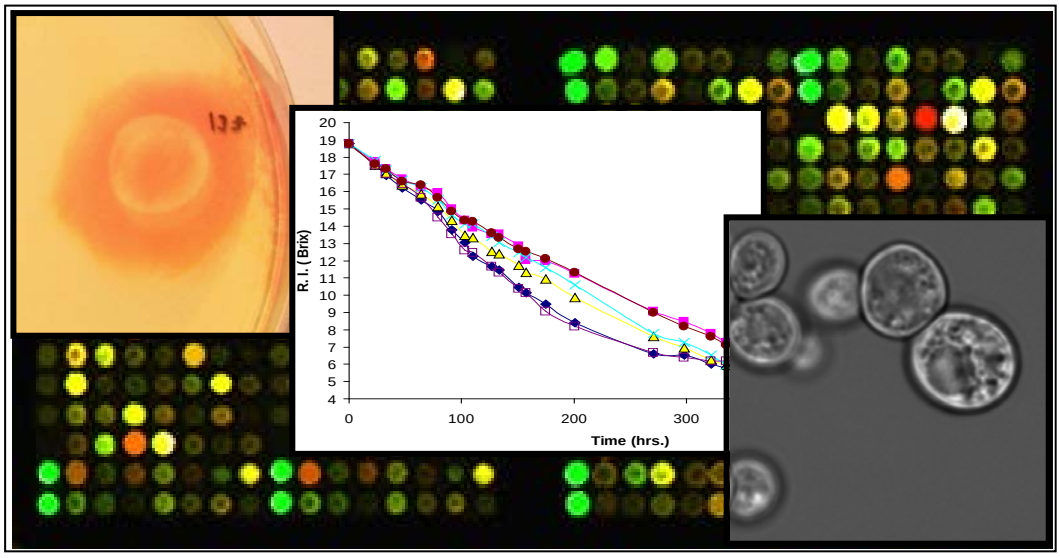
THE UNIVERSITY OF ADELAIDE
AUSTRALIA

Australian Government
Grape and Wine Research and Development Corporation

Microbial Enhancement of Wine Quality and Complexity



FINAL REPORT to

GRAPE AND WINE RESEARCH & DEVELOPMENT CORPORATION

Project Number: **UA 01/04**

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Research Organisation: **University of Adelaide**

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Abbreviations

bp	base pair
CDGJM	Chemically Defined Grape Juice Medium
CORE	Counter-selectable reporter cassette
CPM	Counts per minute
DIG	Dioxigenin
DNA	Deoxyribonucleic acid
DR	Direct repeat
FAN	Free amino nitrogen
GABA	(4-amino butyrate)
HPLC	High performance liquid chromatography
IROs	Integrative recombinant oligonucleotides
LAB	Lactic acid bacteria
LC-MS	Liquid chromatography / mass spectroscopy
MLF	Malolactic fermentation
MRSA	de Mann/Rogosa/Sharp broth medium
NOPA	Nitrogen measured by o-Phthaldialdehyde
*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
NTU	Nephelometric turbidity units
OD	Optical density
ORF	Open reading frame
PCR	Polymerase chain reaction
RPM	Revolutions per minute
Spp.	species
SPS	(Ssy1-Ptr3-Ssy5) sensor
SGD	*Saccharomyces* Genome Database
TCA	Tri-carboxylic acid cycle
UTR	Upstream translated region
VVM	$Volume_{gas}$ per $volume_{medium}$ per minute
YNB	Yeast nitrogen base
YPD	Yeast peptone dextrose
Δ	Gene deletion
::	Gene replacement by subsequent named gene.

Abstract

This report details the outputs from a substantial 4 year research project, including publications and other communications, and highlights areas for further research. Where the findings of this work have been published we have used extracts from such publications to provide the bulk of the material reported herein.

This project has sought to provide a better understanding of the potential influence of the microbial component of winemaking on the process and the resulting wine. We have succeeded in more fully defining the actual or potential contribution of LAB and in particular their enzymatic activities to wine composition. A survey of 40 LAB strains for over 10 enzymatic activities revealed many to be represented. Several such enzymes were minimally inhibited by low pH, temperature and high ethanol and sugar contents, unlike some existing preparations, such as those of fungal origin. These findings go some way to allowing for a more informed application of LAB to winemaking and/or the preparation of enzyme extracts from these organisms for use by the industry.

Use of sophisticated techniques such as adaptive evolution, recombinant DNA technology and classical yeast genetics has allowed several improved yeast strains to be produced and genes important to fermentation reliability and wine composition to be identified. Of particular interest have been strains bearing modifications to *GID7*, *NGR1* and *PUT4*. These strains are either better able to complete fermentation under nitrogen-limited conditions, produce an altered metabolite profile or show enhanced tolerance to stress conditions. Many tens of further strains have been identified through non-recombinant methods. Great interest has been generated in those arising from adaptive evolution as these show superior fermentative capabilities and reduced ethanol yield.

Many of the future directions detailed herein have been incorporated into a new project: UA 05/01, *Better wine from novel and better informed use of microbiology*.

Executive Summary

- A literature review of research on LAB has highlighted enzymatic activities and methods for their quantitation which may be useful in winemaking.
- Screening of 40 *Oenococcus*, *Lactobacillus* and *Pediococcus* strains confirms a widespread and varied occurrence of glycosidase (β- and α-glucosidase, β-xylosidase, α- and β-galactosidase, α-rhamnosidase and α-arabinosidase), lipase, tannase, esterase and polysaccharide-degrading enzymes.
- Unlike other studies which found glycosidase activities to be strongly inhibited by wine conditions, many of those observed amongst LAB in this study were found to be relatively resistant to such inhibition.
- LAB are able to markedly reduce red colour of a malvidin-3 glucoside solution by an as yet undefined mechanism.
- Putative β-glucosidase genes have been cloned and are being sequenced.
- Polysaccharide degrading activities (lichenase, cellulase, β-glucanase) were wide spread amongst *Lactobacillus* and *Pediococcus*, but less so for *Oenococcus*, while no isolate showed xylanase activity.
- Tannase activity was also widely distributed, though its oenological significance awaits definition.
- Esterases were common amongst LAB.
- Sequences for esterase genes in *Lactobacillus* were used to amplify homologous fragments (4) from *Oenococcus*. Homology was high.

- Commercial wine yeasts were demonstrated to sporulate to different degrees, yielding a range of spores of varying viability. Strain L2056 was used in further work.
- An improved system for gene disruption of prototrophic commercial wine yeasts has been developed and shown in L2056 to allow loop-out of the disruption construct leaving essentially no base pair changes let alone foreign sequences at the insertion site.
- A previously constructed strain constitutive for transport of proline has been characterised and found to show almost twice the viability under stress conditions (heat and high salt).
- Cell size was increased in some constitutive proline transporting strains.
- Transposon mutants previously introduced into a laboratory strain and found to ferment low-nitrogen media more effectively were further characterised.
- Such strains generally had reduced nitrogen requirements.
- Disruptants of genes *NGR1* and *GID7* in laboratory and wine yeast derivatives, typically possessed attributes similar to the original transposon mutant through which they were identified.
- Expression pattern for structural genes of enzymes involved in central nitrogen metabolism were altered: most notably *GDH2*, which is expressed at levels 17-fold higher than the wildtype.
- Confirmation of elevated levels of glutamate dehydrogenase is being sought.
- In a parallel screen of transposon mutants in a commercial wine yeast derivative, several further genes of interest were highlighted and are now under investigation.

- Perhaps for the first time, adaptive evolution has been applied to the optimisation of wine yeast.
- We have isolated strains which are much better at fermenting a medium resembling grape juice.
- Microarray analysis has revealed some 20 genes whose pattern of expression differs from that of the parental strains. A selection of these genes is being disrupted or over-expressed (as appropriate) to determine their role in the phenotype of the evolved strains.
- Apart from fermentation kinetics, yields of ethanol, glycerol, acetaldehyde and acetic acid were altered in the evolved strains.
- In order to observe more of the heterogeneity expected to be present several commercial yeast strains, were sporulated, dissected, rediploidised and screened.
- A range of responses to high fructose, high sugar, low nitrogen and high solute media was noted, as were differences in the yield of H_2S.
- Such strains are now being characterised in laboratory fermentations.

- Overall several strains and or genes have been identified whose exploitation and manipulation hold great promise for winemaking efficiency and product composition.

- Communications from this project include 8 peer review papers, over 20 conference presentations and non-peer review articles and nearly 20 public seminar presentations.

Background

Production of good quality wine in a cost effective manner is vital to maximise the economic potential of the Australian wine industry in both local and international markets. Wine consumers are also becoming increasingly informed and selective about their wine preference, particularly as there is a wide range of products currently available. Thus, the production of wines that are the best possible quality and discernibly different, or have noticeably enhanced characters to existing products currently available is an avenue that will continue to capture the interest of consumers. This project has provided winemakers with some novel microbial strategies.

There are at least two key microbial phases that influence and are vital to the quality of wine production, these are the alcoholic and malolactic fermentations. Both can have profound impacts on wine composition processability and shelf-life. 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.

Project Aims and Performance Targets

From the original grant application, the objective of the project was:

> *To establish a comprehensive, mid-term, research project aimed at discovering, characterising and enabling the industrial use of novel lactic acid bacteria (LAB) or yeast strains or their enzymatic activities to bring about value-adding to wine by enhancing complexity and quality.*

This was to be achieved by:

1. An evaluation of novel microbes (wine and food bacteria or their enzymes as well as *S. cerevisiae* yeast) currently under investigation by the group for activities able to modify wine flavour, aroma, mouthfeel and processing efficiency (or others).

2. Broadening the search to identify and evaluate new opportunities for microbiological enhancement of wine quality.

3. Demonstrating the application of above organisms/enzymes to industrial situations with resulting increases in wine quality and or complexity.

Outputs	Performance Target
1. Information on organoleptically and technologically important processes or products of wine and food microorganisms	1. Up-to-date literature review highlighting potential applications of microbes or extracts to the modification of wine processing and organoleptic properties to identify additional targets for investigation.
2. Information on enzymic activities of lactic acid bacteria and isolates/enzyme preparations becoming available.	2. 20 – 30 bacterial strains screened for glycosidic and other activities operating in wine conditions. Impact on flavour/mouthfeel in grape juices defined. Stains or preparations ready for industry trialling.
3. Identification of yeast genes or activities with positive impacts on wine quality or processing	3. 3 – 6 mutations or rate-limiting enzymes that influence wine quality identified and characterised.
4. Mutant or hybrid yeasts produced along with information on their use.	4. 3 – 4 distinct yeast strains with enhanced flavour, mouthfeel or processing characteristics available for industry evaluation. Protocols for industry use.
5. Publications/conference presentations	5. 2 – 3 communications/yr detailing project findings, highlighting potential for application of new strains/ enzymes/treatments to wine quality enhancement.

Results and Discussion from Individual Outputs

OUTPUT 1 Information on organoleptically and technologically important processes or products of wine and food microorganisms

We have fully achieved the Performance Target for this output:

> *Up-to-date literature review highlighting potential applications of microbes or extracts to the modification of wine processing and organoleptic properties to identify additional targets for investigation.*

We undertook a comprehensive survey of the literature to identify reports from studies of LAB in which enzymatic activities of potential winemaking importance were described. Given the number of publications dealing with the impact of LAB in the dairy industry alone, we elected to restrict the survey. Specifically we only included studies related to enzymes that could improve wine quality or complexity and chose to emphasize findings from investigations in which wine LAB were used or which were performed under wine-like conditions. Where no oenological data were available we referred to studies (or aspects thereof) which dealt with those species also found in wine (based on a consensus derived from references (Boulton et al, 1996; Fugelsang, 1997; Ribéreau-Gayon et al, 1998; du Plessis et al, 2004). The activities of greatest interest were those conferred by a single enzyme, ideally one with an extracellular localization. Such enzymes should be most amenable to separation from the cell biomass or preparation as an enzyme-enriched extract, which may be desirable when the originating organism is difficult to grow or is not wanted in grape juice or wine. The following mini-review was published in *Applied and Environmental Microbiology* in 2004 and shortly thereafter was included in their list of "Top Most Requested Articles".

> Matthews, A., Grimaldi, A., Walker, M., Bartowsky, E., Grbin, P., Jiranek, V. (2004) Lactic acid bacteria as a potential source of enzymes for use in vinification. *Applied and Environmental Microbiology* **70**:5715-5731.

An important outcome of the work was that it highlights the great breadth of possible activities which could be pursued experimentally through this project. As a result we elected to conduct a survey of a large collection of LAB isolates for activities which included proteases and peptidases, glycosidases, polysaccharide-degrading enzymes, esterases, ureases and lipases.

OUTPUT 2 Information on enzymic activities of lactic acid bacteria and isolates/enzyme preparations becoming available.

The original Performance Target for this component of the project was stated as:

> *20 – 30 bacterial strains screened for glycosidic and other activities operating in wine conditions. Impact on flavour/mouthfeel in grape juices defined. Stains or preparations ready for industry trialling.*

Much of this work was undertaken by an international student, Mr Antonio Grimaldi, whose scholarship and tuition fees were met by an Adelaide International Scholarship. Project funds originally intended for establishment of a PhD scholarship for Mr Grimaldi were therefore able to be used to appointment an additional PhD student, Ms Angela Matthews, in late 2001. In this way it has been possible to expand the Performance Target to allow a *characterisation and evaluation of 3-4 further activities/attributes* to an extent which was analogous to that intended for the glycosidic activities.
The results under this output are therefore discussed under the headings which demarcate the two PhD projects.

2.1 Glycosidic Activities

2.1.1 Introduction

Grape aglycones include monoterpenes, C_{13}-norisoprenoids, benzene derivatives and aliphatic alcohols, which can occur in free form or as odourless non-volatile 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 growth of lactic acid bacteria (Guilloux-Benatier et al. 1993).

Agents capable of effecting the above hydrolyses have the potential to influence wine composition. Specific glycosidases are 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). Wine microorganisms represent a potential source of the required hydrolytic activities. Of the many studies 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 have focused on LAB, in particular, *Oenococcus oeni*, *Lactobacillus spp*. and *Pediococcus spp*.

At the commencement of this project, there were limited reports of studies of the glycosidase activity of *O. oeni* strains (McMahon et al. 1999; Grimaldi et al. 2000). Also there had been minimal characterisation of glycosidases of *Lactobacillus* and *Pediococcus*. We therefore sought to address this gap in the literature. 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* and nine strains each of *Lactobacillus* and *Pediococcus* under optimised and reproducible conditions. As such, these organisms were grown in defined media, and presented with *p*-nitrophenyl-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.

2.1.2 Methods

Bacterial strains and cultivation: The strains of lactic acid bacteria used in this study are listed in Table 2.1 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%), 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 (*Oenococcus*) or 0.3 (*Lactobacillus* and *Pediococcus*) 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 x *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.

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, 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 bacterial strain prepared in 0.85% NaCl and standardised to

Table 2.1 Strains and sources or lactic acid bacteria used in this study.

Genus/Species	Strain	Origin
Oenococcus oeni	1	Lallemand O.S.U. (VI 77)
	2	Lalvin 4X (VL 92)
	3	Inobacter
	4	Bitec Vino
	5	Lalvin-Inobacter
	6A	Lalvin MT01 Standard
	7	Lallemand 3X (E218)
	8	CHR Hansen Viniflora
	9	CHR Hansen Viniflora
	10	Lalvin EQ54 MBR
	11	Lalvin MCW
	12	Lalvin 3X 1Step
	13	Lalvin IB Standard
	14	Enoferm Alpha
	16	Lalvin 3X Standard
	17	Lalvin OSU MBR
	20	Lallemand No 3[a]
	21	Lallemand No 4[a]
	22	Lallemand No 5[a]
	23	Lallemand No 8[a]
	28	Lallemand No 2[a]
	29	Lallemand No 9[a]
Lactobacillus	Lac6A	Lalvin MT01 Standard
	Lac12	Lalvin 3X 1 Step
	Lac13	Lalvin IB Standard
	Lac15	Lalvin 31 MBR
	Lac16	Lalvin 3X Standard
	Lac24	Experimental starter culture[a]
	Lac25	Experimental starter culture[a]
	Lac19	Commercial olive product
	Lac26	Experimental starter culture[a]
Pediococcus	Ped3	Inobacter
	Ped10	Lalvin EQ54 MBR
	Ped11	Lavin MCW
	Ped15	Lalvin 31 MBR
	Ped16	Lalvin 3X Standard
	Ped17	Lalvin OSU MBR
	Ped24	Experimental starter culture[a]
	Ped27	Experimental starter culture[a]
	Ped18	Commercial olive product

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

yield a final OD_{600} in the assay of 0.5. Substrate solutions (20 µl) were added to give the following final concentrations: *p*-nitro-phenyl 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 (2,500 x *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.

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.

2.1.3 Results and Discussion

A collection of 40 strains was created from commercial LAB preparations and pre-commercial samples and investigated for glycosidic activities. To the best of our knowledge this is the most comprehensive investigation of this type in terms of numbers of strains and substrates and impacting parameters. We have been able to demonstrate that the possession of glycosidic activities is both widespread amongst genera of lactic acid bacteria associated with wine and 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.

Oenococcus oeni: 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; Fig 2.1). The fact that this pH optimum does not correspond with that for the growth of these organisms (~ pH 4.2 – 4.8; van Vuuren and Dicks, 1993) argues against the influence of pH occurring through 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. 2.1). 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; Marasco et al., 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 2.2), 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. While absolute values differed, the responses to temperature were largely consistent for all strains. As such, only selected data are reported here, typically that from strains with highest activities (Fig. 2.2). In general terms, maximum hydrolysis of any of the substrates occurred at temperatures near 40ºC. Although this is outside the typical operating range for winemaking, it becomes more important for alternate processing of small batches of

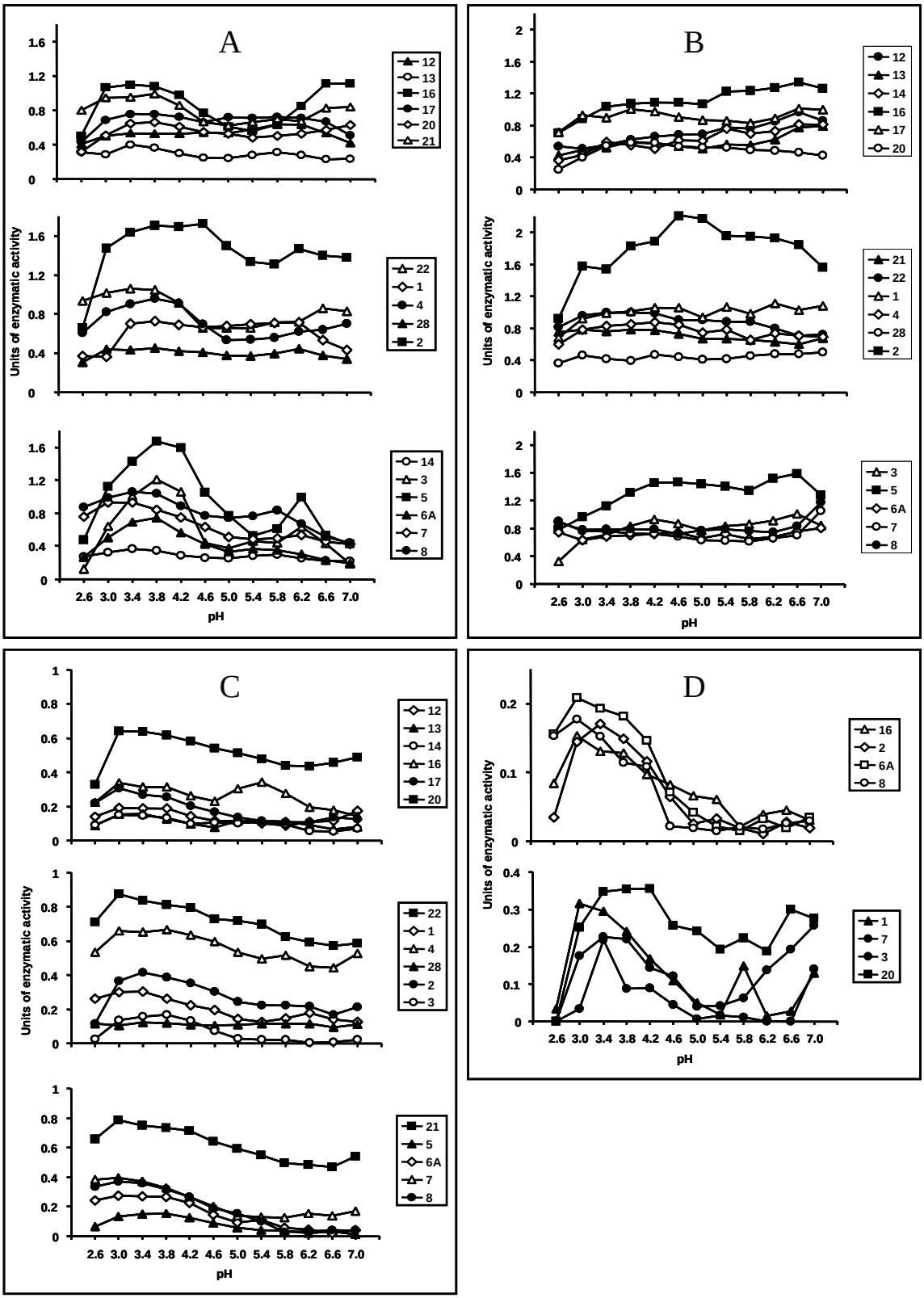


Figure 2.1 Influence of pH on the glycosidase activity of *Oenococcus oeni* strains against *p*NP-βGlu (A), *p*NP-αGlu (B), *p*NP-βXyl (C), *p*NP-αRha (D, open symbols) and *p*NP-αAra (D, close symbols). One unit of activity is defined as mmols of *p*-nitrophenol liberated per min per milligram of cell dry weight. Values are the mean of duplicate determinations.

Table 2.2 Total glycosidic activities for *Oenococcus oeni* strains each of five substrates[a].

Strain	Substrate					TOTAL[b]
	*p*NP-βGlu	*p*NP-αGlu	*p*NP-βXyl	*p*NP-αAra	*p*NP-Rha	
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 Figure 2.1). [b] Measures of total glycosidic activity are derived from the totals determined for each substrate. [c] ND, Not determined.

juice or wine or else other industrial applications (e.g. Mourgues and Bénard, 1982; Girard et al., 1997). Nitrophenol release was also rapidly reduced as the temperature increased to 60ºC, while a more gradual decline was observed at temperatures below the optimum. The retention of some glycosidic activity at temperatures below 20ºC (Fig. 2.2) is of importance for the wine industry, given that wines are usually stored in this range during the MLF.

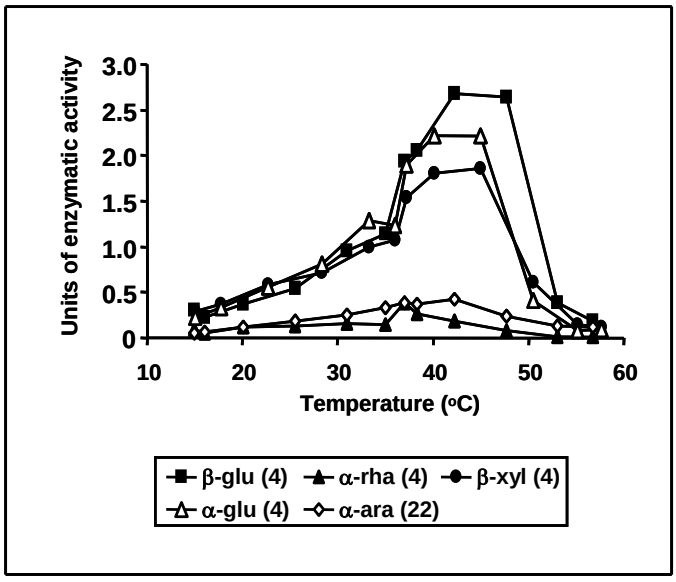


Figure 2.2 Influence of temperature on glycosidic activity for a selection of *Oenococcus oeni* strains and substrates. Values are mean of duplicate determinations.

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.

The response to ethanol was variable and depended on the activity being examined, the strain and concentration at which ethanol was included (Fig. 2.3). At 4% v/v, ethanol resulted in a marked increase in β-D-glucopyranosidase activity by strains 2 and 4, 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. The enhancing effect of ethanol, occurring most often at lower concentrations, 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 *Lactobacillus 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. Inhibition of β-glucopyranosidase activity was also observed for glucose and fructose, even when present at only 0.01% w/v (Fig. 2.3, 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. 2.3, 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 2.3, B2), with the response to fructose being lower (Fig 2.3, C2). The impact of ethanol on β-D-xylopyranosidase activity was modest and variable (Fig. 2.3, A3). At lower concentrations, glucose more so than fructose, produced a modest drop in nitrophenol liberation from *p*NP-Xyl (Fig. 2.3, 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. 2.3, 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. 2.3, C4). Only at the highest concentration (2%) was fructose inhibitory for some strains.

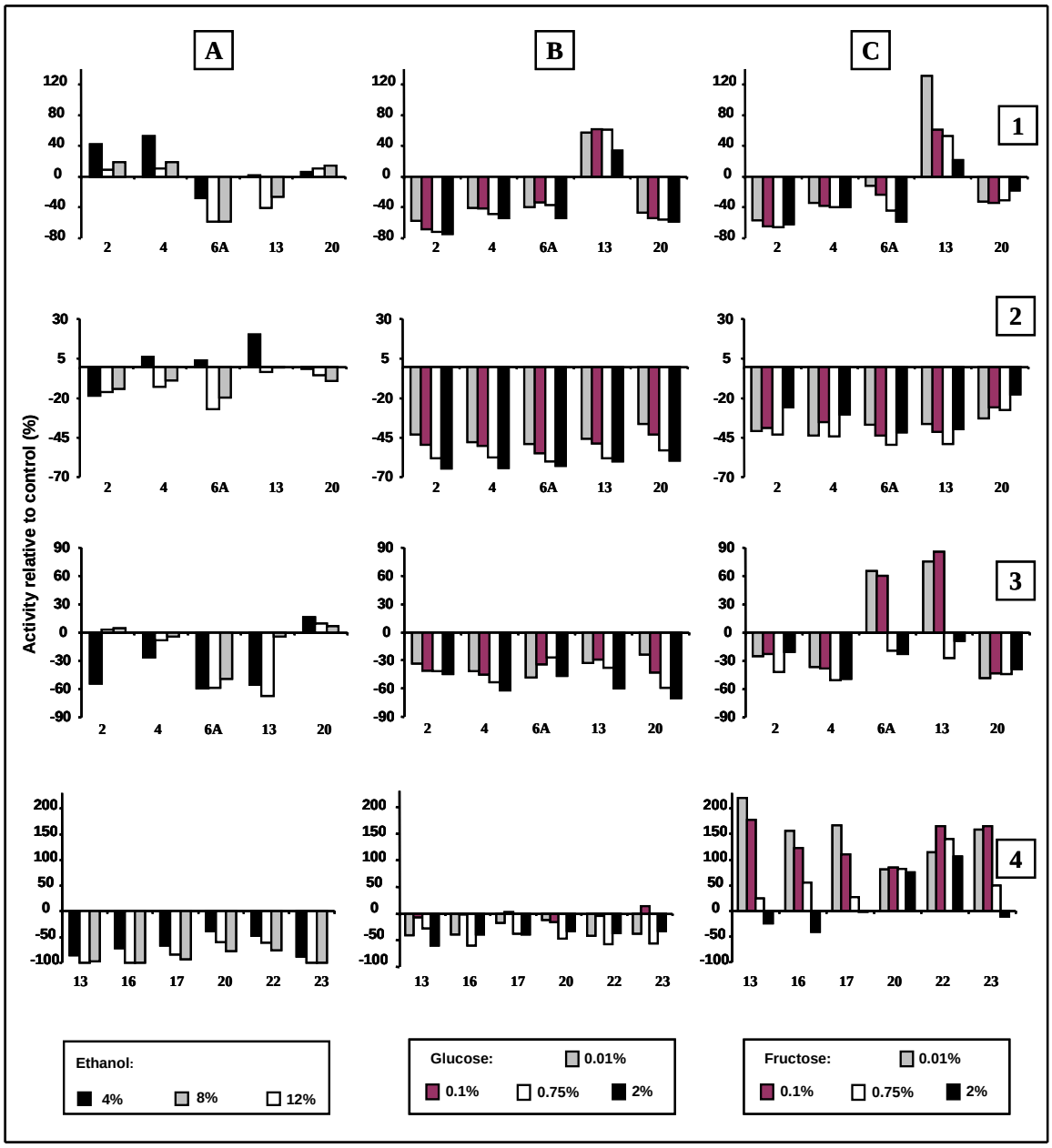


Figure 2.3 Glycosidic activity of *Oenococcus oeni* strains observed in the presence of ethanol (column A), glucose (column B) or fructose (column C) against *p*NP-βGlu (row 1), *p*NP-αGlu (row 2), *p p*NP-βXyl (row 3) and *p*NP-αAra (row 4). Values are relative (%) to the activity observed for the control performed under the same conditions in the absence of either ethanol or added sugars. Values are the mean of duplicate determinations.

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.

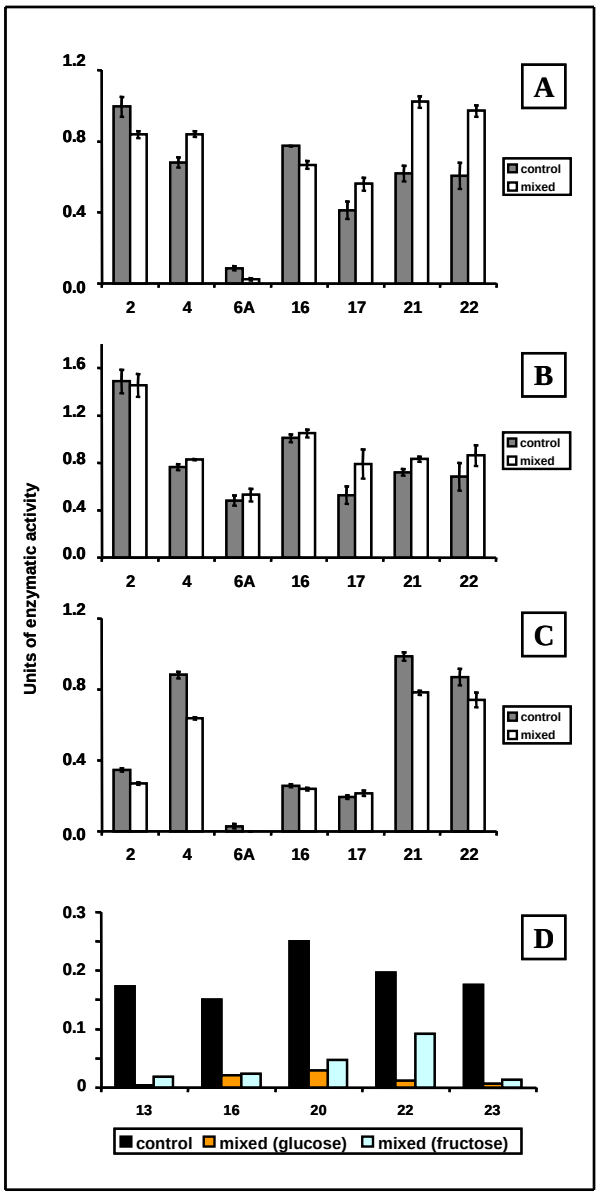


Figure 2.4 Influence of ethanol and glucose (A-C) or ethanol and fructose (D) on the glycosidic activities of selected *Oenococcus oeni* strains against (A) *p*NP-βGlu, (B) *p*NP-αGlu, (C) *p*NP-βXyl and (D) *p*NP-αAra. Values are the mean of triplicate determinations.

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 2.3). 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).

Given the variable and specific nature of the impact of the above parameters when examined individually, the consequences of combinations of these, as would be encountered in wine, was also considered. This multi-factorial approach 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. 2.4). The ability to hydrolyse *p*NP-αAra (and *p*NP-αRha; data not shown) was again found to be weak and, in this 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 and coworkers (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.

Lactobacillus and Pediococcus: 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 malolactic fermentation starters (Pilatte and Prahl 1997), a more detailed characterization was justified. Strains were tested with all the *p*-nitrophenyl forms of the key glycosides of importance in winemaking. Our investigations have shown that unlike earlier work

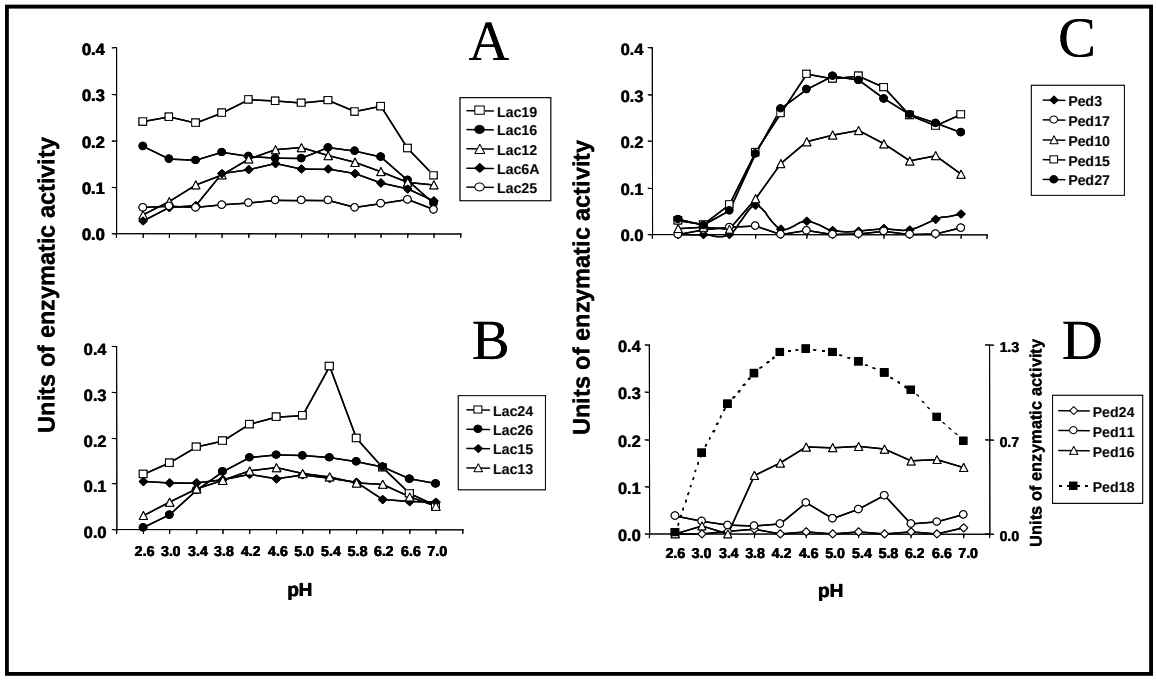


Figure 2.5 Influence of pH on the enzymatic activities of *Lactobacillus* spp. (A and B) and *Pediococcus* spp. (C and D) against *p*NP-βGlu. Values are the means of duplicate determinations. NB: Values for Ped18 are plotted against the right hand axis.

(Grimaldi et al. 2000) and that described above for *O. oeni*, the *Lactobacillus* and *Pediococcus* strains studied here produce little beyond α- and β-D-glucopyranosidase activities (data for other substrates not shown). The effect of pH on the hydrolysis of β-D-glucopyranoside is shown in Figure 2.5. For both *Lactobacillus* spp. (Fig. 2.5A and B) and *Pediococcus* spp. (Fig. 2.5C and 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 (~ pH 3 – 4) *Lactobacillus* spp. retained most of its activity, whereas *Pediococcus* spp. most typically did not affect 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. 2.6A and B), but strain Ped18 was the only *Pediococcus* spp. able to do so (Fig. 2.6C). The range of maximum activities was similar to that seen for *p*NP-βGlu with strong activity being retained at wine pH values.

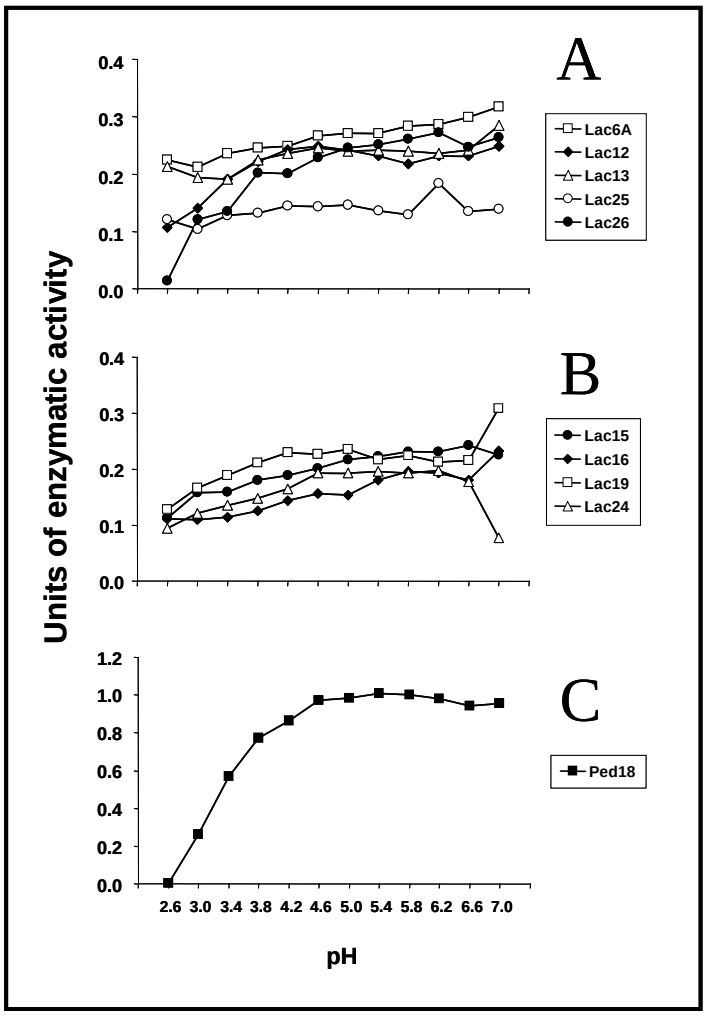


Figure 2.6 Influence of pH on the enzymatic activities of *Lactobacillus* spp. (A and B) and *Pediococcus* spp. (C) against *p*NP-αGlu. Values are the mean of duplicate determinations. NB: a different scale is used in panel C.

These activities were approximately one order of magnitude less than those seen for *O. oeni* in previous work (see above and Grimaldi et al. 2000). Even so, the activities studied here might still have marked impact on bacterial growth and on the flavour profile of wine. Activity against *p*NP-αGlu may be of importance in the liberation of nutrients from yeast-derived macromolecules which can enhance the growth of lactic acid bacteria (Guilloux-Benatier et al. 1993). While β-D-glucopyranosidases can play a role in liberation of sensorialy 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.

As per the characterisation of *O. oeni*, we also examined potential inhibitors individually and in combination. When ethanol was present in the assay medium used in this study, responses were again highly strain-dependent (Fig. 2.7). Nevertheless, β-D-glucopyranosidase activities were typically enhanced amongst *Lactobacillus* spp. (Fig. 2.7A), whereas most often for *Pediococcus* spp., little change was seen (Fig. 2.7C). 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 toward an enhanced activity with increasing amounts of ethanol (Fig. 2.7B), 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. 2.7D).

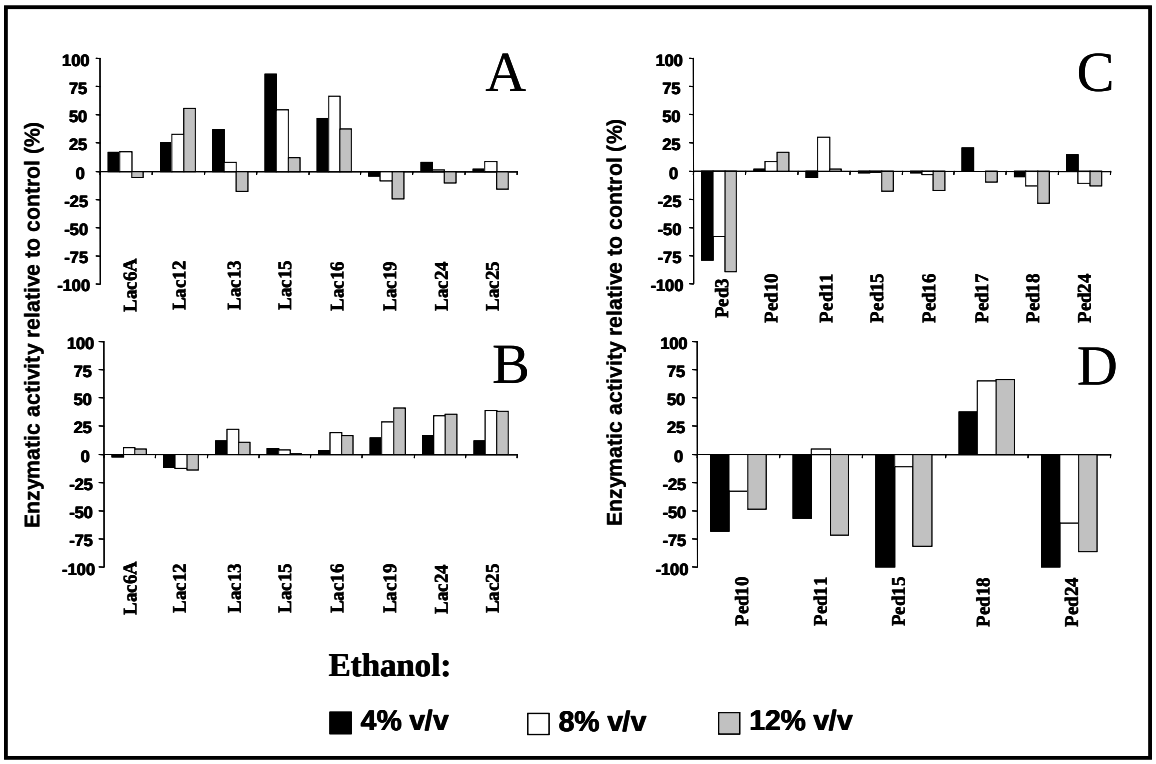


Figure 2.7 Influence of ethanol on the enzymatic activities of *Lactobacillus* spp. against *p*NP-βGlu (A) and *p*NP-αGlu (B), and *Pediococcus* spp. against *p*NP-βGlu (C) and *p*NP-αGlu (D). Values are shown relative (%) to the activity observed for the control performed under the same conditions in the absence of ethanol and are the means of duplicate determinations.

Inclusion of glucose at one of the four concentrations examined in this study (0. 1 – 20 g/L) produced a similar pattern of inhibition for both β-D-glucopyranosidase and α-D-glucopyranosidase activities in *Lactobacillus* spp. (Fig. 2.8A and 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. 2.8C). In strain Ped24 inhibition was minimal while in strain Ped17 the response was one of enhancement with 1 g/L glucose mediating a 125% increase of activity against *p*NP-βGlu compared to the glucose-free control (Fig. 2.8C).

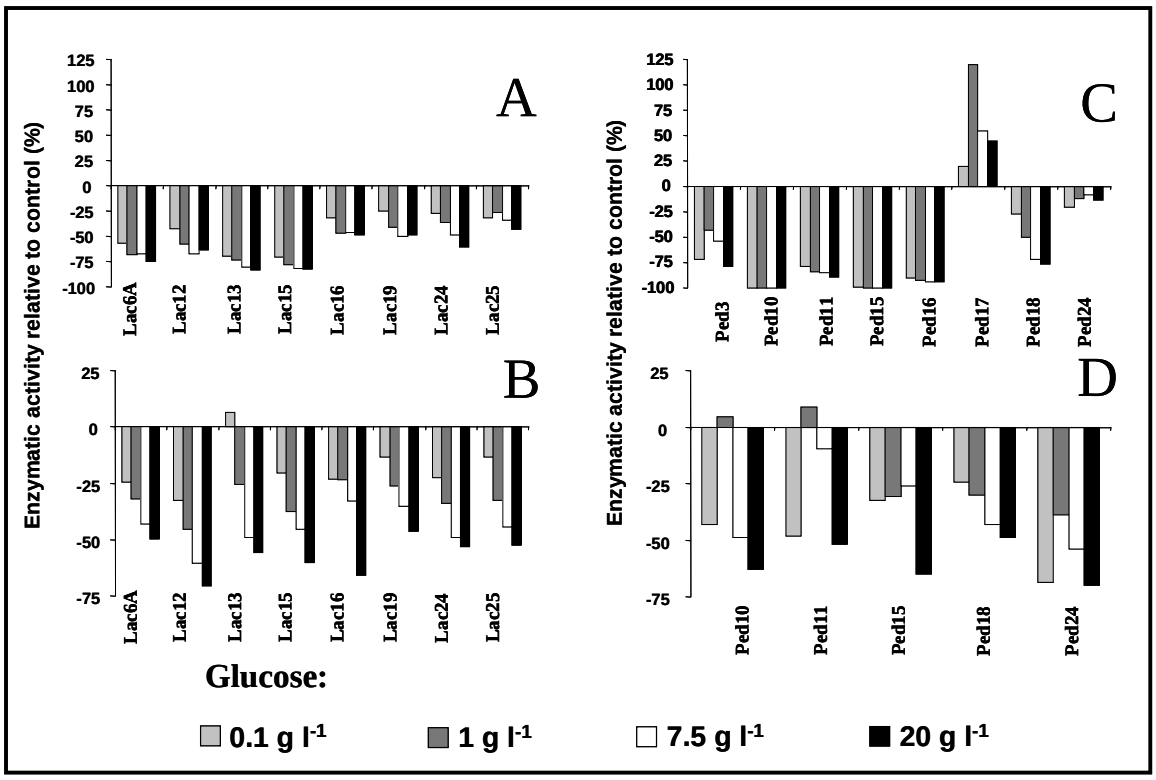


Figure 2.8 Influence of glucose on the enzymatic activities of *Lactobacillus* spp. against *p*NP-βGlu (A) and *p*NP-αGlu (B), and *Pediococcus* spp. against *p*NP-βGlu (C) and *p*NP-αGlu (D). Values are shown relative (%) to the activity observed for the control performed under the same conditions in the absence of ethanol and are the means of duplicate determinations.

The typical response to fructose (Fig. 2.9) 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.

While still showing strong strain dependence, our findings reveal some broad trends: ethanol induces a trend toward enhancement of hydrolysis of both *p*NP-βGlu and *p*NP-αGlu amongst *Lactobacillus* strains compared with a reduction or unaltered hydrolysis by *Pediococcus* strains. The availability of considerable strain variation introduces the possibility of selecting and applying isolates according to 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-βGlu and *p*NP-αGlu with few exceptions. 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).

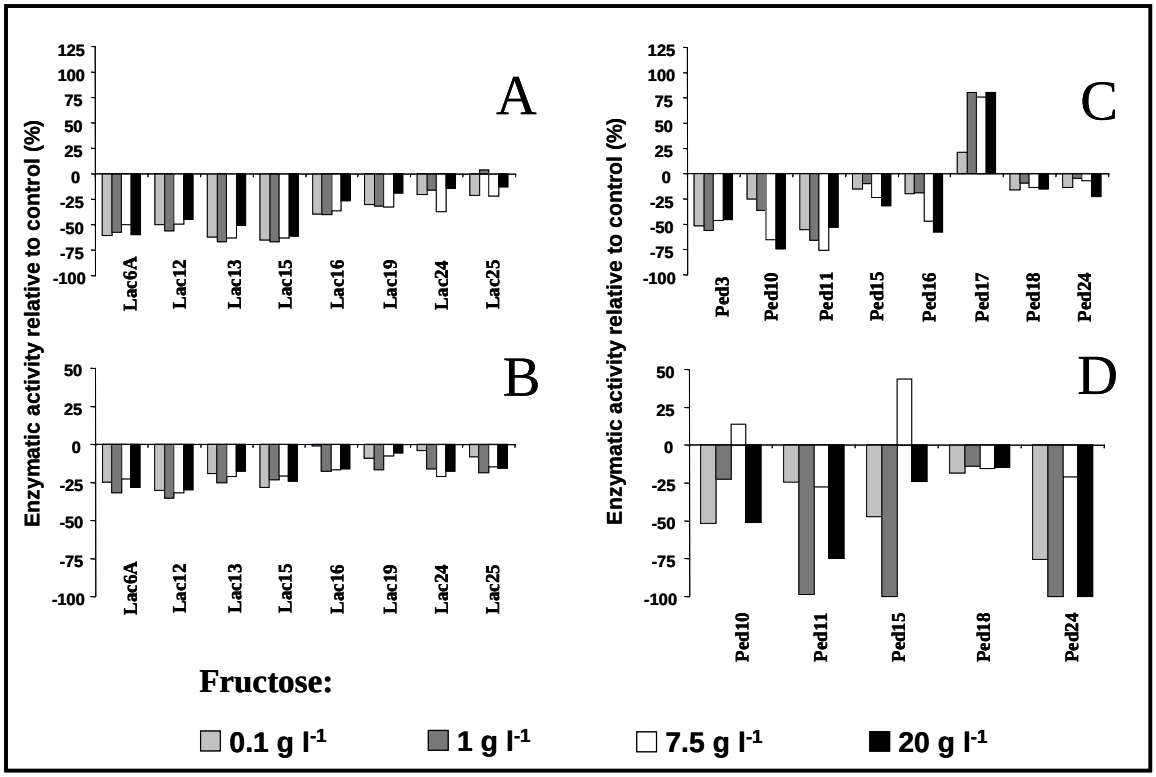


Figure 2.9 Influence of fructose on the enzymatic activities of *Lactobacillus* spp. against *p*NP-βGlu (A) and *p*NP-αGlu (B), and *Pediococcus* spp. against *p*NP-βGlu (C) and *p*NP-αGlu (D). Values are shown relative (%) to the activity observed for the control performed under the same conditions in the absence of ethanol and are the means of duplicate determinations.

Temperature optima for all strains occurred at or above 35ºC for both enzymatic activities (Fig. 2.10). For *Lactobacillus* strains Lac16 and Lac19, maximal hydrolysis of *p*NP-αGlu occurred at around 45ºC. In all cases, as temperatures approached 50ºC both activities were rapidly reduced to zero at 60ºC. At temperatures used in winemaking, significant activities were retained.

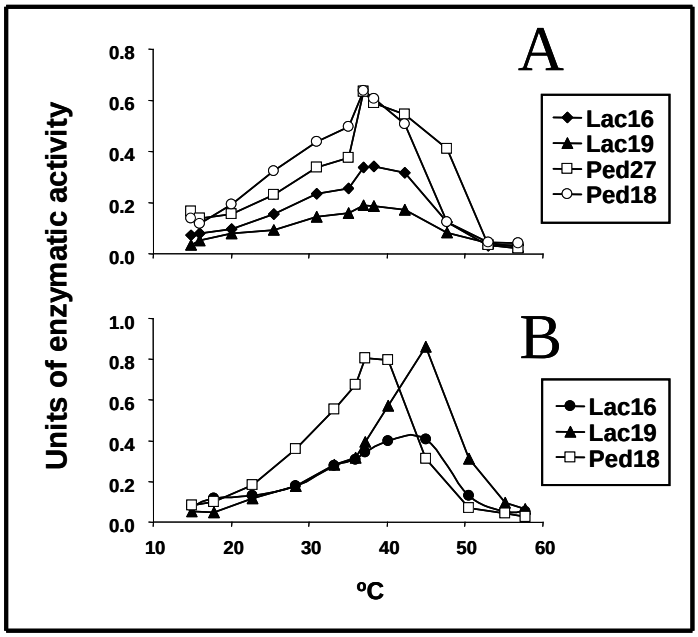


Figure 2.10 Influence of temperature on enzymatic activities of *Lactobacillus spp*. and *Pediococcus spp*. against *p*NP-βGlu (A) and *p*NP-αGlu (B). Values are the mean of duplicate determinations.

Evaluation of strains for hydrolysis of *p*NP-βGlu and *p*NP-αGlu 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 Figure 2.11. Both activities displayed by *Lactobacillus* strain Lac12 and Lac19 showed a small increase when assayed in the presence of 10% ethanol and 2 g/L glucose (Fig. 2.11A and B), suggesting that the response to ethanol (see Fig. 2.7) was the dominant. Meanwhile, β-D-glucopyranosidase activity of *Pediococcus* strain Ped18 was about 30% of that observed for the control (Fig. 2.11A) whereas α-D-glucopyranosidase activity was increased by approximately 100% relative to the control (Fig. 2.11B). 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 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 will be extended (in UA 05/01) 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. Some preliminary data relating to bacterial action on grape glycosides indicates that anthocyanins are either readily hydrolysed by glycosidases, absorbed to bacterial cells or both. This effect is clearly shown in the Figure 2.12. Recent reports have shown a non-*Saccharomyces* glycosidase to be effective in the decolourisation of white wines made from red grapes (Manzanares et al., 2000). Given this potentially detrimental feature of LAB for red wine colour, or alternatively the effectiveness of LAB glycosidases for deliberate decolourisation, we intend to further this work with purified anthocyanins as part of UA 05/01 and/or the Colour project.

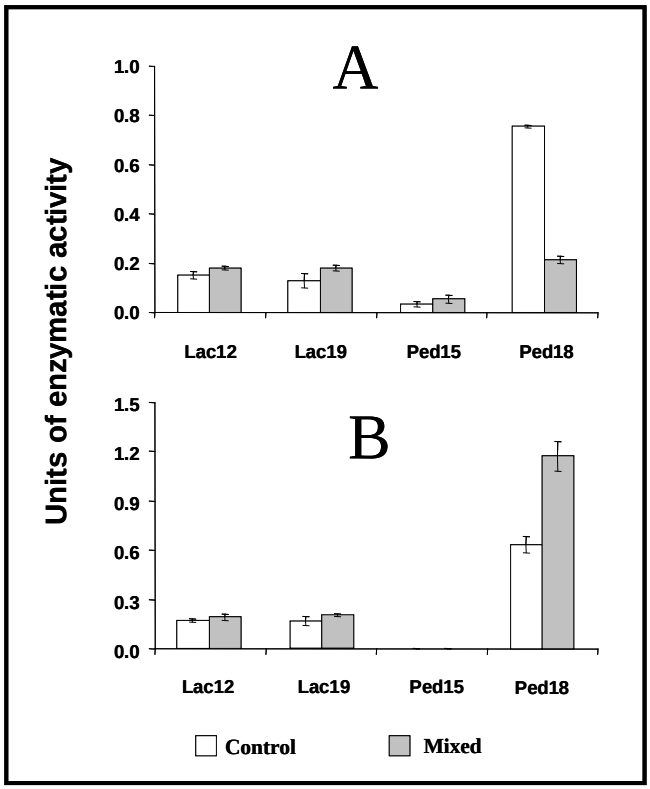


Figure 2.11 Influence of ethanol and glucose on *Lactobacillus spp*. and *Pediococcus spp*. against against *p*NP-βGlu (A) and *p*NP-αGlu (B). Assays were conducted at pH 3.5, in the presence or absence of ethanol (10%, v/v) and glucose (2 g/L). Values are the mean of triplicate determinations ± standard deviation.



Figure 2.12 Appearance of a malvidin–3 glucoside (100 mg/L) solution after 3 days incubation in the absence (left tube) or presence (right tube) of added *O. oeni* biomass. Images show 30 ml tubes.

2.2 Polysaccharide-degrading Enzymes, Tannases, Lipases & Esterases

2.2.1 Introduction

During MLF, lactic acid bacteria decarboxylate malic acid to lactic acid and carbon dioxide. In addition to this prominent enzyme-catalysed action, it is now generally acknowledged that LAB perform a multitude of secondary enzymatic conversions during their growth in wine. In this component of the project we sought to extend our characterisation of the enzymatic properties of LAB beyond their glycosidases to include

other potentially useful enzymes. Initially, the LAB were subjected to a broad screen for the production of such enzymes, including esterases, lipases, tannases and enzymes capable of degrading polysaccharides. A subset of the most promising enzymes were ultimately selected for more detailed characterisation. Specifically, their structural genes were sought ahead of their heterologous expression in *E. coli* and purification.

2.2.2 Methods

Biomass Preparation: Several of the enzyme assays were conducted using liquid-based methods. In such cases, the amount of bacterial cells required to produce an optical density at 600_{nm} 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 (20,000 x *g*, 5 min). An appropriate substrate in pH 5.0 McIlvaine buffer was then added to the cells and the reaction mixture was incubated at 37°C for 2 hours. Subsequent treatments of the samples are described below.

Polysaccharide-degrading enzymes: Four substrates were used to detect polysaccharide-degrading enzymes (Table 2.3). The substrates were added to agar media containing the following per litre: 5 g tryptone, 2.5 g yeast extract, 1 g glucose and 20 g bacteriological agar. Pre-cultured LAB were applied to the plates and incubated at 28°C. Following incubation, producer colonies were identified by staining with Congo Red (0.1% w/v) and destaining with 1M NaCl as described by Teather and Wood (1982), except for xylanases, which were detected using the chromogenic Remazol Brilliant Blue substrate.

Table 2.3 Substrates used to detect polysaccharide-degrading enzymes.

Enzyme	Substrate	Substrate Concentration (w/v)	Reference
Cellulase	carboxymethylcellulose, sodium salt	1%	Strauss et al., 2001
β-Glucanase	barley β-glucan	0.02%	Strauss et al., 2001
Lichenase	lichenan	0.02%	Strauss et al., 2001
Xylanase	Remazol Brilliant Blue Xylan	0.1%	Farkaš et al., 1985

Tannase: Tannase activity was detected using a method based on those described by Osawa and Walsh (1993) and Vaquero et al. (2004). Briefly, bacteria were incubated at 37°C with the substrate methyl gallate for six hours. Following incubation, the reaction mixture was alkalinised for 1 hour and any liberated gallic acid underwent non-enzymatic oxidation, resulting in the development of a green-blue colour, the absorbance of which was measured at 440nm.

Lipase: This 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

wine LAB by using MRSA agar medium adjusted to pH 5.0 prior to autoclaving. Tributyrin at 0.3% (v/v) was emulsified into the medium by sonicating the autoclaved agar for 90 seconds at 70 watts using a Branson B-12 Sonifier immediately prior to pouring. Plates were incubated at 28°C and observed daily for one week. Producer colonies were identified by the formation of a clear halo in the opaque media and the method was confirmed by also growing the known lipase-positive yeast *Yarrowia lipolytica*.

Esterase: To elucidate the esterase systems of wine LAB, our bacterial collection was screened using three *p*-nitrophenyl-linked substrates, *p*-nitrophenyl-acetate (Sigma), -butyrate (Sigma) and -octanoate (Lancaster Synthesis). For each substrate, a stock solution was prepared in an appropriate solvent prior to use. The final 1 mL reaction mixtures contained McIlvaine buffer, bacteria biomass to give an OD_{600} of 0.5 and stock substrate solution to yield 1 mM substrate concentrations, except for *p*-nitrophenyl-octanoate which as used at 0.2 mM. Control assays consisted of the same reaction mixture to which no cells were added. The samples and controls were incubated for 2 hr at 37°C. Following incubation, the samples were centrifuged and the supernatant was then transferred to a 1.5 mL disposable plastic cuvette and 0.5 M NaOH was added for alkalinisation. The absorbance at 410nm was immediately determined spectrophotometrically.

Cloning and heterologous expression of potential esterase genes from* O. oeni*: Based on the available (http://genome.jgi-psf.org/draft_microbes/oenoe/oenoe.home.html) *O. oeni* sequences, PCR primers (Table 2.4) were designed to amplify the open reading frames of four putative esterase genes identified through their sequence homology to known esterase genes of other LAB. Appropriate restriction enzyme recognition sites were incorporated into each primer to enable insertion of the sequences into corresponding sites of vector pET-16b. These primers are shown below in Table 2.4.

Table 2.4 PCR primers used to amplify four esterase genes from *O. oeni*.

Esterase gene	Primers[a]	Restriction enzyme site
A2	**Forward:** GGCGGCATATGGCTTTTTTACAAATAAATTATCATTC	*Nde*I
	Reverse: GCGAGGGATCCTTAATATAATCAATCGGAAGCC	*Bam*HI
A7	**Forward:** GCGCGCATATGGCATTTTTAGAAGTTAATTATTATTCAC	*Nde*I
	Reverse: GCGGCGGATCCCTATGACAAACGTTTTTCTGC	*Bam*HI
B28	**Forward:** GGCGGCCATATGTCAGAAATAGTTTTAAA	*Nde*I
	Reverse: CGGCCCTCGAGTCATTCTACTGATCTGTTAGAAC	*Xho*I
C5	**Forward:** GGCCGCATATGGAAAAGAAATTAACGATATCC	*Nde*I
	Reverse: CGCCGCTCGAGTTATTTACGCAGGAAATCCAG	*Xho*I

[a] Restriction enzyme recognition sites are underlined.

2.2.3 Results and Discussion

Wine polysaccharides are derived from the grape berry, from *Botrytis cinerea* infection of the grape berry (Dubourdieu, 1980) and from wine-borne organisms, including yeast (Doco et al., 1996). The principle polysaccharides present in wine are cellulose (primarily β-glucans), hemicellulose (primarily xylans) and pectic substances (Whitaker, 1990). Collectively, wine polysaccharides can contribute to mouthfeel (Sanchez 1996), influence the perception of some flavours (Hansson and Hills, 1997; Dufour and Bayonove, 1999; Hansson et al., 2001) and cause processing difficulties such as filter blockages and prolonged sedimentation times. The production of enzymes capable of degrading such compounds by LAB could aid in avoiding such processing difficulties.

The LAB studied exhibited cellulase, lichenase and β-glucanase activity, but no activity towards xylan was observed in any of the isolates (Table 2.5). Comparing genera, *O. oeni* strains had much lower frequency of polysaccharide-degradation than both the *Lactobacilli* and *Pediococci*. All *Lactobacilli* were able to hydrolyse the cellulase and lichenase substrates, with the exception of one strain (strain 31), which did not break down either polysaccharide. Similarly, all *Lactobacilli* except one (strain 40) could hydrolyse the β-glucan substrate. Interestingly, the strain unable to degrade lichenan and carboxymethyl cellulose, was able to degrade β-glucan.

All *Pediococci* gave a positive response towards carboxymethyl cellulose. All except one strain was able to degrade barely β-glucan, although some of the responses were observed to be weak towards this substrate, indicated by the formation of a small halo around producer colonies. The activity profile of the *O. oeni* strains suggests that polysaccharide-degrading enzyme activity is highly strain specific. While this may be due to the absence of appropriate enzyme systems in some *O. oeni* strains, the use of media other than MRSA may have prevented sufficient growth of this fastidious bacteria for a response to be detected in some of the strains. Further work will clarify this result.

Tannins play an important role in wine colour, mouthfeel and turbidity. Tannases catalyse the hydrolysis of ester bonds in hydrolysable tannins, yielding gallic acid and glucose (Ramirez-Coronel et al. 2003). All of the LAB tested displayed some tannase activity towards the methyl gallate substrate (Fig. 2.13). As a group, the *Pediococcus* strains generally had lower activity than the remaining LAB. However, some *Pediococcus* showed tannase activity greater than the weaker *Lactobacillus* and *Oenococcus*. Within each genus, there was great variation in activity, suggesting tannase activity is not only genus, but strain dependent. Our findings disagree with those reported by Vaquero et al. (2004) who found that no wine LAB species from the *Lactobacillus*, *Pedicoccus* and *Oenococcus* were capable of hydrolysing methyl gallate, with the exception of *Lactobacillus plantarum*.

Wine lipids can originate from the grape berry (including the skin, seeds and pulp) or from yeasts, particularly during autolysis (Miele et al., 1993). Such lipids include acylglycerols, sterols, phospholipids and glycolipids. The action of lipases on such substrates during wine production could liberate a number of volatile compounds - perhaps most importantly, a range of volatile fatty acids which themselves can be

Table 2.5 Results of the polysaccharide-degrading enzyme screen.

Genus	Strain	Lichenase	Cellulase	β-Glucanase	Xylanase
Lactobacillus	6A	+	+	+	-
	12	+	+	+	-
	13	+	+	+	-
	15	+	+	+	-
	16	+	+	+	-
	19	+	+	+	-
	24	+	+	+	-
	25	+	+	+	-
	26	+	+	+	-
	31	-	-	+	-
	32	+	+	+	-
	33	+	+	+	-
	34	+	+	+	-
	35	+	+	+	-
	39	+	+	+	-
	40	+	+	-	-
Pediococcus	3	+	+	+	-
	10	+	+	weak	-
	11	+	+	weak	-
	15	+	+	+	-
	16	-	+	+	-
	17	+	+	+	-
	18	+	+	-	-
	24	-	+	+	-
	27	-	+	+	-
	42	+	+	+	-
Oenococcus	1	-	-	-	-
	2	-	+	+	-
	3	-	-	nd	-
	4	-	-	-	-
	5	-	-	nd	-
	6A	-	+	+	-
	7	-	-	-	-
	8	-	-	-	-
	9	-	-	-	-
	10	-	-	-	-
	11	-	+	+	-
	12	-	-	-	-
	13	+	+	-	-
	14	-	+	-	-
	16	-	-	+	-
	17	-	-	-	-
	20	+	+	+	-
	21	-	+	+	-
	22	-	-	-	-
	23	-	+	-	-
	28	-	-	-	-
	29	+	+	-	-

nd: not determined, +: positive response, -: negative response, weak: small halo around colony.

converted into important flavour compounds such as esters, ketones and aldehydes (Charpentier and Feuillat, 1993). Previous work has shown LAB are capable of producing lipases, but none of this work was conducted using wine organisms (Matthews et al., 2004).

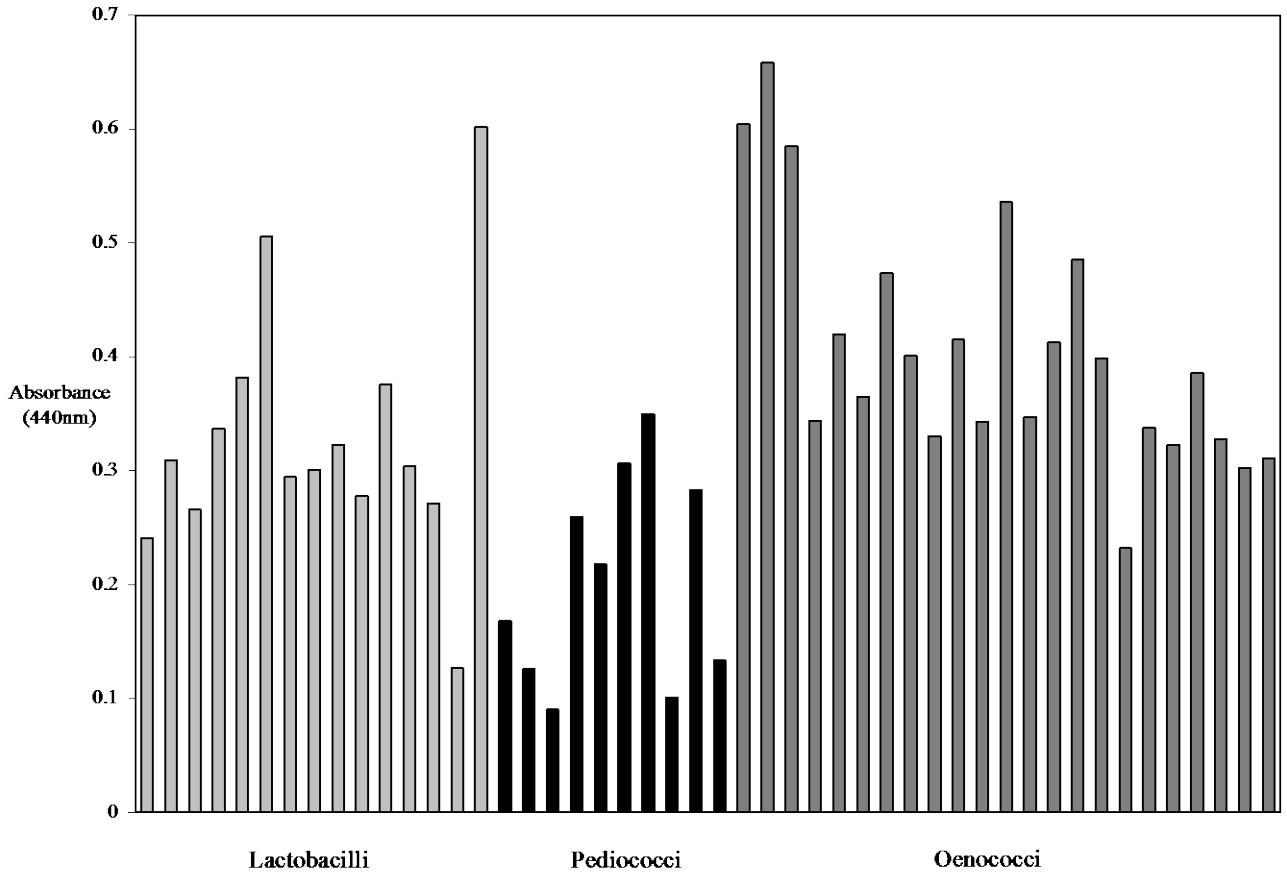


Figure 2.13 Tannase activity of lactic acid bacteria. Assays were performed as described in section 2.2.2. Values are the mean of duplicate determinations and in each case were within 5% of one another.

Three of the *Lactobacillus* strains (No. 25, 16, 15) were found to be able to hydrolyse tributyrin (data not shown). Interestingly, none of the *Pediococcus* or *Oenococcus* possessed lipase activity towards tributyrin. Numerous studies have previously reported lipase activity in *Lactobacillus* and *Pediococcus*, including those with activity towards tributyrin (Katz et al. 2002; El Soda et al. 1986).

Esters are qualitatively one of the most important groups of volatile compounds present in wine. Most esters are produced by yeast during alcoholic fermentation (Younis and Stewart, 1998; Lambrechts and Pretorius, 2000), but some originate from the grape berry (Rapp and Manderay, 1986) and some are formed via chemical esterification of alcohols and acids during wine aging (Etievant, 1991).

Numerous studies have compared the volatile profiles of wines before and after MLF and esters are among those compounds observed to change in concentration. The outcomes of this work suggest wine LAB are capable of both synthesising and degrading esters, although the mechanisms of such changes remains unclear. The esters identified in such studies include ethyl acetate (Maicas et al. 1999; Delaquis et al. 2000), isoamyl acetate (Laurent et al. 1994; Maicas et al. 1999) and ethyl lactate (Delaquis et al. 2000, Gambaro et al. 2001). In this study we sought to measure the activity of LAB against *p*-nitrophenyl-acetate, -butyrate and -octanoate. From Figure 2.14 it can be seen that activities were highly variable amongst and between genera and also according to the substrate used. Activities amongst *Pediococcus* were generally low, while highest activities were observed for *Oenococcus* strains. *Lactobacillus* were generally most active against *p*-nitrophenyl-acetate while for *Oenococcus* greatest activities were seen against *p*-nitrophenyl-butyrate. Generally, activities against *p*-nitrophenyl-octanoate were quite low, with the highest activity observed for *Pediococcus* strains.

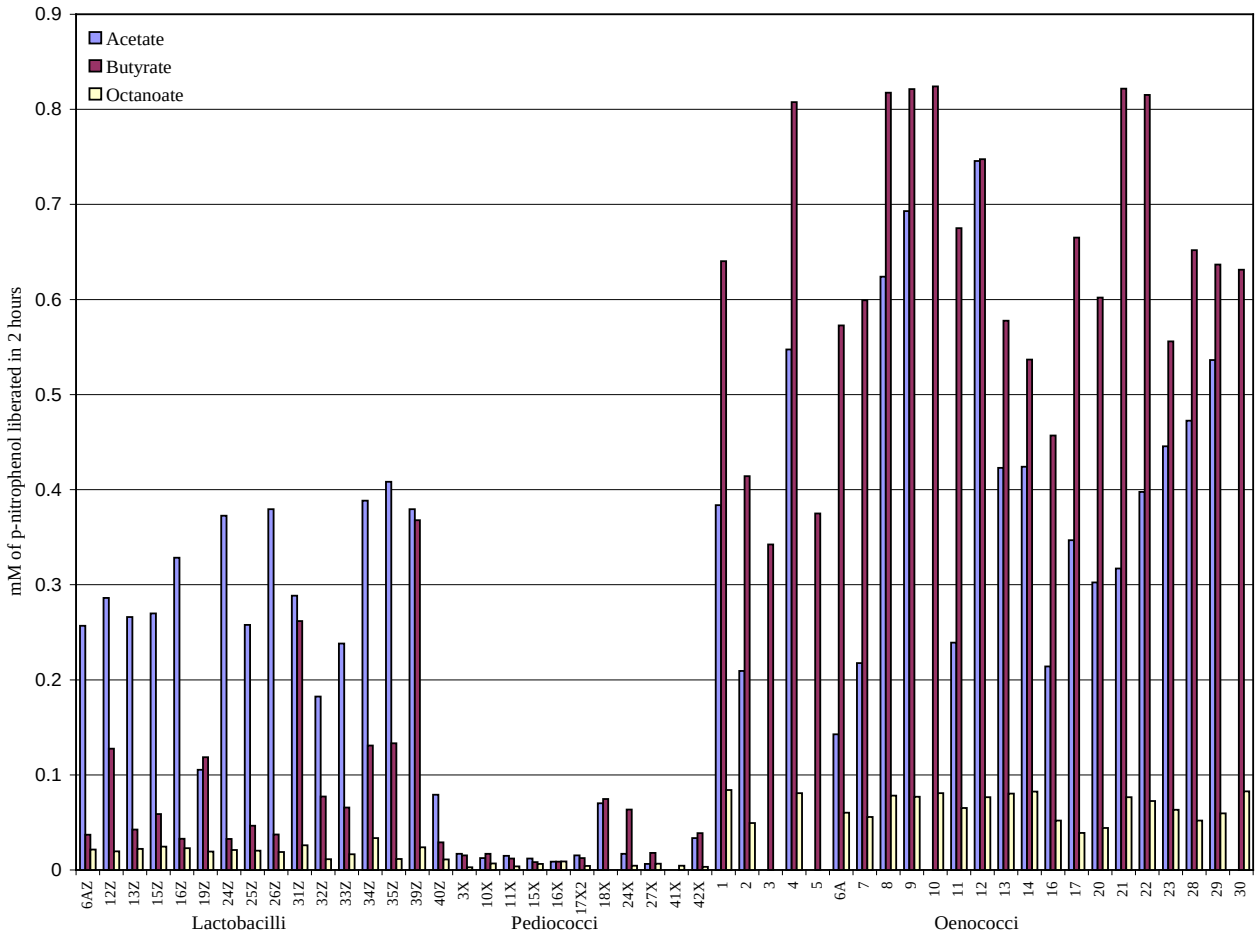


Figure 2.14 Esterase activity of lactic acid bacteria against three *p*-nitrophenol linked esters. Assays were performed as described in section 2.2.2. Values are the mean of duplicate determinations and in each case were within 5% of one another.

Given the wide distribution of esterase activities, particularly amongst *Oenococcus* strains, and their potential contribution to wine composition, attempts were made to clone structural genes for such enzymes as a prelude to their characterisation. Sequences of known esterase genes were found for other LAB and aligned with the *O. oeni* genome sequence. Four regions displaying homology to other LAB esterase genes were found in *O. oeni* (Table 2.5). PCR primers were designed against these regions, amplification reactions performed and the size of the products confirmed by agarose gel electrophoresis (data not shown). Such fragments were isolated, cloned and sequenced. Alignment of the region around the putative active site is shown in Figure 2.15.

Table 2.6 Details of the published esterase gene sequences used in this work.

Organism	Designated gene name in *O. oeni*	Reference
Lactococcus lactis	A2 A7	Fernandez et al. 2000
Lactobacillus casei	B28	Fenster et al. 2003a
Lactobacillus casei	C5	Fenster et al. 2003b

Alignment of the regions flanking the putative active site for each gene is shown below (Fig. 2.15). All putative active sites show similarity to the motif GXSXG commonly found in esterases (Jaeger et al., 1994). Work is now underway to introduce these sequences into *E. coli* for heterologous expression, purification and more detailed characterisation.

A2
YFDAIAHELPKVINNFFPNLSTKREKNFIA**GLSMG**GYGAYRLALGTDYFSYAASLSGVLTFDGME
YFDALTAELFQKVAFMFPEISQKREKHFVA**GLSMG**GYGAFKLAMSTDYFSYAASLSGALMHDFNF

A7
YFDAIAHELPKVINNFFPNLSTKREKNFIA**GLSMG**GYGAYRLALGTDYFSYAASLSGVLPKLYAW
YFDAISQELPRKVASLFPQISTKRKKHFVA**GMSMG**GYGAFKLAFSSSYFSYAASLSGTLPKLYAW

B28
GQIQDIFASLRWLSHFGPQRGFDLDHVLLT**GDSAG**GHLASLVACIQQSEELQELFGVDRVNFNFT
AALEDVNEMMNWLGNHVRDYGLDPQSVFVV**GDSAG**GQLAEQYLTILSNPDFKKLFDYNSPKLRIL

C5
HPKHPFTLENHIEDALAVISKLNLHNFVLY**GGSLG**SYIAEAVATKIPQDLSGLILNVSAAHNPAS
HPKKQFDLEDHINDGIELIK*SKLKDFVLY**GGSLG**SYIVQGTAVRIPEKINKLILNVSAAKNPDS

Figure 2.15 Alignment of amino acid sequences flanking the putative active site (bolded) of known (upper) esterase genes A2, A7, B28 and C5 (see Table 2.5) and the cloned (lower) esterase genes. Regions of homology are shaded.

OUTPUT 3 Identification of yeast genes or activities with positive impacts on wine quality or processing

Performance target:

> *3 – 6 mutations or rate-limiting enzymes that influence wine quality identified and characterised.*

Three times the planned number of candidate mutants have been generated by the contributors to this aspect of the project: Dr Michelle Walker (PDF), Jennie Gardner and Colin McBryde (PhD students), Maria Astorga (MSc student) and Tristan Warren (Honours student). Their areas of investigation come about from the fact that there are many aspects of winemaking that could be improved with the availability of yeast strains with enhanced qualities. For example, strains that more reliably complete fermentation under adverse conditions such as nitrogen and oxygen limitation. At present in the winemaking industry such limitations are overcome by nutrient additions, however, often the timing of such additions is critical and by-products, such as ethyl carbamate, can be undesirable. 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 nutrients, as we have previously shown with efficient use of nitrogen (UA 00/5). This approach can facilitate reliable completion of fermentation and minimise or eliminate the need for additives.

Methods of yeast strain improvement fall into two broad categories; recombinant and non-recombinant approaches. We have used a mixture of these techniques to harness the direct and precise application of recombinant approaches whilst also employing methods which are non-recombinant, as preferred by some consumers groups. The recombinant approaches used here involve direct gene disruption by transposon mutagenesis and directed DNA fragment targeting. The main non-recombinant approaches used here is adaptive evolution.

3.1 Recombinant Strain Improvement Strategies

3.1.1 Introduction

Where improvement strategies utilise recombinant techniques, the prototrophic and higher-ploidy nature of industrial strains precludes the use of traditional 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, from UA 99/1 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 that 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, added sequences are left near the target site and not in their native location (Estruch and Prieto, 2003).

Another system utilises the cre–loxP system (Güldner et al., 1999) yet excision still leaves behind foreign sequences (a single loxP site) and may increase the likelihood of chromosomal rearrangements between these and cryptic loxP 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 needs multiple PCR products (i.e. target-specific CORE and IROs) and two transformations.

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.

Through this project we also sought to more fully characterise and exploit recombinant strains and methods developed in earlier projects. Therefore we additionally aimed to:

1. Characterise strains constitutively expressing the specific proline permease gene, *PUT4*.
2. Utilise the transposon mutagenesis system from UA 00/5 as well as the haploid wine yeast derived from UA 99/1 to identify genes of importance to fermentation completion in a wine yeast background.

3.1.2 Methods

Bacterial and yeast strains and cultivation: The *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 *Saccharomyces cerevisiae* strains used are listed in Table 3.1. Strains TW1-4 were constructed from existing strains/plasmid using standard recombinant techniques (see below). Yeast strains were grown in YPD media (1% yeast extract, 2% bactopeptone, 2% D-glucose), which was solidified with 2% bacto-agar when necessary. Selective growth was achieved either through inclusion of geneticin (Astral Scientific) at 300 mg/L after autoclaving of the YPD medium or through use of the defined medium, Yeast Nitrogen Base (YNB), which lacked or contained the appropriate growth requirements. 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^{o}C. When required, asci were digested, spores dissected and mating carried out using standard classical genetic techniques (Guthrie and Fink, 1991).

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 3.2. Plasmid constructs and PCR products were verified by sequencing (IMVS, Adelaide). *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 3.2). 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 kanamycin (25 mg/L).

***Construction of plasmid pBS418HODR4 for integration at HO and excision of Kan^{r} marker*:** Primers HO1 and HO2 (Table 3.2) 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

Table 3.1 Strains of *Saccharomyces cerevisiae* used in this study.

Strain	Genotype	Source
W303	*ade2-1*, *can1-100*, *his3-11*,*-15 leu2-3*,-112 *trp1-1 ura2-1*, *MAT*a	Susan Henry
L2056	Commercial strain, presumed diploid, wildtype	Lallemand
CY3079	Commercial strain, presumed diploid, wildtype	Lallemand
KP2	Prototrophic derivative of W303, *MAT*α	This study
KP3	Prototrophic derivative of W303, *MAT*a	This study
Lys2 marker	*Lys2*, *MAT*α	Susan Henry
KP12	W303, *MAT*α□*put4::KanMX4*, *ura3::KanMX4*	Kate Poole
TW1	KP12 harbouring pFL38-PUT4	This study
TW2	KP12 harbouring pFL38-PUT4(–160)	This study
TW3	KP12 harbouring pFL38-PUT4(–160, S^{605}A)	This study
KP21	KP12 harbouring pTEF-PUT4	Kate Poole
TW4	KP12 harbouring pTEF-PUT4(S^{605}A)	This study
3-3a	Haploid derivative of L-2056 (*ho*Δ)	This study
41-3	Haploid derivative of L-2056 (*ho*Δ)	This study
1B	Haploid derivative of L-2056 (*ho::KanMX3,MAT*a)	UA99/1
1Ba	Haploid derivative of L-2056 (*ho::KanMX3, ura3::KanMX4, MAT*a)	UA99/1
C9	Haploid derivative of L-2056 (*ho*Δ, *MAT*a)	This study

region (+1965 to + 2030). The direct repeat sequences in the disruption cassette were derived from a 272 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 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 sec; 56.2°C , 45 sec; 72°C, 1 min) and terminated with a 10 min extension at 72°C. The product was purified using *UltraClean* kits (MOBIO 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 and 3' targeting sequence, within a 394-bp sequence adjacent to the *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. 3.1). 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 HO PIV and HO PIIrev, 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 HO PV (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. 3.1).

Table 3.2 Sequences of the primers used in this study.

Primer	**Sequence (5' → 3')**
HO1 [a, b]	TCATgagctcTGACAACATGGTAggcgcgccTGTCTCATGGAAATTGATGCAG
HO2 [a, b]	TTGGggtaccACAATTAAAATATTTGTTTTTCTTTCGCGTAAATATTTAGCTTG ATAACTGTTACTGATAgttaacGTGGACATGACATGAATTTTCGC
HO PIIrev [a, b]	TATTTACATCAACATATCGCGAAAATTCATGTCATGTCCACgttaacATATCA GTAACAGTTATCAAGCTA
HOPCRrev [b, c]	TCAGCAGATGGGTCTTCGAT
HO PIV [a, b]	GTGAACGTggtaccTGTAAAGAAAACACTTTGTTGGGTG
HO PV [a, b]	AGATGgtcgacCCAAGCTGAAGAGTTCAAGGC
HO PVI [b]	TGAAGAGTTCAAGGCTGCCGATG
HO PVIII [b]	GCTGGCATCATTGGGATGTTC
HORga [a, b]	CAAACCTAATGTGACCGTCGCT
HOFga [a, b, c]	TGAAGCTGTTGCTTACGGTGC
HO Fwd [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.

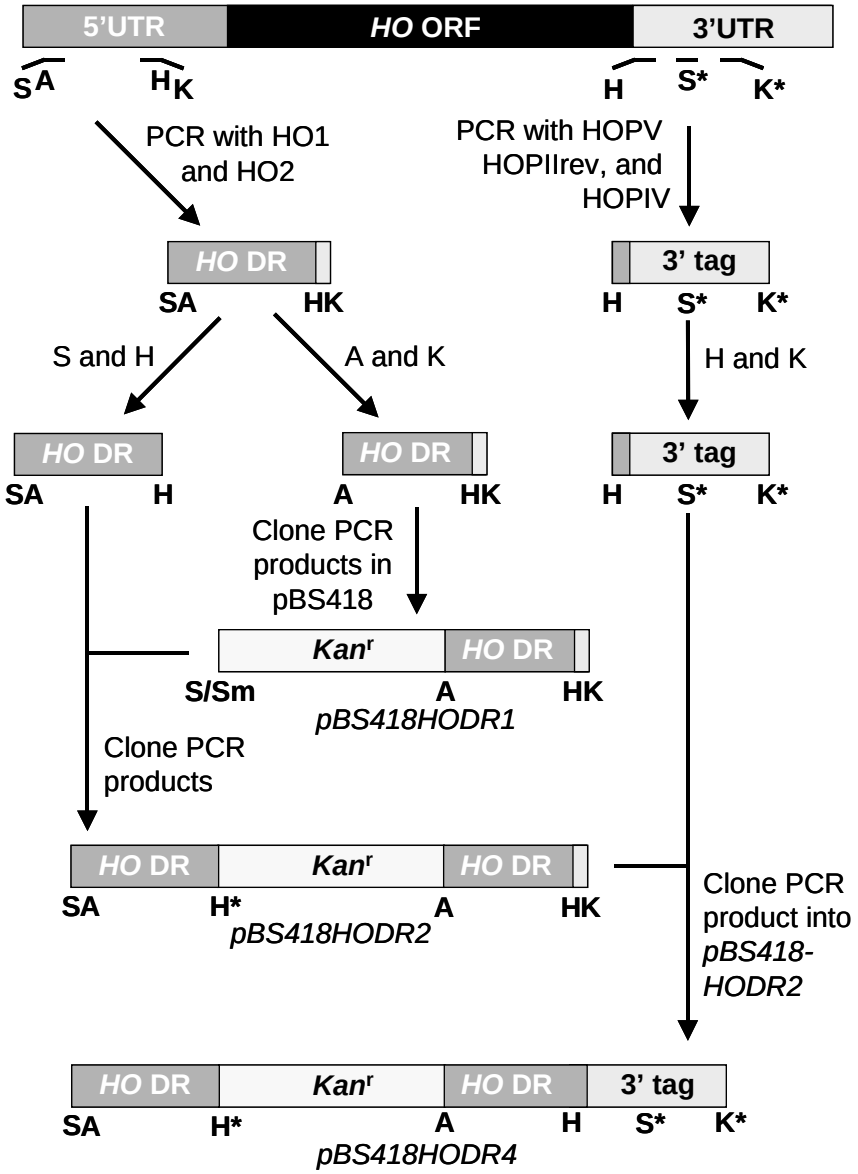


Figure 3.1 Construction of *HO*-disruption cassette (*hodr-Kan*^{r}-*hodr*) in plasmid pBS418HODR4. *HO* ORF is indicated in black, the direct repeat sequences (-670 to -399) amplified from the 5' untranslated region (-724 to -368) are in mid-grey, whilst the 3' tag or targeting sequences of 65 bases (+1965 to + 2030) or 619 bases (+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]).

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.

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 2.3 kb (*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).

Kan^r ***marker loss and deletion of the*** **HO** ***gene:*** Haploid (*ho*) derivatives were identified, following selection of geneticin sensitive sporulation products, and subsequent mating type determination (Huxley et al., 1990). To determine the sequences retained at the disruption/excision site was the region was sequenced. The *HO* genes from the parental wine strains and haploid (*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 3.2). Sequence analysis was performed using the BLAST program, the sequences being compared with laboratory strain S288c (http://genomewww.stanford.edu/Saccharomyces/; or http://www.ebi.ac.uk/Tools/).

Proline accumulation assay**:** YNB media containing 150 g/L glucose, 1 g/L proline, and 0.427 mM [^{14}C]-proline (Amersham Pharmacia, CFB71), was inoculated with yeast cells at 1 x 10^6 cells/ml. Samples (500 µl) taken at 0, 3, 6, 9, 12 and 24 hour intervals were harvested on 0.22 µm nitrocellulose filters, washed twice with sterile deionised water, before addition of 4 ml of scintillation fluid (Starscint, 6013248). [^{14}C]-proline incorporation was counted for one minute and determined as counts per min (CPM) using a Packard scintillation counter. Values were normalized (CPM/mg cells) by dry weight determination of a duplicate sample at the time of sampling.

Evaluation of osmotolerance and thermotolerance: Osmotolerance was assessed by preculturing strains in YNB containing 1 g/L proline as nitrogen source, transfer at 5 x 10^6 cells ml^{-1} to YNB with 64.85 g/L NaCl or 202.26 g/L sorbitol, followed by growth for 24 hours. Growth as turbidity units (NTU) was measured over 24 hrs and viability (colony forming units) at the end of the growth period. To determine thermotolerance, precultured cells were washed in fresh media and subjected to heat, (20 min, 45^{o}C). Viability was measured prior to cells being transferred at 5 x 10^6 cells ml^{-1} to YNB plus 1 g/L proline, and growth monitored as turbidity units (NTU).

Transposon mutagenesis for identification of genes in a wine yeast background: The transposon mutagenesis and screening methodologies established through UA 00/5 was utilise here to screen for potentially useful genes in a haploid wine yeast background, namely strain 1Ba. Screening fermentations were conducted using a chemically define grape juice medium (CDGJM, Henschke and Jiranek, 1993) which contained a mixture or amino acids and ammonium. While the proportions of these nitrogen sources were maintained, the total amount was varied according to the experiment in question.

Fermentations and Analysis: Fermentations and quantitation of key metabolites was carried as described in Walker et al. (2003). Residual nitrogen content was determined initially using NOPA (Dukes and Butzke, 1998) but subsequently by quantitation of individual amino acids by LC-MS analysis (EZ:faast®, Phenomenex, CA, USA).

3.1.3 Results and Discussion

Gene-disruption cassette for seamless modifications in* S. cerevisiae*: Originally we aimed to design gene-specific disruption cassettes possessing 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. 3.1). Changes to overlapping fragment lengths and direct repeats were required to achieve correct targeting, spore viability and expected segregation patterns.

Southern blot analysis confirmed correct localisation of the disruption cassette to the *HO* locus in transformants of L2056 and CY3079 (Fig. 3.2). Haploid (*ho::Kan*^{r}) derivatives of geneticin resistant *HO*/*ho::Kan*^{r} transformants were isolated after sporulation and dissection, with some variation in spore numbers and viability being noted (Table 3.3).

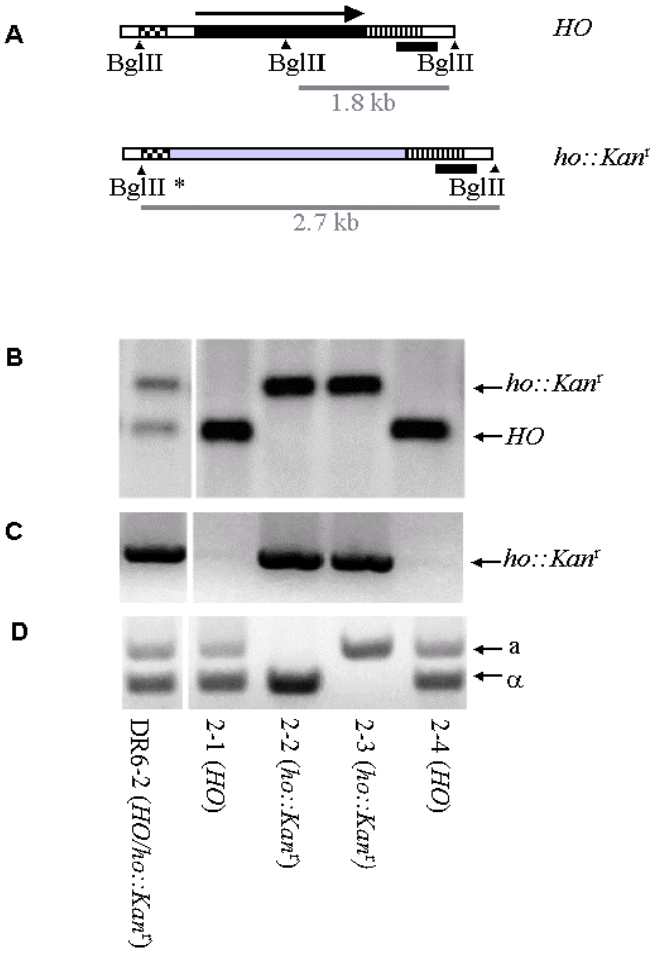


Figure 3.2 Characterisation of *HO*-disruptants by Southern blot and PCR analysis. Block diagram (panel A) depicts the main features of the *HO* and *ho::Kan*^{r} alleles on the chromosome locus. The 5' and 3' *HO* untranslated sequences (open bars), *HO* open-reading (black) and *Kan*^{r} sequence are indicated (grey). The construct has a 5' targeting sequence with single direct repeat generated from the 5' untranslated region (stippled) and a 3' tag or targeting sequence (striped). The direction of *HO* gene transcription is shown (arrow), and solid bars indicate the 671-bp *HO* probe. Labelled (1.8 kb and 2.7 kb) solid grey lines indicate the fragments from the *HO* and *ho::Kan*^{r} alleles upon *Bgl*II digestion and yielding the banding pattern in the Southern blot (panel B) of genomic DNA from ho-disruptant strains hybridised with the 671-bp *HO* probe. The engineered *Sac*I site in the 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 is shown by a 786-bp PCR product using *Kan*^{r} specific primers G418Fwd and G418Rev (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).

Table 3.3 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 *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.

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 (Fig. 3.3). 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 by sequencing and through parallel laboratory fermentation trials.

Sequencing confirmed the loss of the *Kan*^{r} gene and a single DR, and provided the precise nature of any residual sequences (Fig. 3.4). The engineered *Sac*I and *Kpn*I sites were at the 5′ and 3′ ends of the 2.6 kb (hodr–Kanr–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 *ho*Δ strains (Fig. 3.2 and Fig. 3.3). The size of the hybridising band observed in strain 3-3a (*ho*Δ) 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 of foreign sequence) 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.

Classical genetics was used to obtain haploid Δ*ho* derivatives of each mating type for both strains. Findings for derivatives of each strain were comparable, therefore only data for derivatives of L2056 are reported here.

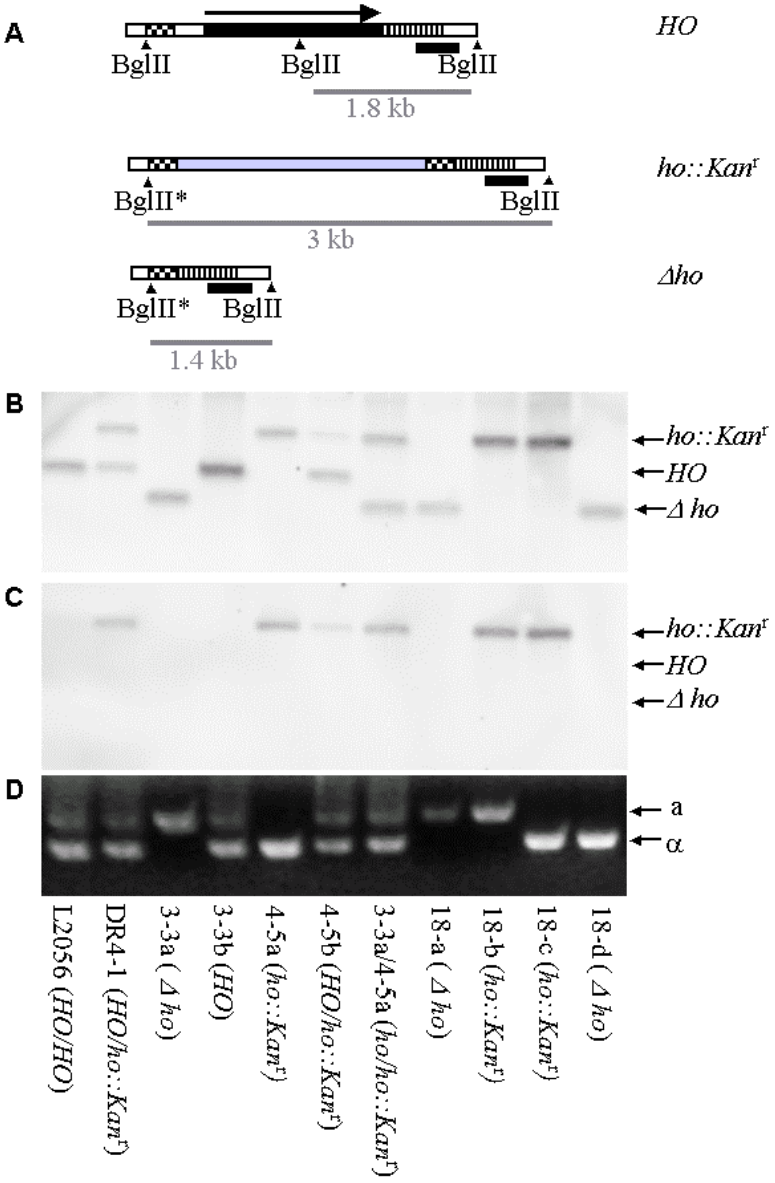
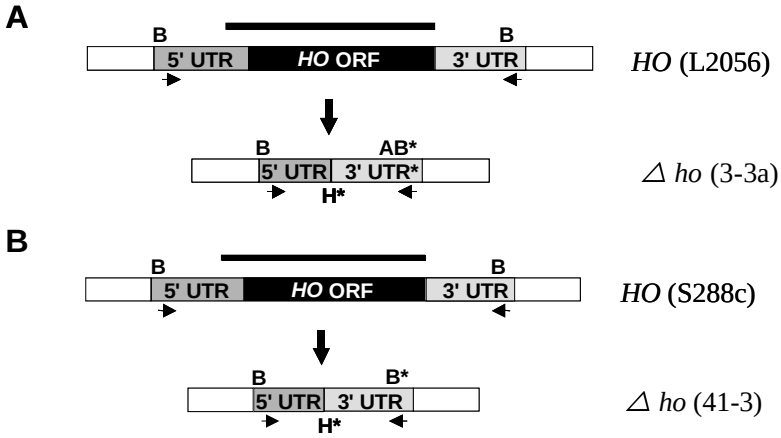


Figure 3.3 Characterisation of haploid *ho::Kan*^{r} and *ho*Δ strains by Southern blot and PCR analysis. Block diagram (panel A) depicts the main features of the *HO*, *ho::Kan*^{r} and *ho*Δ alleles on the chromosome locus. The 5' and 3' *HO* untranslated sequences (open bars), *HO* open reading frame (black), and *Kan*^{r} sequences (grey) are indicated. Direct repeat sequences generated from within the 5' untranslated region (stippled) and the 3' tag or targeting sequence (striped) are shown. 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 *ho*Δ alleles upon *Bgl*II digestion yielding the banding pattern seen in the Southern blot (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 MATα (554-bp) or MATa (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.



C

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3-3a  CAAACCTAAT GTGACCGTCG CTGATTAGCG GATCACGAAA AGTGATCTCG ATACAATTAG AGGATCCACG AAAATGATGT GAATGAATAC ATGAAAGATT   100
41-3  CAAACCTAAT GTGACCGTCG CTGATTAGCG GATCACGAAA AGTGATCTCG 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 TGTGTCACGA AAAGTGAGGT AACTAAATAC ACGATTACCA TGGAAATTAA CGTACCTTTT TTGTGCGTGT ATTGAAATAC   300
41-3  TAAGGGCAAA TGTATTCATG TGTGTCACGA AAAGTGAGGT AACTAAATAC ACGATTACCA 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 GACAACTCTC 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 CTACCAACAG CGTTAGAGAT AGTGATGTTA GAAGACTTAC CGGTAGACTT TTCGACGGCA GTAACCTTCA AGATACCGTT  1000
41-3  CAATTTCTTC AGAAGACAAT CTACCAACAG 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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Figure 3.4 DNA sequence analysis of the *HO* gene after loop out of *Kan*^{r} and direct repeat (DR) sequences. Block diagrams show the sequences retained at the *HO* locus after gene disruption and marker excision by homologous recombination in the commercial L2056 strain (panel A) and the laboratory S288c strain (panel B). Indicated are the deleted *HO* sequence(solid line above the native *HO* gene), *HO* ORF (black), the 5' untranslated region (mid-grey), the 3' untranslated region (light grey), flanking sequences (white). The *Bgl*II sites recognized in Southern blot analysis (Fig. 3.3) 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 (arrows) are also shown. Panel C shows the DNA sequence of the *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 (in bold and underlined) are the *Asc*I site (GgCGcGcc) engineered in the *HO* sequence through Primer HOP1, 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 (underlined lower case) and nucleotide changes (lower case) are indicated

Characterisation of constitutive proline transport strains: Proline is often the predominant amino acid in grape juice (Henschke and Jiranek, 1993), yet it is poorly utilised during winemaking (Ingledew and Kunkee, 1985). The utilisation of proline by *Saccharomyces cerevisiae* is a process reliant on the oxygen dependant conversion of proline to glutamate in a two-step process, within the mitochondrion (Krzywicki and Brandriss, 1984; Wang and Brandriss, 1987). The intracellular levels of proline are in part dependent on the activity of the transport systems for proline. These are tightly regulated since proline is itself a poor nitrogen source, requiring more energy for catabolism than other, preferred nitrogen sources. Uptake is mediated by the high affinity, proline specific permease Put4p, and the general amino acid permease, Gap1p (Horak and Rihova, 1982), although two SPS sensor regulated permeases, Agp1p and Gnp1p, have recently been shown to facilitate proline uptake (Andréasson et al., 2004). The regulation of the *GAP1* and *PUT4* gene products occurs at the transcriptional and post-translational level in response to the quality of the available nitrogen (Courchesne and Magasanik, 1988). Proline transport into the cell occurs only in the presence of poor nitrogen sources, being maximal during early exponential growth, or when the cells are nitrogen starved. In an earlier study (ARC-SPIRT project No: C09943027) strains capable of constitutive transport of proline were constructed in an effort to allow greater of utilisation of proline, particularly in the early stages of fermentation when oxygen may still be present to enable proline catabolism. The goal being to make fermentation of low nitrogen juices more reliable by allowing yeast to utilise an otherwise little exploited source of nitrogen.

Constitutivity was imparted through the use of constitutive heterologous promoters or specific mutations in the promoter (i.e. at position –160), while longevity of the permeases was increased by modification of the coding sequence (Poole, 2002), which was speculated to alter the degree of ubiquitination of the permease and its degradation in the presence of preferred nitrogen sources (Stanbrough and Magasanik, 1995; Roberg et al., 1997; Soetens et al., 2001). Site-directed mutagenesis targeted amino acid residues in the C-terminal region of Put4p that are potentially involved in the ammonia-induced degradation of the permease. From our previous study (Poole, 2002) we observed that the substitution of alanine for the serine residue at amino acid position 605 (designated $S^{605}A$), led to the amelioration of ammonia-induced down regulation of Put4p, compared to the wild type, with an increased restoration of activity upon recovery. Through the present study we sought to more fully characterise strains bearing these modifications, particularly in the context of properties with potential benefit to winemaking.

Figure 3.5 confirms the increased accumulation of proline by strains over-expressing *PUT4* or a variant thereof. TW2 constitutively expressing *PUT4* by virtue of a point mutation in its promoter region took-up marginally more proline than TW1, which harbours a plasmid-borne copy of native *PUT4*. Increasing *PUT4* expression with a stronger *TEF* promoter or stabilising the permease through the $S^{605}A$ modification increased proline uptake dramatically. Possession of plasmid pTEF-PUT4 $S^{605}A$ or the enhanced uptake of proline enabled by it, lead to an altered cell morphology in TW4 (Fig. 3.6). The precise significance of increased cell size in TW4 is yet to be established, but an influence on fermentation kinetics might be expected (Salmon, 1997). Altered proline

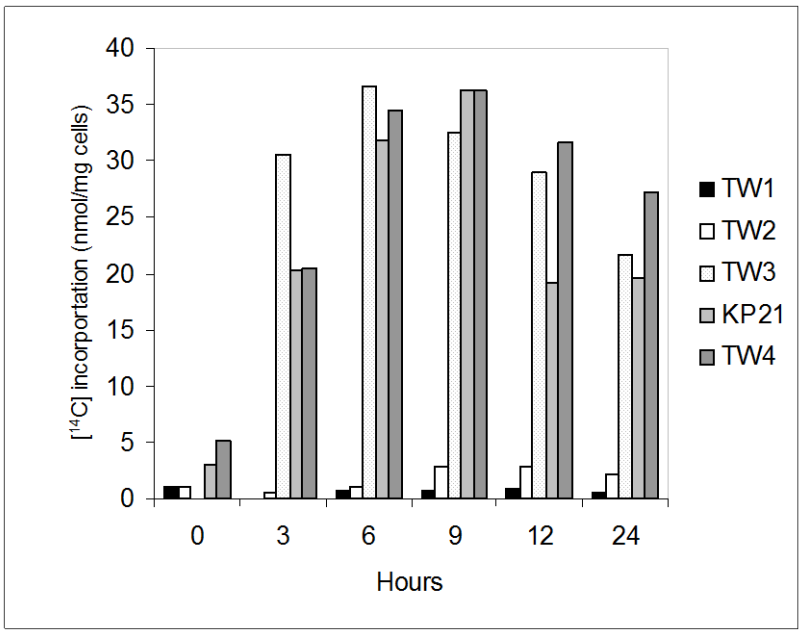


Figure 3.5 Proline accumulation by wild type and constitutive *PUT4* strains. YNB media with ammonium sulphate (5 g/L), proline (1 g/L) and 0.427 mM [^{14}C]-proline was inoculated at 5 x 10^6 cells ml^{-1} and incubated at 30ºC. Incorporation of proline (as [^{14}C] radioactivity) was measured in duplicate at 0, 3, 6, 9, 12 and 24 hrs. Strains TW1 (pFL38-PUT4), TW2 (pFL38-PUT4(–160)), TW3 (pFL38-PUT4(–160, $S^{605}A$)), KP21 (pTEF-PUT4) and TW4 (pTEF-PUT4 $S^{605}A$).

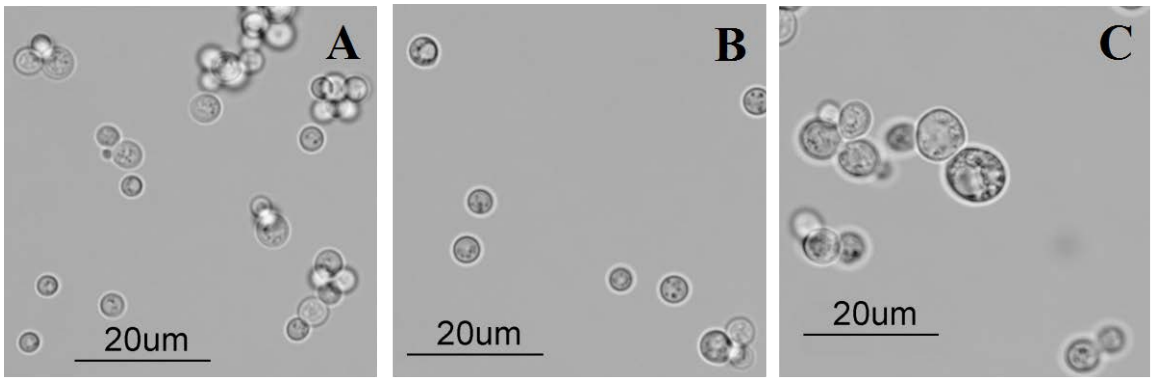


Figure 3.6 Cell morphology of wild type and constitutive *PUT4* strains grown in proline. Strains: TW1 harbouring pFL38-PUT4 (panel A), TW3 harbouring pFL38-PUT4(–160, $S^{605}A$) (panel B) and TW4 harbouring pTEF-PUT4 ($S^{605}A$) (panel C).

uptake did have a significant impact on culture viability following exposure to osmotic or temperature shock. Thus viabilities of the wildtype strain, TW1, ranged between 33 and 40% for the three treatments (Table 3.4). By comparison, the range in viability of all constitutive-*PUT4* strains was between 34 and 64%. Strains in which *PUT4* expression was being driven by the *TEF* promoter, viabilities were typically greater than 50% and up to 64%. The only exception being TW3 during growth in a medium containing sorbitol (i.e. 34%). We next sought to determine whether such enhancement of tolerance to various forms of stress translated into benefits during fermentation. Ideally this phase of work would have been performed using the wine yeast background, as previous studies have shown strains with a W303 background to be poor fermenters. Time

constraints on the Honours student involved in the project meant that only existing strains were used.

Table 3.4 Percentage viability of wild type and constitutive-*PUT4* strains exposed to osmotic stress[a] or heat shock[b].

	TW1	TW2	TW3	KP21	TW4
Sorbitol	38.67 ± 6.23	47.00 ±14.57	33.67 ± 3.84	48.67 ± 3.84	**50.67** ± 2.33
NaCl	32.67 ± 6.74	40.00 ± 1.53	**63.67** ± 5.36	43.33 ± 8.51	**60.00** ± 8.50
Heat	40.00 ± 3.61	38.67 ± 7.13	**63.67** ±10.75	47.33 ± 7.45	**60.00** ± 2.65

Values are the average mean values (± SEM) of triplicate determinations of cell viability. Those of significance are bolded. [a] 20 min at 45°C. [b] 24 hrs in YNB containing 64.85 g/L NaCl or 202.26 g/L sorbitol.

Two points of interest can be made in relation to the observed fermentation kinetics (Fig. 3.7). Firstly, the fermentation rates are generally poor as was expected for a derivative of W303. By comparison, a wine yeast derivative is able to complete a similar fermentation in approximately 70 hours. Secondly, there are no obvious differences between the strains. While it is possible the differences in viability and stress tolerance may be more obvious in the latter stages of fermentation, such comparisons would again be best performed in a wine yeast derivative, which is better able to progress a high-sugar fermentation to these latter stages, thereby enabling the comparison to be made. Further investigation of the merits of these strains will be incorporated into UA 05/01 should a suitable Honours student be identified. Included in such a study would be an assessment of fermentation reliability under conditions of nitrogen limitation and proline availability.

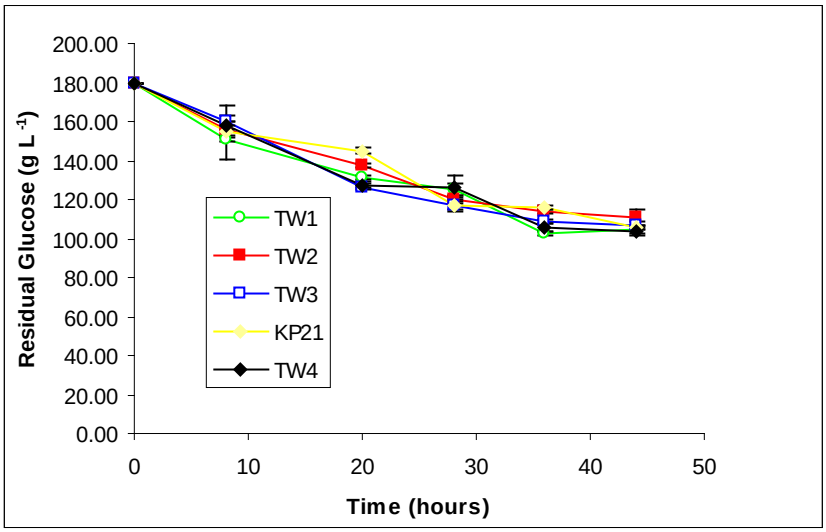


Figure 3.7 Residual glucose concentrations through 44 hours of fermentation by wild-type and constitutive-*PUT4* strains. Data points represent the mean of single determinations of triplicate cultures ± SEM.

Generation and evaluation of mutants with enhanced nitrogen efficiency: Through project UA 00/5 we applied a transposon mutagenesis system and developed a method for rapid screening of large numbers of mutants in order to identify genes which impact the efficiency with which assimilable nitrogen is used by yeast. Mutants displaying improved qualities 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.

A total of some 15,000 transposon mutants have been introduced into haploid yeast and screened. Of this number, an initial 10,000 were produced in a laboratory yeast [as part of project UA 00/5] and screened in media containing ammonium as sole source of nitrogen. A detailed report on this component of the work has been published (Gardner et al., 2005). In parallel work, 5,000 transposon mutants were created in the background of a haploid wine yeast derivative, strain 1Ba (derived from L2056, from project UA 99/1) and screened in a more wine-like environment: CDGJM containing nitrogen as a mix of amino acids and ammonium.

After their initial isolation, the efficacy of the transposon mutants was confirmed in subsequent fermentation trials in nitrogen deficient media (data not shown). We also determined the extent to which the maximum nitrogen demand of these strains was altered. Thus transposon mutants were cultured in a medium of moderate nitrogen content (225 FAN/L supplied as a complex mix of amino acids and ammonium) and the residual nitrogen after the catabolism of all sugars determined by the NOPA method. Strain 1B was used as the control in this experiment because like strain 1Ba, it is a haploid derivative from L2056, but it lacks any auxotrophic markers, which might otherwise complicate determination of nitrogen requirement. The parental strain left 3 mg FAN/L whereas all transposon mutants, except qq23, left a larger residual, ranging from 4 to 20 mg FAN/L (Fig. 3.8). In light of findings supporting the presumed inverse link

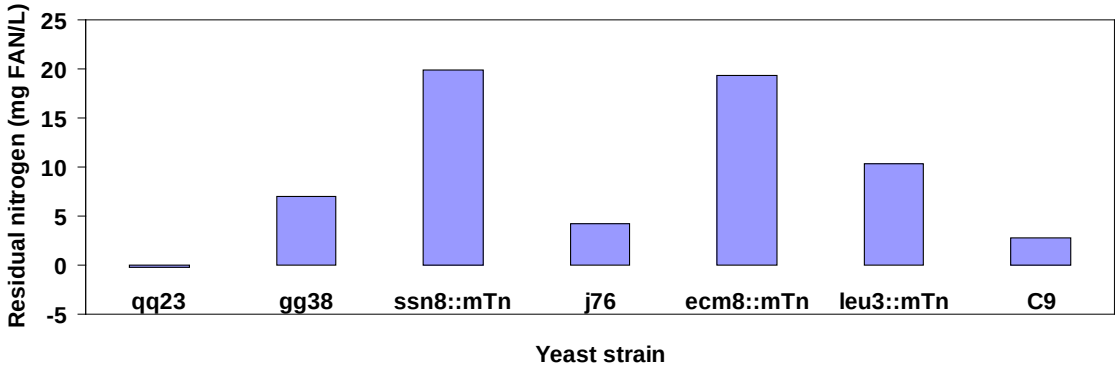


Figure 3.8 Residual nitrogen after fermentation by selected transposon mutants, of CDGJM with 225 mg FAN/L supplied as a complex mix of amino acids and ammonium. Strains are identified by their systematic isolate number or else the target gene, where this has been determined. Nitrogen was measured using the NOPA method.

between fermentation reliability and maximum nitrogen requirement (Jiranek et al., 1992; Gardner et al., 2002), mutants such as *ssn8::mTn* and *ecm8::mTn* might prove to be most effective in fermenting low nitrogen juices. We sought to investigate the nature of the residual nitrogen of all strains with the aim of determining whether differences were due to altered utilisation of specific individual or groups of amino acids.

To achieve this, a new method for quantitation of amino acids was successfully adapted for use with fermentation samples (data not shown). In this way, quantitative and qualitative similarities amongst the mutants were revealed. In keeping with its poor utilisation during growth under oenological conditions (as discussed above) proline was the predominant residual amino acid in all cases (Fig. 3.9). Interestingly, the amounts of proline remaining did vary. Assuming that proline catabolism was minimal due to the near-anaerobic conditions used, such differences may indicate different amounts of direct incorporation of proline. Other abundant residual amino acids were arginine, glycine and histidine, in that order. As has been noted previously (Jiranek et al., 1995a), differences between strains in the amount of nitrogen utilised are not easily attributed to differences in the use of individual amino acids. Rather differences arise out of a proportional change in the extent of utilisation of all amino acids.

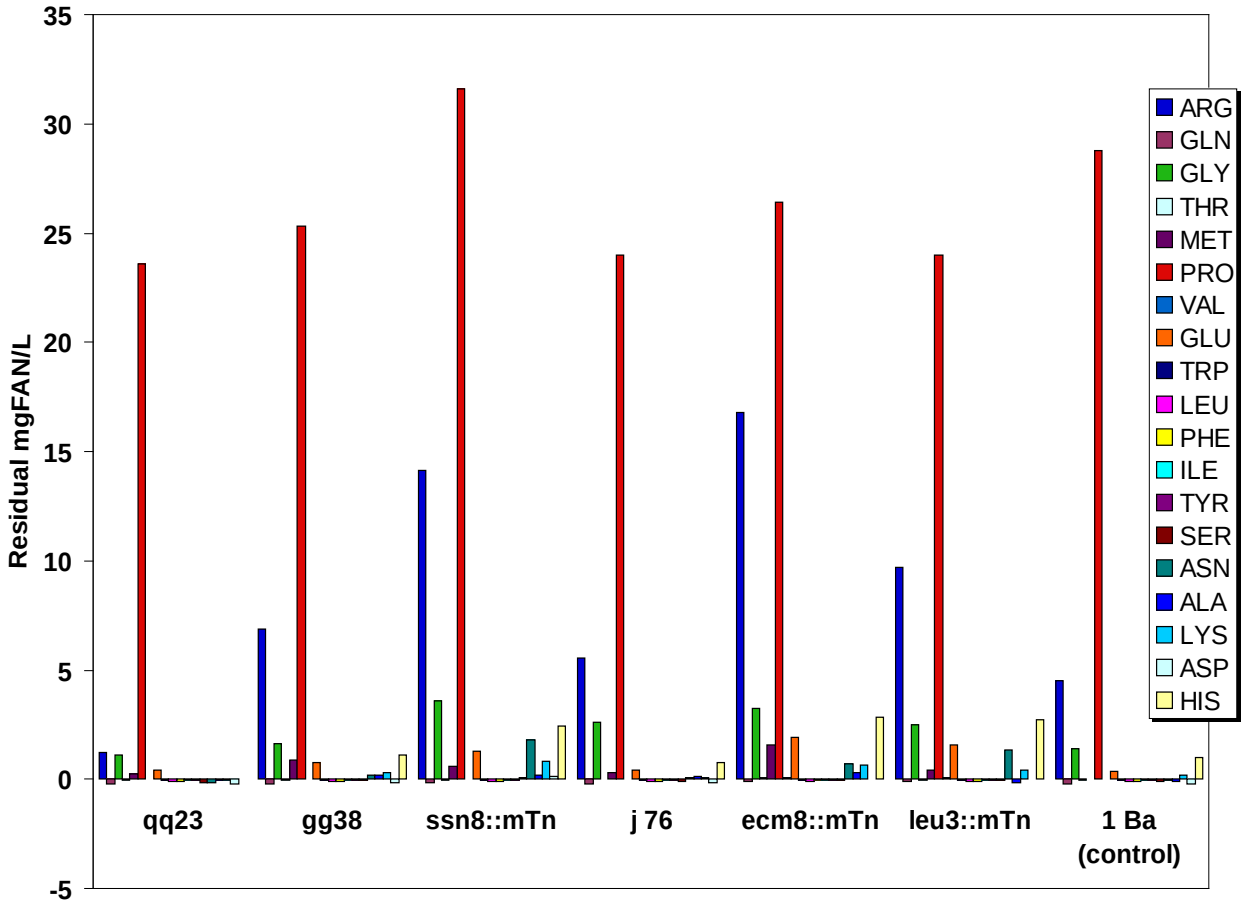


Figure 3.9 The concentration of residual amino acids as measured by LC/MS after fermentation by selected transposon mutants. The media was CDGJM with 225 mg FAN/L supplied as a complex mix of amino acids and ammonium (Jiranek and Henschke, 1993).

In characterising the consequences of such differences in nitrogen metabolism, it must be remembered that a modified utilisation of amino acids has the potential to influence final wine aroma. Amino acids are an important source of volatile wine components such as higher alcohols (Crowell, et al., 1961; Sablayrolles and Ball, 1995; Guitart, et al., 1999).

Having demonstrated that further characterisation of the transposon mutants is justified, the affected genes were identified and inturn disrupted in the wine yeast derivative C9 as detailed previously (Gardner et al., 2005). Such deletants were characterised together with others collected from the screening in the laboratory yeast background, to see how they performed in nitrogen-limited fermentations. Genes of interest include *GID7*, *NGR1* (as reported in UA 00/5), *PUT4*, *LEU3*, *ECM33*, *SSN8*, *PTP1*, *MIP6*, *YDL133W* and *YNL134C*. Deletion mutants of each of these genes have been constructed in both laboratory and wine yeast strains. The fermentation dynamics of a number of these strains have been elucidated under laboratory conditions, and the rest are currently under investigation. A set of these mutants were isolated in a screen dedicated to selecting mutants when only very low (55 mg FAN/L) nitrogen, supplied as a complex mix of ammonia and amino acids, was present. Fermentation profiles of the corresponding gene deletants are shown in Figure 3.10. The more rapid fermentation by all deletants, except *ynl134c* is apparent. This finding suggests that the phenotype observed in the original corresponding transposon mutants arose because of a loss of function of the gene in question. Further work is now underway to further characterise the function of these gene products, particularly their role in nitrogen metabolism.

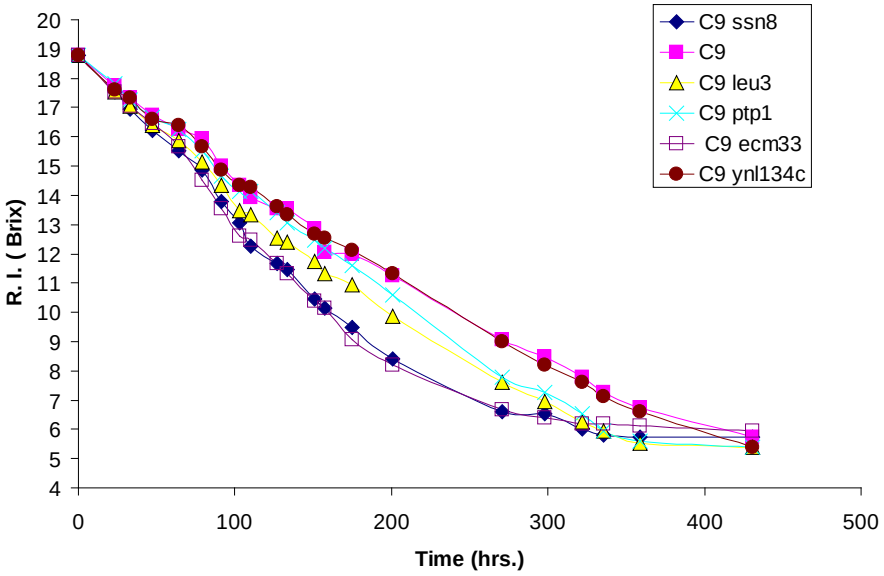


Figure 3.10 Sugar utilisation (estimated by refractive index) by selected mutants in comparison to their parent strain C9. Fermentations were performed in CDGJM with 55 mg FAN/L, supplied as a complex nitrogen source of amino acids and ammonium.

Two genes which have been investigated in some detail are *NGR1* and *GID7*. The most rapid fermentation of a single gene deletion was observed when *NGR1* was deleted from C9, a derivative of the wine yeast strain L2056 (report on UA 00/5). When this strain is grown in a medium of limited, mixed nitrogen, 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 a laboratory yeast also significantly enhanced fermentation, increasing the total glucose catabolised by up to 71% of the parent strain. Unfortunately, so far it has not been possible to produce a wine yeast derivative strain with a combination of both deletions. These efforts are continuing in project UA 05/01.

In the present study we have also begun to investigate other possible effects of manipulation of *NGR1* and *GID7* expression. We have employed Real Time PCR to quantify expression of a number of selected genes. So far, this analysis suggests expression of genes of central nitrogen metabolism (Fig. 3.11) is altered in *GID7*/*NGR1* deletants thereby shifting nitrogen distribution in the cell. For instance deletion of *GID7* causes the gene encoding NAD-dependent glutamate dehydrogenase to be expressed 17 times higher than in the parent strain (Fig. 3.12). The precise benefit of such a proposed shift is yet to be determined. Work is currently underway to determine if the increased expression of *GDH2* is reflected by an increased enzymatic activity and thus modified metabolites of central nitrogen metabolism.

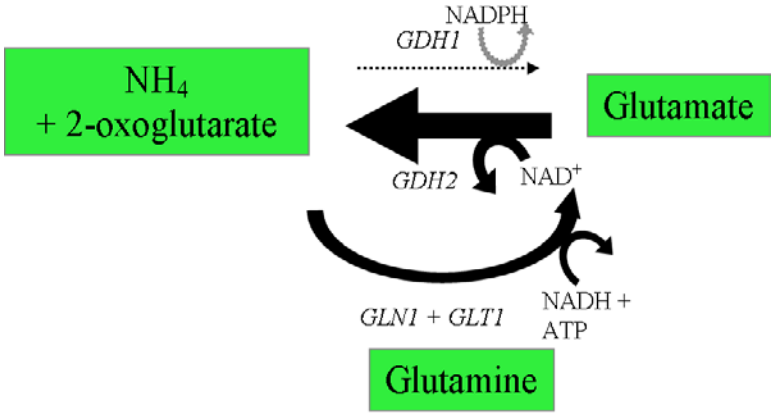


Figure 3.11 Schematic depiction of the alteration of expression of genes (italics) in Central Nitrogen Metabolism. The situation shown is late in fermentation (CDGJM; 75 mg FAN/L) and is based on findings from real-time PCR for *ngr1* and *gid7* strains (Fig. 3.10). Transcript abundance relative to parent strain is either higher (thick arrows) or lower (dotted arrow). *GDH2* up-regulated in KP2 *gid7* only.

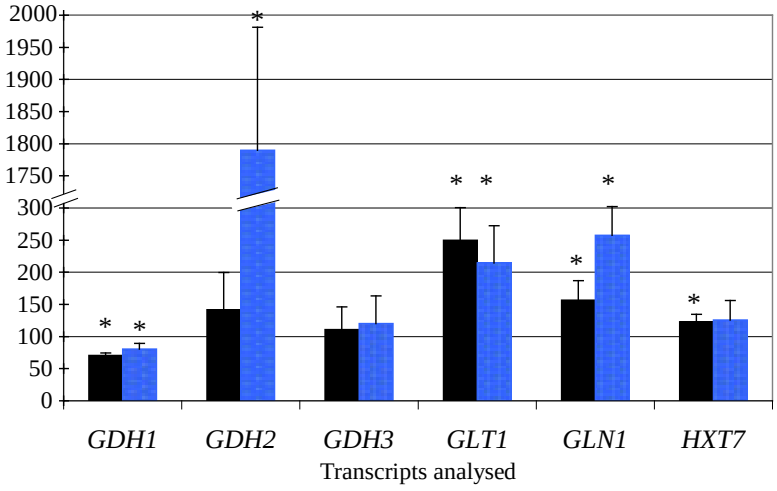


Figure 3.12 Abundance of six transcripts detected by quantitative real-time PCR near the end of fermentation (87.5 % of glucose catabolised) from KP2 *ngr1* (■) or KP2 *gid7* (■), represented as a percentage of that found from in the parent strain KP2. The initial fermentation medium was CDGJM with 75 mg FAN/L supplied as ammonia.

3.2 Non-recombinant Strain Improvement Strategies

3.2.1 Introduction

The construction of optimised strains using the recombinant methods detailed above offers precision and helps highlight the possibilities for strain optimisation. However, some sections of industry and consumer groups are opposed to the use of genetic engineering in foods and beverages. We have therefore also included non-recombinant approaches in our efforts to improve and tailor wine yeasts. An approach which has yet to be widely utilised in development of optimised industrial yeast is adaptive evolution. We have investigated this methods for it suitability for use with wine yeast. In addition, we have used classical genetics and traditional breeding in order to generate novel strains.

Adaptive evolution defines a set of mutations that occur in response to a specific challenge and which are advantageous to the cell under these conditions (Foster, 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 due to natural selection. Adaptive evolution has the advantage of not necessarily requiring previous genetic modification of the strain of choice, application of a complicated method to identify desirable derivatives nor knowledge of the genes involved in the attribute. Furthermore, it has been theorised that the increased ploidy (i.e. aneuploid and polyploid) of wine strains could be well suited to adaptation through this technique (Querol, et al., 2003; Bakalinsky and Snow, 1990). Consequently, adaptive evolution is likely to offer a versatile non-recombinant means for optimising wine yeast.

Classical genetics with wine strains can be difficult because of the lack of selectable markers, poor sporulation rates, incomplete tetrads, low spore viability and mating type switching in spores (Walker et al., 2003). We sought to exploit the tendency of wine yeasts to rediploidise after sporulation and dissection as a means of highlighting any naturally occurring heterogeneity in these strains (e.g. see Takahashi et al., 1980). Such isolates with distinct properties were then to be used as part of a breeding program to generate strains with novel attributes or else new combinations of attributes.

3.2.1 Methods

Strains and cultivation: The strains used in this section of the study are listed in Table 3.5 and were maintained and grown as detailed in Section 3.1.2.

Table 3.5 Strains of *Saccharomyces cerevisiae* used in this study.

Strain	Genotype/properties	Source
L2056	Commercial strain, presumed diploid, wildtype	Lallemand
C9	Haploid Haploid derivative of L2056 (*ho*Δ, *MAT*a)	This study
FM5	Adaptively evolved derivative of C9	This study
FM16	Adaptively evolved derivative of L2056	This study

Adaptive evolution experiments: A starter culture was grown aerobically for 24 h at 30ºC 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 media (CDGJM; Henschke and Jiranek, 1993) in sequential batch fermentations in bench top fermenters (2 L, B Braun Biotech, Melsungen, Germany). The formulation of CDGJM used here differed from that originally reported in that it contained glucose and fructose (each at 100 g/L to replicate carbon sources available in a commercial wine fermentation) and ammonium sulphate (580 mg free amino nitrogen/L) as the sole nitrogen source (to eliminate mutants with amino acid auxotrophies which would be impractical in industry). 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 5×10^6 cells/ml and the inoculated medium aerated using sterile compressed air (0.45 μm, 2 VVM) for a period of four hours with agitation at 350 RPM. Aeration sought to replicate typical industry practices for generation of an inoculum by aerobic cultivation, during which a theoretical pre-accumulation of sterols and unsaturated fatty acids could occur. It was also considered that such an extensive aeration would 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 30ºC with agitation at 150 RPM. Fermentations were considered complete when the concentration of residual sugars was less than 2.5 g/L, at which point, the fermenter was drained. Sufficient culture remained in the fermenter to inoculate a fresh batch of media to approximately 1×10^5 cells/ml. The re-filled fermenter was sparged with compressed air as before, the fermentation lock re-fitted and again the culture was monitored for fermentation completion, before the cycle was repeated.

Analysis of culture isolates: There were approximately 11 generations per fermentation cycle. Culture samples were collected at 50-generation (culture doubling) interims, designated FM1-5 (for C9) and FM10-16 (for L2056) and their fermentation performance compared with the parental strains in separate shake-flack cultures. Upon identification of novel attributes, after some 250 and 350 generations for strains C9 and L2056, respectively, the fermentation performance of both the mixed cultures and 10 single colony clones of each were also 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 prior to inoculation at 1×10^5 cells/ml 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 conducted in 250 mL conical flasks fitted with a fermentation lock and a septum-sealed sidearm port for aseptic and anaerobic sampling. In order to mimic the near-anaerobic conditions of an industrial grape juice fermentation, the head space of each flask was flushed at a rate of approximately 20 VVM for 5 minutes with filtered (0.45 μm), high purity, nitrogen delivered through the flask septum via a 21 gauge needle. Flasks were incubated at 30ºC with shaking at 160 RPM (Walker et al., 2003).

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 pre-dried and pre-weighed 0.22 μm membrane filter, followed by washing, drying and re-weighing. Commercially available kits were used for enzymatic quantitation of glucose and fructose (Boehringer Mannheim). Succinic acid, acetic acid, acetaldehyde, glycerol and ethanol were determined by HPLC analysis as described previously (Walker et al., 2003).

Microarray analysis: Three time-points were used to collect samples for RNA extraction: in early log-phase (1×10^7 cells/ml) at a reference point of divergence in fermentation kinetics (35 and 50 hr for C9 and L2056 strains and derivatives, respectively) and 1 hr before and 2 hr after this time point. RNA was isolated, using the Trizol method outlined by Chomczynski and Sacchi (1987) and indirectly labelled using allyl dUTPs during reverse transcription, in the synthesis of cDNA. The amino-allyl groups act as binding sites for fluorophores Cy3 and Cy5. Control and experimental populations of cDNA are then labelled with either Cy3 or Cy5 before these probes are combined and hybridised to the microarray slide (Eisen and Brown, 1999). The microarray slides used contain a series of spotted 70mer oligonucleotides, where each duplicate spot represents a different gene in the *S. cerevisiae* genome, of which approximately 6000 genes were available for analysis during these experiments. After hybridisation slides are scanned and fluorescence of individual spots indicated transcriptional activity with respect to the other population. Each time point was analysed in a triplicate reverse dye experiment for statistical validity.

3.2.2 Results and Discussion

Numerous stresses are typically imposed on yeast under oenological conditions. It has been implied that this fact may have contributed to the unintentional adaptive evolution of contemporary strains from the less well suited progenitors (Querol et al., 2003). We speculated 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 wine-like conditions. We applied an environment reminiscent of winemaking and which could be considered highly stressful: oxidative stress from pre-fermentation 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, L2056 and, to facilitate later genetic characterisation, a haploid derivate, C9 (Walker et al., 2003). It was hypothesized that mutants 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.

Every 50 generations, aliquots were taken from each chemostat and a number of fermentation parameters were analysed. To achieve this, comparative small-scale batch fermentations of mixed cultures were conducted to allow monitoring of fermentation duration, glucose and fructose utilisation, 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) is proposed to have occurred. That is, by 250 generations under selective pressure for C9 and 350 generations for L2056. The mixed culture, FM16-M, (M denotes the mixed, unpurified culture) derived from L2056 displayed superior fermentative abilities (Fig. 3.13) as well as altered metabolite yields (data not shown). The sluggish catabolism of sugars displayed by L2056, is considered highly undesirable under industrial oenological conditions (Alexandre and Charpentier, 1998; Bisson, 1999; Henschke and Jiranek, 1993) and increases the likelihood of the fermentation becoming stuck. This outcome is a problematic industry-wide issue because residual sugar of even 5 – 10 g L^{-1} can dramatically change the style of a wine, especially where dry styles are the intended

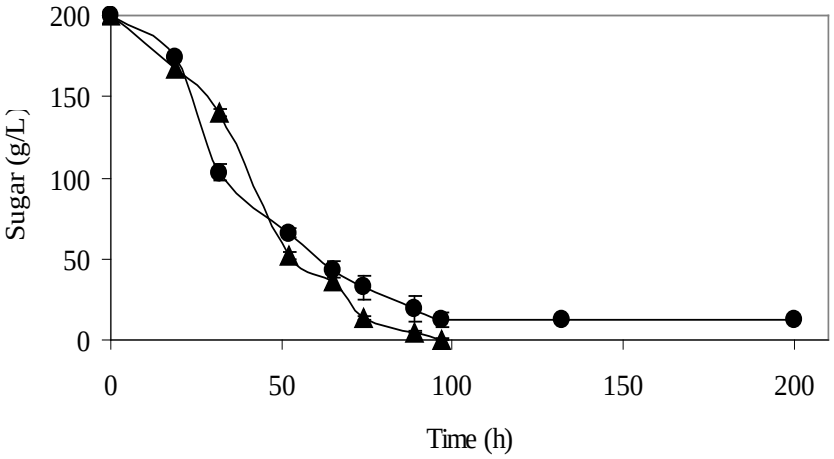


Figure 3.13
Fermentation kinetics of the mixed evolved culture FM16-M (▲) compared to the parental strain L2056 (●). Fermentations were performed in CDGJM. Data points are means of triplicate fermentations ± standard deviation.

product (Iland and Gago, 2002). Additional to this, re-starting fermentations in order to bring the wine within specification can be extremely difficult where arrest has occurred in the later stages when ethanol concentrations are high. Therefore the rapid fermentation seen by evolved isolates may help overcome protracted fermentation. For the wine industry, fermentation reliability is a key yeast attribute (Pretorius, 2000; Pretorius and Bauer, 2002) thus strains with this improvement would be highly prized by winemakers.

Numerous groups have isolated adaptive mutants using strictly continuous culture with defined working volumes and specified dilution rates (Ferea et al., 1999; Sonderegger and Sauer, 2003). Here, the employment of a sequential batch cultivation system ensured that any mutants selected would be suited to fermentation under oenological conditions. In this way selection of mutations that are disadvantageous to general fermentation performance seem to have been eliminated. This system although useful in this application, extends the duration of the selection process considerably. In a similar study investigating the adaptation of a haploid recombinant strain to anaerobic xylose utilisation, Sonderegger and Sauer (2003) isolated an adaptive mutant after 460 generations in a period of 266 days, whereas the isolation of FM5 after approximately 250 generations required 198 days. Given the low inoculation rate, each fermentation was expected to yield approximately 11 generations. The difference in total duration between our batch-fed continuous fermentation and a strictly continuous culture is mainly due to the prevalence of stationary phase under normal fermentation conditions. In a strictly

continuous fermentation this phase can be minimised by dilution of the system with fresh media to ensure that cells are constantly in a logarithmic phase.

The findings reported here do not shed any light on the nature, number or origin of mutations at the basis of the enhanced phenotypes observed. Before any more detailed discussion of these specific phenotypes, it is worth noting that adaptive evolution of the haploid strain to yield a derivative with a desirable phenotype occurred some 100 generations sooner than it did for the commercial strain (presumed to be a diploid, Walker et al., 2003). Ploidy can be reasoned to be important to this outcome (Orr and Otto, 1994; Zeyl et al., 2003) even though this disparity may not be the same in other circumstances. By definition, the occurrence of a new allele requires both a molecular mutation event as well as a fixation process, often requiring many generations (Zeyl, 2004), whereby the new allele replaces all others in the population. While higher ploidy might reduce the waiting period for an adaptive mutation by doubling the number of alleles from which the next adaptive mutation can arise (Paquin and Adams, 1983), the fixing time for non-dominant mutations may be increased, because they confer smaller selective advantages on heterozygotes compared with haploids (Zeyl et al., 2003). The effect that predominates is determined by population size. In a larger population, like in this experiment, it is the fixing time rather than the waiting time for the mutation which takes precedence, consequently haploids evolve faster (Orr and Otto, 1994). Further adaptive evolution experiments with both haploid and diploid wine yeasts will be needed to help determine which ploidy is the most effective route to producing superior strains. populations (Heidenreich et al., 2003).

Enhancement in fermentation performance was also initially observed for cultures derived from C9 (FM5), however in later analysis significant differences were limited to parameters other than fermentation kinetics (Table 3.6). 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.

The increased biomass production (dry weight) demonstrated by mutant isolates at the end of the end of fermentation, might have arisen through a divergence of carbon during glycolysis. In the case of FM5 increased biomass yield could be related, at least in part, to a reduced production of ethanol and/or a response to the toxicity of these metabolites (Aranda and del Olmo, 2003). In the case of FM16-M, the differences between the evolved cultures and the parent were typically greater in magnitude (Table 3.7). FM16-M was able to catabolise all available sugar after 96.5 hours, which is *ca* 69% of the time required by L2056 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 strain FM16-M and less succinic acid produced compared to L2056 (Table 3.7). It was also found that unlike the FM5-M and C9 comparison, there was little deviation between FM16-M and L2056 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 3.6 Metabolite yield and fermentation kinetics of adaptively evolved mutants derived from the parental population of C9.

Parameter	C9	FM5-M	Mean of clones (Range)
Dry weight (mg/ml)	6.236 ± 0.241	108.6	105.4 (92.5-155.6)
Fermentation duration (h)	62	100.0	100.0 (100)
Succinic acid (g/L)	0.22 ± 0.01	97.4	109.4 (89.7-125.1)
Glycerol (g/L)	7.09 ± 0.07	110.3	108.2 (102.2-115.2)
Acetic acid (g/L)	1.39 ± 0.03	109.1	113.9 (97.8-127.6)
Acetaldehyde (g/L)	0.003	120.0	110.0 (90.0-150.0)
Ethanol (g/L)	102.36 ± 1.17	86.5	89.3 (87.1-100.3)

Absolute values (mean of triplicate determinations ± standard deviation) are shown for C9. Data for FM5-M (mixed culture) and a mean derived from nine single isolate clones are expressed as a percentage of C9.

Table 3.7 Metabolite yield and fermentation kinetics of adaptively evolved mutants derived from the parental population of L2056.

Parameter	L2056	FM16-M	Mean of clones (Range)
Dry weight (mg/ml)	4.674 ± 0.071	98.7	95.7 (85.1-101.6)
Fermentation duration (h)	140	68.9	70.3 (55.4-85.0)
Succinic acid (g/L)	0.35 ± 0.05	92.9	79.0 (50.4-109.6)
Glycerol (g/L)	7.54 ± 0.11	112.0	109.2 (98.9-131.2)
Acetic acid (g/L)	1.67 ± 0.02	105.8	98.2 (92.3-105.3)
Acetaldehyde (g/L)	0.01	90.5	106.4 (85.7-128.6)
Ethanol (g/L)	106.28 ± 1.21	96.5	98.8 (94.4-101.1)

Absolute values (mean of triplicate determinations ± standard deviation) are shown for L2056. Data for FM16-M (mixed culture) and a mean derived from nine single isolate clones are expressed as a percentage of L2056.

To further elucidate the phenotypic differences between the two mixed culture evolved strains and their respective parents, ten random single colony representatives from the FM5-M and FM16-M populations were selected to undergo fermentation analysis. The metabolite and fermentation data for these are also shown in Tables 3.6 and 3.7, respectively. In both instances one of the ten analysed clones failed to complete

fermentation within the timeframe of the experiment and therefore the data for this strain was omitted from calculations of means. While there was at times large variation in the production of metabolites and the measured fermentation dynamics for the single clones, for the most part, the mean for the clones from each culture resembled the trends of the corresponding mixed culture. As an example of one such exception, the mean succinic acid yields by the clonal cultures were seen to increase by approximately 10% rather than remaining largely unchanged in the mixed culture derived from FM5. Inter-clonal variation is also highlighted by the measures of dry cell weight, however, again the mean is seen to resemble that of the parent, C9. Metabolite differences between evolved strains and their parent strain which are of greatest industrial importance are perhaps the yields of ethanol and glycerol. Most notably, FM5-M clearly produces more glycerol and less ethanol, with almost all FM5-C strains mimicking this result. One distinct isolate (FM5-C3) demonstrated the most favourable metabolite production with regard to oenological significance, with not only a 15.2% (*ca* 1.1 g/L) increase in glycerol production, but also a 12.9% (*ca* 12.3 g/L) reduction in ethanol production.

Similar patterns can be seen in the FM16 single isolates, the mean values for metabolite yields by these isolates broadly agreeing with those established for the mixed culture. Succinic acid values did however vary widely (Table 3.7). Fermentation duration was dramatically reduced compared to the parent strain, L2056, however the variation between clonal cultures was large (77.5 – 119 h). Glycerol production by the same evolved strains was also consistently increased over the parent strain and the mean of the single isolates is similar to that of the mixed culture. In displaying an elevated glycerol production (31.2% or *ca* 2.4 g l-1 greater than L2056), a mildly reduced ethanol yield (3.5% or *ca* 3.7 g l-1 reduction) and an ability to ferment available sugars in 85% of the time of the parent strain (*ca* 21 hrs less), isolate FM16-C7 is of considerable oenological interest because of the potential sensory and processing significance of these attributes.

Given that such an extended incubation was required to yield a culture with enhanced oenological properties, a clearly predominant, single clone might have been expected (Sonderegger and Sauer, 2003). This however was not the case. Characterisation of clonal isolates from the mixed cultures, FM5-M and FM16-M, revealed clear heterogeneity. It is however noteworthy that the single clone fermentations derived from both FM5-M and FM16-M generally yielded a mean response which was similar to that of the mixed culture populations (Tables 3.6 and 3.7). One advantage of considering individual clones is that the performance of some was a further improvement over the mixed cultures. Clearly clone FM16-C7 yielded a phenotype which would be considered to be of greatest oenological significance, with a reduced fermentation duration, mildly decreased ethanol yield and a dramatically increased glycerol production.

In a winemaking context, a reduced ethanol yield can be a very positive outcome. Ethanol increases the perception of ‘heat’ on the back palate and masks many flavour and aroma compounds of wine (Iland and Gago, 2002). Thus the avoidance of higher ethanol concentrations, particularly those resulting from the use of over-ripened fruit can be most beneficial. There is currently much interest in so-called ‘low-ethanol’ yeasts (Pretorius, 2000; Pretorius and Bauer, 2002) particularly from New World wine producing countries

such as Australia and USA where flavour-ripe grapes are readily achieved and the resulting flavour-intense wines 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). In fact the superior performance of FM5 is likely to at least in part be a result of the lower ethanol concentration of these fermentations.

Glycerol yields were increased by approximately 10% in both mixed mutant cultures. Recombinant strains that over-produce glycerol have previously been shown to also yield elevated amounts of acetic acid due to 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 mutants investigated here is yet to be determined, but mechanism aside, increased diversion of carbon to glycerol is likely to be viewed favourably by winemakers. Glycerol has the potential to enhance the mouthfeel of wine (Nieuwoudt et al, 2002).

In the haploid mutants succinate production was increased, however in the diploid, generally a reduction was observed. The mechanism(s) resulting in this deviation could have arisen in several ways. Diversion of a greater proportion of carbon through the tricarboxylic acid (TCA) pathway is one possibility. During fermentation, the TCA pathway operates in a branched fashion and it is thought that most succinic acid is formed by the reductive pathway (Camarasa et al., 2003). An alternative route arises when glutamate is present in high concentrations. In this case glutamate is catabolised to 2-oxoglutarate, which in turn is oxidatively decarboxylated to succinic acid via succinyl CoA (Albers et al., 1998). However, in these experiments, ammonia was the sole nitrogen source, thus glutamate is actually synthesised with the aid of products of the retrograde genes (The retrograde response) together with early parts of the oxidative branch of the TCA (Tate and Cooper, 2003). Finally, succinic acid can also be formed from the GABA (4-amino butyrate) degradation pathway (Coleman et al., 2001). Again dependent on glutamate, GABA can be produced from glutamate by a glutamate decarboxylase (*GAD1*), followed by transamination (*UGA1*) to form succinate semialdehyde and then a dehydrogenation (*UGA5*) to produce succinate. The significance of this pathway during fermentation is unclear, yet interestingly *GAD1* is required for a normal oxidative stress tolerance (Coleman et al., 2001). These genes will be targeted in further work to reveal whether alterations in associated pathways can account for the modified succinic acid yield of FM5-M, FM16-M and clonal isolates.

Having been produced by non-recombinant techniques, the derivatives of L2056 are ready for immediate larger-scale evaluation. Invitations to do so have already come from industry and we will be following up these possibilities in the near future. Also to further define the nature of modifications in the evolved strains, a microarray analysis was used to begin to identify genes whose expression may have become altered. We conducted microarrays comparing parent and evolved strains, using an indirect-labelling approach. RNA was isolated in early log phase, when cells were thought to share similar

physiological characteristics. Other samples were taken pre- and post- this reference point of divergence (i.e. where fermentation kinetics for parental *vs* evolved strains diverged). Each time point was analysed in a triplicate reverse dye experiment for statistical validity. From this we found a limited number of genes that were significantly differentially regulated (Table 3.8). Interestingly many of these genes are associated with either hexose transport or stress response. Also, we did not find any over expression of genes involved in sterol or unsaturated fatty acid synthesis, which might have been expected if mutants gain their advantage under anaerobic condition by over-accumulation of these oxygen substitutes during the aerobic propagation phase.

Table 3.8 Gene expression differences (fold) ($P < 0.1$) relative to parental strains.

Time Point	Gene	Magnitude of expression	Gene	Magnitude of expression
	FM5 relative to C9		FM16 relative to L-2056	
Early Log (1 x 10^7 cells/ml)	*HXT6*	-5.0	No significant differences	
	ALD6	-3.2		
	GLC3	-2.0		
	HXT7	-3.0		
	INO1	-1.7		
	PDR3	3.5		
	HSP10	1.8		
	ERG25	-1.8		
	LSC1	3.7		
	HAP4	-1.6		
	ERG1	-1.6		
	HXT1	-2.2		
	HXT3	-1.6		
	HXT2	-1.7		
Pre RPD	ADR1	2.4	*HSP26*	3.0
	HXT5	1.8	*HXT7*	-2.0
			AHP1	2.0
			SIP18	2.5
			HXT6	-1.8
Post RPD	No significant differences		*GPH1*	-2.0
			RCK1	-2.0
Pre and Post RPD	*VID24*	1.4	*ACC1*	1.5
			ALD4	-1.4
3-way analysis	*ydr340w*	2.0	*GPH1*	-1.5
	MLH2	2.0		
	PDE1	1.5		
	EXG2	1.7		
	ADH2	1.4		

Reference point of divergence (RPD) is the time point where the rate of sugar catabolism of parental strains subsided compared to the evolved culture. 3-way analysis is a comparison of all three time points, early log, pre- and post-RPD.

We are now in the process of constructing gene deletions or over-expression strains, as appropriate, in order to determine which genes or combination of genes contribute to the

observed phenotype. For instance, genes that were found to be down regulated are being deleted from the corresponding parent strain utilising a PCR-generated KanMX cassette and the fermentation dynamics of such strains will be analysed. Likewise overexpression constructs of genes that were found to be up-regulated are being constructed and are currently being transferred to the appropriate industrial strains. A series of plasmids that allow self-cloning into wine yeast that over-express a gene of interest were constructed by a two step gene replacement, based on that described by Hirosawa et al. (2004). The over-expression strains were constructed by inserting the yeast constitutive promoter of *TDH3*, upstream of the coding region of the gene of interest. The upstream region of the gene of interest was then inserted into the plasmid 5' of the promoter. A series of five backbone vectors (pCMTHD3A, B, C, D, & E) were constructed so as to accommodate the different cloning strategies required for each gene. Linearised plasmids were then transformed into the strain of choice (L2056 or C9) enabling insertion at the gene of interest. Following this counter selection ensured that plasmid integrants that had undergone homologous recombination between the TDH3p-5' upstream sequences result in the inclusion of *TDH3* promoter without any extraneous DNA sequences. Again fermentation trials of the overexpressors will yield valuable information about the merits and possible oenological importance of these strains ad the genes in question.

OUTPUT 4 Mutant or hybrid yeasts produced along with information on their use.

The original Performance Target for this component of the project was stated as:

> *3 – 4 distinct yeast strains with enhanced flavour, mouthfeel or processing characteristics available for industry evaluation. Protocols for industry use.*

4.1 Introduction

The work proposed for this output is in part detailed under Output 3. Thus several strains were generated which have altered or novel properties and have been characterised to varying degrees. Those strains generated by non-recombinant methods are essentially ready to be trialled in industry. The other key area of work within this section is the modification of strains by classical genetic methods.

4.2 Methods

Strains and cultivation: The strains used in this section of the study are listed in Table 4.1 and were maintained and grown as detailed in Section 3.1.2.

Table 4.1 Strains of *Saccharomyces cerevisiae* used in this phase of the study.

Strain	Genotype/properties	Source
QA23	Wine strain, diploid (?), wildtype, β-glucosidase and killer activity.	Lallemand
BDX	Wine strain, diploid (?), wildtype, ethanol tolerant, cold tolerant.	Lallemand
Syrah	Wine strain, diploid (?), wildtype, ethanol tolerant.	Lallemand
D47	Wine strain, diploid (?), wildtype, β-glucosidase activity	Lallemand
S6U	Wine strain, diploid (?), wildtype, high glycerol production.	Lallemand

Sporulation and spore dissection and evaluation: Fresh YPD plate cultures of wine yeast were sporulated as detailed in Section 3.1.2. Asci were digested and spores dissected according to Guthrie and Fink (1991), and germinated on YPD. Individual cultures were then arrayed into a multi-well plate containing YPD and incubated overnight at 30°C. Fermentations were conducted in a fresh multi-well plate containing the indicated medium and inoculated using the appropriate multi-pinned replica plating tool. Fermentation progress was determined from culture refractive index (°Brix). Hydrogen sulphide liberation was assessed by replica plating onto BiGGy agar. B-glucosidase activity was estimated according to that described in Section 2.1.2. Growth on media containing NaCl, sorbitol or glycerol was scored by eye and given a ranking between "-" and "+++".

4.3 Results and Discussion

The generation of a number of potentially useful strains has been described in Output 3. The nature of these strains along with their key attributes is given in Table 4.2. At least 6 yeast strains (mutants of *PUT4*, *GID7*, *NGR1*, *GID7+NGR1*, and evolved strains FM5 and FM16) have been produced, mostly in wine yeast backgrounds. We are working to undertake evaluations on a larger scale. New versions of some of these mutants which contain no foreign sequences are being produced by 'seamless' disruption (UA 99/1; Walker et al., 2005) to facilitate industry trials.

Table 4.2 Novel strains of *Saccharomyces cerevisiae* generated through this study.

Key genetic modification	Background	Notable Properties
Constitutive expression of a modified *PUT4*	Laboratory yeast	Increased uptake of proline and greater viability under salt and heat stress.
GID7 deletion	Laboratory and wine yeast	Enhanced fermentation in nitrogen deficient media.
NGR1 deletion	Laboratory and wine yeast	Enhanced fermentation in nitrogen deficient media.
GID7 + *NGR1* double deletion	Lab yeast	Fermentation performance better than either single deletant.
FM5	Wine yeast derivative	Increased glycerol, acetic acid, acetaldehyde and reduced ethanol yields.
FM16	Wine yeast	Superior fermentation, increased glycerol and reduced acetaldehyde ethanol yields.

In addition, further strains have been produced by classical genetic methods. We have investigated a number of commercial yeast strains, chosen for their distinct properties as a starting point for a breeding program. These strains are BDX, Syrah, QA23, D47 and S6U and their key properties are included in Table 4.1. Strains were sporulated, dissected and allowed to rediploidize. These spore products were then assessed against several criteria including growth on: fructose as sole carbon source, high concentrations of glucose and fructose, low- and moderate assimilable nitrogen, NaCl, sorbitol or glycerol, as well as production of H_2S and β-glucosidase. The results show a wide range of abilities against these criteria (Table 4.3). While we have yet to further characterise the spore products, it is clear even at this stage that the sporulation, dissection and rediploidisation can reveal much heterogeneity. Thus fermentation efficiencies are quite different, with BDX appearing capable of effective fermentation in a fructose-only medium. Similarly, β-glucosidase activity can be seen to range between 20 and 300 μM of liberated *p*NP/10^7 cells/hr. Those strains with most distinct properties are now being evaluated in laboratory fermentations. Furthermore these strains will act as breeding material for Genome Shuffling experiments to magnify their desirable properties.

Table 4.3 Properties of commercial strains of wine yeast and their re-diploidised spores.

Strain/spore number	Fermentation progress (°Brix of terminal sample)[a]					H_2S[g]	α-glucosidase (mM pNP/10^7 cells/hr)	Growth on supplemented media[h]		
	Fructose (100 g/L)[b]	Glucose & Fructose (225 g/L)[c]	Low FAN (75 mg N/L)[d]	Moderate FAN (150 mg N/L)[e]	Total RI[f]			NaCl (1.5 M)	Sorbitol (2 M)	Glycerol (1.5 M)
BDX parent	4.2	7.7	11.0	8.5	31.4	3 - 4	37	++	+++	+++
1-1	4.9	7.0	10.4	8.0	30.3	5	33	-	-	+++
1-2	8.6	15.1	13.0	9.1	45.8	5	65	++	-	+++
2-1	6.5	12.4	12.3	9.0	40.2	3	123	-	-	+++
2-3	6.2	13.5	11.9	8.7	40.3	4	87	-	-	+++
3-1	5.6	12.3	11.5	8.6	38.0	5	147	-	-	+++
3-3	6.0	14.0	14.3	10.1	44.4	3	52	+++	-	+++
4-1	4.7	8.2	13.1	9.0	35.0	4	102	-	-	+++
4-2	5.6	12.1	11.7	9.5	38.9	5	52	-	-	+++
5-1	6.5	16.2	11.2	8.2	42.1	4	130	-	-	+++
5-2	6.8	14.1	13.5	9.1	43.5	3	65	++	-	+++
6-2	13.2	18.1	15.1	ND	ND	3	ND	-	-	+++
6-3	7.4	15.5	11.9	9.6	44.4	4	72	-	-	+++
7-1	7.5	12.6	13.5	7.5	41.1	3	86	-	-	+++
8-1	5.6	12.0	11.5	8.5	37.6	3	40	-	+++	+++
8-2	5.5	10.8	14.2	9.2	39.7	3	95	-	-	+++
9-1	3.5	5.5	11.1	8.5	28.6	5	32	-	-	+++
10-2	6.2	16.2	13.1	10.2	45.7	4	68	-	-	+++
11-1	4.7	8.3	11.3	8.1	32.4	4	40	-	-	+++
11-2	5.0	11.7	9.0	8.7	34.4	5	19	-	-	+++
13-2	7.0	12.1	9.8	8.2	37.1	ND	36	-	-	+++
14-2	6.5	12.1	ND	8.8	ND	1	49	-	-	+++
15-1	5.7	12.7	ND	9.0	ND	0	52	-	+++	+++
Syrah parent	6.8	6.0	10.9	7.2	30.9	3 - 4	60	+++	+++	+++
1-1	5.8	6.8	10.3	6.0	28.9	4	41	-	-	+++
1-2	5.6	6.9	10.9	7.3	30.7	3	56	-	-	+++
1-3	11.3	14.0	14.2	12.5	52.0	4	55	-	-	+++
1-4	6.5	8.0	8.6	7.0	30.1	4	52	-	-	+++
2-1	8.6	12.3	10.3	7.7	38.9	3	47	-	-	+++
2-2	9.0	12.8	11.2	7.3	40.3	3	42	-	-	+++
2-3	8.3	12.3	10.4	6.3	37.3	4	59	-	-	+++
2-4	6.5	9.1	9.9	7.8	33.3	3	140	-	-	+++
3-1	7.8	10.8	10.8	7.6	37.0	3	113	-	+++	+++
3-2	7.4	11.1	10.2	6.3	35.0	4	73	-	-	+++
3-3	7.9	10.6	10.4	8.2	37.1	3	42	-	-	+++
3-4	9.4	12.2	9.6	9.0	40.2	4	55	-	-	+++
4-1	10.3	14.6	11.5	8.2	44.6	2	46	-	-	+++
4-2	9.0	11.9	13.4	9.6	43.9	3	43	-	-	+++
4-3	6.1	6.7	11.5	6.7	31.0	4	51	-	-	+++
4-4	5.7	8.4	9.1	6.9	30.1	4	78	-	-	+++
5-1	6.9	10.5	10.3	7.3	35.0	3	54	-	++	+++
5-2	5.8	8.0	10.4	6.3	30.5	3	83	-	+++	+++
5-3	9.0	13.8	13.3	9.8	45.9	2	60	-	++	+++
5-4	8.5	9.8	11.2	7.7	37.2	2	45	++	-	+++
S6U parent	6.4	13.1	12	ND	31.5	3 - 4	60	ND	ND	ND
1-1	6	14.9	12.3	ND	ND	3 - 4	55	+++	++	+++
2-1	6.2	15.3	12.4	ND	ND	3 - 4	52	+++	++	+++
3-1	6.4	14.6	11.6	ND	ND	3 - 4	61	+++	++	+++
4-1	5.6	14.8	11.7	ND	ND	3 - 4	48	+++	++	+++
5-1	5.6	13.3	12	ND	30.9	3 - 4	56	+++	++	+++
6-1	5.5	12	11.6	ND	29.1	3 - 4	75	+++	++	+++
6-2	6	12.6	11.8	ND	ND	3 - 4	55	+++	++	+++
7-1	5.9	ND	11.9	ND	ND	3 - 4	57	+++	++	+++

[a] Refractive index of culture supernatants at the experiment end, i.e.: [b], 88.5 h; [c], 161 hr; [d], 185.5 hr; [e], 92.5 hr; [f], sum of supernatant RIs for [b-e]; [g], low (1) to high (5) liberation of H_2S; [h], no (-), low (+) or rich (+++) growth. ND, not determined.

Table 4.3 (cont.).

Strain/spore Number	Fermentation progress (°Brix of terminal sample)[a]					H_2S	β-glucosidase (mM *p*NP/10^7 cells/hr)	Growth on supplemented media		
	Fructose (100 g/L)[b]	Glucose & Fructose (225 g/L)[c]	Low FAN (75 mg N/L)[d]	Moderate FAN (150 mg N/L)[e]	Total RI[f]			NaCl (1.5 M)	Sorbitol (2 M)	Glycerol (1.5 M)
QA23 parent	6	13.6	13.4	8.7	41.7	3 - 4	34	ND	ND	+++
1-1	8.7	20	13.4	8.3	ND	3	35	+	+	+++
2-1	6.8	17.1	7.9	6.2	38	2	23	-	+	+++
3-1	12	20.5	14.7	10.9	ND	3	25	-	+	+++
3-2	9.3	17.3	12.8	10.2	ND	3	36	-	+	+++
4-1	7.8	17.5	13.3	10.5	ND	3	42	-	+	+++
4-2	17.3	28.1	14.9	9.7	ND	5+	49	-	+	+++
5-1	10.3	18.3	13.6	9.6	ND	2	46	-	+	+++
5-2	8.4	17.6	12.6	ND	ND	5+	ND	-	+	+++
6-1	7.5	16.7	8.1	6.3	38.6	2	45	-	+	+++
6-2	11.1	19.5	13.8	9	ND	4	68	+	+	+++
7-1	14.2	24	12.8	8.8	ND	3	60	-	+	+++
8-1	8.5	17	13.1	6	ND	4	51	-	+	+++
9-1	9.3	17.9	14.1	9.4	ND	5	89	-	+	+++
10-1	6	15.7	10	6.3	38	2	55	-	+	+++
11-1	10.3	18.3	13.9	9.8	ND	4	29	-	+	+++
12-1	13	20.3	14.4	10.8	ND	1	64	-	+	+++
12-2	12.4	20.3	16.5	11.1	ND	5	28	-	+	+++
D47 parent	7.2	12.1	12.5	7.8	39.6	3	36	++	+++	+++
1-1	5.5	11.1	10.4	5.7	32.7	3	43	-	-	+++
1-2	11.5	22.2	13.5	11.7	ND	3	37	-	-	+++
2-1	5.9	10.8	13	7.8	37.5	3	50	+++	-	+++
3-1	6.5	12	12.1	7.2	37.8	3	51	-	-	+++
3-2	12.5	23.6	13	10.1	ND	3	295	-	-	+++
4-1	15.1	26	12.9	12.3	ND	3	174	+	-	+++
4-2	5.1	8.1	10.6	6.1	29.9	3	31	-	-	+++
5-1	12.5	23.6	12.8	11.6	ND	3	157	++	-	+++
5-2	16.3	25.1	12.4	12.2	ND	3	130	+++	-	+++
6-1	11.3	19.2	11.9	8.8	ND	3	76	++	-	+++
6-2	14.9	26.3	13.5	12	ND	3	202	+++	-	+++
7-1	6.1	12.2	12.7	6.7	37.7	3	44	+	-	+++
7-2	9	17.6	11.6	8.2	ND	3	59	+	+	+++
8-1	5.3	6.9	10.7	6	28.9	4	38	++	-	+++
8-2	13.5	21.9	12.7	10.1	ND	4	95	-	-	+++
9-1	6.9	14.5	12.4	7.7	41.5	3	61	++	-	+++
9-2	8.3	16.5	10.6	6.7	ND	3	39	+++	+	+++
10-1	8.1	16.1	12.5	7.3	ND	3	48	+++	++	+++
10-2	16.5	25.3	11.5	12.3	ND	3	140	++	++	+++
11-1	18.1	ND	12.4	11	ND	3	174	+++	-	+++
11-2	8.5	16.3	13.3	7.7	ND	3	39	-	-	+++
12-1	7.9	16	13.3	7.8	ND	3	33	-	-	+++
13-1	8.6	18.2	12.2	7.3	ND	3	47	-	-	+++
13-2	8.5	16.6	11.5	6.6	ND	3	46	-	-	+++
14-1	10.4	20.2	12.5	8.5	ND	3	72	-	-	+++
15-1	8.4	14.6	13	7.5	ND	3	46	-	-	+++

[a] Refractive index of culture supernatants at the experiment end, i.e.: [b], 88.5 h; [c], 161 hr; [d], 185.5 hr; [e], 92.5 hr; [f], sum of supernatant RIs for [b-e]; [g], low (1) to high (5) liberation of H_2S; [h], no (-), low (+) or rich (+++) growth. ND, not determined.

OUTPUT 5 Publications/conference presentations

We have exceeded the Performance Target for this output:

> *2 – 3 communications/yr detailing project findings, highlighting potential for application of new strains/enzymes/treatments to wine quality enhancement.*

5.1 Refereed Journal Papers

1. McBryde, C., Gardner, J.M., de Barros Lopes, M., and Jiranek, V. (2005) Generation of novel wine yeast strains by adaptive evolution. ***Appl. Environ. Microbiol***. (in revision).
2. Grimaldi, A., Bartowsky, E., and Jiranek, V. (2005) Screening of *Lactobacillus* spp. and *Pediococcus* spp. for glycosidase activities that are important in oenology. ***J. Appl. Microbiol.*** **99:**1061-9.
3. Walker, M., Vystavelova, A., Pedler, S., Eglinton, J., and Jiranek, V. (2005) PCR-based gene disruption and recombinatory marker excision to produce modified industrial *Saccharomyces cerevisiae* without added sequences. ***J. Microbiol. Meth.*** **63:**193-204.
4. Grimaldi. A., Bartowsky, E., and Jiranek, V. (2005) A survey of glycosidase activities of commercial strains of *Oenococcus oeni*. ***Internat. J. Food Microbiol.*** **105:**233-44.
5. Gardner, J., McBryde, C., Vystavelova, A., de Barros Lopes, M., Jiranek, V. (2005) Identification of genes affecting glucose catabolism in nitrogen limited fermentation. ***FEMS Yeast Res.*** **5:**791-800.
6. Matthews, A., Grimaldi, A., Walker, M., Bartowsky, E., Grbin, P.R. and Jiranek, V. (2004) Lactic acid bacteria as a source of enzymes for use in vinification. ***Appl. Environ. Microbiol.*** **70:**5715-31.
7. Walker, M. E., J. M. Gardner, A. Vystavelova, C. McBryde, M. de Barros Lopes, and V. Jiranek (2003) Application of the reuseable, *KanMX* selectable marker to industrial yeast: construction and evaluation of heterothallic wine strains of *Saccharomyces cerevisiae*, possessing minimal foreign DNA sequences. ***FEMS Yeast Res.*** **4:**339-47.
8. Gardner, J.M., K. Poole, V. Jiranek (2002) Practical significance of relative assimilable nitrogen requirements of yeast: a preliminary study of fermentation performance and liberation of H_2S. ***Aust. J. Grape Wine Res.*** **8:**175-9.

5.2 Conference presentations/posters/non-refereed papers (invited papers underlined)

1. Jiranek, V. (2006) Making yeast make the most of nitrogen during fermentation. *Internat. Wine Microbiol. Conf.* April, 2006, Yosemite. (In preparation).
2. Gardner, J, de Barros Lopes, M., and Jiranek, V. (2005) Identification of genes contributing to a 'High Nitrogen Efficiency' (HNE) phenotype in yeast. *Proc. XXIInd Internat. Conf. Yeast Genet. Molec. Biol.* August, 2005, Bratislava, Slovak Republic. ***Yeast*** **22S**:38.

3. McBryde, C., de Barros Lopes, M., and Jiranek, V. (2005) Adaptive evolution in enhancing wine yeast. *Proc. XXII[nd] Internat. Conf. Yeast Genet. Molec. Biol.* August, 2005, Bratislava, Slovak Republic. ***Yeast* 22S**:38.
4. Gardner, J., McBryde, C., de Barros Lopes, M., and Jiranek, V. (2004) Identification of genes that contribute to a 'High Nitrogen Efficiency' (HNE) phenotype in yeast. *Proc. 3[rd] Aust. Conf. Yeast: Prod. & Discov.* April, 2004, Barossa Valley. p 39a.
5. Jiranek, V. (2004) Recent efforts to optimise wine yeast and their fermentation performance. *Proc. 12[th] Aust. Wine Ind. Tech. Conf.* July, 2004, Melbourne. pp 123-125.
6. Grimaldi, A., Bartowsky, E., and Jiranek, V. (2004) Characterisation of glycosidase enzymes of wine Lactic Acid Bacteria. *Proc. 12[th] Aust. Wine Ind. Tech. Conf.* July, 2004, Melbourne. p 290.
7. Grimaldi, A., Bartowsky, E., and Jiranek, V. (2004) Characterisation of glycosidase enzymes of wine Lactic Acid Bacteria. *155[th] Meet. Soc. Gen. Microbiol.* Sept, 2004, Dublin, Ireland.
8. Matthews, A., Grbin, P., and Jiranek, V. (2004) The enzymatic activities of wine lactic acid bacteria. *155[th] Meet. Soc. Gen. Microbiol.* Sept, 2004, Dublin, Ireland.
9. McBryde, C., de Barros Lopes, M., and Jiranek, V. (2004) Adaptive evolution in enhancing wine yeast. *Proc. 3[rd] Aust. Conf. Yeast: Prod. & Discov.* April, 2004, Barossa Valley. p 29.
10. Petropoulos, S., Grbin, P.R., and Jiranek, V. (2004) Heat-shock selection of yeast for altered formation of metabolites. *Proc. 12[th] Aust. Wine Ind. Tech. Conf.* July, 2004, Melbourne. p 290.
11. Walker, M., Vystavelova, A., Pedler, S., Eglinton, J., and Jiranek, V. (2004) Towards tools for 'seamless' modification of genes in industrial yeast. *Proc. 12[th] Aust. Wine Ind. Tech. Conf.* July, 2004, Melbourne. p 290.
12. Jiranek, V. (2003) Nutrition management for sound fermentation practices. Wash. Assoc. Wine Grp. Gwrs. Ann. Meet. February, 2003, Yakima, Washington.
13. Jiranek, V. (2003) Yeast nutrition and fermentation kinetics. *Proc. IX[th] Lat. Am. Congr. Vitic. Enol.* November, 2003, Santiago, Chile. pp 129-136.
14. Jiranek, V. (2002) Causes of hydrogen sulfide formation in winemaking. *Vinidea.Net - Wine Internet Technical Journal*, No 3.
15. Poole, K., de Barros Lopes, M., Jiranek, V. (2002) Potential for yeast exploitation of proline in grape juice to facilitate fermentation completion. *30[th] Ann. Tech. Issue. Aust. N.Z. Grpgrwr. Wnmkr.* **461a:**121-123.
16. Walker, M.E., Gardner, J.M., de Barros Lopes, M., Jiranek, V. (2002) Wine yeast as tools for oenological research and strains improvement by genetic techniques. 30[th] *Ann. Tech. Issue. Aust. N.Z. Grpgrwr. Wnmkr.* **461a:**109-114.
17. Poole K., Gardner, J.M., de Barros Lopes, M., Jiranek, V. (2002) Strategies for avoiding problems associated with nitrogen limitation during fermentation. *Proc. 2[nd] Aust. Conf. Yeast: Prod. & Discov.* November, 2002, Melbourne. pp. 22.
18. Gardner, J., Wenk, M., de Barros Lopes, M., Jiranek, V. (2001) Identification of genes contributing to a 'High Nitrogen Efficiency' (Hne) phenotype in a modified wine yeast. *Proc. 11[th] Aust. Wine Ind. Tech. Conf.* October, 2001, Adelaide. p 242.

19. Poole, K., de Barros Lopes, M., Jiranek, V. (2001) Modification of *Saccharomyces cerevisiae* to utilise proline as a nitrogen source during oenological fermentation. *Proc. 11th Aust. Wine Ind. Tech. Conf.* October, 2001, Adelaide. p 247.
20. Poole, K., de Barros Lopes, M., Jiranek, V. (2001) Modification of *Saccharomyces cerevisiae* to enable proline utilisation during oenological fermentation. *Proc. XXth Internat. Conf. Yeast Genet. Molec. Biol.* August, 2001, Prague. *Yeast* 18S:S289.
21. Walker, M., Gardner, J., Pedler, S., Jiranek, V. (2001) Construction and evaluation of haploid wine yeast for research purposes. *Proc. 11th Aust. Wine Ind. Tech. Conf.* October, 2001, Adelaide. p 241-242.

5.3 Other

In addition to the above, the group has collectively made 4-6 seminar presentations each year to the campus community. The presentations have formed part of the Research Seminar Series, the Postgraduate Seminar Series or the Waite Precinct Plant Researchers Seminars, all of which are open and widely advertised.

Summary

This project has succeeded in more fully defining the actual or potential contribution of microbes to the winemaking process and thereby allowing microbes to be used to enhance wine quality and complexity. Essentially all the project performance targets have been met or exceeded. Any outstanding items or targets relate to the pilot or industrial scale trails of microbes or their extracts.

A detailed literature review has been prepared and published and used to highlight likely enzymes for pursuit in this study. In addition, this review allowed us to identify suitable methods for detection of relevant enzymatic activities. In many cases these methods were tailored to suit the wine strains used or the growth conditions and media needed to cultivate these. The enzymatic activities ultimately investigated amongst 40 *Oenococcus, Lactobacillus* and *Pediococcus* strains were glycosidase (β- and α-glucosidase, β-xylosidase, α- and β-galactosidase, α-rhamnosidase and α-arabinosidase), lipase, tannase, esterase and polysaccharide-degrading enzymes. All activities were detected to varying extents and in a highly strain dependent manner. The glycosidic activities were characterised to the greatest degree, with several strains displaying high levels of activity, even under wine-like conditions of low pH and in the presence of ethanol and residual sugars. Of special interest was the fact that LAB may have a marked influence on anthocyanin contents. Several strains can now be targeted for selection as strong glycosidase producers and their precise impact on wine composition and quality evaluated. To assist the purification and specific characterisation of these enzymes, work has progressed toward cloning and heterologous expression of genes encoding putative β-glucosidases and esterases from various LAB.

In terms of achieving better outcomes from the use of yeast, work was undertaken to produce more effective tools for seamless strain modification as well as to characterise yeast genes important to efficient use of assimilable nitrogen. Enhanced or novel strains were sought by both recombinant and non-recombinant methods. Through this work we improved upon existing methods for modifying genes in commercial strains of yeast with a particular emphasis on simplified construction of the disruption cassette and an ability to remove any foreign sequences (including the selectable marker). We also progressed earlier efforts to identify genes from both laboratory and wine strains involved in efficient use of assimilable nitrogen. We have now identified close to 20 such genes and have investigated two, *GID7* and *NGR1* in some detail. So far we have seen that genes in central nitrogen metabolism do in fact show an altered degree of expression, suggesting a different fate for intracellular nitrogen. Strains in which these genes have been disrupted are now being evaluated under model oenological conditions to better define their benefits for fermentation reliability and wine composition. Similarly, strains capable of constitutive uptake of the plentiful grape juice amino acid, proline, were confirmed to possess superior stress tolerance with viabilities almost twice that of the parent strain. Such data provide strong justification for efforts to include constitutive proline transport as a part of modifications to improve wine yeast.

In attempts to improve yeast by non-recombinant methods we have applied, to our knowledge for the first time, the technique of adaptive evolution to wine strains of yeast.

In this way derivatives of both a commercial wine yeast and a haploid derivative thereof were isolated. These evolved strains are better able to complete a model oenological fermentation and/or yield a fermented medium of altered content of key metabolites, including ethanol and glycerol. Microarray anaylsis of evolved strains has identified several genes whose expression may have become altered. These findings are beginning to highlight metabolic processes or cellular functions which may impact on yeast performance under the stresses of a winemaking fermentation. Perhaps most importantly, such strains require no special approval for trialling by industry – a key goal of future work. Further novel yeast strains have been generated by classical genetic techniques. Sporulation, dissection and rediploidisation of industrial strains has revealed marked heterogeneity in the spore products. Attributes investigated include fermentation in high sugar, high fructose or low nitrogen media as well as increased tolerance to stresses or reduced H_2S production. The generation of hybrids between those strains showing most novel or useful attributes will now be undertaken.

We have communicated the findings of this study through seminar presentations, over 20 conference or non-refereed papers and 8 papers in refereed journals. Four PhD students are due to submit their thesis in the next quarter. Five Honours students have completed their studies and one MSc students has upgraded to a PhD.

Recommendations

1. *Characterisation of glucosidase enzymes from lactic acid bacteria (LAB) and development of extracts or protocols for their informed application in winemaking.* Our work over 4-5 years shows that glycosidases from LAB are superior to enzymes derived from other sources in terms of their tolerance of low pH and temperatures, and high residual sugar and ethanol contents. While cloning and biochemical characterisation of β-glucosidases from *Oenococcus oeni* is well advanced, the same is true for the remaining glucosidases. Yet these remaining activities are important in flavour liberation because the precursors from grapes are often linked to several glycosides. Characterisation of non-glucosidase enzymes should be undertaken. Also, the efficacy of the purified enzymes or bacterial biomass in flavour liberation should be confirmed using natural precursors, both in purified form or in grape juices and wines.

2. *Large scale trials of enzyme preparations or selected LAB strains for enhancing wine composition.* In addition to work on glycosidases of LAB, we have also completed perhaps to most comprehensive survey of LAB for other enzymatic activities with potential applications in winemaking. Several of these activities, warrant further investigation, for example on account of their potential for increasing wine wholesomeness (e.g. urease removal of urea), altering wine ester profile (i.e. esterases) or facilitating clarification of wine or colour extraction (polysaccharide degrading enzymes).

3. *Use of adaptive evolution techniques to produce new yeast strains with specific enhancements*. Our data suggests that adaptive evolution has the ability to not only yield markedly improved strains, but strains which may be trialled immediately on a larger scale or else by industry because of their non-GM status. Essentially the only limitation as to which yeast properties can be targeted and improved by adaptive evolution, is whether or not a selective screen can be designed in which the desired isolate has a growth advantage. Certainly phenotypes such as tolerance to SO_2, ethanol, low or high temperatures, nutritional or oxygen deficiencies, are straightforward to screen for by adaptive evolution.

4. *Development of novel yeast by classical genetic manipulation of commercial wine yeasts.* While non-*Saccharomyces* yeasts can distinguish themselves by producing novel metabolites or else yields thereof, their fermentative properties are often inferior. Our studies have revealed marked phenotypic heterogeneity amongst the sporulation products of commercial yeast strains. This introduces the possibility of using classical genetic approaches to generate strains with novel attributes, but which are likely to retain good fermentative abilities. Work currently underway to produce such yeasts should be continued and expand. Presently, individual spores are being isolated and evaluated as pure cultures. We intend however to increasing rely on high-throughput screening methods to isolate strains of interest from large populations of spores or mass spore matings.

5. *Exploitation of identified genes or mutants with enhanced fermentative capacities or metabolite yields.* Detailed above are several genes (or rather deletions or modifications thereof) as well as mutants which are better able to complete fermentation under anaerobic conditions, in limited nitrogen media, or else to produce key metabolites in altered amounts. Future work should aim to i) produce strains combining 2 or more of the phenotypes identified to date, ii) identify analogous spontaneous mutant (i.e. non-GM) versions of those produced by recombinant means, or iii) introduce other sets of genetic changes to produce strains enhanced in several properties.

Appendix 1: Communications

See Output 5.

Appendix 2: References

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Appendix 3: Staff

The following staff contributed to this project.

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