, did not inhibit EstB28 until it was present at high concentrations (10 $g \cdot liter^{-1}$), higher than those found in wine. During fermentation, yeast can produce 0.01 to 0.03 $g \cdot liter^{-1}$ of SO_2, and sulfites are often added by the winemaker in the form of potassium metabisulfite, resulting in final levels of 0.1 to 0.2 $g \cdot liter^{-1}$ SO_2 in the wine. Inactivation of the *O. oeni* Ooeni28 esterase by PMSF and DEPC suggests the involvement of a serine and a histidine at the active site of the enzyme. The serine residue of the pentapeptide GXSXG is well known as the active catalytic residue that is modified by PMSF (9). EstB28 sensitivity to PMSF was similar to that of EstB from *Lactobacillus casei* LILA (18). PGO caused an initially unexpected 82% decrease in activity of EstB28. However, on closer inspection of the deduced amino acid sequence of EstB28 (Fig. 1), it is evident that the arginine residue (R) is close enough to interfere with the binding of the catalytic histidine (H) with the substrate. EstB28 esterase was only partially inhibited by PAO and β-ME, suggesting that an accessible cysteine is not necessary for activity and disulfide bonds are not present. No disulfide bonds were predicted from the EstB28 protein sequence using DISULFIND (8). This correlates with the lower levels of inhibition of the esterase by β-ME and PAO, which would act through disruption of any disulfide bonds.

The substrate specificity of *O. oeni* Ooeni28 esterase revealed greater selectivity for *p*NP esters of short-chain fatty acids and to a lesser extent *p*NP esters of medium-chain fatty acids. Increases in fatty acid chain lengths resulted in an increase in K_m; however, the effect on catalytic efficiency is seen only when the chain length is $\geq C_8$. The specificity of the esterase for *p*NP-acetate and *p*NP-butanoate is most similar to that of EstC from *Lactobacillus casei* LILA (17), which is also mainly specific for short-chain fatty acids. Examples of such esters in wine include ethyl acetate and ethyl butanoate. The removal of these from wine is not necessarily desirable, as they can contribute to the fruity aroma of wine at low concentrations (37, 52a). However the removal of excess ethyl acetate, which at high concentrations ($\geq$100 mg ml^{-1}) contributes a solvent-like aroma to wine, would be an advantage. The role that EstB28 plays in ester synthesis will also need to be investigated to determine the usefulness of adding whole cells of selected strains or else a preparation of the purified enzyme to wine with a view to selectively modifying the ester profile.

EstB28 is the first esterase from the wine-associated species *O. oeni* to be characterized and is believed to be the first LAB esterase demonstrated to retain activity under conditions relevant to winemaking. Based on the findings reported for the artificial substrates used in this study, it appears that EstB28 will retain at least partial activity under wine-like conditions of pH, temperature, ethanol, and sulfite concentration. The *O. oeni* Ooeni28 esterase is a potential candidate for altering the ester profile of wine and could play an important role in ester profile modifications during the malolactic fermentation. As most esterases are also capable of ester synthesis, investigation of the role that this enzyme might play in wine would be of interest.

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Review

Microbial modulation of aromatic esters in wine: Current knowledge and future prospects

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ABSTRACT

This review focuses on the considerable amount of research directed at defining the accumulation of esters during fermentation and their contribution to aromas in foods and beverages. From this research it is clear that esters are extremely important for the aroma profile of fermented beverages and various dairy products. A large amount of this research is focused on wine and has yielded the genes involved in ester synthesis and hydrolysis in organisms such as *Saccharomyces* sp. It is also clear from recent research in both the fermented beverage and dairy context that lactic acid bacteria possess an extensive collection of ester synthesising and hydrolysing activities. This review describes the major esters reported in wine and the enzymes responsible for their hydrolysis and synthesis. Ester impact on wine aroma and formation during primary and malolactic fermentation is also evaluated. Finally the potential applications of current knowledge are outlined.

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1. 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. The aroma of wine has received much research attention over recent decades, with numerous components being identified as playing a role in specific sensory notes. The distinctive flavour and aroma of wine is determined by many variables, including grape variety, viticultural and winemaking practices and wine maturation and storage conditions. A range of microorganisms come into contact with wine during its production, and thus their metabolic activities and synthetic and degradative enzymes, may influence wine aroma. In this review we examine the types and concentrations of one important group of aroma-active compounds, the esters, and consider their sensory contribution, the impact of wine-associated yeast and bacteria on wine ester makeup and explore the potential for modulation of this profile by selective use of microbes or their derivatives. Where wine-related information is not available, this review will draw on findings from other food-related organisms involved in similar processes.

2. Esters in wine

Esters are flavour compounds that occur widely in a variety of food products (Gatfield, 1992). In fermented beverages such as wine and beer, they are frequently in trace amounts, such that individually they are often below aroma threshold concentrations, and collectively they generally do not exceed concentrations of 100 mg/l. Nonetheless, as a group these compounds are the next major constituents in wine (Fig. 1) after water, ethanol and fusel alcohols (Etiévant, 1991), and are the primary source of fruity aromas (Gurbuz, Rouseff, & Rouseff, 2006). As such, esters are extremely important for the flavour profile of fermented beverages, with the presence of different esters often having a synergistic effect, impacting on the individual flavours well below their individual threshold concentrations. The fact that most esters are present in concentrations around their threshold value implies that modest concentration changes might have a dramatic effect on wine flavour. An understanding of ester hydrolysis/synthesis is therefore essential in aiding the winemaker in achieving the best possible winemaking outcome.

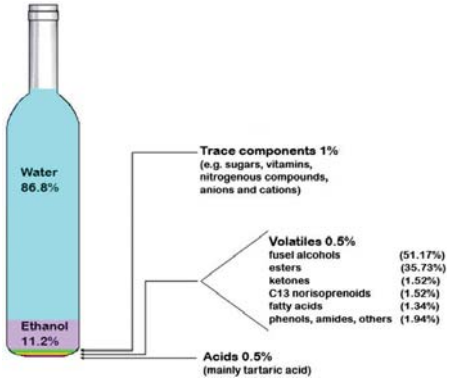


Fig. 1. Chemical composition of wine and average quantities of major wine components. Values vary according to the wine type assayed and the extraction technique. Ethanol is represented as weight per volume (w/v), as are all other components, and is equivalent to 14% (v/v). In red wine tannins would also be present at concentrations up to 0.4% (w/v). Data taken from Ferreira et al. (2000), Girard, Kopp, Reynolds, and Cliff (1997), Koundouras, Marinos, Gkoulioti, Kotseridis, and van Leeuwen (2006), Rocha et al. (2004).

2.1. Ester formation

Esters are formed when alcohol and carboxylic acid functional groups react, and a water molecule is eliminated. In wine, esters can be classified into two groups, those formed enzymatically and those formed during wine ageing, by chemical esterification between alcohol and acids at low pH (Margalit, 1997). Enzymatic ester synthesis is catalysed by esterases and lipases, which are produced by many food microorganisms (Choi, Miguez, & Lee, 2004; Fenster, Parkin, & Steele, 2000, 2003a, 2003b; Fernandez et al., 2000; Gobbetti, Fox, & Stepaniak, 1997; Nardi, Fiez-Vandal, Tailliez, & Monnet, 2002). Ester synthesis can also be catalysed by alcohol acetyltransferases (Lilly, Lambrechts, & Pretorius, 2000; Liu et al., 2004; Mason & Dufour, 2000). Examples of enzymatically-formed esters include ethyl acetate, ethyl butanoate, ethyl hexanoate and ethyl octanoate. Due to its complex nature, wine is subject to continuous changes in composition during storage and even after bottle opening. This may be because of hydrolysis and esterification or could be caused by ester oxidation by hydroxyl radical-related processes (Ramey & Ough, 1980).

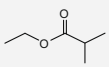
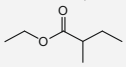
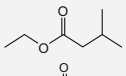
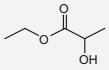
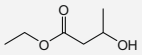
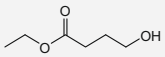
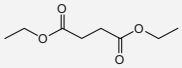
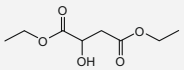
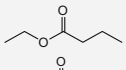
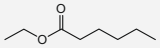
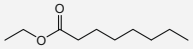
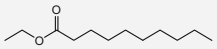
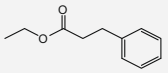
With a large number of different acids and alcohols in wine there is considerable potential for the formation of a wide range of esters, many of which are in fact found in wine (Table 1). The C_4–C_{10} ethyl esters of organic acids, ethyl esters of straight chain fatty acids (ethyl esters of branched chain fatty acids to a lesser degree) and acetates of higher alcohols are largely, if not exclusively, responsible for the fruity aroma of wine (Ebeler, 2001) and are particularly pronounced in young wines. The ethyl esters comprise of an alcohol group (ethanol) and an acid group (medium-chain fatty acid) (Saerens et al., 2008), and include ethyl hexanoate, ethyl octanoate and ethyl decanoate (Table 1). The acetate esters are comprised of an acid group (acetate) and an alcohol group which is either ethanol or a complex alcohol derived from amino acid metabolism (Saerens et al., 2008), and includes esters such as ethyl acetate and isoamyl acetate (Table 1). Malolactic fermentation (MLF) can also impact on the ester profile of the final wine product (see Section 4 for an explanation of MLF). The aroma compound that is produced the most during MLF is the ester ethyl 2-hydroxypropanoate (ethyl lactate) (Maicas, Gil, Pardo, & Ferrer, 1999). Ethyl 2-hydroxypropanoate production is coupled to lactic acid formation and its synthesis can be correlated with the percentage of degradation of malic acid (de Revel, Martin, Pripis-Nicolau, Lonvaud-Funel, & Bertrand, 1999).

2.2. Impact on wine aroma

Ethyl esters as a group have a strong influence on wine aroma (Gomez-Miguez, Cacho, Ferreira, Vicario, & Heredia, 2007), with ethyl acetate being qualitatively the most common ester in wine, due to its ready formation from the predominant ethanol and acetic acid (Ribereau-Gayon, Glories, Maujean, & Dubourdieu, 2000). This is due to the large quantities of ethanol present and the fact that primary alcohols are the most reactive. It is therefore often an important contributor to wine aroma; at low concentrations ($\leqslant$100 mg/l) giving a desirable and fruity character to the wine, however, at higher concentrations it can impart a solvent/nail varnish-like aroma (Table 1).

Esters that have been reported to be present in wine at concentrations above their aroma threshold include ethyl 2-hydroxypropanoate (after MLF), diethyl butanedioate, ethyl butanoate,

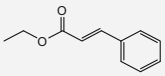
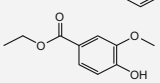
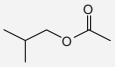
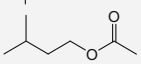
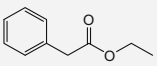
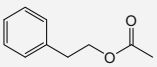
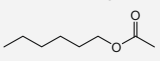
Table 1
A summary of the major esters reported in wine: their structure, aroma characteristics, concentration in wine and aroma thresholds.

Compound (common name)	Structure	CAS-RN	Reported aroma characteristics[a]	Reported concentration in wine (mg/l)[b]	Aroma threshold (mg/l)[c]
Ethyl esters					
Ethyl 2-methylpropanoate (ethyl isobutyrate)		97-62-1	Fruity, strawberry, lemon	0.01–0.48[3,4,5]	0.001, 0.015[a, s], 5.0[b]
Ethyl 2-methylbutanoate		7452-79-1	Apple, strawberry, berry, sweet, cider, anise	Trace-0.03[3,4]	0.0001, 0.001[a], 0.018[s]
Ethyl 3-methylbutanoate (ethyl isovalerate)		108-64-5	Sweet fruit, pineapple, lemon, anise, floral	Trace-0.07[1,3,4,5]	0.0001, 0.003[a, s], 1.3[b]
Ethyl 2-hydroxypropanoate (ethyl lactate)		97-64-3	Milk, soapy, buttery, fruity	3.05–297.5[1,4,6]	0.05–0.2, 150[w]
Ethyl 3-hydroxybutanoate		5405-41-4	Fruity (winey), green, marshmallow	0.05–0.58[4,6]	20
Ethyl 4-hydroxybutanoate		999-10-0	Caramel	6.61[6]	NR
Diethyl butanedioate (diethyl succinate)		123-25-1	Fruity, fermented, floral	1.21–61.11[1,4,6]	NR
Diethyl hydroxybutandioate (diethyl malate)		7554-12-3	Brown sugar, sweet	0.81[6]	NR
Ethyl butanoate		105-54-4	Floral, fruity, strawberry, sweet	0.07–0.53[1,3,4,5]	0.001, 0.02[a], 0.4[b]
Ethyl hexanoate		123-66-0	Fruity, strawberry, green apple, anise	0.15–1.64[1,3,4,5]	0.005[a, s], 0.08[w], 0.85[w]
Ethyl octanoate		106-32-1	Sweet, fruity, ripe fruit, burned, beer	0.14–2.61[1,3,4,5,6]	0.002[s], 0.005[a], 0.012, 0.58[w]
Ethyl decanoate		110-38-3	Oily, fruity (grape), floral	0.01–0.70[1,3,4,6]	0.2[s], 0.012, 0.51[w]
Ethyl 3-phenylpropanoate (ethyl dihydrocinnamate)		2021-28-5	Flower	Trace-0.003[3,4]	0.002[s]

(*continued on next page*)

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Table 1 (*continued*)

Compound (common name)	Structure	CAS-RN	Reported aroma characteristics[a]	Reported concentration in wine (mg/l)[b]	Aroma threshold (mg/l)[c]
Ethyl 3-phenylprop-2-enoate (Ethyl cinnamate)		103-36-6	Honey, cinnamon	Trace-0.01[3,4,5]	0.001[a, s], 0.048[w]
Ethyl 4-hydroxy-3-methoxybenzoate (Ethyl vanillate)		617-05-0	Flower, fruit, sweet, vanilla	0.46[6]	NR
Acetates					
Ethyl acetate		141-78-6	Fruity (at > 100 mg ml^{-1})[2], solvent, balsamic	5.0–63.5[4,5]	7.5[a], 60, 12.27[w]
2-Methylpropyl acetate (isobutyl acetate)		110-19-0	Fruity, apple	Trace-0.17[4]	1.6[b]
3-Methylbutyl acetate (isoamyl acetate)		123-2-2	Banana, fruity	0.03–5.52[1,3,4,5]	0.03[a], 0.16[w]
Ethyl 2-phenylacetate		101-97-3	Rose, floral	0.03–0.39[1,4]	NR
2-Phenylethyl acetate		103-45-7	Flowery, rose	Trace-0.26[3,5,6]	0.25[a], 0.65, 1.80[w]
Hexyl acetate		142-92-7	Green, herbaceous, fruit, grape	Trace-3.90[1]	0.002–0.48, 0.67/2.4[w]

[a] Aroma characteristics collated from: Aznar and Arroyo (2007), Clarke and Bakker (2004), Gomez-Miguez et al. (2007), and Gurbuz et al. (2006).
[b] Concentration range derived from indicated sources: [1]Aznar and Arroyo (2007), [2]Clarke and Bakker (2004), [3]Ferreira et al. (2000), [4]Gomez-Miguez et al. (2007), [5]Guth (1997), and [6]Rocha et al. (2004).
[c] Aroma thresholds collated from indicated sources. Values determined in water except where specified ([a], 10% (v/v) aqueous ethanol; [b], beer; [s], synthetic wine [11% (v/v) ethanol, 7 g/l glycerol, 5 g/l tartaric acid, pH 3.4]; [w], wine).

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ethyl hexanoate, ethyl octanoate, ethyl decanoate, ethyl acetate, ethyl 2-methylpropanoate, ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, ethyl cinnamate, 3-methybutyl acetate, 2-phenylethyl acetate, and hexyl acetate (Table 1). As the length of the hydrocarbon chain increases, a more soap-like odour develops, with esters formed from C_{16} and C_{18} fatty acid hydrocarbon chains having been described as lard-like (Jackson, 1994).

2.3. Formation during primary fermentation

The formation of esters during fermentation has been described as a dynamic process (Lee, Rathbone, Asimont, Adden, & Ebeler, 2004), with numerous variables interacting. Variables that are known to affect ester production and their concentration include the quantity of esters or their precursors originally present in the grape, the temperature of fermentation, the yeast strain that predominates and the nutrients present, especially the concentration of nitrogen compounds and must solids (Boulton, Singleton, Bisson, & Kunkee, 1996; Guitart, Orte, Ferreira, Pena, & Cacho, 1999; Killian & Ough, 1979; Miller, Wolff, Bisson, & Ebeler, 2007; Nykänen, 1986). The average ester production and the relative proportions of each ester are highly dependent on the yeast strain and the influence of other parameters, such as temperature, oxygen and nitrogen, may also be strain-dependent (Vilanova et al., 2007). Strain-specific differences may also be due to differences in expression of genes involved in ester synthesis.

The effect of different variables on wine composition is well documented. For example, a recent study using a commercial wine yeast strain reported that there were higher concentrations of fresh and fruity aromas after fermentation at 15 °C as opposed to a 28 °C fermentation, which produced higher concentrations of compounds with flowery aroma (Molina, Swiegers, Varela, Pretorius, & Agosin, 2007). The overall volatile composition of most grape varieties is similar despite clear differences in their aromas. Most varietal differences occur from changes in relative ratios of volatile compounds. There is considerable variability in ester content amongst different grape cultivars. Ferreira, Lopez, and Cacho (2000) reported that the yeast-derived esters are strongly linked to the variety of grape. Gurbuz et al. (2006) have also reported that Australian Merlot had a higher proportion of esters (83%) amongst the identified volatiles, compared to a Californian Merlot (60%) and Cabernet Sauvignon from the same sources. This study used commercially available finished wines but from the same region (Barossa Valley in Australia and Napa Valley in the USA), however differences in winemaking practice were not taken into account.

2.4. Formation during malolactic fermentation

Depending on the style of wine that the winemaker seeks to achieve, MLF may be carried out. 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 spoilage organisms after the wine is bottled (Davis, Wibowo, Eschenbruch, Lee, & Fleet, 1985). This secondary fermentation is typically carried out by one or more populations of lactic acid bacteria (LAB) (Versari, Parpinello, & Cattaneo, 1999). The groups of LAB most commonly associated with MLF are *Oenococcus oeni*, *Lactobacillus* sp. and *Pediococcus* sp. which have been shown to modify and possibly synthesise fruity aromas during MLF (D'Incecco et al., 2004; Liu, 2002; Maicas et al., 1999; Matthews et al., 2004), therefore they have enormous potential to impact wine composition.

2.5. Formation during wine storage

After the significant modifications in composition during fermentation, chemical constituents generally react slowly during ageing to move to their equilibrium position, resulting in gradual changes in flavour (Garcia-Falcon, Perez-Lamela, Martinez-Carballo, & Simal-Gandara, 2007; Lilly et al., 2000; Ramey & Ough, 1980; Ribereau-Gayon, Boidron, & Terrier, 1975; Sivertsen, Figenschou, Nicolaysen, & Risvik, 2001). There is a wide variability in the results on the development of esters during wine maturation (Moreno & Azpilicueta, 2006; Ramey & Ough, 1980). Due to their gradual hydrolysis over time, volatile esters are sometimes considered unimportant in the favourable effects of wine. In fact, depending on the acid–ester equilibrium, branched fatty acid ethyl esters can increase during wine ageing (Díaz-Maroto, Schneider, & Baumes, 2005). The branched fatty acid ethyl esters are less volatile than their straight-chain analogues, however, they are also important odourants of wine (Table 1). The ethyl esters of diprotic acids (e.g. diethyl butanedioate; Table 1) have also been shown to increase significantly with time (Câmara, Alves, & Marques, 2006), due to chemical esterification during wine ageing. Because of a tendency of esters to return to their equilibrium levels, any effect of microorganisms on the ester profile of wine will mostly be advantageous for young 'fruity' wines. A recent study by Roussis, Lambropoulos, and Tzimas (2007) tested the inhibition of volatile ester degradation during storage of wine using caffeic acid or glutathione. Addition of caffeic acid protected several important esters involved in wine aroma during storage, including isoamyl acetate, ethyl hexanoate, ethyl octanoate and ethyl decanoate (Roussis et al., 2007). This could prove to be a useful method to extend a wine's fruity character. A decrease in ester hydrolysis during storage can also be seen with the addition of SO_2 (Garde-Cerdán & Ancín-Azpilicueta, 2007).

3. Enzymatic synthesis and hydrolysis of esters

The enzymatic accumulation of esters in wine during fermentation is known to be the result of the balance of the enzymatic synthesis and hydrolysis reactions involving esterases (EC 3.1.1.1) and synthesis reactions involving alcohol acetyltransferases (EC 2.3.1.84) (Lilly et al., 2000; Mason & Dufour, 2000; Matthews, Grbin, & Jiranek, 2007; Verstrepen et al., 2003c). Wine microflora possess these enzymes and enzymatic activity in microbial strains used during the production of wine is of great importance when determining how to best enhance the varietal characteristics of wine. Esterases and lipases are serine hydrolases capable of synthesising or hydrolysing esters depending on the physicochemical conditions, while alcohol acetyltransferases only participate in ester synthesis. Substrates for these enzymes are alcohols or thiols and fatty acids (or their acyl CoA-activated forms). These substrates are produced during lipid, sugar and amino acid metabolism (Molimard & Spinnler, 1996; Yvon & Rijnen, 2001).

3.1. Lipolytic enzymes

Esterases and lipases are widely present in various organisms from bacteria to higher eukaryotes (Horton & Bennett, 2006; Moreira, Mendes, Hogg, & Vasconcelos, 2005) and belong to the general class of carboxylic ester hydrolases (EC 3.1.1). Lipolytic enzymes are important biocatalysts for various industrial applications, due to critical features, such as no requirement for co-factors, stability in organic solvents, and broad substrate specificity (Jaeger, Dijkstra, & Reetz, 1999). It was initially believed that all lipases and esterases contained the structural motif, G-X-S-X-G (where X represents an arbitrary amino acid residue), which contains the active

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serine residue (Jaeger et al., 1994). This motif forms an extremely sharp turn in the protein tertiary structure, called a nucleophilic elbow, and is usually located between a β-strand and an α-helix. While most lipolytic enzymes do indeed contain this motif, more recent research has revealed that other motifs do exist (Arpigny & Jaeger, 1999; Jaeger et al., 1999). A common characteristic of these enzymes is that they usually contain a catalytic triad composed of serine, histidine and aspartic acid or glutamate (Dodson & Wlodawer, 1998; Jaeger et al., 1994). There is however a reported exception to this as well. An esterase characterised from *Streptomyces scabies* contains a GDSL structural motif and it also contains a catalytic Ser-His dyad instead of the common Ser-Asp-His triad (Wei et al., 1995). The enzyme also has an α/β-tertiary fold, which differs substantially from the α/β-hydrolase fold. Other esterases in this GDSL structural motif group include those from *Pseudomonas aeruginosa* (Wilhelm, Tommassen, & Jaeger, 1999) and *Salmonella typhimurium* (Carinato et al., 1998).

Lipolytic enzymes also contain an oxyanion hole which consists of two residues that donate their backbone amide protons to stabilise the substrate in the transition state (Arpigny & Jaeger, 1999; Heikinheimo, Goldman, Jeffries, & Ollis, 1999; Jaeger et al., 1999; Pleiss, Fischer, Peiker, Thiele, & Schmid, 2000). The oxyanion hole residues (in bold) have been divided into two groups termed G**X** and GG**G**X with the glycine residue and a hydrophobic residue (X) being highly conserved (Pleiss et al., 2000). The distinction between lipases and esterases is based on three main characteristics:

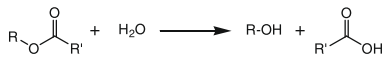
(1) Length of the hydrolysed acyl ester chain
(2) Physicochemical nature of the substrate
(3) Enzymatic kinetics

Esterases show greater specificity towards substrates with chain lengths of 2–10 carbon atoms, hydrolyse soluble, monomeric substrates in aqueous solutions, and conform to Michaelis–Menten kinetics. Lipases show greater specificity towards substrates with chain lengths of 10 or more carbon atoms, hydrolyse emulsified substrates and display 'interfacial Michaelis–Menten' kinetics. Hence, lipases are described as acting at the interface between hydrophobic and hydrophilic regions (Holland et al., 2005; Jaeger et al., 1999; Wilhelm et al., 1999; Yahya, Anderson, & Moo-Young, 1998). Using this information and various protein and gene databases it has been proposed, based on known conserved sequence motifs and biological properties, that there are eight different families within the lipolytic enzymes (Arpigny & Jaeger, 1999).

3.2. Esterases

Esterases are broadly defined as enzymes that catalyse the hydrolysis of esters of organic acids, regulating the equilibrium between esters and free acids. Esterases (EC 3.1.1.1) can exist as either monomers or oligomers with subunit weights (MW) that range from 25 kDa to 85 kDa (Elmi et al., 2005; Fenster et al., 2000, 2003a, 2003b; Gobbetti, Fox, Smacchi, Stepaniak, & Damiani, 1996; Gobbetti, Smacchi, & Corsetti, 1997; Tsakalidou & Kalantzopoulos, 1992). The primary reaction catalysed by esterases and lipases is hydrolysis:

(1) hydrolysis



$(R_1\text{–}COO\text{–}R_2 + H_2O \rightarrow R_1\text{–}COOH + R_2\text{–}OH)$.

Both esterases and lipases may also catalyse four different ester-synthesis reactions dependent on conditions (Holland et al., 2005):

(2a) Esterification

$$(R_1 - COOH + R_2 - OH \rightarrow R1 - COO - R_2 + H_2O),$$

(2b) Alcoholysis

$$(R_1 - COO - R_2 + R_3 - OH \rightarrow R_1 - COO - R_3 + R_2 - OH),$$

(2c) Acidolysis

$$(R_1 - COO - R_2 + R_3 - COOH \rightarrow R_3 - COO - R_2 + R_1 - COOH),$$

(2d) Trans-esterification

$$(R_1 - COO - R_2 + R_3 - COO - R_4 \rightarrow R_1 - COO - R_4 + R_3 - COO - R_2)$$

The proposed mechanism of catalysis involves binding of the substrate and nucleophilic attack by the catalytic serine hydroxyl on the carbonyl carbon of the scissile bond, yielding a tetrahedral intermediate (Cygler et al., 1993; Heikinheimo et al., 1999). The nucleophilicity of the hydroxyl group is enhanced by a nucleophile (water in hydrolysis, alcohol or ester in trans-esterification) and the reactive intermediate is stabilized through hydrogen bonding to the imidazole group of the catalytic histidine. This in turn is stabilized by the carboxyl group of the acidic member of the catalytic triad (Dodson & Wlodawer, 1998) yielding the product (acid or ester) and free enzyme. Esterases may therefore enhance or degrade wine quality depending on the ester metabolised.

3.3. Alcohol acetyltransferases

Esterases have been shown to have the ability to synthesise esters and act as alcohol acyltransferases, *via* reaction (2b). However, as these enzymes can also hydrolyse esters and have been described above, this section only describes alcohol acetyltransferases (AATases) belonging to the transferase enzyme grouping (EC 2). This group contains enzymes that transfer a group from a donor compound to an acceptor compound. In many cases, the donor is a

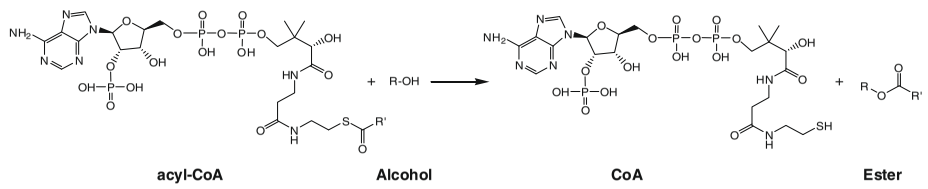


Fig. 2. AATase enzyme biosynthetic pathway for ester synthesis.

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cofactor (coenzyme), carrying the group to be transferred. In relation to esters the AATases are sulfhydryl enzymes capable of synthesising esters using higher alcohols and acetyl-CoA as substrates (Fig. 2) (Liu, Holland, & Crow, 2004). All the identified putative AATases are categorised in the pfam07247 family, which contains a number of alcohol acetyltransferases (EC 2.3.1.84), each approximately 500 amino acids long (Finn et al., 2006). AATases are membrane-bound enzymes inhibited by several kinds of lipids, including unsaturated fatty acids (Yoshioka & Hashimoto, 1981). Ester formation is also influenced by the fatty acid content of the membrane (Yoshioka & Hashimoto, 1983).

ATF1 and *ATF2* were the first group of acetyltransferase structural genes to be characterised in *Saccharomyces cerevisiae* and it was hypothesised that the highly conserved region WRLICLP (exclusive to these proteins over the entire *S. cerevisiae* genome) was part of the active site of these proteins (Mason & Dufour, 2000; Nagasawa, Bogaki, Iwamatsu, Hamachi, & Kumagai, 1998). This hypothesis was further supported by the presence of a (potentially reactive) cysteine residue in this region and the fact that AATases are inhibited by metal ions and sulfydryl reagents, which are known to interact with cysteine residues (Malcorps & Dufour, 1992; Yoshioka & Hashimoto, 1983). However this putative active site is not conserved throughout all ascomycetous fungi. A recent study analysed multiple ascomycetous fungi for orthologues of these AATases and found a second region of interest, described as the putative active site H-X-X-X-D (Van Laere et al., 2008). This putative active site was found to be conserved throughout all sequences of the yeast AATases (even in the more divergent sequences in the pfam07247 AATase family) and is located in the *S. cerevisiae* Atf1p amino acid sequence from residue 198 to 202 (Van Laere et al., 2008). Further research, including mutagenic analysis, could prove interesting in investigating the physiological role of the alcohol acetyltransferases and their importance in ester biosynthesis.

4. Microbes and aroma

As stated, the initial conversion of grape must into wine occurs *via* an alcoholic fermentation largely carried out by one or more strains of yeast, typically *S. cerevisiae*. Ethanol produced during the primary fermentation progressively limits the growth of other microbes that are potentially undesirable, thereby helping to protect the wine from spoilage (Renouf, Claisse, & Lonvaud-Funel, 2007). Nevertheless, without their deliberate elimination, bacteria and other yeasts, including non-*Saccharomyces* species, will be present in all wine fermentations and potentially impact wine quality. These indigenous yeasts are not only metabolically active (Jolly, Augustyn, & Pretorius, 2006; Strauss, Jolly, Lambrechts, & van Rensburg, 2001) but can impart desirable and distinct regional characteristics integral to the authenticity of some wines (Lema, Garciajares, Orriols, & Angulo, 1996).

Of the aroma-active compounds found in wine, the esters can be produced as secondary metabolites, along with other sensorily important compounds, by many yeast species during the alcoholic fermentation. The physiological role of esters in yeast metabolism is not yet fully understood. Current literature suggests that ester biosynthesis is a result of the detoxification of long-chain fatty acids and is also a metabolic process used to balance the acetyl-CoA/CoA ratio (Mason & Dufour, 2000). *S. cerevisiae* produces not only ethyl esters of short to medium-chain fatty acids but also acetate esters of various alcohols (Plata, Millan, Mauricio, & Ortega, 2003; Verstrepen et al., 2003c). Research by several groups has reiterated the importance of strain choice in winemaking and the ester profile of the finished product (Fleet, 2003; Miller et al., 2007; Rojas, Gil, Pinaga, & Manzanares, 2001; Romano, Fiore, Paraggio, Caruso, & Capece, 2003; Soles, Ough, & Kunkee, 1982; Swiegers, Bartowsky, Henschke, & Pretorius, 2005).

The utilisation of LAB to conduct MLF can modify and possibly synthesise fruity aromas (D'Incecco et al., 2004; Liu, 2002; Maicas et al., 1999; Matthews et al., 2004). Aroma compounds that can increase or decrease during MLF include esters (Pozo-Bayón et al., 2005), aldehydes (Osborne, Mira de Orduna, Pilone, & Liu, 2000), glycosylated compounds (including 2-phenylethanol, terpenols, and C13-norisoprenoids (Boido, Lloret, Medina, Carrau, & Dellacassa, 2002), and 2,3-butanedione (diacetyl; Bartowsky & Henschke, 2004). It is clear therefore that yeast and bacteria have the ability to alter wine volatile composition and specifically the profile of esters.

4.1. Ester synthesis and hydrolysis by Saccharomyces spp.

The ester synthesis and hydrolysis activities of several wine-associated yeast species have been described and a number of alcohol acetyltransferases and esterases have been purified. Volatile esters are of particular interest as the presence of these compounds determines the fruity aroma of wine. Aroma-active esters are formed intracellularly by fermenting yeast cells, but since they are lipid-soluble, ethyl esters can diffuse through the membrane into the fermenting medium (Saerens et al., 2008). The transfer of ethyl esters to the medium appears to decrease drastically with increasing chain length, and ranges from 100% for ethyl hexanoate to 8–17% for ethyl decanoate (Verstrepen et al., 2003b). Alcohol acetyltransferase and esterase genes that have been identified in the synthesis and hydrolysis of esters in yeast species are described in Table 2.

To date six distinct proteins have been isolated and characterised as having ester synthesis or hydrolysis activity, with AATase I (or ATF1), encoded by *ATF1*, having the greatest activity and being

Table 2
Genes reported to be involved in ester synthesis and hydrolysis in *Saccharomyces* sp.

Gene	Mechanism	Metabolic pathway	Localisation	Source	References
ATF1 (YOR377W)	Alcohol acetyl-transferase	Acetate ester synthesis	Intracellular – in lipid particles	*S. cerevisiae*	Fujii et al. (1994), Verstrepen et al. (2004)
Lg-*ATF1*	Alcohol acetyl-transferase	Acetate ester synthesis	Unknown	*S. uvarum* and *S. pastorianus*	Fujii et al. (1994), Yoshimoto et al. (1998)
ATF2 (YGR177C)	Alcohol acetyl-transferase (isoamyl alcohol acetyl-transferase)	Acetate ester synthesis and response to toxin	Integral to endoplasmic reticulum membrane	*S. cerevisiae*	Cauet et al. (1999), Nagasawa et al. (1998), Tiwari et al. (2007)
EHT1 (YBR177C)	Ethanol hexanol -transferase	Medium-chain fatty acid ethyl esters synthesis and hydrolysis	Localises to lipid particles and the mitochondrial outer membrane	*S. cerevisiae*	Saerens et al. (2006), Zahedi et al. (2006)
EEB1 (YPL095C)	Ethanol acyltransferase /ethyl ester hydrolase	Medium-chain fatty acid ethyl esters synthesis and hydrolysis	Unknown	*S. cerevisiae*	Saerens et al. (2006)
IAH1 (Est2) (YOR126C)	Esterase	Acetate ester hydrolysis	Unknown	*S. cerevisiae*	Fukuda et al. (2000)

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the most studied (Cauet, Degryse, Ledoux, Spagnoli, & Achstetter, 1999; Fujii, Yoshimoto, & Tamai, 1996; Fujiwara, Kobayashi, Yoshimoto, Harashima, & Tamai, 1999; Horton & Bennett, 2006; Verstrepen et al., 2003a, 2003c). The *ATF1* gene was first cloned from *S. cerevisiae*, which has only one *ATF1* gene, and the brewery lager yeast *Saccharomyces uvarum*, which has one *ATF1* gene and another, homologous gene (Lg-*ATF1*) (Fujii et al., 1994). This study reports that yeast strains carrying multiple copies of the *ATF1* gene or the Lg-*ATF1* gene exhibited high AATase activity and produced greater concentrations of acetate esters than the control. There was a 27-fold increase in 3-methylbutyl acetate (isoamyl acetate) concentration and a nine-fold increase in ethyl acetate concentration. Manipulation of the expression of *ATF1* has also been shown to significantly alter ester production during wine fermentation (Lilly et al., 2006).

Deletion of *ATF1* produces a large decrease in ester production, while its over-expression results in more than a 10–200-fold increase in various esters. Lilly et al. (2000) constitutively expressed *ATF1* from *S. cerevisiae* in three commercial wine yeast strains, using the *PGK1* promoter and terminator. This resulted in higher concentrations of several esters with the level of ester increase being dependent on the fermentation temperature, cultivar and yeast strain used. The levels of ethyl acetate increased 3–10-fold (fruity at >100 mg/l), 3-methylbutyl acetate increased 3.8–12-fold (banana, fruity), and 2-phenylethyl acetate increased 2–10-fold (flowery, rose) with other esters only showing minor changes (Lilly et al., 2000). The ester synthesis rate is also determined by the concentration of available substrates, e.g. carbon (Verstrepen et al., 2003a), nitrogen (Yoshimoto, Fukushige, Yonezawa, & Sone, 2002), and fatty acid metabolism (Fujii, Kobayashi, Yoshimoto, Furukawa, & Tamai, 1997), and total enzymatic activity (determined by gene expression). For example, Yoshimoto et al. (2002) overexpressed a branched-chain amino acid aminotransferase (*BAT2*) gene in *S. cerevisiae* and reported that an increase in the *BAT2* gene product resulted in about 1.3-fold more 3-methylbutyl alcohol and 1.5-fold more 3-methylbutyl acetate. This suggests that the production of esters is affected by the availability of fusel alcohols. However this is not the only factor influencing ester formation in *Saccharomyces* spp.

ATF1 is repressed by both oxygen and unsaturated fatty acids (UFAs) (Fujii et al., 1997; Fujiwara, Yoshimoto, Sone, Satoshi, & Tamai, 1998; Fujiwara et al., 1999), and may play a physiological role in fatty acid metabolism (Mason & Dufour, 2000). Regulation is mediated at the transcriptional level and when *ATF1* is constitutively expressed no reduction in AATase activity is observed (Fujii et al., 1997). More recently, in a study using the industrial ale strain *S. cerevisiae* CMBS SS01, UFAs added to a standard low-UFA wort resulted in a decrease in the production of all ethyl esters, with ethyl decanoate concentration reduced by 50% (reduced from 0.2 mg/l to 0.1 mg/l), ethyl hexanoate by 33% and ethyl octanoate by 25% (Saerens et al., 2008). These decreases have the potential to affect aroma perception, as a 50% reduction in ethyl decanoate, for example, could place its concentration below the reported aroma threshold level (Table 1).

Further investigations of *ATF1* gene regulation have demonstrated that *ATF1* and the gene encoding Δ9 fatty acid desaturase, *OLE1*, are co-regulated through a Low-Oxygen Response Element (LORE), which is activated under hypoxic conditions and selectively repressed by unsaturated fatty acids (Fujiwara et al., 1998, 1999; Vasconcelles et al., 2001). The Ras/cAMP/PKA nutrient signalling pathways are also involved in the rapid induction of *ATF1* and Lg-*ATF1* when glucose is added to carbon-starved cells (Tiwari, Köffel, & Schneiter, 2007). However a persistent level of expression is not achieved unless a nitrogen source is present (Tiwari et al., 2007). Ras proteins are small, GTP-binding proteins that oscillate between an active GTP-bound form and an inactive GDP-bound form and act as molecular switches (Bokoch & Der, 1993). *S. cerevisiae* has two Ras proteins directly interacting with the enzyme adenylate cyclase, Ras1p and Ras2p (Toda et al., 1985). This interaction produces higher intracellular levels of cAMP and also results in an increase in protein kinase A (PKA) activity (Toda, Cameron, Sass, Zoller, & Wigler, 1987). It has been hypothesised that *ATF1* and *Lg-ATF1* are regulated by a 'fermentable growth medium' pathway (Tiwari et al., 2007) which ensures the regulation of PKA targets.

A second alcohol acetyltransferase, AATase II (or ATF2) encoded by *ATF2* (Table 2), has also been characterised (Cauet et al., 1999; Nagasawa et al., 1998). The *ATF2* gene was originally cloned from a λ-EMBL3 phage library of *S. cerevisiae* Kyokai No. 7 (sake yeast) genomic DNA. Oligonucleotide probes were designed on the basis of internal peptide sequences, obtained following purification of an enzyme with 3-methylbutyl alcohol acetyltransferase activity and 36.9% identity to Atf1p at the amino acid level (Nagasawa et al., 1998). Sequence alignments of ATF1, LgATF1 and ATF2 with closely related protein sequences from other ascomycetous fungi show only moderate sequence conservation across the entire length of the protein. The potential catalytic cysteine residue is not conserved in *Kluyveromyces lactis* and an alternative conserved region exists (Van Laere et al., 2008). ATF2, along with ATF1, is responsible for the production of ethyl acetate and 3-methylbutyl acetate during fermentation (Lilly et al., 2006; Malcorps & Dufour, 1992; Yoshioka & Hashimoto, 1983). A study using over-expression of a recombinant ATF2 in *E. coli*, with mutations in other pathways that compete for acetyl-CoA, showed that 3-methylbutyl acetate production was linked to intracellular acetyl-CoA levels (Vadali, Horton, Rudolph, Bennett, & San, 2004). This correlates with the proposed biosynthetic pathway for AATases (Fig. 2). Tiwari et al. (2007) recently reported that ATF2 is responsible for the formation of acetylated sterols and may also play a role in steroid detoxification. This data correlates with an earlier report, which showed that pregnenolone (a steroid precursor) is acetylated by a protein, which was later identified as ATF2 (Cauet et al., 1999). Deletion/disruption of *ATF2* eliminated all detectable pregnenolone acetylation activity with no other discernable affect on phenotype, with activity restored when the null mutant was transformed with *ATF2* on a multicopy plasmid.

More recently two new proteins with both medium-chain fatty acid (MCFA) ethyl ester synthase and esterase activity have been reported (Saerens et al., 2006). These proteins are designated EHT1 and EEB1 (Table 2) and belong to a three-member gene family, with the function of the third gene YMR210w not yet confirmed. How the balance between MCFA ethyl ester synthesis and hydrolysis is regulated by these proteins *in vivo* has not been determined. Sequence alignments of EEB1 and ETH1 with closely related protein sequences from other ascomycetous fungi show high sequence conservation across the entire length of the protein. The potential catalytic residues and nucleophilic elbow motif are also conserved. Both EHT1 and EEB1 contain the characteristic esterase catalytic triad (Ser-Asp-His) and a putative G-X-S-F-G nucleophilic elbow motif. *EHT1* has been suggested to encode an ethanol hexanoyl transferase, which generates ethyl hexanoate from ethanol and hexanoyl-CoA, playing a minor role in medium-chain fatty acid ethyl ester biosynthesis and possessing short-chain esterase activity (Saerens et al., 2006). *EEB1* encodes an ethanol acyltransferase responsible for the major part of medium-chain fatty acid ethyl ester biosynthesis during fermentation. EEB1 also possesses short-chain esterase activity and may be involved in lipid metabolism and detoxification. Saerens et al. (2006) also hypothesised that yeast cells must have one or more additional enzymes responsible for MCFA ethyl ester synthesis, due to a double deletion mutant retaining some MCFA ethyl ester synthesis ability (dependent on the substrate).

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Whereas expression level seems to be the limiting factor for *ATF1* gene regulation, it has recently been suggested that precursor availability and not the expression level of the ethyl ester biosynthesis genes *EEB1* and *EHT1* is the limiting factor for ethyl ester production (Saerens et al., 2006). Over-expression of *EEB1* and *EHT1* does not cause a significant increase in ethyl ester production. This is most likely due to the balance between their ester synthesis and ester hydrolysis activities and might not be a true reflection of the effect of gene expression. Saerens et al. (2008) also investigated the effect of MCFA addition to the fermentation medium on *EEB1* and *EHT1* transcription and reported that only octanoic acid (and not hexanoic acid) was able to induce expression of *EEB1* and *EHT1*. It has been previously reported that the addition of hexanoic acid or octanoic acid to the fermentation medium causes a strong increase in the formation of the corresponding ethyl ester and suggested that the cellular precursor concentration is rate limiting for ethyl ester synthesis (Saerens et al., 2006). It would therefore be of interest to investigate factors that inhibit *EEB1* and *EHT1* gene expression.

IAH1 is the final esterase gene reported to be involved in acetate ester hydrolysis (Table 2) with a putative active site motif comprising Ala85-Cys86-Ser87-Ala88-Gly89 (Fukuda et al., 1996, 1998). The reporting of this area of IAH1 as the active site was based on inhibition studies, which suggested that both a serine and a cysteine residue were involved in the catalytic activity of this enzyme. It has been suggested that blocking the serine residue adjacent to the cysteine residue causes the inhibition (Fukuda et al., 2000). However, *in silico* analysis of IAH1 with closely related protein sequences shows that although there is high conservation across the entire length of the protein only the cysteine residue in this region of sequence is conserved throughout other ascomycetous fungi. This data suggests that the esterase contains an active site thiol and can be potentially classified as an arylesterase. An *IAH1Δ* mutant showed an increase in 2-methylpropyl acetate (isobutyl acetate), and 3-methylbutyl acetate (isoamyl acetate), relative to the wild-type yeast strain (Fukuda et al., 1996, 1998). Fukuda et al. (2000) reported purification and characterisation of the *IAH1* gene product. They observed that IAH1 is most active towards 2-methylpropyl acetate and suggested that activity was based upon the length of the main chain of the aliphatic group rather than its structure (i.e. straight or branched chain). It was also reported that the k_{cat} for the IAH1 reverse reaction (acetate ester synthesis) was significantly lower than for acetate ester hydrolysis (Fukuda et al., 2000).

The genes reported to be involved in ester synthesis and hydrolysis in yeast have been well characterised and there is the potential to further utilise this knowledge. There are currently four proteins from *S. cerevisiae* implicated in ester synthesis (ATF1, ATF2, ETH1, and EEB1) and one protein (IAH1) which is primarily involved in hydrolysis of acetate esters. There are however still some gaps in knowledge regarding these proteins. For example, the catalytic sites of ATF1 and ATF2 and the protein folding and structure are yet to be determined. The role of ATF1 and ATF2 in acetate ester synthesis is clear, however the role of ETH1 and EEB1 in ethyl ester synthesis needs to be more clearly defined. There are many orthologues of these proteins in ascomycetous fungi that also have the potential to be involved in ester synthesis and hydrolysis if present and active during the fermentation.

4.2. Ester synthesis and hydrolysis by lactic acid bacteria

LAB are traditionally defined as a group of microaerophilic, Gram-positive organisms that ferment hexose sugars to produce lactic acid (Makarova et al., 2006). The LAB consist of a number of genera, including *Leuconostoc*, *Oenococcus*, *Lactobacillus*, *Lactococcus* (lactic *Streptococci*), *Enterococcus*, *Streptococcus* and *Pediococcus* species, and are often associated with plants, meat and dairy products (Carr, Chill, & Maida, 2002; Thunell, 1995). Although LAB have beneficial effects in the food industry, they can be problematic, producing contaminants which have the potential of forming off-flavours. Esterase activity is well documented for dairy LAB and has been shown to be strongly inhibited by phenylmethylsulfonyl fluoride, suggesting that, as previously discussed, a serine residue is involved in the catalytic mechanism of these enzymes (Table 3). Alongside these characterised genes there is an abundance of genomic sequence data available for LAB in the public domain.

The results of an NCBI blast search (www.ncbi.nlm.nih.gov/BLAST) against the five published esterase sequences in LAB are given as a CLUSTAL W alignment (Thompson, Higgins, & Gibson, 1994). The nucleophilic elbow motif G-X-S-X-G is conserved amongst all alignments with EstA from *Lc. lactis*, Est B and Est C from *Lb. casei* LILI and EstI from *Lb. casei* CL96. However EstA from *Lb. helveticus* has a unique putative nucleophilic elbow motif, designated GASI, and based on alignments is most closely related to the GDSL group (Arpigny & Jaeger, 1999) of lipolytic enzymes. This location of the active site is supported by site-directed mutagenesis experiments (Fenster et al., 2000). Also of note is the complete lack of homology of EstI from *Lb. casei* CL96 with any other closely related LAB. The origin of this esterase warrants further investigation. These results are all derived from dairy-associated LAB and their activity is well documented. Although all the published esterase sequences lack a classical secretion signal sequence there are reports of cell surface-associated esterases (Gobbetti, Smacchi, et al., 1997).

A broad range of dairy LAB can form ethyl butanoate, with varying ability between strains within this species (Liu, Holland, & Crow, 2003a). Ester synthesis by dairy LAB in an aqueous environment is thought to be mainly due to a transferase reaction (alcoholysis), in which the fatty acyl group of a glyceride is transferred to an alcohol (Inoue et al., 1997; Liu, Holland, & Crow, 2003b). There are no reports of cofactor requirement for this reaction. This mechanism is particularly relevant to wine, not only because of the aqueous environment, but also because a greater ethanol availability increases transferase activity (Holland et al., 2005). For this transferase reaction Liu, Holland, and Crow (2003a) showed that esterases from 19 dairy LAB were active against mono- and diglycerides of C_6–C_{10}, but not active towards triglycerides, except tributyrin. This has also been demonstrated with esterases purified from other LAB (Gobbetti, Fox, et al., 1997; Holland et al., 2005; Liu, Holland, & Crow, 2001). Studies, with other LAB, show that while some strains have only one gene encoding an active esterase, there are also some strains that contain multiple esterase enzymes. In order to understand the role these genes play in ester formation, the genes involved must first be fully characterised.

Table 3
Published sequences for esterases from LAB. See Matthews et al. (2004) for an overview of published activities for LAB.

Esterase	Nucleophilic elbow motif	Mechanism	Localisation	Source	References
EstA (AF157601)	GLSMG	Arylesterase (3.1.1.2)	Intracellular	*Lc. lactis* subsp. lactis (B1014)	Fernandez et al. (2000), Fenster et al. (2003a)
EstB (AF494421)	GDSAG	Arylesterase (3.1.1.2)	Intracellular	*Lb. casei* LILI	Fenster et al. (2003b)
EstC (AF506279)	GGSLG	Esterase (3.1.1.1)	Intracellular	*Lb. casei* LILI	Fenster et al. (2003a)
EstI (AY251019)	GHSMG	Esterase (3.1.1.1)	Intracellular	*Lb. casei* CL96	Choi et al. (2004)
EstA (AF136284)	GASI	Arylesterase (EC 3.1.1.2)	Intracellular	*Lb. helveticus* (CNRZ32)	Enster et al. (2000), Fenster et al. (2003b)

Most evidence that wine LAB possess esterase activity has been from wine volatile profiling studies which have investigated the changes in concentrations of individual esters during MLF (Delaquis et al., 2000; Maicas et al., 1999; Ugliano & Moio, 2005). In a more recent study, Matthews, Grbin, and Jiranek (2006) surveyed the esterase activities of 16 *Lactobacillius* spp., 11 *Pediococcus* spp. and 23 *O. oeni* strains and found variations within species, and as expected, far more variation between the genera. *O. oeni* showed the greatest amount of esterase activity towards the *p*-nitrophenyl-linked substrates tested. Current reports in the literature show that the changes in ester concentration during MLF are strain specific (Table 4). General increases are seen in ethyl 2-methylpropanoate (fruity, strawberry, lemon), ethyl 2-methylbutanoate (apple, berry, sweet, cider, anise), ethyl 3-methylbutanoate (sweet fruit, pineapple, lemon, anise, floral), ethyl 2-hydroxypropanoate (milk, soapy, buttery, fruity), ethyl 3-hydroxybutanoate (fruity, green, marshmallow), ethyl hexanoate (fruity, strawberry, green apple, anise), 3-methybutyl acetate (banana, fruity), ethyl 2-phenylacetate (rose, floral), 2-phenylethyl acetate (flowery, rose) and hexyl acetate (green, herbaceous, fruit, grape). Other esters show a mixed response and may either increase or decrease in a strain-specific manner. These changes have the potential to greatly affect the final aroma of the wine. It would therefore be of interest to relate this data to odour-active values in wine and determine which of these compounds has an effect on the final aroma or if there is a synergistic effect between certain compounds.

4.2.1. O. oeni

The genus *Oenococcus*, first described by Dicks, Dellaglio, and Collins (1995), contains the sole species *O. oeni*, formerly known as *Leuconostoc oenos*. *O. oeni* is acidophilic, indigenous to wine and similar environments, and remains viable at high ethanol concentrations. The genome of *O. oeni* strain PSU-1, a commercially available MLF strain, has been sequenced and a genomic analysis has recently been carried out (Mills, Rawsthorne, Parker, Tamir, & Makarova, 2005). This information can potentially be used to target specific genes that may be involved in esterase activity. *In silico* analysis performed for this review shows that there are at least four potential esterase genes in *O. oeni*. These genes are most closely related to EstA from *Lc. lactis* subsp. *lactis* (B1014), EstB from *Lb. casei* and EstC from *Lb. casei*. A putative acyltransferase with homology to EstC from *Lb. casei* was also identified. Evidence of esterase activity in *O. oeni* exists and there are a number of reports showing changes in the volatile aroma composition of wines after MLF with *O. oeni* (Table 4), a recent study has demonstrated that *O. oeni* can hydrolyse artificial *p*-nitrophenyl-linked ester substrates (Matthews et al., 2006).

4.2.2. Lactobacillus and Lactococcus sp.

The esterase activities of several dairy-associated lactobacilli have been described and a number of esterases purified from them, namely, *Lb. casei* (Castillo, Requena, De Palencia, Fontecha, & Gobbetti, 1999; Choi et al., 2004; Fenster et al., 2003a, 2003b), *Lb. plantarum* (Gobbetti, Fox, et al., 1997), *Lb. helveticus* (Fenster et al., 2000), *Lb. fermentum* (Gobbetti, Smacchi, et al., 1997), and *Lc. lactis* (Chich, Marchesseau, & Gripon, 1997; Fernandez et al., 2000; Holland & Coolbear, 1996). These esterases were found to have varying activity towards *p*-nitrophenyl-linked substrates. For example, Choi et al. (2004) described a novel esterase, designated *estI* from *Lb. casei* CL96 (Table 3) with highest activity towards *p*-nitrophenyl octanoate. Some LAB have been reported to have only a single gene encoding an active esterase. For example, Nardi et al. (2002) used an esterase-negative mutant of *L. lactis* to determine that the *estA* esterase is the only enzyme in *L. lactis* to synthesise short chain fatty acids *in vitro*. Interestingly, the presence of only one esterase gene seems to be a widespread attribute in *Lactococcus* species (Fernandez et al., 2000; Holland & Coolbear, 1996). Nevertheless, some LAB also possess multiple esterase proteins. For instance, Fenster et al. (2003a, 2003b) reported the nucleotide sequences of arylesterase (*estB*) and intracellular esterase (*estC*) from *Lb. casei* LILA. Amino acid sequence analysis revealed that the mature proteins possess the GXSXG motif at the active site (Table 3). *EstB* selectivity for *p*-nitrophenyl esters was greatest for C_5 and C_6 compounds, whereas *estC* was most selective for C_3 and C_4 compounds.

4.2.3. Pediococcus spp.

Pediococcus spp. commonly occur in milk and cheese (Tzanetakis & Litopoulou-Tzanetaki, 1989) and can also be found in wine (Carr et al., 2002; Grimaldi, Bartowsky, & Jiranek, 2005). There are less data on the esterase activity of *Pediococcus* spp. compared to *Lactobacillus* and *Lactococcus* spp. Where esterase activity has been described it seems to be less significant than for other LAB. Bhowmik and Marth (1989) examined two different species of *Pediococcus* for esterase activity and found that there was either no esterase activity or the activity was low. The esterase activity that was present was primarily towards α-naphthyl acetate. Liu, Holland, and Crow (1998) also showed that synthesis of ethyl butanoate was weaker in the *Pediococcus* strains tested relative to other LAB. This has been confirmed by Matthews et al. (2006) with only one of 11 *Pediococcus* strains examined displaying significant esterase activity, compared to *Lactobacillius* and *Oenococcus* sp.

5. Impact of ester synthesis and hydrolysis on wine

Esterolytic activity during wine production has the potential to either increase or decrease the amount of esters present in wine and therefore its perceived quality. The ester-associated aroma profile does depend on the esters involved, but also the compounds liberated by the esterases (for example fatty acids and higher alcohols). There is a large variation in ester production between yeast strains used for the primary fermentation and investigations into structural changes in the expressed esterase protein or changes in induction and signalling pathways would be of interest. Strain-specific variation is expected to have a greater affect on younger wines and further investigation into the role of esters in the aroma profile of aged wines, where the branched chain esters (Table 1) may play a greater role, would also be of interest.

As previously reported, most strains of wine LAB, *Oenococcus*, *Pediococcus* and *Lactobacillus*, have esterase activities. However the majority of studies on esterolytic activity have been conducted for the dairy industry, where such enzymes contribute to the characteristic flavours and defects of cheeses (Fenster, Rankin, & Steele, 2003; Holland et al., 2005). Dairy LAB have been reported to contain esterases that can hydrolyse mono-, di- and triacylglycerols (Gobbetti, Lowney, et al., 1997; Liu et al., 2001). The appearance of esterase activity in association with whole cells, or in culture supernatants of some LAB, implies that growth in grape juice or wine of these species may modify the ester profile of the beverage. Where intracellular esterase activities are reported, cell disruption would presumably be required in order for these activities to have an impact on wine. However, there is a lack of knowledge of the mechanism of ester formation by wine LAB during MLF.

Most investigations up until now have focused on the metabolism of esters by LAB, although it is suspected that the responsible

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Table 4
A summary of reported strain-specific concentration changes of esters in wine following MLF. For structures and CAS reference numbers see Table 1.

Compound	Inoculated Malolactic Strain[a]					Overall trend[b]
	Oenococcus oeni	*Lactobacillius* sp.	*Pediococcus* sp.	*Leuconostoc* sp.	Unidentified/mixed cultures	
Ethyl esters						
Ethyl 2-methylpropanoate (ethyl isobutyrate)	–	–	–	–	↑X-3[2], Inobacter[2]	↑
Ethyl 2-methylbutanoate	⟺ Lalvin 31[6], EQ54[6], Lalvin O.S.U[6], Uvaferm Alpha[6]	–	–	–	↑X-3[2], Inobacter[2]	⟺/↑
Ethyl 3-methylbutanoate (ethyl isovalerate)	⟺ Lalvin 31[6], EQ54[6], Lalvin O.S.U[6], Uvaferm Alpha[6]	–	–	–	↑X-3[2], Inobacter[2]	⟺/↑
Ethyl 2-hydroxypropanoate (ethyl lactate)	↑ML-D (Uvaferm)[3], BM3[4], MA4[4], VV5[4], TE3[4],IS-18[6],IS-159[6], Lalvin 31[6], EQ54[6], Lalvin O.S.U[6], Uvaferm Alpha[6]	↑CH4[4], *Lb. delbrueckii*[5], *Lb. buchneri*[5], *Lb. brevis*[5],J-39[6], J-51[6]	↑*Pd. cerevisiae*[5]	↑*Leuc. citrovorum*[5]	↑Uninoculated[3]	↑
Ethyl 3-hydroxybutanoate	↑Lalvin 31[6], EQ54[6], Lalvin O.S.U[6], Uvaferm Alpha[6] ⟺ BM3[4], MA4[4], VV5[4], TE3[4]	⟺ CH4[4]	–	–	–	⟺/↑
Ethyl 4-hydroxybutanoate	↑Lalvin 31[6], EQ54[6], Uvaferm Alpha[6] ⟺ Lalvin O.S.U[6] ↓BM3[4], MA4[4], VV5[4], TE3[4]	↓CH4[4]	–	–	–	⇳
Diethyl butanedioate (diethyl succinate)	↑ML-D (Uvaferm)[3], BM3[4] TE3[4],IS-18[6],IS-159[6], Lalvin 31[6], EQ54[6], Lalvin O.S.U[6], Uvaferm Alpha[6] ↓MA4[4], VV5[4]	↑CH4[4], *Lb. buchneri*[5], *Lb. brevis*[5]J-39[6], J-51[6] ⟺ *Lb. delbrueckii*[5]	↑*Pd. cerevisiae*[5]	⟺ *Leuc. citrovorum*[5]	↑ Uninoculated[3]	⇳
Ethyl butanoate	↑Lalvin 31[6], EQ54[6], Uvaferm Alpha[6] ⟺ Lalvin O.S.U[6] ↓ML-D (Uvaferm)[3]	–	–	–	↓ Uninoculated[3]	⇳
Ethyl hexanoate (ethyl caproate)	↑ER1a[1], Lalvin 31[6], EQ54[6], Lalvin O.S.U[6], Uvaferm Alpha[6] ⟺ BM3[4], MA4[4], VV5[4], TE3[4],IS-18[6],IS-159[6]	⟺ CH4[4],J-39[6], J-51[6]	–	–	↑X-3[2], Inobacter[2]	⟺/↑
Ethyl octanoate (ethyl caprylate)	↑Lalvin 31[6], EQ54[6], Lalvin O.S.U[6], Uvaferm Alpha[6] ⟺ IS-159[6] ↓IS-18[6]	⟺ *Lb. delbrueckii*[5], *Lb. buchneri*[5], *Lb. brevis*[5] ↓J-39[6], J-51[6]	⟺ *Pd. cerevisiae*[5]	⟺ *Leuc. citrovorum*[5]	↑X-3[2], Inobacter[2]	⇳
Ethyl decanoate (ethyl caprate)	↑Lalvin 31[6], EQ54[6], Lalvin O.S.U[6], Uvaferm Alpha[6] ↓IS-18[6],IS-159[6]	↑*Lb. buchneri*[5] ⟺ *Lb. delbrueckii*[5], *Lb. brevis*[5] ↓J-39[6], J-51[6]	⟺ *Pd. cerevisiae*[5]	↑*Leuc. citrovorum*[5]	–	⇳
Acetates						
Ethyl acetate	↑VV5[4], TE3[4], IS-18[6], IS-159[6] ⟺ ML-D (Uvaferm)[3] ↓BM3[4], MA4[4]	↑CH4[4], J-39[6], J-51[6]	–	–	↑X-3[2], Inobacter[2] ⟺ Uninoculated [3]	⇳
2-Methylpropyl acetate (isobutyl acetate)	⟺ ML-D (Uvaferm)[3]	–	–	–	⟺ Spontaneous [3]	⟺
3-Methylbutyl acetate (isoamyl acetate)	↑ML-D (Uvaferm)[3], VV5[4], Lalvin 31[6], EQ54[6], Lalvin O.S.U[6], Uvaferm Alpha[6] ⟺ BM3[4], MA4[4], TE3[4]	↑CH4[4]	–	–	↑X-3[2], Inobacter[2], Uninoculated [3]	⟺/↑
Ethyl 2-phenylacetate	↑BM3[4], MA4[4], VV5[4] ⟺ TE3[4], Lalvin 31[6], EQ54[6], Lalvin O.S.U[6], Uvaferm Alpha[6]	↑CH4[4]	–	–	–	⟺/↑
2-Phenylethyl acetate	↑Lalvin 31[6], EQ54[6], Lalvin O.S.U[6], Uvaferm Alpha[6]	–	–	–	–	↑
Hexyl acetate	↑ER1a[1]	–	–	–	⟺ X-3[2], Inobacter[2]	⟺/↑

Source: [1]Avedovech, Mcdaniel, Watson, and Sandine (1992), [2]Delaquis et al. (2000), [3]Herjavec, Tupajić, and Majdak (2001), [4]Maicas et al. (1999), [5]Pilone, Kunkee, and Webb (1966), [6]Pozo-Bayón et al. (2005), [7]Ugliano and Moio (2005).

[a] Data collected from the studies as listed, ↑ significant increase in concentration, ⟺ no significant increase in concentration, ↓ significant decrease in concentration. –, indicates no reported data for these parameters.

[b] General trend ↑ all MLF strains increased ester concentration, ⟺/↑ ester concentration remained unchanged for some MLF strains but increased for others, ⇳ large strain-specific differences and no noticeable trend in the effect of the MLF strains on the ester concentration.

enzymes have the ability to not only synthesise, but also hydrolyse esters. Evidence is available that ethyl esters, such as ethyl acetate, ethyl lactate, ethyl hexanoate and ethyl octanoate, are formed during MLF (De Revel et al., 1999; Delaquis et al., 2000; Pozo-Bayón et al., 2005; Ugliano & Moio, 2005). This suggests that wine LAB possess the ability to synthesise esters, but this needs to be verified. At this point, the characterisation of wine LAB esterases has not been reported. However, it has been established that during MLF, some individual ester concentrations change, and this can be strain-dependent (Table 4). For example, Maicas et al. (1999) observed an increase in ethyl lactate for all *O. oeni* tested, and an increase in isoamyl acetate for only one *O. oeni* strain. Ethyl acetate was also shown to either increase or decrease in a strain-dependent manner. These results suggest that esterases of wine LAB are involved in both synthesis and hydrolysis of esters. A recent investigation from Sumby, Matthews, Grbin, and Jiranek (2009) is the first report of characterisation of such enzymes at the biochemical or genetic level.

6. Methods of measuring esters and esterase activity

The general method in the literature for measuring substrate activities of esterases uses a series of *p*-nitrophenyl esters of C_2–C_{16} fatty acid compounds, and substituted ethyl and acetic acid ester compounds (Bendicho, Trigueros, Hernandez, & Martin, 2001; Fenster et al., 2003a, 2003b; Matthews et al., 2006, 2007). The *p*-nitrophenyl-linked substrates and rates of *p*-nitrophenyl release are easily quantified by measurement of absorbance at 410 nm. Assays with ethyl esters of C_2–C_6 fatty acids and C_2–C_6 alkoxy esters of acetate, phenylacetate and phenylthioacetate have also been employed to test esterase activity. Reaction rates were quantified on the basis of release of ethanol or acetate (Fenster et al., 2003b).

There have been a number of reports describing methods for measuring volatile compounds in wine (for a detailed review see Polášková, Herszage, and Ebeler (2008)). Most recent publications have focused on solid-phase microextraction with gas chromatography-mass spectrometry (SPME–GC–MS) (Campo, Cacho, & Ferreira, 2007; Rocha, Coutinho, Delgadillo, & Coimbra, 2007; Rodríguez-Bencomo, Conde, Rodríguez-Delgado, García-Montelongo, & Pérez-Trujillo, 2002; Siebert et al., 2005). Solid-phase microextraction (SPME) utilises the partitioning of organic components between a bulk aqueous or vapour phase and a thin polymeric film coated onto fused silica fibres. SPME allows for rapid solventless extraction of volatile and semi-volatile organic compounds and is widely used in analytical laboratories for either sample extraction or sample clean-up procedures. Each component will behave differently, depending on the volume of the headspace, volume of the sample and the temperature, pH, polarity and volatility of the measured components. However, excellent quantitative correlations can be made when an internal standard is incorporated into the matrix and specific sampling times are adhered to. Coupled with gas chromatography and mass spectrometry, this method provides an accurate picture of the aroma profile of wine. This technique will not provide an accurate picture of aroma precursors that may give rise to esters upon maturation in bottle; however, high performance liquid chromatography (HPLC) analysis could be used to analyse these compounds.

6.1. Aroma thresholds and the importance of sensory studies

As can be seen from Table 1, aroma thresholds in alcoholic beverages (beer and wine) are higher than those reported in either synthetic wine or ethanol. This is most likely because aroma volatility and perception can be significantly impacted by the non-volatile matrix composition and the more complex nature of the fermented beverages. Therefore detection of a specific aroma requires it to break the 'aroma buffer' of the particular beverage (Escudero et al., 2004; Ferreira, Escudero, Campo, & Cacho, 2007). Volatile compounds can also interact with macromolecules, such as proteins and carbohydrates, potentially leading to a change in aroma impact (Guth & Fritzler, 2004; Voilley, Lamer, Dubois, & Feuillat, 1990). The perceived flavour is the result of specific ratios of many compounds, rather than one single aroma compound (Etiévant, 1991). For example, Escudero and coworkers have reported that the fruity notes of red wine are a complex interaction between wine volatiles, including fruity esters, ethanol, nonisoprenoids and dimethyl sulphide (Escudero, Campo, Farina, Cacho, & Ferreira, 2007). Therefore ester aroma perception may be influenced by vineyard or winemaking practices that affect the concentrations of various matrix components (such as proteins, carbohydrates, ethanol and other volatile compounds), even if no other changes in odourant concentrations occur.

Taste and smell senses can also interact so that a sub-threshold taste combined with a sub-threshold odour can be detected (Dalton, Doolittle, Nagata, & Breslin, 2000). To address the question of how well odour threshold values relate to odour impact, most recent research involves the use of odour activity values (OAV) in the matrix tested (concentration/threshold level) (Rocha, Rodrigues, Coutinho, Delgadillo, & Coimbra, 2004; Vilanova & Martínez, 2007). However aroma compounds having a high OAV do not always have an effect on the aroma of wine and this information only shows the potential aroma contribution. The absence of an individual ester, previously present at above threshold levels, can be masked by the presence of other related esters (Van Der Merwe & Van Wyk, 1981). The ability of a compound to change wine aroma is due to the type of aroma and its differentiation from other wine aromas (Escudero et al., 2004). GC-olfactometric studies are also useful to confirm the impact of each aroma. Therefore, despite the extensive information published on flavour chemistry, odour thresholds, and aroma descriptions, the flavour of complex products such as wine cannot be easily predicted and sensory studies are still important when determining odour impact.

7. Potential applications of current knowledge

There are several ways in which the findings reported to date could be utilised in order to achieve outcomes for fermented beverage production. These include:

- Strain selection, including non-*Saccharomyces* yeast, to positively enhance the ester profile of wine. Strategies for extending the survival of non-*Saccharomyces* with favourable effects on ester production, might also be considered.
- Preparation of esterase enzyme extracts that are better able to function under the harsh and changing environmental conditions of wine fermentation and maturation.
- Use of an 'enzyme–nanofibre composite' (Lee et al., 2007) or cell surface expression of esterases (Breinig et al., 2006) as an immobilisation tool to specifically alter the aromatic ester profile of wine.
- Use of available sequence data, including knowledge of putative catalytic sites, to identify new gene targets for further characterisation.
- Rational Protein Design, using computer modelling, to understand the enantioselectivity and substrate specificity of esterases and other wine-related enzymes, to design enzymes with improved activity and selectivity.
- Directed evolution to alter substrate specificity or improve stability and activity of microbial esterases (Neuenschwander, Butz, Heintz, Kast, & Hilvert, 2007; Schrag et al., 1995).

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8. Conclusions

A considerable amount of research has been directed at the accumulation of esters during fermentation and their role in the fruity aroma of wine. From this research it is clear that esters are extremely important for the flavour profile of wine. Much of this work has focused on formation during the primary fermentation and a large amount of the research focus has been on the determination of the genes involved in ester synthesis and hydrolysis in *Saccharomyces* sp. and the environmental parameters affecting this. Summarising these observations it can be deduced that ester formation is significantly reduced by dissolved oxygen and the presence of unsaturated fatty acids in the fermenting medium. Oxygen directly represses *ATF1* transcription *via* the LORE (Low-Oxygen Response Element) in the *ATF1* promoter sequence. This induction is yet to be fully characterised and further research in this area is required. The yeast genome encodes five known genes that affect the ester profile of wine and other fermented beverages. Therefore, there is enormous potential for the application of current knowledge on alcohol acetyltransferases and esterases to improve wine quality.

There are more limited data on the role of LAB isolated from a variety of sources in ester synthesis and hydrolysis. During their growth in wine, LAB ferment residual sugars left by yeasts and transform numerous other wine components. The esterase enzymes of LAB represent a diverse group of enzymes that may have multiple activities depending on the substrate available and on the environment in which the enzyme is operating. Wine LAB have previously been shown to hydrolyse synthetic *p*-nitrophenyl-linked substrates. It is clear from this research that these organisms possess an extensive collection of ester synthesising and hydrolysing activities, many of which have the potential to influence wine composition and therefore the processing, organoleptic properties, and quality of wine. However, with no detailed analysis of esterase activity and characterisation of wine LAB in this context, there is a need for further research. This investigation will ideally target the underlying molecular mechanisms involved in the hydrolysis and synthesis of esters by wine LAB and the effect on the aroma profile of wine. Given the major impact of LAB on the sensory properties of wine, such research stands to be of great benefit and interest to the wine industry.

Several genes responsible for esterase synthesis and hydrolysis have been cloned and sequenced. Using information readily available in the public database it is possible to identify new esterases that might be significant not just in the wine context. Genetic engineering also offers potential for further control of wine aroma, including inactivation or over-expression of esterase and alcohol acetyltransferase genes. Once a better understanding of these genes is achieved, new techniques for altering aroma profile in wine could be devised. For example, immobilisation or addition of enzymes in the wine to target problems such as high levels of ethyl acetate.

The potential of wine-associated microbes that has been highlighted in this review will certainly stimulate fuller characterisation of these organisms. This will allow potential applications to be investigated and hopefully be applied by the winemaker in a more informed manner. In addition, further sensory and GC–MS analysis of esters will allow an improved understanding of the interactions between esters and other wine components and may lead to better prediction and optimisation of ester release in wine.

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β-Glucoside metabolism in *Oenococcus oeni*: Cloning and characterisation of the phospho-β-glucosidase bglD

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ABSTRACT

The structural gene for a phospho-β-glucosidase from the oenologically important lactic acid bacterium (LAB) *Oenococcus oeni* has been cloned and its protein product characterised. This gene is found in a putative β-glucosidase operon of 2178 base pairs encoding 4 genes designated bglA to bglD. The bglA, B and C genes were not cloned and characterised, however, are thought to be phosphoenolpyruvate dependent phospho transferase system (PEP-PTS) components IIC, IIA and IIB which regulate the uptake, phosphorylation and translocation of β-glucosides across the cytoplasmic membrane. The cloned bglD was sequenced and expressed in *Escherichia coli* followed by purification. The purified bglD protein has 480 residues, a molecular mass of 55.5 kDa and shows high homology to known phospho-β-glucosidases. bglD exhibited high activity towards the phosphorylated β-glucoside para-nitrophenol-β-D-glucopyranoside-6-phosphate with a pH optimum of 5.5 and maintained similar levels of activity between temperatures of 4 °C and 40 °C. The enzyme was not active against non-phosphorylated β-glucosides.

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1. Introduction

The lactic acid bacterium *Oenococcus oeni* is important in winemaking due to its role in malolactic fermentation (MLF), a process which deacidifies wine through decarboxylation of malic acid to lactic acid. The primary alcoholic fermentation is carried out principally by *Saccharomyces cerevisiae* (Alexandre, Costello, Remize, Guzzo, & Guilloux-Benatier, 2004), whilst MLF is a secondary fermentation normally occurring after the alcoholic fermentation is complete. Studies have demonstrated that the aroma profile of a wine can be altered during MLF due to the production of various aroma active compounds (D'Incecco et al., 2004; de Revel, Martin, Pripis-Nicolau, Lonvaud-Funel, & Bertrand, 1999). It is thought that enzymes native to *O. oeni* and other lactic acid bacteria (LAB) are largely responsible for this phenomenon. Matthews et al. (2004) reviewed the marked potential for LAB to influence wine in this way based on reports of enzymatic activities from these organisms under oenological or wine-like conditions. One such enzyme, β-glucosidase, has a particularly significant role in wine, as many aroma compounds in wine and must are found in the glycosidic form (i.e. linked to a sugar moiety) and thereby rendered unperceivable (Maicas & Mateo, 2005). While much research has looked at the effect of β-glucosidases on aroma in juice and wine, there has been limited attention dedicated to β-glucosidase activity on colour pigments in wine. Studies on various fruit juices (Wightman & Wrolstad, 1995) and Sicilian blood oranges show that there is a marked breakdown of anthocyanins with increasing β-glucosidase activity (Barbagallo, Palmeri, Fabiano, Rapisarda, & Spagna, 2007). However, little or no pigment loss was observed in wines when utilising commercial enzyme preparations with β-glucosidase activity applied at the recommended doses (Wightman & Wrolstad, 1996). It can be stated that the deliberate application of β-glucosidases has been shown to enhance aroma intensity (Mateo & di Stefano, 1997; Ribereau-Gayon, Boidron, & Terrier, 1975) and potentially modify wine colour (Wightman, Price, Watson, & Wrolstad, 1997). Most commercial preparations used in winemaking are derived from fungi rather than bacteria. Commercial preparations can be limited to one enzyme and one main function (e.g. pectinases for maceration, glycosidases for aroma liberation and glucanases for settling) or possess a multitude of enzymes and thus have combined effects. These can be utilised at different stages of the winemaking process depending on the desired effect. LAB have a lower optimum pH than many yeast, and a greater tolerance to high levels of ethanol (up to 16%). The very fact that wine associated LAB are able to grow post-alcoholic fermentation is evidence of this. It therefore seems likely that enzymes derived from wine associated bacteria as opposed to yeast, may be more suited to wine-like conditions.

The breakdown of β-glucosides can occur in bacteria through a number of systems. Extracellular or cell wall associated β-glucosidases are commonly responsible. Michlmayr et al. (2009) characterised a glycosyl hydrolase family (GHF) 3 β-glucosidase from *O. oeni*. To date none of the more common GHF1 β-glucosidases have been identified in *O. oeni*. The breakdown of β-glucosides in whole bacterial cells can also be explained by the presence of intracellular phospho-β-glucosidases and the phosphoenolpyruvate dependent phospho transferase system (PEP-PTS) which allows bacterial cells to grow on various carbon sources including β-glucosides (Deutscher, Francke, & Postma, 2006).

The PEP-PTS is well characterised as a bacterial transport system. It is composed of two generic cytoplasmic components (HPr and CI) common to all PEP-PTS's, combined with a carbohydrate specific EII complex (Deutscher, 2008; Deutscher et al., 2006). The PEP-PTS's in *Escherichia coli* and *Bacillus subtilis* are extremely well defined and each contains over 15 distinct EII complexes (Deutscher et al., 2006; Reizer et al., 1999). EII complexes can either contain up to four distinct proteins or single proteins with multiple domains. It is thought that *O. oeni* contains upwards of 15 EII complexes based on sequence homology to a number of characterised EII proteins from other bacteria.

β-Glucosidase activity has been observed in whole LAB cells (Barbagallo, Spagna, Palmeri, & Torriani, 2004; Grimaldi, McLean, & Jiranek, 2000; Guilloux-Benatier, Son, Bouhier, & Feuillat, 1993) of numerous commercial preparations of *O. oeni*, as well as amongst *Lactobacilli* spp. and *Pediococci* spp. Whole *O. oeni* cells have also demonstrated activity against β-D- and α-D-glucopyranosides as well as β-D-xylopyranoside, α-L-rhamnopyranoside and α-L-arabinofuranoside substrates (Grimaldi, Bartowsky, & Jiranek, 2005a). Such activities exhibited strain dependence and a high degree of tolerance to wine-like conditions. As an extension of this work, a putative β-glucoside operon was identified in *O. oeni* based on the sequenced genome PSU-1(CP000411) (Mills, Rawsthorne, Parker, Tamir, & Makarova, 2005), and designated bgl. It is 2178 base pairs in length and encodes 4 genes. In sequential order, the genes bglA, B and C encode PEP-PTS components EIIC, EIIA and EIIB, while bglD encodes a phospho-β-glucosidase (Table 1). This annotation is based on sequence homology to characterised components of the PEP-PTS from multiple bacteria (Table 2). In this study we report the cloning, sequencing, expression and purification of the phospho-β-glucosidase bglD in *E. coli* in a catalytically active state.

2. Materials and methods

2.1. Bacterial strains and growth

The commercially available *O. oeni* strain Lalvin 4X (VL92) was used throughout the experiment since previous reports showed it to have high β-glucosidase activity (Grimaldi, Bartowsky, & Jiranek, 2005b; Grimaldi et al., 2000). The *E. coli* strain DH5α (Invitrogen Australia Pty Limited, 30–32 Compark Circuit, Mulgrave Victoria 3170) was used for cloning and sequencing. The *E. coli* strain BL21 (DE3) plysS (Novagen, distributed by Merck Serono Australia Pty Ltd.) was used for protein expression. Both strains were maintained in 26% glycerol at −80 °C, with VL92 cultivated on MSR supplemented with 10% (v/v) filter sterilized apple juice at 30 °C and DH5α being grown on Terrific Broth (Sambrook & Russell, 2001) at 37 °C.

Table 1
Gene arrangement of the bgl region of the *O. oeni* genome, derived from the published genome sequence with proposed new gene names.

ORF	Proposed gene name	Function
OEOE_0221	bglA	PTS system IIC component
OEOE_0222	bglB	PTS system IIA component
OEOE_0340	bglC	PTS system IIB component
OEOE_0341	bglD	6-Phopho-β-glucosidase

2.2. PCR amplification and cloning of BglD

Genomic DNA was isolated from VL92 after approximately 5 days of growth using Ultraclean™ Microbial DNA Isolation kit (Mo bio Laboratory, distributed by GeneWorks Ltd. 39 Winwood Street Thebarton SA 5031, Australia) according to manufacturer's instructions and verified on a 1% (w/v) agarose gel stained with GelRed™ (Biotium, distributed by Jomar Diagnostics P/L PO Box 385 Stepney SA 5069 Australia). BglD, a 1443 bp fragment, was amplified by PCR using primers AC002 and AcbG2b (Table 3) sourced from Sigma–Aldrich (Sigma–Aldrich PO Box 970 Castle Hill, NSW 1765 Australia), with *Nde* I and *Xho* I restriction sites incorporated. The restriction enzymes were sourced from New England Biolabs (distributed by Genesearch Pty. Ltd. 14 Technology Drive Arundel, Queensland 4214, Australia) and used in accordance with the recommended protocol. The PCR fragment was digested with *Nde* I and *Xho* I and cloned into a previously *Nde* I-*Xho* I digested pET 14.b plasmid (Novagen). PCR reactions to amplify the target ORF used Pfu Ultra II fusion Hotstart DNA Polymerase (Integrated Sciences, Pty.Ltd. 54 Gibbes Street Chatswood NSW 2067, Australia) in accordance with the manufacturer's instructions. The plasmid harbouring bglD was transformed into the *E. coli* strain DH5α. DNA was extracted using the Wizard® Plus SV Minipreps DNA Purification System (Promega Corporation, Sydney, Australia) and sequenced by the Australian Genome Research Facility (AGRF, Brisbane Node Level 5 Gehrmann Laboratories University of Queensland Research Road St Lucia QLD 4072). The same plasmid was subsequently transformed into the *E. coli* strain BL21 (DE3) plysS for expression.

2.3. Expression of bglD in E.coli

BL21 (DE3) plysS with pET 14.b-bglD was grown in 200 ml of Terrific Broth supplemented with 34 μg/ml chloramphenicol (Fluka, distributed by Sigma–Aldrich, Australia) and 50 μg/ml of ampicillin sodium salt (Sigma–Aldrich, Australia) in 500 ml Erlenmeyer flasks to an optical density at 600 nm of approximately 0.6. The cultures were cooled to approximately 15 °C for 15 min and induced with 400 μl of 100 mM isopropyl-D-thiogalactopyranoside (IPTG) (Sigma–Aldrich, Australia). The flasks were left on a shaker at 160 rpm. at 10 °C for 48 h. The cells were harvested by centrifugation (13,000g for 10 min at 5 °C) and lysed with amine-free Bugbuster Mastermix (Novagen) according to the manufacturer's instructions.

2.4. Purification of bglD

The construct pET 14.b-bglD encoded a polyhistidine-tag at the N terminus. The resulting fusion proteins were purified using immobilized metal-affinity chromatography with Talon® cobalt metal affinity resin (Clontech, distributed by Scientifix Pty. Ltd. PO Box 18 Southland Centre Cheltenham Victoria, 3192, Australia) according to manufacturer's instructions. 20 Protein fractions of 0.5 ml were eluted with McIlvane buffer at pH 4.5 in the presence of 10 mM β-mercaptoethanol. Fractions containing pure recombinant protein as visualised on the SDS–PAGE described by Laemmli (1970) were pooled, quantified (Bradford, 1976), aliquoted and stored at 4 and −80 °C in 10% (v/v) glycerol. The protein retained 100% activity for two months when stored at either 4 or −80 °C. All bioconversion of the substrate was performed using the purified recombinant enzyme from a single protein band.

2.5. Assay of β-glycosidase and phospho-β-glucosidase activity

β-Glycosidase activity was determined by measuring para-nitrophenol (pNP) liberated from pNP-α-D-glucopyranoside,

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Table 2
Sequence alignment of bglA, bglB and bglC from the sequenced *O. oeni* genome PSU-1 (CP000411) to characterised PEP-PTS components and bglD to characterised phospho-β-glucosidases.

O.oeni protein	Putative homologues		Percentage (%)		Accession Number	References
			Positives	Identity		
bglA	*Streptococcus mutans*	celE	68	51		Old, Lowes, and Russell (2006); Zeng and Burne (2009)
	Pectobacterium carotovorum	celF	47	28	DQ987482	Hong et al. (2007)
		bglF	49	31	AY769096	Hong et al. (2006)
bglB	*Streptococcus mutans*	celD	70	45	NC_004350	Old et al. (2006); Zeng and Burne (2009)
	Pectobacterium carotovorum	bglI	59	34	AY769096	Hong et al. 2006
bglC	*Pectobacterium carotovorum*	celE	69	49	DQ987482	Hong et al. 2007
		bglE	65	48	AY769096	Hong et al. 2006
bglD	*Escherichia coli*	bglB	65	51	M16487	Mahadevan, Reynolds, and Wright (1987); Schnetz, Toloczyki, and Rak (1987)
		ascB	66	47	M73326	Hall and Xu (1992)
		bglA	71	58	Q46829	Schnetz et al. (1987)
	Clostridium longisporum	abgA	66	51	L49336	Brown and Thomson (1998)
	Erwinia chrysanthemi	arbB	65	52	M81772	El Hassouni, Henrissat, Chippaux, and Barras (1992)
	Bacillus subtilis	bglA	72	58	P42973	Zhang and Aronson (1994)
		bglH	68	55	Z34526	Le Coq, Lindner, Kruger, Steinmetz, and Stulke (1995)
	Lactobacillus plantarum	bglH	64	51	AJ250202	Marasco, Salatiello, De Felice, and Sacco (2000)
	Streptococcus mutans	bglA	66	49		Cote & Honeyman (2002)
	Pectobacterium carotovorum	celA	81	66	NC_004350	Old et al. (2006); Zeng and Burne (2009)
		ascB	65	48	AY622309	An et al. (2005)
		celG	47	31	DQ987482	Hong et al. (2007)
		bglB	66	53	AY542524	An et al. (2004)
		bglA	71	57	AY769096	Hong et al. (2006)

Table 3
Primers used for cloning and sequencing bglD.

Primer		Restriction site
AC002	GCGCGGGAATTCCATATGTTGAATAAACTTTTTTTGCCGA	Nde I
AcbG2b	CCGCTCGAGTTAATCTAATTGACTGCCGTTTGACTTAATAACCT	Xho I
BG5Fc	GGCTGCATGATCAACCATGAC	na[a]
BG5Rc	GTCATGTTGATCATGCAGCC	na[a]

[a] Used for sequencing. No restriction site required.

ortho-nitrophenol-β-D-glucopyranoside, pNP-β-D-galactopyranoside and pNP-α-D-galactopyranoside. All pNP linked reagents were obtained from Sigma–Aldrich and were of analytical grade or higher. Unless otherwise stated, the assay involved incubation of the enzyme with a substrate concentration of 2 mM in McIlvaine buffer (pH 5.5) for 30 min in a total volume of 50 µl at 37 °C. The amount of pNP released was determined by measuring the absorbance of the reaction mixture at 400 nm (An et al., 2005). Phospho-β-glucosidase activity was measured using the same assay conditions with the phosphorylated substrate pNP-β-D-glucopyranoside-6-phosphate (pNPβG6P). pNPβG6P was prepared from the non-phosphorylated pNP-β-D-glucopyranoside (Wilson & Fox, 1974).

The effects of pH and temperature on β-glycosidase activity of purified recombinant enzyme were examined. pH values ranging from 3.0 to 7.5 achieved using 0.1 M McIlvaine buffer were utilised. Following incubation, 50 µl of 0.1 M disodium hydrogen phosphate were added to the pH 3.0 and 3.5 samples and 20 µl of 0.1 M disodium hydrogen phosphate were added to the pH 4.0 and 4.5 samples for alkalinisation prior to absorbance measurement. To determine the effect of temperature on the enzymatic activity, buffer and substrate were pre-incubated for 10 min in 0.2 ml tubes in a PCR machine at the chosen assay temperature prior to the addition of enzyme. Assays were incubated for 30 min at temperatures ranging from 5 to 70 °C before transfer to a 96 well microtitre plate and their absorbances read at 400 nm. The kinetic constants reported, K_m and V_{max} correspond to β-glycosidase activity at 25 °C. The effects of the metal ions Mn^{2+}, Mg^{2+}, Cu^{2+}, Co^{2+} and Ca^{2+} at a concentration of 10 mM were also investigated. All assays were performed in triplicate and were repeated at least three times.

3. Results and discussion

3.1. Sequence comparison of bglD with other family 1 glycosyl hydrolases

bglD showed high homology to the following characterised genes: bglB in *E. coli*; bglA and bglH in *B. subtilis*; arbB in *Erwinia chrysanthemi*; abgA in *Clostridium longisporum*; ascB, bglA, bglB in *Pectobacterium carotovorum*; and celA in *Streptococcus mutans* (Fig. 1). The hydrolysis of the β-glucosidic bond is a two-step process in GHF 1 enzymes which have a retaining mechanism, whereby the product has the same anomeric configuration as the substrate (Koshland, 1953; McCarter & Withers, 1994) involving a highly conserved catalytic proton donor/base and a catalytic nucleophile (Davies & Henrissat, 1995). Phospho-β-glucosidases from GHF 1 have high sequence homology to characterised β-glucosidases from GHF 1. In fact, there appear to be very few differences between them. A consensus sequence for a GHF 1 β-glucosidase has been established for the active site as YITENG, the glutamic acid residue being the active residue. The active site in bglD, as with other characterised GHF 1 phospho-β-glucosidases, would appear to be M/FI-VENG (Fig. 1), with the active glutamic residue in position 394 (bglD numbering) acting as the catalytic nucleophile. It is likely that the proton donor/base is found in the glutamic acid residue at position 186 in the conserved region TFNEI. It has been demonstrated by site-directed mutagenesis of the β-glucosidase from *Pyrococcus furiosus* (Hill & Reilly, 2008) that when the glutamic acid residue in position 471 was changed to a serine, the activity of the enzyme was shifted towards the hydrolysis of phosphorylated glycosides. In light of this it is believed that the serine in position 453 (bglD numbering) is principally responsible for the enzyme's affinity for phosphorylated glycosides. Also of interest is the leucine codon TTG which serves as start codon for bglD. Although not common in bacteria, leucine is able to function as a start codon as opposed to methionine. When bglD was expressed with methionine incorporated as a start codon, the protein remained soluble and there was no difference in activity (data not shown).

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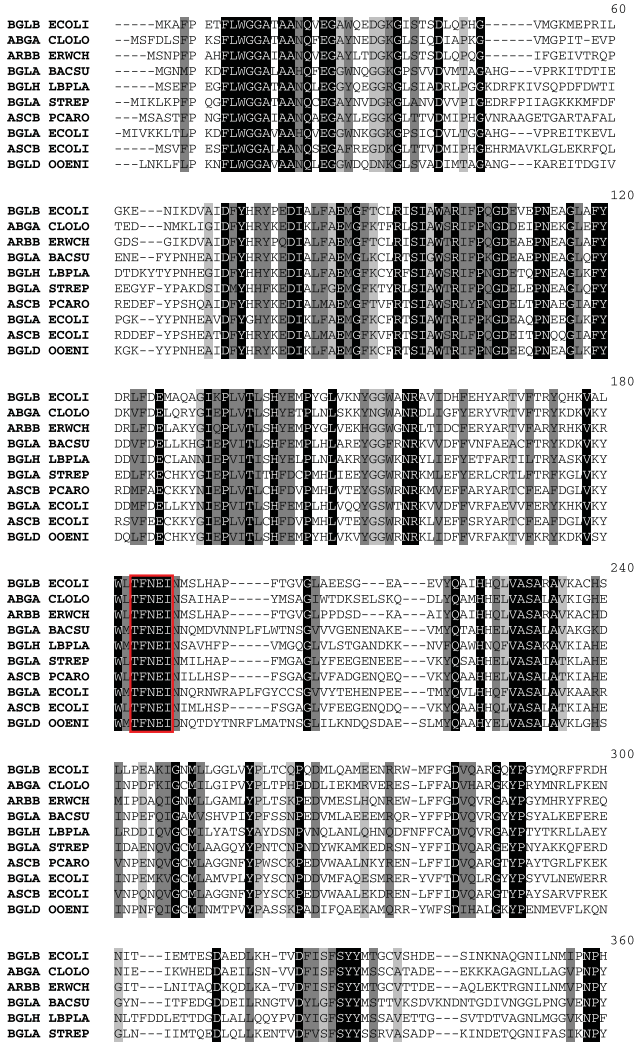


Fig. 1. Alignment of the amino acid sequences of phospho-β-glucosidases from characterised GHF 1: **BGLD OOENI**, *O. oeni* BglD. **BGLB ECOLI**, *E. coli* BglB. **ABGA CLOLO**, *Clostridium longisporum* AbgA. **ARBB ERWCH**, *Erwinia chrisanthemi* ArbB. **BGLA BACSU**, *Bacillus subtilis* BglA. **BGLH LBPLA** *Lactobacillus plantarum* BglH. **BGLA STREP,** *Streptococcus mutans* BglA. **BGLH BACSU**, *Bacillus subtilis* BglH. **ASCB PCARO**, *Pectobacterium carovatorum* AscB. **BGLA ECOLI**, *E. coli* BglA. **ASCB ECOLI**, *E. coli* AscB. Identical residues are highlighted black on white, highly conserved residues are highlighted with dark grey on black and conserved residues are represented by light grey on black. Putative catalytic residues and conserved regions are marked by a box. The numbers to the right denote residue positions.

3.2. Expression, purification and characterisation of bglD

bglD was PCR amplified, cloned into pET 14b and transformed in *E. coli* BL21 (DE3) plysS. In the presence of IPTG, large amounts of a protein were produced at a molecular mass of 55.5 kDa, corresponding to that expected for the full length polypeptide encoded by bglD (Fig. 2). When whole cells were lysed in a buffer solution that did not contain detergent, the bglD protein could not be found in the soluble fraction (data not shown). However, when the whole cells were lysed in a buffer containing detergent (e.g. amine-free Bug Buster) the protein was soluble and catalytically active. Purification of bglD was achieved by immobilized metal-affinity

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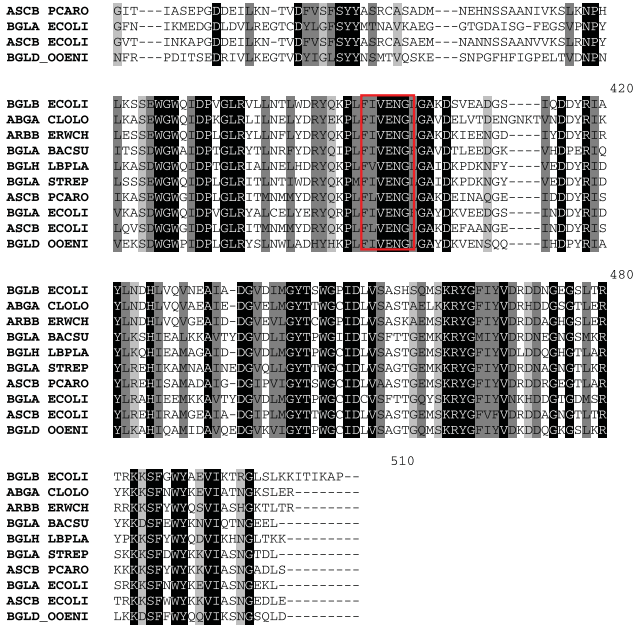


Fig. 1 (*continued*)

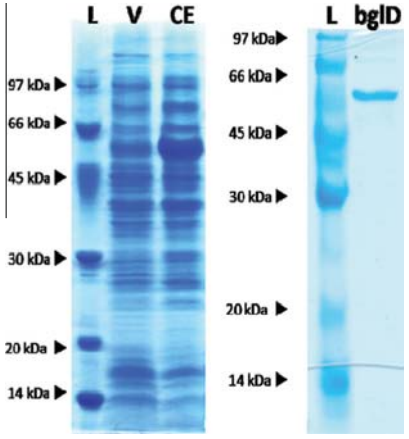


Fig. 2. Electrophoretic analysis of the purified phospho-β-glucosidase bglD on a 12% (w/v) SDS–polyacrylamide gel. **L**; Low molecular weight marker, **V**; Crude extract of vector pET 14.b without the bglD gene inserted, **CE**; Crude extract of vector pET 14.b with the bglD gene inserted, **bglD**; purified bglD. The gel was stained with 0.025% Coomassie blue.

chromatography and only the purified form of the enzyme was used for activity assays. The resulting protein had a predicted molecular mass of 55.5 kDa and a *pI* of 6.58. No activity was detected when assaying bglD against non-phosphorylated β-glucosides (data not shown). However, bglD was active towards the phosphorylated chromogenic substrate pNPβG6P, indicating that bglD is a phospho-β-glucosidase (EC 3.2.1.86). The protein retained 100% activity for two months when stored at either 4 or −80 °C. bglD has maximal phospho-β-glucosidase activity at 40 °C, however, it retained activity between 5 and 40 °C (Fig. 3A). Activity was reduced to 21% at 50 °C and zero at 60 °C. bglD had greatest activity at pH 5.0, with 9% activity at pH 3.5 and 12% activity at pH 7.0 (Fig. 3B). Typical wine conditions range from approximately pH 3.4–4.0 and temperatures during the most extreme winemaking conditions will vary from 10 °C to a maximum of 45 °C in red wine ferments, enabling bglD to retain activity. Metal ions have previously been shown to impact phospho-β-glucosidase activity (An et al., 2004, 2005) and are also important in a winemaking context. The addition of 10 mM Mn^{2+} or Co^{2+} decreased phospho-β-glucosidase activity by approximately 28% and 45% respectively, while the addition of 10 mM Cu^{2+} resulted in near complete inhibition of phospho-β-glucosidase activity (Fig. 3C). By comparison, 10 mM Ca^{2+} or Mg^{2+} showed a significant increase in phospho-β-glucosidase activity. One consequence of the presence of metal ions in wine is with regard to tartrate stability. In this context the concentration of calcium ions is the second highest contributor (Ribereau-Gayon, Branco, & Rychlewski, 2006) after that of potassium ions. Divalent calcium ions mostly originate from deacidification treatments with $CaCO_3$, as well as some vineyard treatments. Magnesium is found in similar amounts to calcium

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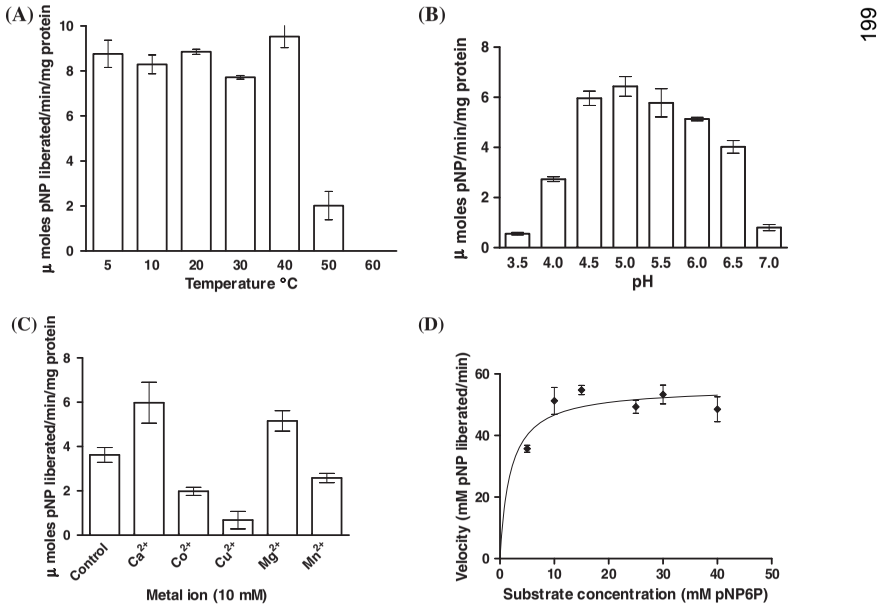


Fig. 3. Effect of pH (A), temperature (B) and metal ions (C) on the activity of bglD. Enzyme activity was assayed at 37 °C for 30 min in McIlvaine buffer of indicated pH. (B) Effect of temperature on the specific activity of bglD. Enzyme activity was assayed at pH 5.5 for 30 min at the indicated temperature. The buffer was brought to temperature 10 min prior to commencement of the assay in a PCR machine. (C) Effect of 10 mM addition of Ca^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+} on the specific activity of bglD. Enzyme activity was assayed at pH 5.5 for 30 min. (D) Effect of substrate concentration on enzyme kinetics, analysed with Michaelis–Menten non-linear regression analysis.

(up to 150 mg/l for both cations) and remains in the wine forming soluble salts (Ribereau-Gayon et al., 2006). Copper ions can be found in wine due to copper fining treatments but if present are found in trace amounts as with manganese. It can be deduced that there would be no detrimental effect on bglD in whole cell form from any of these cations based on the amounts they are found in wine. In the absence of enzyme, no chemical hydrolysis of pNP was evident, when incubated in the presence of 10 mM metal ions for 1 h at 37 °C (data not shown). The kinetic data from bglD produced a Michaelis–Menton curve, with a V_{max} of 55.67 mM pNP liberated/min/mg protein and a K_m of 1.938 mM (Fig. 3D).

4. Conclusions

In a wine context, *O. oeni* is an important microorganism due to its role in MLF. Many aroma and colour components in wine are found in the glycosidic form, and their sensorial importance can change when they are deglycosylated (Maicas and Mateo, 2005). In an attempt to identify the genes responsible for the β-glucosidase activity of whole cells previously reported by this laboratory (Grimaldi et al., 2005b, 2000), the sequences corresponding to reported β-glucosidases and phospho-β-glucosidases were sought. In this way a phospho-β-glucosidase bglD was purified and characterised. bglD is thought to be a fundamental component of the phosphoenolpyruvate dependent phospho transferase system (PEP-PTS) which allows bacterial cells to grow on various carbon sources including β-glucosides (Deutscher et al., 2006).

A putative β-glucoside utilising operon has been identified in *O. oeni* by sequence alignment with characterised β-glucoside operons from other species. bglD was only soluble in a hydrophobic environment suggesting that this enzyme may be localised on the cell membrane in *O. oeni* (Schey, 1996). The purified enzyme was stable for extended periods at 4 and −80 °C. bglD had an optimum pH of 5.0, retained activity from 5 to 40 °C and divalent metal ions were found to have an impact on phospho-β-glucosidase activity as previously suggested (An et al., 2004, 2005). bglD showed a decrease in activity at wine like pH 3.5–4.0 and high activity in temperatures which are routinely adopted for many winemaking manipulations such as alcoholic and malolactic fermentation, pre-fermentation cold maceration, extended maceration on skins for red wines and oak maturation.

It is clear that the hydrolysis of β-glycosides is able to change the sensory profile of a wine. There is an increasing demand for competitively priced wines with specific aroma profiles depending on the desired style of wine. It is hoped that the identification and characterisation of β-glycosidases from LAB, species with a higher tolerance to harsh wine conditions, will aid winemakers in tailoring wine aroma, colour and overall complexity.

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β-Glucoside metabolism in *Oenococcus oeni*: Cloning and characterization of the phospho-β-glucosidase CelD

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ABSTRACT

In previous work, we reported characterization of a phospho-β-glucosidase gene *bglD* in a β-glucoside metabolizing operon in the oenologically important lactic acid bacterium *Oenococcus oeni*. Here we report a second phospho-β-glucosidase gene *celD* which has been cloned and expressed in *Escherichia coli*. This gene is found in a putative operon 6043 bp long encoding six genes designated *celA* to *celF*. Comparative sequence analyses of lactic acid bacteria suggest that the open reading frames of *celA*, *B* and *F* from the sequenced *O. oeni* PSU-1 encode phosphoenolpyruvate dependent phosphotransferase system (PEP-PTS) components IIB, IIA and IIC, respectively, which regulate the uptake and phosphorylation of β-glucosides across the cytoplasmic membrane. *celE* is speculated to have a regulatory function. *celD* was cloned and expressed in *E. coli* followed by purification of the gene product. The purified protein His-tagged CelD (485 residues, Mw = 55.8 kDa) has high homology to known phospho-β-glucosidases and has high activity towards the phosphorylated β-glucoside para-nitrophenol-β-D-glucopyranoside-6-phophate (pNPβG6P). CelD has an optimum pH between 4.0 and 5.0 and is most active at 40 °C. The gene *celC* was cloned, heterologously expressed and purified (481 residues, Mw = 55.7 kDa) but showed no significant activity towards pNPβG6P despite high sequence homology to *celD* and characterized phospho-β-glucosidases. Neither CelC nor CelD are active against non-phosphorylated β-glucosides.

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1. Introduction

Lactic acid bacteria (LAB) are important in winemaking subsequent to the alcoholic fermentation. LAB deacidify wine in the malolactic fermentation (MLF) by converting malic acid to lactic acid. MLF is carried out principally by *Oenococcus oeni*, but also *Lactobacillus* spp. and *Pediococcus* spp. [1]. It is of interest to understand β-glucosidase metabolism in *O. oeni* as many aroma compounds in wine and must are found in the glycosidic form (i.e. linked to a sugar) yet are only perceivable in their non-glycosidic form [2]. Other glycosylated compounds found in wine, including many phenolics, are suggested to have a health effect, whilst the deglycosylated form of some may be even more beneficial [3,4]. Anthocyanins, which contribute to wine colour, are found in the glycosidic form in young wines, however, it is not clear what effect, if any, anthocyanin deglycosylation has on colour and its stability during the maturation and aging of wine. Studies on various fruit juices [5] and Sicilian blood oranges [6] show that there is a marked breakdown of anthocyanins with increasing β-glucosidase activity. However, Wightman and Wrolstad [7] demonstrated that there is no loss of pigmentation when commercial juice processing enzyme preparations of β-glucosidases are used in recommended doses using boysenberry juice as a substrate. Commercial enzymes for oenological use are mostly isolated from fungal rather than bacterial sources. Bacteria have a lower optimum pH for growth than fungi, and are able to grow in harsh post-alcoholic fermentation conditions with up to 15% ethanol (v/v) [8]. This suggests that enzymes originating from bacteria may be better suited to use in wine-like conditions.

The most common pathway for β-glucoside utilization in bacteria is dependent on extracellular or cell wall-associated β-glucosidases [9]. β-Glucosidase activity has been detected in whole cells [10,11] of 22 commercial wine strains of *O. oeni*. Spano et al. [12] identified a putative β-glucosidase gene from *O. oeni*, however, it bore low homology to the published *O. oeni* sequence [13]. Michelmyer identified a glycosyl hydrolase family (GHF) 3 β-glucosidase [14] from *O. oeni*. The presence of intracellular phospho-β-glucosidases and the phosphoenolpyruvate dependent phosphotransferase system (PEP-PTS) [15] can also be responsi-

Abbreviations: PEP-PTS, phosphoenolypyruvate-phospho transferase system; *p*NPβG6P, para-nitrophenol-β-D-glucopyranoside-6-phosphate; *p*NP, para-nitrophenol; LAB, lactic acid bacteria; MLF, malolactic fermentation; GHF, glycosyl hydrolase family; IPTG, isopropyl-D-thiogalactopyranoside.

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Table 1
Primers used for cloning and sequencing celD and celC.

Gene	Primer	Oligonucleotide sequence (5′ to 3′)	Restriction site
celD (Fwd)	AC003	CCGCTCGAGATGACTGAAACAACAAAAAGTGGATTA	XhoI
celD (Rev)	AC004	ATCGGGATCCTCAGTCTAAGTCTAATCCGTTCGAA	BamHI
celC (Fwd)	AC005	GGGAATTCCATATGAGTGAGGGAATTCAAATGCCCAAAGGT	NdeI
celC (Rev)	AC006	CCGCTCGAGCTATATGTCTAGATCTTTACCATTTGATTC	XhoI
celD (Fwd)	ORF2fwdse	GGTGATGAAACGGAACC	na[a]
celD (Rev)	ORF2bwds	CCATGGAGTGTAACCCA	na[a]
celC (Fwd)	ORF3fwdse	CAATGGTGATGAAACGG	na[a]
celC (Rev)	ORF3bwds	ACCCCATGGAGTGTAACC	na[a]

[a] Used for sequencing. No restriction site required.

ble for any whole-cell β-glucosidase activity. The PEP-PTS allows bacterial cells to grow on various carbon sources (including β-glucosides) by the simultaneous uptake and phosphorylation of the substrate. The PEP-PTS has two main cytoplasmic components, HPr and EI, which are utilized by all systems, and an EII complex specific to the substrate and normally localized in the membrane [15,16].

The *bgl* operon has already been identified in *O. oeni* by this laboratory [17] by sequence analysis and cloning and characterization of the phospho-β-glucosidase BglD. Here we report on a second β-glucoside operon to be identified in *O. oeni* by sequence analysis [13,18] from the published genome of PSU-1 (CP000411) [13]. According to comparative sequence analyses of lactic acid bacteria [18] six open reading frames (ORF) between 329032 and 333480 (PSU-1 numbering) were putatively designated as encoding components of the PEP-PTS, IIB, IIA and IIC and two (phospho-)β-glycosidases. Components IIA–IIC regulate the uptake and phosphorylation of β-glycosides across the cytoplasmic membrane [19]. No function has been assigned to the sixth ORF in this operon, *celE*.

We propose that *O. oeni* are able to take up β-glucosides via a PEP-PTS involving phosphorylation and subsequent hydrolysis of the phosphorylated glucosides in the cytoplasm through the action of phospho-β-glucosidases. The objective of this study was to gain knowledge on β-glucoside metabolism in *O. oeni*. Accordingly the ORFs putatively encoding (phospho-)β-glycosidases, designated *celC* and *celD* were heterologously expressed in *E. coli* followed by purification and characterization. The genes *celC* and *celD* were sequenced from the wine strain Lalvin 4X (VL92) to identify any sequence changes from the published genome PSU-1. This report details findings for CelD relating to substrate specificity, pH and temperature optima, kinetic data and metal ion dependency. CelC was cloned and purified however it was not further characterized due to its low specific activity towards both phosphorylated and non-phosphorylated substrates. Only the cloning strategy and protein purification is therefore shown for CelC.

2. Materials and methods

2.1. Bacterial strains and growth

Previous work in this laboratory [10,11] demonstrated the presence of β-glucosidase activity in whole *O. oeni* cells. The strain with the highest activity, Lalvin 4X (VL92) was used for all subsequent DNA work. The strain was cultivated from a glycerol stock of the freeze dried commercial form in de Man, Rogosa and Sharpe broth (Amyl Media, Dandenong, VIC Australia) supplemented with 10% (v/v) filter sterilized preservative free apple juice and grown at 30 °C for approximately 4–5 days. The *E. coli* strain DH5α (Invitrogen Australia Pty Limited, Australia) [20] was used for cloning and sequencing (Sequencing by the Australian Genome Research Facility, Brisbane Node, QLD, Australia). The *E. coli* strain BL21 (DE3) pLysS (Novagen, USA) was used for protein expression. Both strains were maintained in 26% glycerol (v/v) at −80 °C and cultivated in Terrific Broth [21] at 37 °C overnight. When necessary the Terrific Broth was supplemented with 34 μg/mL chloramphenicol and/or 50 μg/mL of ampicillin sodium salt.

2.2. PCR amplification and cloning of celD and celC

celD, a 1458 bp fragment, was amplified by PCR using primers AC003 and AC004 (Table 1). The PCR fragment was cleaned with the Wizard® Plus PCR cleanup kit (Promega, Corporation, Sydney, Australia) digested with XhoI and BamHI (New England Biolabs, USA) in accordance with the recommended protocols and cloned into a previously digested pET 14.b plasmid (Novagen) to yield plasmid pET 14.b-*celD*. Pfu polymerase (Integrated Sciences, NSW, Australia) was used according to the manufacturer's instructions to amplify the ORF. *celC* was initially amplified by PCR with primers AC005 and AC004 (Table 1) to obtain a 2919 bp fragment and then cleaned using DNA PCR wizard clean up kit (Promega) according the manufacturer's instructions. This PCR fragment was used as template DNA and re-amplified by PCR using primers AC005 and AC006 to obtain a 1446 bp fragment, which encodes the *celC* gene. This fragment was digested with NdeI and XhoI and cloned into similarly digested plasmid pET 14.b. Plasmid DNA was extracted using the Wizard® Plus SV Minipreps DNA Purification System (Promega). Plasmid DNA was sequenced directly using primers listed in Table 1 three times for each gene to ensure PCR fidelity.

2.3. Expression of celD and celC in E. coli

Overnight cultures of pET 14.b-*celD* and pET 14.b-*celC* in BL21 (DE3) pLysS were inoculated (1/100 dilution) into Terrific Broth and grown at 37 °C until reaching an optical density of 0.6 at 600 nm. The temperature was then lowered to 15 °C for approximately 15 min and subsequently isopropyl-D-thiogalactopyranoside (IPTG) (Sigma–Aldrich, Australia) was added to a final concentration of 1 mM. Flasks were left on a shaker at 160 r.p.m. for 48 h at 10 °C. The cells were harvested by centrifugation (13,000 × *g* for 10 min at 5 °C) and lysed with amine free Bugbuster Mastermix (Novagen) according to the manufacturer's instructions.

2.4. Purification of CelD and CelC

The pET 14.b-*celD* and pET 14.b-*celC* constructs both incorporated a polyhistidine-tag at the N terminus. The resulting fusion proteins were purified using immobilized metal-affinity chromatography with Talon® cobalt resin (Clontech, USA) according to the manufacturer's instructions. Protein fractions from the purifications were eluted with McIlvaine buffer [22] at pH 4.5 and analyzed by SDS-PAGE [23] and quantified by the method of Bradford [24]. Long term storage was achieved at −80 °C, and short term (2 months) at 4 °C in 10% (v/v) glycerol.

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Table 2
Gene arrangement of the *cel* operon of *O. oeni*, derived from the published genome sequence PSU-1 [13] with proposed gene names.

ORF	Gene name	Function
OEOE 0338	*celA*	PTS system IIB component
OEOE 0339	*celB*	PTS system IIA component
OEOE 0340	*celC*	Putative phospho-β-glycosidase
OEOE 0341	*celD*	6-Phospho-β-glucosidase
OEOE 0342	*celE*	Putative transcriptional regulator
OEOE 0343	*celF*	PTS system IIC component

2.5. Assay of β-glucosidase and phospho-β-glucosidase activity

β-Glucosidase enzyme activity was quantitatively determined by measuring para-nitrophenol (*p*NP) released from *p*NP-β-D-glucopyranoside, *p*NP-α-D-glucopyranoside, ortho-nitrophenol-β-D-glucopyranoside, *p*NP-β-D-galactopyranoside, *p*NP-α-D-galactopyranoside, *p*NP-α-xylopyranoside, *p*NP-α-L-rhamnopyranoside and *p*NP-α-L-arabinofuranoside. Unless otherwise stated the standard assay consisted of incubation of the purified histidine tagged enzyme with a final substrate concentration of 2 mM in McIlvaine buffer at pH 5.5 for 30 min in a total volume of 50 μL at 37 °C. Phospho-β-glucosidase activity was measured in assay conditions identical to those described above, using the phosphorylated substrate *p*NP-β-D-glucopyranoside-6-phosphate (*p*NPβG6P). This substrate was prepared from the non-phosphorylated glycoside by the method of Wilson and Fox [25]. The amount of *p*NP released was determined by measuring the absorbance of the reaction mixture at 400 nm [26]. All assays were blanked against ultra pure water (18.2 MΩ cm) and had the control (substrate minus enzyme) taken away from the absorbances read at 400 nm. BglD, a phospho-β-glucosidase reported previously [17] and a β-glucosidase from *Aspergillus niger* (Sigma–Aldrich, Australia) were used as positive controls under standard assay conditions. Specific activity was expressed in μmole of *p*NP liberated per minute per mg of enzyme under the standard assay conditions.

The pH and temperature optima of the purified CelD were determined. Accordingly, pH values ranging from pH 3.0 to 7.5 were used in the standard assay, whilst the effect of temperature was determined by pre-incubating buffer and substrate for 10 min in a PCR machine in 0.2 mL PCR tubes at the chosen assay temperature prior to the addition of enzyme. Assays were incubated for 30 min at temperatures ranging from 4 °C to 70 °C, transferred to a 96-well microtitre plate and absorbance at 400 nm recorded. The kinetic constants, K' and V_{max} correspond to the activity determined at 25 °C. The effect of the metal ions Mn^{2+}, Mg^{2+}, Cu^{2+}, Co^{2+} and Ca^{2+} at a concentration of 10 mM was also investigated. All assays were performed in triplicate and were repeated at least three times.

3. Results and discussion

3.1. Sequence analysis of the cel operon

From the published *O. oeni* PSU-1 genome sequence (CP000411) [13] and comparative studies on genomes of lactic acid bacteria, six genes were identified that are transcribed in the same direction [18]. Sequence homology of the translated DNA in conserved regions to characterized bacterial genes indicates that they form components of a PEP-PTS operon in this organism (Table 2). This 6043 bp region we have designated *cel*, based on sequence similarity to *cel* genes from various organisms (Table 3) and to distinguish it from the *bgl* operon in *O. oeni* previously characterized in this laboratory [17]. The *cel* operon is speculated to function by phosphorylation and uptake of β-glycosides for use as alternative carbon sources.

celC and *celD* were both sequenced, demonstrating two amino acid changes in *celD* and one in *celC* (underlined in Fig. 1) from the published PSU-1 sequence, all of which are found in non-conserved and non-catalytic regions. *celD*, a putative (phospho-)β-glycosidase, is assigned to the glycosyl hydrolase family (GHF) 1 [27–29] based on sequence homology and highly conserved signature sequences (Fig. 1). *celD* is found on the *O. oeni* genome 17 bp from a putative (phospho-)β-glycosidase *celC*. There are few differences between GHF 1 phospho-β-glucosidases and GHF 1 β-glucosidases, which can cause confusion when attempting to infer enzyme function from the sequence alone. One principal difference appears to be the serine located in position 453 (numbers according to Fig. 1), which has been shown to be at least partially responsible for the affinity of GHF 1 phospho-β-glucosidases for phosphorylated substrates [30]. In characterized GHF 1 β-glucosidases the serine in position 453 is typically replaced by a glutamic acid residue. The one known exception being from *Pectobacterium carotovorum*, in which the characterized GHF 1 β-glucosidase/phospho-β-glucosidase AscB has an arginine in position 453 and demonstrates an affinity for both phosphorylated and non-phosphorylated substrates.

Both the putative (phospho-)β-glycosidase CelC and the (phospho-)β-glycosidase CelD characterized in this study show high homology to the characterized proteins: BglB in *E. coli*, BglA and BglH *in Bacillus subtillis*, ArbB in *Erwinia chrysanthemi*, AbgA in *Clostridium longisporum*, CelA in *Streptococcus mutans* and AscB, BglA, BglB in *P. carotovorum* (Table 3). The hydrolysis of a glucosidic bond involves two catalytic residues: a proton donor (acid) and a nucleophile (base) [31]. Based on sequence comparison with characterized phospho-β-glucosidases the active site for CelD is located in position 390–399 and comprises the residues FIVENGLGA with the glutamic acid (position 392) residue acting as the catalytic nucleophile [30,32]. CelD also shows a highly conserved region at position 183–188 containing TFNEIN whereby a second glutamic acid (position 186) has been demonstrated in other species [30] to be the active proton donor residue (Fig. 1).

BLAST analysis demonstrates that CelE has homology to the DNA binding HTH region of *Bacillus subtilis* GlvR and YebF from *Lactococcus lactis* regulatory gene. GlvR was found to be fundamental for the transcription of the *glv* operon [33], and the interruption of YebF demonstrated complete lack or severe inhibition of growth in cellobiose- and/or lactose-containing media, suggesting that YebF is involved in the metabolism of cellobiose and lactose through the PEP-PTS system [34]. To gain some insight into the biological function of CelC and CelD, the products of these gene products were expressed and characterized in *E. coli*.

3.2. Expression, purification and characterization

CelC showed no significant activity towards either phosphorylated or non-phosphorylated β-glycoside substrates (data not shown), and thus only data relating to the cloning and characterization of CelD are presented. No activity has since been defined (<0.27 μmole *p*NP/min/mg), referring to the greatest specific activity found for CelC (data not shown) whereby mg rather than pg of enzyme were used, thus lowering the specific activity to a point which was deemed not active. Only the cloning strategy and protein purification has been shown for CelC. pET 14.b-*celD* and pET 14.b-*celC* in BL21 (DE3) pLysS produced large amounts of soluble IPTG-inducible protein whose estimated molecular weight, 55.8 and 55.7 kDa, respectively, corresponded to that expected for the full length polypeptides encoded by CelD and CelC. Purification was achieved as described in Section 2. Protein fractions analyzed by SDS-PAGE revealed the presence of a single protein band for both CelD and CelC (~55 kDa, Fig. 2). Fractions that contain these proteins were pooled, quantified, aliquoted and stored at 4 °C and

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Table 3
Degree of identity to known sequences of the genes in the cel operon.

Protein	Putative homologues		Percentage (%)		Accession number	Reference
			Positives	Identity		
CelA	*Pectobacterium carotovorum*	BglE	66	47	AY769096	[38]
		CelE	69	50	DQ987482	[39]
	O.oeni	BglC	84	73		[17]
CelB	*Streptococcus mutans*	CelD	72	43		[40,41]
	Pectobacterium carotovorum	BglI	62	36	AY769096	[38]
	O.oeni	BglB	82	64		[17]
CelC	*O.oeni*	BglD	77	63		[17]
	E.coli	BglA	72	58	Q46829	[42]
		AscB	66	47	M73326	[43]
		BglB	65	51	M16487	[42,44]
	Bacillus subtillis	BglA	73	58	P42973	[45]
		BglH	68	54	Z34526	[46]
	Erwinia chrysanthemi	ArbB	65	50	M81772	[47]
	Clostridium longisporum	AbgA	66	49	L49336	[48]
	Pectobacterium carotovorum	AscB	63	45	AY622309	[26]
		BglA	73	57	AY769096	[38]
		BglB	67	50	AY542524	[49]
		CelG	47	30	DQ987482	[39]
	Streptococcus mutans	CelA	81	66	NC_004350	[40,41]
	Lactobacillus plantarum	BglH	64	50	AJ250202	[50]
CelD	*O.oeni*	BglD	85	73		[17]
	E.coli	BglA	73	58	Q46829	[42]
		AscB	66	47	M73326	[43]
		BglB	64	50	M16487	[42,44]
	Bacillus subtillis	BglA	73	58	P42973	[45]
		BglH	67	53	Z34526	[46]
	Erwinia chrysanthemi	ArbB	65	50	M81772	[47]
	Clostridium longisporum	AbgA	66	49	L49336	[48]
	Pectobacterium carotovorum	AscB	65	48	AY622309	[26]
		BglA	73	59	AY769096	[38]
		CelG	43	29	DQ987482	[39]
		BglB	68	52	AY542524	[49]
	Streptococcus mutans	CelA	81	66	NC_004350	[40,41]
	Lactobacillus plantarum	BglH	65	51	AJ250202	[50]
CelE	*Bacillus subtilise*	GlvA[a]	54	34	P54717	[33]
	Lactococcus lactis	YebF[a]	69	44	NP_266568	[51]
CelF	*Streptococcus mutans*	CelE	53	33		[40,41]
	Pectobacterium carotovorum	CelF	55	35	DQ987482	[39]
	Pectobacterium carotovorum	BglF	56	33	AY769096	[38]
	O.oeni	BglA	52	33		[17]

[a] Homologous to the HTH domain, rpiR family; pfam01418.

−80 °C. CelD maintained 100% activity when stored at 4 °C for up to two months or at −80 °C in 10% (v/v) glycerol for up to 2 years (data not shown).

No activity was detected when assaying CelD against non-phosphorylated β-glucosides (data not shown), where the enzyme, buffer and substrate were added did not show any variation from the control (substrate plus buffer minus enzyme). The effect of pH on the activity of CelD against *p*NPβG6P was determined at 37 °C in buffers ranging in pH from 3.0 to 7.5 (Fig. 3A).

CelD had an optimum pH of 4.0–5.0, and retained high levels of activity (above 78%) at pH values of up to 6.0. Typically wines range from pH 2.9 to 3.4 for whites and pH 3.4 to 3.6 for reds [35]. The application of CelD in the modification of wine aroma is not necessarily precluded by such pH values as the enzyme requirement for phosphorylated substrates dictates that it be applied in whole cell form where higher pH values are likely.

The temperature dependence of CelD was determined using *p*NPβG6P at an assay pH of 5.5. This pH was selected because CelD demonstrated high activity at this value (above 80%) and absorbance readings could be taken without prior dilution or pH adjustment. CelD exhibited a temperature optimum of 40 °C, below which activity was largely retained such that, as much as 74% was seen at 4 °C (Fig. 3B). By comparison, at values above 40 °C, activity declined rapidly so that only 2% was retained at 60 °C and none at 70 °C. Winemaking conditions classically vary from approximately 10 °C for pre-fermentation cold maceration and up to maxima around 33 °C in red wine fermentations in the middle of the cap [36], a temperature range across which CelD has high activity.

The activity of CelD was measured in the presence of various divalent metal ions (Fig. 3C). In a winemaking context, calcium and magnesium cations are present in the greatest amounts (up to 150 mg/L; thus 3.75 mM Ca^{2+} and 6.25 mM Mg^{2+}) whilst cobalt, manganese and copper are found in trace amounts [35]. Typical concentrations of copper in wine are 0.3–0.4 mg/L [35] which equates to approximately 6 nM. Calcium is derived from vineyard and deacidification treatments, but forms insoluble salts which tend to precipitate out of the wine. Magnesium is present in the grape and remains in wine in the form of soluble salts, whilst copper can originate from copper fining treatments. In the absence of enzyme, no chemical hydrolysis of pNP was evident when incubated in the presence of 10 mM metal ions for 1 hour at 37 °C. The phospho-β-glucosidase activity seen in the presence of enzyme was significantly increased by the addition of 10 mM Ca^{2+} or Mg^{2+}. By comparison 10 mM Mn^{2+} or Co^{2+} resulted in activities 20% lower than seen in the absence of these ions, both of which were not statistically significant, ($p > 0.05$) to the control. Near complete inhibition was seen with 10 mM Cu^{2+}. Whilst the latter three metal ions were inhibitory in our assay this might not necessarily be the case at the lower concentrations typically found in wine. Some support for this possibility comes from the fact that the highly structurally

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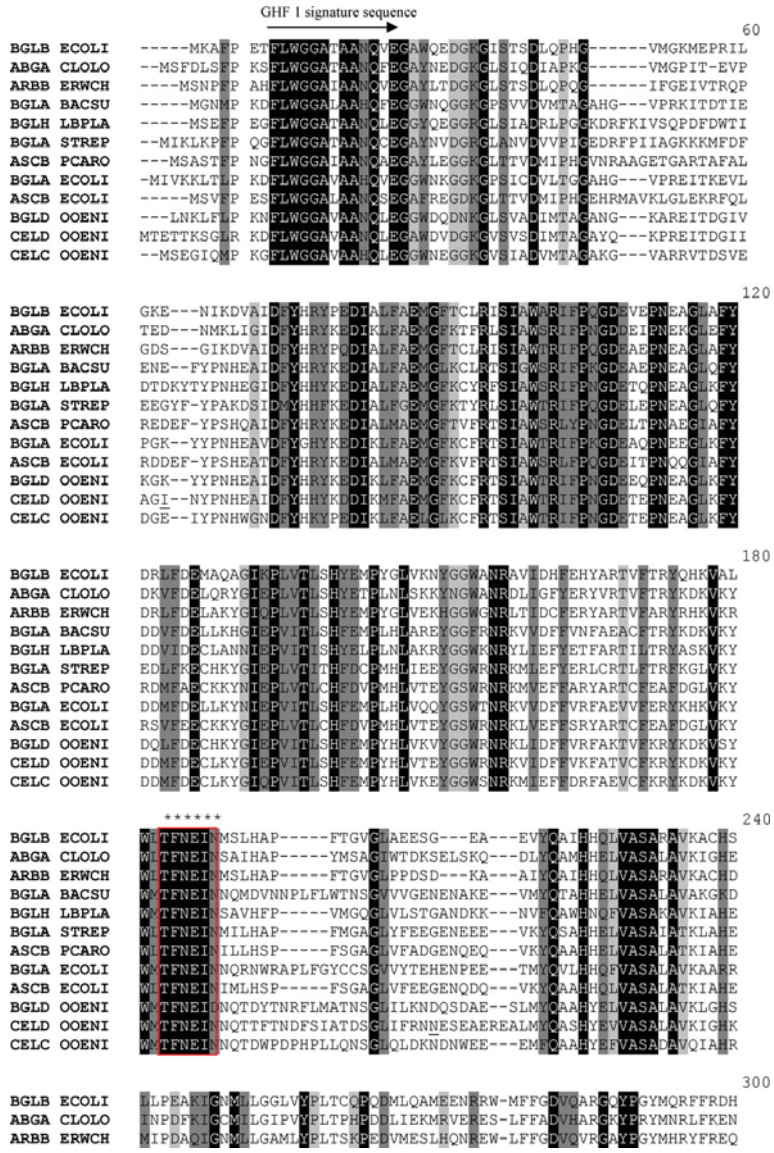


Fig. 1. Alignment of the amino acid sequences of phospho-β-glucosidases from characterized GHF 1: BGLB ECOLI, *E. coli* BglB. ABGA CLOLO, *Clostridium longisporum* AbgA. ARBB ERWCH, *Erwinia chrisanthemi* ArbB. BGLA BACSU, *Bacillus subtilis* BglA. BGLH LBPLA, *Lactobacillus plantarum* BglH. BGLA STREP, *Streptococcus mutans* BglA. BGLH BACSU, *Bacillus subtilis* BglH. ASCB PCARO, *Pectobacterium carovatorum* AscB. BGLA ECOLI, *E. coli* BglA. ASCB ECOLI, *E. coli* AscB. CELD OOENI, *O. oeni* CelD. CELC OOENI, *O. oeni* celC. BGLD OOENI, *O. oeni* bglD. CELA STREP, *Streptococcus mutans* celA. Identical residues are highlighted white lettering on a black background, highly conserved residues are highlighted with black lettering on dark grey and conserved residues are represented by black lettering on light grey. The numbers to the right denote residue positions, asterisks denote the conserved active sites and the numbers above the residues refer to alignment positions. Amino acid changes in celC and celD from the sequenced genomic strain PSU-1 are underlined.

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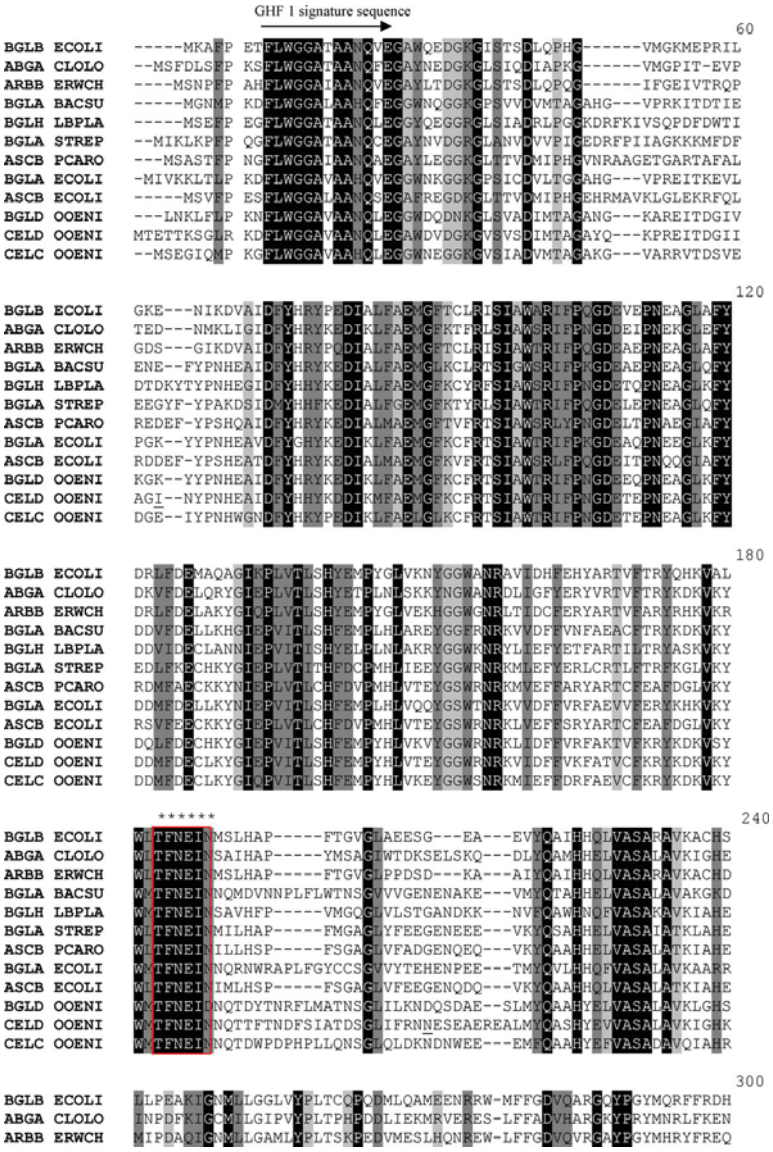


Fig. 1. (*Continued*).

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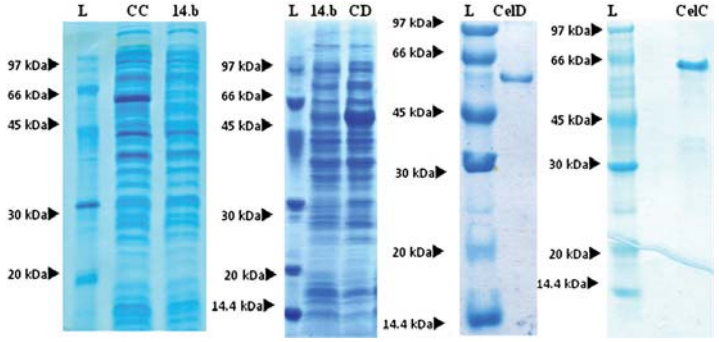


Fig. 2. Electrophoretic analysis of the purified phospho-β-glucosidase CelD and CelC. **L**, Low molecular weight marker. **14.b**, Crude extract of vector pET 14.b without CelD or CelC inserted. **CD**; Crude extract of vector pET 14.b with the *celD* gene inserted. **CC**; Crude extract of vector pET 14.b with the *celC* gene inserted. **celD**; purified CelD. **celC**; purified CelC. Separation was performed on a 12% (w/v) SDS-polyacrylamide gel. The gel was stained with 0.025% (w/v) Coomassie blue.

similar phospho-β-glucosidase isolated from *P. carovotorum*, *ascB*, (Table 3) exhibits losses of only about 10% when assayed with 5 mM Cu^{2+}, Co^{2+} and Mn^{2+}, as well as comparable increases in the presence of Mg^{2+} and Ca^{2+} [26]. Of course, further experimental work under wine conditions at appropriate cation conditions is required to confirm this.

The kinetic data from CelD produced a sigmoidal curve, with a V_{max} of 176.7 μmole pNP liberated/min/mg protein, K' of 1211 μmole pNPβG6P, a Hill slope of 2.831 and an R^2 coefficient of 0.987 (Fig. 3D), indicating positive cooperativity [37]. This suggests a potential regulatory role for CelD, in which its activity is modulated by interaction with an allosteric regulator namely the

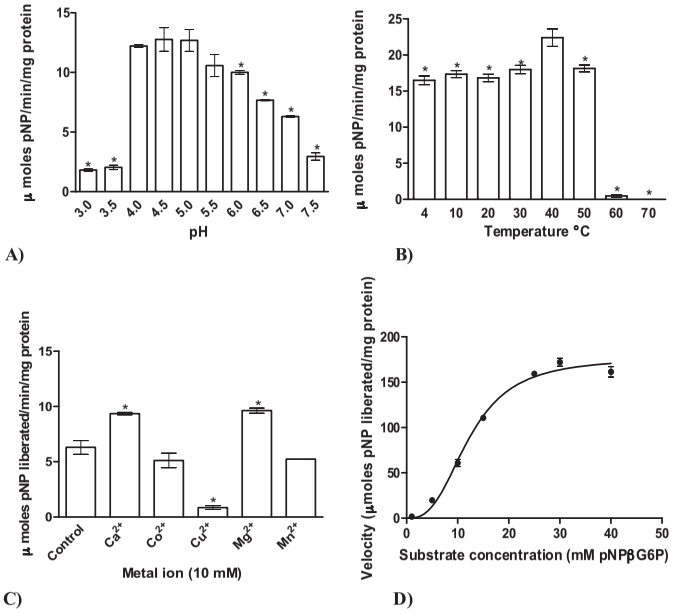


Fig. 3. Effect of pH, temperature and metal ion on the activity of CelD. (A) Effect of pH on the specific activity of CelD. Enzyme activity was assayed at 37 °C for 30 min. in McIlvaine buffers of indicated pH. (B) Effect of temperature on the specific activity of CelD. Enzyme activity was assayed at pH 5.5 for 30 min at the indicated temperature. The buffer was brought to temperature 10 min prior to commencement of the assay in a PCR machine. (C) Effect of 10 mM addition of Ca^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+} on the specific activity of CelD. Enzyme activity was assayed at pH 5.5 for 30 min. (D) Effect of substrate concentration on enzyme kinetics (CelD), analyzed with allosteric sigmoidal non linear regression. *Significant differences compared to control on each panel (one-way ANOVA with Tukey's multiple comparison post test, $p < 0.05$).

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substrate. It is of interest to note however, that the product of CelD, glucose-6-phosphate, is a direct product of the first enzyme of glycolysis and therefore it may be that factors regulating glycolysis are active also in the regulation of CelD activity [15].

We have characterized the phospho-β-glucosidase CelD in a putative PEP-PTS operon from a wine strain of *O. oeni*. The enzyme has a molecular weight of 55.8 kDa, exhibits positive cooperativity, pH and temperature optima of 4.0–5.0 and 40 °C respectively. Calcium and magnesium ions increased activity. Based on the characterization of other PEP-PTSs and their components in other bacteria, CelD is proposed to be an intracellular enzyme that acts on phosphorylated β-glucosides. Further experimental work needs to be undertaken in order to understand the specific role of CelD, a second putative phospho-β-glycosidase CelC and the other components of the *cel* operon involved in β-glucoside metabolism in *O. oeni*. Given the potential impact of β-glycosidases on the sensory profile of wine, it is hoped that the characterization of β-glycosidase systems from LAB, will provide information to aid winemakers in tailoring wine aroma, colour and overall complexity.

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Appendix 2: Intellectual Property

Key, valuable IP centres on the identification of yeast mutants which have a marked influence on fermentation including traits such as efficient nitrogen uptake and utilisation, tolerance to fermentation stresses e.g. tolerance to ethanol and high osmolarity (sugar). Further work is however required to more fully characterise these mutants/genes to the level where IP protection might be sought. The work being undertaken around the protection of the evolved wine yeasts through PBR has been detailed above.

Lactic acid bacteria exhibiting high esterase activity under wine-like conditions (low pH and high ethanol) are valuable intellectual property. Such strains have 'industry ready' application and do not require approval from Food Standards Australia New Zealand (http://www.foodstandards.gov.au/). Further IP potential exists in the method of application of these food-safe bacteria (either in free or immobilised form).

In this project, LAB-derived esterases have been expressed and purified from *Escherichia coli* as recombinant proteins (poly-histidine tagged chimeric proteins). Being recombinant, these enzymes do not have approval for use in the food and beverage industry. However, they have been shown to act upon a broad spectrum of fatty acyl esters (C2-C8) and exhibit high activity in the synthesis and hydrolysis of esters under wine making conditions (low pH and high ethanol). IP potential resides firstly, in the purification of such enzymes in the native form from LAB, and secondly, their potential impact on the wine sensory profile during vinification.

Opportunities around these outcomes are to be explored through UA 11/01.

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The following staff contributed to this project:

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Appendix 5: Supplementary Data

Supplementary Table 1: 2% Lactate media

Component	Per Litre
Yeast extract	1 g
Glucose	3 g
KH_2PO_4	1 g
NH_4Cl	1g
$CaCl_2.2H_20$	0.5 g
NaCl	0.5 g
$MgSO_4.7H\ H_20$	0.73 g
88 % DL lactic acid	23 mL
pH to 6 with NaOH	volume to 1L

Autoclave media and store at ambient temperature.

Supplementary Table 2. Microsatellite analysis of wine yeast L2056 and haploid derivatives

Analysis 1	AT2-fam	AT4-fam	C3-fam	C4-hex	C5-hex	C8-ned	ORF2-hex	ORF3-fam	SCAAT1-ned	SCAAT3-ned	a-hex	alpha-fam	comments
L2056	357	277, 292	120,123	250,265	121	146,150	395	393,452	275, 324	400	492	468	diploid wine yeast
L2056	357	276, 291	121,124	249,264	161	146,150	395	393,451	274, 324	400	492	468	diploid wine yeast
1B	357	277	120	265	161	149	395	393	324	400	492	nd	MATa, Δho::kanMX
1D	357	277	123	250	161	146	395	452	274	400	nd	468	MATα, Δho::kanMX
F3	357	277	123	265	161	146	395	393	275	400	492	nd	MAT a, Δho
C9	357	276	123	265	161	150	395	393	274	400	492	nd	MAT a, Δho
C91D	357	277, 292	123	250,265	161	146,150	395	393,452	274, 324	400	492	468	diploid cross C9/1D
C911a	357	277	123	250	161	146	395	452	274	400	nd	468	MATα, Δho, from tetrad 1 - sporulation of C9/1D
C911b	357	277	123	250	161	150	395	393	275	400	492	nd	MATa, Δho::kanMX, from tetrad 1 - sporulation of C9/1D
C911c	357	277	123	265	161	146	395	452	275	400	nd	468	MATα Δho::kanMX, from tetrad 1- sporulation of C9/1D
C911d	357	277	123	265	161	150	395	393	274	400	492	nd	MATa, Δho, from tetrad 1 - sporulation of C9/1D
iso C9a	357	276	124	264	161	150	395	393	274	400	492	nd	MAT a, Δho
iso C9b	357	276	124	264	161	150	395	393	274	400	nd	468	MATα, Δho
iso C9c	357	276	124	264	161	150	395	393	274	400	nd	468	MATα, Δho
iso C9d	357	276	124	264	161	150	395	393	274	400	492	nd	MATa, Δho
Analysis 2	**AT2-fam**	**AT4-fam**	**C3-fam**	**C4-hex**	**C5-hex**	**C8-ned**	**ORF2-hex**	**ORF3-fam**	**SCAAT1-ned**	**SCAAT3-ned**	**a-hex**	**alpha-fam**	**comments**
L2056	357	277, 292	120,123	250,265	121	146,150	395	393,452	275, 324	400	492	468	diploid wine yeast
DR4-1	357	277, 292	120, 123	249	121	146, 149	395	393, 452	275, 324	400	492	468	L2056 diploid
3-3a	355	277	123	250	121	146	395	452	275	400	492	nd	MATa, Δho, sporulation from diploid DR4-1
4-5a	357	292	123	249	121	146	395	393	275	400	nd	468	MATα, Δho::KanMX sporulation from diploid DR4-1
3-3a/4-5a	357	277, 292	123	249	121	146	395	393, 452	275	400	492	468	diploid cross 3-3a/4-5a
18a	357	277	123	249	121	146	395	452	275	400	492	nd	MATa, Δho, from tetrad 1 -sporulation of 3-3a/4-5a
18b	357	292	123	249	121	146	395	452	275	400	492	nd	MATa, Δho::KanMX, from tetrad 1 -sporulation of 3-3a/4-5a
18c	357	277	123	249	121	146	395	393	275	400	nd	468	MATα, Δho::KanMX, from tetrad 1 -sporulation of 3-3a/4-5a
18d	357	292	123	249	121	146	395	393	275	400	nd	468	MATα, Δho, from tetrad 1 -sporulation of 3-3a/4-5a

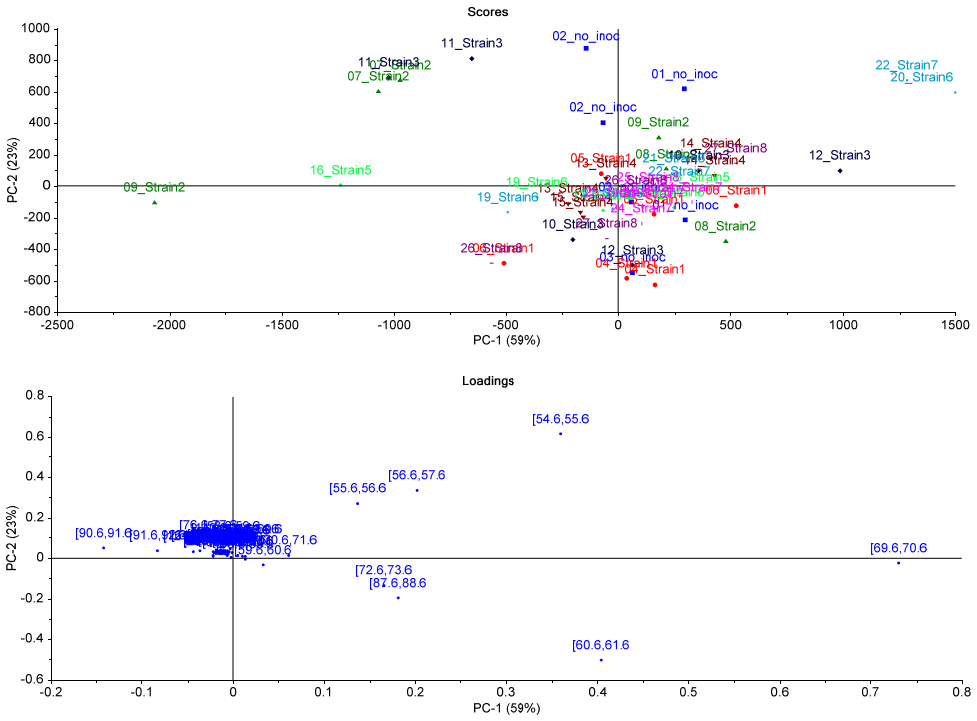
Microsatellite analysis of 10 loci and mating type loci MATa and MATα was performed in two separate analyses. Analysis 1 was of the strains reported in Walker *et al.*, (2003) whilst Analysis 2 was of the strains reported in Walker *et al.*, (2005). Single nucleotide differences noted are considered artefacts of peak size scoring and differences due to the capillary sequencers used. Analyses 1 and 2 are composites of three microsatellite experiments performed by Michelle Walker and Keith Richards (University of Auckland).

Supplementary Table 3: PCR reaction utilized to amplify cDNA

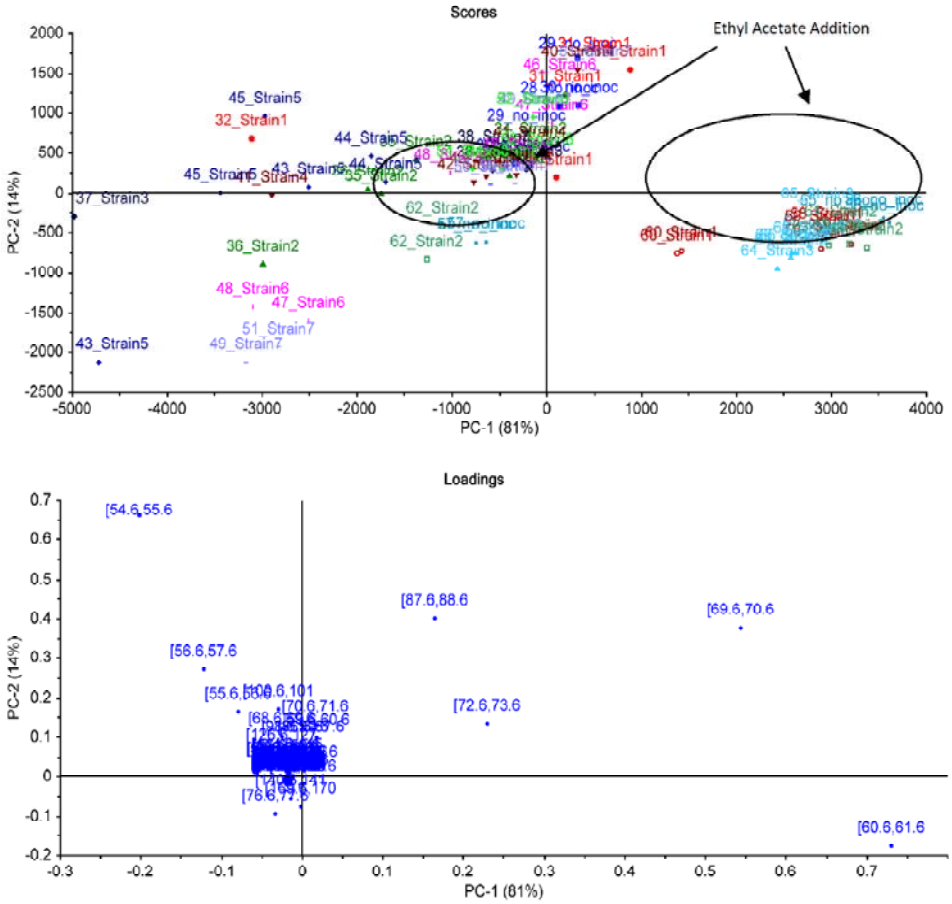
PCR components	Stock solution	Final concentration	Volume (µL)
Forward primer	10 µM	0.5 µM	2.5
Reverse primer	10 µM	0.5 µM	2.5
Template cDNA	~1 ng/µL	1 pg – 10 ng	0.5
Buffer with $MgCl_2$	10 X	1 X	5
dNTPs	10 mM	200 µM	1
MQ	-	-	38
DyNAzyme ext	1 unit/µl	1 unit	1
Total volume	**-**	**-**	**50 µL**

Supplementary Table 4: Conditions for PCR amplification of cDNA

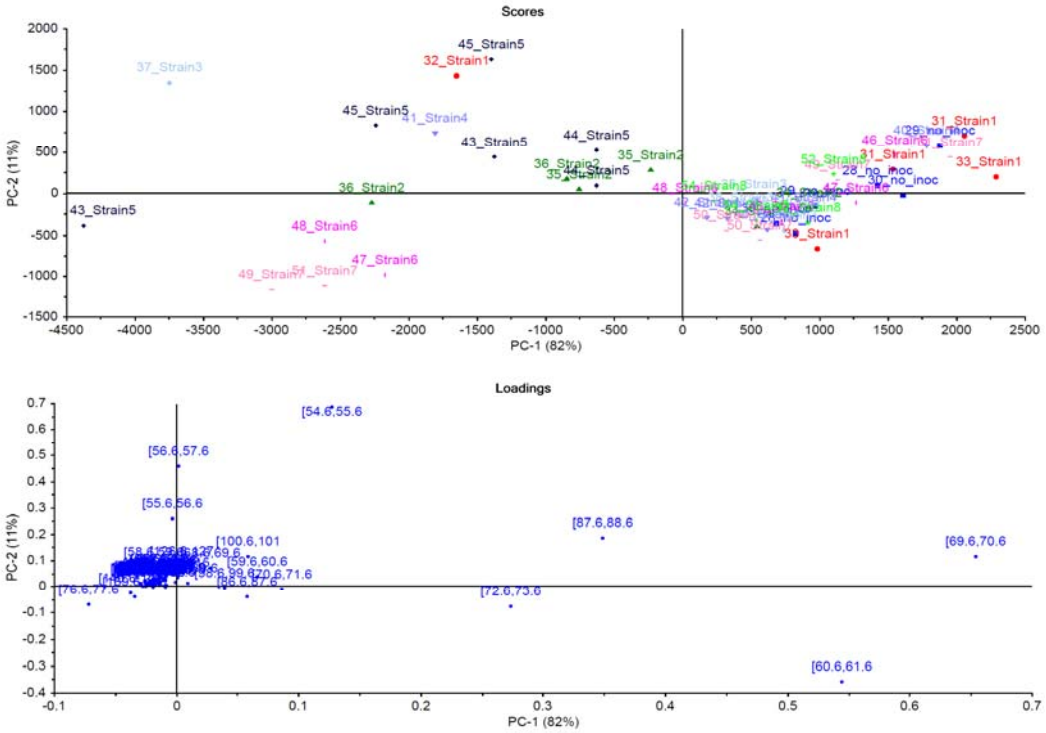
Number of cycles	Cycle step	Temperature (°C)	Duration
x 1	Initial denaturation	94	2 minutes
x 25	Denaturation	94	30 seconds
	Annealing	58	30 seconds
	Extension	72	40 seconds
x 1	Final extension	72	10 minutes
		4	Hold



Supplementary Figure 1: Scores and Loading Plot from the PCA of Cabernet Sauvignon samples analysed for impact of MLF on volatile profile, (PC1 and PC2).



Supplementary Figure 2: Scores and Loading Plot from the PCA of all Chardonnay samples (including those spiked with ethyl acetate) analysed for impact of MLF on volatile profile, (PC1 and PC2).



Supplementary Figure 3: Scores and Loading Plot from the PCA of Chardonnay samples analysed for impact of MLF on volatile profile, (PC1 and PC2).

```
   1 - ATGACCATGATTACGGATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCT - 60
   1 - M  T  M  I  T  D  S  L  A  V  V  L  Q  R  R  D  W  E  N  P   - 20

  61 - GGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGC - 120
  21 - G  V  T  Q  L  N  R  L  A  A  H  P  P  F  A  S  W  R  N  S   - 40

 121 - GAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGCGC - 180
  41 - E  E  A  R  T  D  R  P  S  Q  Q  L  R  S  L  N  G  E  W  R   - 60

 181 - TTTGCCTGGTTTCCGGCACCAGAAGCGGTGCCGGAAAGCTGGCTGGAGTGCGATCTTCCT - 240
  61 - F  A  W  F  P  A  P  E  A  V  P  E  S  W  L  E  C  D  L  P   - 80

 241 - GAGGCCGATACTGTCGTCGTCCCCTCAAACTGGCAGATGCACGGTTACGATGCGCCCATC - 300
  81 - E  A  D  T  V  V  V  P  S  N  W  Q  M  H  G  Y  D  A  P  I   - 100

 301 - TACACCAACGTAACCTATCCCATTACGGTCAATCCGCCGTTTGTTCCCACGGAGAATCCG - 360
 101 - Y  T  N  V  T  Y  P  I  T  V  N  P  P  F  V  P  T  E  N  P   - 120

 361 - ACGGGTTGTTACTCGCTCACATTTAATGTTGATGAAAGCTGGCTACAGGAAGGCCAGACG - 420
 121 - T  G  C  Y  S  L  T  F  N  V  D  E  S  W  L  Q  E  G  Q  T   - 140

 421 - CGAATTATTTTTGATGGCGTTAACTCGGCGTTTCATCTGTGGTGCAACGGGCGCTGGGTC - 480
 141 - R  I  I  F  D  G  V  N  S  A  F  H  L  W  C  N  G  R  W  V   - 160

 481 - GGTTACGGCCAGGACAGTCGTTTGCCGTCTGAATTTGACCTGAGCGCATTTTTACGCGCC - 540
 161 - G  Y  G  Q  D  S  R  L  P  S  E  F  D  L  S  A  F  L  R  A   - 180

 541 - GGAGAAAACCGCCTCGCGGTGATGGTGCTGCGTTGGAGTGACGGCAGTTATCTGGAAGAT - 600
 181 - G  E  N  R  L  A  V  M  V  L  R  W  S  D  G  S  Y  L  E  D   - 200

 601 - CAGGATATGTGGCGGATGAGCGGCATTTTCCGTGACGTCTCGTTGCTGCATAAACCGACT - 660
 201 - Q  D  M  W  R  M  S  G  I  F  R  D  V  S  L  L  H  K  P  T   - 220

 661 - ACACAAATCAGCGATTTCCATGTTGCCACTCGCTTTAATGATGATTTCAGCCGCGCTGTA - 720
 221 - T  Q  I  S  D  F  H  V  A  T  R  F  N  D  D  F  S  R  A  V   - 240

 721 - CTGGAGGCTGAAGTTCAGATGTGCGGCGAGTTGCGTGACTACCTACGGGTAACAGTTTCT - 780
 241 - L  E  A  E  V  Q  M  C  G  E  L  R  D  Y  L  R  V  T  V  S   - 260

 781 - TTATGGCAGGGTGAAACGCAGGTCGCCAGCGGCACCGCGCCTTTCGGCGGTGAAATTATC - 840
 261 - L  W  Q  G  E  T  Q  V  A  S  G  T  A  P  F  G  G  E  I  I   - 280

 841 - GATGAGCGTGGTGGTTATGCCGATCGCGTCACACTACGTCTGAACGTCGAAAACCCGAAA - 900
 281 - D  E  R  G  G  Y  A  D  R  V  T  L  R  L  N  V  E  N  P  K   - 300

 901 - CTGTGGAGCGCCGAAATCCCGAATCTCTATCGTGCGGTGGTTGAACTGCACACCGCCGAC - 960
 301 - L  W  S  A  E  I  P  N  L  Y  R  A  V  V  E  L  H  T  A  D   - 320

 961 - GGCACGCTGATTGAAGCAGAAGCCTGCGATGTCGGTTTCCGCGAGGTGCGGATTGAAAAT - 1020
 321 - G  T  L  I  E  A  E  A  C  D  V  G  F  R  E  V  R  I  E  N   - 340

1021 - GGTCTGCTGCTGCTGAACGGCAAGCCGTTGCTGATTCGAGGCGTTAACCGTCACGAGCAT - 1080
 341 - G  L  L  L  L  N  G  K  P  L  L  I  R  G  V  N  R  H  E  H   - 360

1081 - CATCCTCTGCATGGTCAGGTCATGGATGAGCAGACGATGGTGCAGGATATCCTGCTGATG - 1140
 361 - H  P  L  H  G  Q  V  M  D  E  Q  T  M  V  Q  D  I  L  L  M   - 380

1141 - AAGCAGAACAACTTTAACGCCGTGCGCTGTTCGCATTATCCGAACCATCCGCTGTGGTAC - 1200
 381 - K  Q  N  N  F  N  A  V  R  C  S  H  Y  P  N  H  P  L  W  Y   - 400

1201 - ACGCTGTGCGACCGCTACGGCCTGTATGTGGTGGATGAAGCCAATATTGAAACCCACGGC - 1260
 401 - T  L  C  D  R  Y  G  L  Y  V  V  D  E  A  N  I  E  T  H  G   - 420

1261 - ATGGTGCCAATGAATCGTCTGACCGATGATCCGCGCTGGCTACCGGCGATGAGCGAACGC - 1320
 421 - M  V  P  M  N  R  L  T  D  D  P  R  W  L  P  A  M  S  E  R   - 440

1321 - GTAACGCGAATGGTGCAGCGCGATCGTAATCACCCGAGTGTGATCATCTGGTCGCTGGGG - 1380
 441 - V  T  R  M  V  Q  R  D  R  N  H  P  S  V  I  I  W  S  L  G   - 460

1381 - AATGAATCAGGCCACGGCGCTAATCACGACGCGCTGTATCGCTGGATCAAATCTGTCGAT - 1440
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 461 - N  E  S  G  H  G  A  N  H  D  A  L  Y  R  W  I  K  S  V  D   - 480

1441 - CCTTCCCGCCCGGTGCAGTATGAAGGCGGCGGAGCCGACACCACGGCCACCGATATTATT - 1500
 481 - P  S  R  P  V  Q  Y  E  G  G  G  A  D  T  T  A  T  D  I  I   - 500

1501 - TGCCCGATGTACGCGCGCGTGGATGAAGACCAGCCCTTCCCGGCTGTGCCGAAATGGTCC - 1560
 501 - C  P  M  Y  A  R  V  D  E  D  Q  P  F  P  A  V  P  K  W  S   - 520

1561 - ATCAAAAAATGGCTTTCGCTACCTGGAGAGACGCGCCCGCTGATCCTTTGCGAATACGCC - 1620
 521 - I  K  K  W  L  S  L  P  G  E  T  R  P  L  I  L  C  E  Y  A   - 540

1621 - CACGCGATGGGTAACAGTCTTGGCGGTTTCGCTAAATACTGGCAGGCGTTTCGTCAGTAT - 1680
 541 - H  A  M  G  N  S  L  G  G  F  A  K  Y  W  Q  A  F  R  Q  Y   - 560

1681 - CCCCGTTTACAGGGCGGCTTCGTCTGGGACTGGGTGGATCAGTCGCTGATTAAATATGAT - 1740
 561 - P  R  L  Q  G  G  F  V  W  D  W  V  D  Q  S  L  I  K  Y  D   - 580

1741 - GAAAACGGCAACCCGTGGTCGGCTTACGGCGGTGATTTTGGCGATACGCCGAACGATCGC - 1800
 581 - E  N  G  N  P  W  S  A  Y  G  G  D  F  G  D  T  P  N  D  R   - 600

1801 - CAGTTCTGTATGAACGGTCTGGTCTTTGCCGACCGCACGCCGCATCCAGCGCTGACGGAA - 1860
 601 - Q  F  C  M  N  G  L  V  F  A  D  R  T  P  H  P  A  L  T  E   - 620

1861 - GCAAAACACCAGCAGCAGTTTTTCCAGTTCCGTTTATCCGGGCAAACCATCGAAGTGACC - 1920
 621 - A  K  H  Q  Q  Q  F  F  Q  F  R  L  S  G  Q  T  I  E  V  T   - 640

1921 - AGCGAATACCTGTTCCGTCATAGCGATAACGAGCTCCTGCACTGGATGGTGGCGCTGGAT - 1980
 641 - S  E  Y  L  F  R  H  S  D  N  E  L  L  H  W  M  V  A  L  D   - 660

1981 - GGTAAGCCGCTGGCAAGCGGTGAAGTGCCTCTGGATGTCGCTCCACAAGGTAAACAGTTG - 2040
 661 - G  K  P  L  A  S  G  E  V  P  L  D  V  A  P  Q  G  K  Q  L   - 680

2041 - ATTGAACTGCCTGAACTACCGCAGCCGGAGAGCGCCGGGCAACTCTGGCTCACAGTACGC - 2100
 681 - I  E  L  P  E  L  P  Q  P  E  S  A  G  Q  L  W  L  T  V  R   - 700

2101 - GTAGTGCAACCGAACGCGACCGCATGGTCAGAAGCCGGGCACATCAGCGCCTGGCAGCAG - 2160
 701 - V  V  Q  P  N  A  T  A  W  S  E  A  G  H  I  S  A  W  Q  Q   - 720

2161 - TGGCGTCTGGCGGAAAACCTCAGTGTGACGCTCCCCGCCGCGTCCCACGCCATCCCGCAT - 2220
 721 - W  R  L  A  E  N  L  S  V  T  L  P  A  A  S  H  A  I  P  H   - 740

2221 - CTGACCACCAGCGAAATGGATTTTTGCATCGAGCTGGGTAATAAGCGTTGGCAATTTAAC - 2280
 741 - L  T  T  S  E  M  D  F  C  I  E  L  G  N  K  R  W  Q  F  N   - 760

2281 - CGCCAGTCAGGCTTTCTTTCACAGATGTGGATTGGCGATAAAAAACAACTGCTGACGCCG - 2340
 761 - R  Q  S  G  F  L  S  Q  M  W  I  G  D  K  K  Q  L  L  T  P   - 780

2341 - CTGCGCGATCAGTTCACCCGTGCACCGCTGGATAACGACATTGGCGTAAGTGAAGCGACC - 2400
 781 - L  R  D  Q  F  T  R  A  P  L  D  N  D  I  G  V  S  E  A  T   - 800

2401 - CGCATTGACCCTAACGCCTGGGTCGAACGCTGGAAGGCGGCGGGCCATTACCAGGCCGAA - 2460
 801 - R  I  D  P  N  A  W  V  E  R  W  K  A  A  G  H  Y  Q  A  E   - 820

2461 - GCAGCGTTGTTGCAGTGCACGGCAGATACACTTGCTGATGCGGTGCTGATTACGACCGCT - 2520
 821 - A  A  L  L  Q  C  T  A  D  T  L  A  D  A  V  L  I  T  T  A   - 840

2521 - CACGCGTGGCAGCATCAGGGGAAAACCTTATTTATCAGCCGGAAAACCTACCGGATTGAT - 2580
 841 - H  A  W  Q  H  Q  G  K  T  L  F  I  S  R  K  T  Y  R  I  D   - 860

2581 - GGTAGTGGTCAAATGGCGATTACCGTTGATGTTGAAGTGGCGAGCGATACACCGCATCCG - 2640
 861 - G  S  G  Q  M  A  I  T  V  D  V  E  V  A  S  D  T  P  H  P   - 880

2641 - GCGCGGATTGGCCTGAACTGCCAGCTGGCGCAGGTAGCAGAGCGGGTAAACTGGCTCGGA - 2700
 881 - A  R  I  G  L  N  C  Q  L  A  Q  V  A  E  R  V  N  W  L  G   - 900

2701 - TTAGGGCCGCAAGAAAACTATCCCGACCGCCTTACTGCCGCCTGTTTTGACCGCTGGGAT - 2760
 901 - L  G  P  Q  E  N  Y  P  D  R  L  T  A  A  C  F  D  R  W  D   - 920

2761 - CTGCCATTGTCAGACATGTATACCCCGTACGTCTTCCCGAGCGAAAACGGTCTGCGCTGC - 2820
 921 - L  P  L  S  D  M  Y  T  P  Y  V  F  P  S  E  N  G  L  R  C   - 940
```

```
2821 - GGGACGCGCGAATTGAATTATGGCCCACACCAGTGGCGCGGCGACTTCCAGTTCAACATC - 2880
 941 - G  T  R  E  L  N  Y  G  P  H  Q  W  R  G  D  F  Q  F  N  I  - 960

2881 - AGCCGCTACAGTCAACAGCAACTGATGGAAACCAGCCATCGCCATCTGCTGCACGCGGAA - 2940
 961 - S  R  Y  S  Q  Q  Q  L  M  E  T  S  H  R  H  L  L  H  A  E  - 980

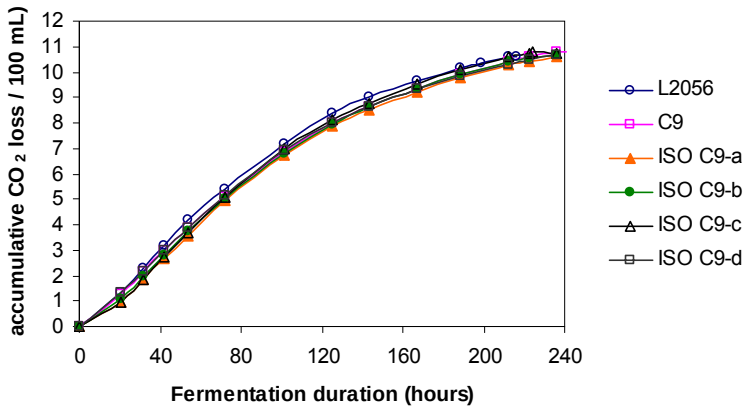
2941 - GAAGGCACATGGCTGAATATCGACGGTTTCCATATGGGGATTGGTGGCGACGACTCCTGG - 3000
 981 - E  G  T  W  L  N  I  D  G  F  H  M  G  I  G  G  D  D  S  W  - 1000

3001 - AGCCCGTCAGTATCGGCGGAATTCCAGCTGAGCGCCGGTCGCTACCATTACCAGTTGGTC - 3060
1001 - S  P  S  V  S  A  E  F  Q  L  S  A  G  R  Y  H  Y  Q  L  V  - 1020

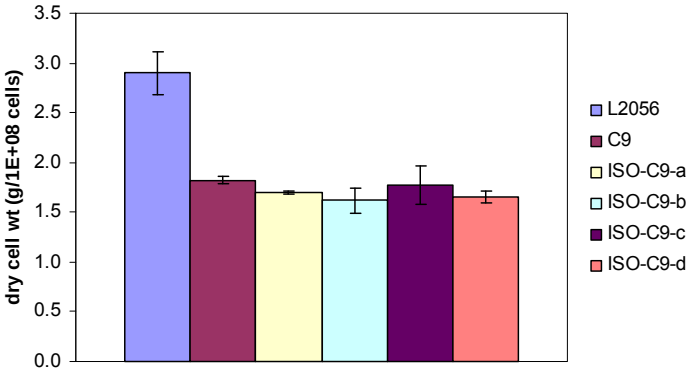
3061 - TGGTGTCAAAAATAATAATAA
1021 - W  C  Q  K  *  *  *
```

Supplementary Figure 4: β-galactosidase (*LACZ*) gene in plasmid pT161T

A.



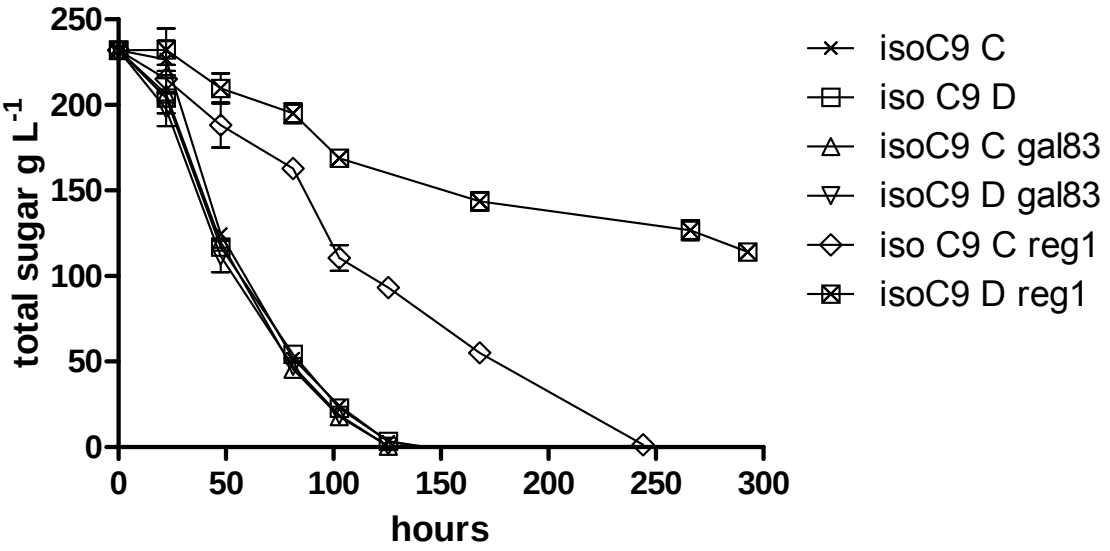
B.



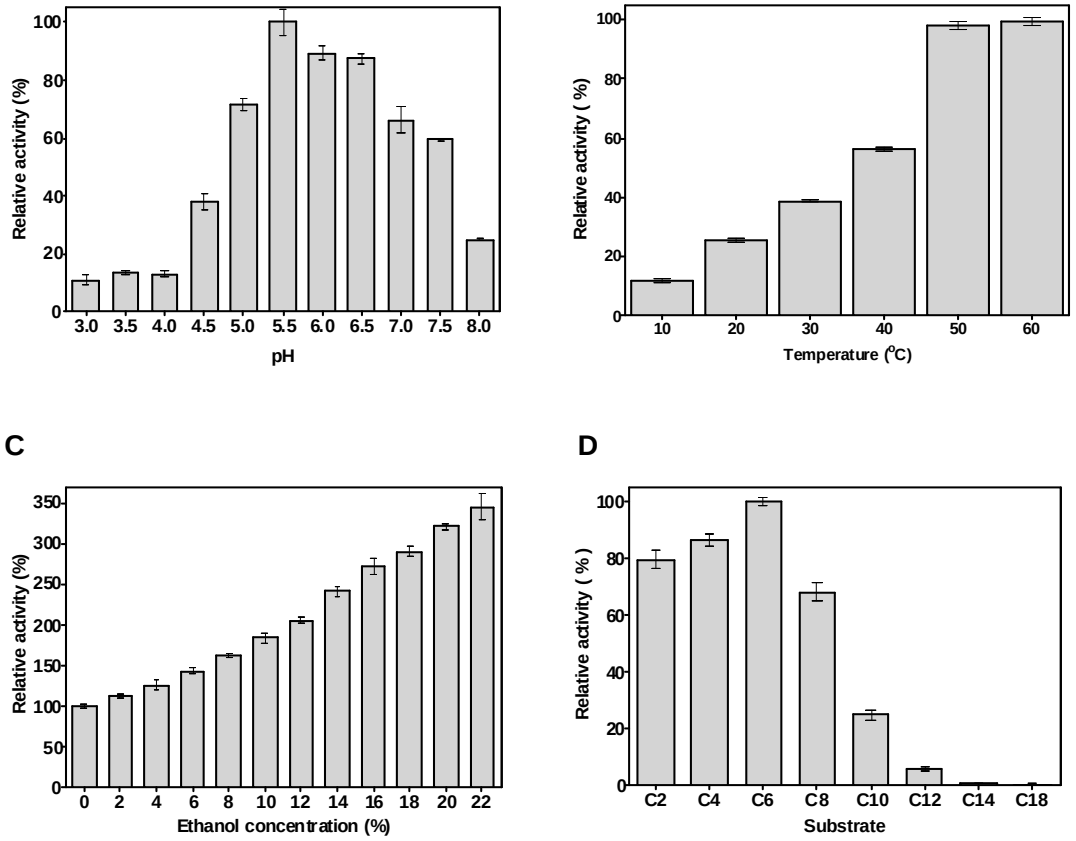
C.

Sample ID	Citric Acid	Tartaric Acid	Malic Acid	Succinic Acid	Lactic Acid	Acetic Acid	Glycerol	Acetald-ehyde	Ethanol
L2056	0.68±0.15	1.49±0.06	0.89±0.03	0.87±0.03	0.47±0.05	0.47±0.01	8.1±0.3	0.007±0.001	136±3
C9	0.74±0.08	1.14±0.03	0.92±0.02	0.52±0.07	0.52±0.07	0.54±0.02	7.4±0.3	0.007±0.001	132±8
ISO C9-a	0.76±0.07	1.14±0.05	0.92±0.02	0.52±0.01	0.52±0.01	0.56±0.09	7.3±0.6	0.006±0.002	132±6
ISO C9-b	0.79±0.05	1.11±0.05	0.92±0.00	0.92±0.04	0.50±0.04	0.50±0.05	7.5±0.6	0.019±0.001	137±1
ISO C9-c	0.78±0.05	1.16±0.05	0.91±0.02	0.94±0.04	0.52±0.01	0.57±0.10	7.0±0.2	0.006±0.000	132±5
ISO C9-d	0.85±0.03	1.16±0.04	0.92±0.00	0.94±0.01	0.54±0.01	0.58±0.01	8.3±0.2	0.005±0.001	137±2

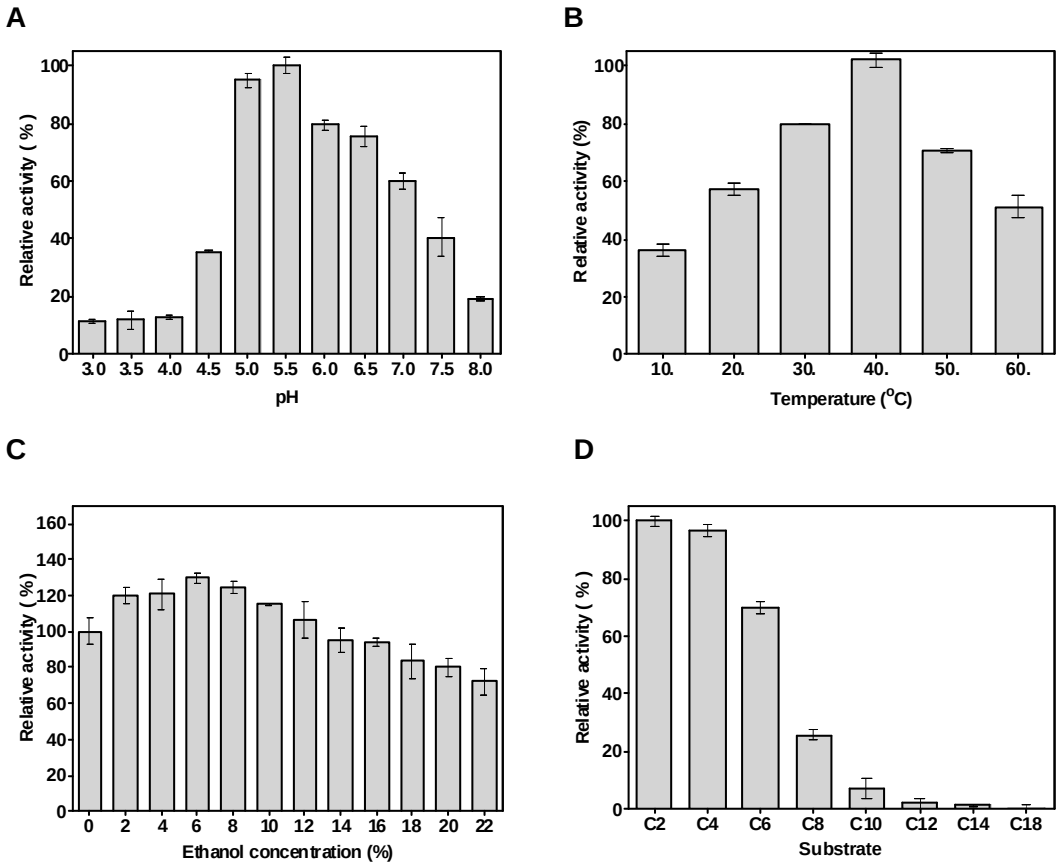
Supplementary Figure 5: Fermentation performance of wine yeast L2056 and haploid derivatives. Panel A: fermentation performance was evaluated in CDGJM (220 g L^{-1} sugar, 125 mg N L^{-1} as ammonium sulphate, 25°C). Fermentation was monitored by accumulative weight loss of fermentation flasks due to CO_2 evolution. Wine yeast L2056 and the haploid derivative C9 and four isogenic haploid derivatives isoC9a to isoC9d were cultured as described in the text. All values are the mean of three replicate fermentations per strain. 30°C. Panel B: Dry cell weight determined for duplicate 10 mL cultures per strain and expressed as grams per 1 x 10^8 cells. Values are the mean of 6 values per strain. Error bars representing the standard deviation (SD) are included. Panel C: Concentration (g L^{-1} ± SD) of organic acids, glycerol and ethanol were determined by HPLC analysis.



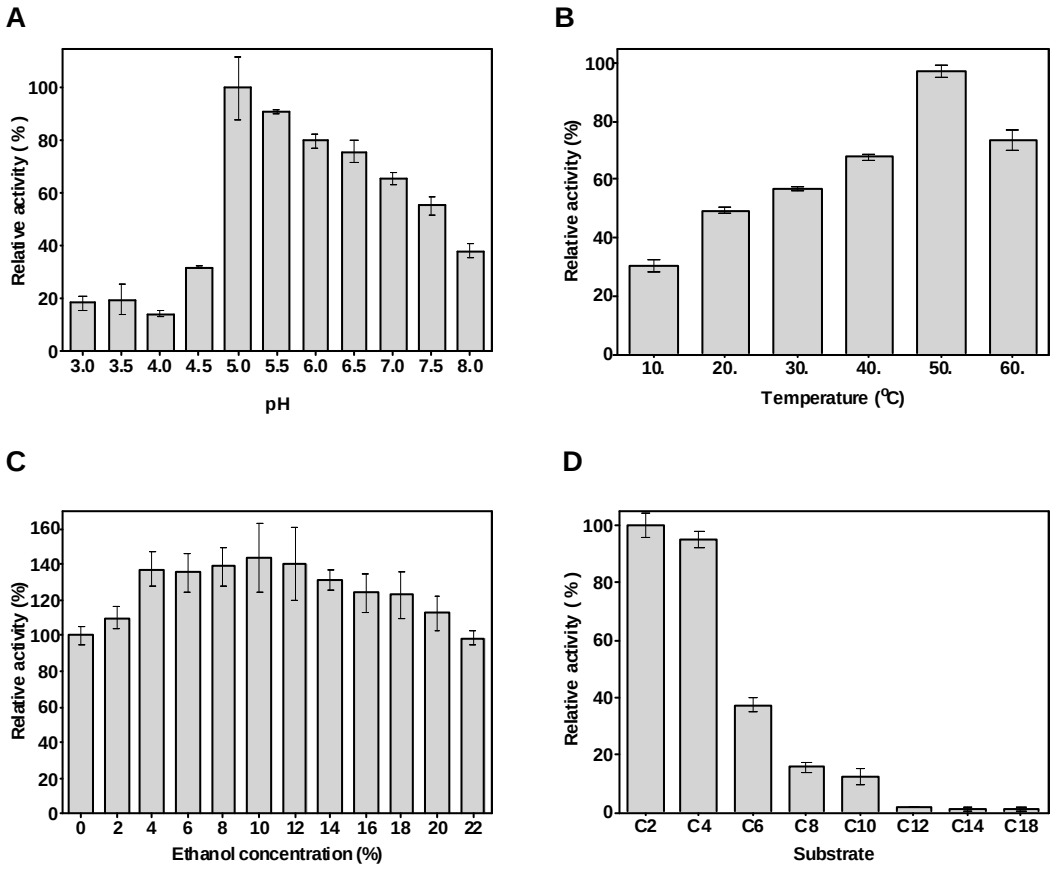
Supplementary Figure 6: Fermentation performance of parental strains isoC9c and isoC9d and their Δ*gal83* and Δ*reg1* deletants. Fermentation performance was evaluated in CDGJM (200 g L^{-1} sugar, 450 mg N L^{-1} FAN) in shake flasks at 30°C. Fermentation was monitored by refractive index (°Brix). Residual sugar was determined by enzymatic analysis of glucose and fructose. Over-estimation of fructose concentrations resulted in inaccurate measurement of total sugar for 0 to 50 h time points only. All values are the mean of three replicate fermentations per strain. Error bars representing the standard deviation (SD) are included.



Supplementary Figure 7: Characterization of EstA2. Values are the means of triplicates ± standard deviations. Values are shown relative to the highest observed activity, which was arbitrarily designated 100%, except for panel C, where values are shown relative to the activity observed in the absence of ethanol, which was arbitrarily designated 100%. (A) Influence of pH on esterase activity of EstA2. (B) Influence of temperature on esterase activity of EstA2. (C) Influence of ethanol on esterase activity of EstA2. (D) Substrate specificity of EstA2 (*p*NP-acetate C2, *p*NP-butanoate C4, *p*NP-hexanoate C6, *p*NP-octanoate C8, *p*NP-decanoate C10, *p*NP-dodecanoate C12, *p*NP-tetradecanoate C14, *p*NP-octadecanoate C18).



Supplementary Figure 8: Characterization of EstCOo8. Values are the means of triplicates ± standard deviations. Values are shown relative to the highest observed activity, which was arbitrarily designated 100%, except for panel C, where values are shown relative to the activity observed in the absence of ethanol, which was arbitrarily designated 100%. (A) Influence of pH on esterase activity of EstCOo8. (B) Influence of temperature on esterase activity of EstCOo8. (C) Influence of ethanol on esterase activity of EstCOo8. (D) Substrate specificity of EstCOo8 (*p*NP-acetate C2, *p*NP-butanoate C4, *p*NP-hexanoate C6, *p*NP-octanoate C8, *p*NP-decanoate C10, *p*NP-dodecanoate C12, *p*NP-tetradecanoate C14, *p*NP-octadecanoate C18).



Supplementary Figure 9: Characterization of EstC34. Values are the means of triplicates ± standard deviations. Values are shown relative to the highest observed activity, which was arbitrarily designated 100%, except for panel C, where values are shown relative to the activity observed in the absence of ethanol, which was arbitrarily designated 100%. (A) Influence of pH on esterase activity of EstC34. (B) Influence of temperature on esterase activity of EstC34. (C) Influence of ethanol on esterase activity of EstC34. (D) Substrate specificity of EstC34 (*p*NP-acetate C2, *p*NP-butanoate C4, *p*NP-hexanoate C6, *p*NP-octanoate C8, *p*NP-decanoate C10, *p*NP-dodecanoate C12, *p*NP-tetradecanoate C14, *p*NP-octadecanoate C18).