Increasing fermentation reliability and flavour compound formation by wine yeast

FINAL REPORT to

GRAPE AND WINE RESEARCH & DEVELOPMENT CORPORATION

Project Number: **UA 00/5**

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Research Organisation: **University of Adelaide**

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Abbreviations: CDGJM, chemically defined grape juice medium, FAN, free amino nitrogen, HNE, high nitrogen efficiency, PCR, polymerase chain reaction, ?, deletion, HPLC, high performance liquid chromatograph.

Background

The availability of a variety of wine yeast strains allows the winemaker to have greater control over the characteristics of oenological fermentation. Even so problem fermentations, specifically those which are sluggish or fail to complete are common. With the recognition of the central role of juice nitrogen deficiencies in many of these problem ferments, the use of nitrogen supplements has become widespread and routine. Nevertheless this strategy is not always successful. Nitrogen additions are a valuable tool for prevention and control of stuck fermentations, yet the timing of addition is critical (Bely *et al.*, 1990) and the presence of residual nitrogen might favour microbial spoilage (Henschke and Jiranek, 1993) and formation of a carcinogen, ethyl carbamate (An and Ough, 1991). At any rate it is desirable to reduce additives made to premium wines.

Another option for the prevention of stuck fermentation, which has yet to be employed is the use of "nitrogen efficient" wine yeast strains (Jiranek *et al.*, 1991, 1995, Gardner *et al.*, 2002). This study aims to develop a new and alternative strategy for dealing with low nitrogen juices based on such strains. Specifically we seek to identify and characterise genetic differences between yeast, which confer a greater efficiency of nitrogen utilisation during fermentation. Nitrogen efficiency is a measure of the relative amount nitrogen utilised during the catabolism of a given amount of sugar. Therefore yeasts that are able to catabolise more sugar for a given amount of

nitrogen are considered more nitrogen efficient. Genes found to be responsible for a "high nitrogen efficiency" (HNE) phenotype are presently being identified ahead of being exploited to further manipulate and understand yeast nitrogen requirements.

A better understanding of nitrogen metabolism, particularly as it relates to oenologically important processes such as flavour compound formation, will be of both fundamental and applied significance. In terms of the latter, such information will introduce the possibility of the manipulation of yeast nitrogen metabolism to tailor the profile of aroma compounds formed during fermentation.

Proposed Project Outputs

1. *Information on genetic basis for varying nitrogen (N)-efficiency and flavour formation in yeast.*
2. *Information on consequences of altered N-efficiency for winemaking properties of yeast.*
3. *Yeast strains showing potential for improved fermentation performance and wine quality.*
4. *Publications/conference presentations.*
5. *Trained individual with industry-relevant expertise.*

Results

Isolation of HNE strains

Traditionally, mutants with altered nitrogen deficiency would be produced by a process of chemical mutagenesis that generates random point mutations. Such mutants/mutations are only apparent because of the altered phenotype they possess/produce. In the case of nitrogen efficiency, this phenotype must, by definition, be evaluated during a fermentation. As such, the routine identification of mutated genes becomes quite laborious as it requires relative nitrogen efficiency to be determined through fermentation trials. The alternative system of transposon mutagenesis (Ross MacDonald *et al.*, 1997) is equally suited to the generation of mutants with altered nitrogen efficiency but more importantly, produces mutations that are genetically tagged (Fig.1). Therefore, after the presence of a *hne* mutation has been initially demonstrated through

fermentation trials, it can more conveniently be tracked through subsequent experiments and genetic manipulations by PCR. Several other characteristics of this system made it suitable for

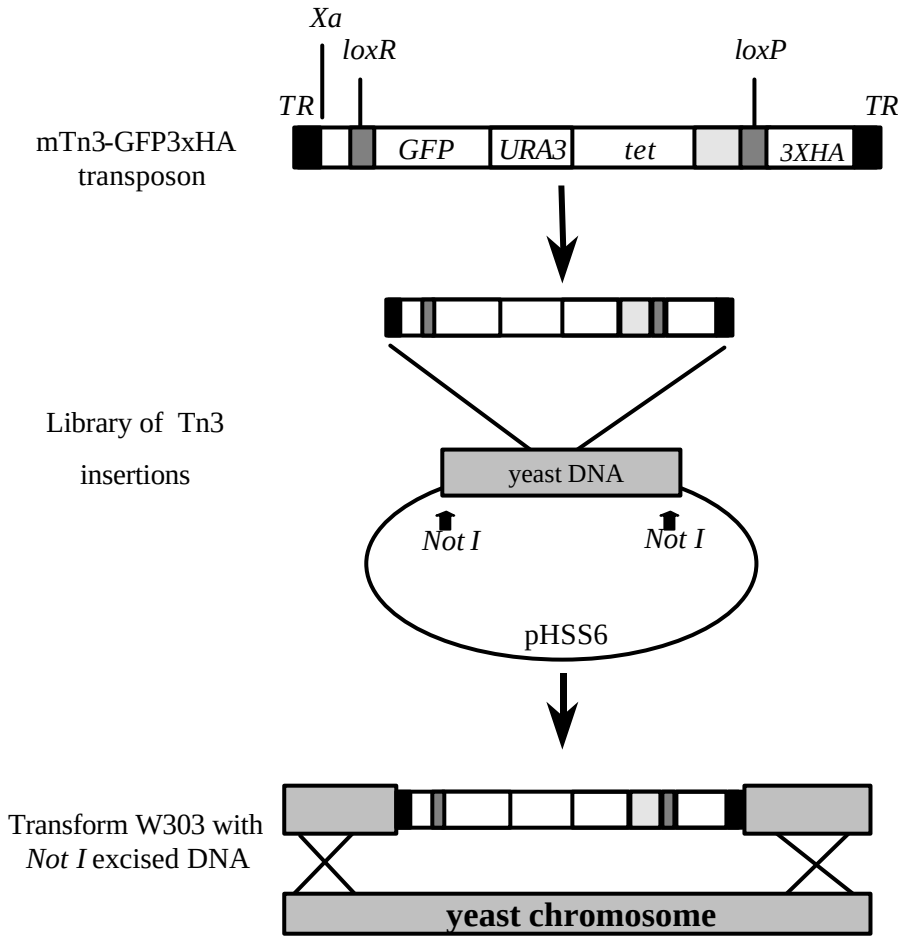


Figure 1. Transposon mutagenesis of strain W303
The mTn3-HAGFP library was obtained from Dr M. Snyder, Yale University (Ross McDonald *et al.*, 1997). *Not I* restriction fragments of yeast genomic DNA containing the Tn3 transposon were transformed into laboratory yeast strain W303. *TR* (terminal inverted repeats), *Xa* (a cleavage recognition site), *GFP* (green flourescent protein), *tet* (tetracyclin resistance gene), *HA* (heamagglutinin epitope tag) *loxR* and *loxP* (lox sites, target for Cre-recombinase).

this study: i) the single insertion mutations are introduced in a close to random manner across the genome, ii) the insertions are themselves non-toxic to the yeast cell, and iii) the inserted transposon also contains useful tools such as the *GFP* reporter gene, HAT tag fusion, and a reducibility of the insertion to a minimal HAT tag via the *cre-lox* system. The mTn-3xHA/GFP-mutagenised library came in the form of 18 pools of pHSS::mTn 3xHA/GFP plasmid DNA containing the mutagenic transposon inserted randomly into fragments of a *Sacch. cerevisiae* S288C genomic library. Single insertional mutations were introduced into a laboratory yeast (W303) by transformation with *NotI* digested pHSS::mTn-3xHA/GFP fragments (see Fig. 1). As a genetic background, a haploid wine yeast would have been preferred over a laboratory strain, however efforts to produce such a strain failed to yield one at the commencement of this work. As an alternative, the laboratory strain W303 was chosen for this screen based on the fact that it was able to ferment the high sugar-content media used here and that its was relatively resistant to ethanol (data not shown). The use of a genomic library from the laboratory strain, S288C, rather than from a wine yeast may have reduced the likelihood of finding relevant wine-yeast-specific genes. Even so, apart from the non-availability of an alternative, it was further reasoned that most genes of interest would be common to both laboratory and wine yeast strains. In fact, mutations identified from the S288C library and introduced into a wine yeast derivative (once it became available) retained the HNE phenotype, confirming the validity of this approach (see below).

From a pool of some 5000 mutants containing various Tn-disrupted sequences, which collectively cover most of the yeast genome, nitrogen efficient mutants were selected by way of a tailored fermentation screen. The screen sought to highlight those mutants that were able to catabolise a greater amount of glucose under nitrogen-limited conditions. Work by Wenk (2000) indicated that *hne* mutants selected under nitrogen-limited conditions were also nitrogen efficient in media containing excess nitrogen. This fact was exploited to simplify analysis of the large number of fermentations required as part of the primary screen of the mutant library. Thus, the

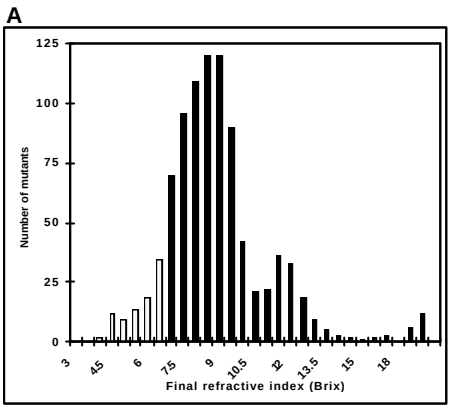


Figure 2. Histograms showing the distribution of final refractive index values for individual fermentations performed by approximately 1000 transposon mutants (A) or approximately 50 W303 (B) in CDGJM containing 100 mg FAN/L as ammonia. Values are derived from a typical experiment for a subset of the total 5000 mutants examined. The unshaded histograms (Panel A) correspond to the mutants selected for further investigation.

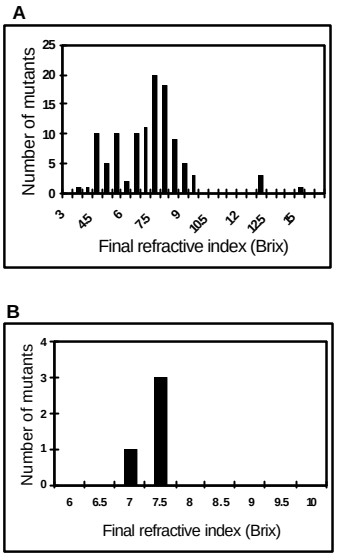


Figure 3. Histograms showing the distribution of average final refractive index values for triplicate 5ml fermentations by each of the selected 110 mutants (A) from Figure 2 or W303 (B) in CDGJM containing 75 mg FAN/L as ammonia.

degree of glucose catabolism in these fermentations was estimated from the refractive index of the medium. Screening fermentations were performed aerobically with CDGJM containing 75-100 mg FAN/L as ammonia, in a volume of 1 mL in 48-well plates. Of the approximately 5,000 mutants screened, 110 were selected for further investigation (Fig. 2). In a secondary screen the enhanced nitrogen efficiency of the selected 110 mutants was confirmed in triplicate 5 mL fermentations in CDGJM with 75 mg FAN/L as ammonia, similar to the primary screen (Fig. 3). Forty of these mutants (36 %) were shown to catabolise more glucose than the parent (non-mutagenised) strain, proving the suitability of the method.

As grape juices typically contain assimilable nitrogen as a complex mixture of compounds, primarily amino acids and ammonia, it was of interest to evaluate the nitrogen efficiency of the

selected mutants during growth on a complex nitrogen mix of 75 mg FAN/L compared with the same amount of nitrogen supplied as ammonium. It can be seen in Figure 4, that the extent of sugar catabolism is generally decreased when the limited nitrogen is supplied as amino acids and ammonium compared with ammonium alone. This finding may be a reflection of the fact that the original screen sought nitrogen efficiency during growth in an ammonium-containing medium. Alternatively, these findings may indicate the greater relative efficiency of growth or sugar catabolism when nitrogen is supplied as ammonium.

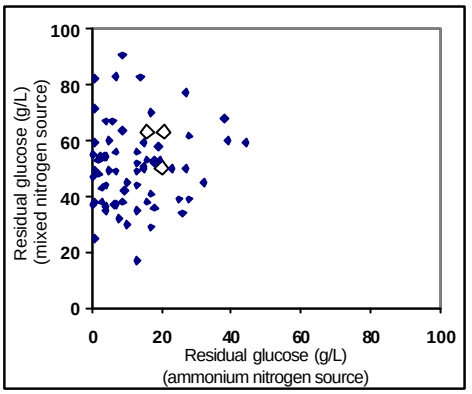


Figure 4 Comparison of residual glucose contents of fermentations performed by selected transposon mutants (◆) and W303 (◇) when grown in CDGJM containing 75 mg FAN/L supplied as ammonium or as an ammonium and amino acid (mixed) nitrogen source.

The ultimate objective of the mutagenesis screen used here was the isolation of mutants with an increased efficiency of nitrogen utilization. Specifically, we sought to identify mutants that are analogous to existing nitrogen efficient wine yeasts (Jiranek *et al.*, 1991, 1995), in that they use less nitrogen to ferment a given amount of sugar even in a nitrogen excess condition. Selected mutants were therefore grown in CDGJM containing excess nitrogen (750 mg FAN/L). As can be seen in Figure 5, only a few strains tested used less nitrogen than the parental strain. However, even mutants that failed to show nitrogen efficiency under excess-nitrogen conditions are nevertheless likely to be useful in the conditions under which they were originally isolated, that is, during nitrogen limitation.

Ten mutants were selected from the above screens conducted under conditions of limited-nitrogen according to an increased nitrogen efficiency that was largely independent of the nature of the supplied nitrogen (amino acids and ammonium or ammonium alone, Fig. 4). The fermentation kinetics and extent of glucose catabolism of these mutants during anaerobic growth in CDGJM containing 75 mg FAN/L as amino acids and ammonium were determined (Table 1). Where appropriate, mutants were classified as *hne* and selected for further characterization according to the key criterion of their being able to catabolise more glucose than the parental strain under the test conditions.

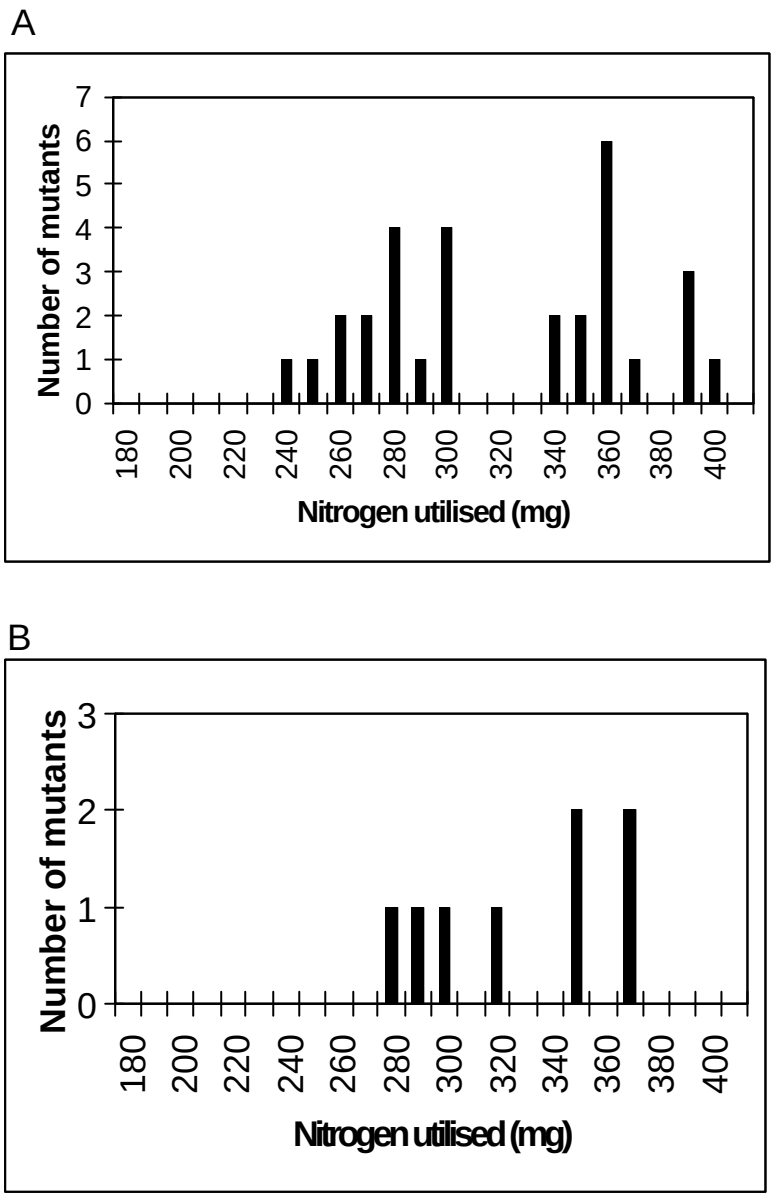


Figure 5. Histograms showing the distribution of quantities of nitrogen utilised by selected transposon mutants (A) and W303 (B) after fermentation of CDGJM containing 750 mg FAN/L as ammonia. Residual nitrogen was measured in clarified fermentation samples with an ammonium electrode.

A

Mutant/Strain	Total weight loss (g)	Relative change in total weight loss (%)	Residual glucose (g/L)	Increased glucose consumption (%)	Affected gene
W303	5.75 +/- 0.23	0	46.2 +/- 0.8	0	-
mTn82	6.55 +/- 0.19	13.9	41.5 +/- 5.7	3.1	*TIF4632*
mTn113	6.65 +/- 0.14	15.9	35.0 +/- 4.7	7.3	*NGR1*
mTn63	6.93 +/- 1.12	20.5	36.8 +/- 3.8	6.1	*RDN37-1*
mTn77	6.32 +/- 0.12	9.9	43.1 +/- 1.0	2.0	*RDN37-1*
mTn125	6.45 +/- 0.05	12.2	46.3 +/- 2.2	0	*RDN37-1*
mTn130	5.80 +/- 0.18	0.87	49.7 +/- 5.8	-2.3	*RDN37-1*

B

W303	7.79 +/- 0.95	0	76.13 +/- 4.27	0	-
mTn98	6.92 +/- 0.20	-11.1	65.67 +/- 3.47	8.4	*PUT4*
mTn72	7.38 +/- 0.18	-5.3	63.87 +/-5.50	9.9	Not identified
mTn78	7.05 +/- 0.32	9.5	72.10 +/- 7.39	3.3	YDL133W
mTn120	7.22 +/- 0.11	-7.3	57.00 +/- 12.6	15.4	YCL039W

Table 1. Final cumulative weight loss and residual glucose content (extent of fermentation) of fermentations performed by transposon mutants. Fermentation were carried out over the course of two separate experiments (reported in panels A and B above) under anaerobic conditions in 100mL of CDGJM with 75 mg FAN/L as a mix of amino acids and ammonium. Data points are the average of triplicate fermentations (+/- standard deviation).

Identification of genes influencing nitrogen efficiency

Sequencing, using inverse PCR (Martin *et al.,* 1999), of the inserted transposon and its adjacent regions enabled the determination of the genomic location of the insert as well as the identity of the affected gene for each *hne* mutant. Gene identity was determined by comparison of sequences to the yeast genome database (http://genome-www.stanford.edu/Saccharomyces/) using the homology search engine BLAST (http://genome-www2.stanford.edu/cgi-bin/SGD/nph-blast2sgd). The identity of the affected gene in each of the selected mutants is shown in Table 1.

The transposon insertion of mutant 113 is very close to a glutamine rich region at 1491bp of the 2018bp *NGR1* gene. The specific biological function of *NGR1* (negative growth regulator 1) is unknown, although it has been hypothesized to be involved in regulation of growth rate and mRNA stability and/or processing (Lee and Moss, 1993). Deletion of the open reading frame of *NGR1* confers a 30% increase in growth rate in the early log phase during growth on glucose, or an increase of 60% or 75% during growth on galactose or glycerol, respectively. Supporting this *NGR1* (*RBP1*) was also isolated from a screen selecting for genes that negatively regulate growth when overexpressed (Akada *et al.,* 1997). *NGR1* has also been shown to be glucose repressible (Lee and Moss, 1993). More recently it has been suggested that *NGR1* is involved in the response to high sugar stress, as microarray data showed that *NGR1* mRNA is upregulated 3.7 fold when cells are exposed to high (400 g/L) sugar (Erasmus *et al.,* 2002).

The mutant strain 82 has a transposon inserted at 351bp of the 914bp *TIF4632* gene, encoding the eIF4G (eukaryotic initiation factor 4 gamma), a global translation initiation factor. eIF4G is known to mediate the binding of several factors to the yeast nuclear cap binding complex as a part of cap-dependent mRNA translation initiation. There are two isoforms of eIF4G in yeast, eIF4G1 and eIF4G2, encoded by *TIF4631* and *TIF4632*. A 320-amino acid stretch in the carboxy terminal half of these genes is 80% identical (Goyer *et al.,* 1993). These two proteins do have functional differences, yet their essential functions are redundant (Tarun *et al.,* 1997). eIF4G is proposed to be affected by the nutritional status of the cell, as eIF4G is rapidly degraded when cells enter the diauxic growth phase or when treated with rapamycin (Berset *et al.,* 1998). It is thought that this effect is mediated by the TOR signal transduction pathway by regulating the stability of eIF4G.

The transposon in mutant 98 is inserted at –357bp in the promoter of the *PUT4* gene. *PUT4* encodes the proline permease, put4p. The inserted transposon could possibly disrupt binding of upstream regulators, such as SWI5, as the inserted transposon is located 4 base pairs away from such a putative binding site. Disruption of the regulation of *PUT4* could lead to constitutive expression. Work in this laboratory has located sequences within the 3' region of *PUT4* (-90, -160, -213 and -708) whose disruption allows constitutive expression under normally repressing conditions (Poole, 2002). Normally *PUT4* is nitrogen catabolite repressed in the presence of a rich nitrogen source such as ammonia. De-repression of put4p should allow the cell to utilise more of the abundant supply of proline present in CDGJM and thus have access to a greater supply of nitrogen. The degree of expression of *PUT4* in mutant 98 under the experimental conditions used will be determined in an attempt to explain the high nitrogen efficiency of this mutant.

RDN25-1 and *RDN37-1* are sections encoding the ribosomal RNA of which there are 100-200 tandem repeats in a 1-2Mb region on the right arm of chromosome XII. As these mutants have been isolated in our laboratory with other screens we believe that they are likely to be false positives, a feature not uncommon to these libraries (M. Snyder pers. comm.).

YDL133W and *YCL039W* are hypothetical open reading frames located on chromosomes IV and III, respectively, and no function has been assigned to these sequences. The putative protein of *YCL039W* is predicted to contain seven WD40 repeats (SWISS-PROT). The WD40 domain is thought to mediate protein-protein interactions, and is found in many regulatory proteins such as STE4p, encoding the ß subunit of the heterotrimeric G-protein GTPase (Fong *et al.*, 1986).

Further work is clearly required to determine the precise mechanism by which disruption (or modification) of the above genes alters nitrogen efficiency. Aside from this beneficial impact on nitrogen metabolism, this work will also determine the extent to which modification of the above genes has a detrimental effect, if any, on the fermentative performance of the strains. For a selection of the more interesting or most promising mutations/genes identified so far, such further characterization has already been initiated. Thus, the genes of interest were disrupted in a wine yeast background so as to more effectively assess the impacts under oenological conditions.

Modification of wine yeast to induce high nitrogen efficiency

Wine yeast are valued for their ability to rapidly ferment sugars without formation of significant amounts of undesirable flavours and aromas, their tolerance to ethanol, and resistance to sulphur dioxide (Rankine, 1977). It would be of great benefit to the wine industry to have access to strains that not only show such desirable traits but also greater nitrogen efficiency. For this reason *hne* genes were deleted in the haploid wine yeast strain, C911D, produced by this laboratory (Walker *et al.*, 2002; GWRDC project UA 99/1). This strain was produced from the commercial wine yeast strain L-2056 (Lallemand Australia), by disruption of the *HO* gene through gene replacement with *KANMX3* (Wach *et al.*, 1994), followed by removal of the *KANMX3* cassette by loop-out technology.

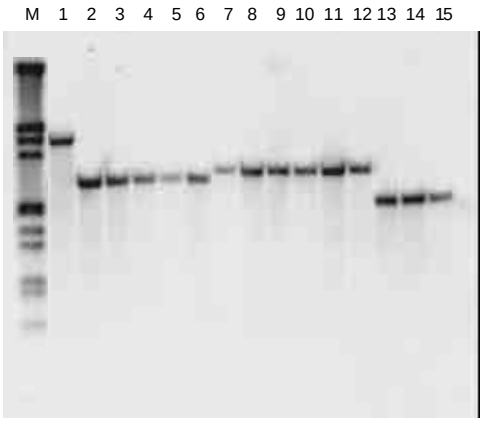


Figure 6. Southern blot analysis to confirm deletion of *TIF4632*, *TIF 4631* and *NGR1* in haploid wine yeast strain C911D. *EcoRI* cut genomic DNA was prepared from mutants from the yeast deletion project (lane 1, #4417, *tif4632?*; lane7, #7284, *tif4631?*; lane 13, #3352? , *ngr1?*), deletion mutants in haploid wine yeast C911D of *TIF4632*-delete strains (lanes 2-6), *TIF4631*-delete strains (lanes 8-12) and *NGR1*-delete strains (lanes 14-15), hybridised with a probe specific for the *KANMX* cassette. Lane M corresponds to a molecular weight marker.

Thus far, genes *TIF4632*, *TIF4631* (encoding the isomer of the TIF4632 protein), *NGR1* and *YCL039W*, have been deleted from C911D by replacing their open reading frames with *KANMX4* by homologous recombination. Similarly these genes have also been deleted from the laboratory strain W303, to confirm that the observed phenotype is due to disruption of the identified genes. Disruption constructs were generated by PCR amplification with primer sets *TIF4632*A, *TIF4632*D, *TIF4631*A, *TIF4631*D, *NGR1*A, *NGR1*D, *YCL039W*A and *YCL039W*D from strains #4417, #7284, #3352 and #3446, respectively, obtained from the yeast deletion project (http://www-deletion. stanford.edu/cgi-bin/deletion/search3.pl). This method yields long homologous flanking regions, which offer efficient homologous recombination. Southern blot

analysis confirmed the deletion of each gene (Fig. 6and 7). Transformants were selected that were shown to carry the *KANMX* cassette indicating incorporation of the disruption construct.

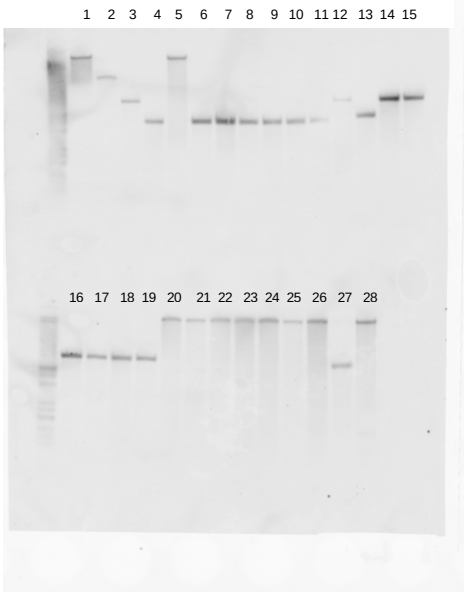


Figure 7. Southern blot analysis used to confirm deletion of *TIF4631*, *TIF4632*, *NGR1* and *YCL039W* in laboratory yeast strain W303 or haploid wine yeast strain C911D. *EcoRI* cut genomic DNA was prepared from yeast deletion mutants from the yeast deletion project [lanes 1, 5 and 24, #3446 (*ycl039w?*), lane 2, #7284, (*tif4631?*), lane3, #4417 (*tif4632?*), lane 4, #3352? (*ngr1?*)], and yeast deletion mutants in W303 of *NGR1* (lanes 6-11), *TIF4631* (lanes 12 and 14), *TIF4632* (lanes 15-19), *YCL039W* (lanes 22-26) and in C911D of *YCL039W* (lane 28), and hybridised with a probe specific for the *KANMX* cassette.

To determine the effect of the *ngr1?*, *tif4631?*, *tif4632?* and *ycl039w?* mutations, fermentations were performed under anaerobic conditions with 300, 150 and 75mg FAN/L as complex amino acids and ammonium (Figures 8 and 9, data for 150mg FAN/L fermentations not shown). Deletion of *NGR1* from W303 resulted in an increased rate of glucose catabolism in a medium of limiting nitrogen supplied as ammonia (Fig. 8 A), confirming the identity of the disruption in the mTn82 strain. Over 400 hrs, W303*ngr1* was found to catabolise 195.7 g/L of glucose whereas W303 only catabolised 186.6g/L. When nitrogen is present as a mixture of amino acids and nitrogen, deletion of *NGR1* from W303 appears to have no affect upon the rate of glucose catabolism (Fig. 8 C, D). Deletion of *NGR1* from C911D resulted in a significant shortening of total fermentation time in the presence of both limiting and non-limiting amounts of complex nitrogen and limiting amounts of ammonia (Fig. 8 E, G, H). More specifically, when 75 mg FAN/L were available as ammonia *ngr1?* catabolised 200g/L of glucose in 85% (*ca.*145 h) of the time required by C911D (*ca.*170 h). Similarly when a mixture of amino acids and ammonia were available, *ngr1?* catabolised 200g/L of glucose in 73% (*ca*. 140 h) of the time required by C911D (*ca.* 190 h). Alike to when 150 (data not shown) and 300 mg FAN/L are available, *ngr1?* completed fermentation in 83% and 73% of the time of C911D, respectively. Surprisingly when

300 mg FAN/L is available as ammonia, fermentation by W303*ngr1* seems to stall after 100g/L of glucose is catabolised, this experiment will be repeated to confirm this result.

The C911D *ngr1?* strain also produced a smaller final dry cell weight of between 75-80% of that of the wildtype C911D (Fig. 10), independent of the amount of available nitrogen when supplied as complex nitrogen. When a low concentration of nitrogen is supplied as ammonia C911D *ngr1?* also produces only 85% of the final dry weight of the parental, yet when 150 or 300 mg FAN/L of ammonia is present a greater dry weight (118-122%) is produced. The cell number yield of these strains under these conditions however appears to be unaltered (data not shown). This result indicates some differences in the way that inorganic *vs* organic nitrogen sources are used by C911D *ngr1?* and that growth with ready availability of ammonium induces increased biomass yields (as larger rather than more numerous cells). Therefore, the nitrogen efficiency conferred upon the C911D *ngr1?* strain might arise as a result of a sacrifice of the production of particular cellular metabolites (reserve compounds) thereby enabling a faster or extended fermentative activity.

Deletion of *YCL039W* from W303 confers the ability to catabolise glucose faster when nitrogen is present as ammonia or as a mixture of amino acids and ammonia (Fig. 9 I-L). Thus, W303 *ycl039w?* is able to catabolise 161.8g/L of glucose when 75 mg FAN/L is available as ammonia whereas W303 can only catabolise 137.9g/L, that is, under these conditions the deletion is able to catabolise 17% more glucose than the parental. Similarly, when a low concentration of complex nitrogen is present, deletion of *YCL039W* from W303 shortens the time required for fermentation of 200g/L of glucose by 33.5 hrs, that is the total fermentation duration is 92% of the parental strain (*ca*.298.5h vs *ca.* 326h). In media with a high concentration of ammonia or complex nitrogen, fermentation duration is also reduced (90% and 95.2% respectively).

Interestingly, W303 *ycl039w?* behaves like C911D *ngr1?*, in that it also has a smaller final dry weight (82% of the parental, data not shown) when grown in media containing low concentrations of complex nitrogen. Deletion of *YCL039W* from C911D also reduces fermentation duration on mixed nitrogen (Fig. 9 O and P, 87% in both high and low nitrogen), yet does not have any affect when ammonia is the nitrogen source (Fig. 9 M and N).

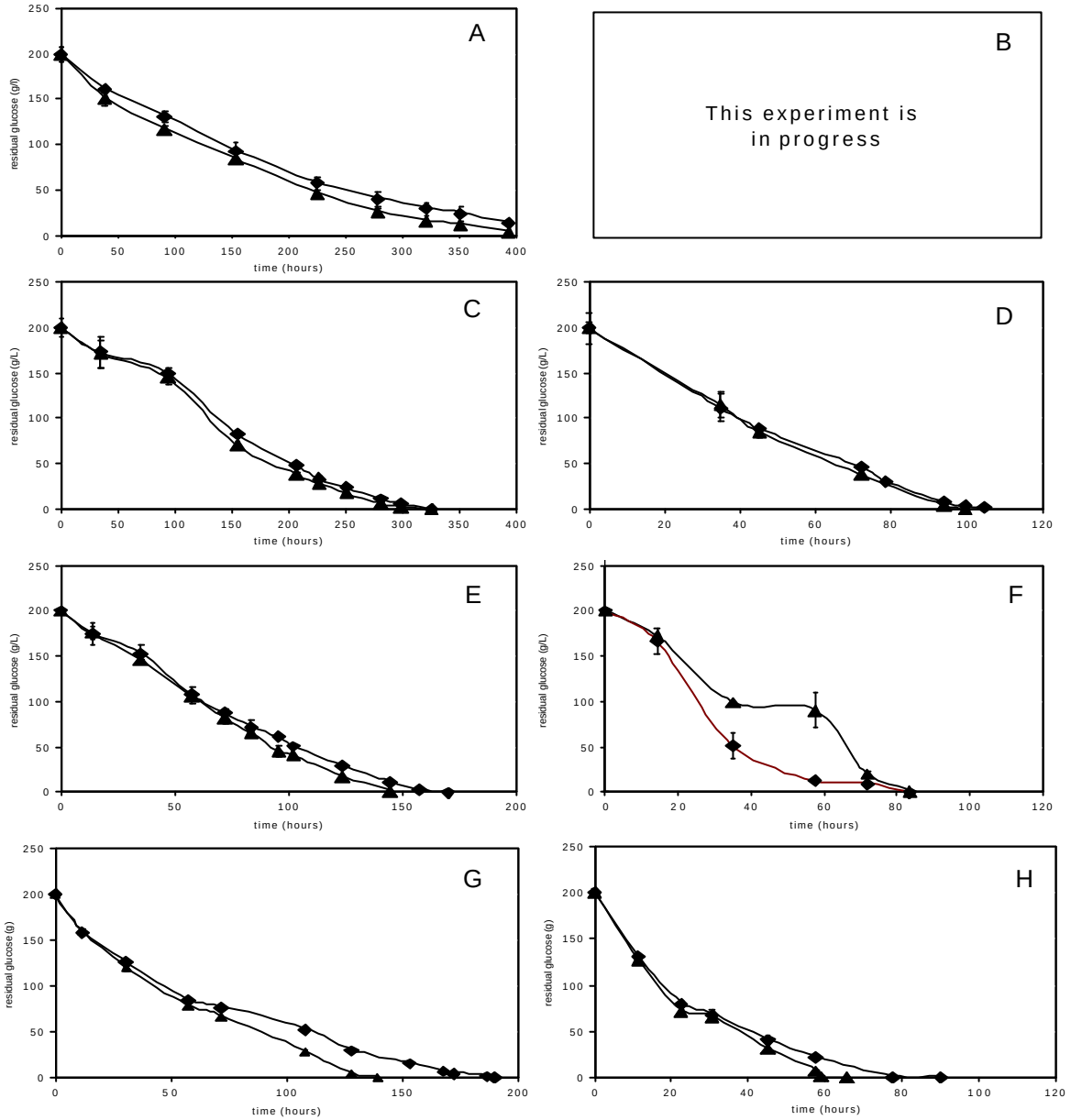


Figure 8. Catabolism of glucose by yeast *NGR1*-deletion (▲) and wildtype (◆) strains. *NGR1* was deleted in a laboratory yeast strain W303 (A-D) and a wine yeast derivative strain C911D (E-H). Fermentations were performed in CDGJM with 75 (A, C, E, G) or 300 mg FAN/L (B, D, F, H) supplied either as ammonia (A, B, E, F) or as a mixture of amino acids and ammonia (C, D, G, H), in 100mL under anaerobic conditions.

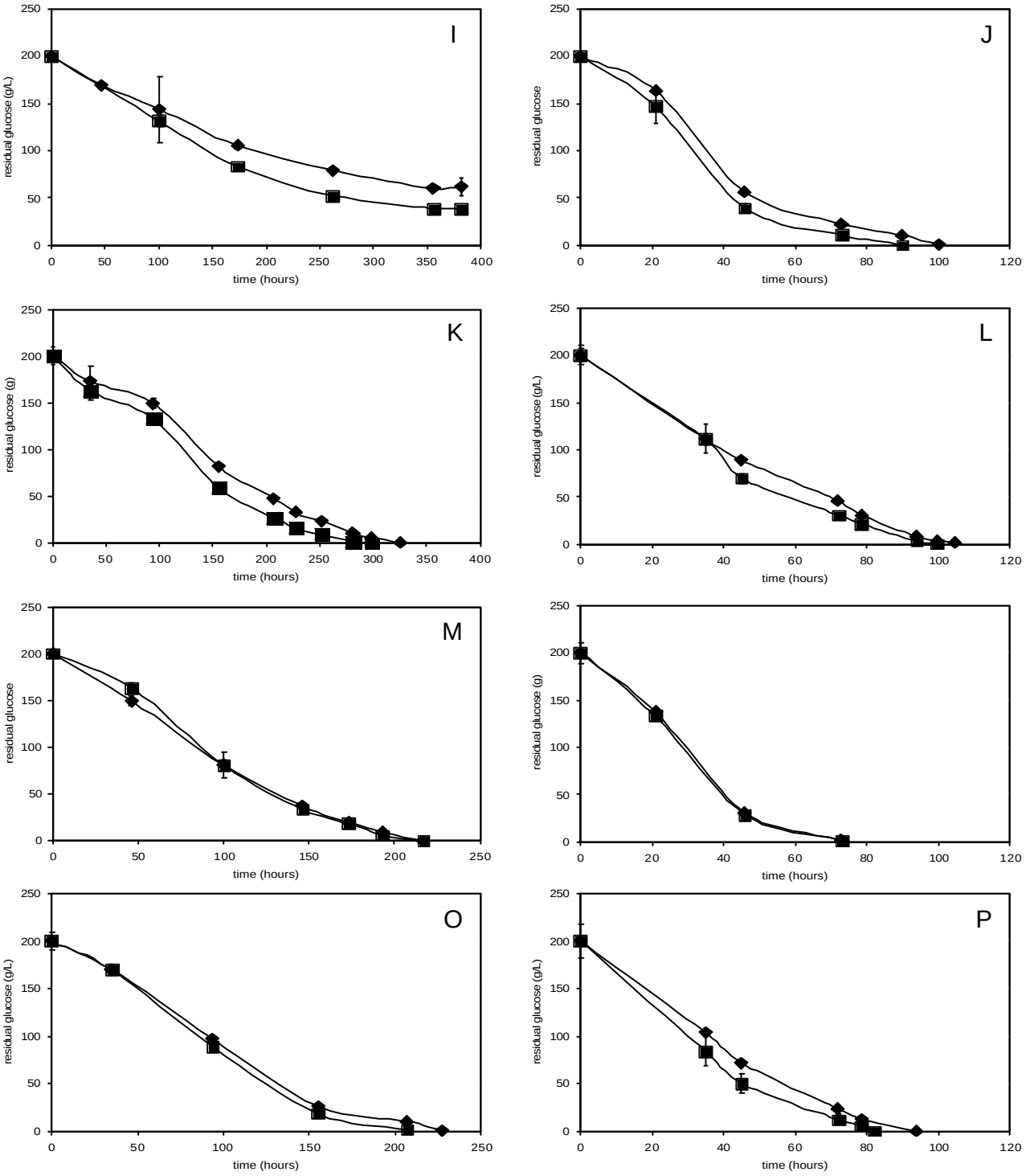


Figure 9. Catabolism of glucose by yeast deletion strains of *YCL039W* (¦) and wildtype (◆). *YCL039W* was deleted in a laboratory yeast strain W303 (I-L) and a wine yeast strain C911D (M-P). Fermentations were performed in CDGJM with 75 (I, K, M, O) or 300 mg FAN/L (J, L, N, P) supplied either as ammonia (I, J, M, N) or as a mixture of amino acids and ammonia (K, L, O, P), in 100mL under anaerobic conditions.

The accumulation of fermentation metabolites at the end of fermentation, some of which are flavour active, is also being examined. So far, ethanol, glycerol, acetic acid, succinic acid, lactic acid, citric acid, acetaldehyde and tartaric acid have been analysed by high performance liquid chromatography (data not shown). A small but reproducible increase in glycerol accumulation is seen for both *ngr1?* and *ycl039w?* compared to the corresponding parental strains when grown in low nitrogen (Fig. 11). Glycerol is one of the predominant products of fermentative metabolism. It is of great interest to the wine industry as it can contribute to a desirable "mouth-feel" of wine. Glycerol is produced during the regeneration of NAD+, a requirement for anabolic metabolism, particularly during anaerobiosis. It is also known to be important in the balance of osmotic pressure (Taherzadeh *et al.*, 2002). It is unknown how the deletion of *NGR1* or *YCL039W* affects glycerol production, but perhaps alteration of these genes affects the redox balance of the cell. Remize et al (1999) noted that a glycerol overproducing strain, produced by overexpressing *GPD1*, had a faster fermentation rate at stationary phase. They hypothesise that this is could be due to an enhanced release of inorganic phosphate.

Contrary to results of Lee and Moss (1993), in our hands, an *ngr1?* does not appear to have an increased growth rate (data not shown). Such differences may however be attributable to differences in the culture methodology (lab fermentations *vs* model oenological fermentations) or the genetic background of the strain bearing the *ngr1?*.

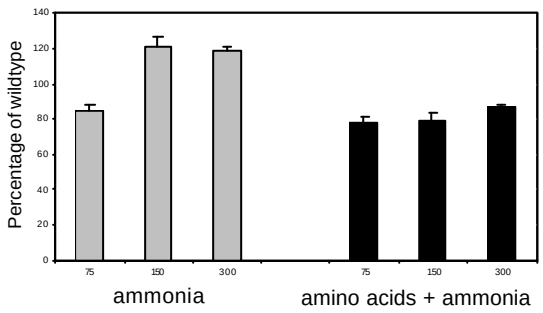


Figure 10 . The dry cell weight at the end of fermentation of yeast strain C911D*ngr1 ?*. Fermentations were performed in CDGJM with 75, 150 or 300 mg FAN/L supplied as ammonia or a mixture of amino acids and ammonia. Measurements were made of 10 ml of media and are derived from triplicate fermentations.

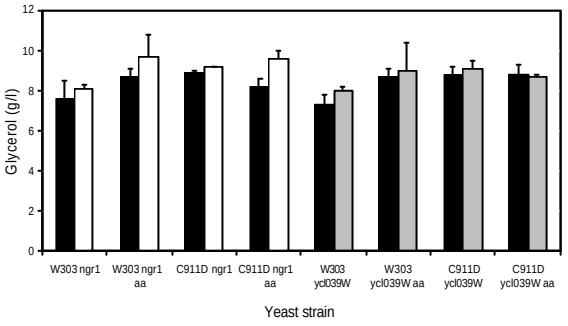


Figure 11. The concentration of glycerol at the end of fermentation produced by yeast deletion mutants of *NGR1* (?) or *YCL039W* (¦) in W303 or C911D in comparison to the wildtype ( ). Fermentations were performed in CDGJM containing low nitrogen (75 mg FAN/L) as either ammonia or as a mixture of complete amino acids and ammonia. Glycerol was measured from triplicate fermentations by HPLC analysis using an Aminex HPx-87H column and a Shimadzu RI detector.

Further charactersation of the 5 mutants of greatest interest is now being carried out. Specifically we are seeking to determine the response of these strains in phenotype assays to a variety of physicochemical stressors. Responses are expected in terms of their growth and/or morphological response. More detailed investigations e.g. involving comparisons of global gene expression in the wildtype compared to the mutant backgrounds will provide additional clues as to the precise role of the genes in question in nitrogen metabolism. With the further information coming form this work, it is hoped that additional modification targets or strategies will emerge that can be used to improve nitrogen efficiency or flavour complexity of wine.

Summary

A transposon mutagenesis approach has been used to identify yeast genes that have an impact on the efficiency with which assimilable nitrogen (ammonium and/or amino acids) is utilized under model oenological conditions. Nine such genes have been sequenced and identified, of which four have been disrupted in both a laboratory and wine yeast background. In all cases, the nitrogen efficiency of the disruptants was improved under some or all test conditions, and the yield of sensorially important compounds (e.g. glycerol) was altered, thereby exceeding Performance Target 1 (*i.e. 1 – 3 genes identified which impact on N-efficiency and/or formation of flavour compounds*).

Deletion of one of the identified genes, *NGR1*, in a wine yeast significantly reduced the time required to complete fermentation, when complex nitrogen was present in low or even in high concentrations, or in low concentrations of ammonia. Disruption of *YCL039W* in W303 also reduces the time required to complete fermentation in all conditions tested. Deletion of this sequence in the wine yeast strain C911D only lead to enhanced glucose catabolism when complex nitrogen was supplied. As for *ngr1?* strains, *ycl039w* strains also produced greater amounts (albeit to a lesser degree) of the sensorially important metabolite, glycerol.

As such these strains show great promise for decreasing the incidence of problem fermentations due to insufficient nitrogen. Therefore, a mutant such as *ngr1* satisfies Performance Target 2 (*i.e. Strategies for optimal use of supplements/strains for greater fermentation reliability/flavour complexity*). Work is still underway to determine how exactly deletion of the *NGR1* gene or

YCL039W enables greater nitrogen-efficiency, and what other effects that deletion may have on the finished wine, for example, in terms of aroma and flavour. So far a reduction in the production of dry weight and a slight increase in glycerol production have been noted, suggesting that major metabolic pathways have been altered to enable the increased catabolism of glucose. However, other benefits for wine flavour beyond increased glycerol concentrations are yet to be demonstrated. A related "benchmarking" exercise intended to help answer this question, provides crucial clues. Before the various gene disruptants became available, existing wine yeast strains of high and low nitrogen efficiency were compared to reveal the consequences of nitrogen efficiency under model oenological conditions. In one of the publications arising from this study we demonstrated that highly nitrogen efficient strains don't just complete nitrogen-limited fermentations more effectively, but are likely to show an enhanced flavour profile due to a reduce degree and duration of hydrogen sulfide formation (Gardner et al., 2002). Similar work is now being carried out the *NGR1* and *YCL039W* disruptants.

Performance Target 3 (*1 – 2 novel strains able to complete fermentation with minimal need for supplements/intervention and novel/improved flavour production*) will best be met through non-recombinant or "minimally-modified" versions of strains bearing mutations such as *ngr1*. Again, mutation of *NGR1* as well as other genes has been shown to be beneficial in both wine and laboratory yeast backgrounds. We are currently looking at approaches to combine such mutations in a single cell as well as develop such mutants through non-recombinant means. Further analysis of the sensorial consequences of these individual and combined mutations will be performed through grape juice fermentations this vintage.

One refereed paper, one industry journal article and four conference presentations have been made on this topic in the last 3 years or so, thereby satisfying Performance Target 4 (*2 reports on characterisation of genes affecting N-efficiency/flavour formation. Report on benefits of optimised yeast/fermentation strategies*). Further reports will be forth coming as the work is finalized (in the next few months) and expanded upon in GWRDC Project UA 01/04 – *Microbial Enhancement of Wine Quality and Complexity*.

Ms Jennie Gardner has intermitted her studies to undertake a vintage from March – April 2003. Upon her return she will complete and submit her thesis for examination. This process will mark the completion of her high level training in an area of research closely aligned with the wine industry (i.e. Performance Target 5 - *1 PhD trained in the oenology area with expertise to aid an innovation and technology-driven industry*).

Publications and Reports Arising From This Project.

Gardner, J. M., Poole, K., Jiranek, V. (2002) Practical significance of relative assimilable nitrogen requirements of yeast: a preliminary study of fermentation performance and liberation of H_2S. *Aust. J. Grp. Wine Res.* 8:175-179.

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. 30th Annual Technical Issue. *Aust. N.Z. Gregrwr Winemkr.* **461a**: 109-114.

Poole K., Gardner, J. M., de Barros Lopes, M., Jiranek, V. (2002) Strategies for avoiding problems associated with nitrogen limitation during fermentation. *Proc. 2nd Aust. Conf. Yeast: Prod. & Discov*. November 2002. Melbourne, Australia. pp. 22.

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. 11th Aust. Wine Ind. Tech. Conf.*, October 2001. Adelaide, South Australia. pp. 242.

Gardner, J. M., Wenk, M., de Barros Lopes, M., and Jiranek, V. (2000) Identification of genes contributing to a "High Nitrogen Efficiency" (Hne) phenotype in a modified wine yeast. *Proc. 1st Aust. Conf. Yeast: Prod. & Discov.* June/July, 2000. Couran Cove, Australia. pp. 11.

Poole, K., Gardner, J., Wenk, M., de Barros Lopes, M., and Jiranek, V. (2000) New strategies for fermenting low nitrogen musts: HNE mutants and proline utilization. *Proc. 8th Lallemand Tech. Meet.* May, 2000. Krems, Austria. pp. 15-20.

References

Akada R., Yamamoto J., and Yamashita J. (1997) Screening and identification of yeast sequences that cause growth inhibition when overexpressed. *Mol. Gen. Genet.* **254**: 267-274.

An, D., and C. S. Ough. (1993) Urea excretion and uptake by wine yeasts as affected by various factors. *Am. J. Enol. Vitic.* **44**:35-40.

Bely, M., J._M. Sablayrolles, and P. Barre. (1990) Automatic detection of assimilable nitrogen deficiencies during alcoholic fermentation in enological conditions. *J. Ferm. Bioeng*. **70**:246-252.

Berset, C. H., Trachsel, H., Altmann, M. (1998) The TOR (target of rapamycin) signal transduction pathway regulates the stability of translation initiation factor eIF4G in the yeast *Saccharomyces cerevisiae*. *PNAS USA* **95:** 4264-9.

Erasmus D.J., van der Merwe G.K., van Vuuren H.J.J. (2002) Genome wide expression analyses: Metabolic adaptation of *Saccharomyces cerevisiae* to high sugar stress. *FEMS Yeast. Res.* 1540: 1-25.

Fong, H.K., Hurley, J.B., Hopkins, R.S., Miake-Lye, R., Johnson, M.S., Doolittle, R.F. and Simon, M.I. (1986) Repetitive segmental structure of the transducin ß subunit: homology with the CDC4 gene and identification of related mRNAs. *PNAS USA* **83**, 2162-2166.

Gardner, G. M., K. Poole, and 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. Grp. Wine Res.* 8:

Goyer C., Altmann M., Lee H. S., Blanc A., Deshmukh M., Woolford J. L. Jr, Trachsel H., Sonenberg N. (1993) TIF4631 and TIF4632: two yeast genes encoding the high-molecular-weight subunits of the cap-binding protein complex (eukaryotic initiation factor 4F) contain an

RNA recognition motif-like sequence and carry out an essential function. *Molec. Cell. Biol.* **13:**4860-74.

Henschke, P. A., and V. Jiranek. (1993). Yeasts – Metabolism of nitrogen compounds. In: Wine Microbiology and Biotechnology. G. H. Fleet. Ed. Harwood Academic Publishers, Chur, Switzerland, pp 77-164.

Jiranek, V., Langridge, P. and Henschke, P. A. (1991) Yeast nitrogen demand: selection criterion for wine yeasts for fermenting low nitrogen musts. *Proc. Internat. Symp. Nitrgn. Grp. Wine.*, Seattle, Washington. (Am. Soc. Enol. Vitic.: Davis) pp. 266-269.

Jiranek, V., Langridge, P. and Henschke, P. A. (1995) Amino acid and ammonium utilization by *Saccharomyces cerevisiae* wine yeasts from a chemically defined medium. American Journal of Enology and Viticulture **46,** 75-83.

Lee, F. J., and Moss, J. (1993) An RNA-binding protein (RBP1) of *Saccharomyces cerevisiae* encodes a putative glucose-repressible protein containing two RNA recognition motifs. *J. Biol. Chem.* **268:**15080-7.

Martin, V. J. J. and W. W. Mohn. (1999) An alternative inverse PCR (IPCR) method to amplify DNA sequences flanking Tn5 transposon insertions. *J. Microbiol. Meths.* **35:**163-166.

Poole, K. (2002) Enhancing yeast performance under oenological conditions by enabling proline utilization. PhD Thesis, The University of Adelaide.

Rankine, B. C. (1977) Modern developments in selection and use of pure yeast cultures for winemaking. *Internat. Oenol. Symp. Bordeaux, France.*

Remize, F., Andrieu, E., and S. Dequin (2000) Engineering of the pyruvate dehydrogenase bypass in *Saccharomyces cerevisiae*: Role of the cytosolic Mg^{2+} and mitochondrial K^{+}

acetaldehyde dehydrogenases Ald6p and Ald4p in acetate formation during alcoholic fermentation. *Appl. Environ. Microbiol.* **66**: 3151-3159

Ross-Macdonald, P., Sheehan, A., Roeder, G. S. and Snyder, M. (1997) A multipurpose transposon system for analysing protein production, localisation and function in *Saccharomyces cerevisiae. PNAS USA* **94:** 190-195.

Tarun, S. Z., Wells, S. E., Deardorff, J. A. and Sachs, A. B. (1997) Translation initiation factor elF4G mediates in vitro poly(A) tail-dependent translation. *Biochem.* **94:** 9046-9051.

Taherzadeh, M.J., Alder L., Liden G. (2002) Strategies for enhancing fermentation production of glycerol- a review. *Enz. Microbiol. Tech.* **31**:53-66.

Wach, A., Brachat, A., Pohlmann, R. and Philippsen, P. (1994) New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. *Yeast* **10:** 1793-808.

Walker, M., Gardner, J. M., de Barros Lopes, M. and Jiranek, V. (2002) Wine yeasts as tools for oenological research and strain improvement by genetic techniques. *Aust. N. Z. Grpgrwr. Winemkr.* **461a:** 109-114.

Wenk, M. (2000) Isolation and characterisation of yeast mutants with increased nitrogen efficiency. Honours Thesis, The University of Adelaide.