

Wine yeasts as tools for oenological research and strain improvement by genetic techniques

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Research Organisation: Adelaide University

Principal Investigator: Dr Vladimir Jiranek
Department of Horticulture,
Viticulture & Oenology,
PMB 1 Glen Osmond, SA 5064.
Ph. (08) 8303 6651.

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Abstract

Wine yeast make a substantial contribution to the composition and hence quality of a wine. A detailed understanding of how the environment of the grape juice fermentation influences wine yeast behaviour is lacking. Yet such information is essential for the manipulation of the interaction of yeast with grape juice to enhance wine quality. The dissection of metabolic processes and their regulation is relatively straight forward in laboratory strains of yeast. Being available as haploids with a number of selectable markers, such strains have a simple genetic constitution and are therefore readily manipulated. A major obstacle to understanding the metabolism of wine yeast under oenological conditions is the genetic complexity of such industrial strains. Through this project we have undertaken to construct wine yeast derivatives that are of simpler genetic constitution. Furthermore we have developed a set of disruption vectors for use with these strains, which take advantage of dominant selectable markers and, in specific cases, are designed to be lost from the genome when not required, leaving minimal or no foreign sequences.

Specifically we have produced haploid strains from diploid (or potentially higher) commercial wine yeast strains (L2056, O16 and CY3079). This was achieved through disruption of the *HO* gene, which is responsible for mating type switching and hence spontaneous re-diploidisaton of haploids. These *ho* strains display fermentation kinetics and key metabolite formation, which closely resembles that of parental wine yeast strains.

The *ho* strains are intended as a) tools for oenological research and b) as vehicles for the construction of new optimised wine yeasts. In line with the former, we have shown these yeast to be fully amenable with classical genetic techniques and chemical mutagenesis. The introduction of additional selectable markers has also been accomplished thereby enhancing the versatility of these strains for research purposes. In particular, a *ura3* marker has been introduced which now allows the screening of a transposon mutant library in the wine yeast background of these strains. This possibility, to be taken up in a subsequent project, will be extremely useful in identifying genes of importance to key wine yeast attributes.

As part of a strategy to produce an optimised wine yeast destined for industrial use, we are finalising characterisation of genes from related projects as candidates for modification. Our ability to make modifications with minimal extraneous foreign sequences or additional modifications is also being confirmed. The development of a disruption vector from which the selectable marker, *KanMX* (geneticin resistance), can be removed after gene disruption is instrumental to this goal. Future work will centre on the exploitation of the tools developed in this project as well as their fuller evaluation in terms of winemaking and organoleptic criteria.

Abbreviations

Genetic Nomenclature: For *Saccharomyces cerevisiae*, genes are identified by way of a 2 or 3 letter designation, which is italicised. A number following this designation refers to the specific gene with a family of related genes, i.e. related biosynthetic pathway. Where gene designation are written in upper case letters the gene is functional or wild type. The use of lower case letters indicates a loss of function of the gene in question.

CDGJM, Chemically defined grape juice medium
DIG, digoxigenin
EMS, Ethyl methanesulfonate
FOA, 5 fluoro-orotic acid
Geneticin, antibiotic G418
HPLC, high performance liquid chromatography
MYPG, malt extract, yeast extract, peptone, glucose medium
dNTPs, deoxy nucleotide triphosphates
PCR, polymerase chain reaction
PFGE-CHEF, pulsed field gel electrophoresis contour-clamped homogeneous electric field
PVDF, polyvinylidene fluoride
SD, synthetic dextrose medium
YEPD/YPD, yeast extract peptone dextrose medium

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Dr Michelle Walker (Adelaide University, Project PDF [0.5], 2000-2001)
Ms Jennie Gardner (Project PhD student [0.4], 1999-2001)
Ms Andrea Vystavelova (Project Technician [0.8], 2001)

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Summary

We have succeeded in addressing all or most of the outputs under the objectives listed below. Additional work was undertaken or is warranted in light of new findings reported during the course of this study, or else due to unforeseeable technical difficulties. Outstanding items are either near completion or else have been incorporated for completion or expansion in UA 01/04 - *Microbial enhancement of wine quality and complexity* and UA 00/5 - *Increasing fermentation reliability and flavour compound formation by wine yeast.*

Objective 1: *Produce a haploid, heterothallic wine yeast which retains good winemaking and organoleptic properties in addition to good genetic properties and is thereby suited to physiological studies.*

We have successfully produced haploid derivatives of both mating types of three commercial wine yeasts, L2056, O16 and CY3079. Fermentation trials show no major deviation from the fermentative behaviour of the parental strains and no adverse impact on the final concentration of organoleptic compounds studied i.e. residual sugar, ethanol, glycerol, acetic acid, succinic acid, and lactic acid. Strains were shown to be readily subject to classical genetic manipulation (mating, sporulation, tetrad dissection, etc.) and use as a vehicle for genetic modification (i.e via mutagenesis) or further gene disruption (i.e. introduction of *ura3*).

Objective 2: *Adapt to wine yeasts existing systems for "knocking out" specific genes with the introduction of minimal foreign sequences (ideally use dominant and removable markers, e.g. G418 resistance).*

In producing the above strains, we successfully adapted *KanMX*, the G418-resistance gene, to the disruption of the *HO* gene. An analogous construct has been prepared and successfully applied for the introduction of a *ura3* marker (uracil auxotrophy) into wine yeast. We have also developed a novel "one-PCR/two-cloning" method for the construction of alternate *KanMX*-based disruption vectors which should leave no traces of foreign sequences upon loop-out of the marker gene.

Objective 3: *Use above to introduce selectable markers into heterothallic wine yeast to produce research strains into which modified or new genes can be precisely introduced and the physiological effects studied.*

As stated above, we have successfully introduced a *ura3* selectable marker into a wine yeast. This has been achieved directly into an otherwise unmodified (diploid) wine yeast. In addition, through mating of the strains independently carrying *ho* and *ura3* we have isolated heterozygous diploids bearing these mutations in combination (i.e. *HO*/*ho::KanMX*, *URA3*/*ura3*). Sporulation and tetrad dissection will yield the corresponding haploids of both mating types.

Objective 4: *Once above outcomes attained, use these developments to produce a recombinant strain in a wine yeast background with improved properties e.g. increased nitrogen-efficiency as*

per GWRDC Project UA 00/5 Increasing fermentation reliability and flavour compound formation by wine yeast.

Project UA 00/5 has recently succeeded in identifying genes from a laboratory yeast that appear to influence the efficiency with which assimilable nitrogen is utilized during fermentation. Confirmation of the role of these *HNE* genes will be obtained by comparing *hne* mutants with wild type laboratory as well as wine yeasts. Disruption vectors developed in this study as well as the *ho*, *ura3* wine yeasts will be instrumental in this process. Other enhancements of wine yeasts may include the enabling of proline utilisation by wine yeast. This Objective will be largely taken up as indicated as part of UA 01/01 - *Microbial enhancement of wine quality and complexity*.

Publications/Presentations arising from this project:

1. Walker, M., Gardner, J., Pedler, S., Jiranek, V. (2001) Construction and evaluation of haploid wine yeast for research purposes. *11th Aust. Wine Indust. Tech. Conf*. Adelaide, OCT, 2001.
2. Gardner, J. 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*. JUN/JUL, 2000. Couran Cove, Queensland.
3. Gardner, J. M., de Barros Lopes, M. and Jiranek, V. (2000) Isolation and characterisation of mutants with increased nitrogen efficiency. *5th Internat. Symp. Cool Climate Vitic. Oenol.* JAN, 2000. Melbourne, Victoria.
4. Jiranek, V**.** (1999) Investigations of yeast nitrogen metabolism aimed at minimizing fermentation problems. *Proc. 12th Internat. Oenological Symposium,* MAY/JUN, 1999. Montreal, Canada.

Background

Yeast metabolic activity is a major contributor to the chemical and sensory properties of wine. A greater understanding of those activities that produce oenologically important outcomes will therefore lead to a greater degree of predictability, control and tailoring of the winemaking process. In this way, yeast performance could be improved to reduce the incidence of sub-optimal ferments and the need for additives or to increase the complexity of the finished product.

Although the yeast *Saccharomyces cerevisiae* is a relatively well characterised organism, most of this information has been derived from studies of laboratory strains. Furthermore, the impact of winemaking conditions is rarely considered, as are the metabolic processes yielding the quantitatively minor but sensorily important flavour and aroma compounds. Thus, in order for the capabilities of wine strains to be fully understood and exploited, detailed characterisation of the sort seen for lab strains are needed for wine strains.

The disparity in available knowledge about laboratory yeast *vs* wine yeasts is largely due to the ease with which laboratory yeast can be genetically manipulated. In the haploid state, single genes can be disrupted or introduced and the metabolic consequences studied. This is not possible in diploid or polyploid wine strains since multiple copies of the same gene typically mask such genetic modifications. Even when haploid wine strains of a single mating-type are isolated, the population is unstable and a mating-type switch in some cells results in restoration of the diploid state.

Disruption in a wine strain of the *HO* gene responsible for mating-type switching followed by spore isolation would provide a stable (HO^-) haploid population. Confirmation that the HO^- haploid retains the desirable winemaking properties of the parental diploid strain would be the final step in providing an invaluable tool for future research. Such a strain could then be subjected to the powerful classical genetic techniques so well established for laboratory strains in order to identify, isolate and determine the functions of genes of importance to winemaking. More importantly this work would be carried out in a wine strain background.

To further strengthen the research potential of a HO^- wine strain, a series of selectable markers (typically nutritional auxotrophies) could be introduced by way of gene disruptions. With these markers it is then possible to introduce and particularly track DNA fragments (vectors or inserts) containing complementary markers together with any other desired sequence. Such capabilities allow isolation and identification of modified strains during initial construction as well as their subsequent evaluation in fermentations.

The construction of yeasts suited to industrial use will follow on from findings from these trails but must take into account the release approval process. Minimising the amount of “foreign” sequences in the modified yeasts will be crucial for a favourable response to an application for release. Accordingly, the vectors that are designed for the introduction of sequences into wine yeasts will be prepared with this goal in mind. Key requirements of the transformation process used for yeast destined for release will include: 1) selectable markers that are both dominant (hence obviating the need for selectable markers in the recipient genome) and can be lost once they are no-longer needed, 2) transformation by linear DNA to encourage integrative

recombinantion (*cf* transformation with an entire episomal vector which may also contain bacterial sequences) and 3) where ever possible, use of *S. cerevisiae* sequences. In this way it is hoped that changes to recombinant yeasts can be limited to copy-number increases or disruptions of native target genes.

The ability to more precisely determine the basis for a given physiological response/behavior by yeast during winemaking stands to be of high value to the industry. It is expected that completion of this project will enable a more rapid and accurate delineation of yeast metabolic processes and thereby development of strategies for the more effective use of the available yeasts. Also, the construction of improved recombinant wine yeasts (and hence progress of previous/future constructs to an "industry-usable" stage) will be greatly aided by the strains and vectors generated by this project.

Objectives (from project application)

- Produce a haploid, heterothallic wine yeast which retains good winemaking and organoleptic properties in addition to good genetic properties and is thereby suited to physiological studies.

- Adapt to wine yeasts existing systems for "knocking out" specific genes with the introduction of minimal foreign sequences (ideally use dominant and removable markers, e.g. G418 resistance).

- Use above to introduce selectable markers into heterothallic wine yeast to produce research strains into which modified or new genes can be precisely introduced and the physiological effects studied.

- Once above outcomes attained, use these developments to produce a recombinant strain in a wine yeast background with improved properties e.g. increased nitrogen-efficiency as per GWRDC Project UA 00/5 *Increasing fermentation reliability and flavour compound formation by wine yeast.*

Technical Introduction

Few previous studies have utilised genetic techniques as part of a characterisation of yeast physiology as seen under winemaking conditions. Where these techniques have been used, they have been applied largely to laboratory strains and not wine strains. Similarly, many recent reports detailing the construction of improved recombinant yeasts have been limited to work carried out in laboratory strains using traditional bacterial/yeast shuttle vectors (Perez-Gonzalez *et al.* 1993, Volschenk *et al*. 1997, Remize 1999). As a consequence, these constructs contain bacterial sequences, antibiotic resistance genes and selectable markers that make their acceptance by regulatory authorities for commercial use highly unlikely.

To date most progress in terms of the construction of new yeast strains has been achieved via traditional breeding techniques (Romano *et al*. 1985). These methods are highly time consuming and, since they involve the fusion of genomes from two strains, lack the precision of molecular genetic techniques.

We have acquired plasmids containing the *KanMX* resistance marker (confers resistance to the antibiotic G418) flanked by inverted direct repeats (IDR's). These plasmids (Wach *et al*. 1994) are being used as a template for the PCR synthesis of the construct pictured in Figure 1.

The outer flanking sequences bearing homology to *HO* will result in targeting and replacement of the of the native *HO* gene with the above non-functional *HO* truncation. By replacing the *HO* sequences in the flanking regions of the proposed disruption construct, with those from an alternate gene, other genes can easily be targeted for disruption by this method.



Figure 1. Construct used to disrupt the native *HO* gene in yeast. The construct contains the *KanMX* resistance marker (which confers resistance to the antibiotic geneticin or G418) flanked by inverted direct repeats (IDRs). The *HO* coding sequence has been replaced by the *KanMX* module.

Systems for the ready production of "knock-out" strains in which specific genes have been disrupted have been described (Alani *et al*. 1987, Wach, *et al.* 1994). These strategies will be adapted to this project to allow their use with wine yeasts lacking auxotrophic markers.

Research Methodology

- From a selection of commercial wine yeasts, 3 strains suitable for use in this study were identified. The criteria for their selection being the ability to show a high degree of sporulation with the generation of complete tetrads of viable spores. Although such spores re-diploidised, they were evaluated for basic fermentation properties to establish the possible importance of heterogeneity to such attributes.

- The PCR-generated construct depicted in Figure 1 was used to replace the native *HO* gene in a number of commercial wine strains with a truncated sequence disrupted by the G418 (geneticin) resistance gene (*KanMX*) (Wach *et al.,* 1994). Diploid transformants were isolated on antibiotic-containing media before being sporulated and dissected (Guthrie and Fink 1991). Once isolated, heterothallic haploids of each mating type were induced to loose the non-*Saccharomyces* sequences by their "looping out" under non-selective conditions (Wach *et al.,* 1994, J.-M. Salmon, *per. comm*.). Presence or absence of the truncated *HO-KanMX* construct was checked by PCR and Southern blot analysis.

- Further selectable markers were introduced by knock-out strategies. Auxotrophy for uracil, i.e. *ura3*, was considered particularly useful due to the possibility of using both positive and negative selective pressures to *URA3* and *ura3* strains, respectively. Preparation of a collection of yeast with traditional auxotrophic markers will allow use of existing vectors without modification (for research purposes only).

- The *ho* strain was used to perform a chemical mutagenesis to demonstrate its effectiveness for genetic studies. Strains were subjected to classical genetic manipulation (mating, sporulation and dissection), as well as transformation.

- A haploid (*ho*) wine yeast that also possesses a *ura3* marker was prepared by mating of cells carrying alternate mutations. Strains isolated on selective media, have been genotyped by checking growth requirements, mating behaviour and confirmed by PCR.

- A gene whose disruption or altered expression is known to improve on the winemaking or technological properties of a wine yeast would then be appropriately modified in the *ho*, *ura3* strain. Fermentation characteristics of the modified yeast would be evaluated (largely as part of the follow-on project, UA 01/04).

Results

Identification of candidate wine yeasts for *HO* disruption.

Ten commercial strains (see Appendix Table 1a) were evaluated to identify those suited for construction of a haploid, stable mating-type (*HO*-disruptant) derivative. Strains were subjected to repeated rounds of sporulation, tetrad dissection and spontaneous re-diploidisation of individual spores. Spore viability was determined at each round of tetrad dissection. Three strains, L2056, 016 and CY3079 (Lallemand) were found to possess the required genetic properties, that is, good sporulation frequency (*ca* 40%) and spore viability (*ca* 100% in 40 – 80% of tetrads) during repetitive rounds of sporulation (Table 1). Such properties ensure that when conducting genetic analyses of such strains, sporulation occurs at a satisfactory rate, thereby simplifying the microscopic search for tetrads and spores.

The generation of homogeneous strains through repeated spore re-diploidisation was used to investigate the importance of any genetic heterogeneity of the parental strains to their fermentative properties. This issue was considered to gauge the extent to which a strain derived from half the genetic material of the parent wine yeast, truly reflected the genetic makeup and hence phenotype of that parent. A comparison of fermentation kinetics and the profile of key metabolite formation revealed no large differences between derivative strains and the L2056 parent (data not shown). These findings indicate that of the fundamental fermentative properties of the parent strains examined here, none were the result of genetic heterogeneity.

Table 1. Sporulation performance of 10 commercial wine yeast strains.

Strains	Percentage of cells which sporulate	Percentage of asci as 4 spore tetrads	Percentage of tetrads with 4/4 spore viability
Premier Cuvee	45.5	17.0	0
Pasteur Red	15.0	7.0	0
Burgundy	27.8	<0.1	40
L2056	**35.8**	**27.9**	**62**
Flor Sherry	14.9	1.7	10
R2	16.3	2.0	13
EC1118	46.0	2.0	0
CY3079	**42.9**	**1.7**	**40**
O16	**49.4**	**3.0**	**83**
CEG	ND	-	-
Cote des blanc	ND	-	-

Yeast strains were grown 24h (30°C) on YPD plates (2% bactopeptone, 1% yeast extract, 2% glucose), before being patch-streaked onto sporulation plates (1% potassium acetate, 0.1% yeast extract, 0.05% glucose) and incubated for 8 days (30°C). The number of sporulating cells and percentage as 4 spore asci was determined. Tetrads were dissected and grown over two nights on YPD plates. Spore viability was scored for 7-10 tetrad sets per strain. Three rounds of re-diploidisation and sporulation were performed for each strain. ND, not detected.

Construction of *ho::KanMX* disruptants

HO was disrupted by the one-step gene disruption method (Rothstein, 1983). Primers HOFga and HORga (see Appendix Table 1b), designed according to the laboratory yeast genomic sequence database (SGD, Accession number 9169867) (Jacq *et al.,* 1997), were used to amplify 3704 bp of genomic sequence containing the *HO* gene. The fragment was inserted into pGEMT (see Appendix Table 1c) by TA cloning (Promega), yielding plasmid pGEMT-HO. The 1245 bp *BbrPI-BamHI* fragment (containing the *HO* coding sequence) was excised from pGEMT-HO and replaced with the 2562 bp *HpaI-BglII KanMX3* module from pFA6-lacKanMX3 (Wach *et al.,* 1994) to give the recombinant plasmid pGEMTho::KanMX. The plasmid was cut with *NspI*, and the isolated 4.9 kb *NspI* fragment was used to transform diploid wine yeast by the lithium acetate method (Ito *et. al.*, 1983), which included a 2 - 4 hr pre-incubation step at 30^{o}C in YPD prior to plating. The latter provided transformants with an opportunity to express the resistance *KanMX* gene and acquire resistance to the antibiotic. Transformants were selected on YPD supplemented with geneticin (Astral Scientific) at 300 μg/mL (for L2056) and 400 μg/mL geneticin (for CY3079 and O16) as determined from evaluations of the relative sensitivity of the parental strains to geneticin.

Confirmation of *HO* disruption

The disruption of the chromosomal *HO* gene in the wine strains was confirmed by Southern blot analysis. The method use would also highlight transformants in which a multiple integration event had occurred. Genomic DNA was prepared from the parental wine strains as well as the geneticin-resistant transformants (Adams *et. al*. 1997). The DNA was digested with an enzyme that cuts outside the pGEMTho::KanMX construct (*BglII*), and the digests separated on a 1% agarose gel in TAE (Tris-acetate) buffer. The DNA was denatured, transferred to a *Nytran* membrane and fixed by UV cross-linking (Amersham). The filters were prehybridised for 2 hours at 42^{o}C and subsequently probed with a 564 bp DIG–labelled PCR 3' *HO* product (overnight at 42^{o}C). The filters were washed in 0.5 x SSC/0.1% SDS before chemiluminescent detection (van Miltenburg *et al*., 1995).

Southern blot analysis of the parental wine strains O16, CY3079 and L2056 and the geneticin transformants confirmed the disruption of *HO* gene in several transformants (Figure 2). In the case of the wild-type chromosomal *HO* gene, a single ~1.8 kb band was detected with the probe. Two bands are detectable in the transformants, which at this stage were still diploid and thereby typically still retained one native copy of the *HO* gene. Accordingly, such transformants possessed the 1.8 kb wild type gene and a larger, disrupted *HO* gene (*ho::KanMX*). The size of the disrupted gene varied depending on whether a single (~5.3 kb) or double (~10.1 kb) insertional event of the 4.9 kb *NspI* fragment had occurred.

The *HO*-disrupted, diploid transformants were sporulated, their tetrads dissected and grown on YPD. Again, disruption of *HO* was confirmed by Southern blot analysis as described above. Thus in the case of the diploid transformants from the strains O16 (#5, #6) and CY3079 (#8), bands corresponding to the native *HO* (~1.8 kb) gene are seen together with a larger (~5.3 kb/~10.1 kb) band corresponding to the *ho::KanMX* insertion (Figure 3). More importantly, the expected 2:2 pattern of segregation of *HO* and *ho::KanMX* genes was observed in the dissected spores. The analysis clearly demonstrated that for #8 (derived from CY3079) and its haploid

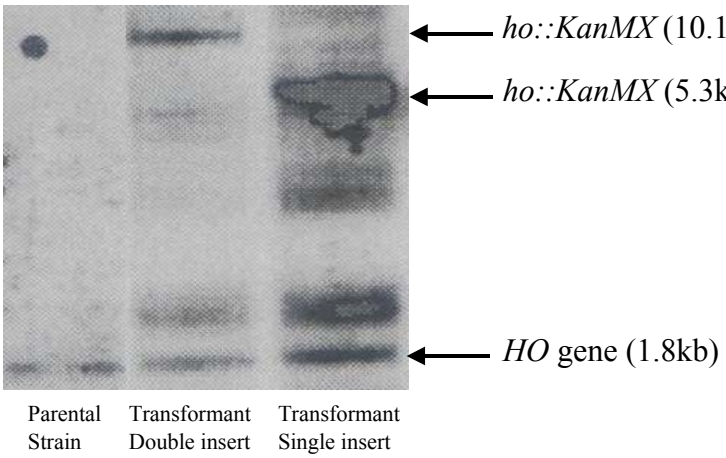


Figure 2. Southern blot analysis showing examples of the banding pattern seen for genomic DNA preparations from a parental wine yeast and transformants (*HO*-disruptants), probed with a fragment of the *HO* gene. Disruptants were prepared as described using the One-step disruption method.

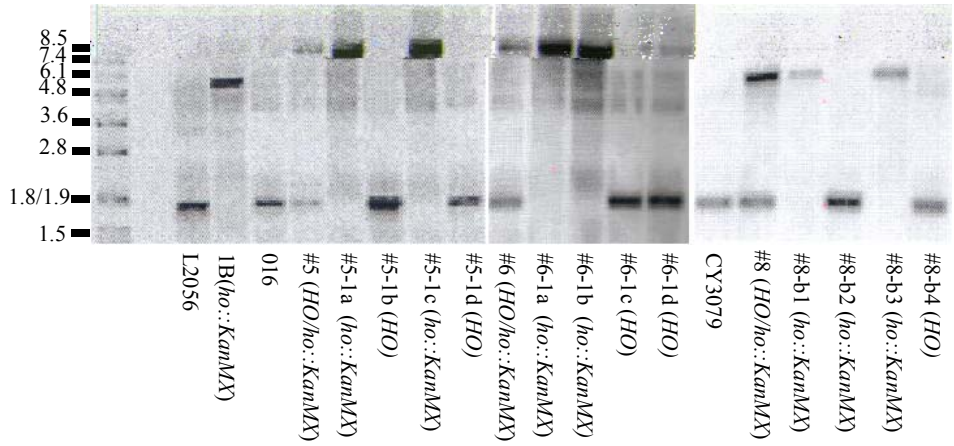


Figure 3. Southern blot analysis revealing segregation patterns of *HO* and *ho::KanMX* in *HO*-disruptant diploids and their haploid derivatives. Samples were prepared as described for Figure 2.

disrupted spores #8-b1 and #8-b1, there was a single insertion into the *HO* gene, whereas a double insertion was observed for the O16 transformants, #5 and #6, and their haploid spores, #5-1a and #5-1c, and #6-1a and #6-1b, respectively. The number of insertions was not strain related, as double insertions were also observed in two other transformants of CY3079; #9 and

#10 (data not shown). For strain L2056, only one haploid disruptant, 1B (*ho::KanMX, MAT*α*)* is shown, although 1D (*ho::KanMX, MAT*a) was also constructed (see Appendix Table 1a).

Determination of mating type of haploid *HO*-disruptants

To facilitate genetic studies, non-switching haploid wine yeast derivatives should ideally be available in both mating types. As such it was necessary to determine the mating types of the haploid *ho*-disruptants isolated so far. Multiple methods were used. PCR using *MAT-, MAT*a- and *MAT*α-specific primers (Huxley *et al.,* 1990) (Appendix Table 1b) was applied. PCR was performed using ~250 ng genomic DNA as template in a 50 μl reaction, according to Huxley *et al.,* (1990). *MAT*a cells demonstrated a 544 bp PCR product, and *MAT*α cells, a 404 bp product, while diploid cells contain both. Figure 4 shows the presence of both mating type loci in the diploid transformants (*HO/ho::KanMX*). Further, for each set of tetrads examined, two spores each possessing one only of the two *MAT* loci are evident. This pattern attests to the haploid state of these spores. By comparison, the remaining pair of spores from each tetrad show both *MAT* bands, thereby being indicative of their having a functional *HO* gene and having already switched mating type and self-diploidised. The haploid state of putative haploid spores (single band by *MAT* PCR) was confirmed by their inability to sporulate. Furthermore, the identity of such haploids was further defined through their appropriate mating response with the tester strains KP2 (*MAT*α) and KP3 (*MAT*a) (Appendix Table 1a) and subsequent ability to undergo sporulation (data not shown). The ability of these strains to undergo this process with predictable outcomes, in itself demonstrates the efficacy of these haploid strains as the basis for a genetic investigation in a wine yeast background.

Flocculation minimization

A further indirect indication of the haploid state of spores derived from the *HO*-disruptants was their flocculation, that is the formation of aggregates of cells not seen in the parental strain. Flocculent tendencies are typical of the haploid state. The desirability of flocculation is situation dependent. Thus while flocculation greatly simplifies post-fermentation clarification, this attribute makes it difficult the keep the culture in suspension and therefore fermenting at an optimal rate without agitation. From a research point of view, flocculent strains are more difficult to work with since the accurate determination of viable cell numbers by plating as well as culture cell or optical density is not possible. We therefore sought to demonstrate that the degree of flocculation by a haploid *HO*-disruptant could be reduced relatively simply. We approached this by way of the generation of a non-flocculent mutant following chemical mutagenesis with EMS (Adams *et al*. 1997). In the process the suitability of the *HO*-disruptant haploid to use in a basic genetic operation, i.e. the isolation of mutants, was also proven.

Strain 1B, the haploid derivative of L2056, was exposed to the mutagen EMS before being grown in CDGJM without agitation. Once the culture was well established (*ca* 2 – 3 days) samples was taken from the top of the ferment and sub-cultured into fresh media. This process was then repeated 2 – 3 times. In this way it was possible to select for the sub-population of the culture whose cells tended not to flocculate and hence remained in suspension. Microscopic examination confirmed that cells selected via this passaging technique did in fact tend to occur as single cells or at least formed smaller and fewer aggregates (Figure 5). A preliminary

investigation revealed that the degree of flocculation increased progressively over subsequent incubations without selection. However, despite this occurring, flocculation could be reduced to a low level for long enough to allow the required single cell manipulations and determinations to be performed (data not shown). Accordingly this attribute is considered sufficiently manageable for the research-related use of such haploids.

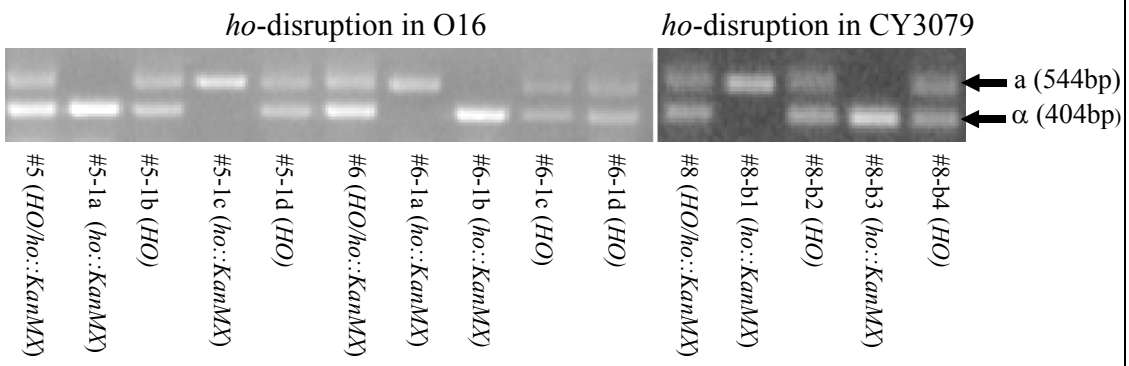
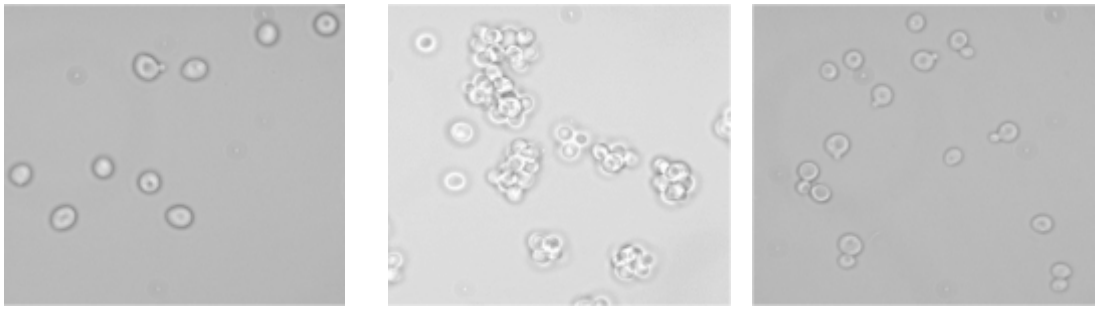


Figure 4. PCR analysis of genomic DNA derived from O16 and CY3079 disruptants and their haploid progeny. Haploids are designated 'a' – 'd' for each disruptant.



Parental L2056 | **Haploid *HO*-disruptant 1B** | **Selected, non-flocculant 1B**

Figure 5. Photomicrographs showing the arrangement of cells derived from L2056 after passaging. Non-flocculent cells from 1B were selected following EMS mutagenesis and several passages through non-agitated media. All images were taken at the same magnification.

Small-scale fermentation trials of parental and haploid wine yeast

At this point it was important to undertake an evaluation of the fermentative properties of the haploids that had been isolated. Small-scale (100 mL) fermentation trials were carried out to determine whether the genetic modification of any of the wine yeasts, that is the disruption of their *HO* genes, had had any adverse affects on fermentation.

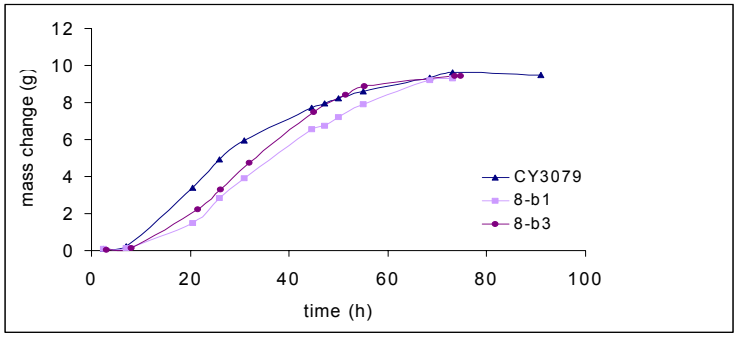
Strains were cultured according to Jiranek *et al.,* (1995) except MYPG medium was substituted with YPD medium. Fermentations were conducted in duplicate in 250 mL screw-cap conical flasks fitted with a sidearm port, sealed with a rubber septum for aseptic and anaerobic sampling and supplementation. CDGJM medium (100 mL) was inoculated with yeast at 5 x 10^6 cells/mL, fermentation locks fitted and the headspace flushed for 3 minutes with nitrogen delivered via a 21-guage needle inserted through the flask septum. Flasks were incubated with shaking at 30^{o}C. Fermentation progress was monitored by determination of the cumulative change (reduction) in flask weight that occurred through the evolution of CO_2. In addition, samples were analysed for residual glucose using enzymatic test kits (Boehringer Mannheim). Once the sugar of the medium had been depleted, terminal samples were collected, clarified by centrifugation and frozen for subsequent metabolite determination by HPLC.

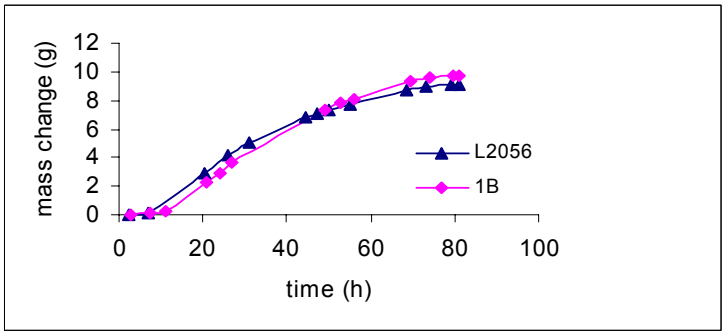
Analysis was carried out on ferment samples to determine residual glucose, as well as L-lactic acid, succinic acid, acetic acid, glycerol and ethanol. Frozen ferment samples were thawed and 500 μl aliquots filtered through 0.45 μm PVDF syringe filters (Millipore). HPLC analysis was performed on undiluted and diluted (1:20) samples on a Benson Carbohydrate SS column (Alltech). Elution was performed at 60^{o}C with 2.5 mM H_2SO_4 at a flow rate of 0.5 mL/min. Detection was performed by means of a RID-10A refractive index detector (Shimadzu). Quantitation was achieved by comparison with prepared standards in CDGJM (without glucose) and integration software (Deltaware).

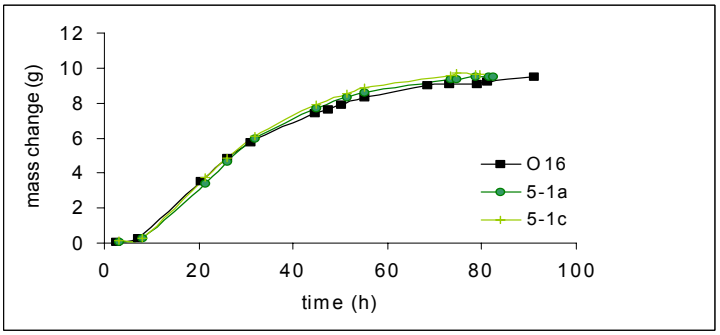
Generally, the *HO*-disruptants were found to behave similarly to the parental wine strains in terms of fermentation kinetics (Figure 6). Accordingly, growth curves of all disruptants, excluding #8-b1 and #8-b3, closely matched that of the corresponding parental wine yeast strain. In the case of #8-b1 and #8-b3, where fermentation appeared initially slower compared to CY3079, the possibility that such a difference might be due to inoculation rate errors caused by the flocculent nature of the haploids has not yet been excluded. The analysis of the fermented media showed only minor differences in the concentrations of key metabolites (Table 2).

"Loop-out" of non-*Saccharomyces* sequences from the genome of haploid *ho* wine yeast.

A key objective of this project was to keep genetic modifications and the introduction of foreign sequences to a minimum. In this way, the likelihood of securing approval for the release of such strains for pilot-scale trials (i.e. greater that 10 L) was increased. The rationale behind the use of a dominant selectable marker such as *KanMX* was that the target strain did not first need to have a selectable marker introduced into it. A further benefit of the gene disruption system based on the *KanMX* construct reported by Wach *et al*. (1994) was the incorporation of inverted direct repeat sequences (IDRs) which allowed "looping-out" and hence removal of the selectable marker once no longer required. This phenomenon was said to occur at a frequency of 10^{-3} to 10^{-4} under non-selective conditions.


mass change (g)
time (h)
CY3079
8-b1
8-b3


mass change (g)
time (h)
L2056
1B


mass change (g)
time (h)
O16
5-1a
5-1c

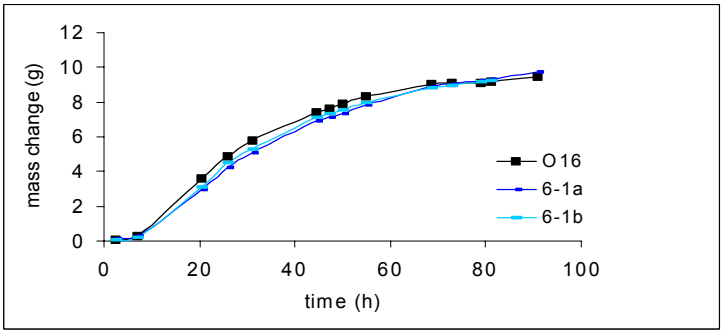

mass change (g)
time (h)
O16
6-1a
6-1b

Figure 6. Fermentation performance of wine yeasts and their haploid, *ho* derivatives. Fermentations were performed as described in the text.

Table 2. HPLC analysis of supernatants from parental wine yeast and haploid *HO*-disruptant fermentations.

	Strain				
Metabolite (g/L)	**CY3079**	**#8-b1(*MAT*a)**	**#8-b3(*MAT*α)**		
Glucose	0	0	0		
Succinic acid	0.24	0.26	0.27		
Lactic acid	0.53	0.41	0.31		
Glycerol	7.06	6.37	6.97		
Acetic acid	0.68	0.70	0.70		
Ethanol	104.2	104.3	108.2		
	Strain				
Metabolite (g/L)	**L2056**	**1B (*MAT*a)**			
Glucose	0.09	0.65			
Succinic acid	0.22	0.19			
Lactic acid	0.26	0.18			
Glycerol	8.42	7.35			
Acetic acid	1.04	1.21			
Ethanol	95.7	101.5			
	Strain				
Metabolite (g/L)	**016**	**#5-1a (*MAT*α)**	**#5-1c (*MAT*a)**	**#6-1a (*MAT*a)**	**#6-1b (*MAT*α)**
Glucose	0.17	0.22	0.07	0.03	0
Succinic acid	0.24	0.22	0.22	0.22	0.2
Lactic acid	0.53	0.44	0.41	0.46	0.47
Glycerol	7.67	6.95	6.65	6.93	6.79
Acetic acid	0.78	0.75	0.77	0.71	0.65
Ethanol	104.6	100.1	102.5	103.3	107.8

Values are the average of single determinations from duplicate fermentations.

Extensive attempts were made to induce the haploid 1B (derived from L2056) to loose the non-*Saccharomyces* DNA (IDRs and *KanMX*) by their looping-out under non-selective conditions. The strain was grown at 30^{o}C overnight, in 10 mL YPD from a single colony, and subcultured daily into fresh YPD (1:100 dilution into 100 mL medium). Sub-culturing conditions varied from 10 to 18 passages, in fresh YPD containing 4% glucose, at a dilution rate of between 1:100 and 1:400. Approximately 6000 colonies were plated onto YPD, and grown up prior to replica streaking onto (i) YPD and (ii) YPD containing geneticin at 400 μg/mL, and grown overnight at 30^{o}C. Putative loop-outs were screened by PCR for the presence of the kanamycin resistance gene (*KanMX*), as a 786 bp product.

Each 50 µl PCR reaction contained 10 mM Tris HCl pH 8.3, 500 mM KCl, 0.001% gelatin, 2 mM $MgCl_2$, 80 µM dNTPs, 100 pmols of G418 Fwd and G418 Rev primers (Appendix Table 1b) and 1unit of Taq DNA polymerase (Sigma). A small amount of colony mass was used as template. Hot start amplification was initiated with a 1.5 min 94°C denaturation, followed by 30 amplification cycles (94°C 30 sec, 60°C 1min, 72°C 1 min), and terminated with a 10 min 72°C extension.

Unfortunately, our work revealed that the looping-out did not function as described. Despite screening large numbers of cells, no *HO*-disruptants now sensitive to geneticin could be isolated. Figure 7 shows typical results of the PCR screening of potential loop-outs. Discussions with a collaborator from INRA in France revealed that the failure of looping-out was not limited to our group (J.-M. Salmon, *pers. comm*.). In fact, correspondence with the originating group revealed that the published data represented the very best case scenario for a standard laboratory strain (P. Philipsen, *pers. comm*.).

The low frequency of loss of the *KanMX* module (Wach *et al.,* 1994) and our lack of success may be a consequence of the direct repeats being derived from non-*Saccharomyces* sequences, namely those from the filamentous fungi *Ashyba gossypii.* In recognition of this possibility and the overriding principle of minimal foreign sequences in the construction of optimised strains for large–scale trials, work was commenced on an improved vector for *ho::KanMX* disruption.

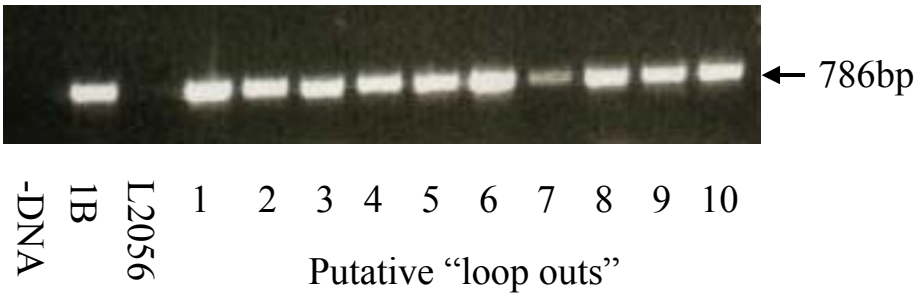


Figure 7. PCR analysis for the retention of the kanamycin resistance gene (*KanMX*) by putative loop-out isolates of strain 1B. Loop-outs were selected from over 6000 colonies based on their having apparently become sensitive to geneticin. All proved to be false positives by retaining the *KanMX* as indicated by the presence of a 786 bp band.

Construction of an improved *ho::KanMX* disruption vector

A modified *HO*-disruption vector was designed which retained the dominant *KanMX* resistance marker (conferring resistance to the antibiotic geneticin or G418). However, instead of foreign sequences from *Ashyba gossypii*, the direct repeats required for looping-out were derived from native *Saccharomyces cerevisiae* sequences (5' untranslated region from the *HO* gene in L2056).

This construct was anticipated to have the advantage of an enhanced ability to loop-out thereby facilitating loss of the *KanMX* from the genome as well as part of the repeat sequences. The remainder of the latter should be only native sequences in their correct genomic location having being originally derived from the target/disrupted gene by design. Furthermore, alternate versions of this vector would be adaptable for disruption of other target genes by simply replacing the targeting and direct repeat sequences.

Plasmid pBS418, a pBluescript SK-(Stratagene) vector was modified by the addition of direct repeat sequences amplified from the *HO* gene (-399 to -670) in L2056. A 60 bp sequence complementary to region (+1963 to +2023) was included for 3'- targeting of the construct during homologous recombination with the chromosomal *HO* gene (Figure 8).



Figure 8. Schematic depiction of the improved construct used to disrupt the native *HO* gene in wine yeast. The construct contains the *KanMX* resistance marker, flanked by direct repeats (DR) from native 5' untranslated sequence of the *HO* gene as amplified from L2056. The 5' sequence at the 5' end of the construct, along with 60 bp of 3' sequence (3' target) at the 3' end of the construct enables targeting to the *HO* gene.

The repeat sequences were generated by a novel process involving alternate cloning steps of a single 425 bp PCR product (amplified from L2056 genomic DNA) into pBS418. PCR was performed using 250 ng genomic DNA as template in a 50 µl reaction. Each reaction contained 50 mM Tris HCl pH 9.0, 3.5 mM $MgCl_2$, 15 mM $(NH_4)_2SO_4$ and 0.1% Triton X-100, 80 µM dNTPs, 50 µM each of primer HOP1 and HOP2 (Appendix Table 1b) and 1unit of Dynazyme EXT DNA polymerase (Finnzymes). Hot start amplification was initiated with a 2 min 94°C denaturation, followed by 30 amplification cycles (94°C 30 sec, 56.2°C 45 sec, 72°C 1 min), and terminated with a 10 min 72°C extension. The PCR product was isolated on a 1% gel in TAE (Tris-acetate) buffer, and purified by UltraClean (MioBio). The PCR product was digested with *AscI* and *KpnI* and cloned into the multiple cloning site of pBS418 to give the recombinant plasmid pBS418-HODR1. *Escherichia coli* transformants containing pBS418-HODR1 were identified by colony hybridisation using a 425 bp DIG-labelled PCR product (Maniatis *et al.* 1982). The purified plasmid (~4.67 kb) was characterised by restriction digestion (Figure 9) and sequencing (data not shown).

The presence of the cloned PCR fragment was revealed in the restriction digest, by the presence of two bands (*HpaI* & *AscI* digest) and (*HpaI* & *SacI* digest) as a consequence of a *HpaI* site in the PCR product. The plasmid, pBS418 (4.346 bp), itself has no *HpaI* site. Sequencing of the plasmid pBS418-HODR1 using primers HOP1 and HOP2 confirmed the complete integrity of the construct sequence and showed that the cloned PCR product contained the direct repeat

sequence and the 60bp 3' targeting sequence (data not shown). In order to construct the second direct repeat, the PCR product was digested with *HpaI* and *SacI* and cloned into pBS418-HODR1 (digested with *SmaI* and *SacI*). The recombinant plasmid pBS418-DR2 was identified from *E. coli* transformants by *AscI* digestion and sequencing. Digestion of pBS418-DR2 with *AscI* resulted in a 3.2 kb and a 1.8 kb fragment, due to the *AscI* site (close to the *SacI* site) in the second PCR fragment, which was cloned as a *HpaI-SacI* insert. Another clone, pBS418-DR21 was shown to have a smaller PCR insert (Figure 10). Both pBS418-DR21 and pBS418-DR2 were sequenced using the primer M13-20mer (Appendix Table 1b). Sequencing confirmed the presence of the complete second direct repeat sequence in pBS418-DR2, whilst pBS418-DR21 contained only 158 bp of the insert.

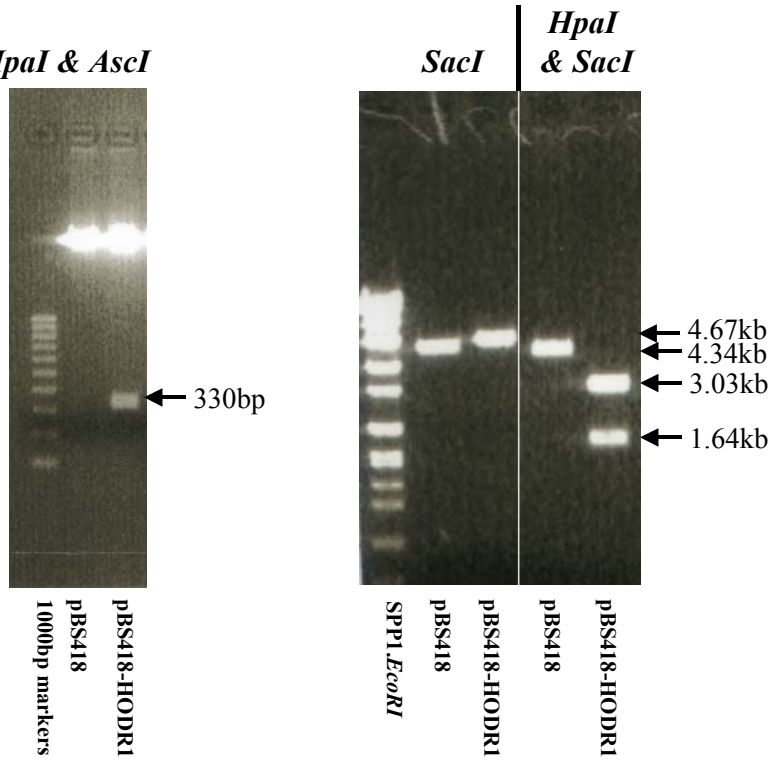


Figure 9. Restriction digestion analysis of pBS418 and pBS418-HODR1.

The wine strain L2056 was transformed with a 2086 bp *SacI-KpnI ho::KanMX* fragment isolated from pBS418-HODR2, and geneticin resistant transformants were isolated on YPD containing 300 μg/mL geneticin. Characterisation of the *HO*-gene disruption and efficacy of loop-out of the foreign *KanMX* sequences should be completed by early 2002.

Construction of haploid *ura3::KanMX* disruptants in wine yeast

The construction of a *ho, ura3* disruptant in a wine yeast background was a specific goal of this project. Such a strain would provide a useful tool for studying yeast physiology in a genetic

background relevant to winemaking. Specifically, the strain would be used to perform transposon mutagenesis (as part of the project: UA 00/5, *Characterisation and adoption of highly nitrogen-efficient yeasts for increased fermentation reliability*) to isolate for example, mutants with increased nitrogen efficiency. Mutants isolated by transposon mutagenesis (Ross-MacDonald *et al.* 1998) would be subjected to classical genetic manipulation (mating, sporulation and dissection), as well as transformation for the isolation of genes of interest. Such genes would then be isolated and characterised by inverse-PCR and sequencing (Martin and Mohn, 1999, Huang *et al*. 2000).

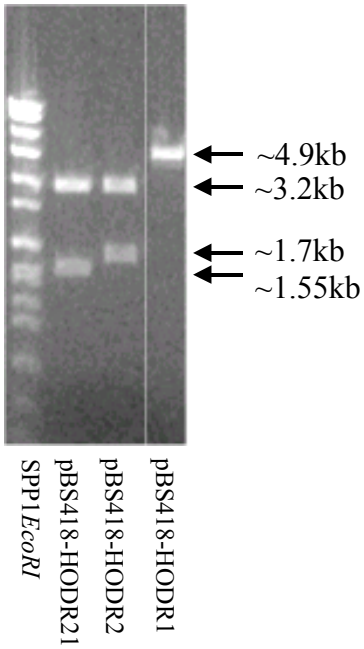


Figure 10. ***AscI*** **restriction digestion analysis of pBS418-HODR1, pBS418-HODR2 and pBS418-HODR21.** Banding patterns indicate the presence of the complete second direct repeat sequence in pBS418-DR2 and only an aberrant 158 bp insert in pBS418-DR21.

Our strategy aimed at avoiding the need to introduce a further selectable marker to allow integrative disruption of genes, was to utilise the *KanMX* dominant selectable marker. The possibility of using this marker in a *HO*-disruptant, for which *KanMX*-based selection had already been used, relied on the looping-out and hence removal of this selectable marker. Fulfillment of this goal has proven difficult within the time frame of the project, because of the specific unforeseeable problems outlined above (i.e. failure of looping-out of the *KanMX* marker and hence inability to re-use this marker as planned). Nevertheless, transposon mutagenesis in a laboratory yeast strain (*ura3::kanMX* disruptant in haploid W303) has been successful. A number of mutants have been isolated with improved nitrogen efficiency, from which, a number of genes have been characterised (J. Gardner, *pers. comm*.). It is anticipated that upon construction of a haploid *ura3* wine strain, disruption of specific *HNE* genes to achieve an

optimised yeast will be a straight forward procedure. This aspect of the work is in fact to be continued and extended as part of the recently awarded project UA 01/04 *Microbial enhancement of wine quality and complexity*.

Construction of *URA3* (*ura3::KanMX*) disruptants in strain 1B

The *ura3::KanMX* disruption vector pDR1123 (Replogle *et al.* 1999) was used for the disruption of the *URA3* gene in 1B, the haploid (*ho::KanMX)* derivative of L2056. The inability of the *KanMX* selectable marker to be lost, prevented its reuse as a selection system, and as an alternative strategy, the selection of *ura3::KanMX* disruptants was based on the use of the uracil analogue, 5 fluoro-orotic acid (FOA).

The yeast was transformed with a 2.5 kb *SacII-KpnI ura3::KanMX4* fragment using the lithium acetate method (Ito *et al*. 1983), and the ura$^-$ transformants were positively selected for on minimal yeast medium containing 0.1% FOA and 50 μg/mL uracil. Six transformants were isolated, which were shown to have a requirement for uracil when grown on minimal SD medium. Eight colonies of each transformant were replica streaked onto SD minus uracil and SD plus 100 mg/L uracil, and grown overnight at 30°C. Figure 11 shows representative results.

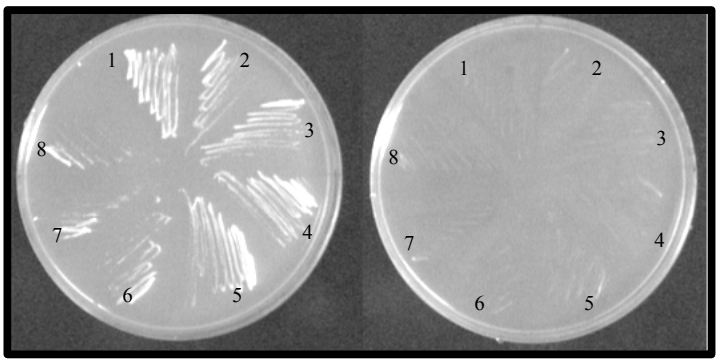


Figure 11. Requirement for uracil for growth by 8 isolates of the putative *ura3::KanMX* transformants strain 1B#4.

Uracil-requiring transformants were analysed by PCR using primers URAR and URA3F, which are complementary to *URA3* gene sequences (Table 1b). PCR was carried out on yeast cells in a 50 μl reaction, 10 mM Tris HCl pH 8.3, 500 mM KCl, 0.001% gelatin, 2 mM $MgCl_2$, 80 μM dNTPs, 100 pmols of URAR and URA3F primers and 1unit of Taq DNA polymerase (Sigma). Interestingly, none of the isolated uracil-requiring mutants appeared to have acquired uracil auxotrophy due to disruption of *URA3* by the *ura3::KanMX* construct (Figure 12). All strains retained a *URA3* gene whose size appeared to match that of the untransformed controls, 1B and L2056. This finding was taken to indicate the isolation of spontaneous mutants, identified through the selection protocol used. Such mutants are likely to carry point mutations (single base changes) in *URA3* or a related gene, thereby accounting for their apparently wild type banding

pattern by PCR. Attempts to complement the mutation by transformation with Yep24, a plasmid containing a functional *URA3* gene, however, proved unsuccessful (data not shown). The latter supports the possibility that an alternate gene in the uracil biosynthetic pathway is involved.

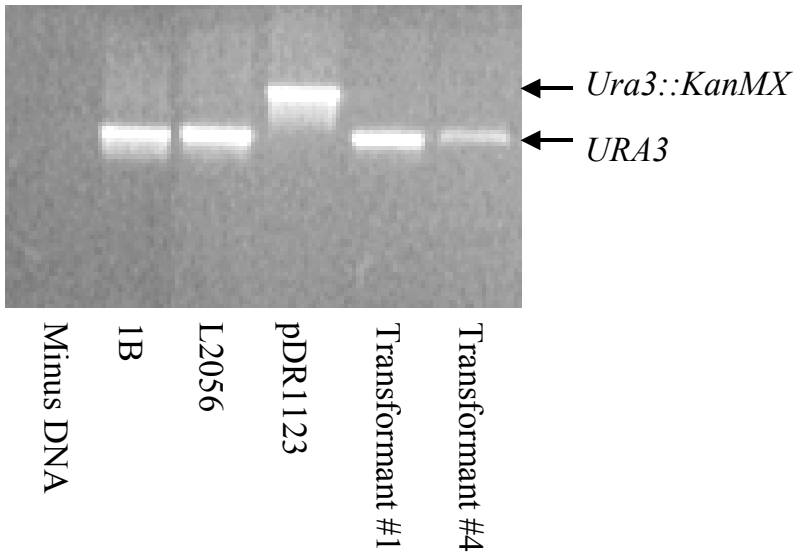


Figure 12. PCR determination of the presence of the *URA3* gene in L2056, haploid 1B and putative *ura3::KanMX* transformants, #1 and #4. Note that both transformants appear to retain the native *URA3* sequence rather than the large *ura3::KanMX* insert (indicated through the use of plasmid pDR1123 bearing this insert).

Construction of diploid *ura3::KanMX* disruptants in L2056

Efforts to produce a strain with both *ho* and *ura3* mutations have continued and required an alternative strategy for selection of mutants to that using fluoro-orotic acid. It was decided to construct a *ura3* mutant in wild type L2056 thereby allowing use of the *ura3::KanMX* construct and associated selection for resistance to geneticin. Once generated, this diploid, heterozygous (*ura3/URA3*) L2056 strain would be sporulated, dissected and allowed to rediploidise to produce a homozygous (*ura3/ura3*) strain before resporulation and mating with 1B. Upon sporulation and dissection, spores of each mating type and bearing *ura3* and *ho* would be isolated.

Transformants of L2056 bearing the *ura3::KanMX* fragment (i.e. *URA3*/ *ura3::KanMX*) were isolated. Two examples of these heterozygous diploids, #40 and #41, were sporulated and their tetrads dissected. PCR analysis using the primers URAR and URA3F (Appendix Table 1b) confirmed the 2:2 segregation of the uracil biosynthetic gene. Specifically, two spores possessed the native *URA3* gene while two others bore the *ura3::KanMX* fragment (Figure 13). Plating of all spores on uracil-minus media revealed the appropriate pattern of uracil auxotrophy (Figure 14).

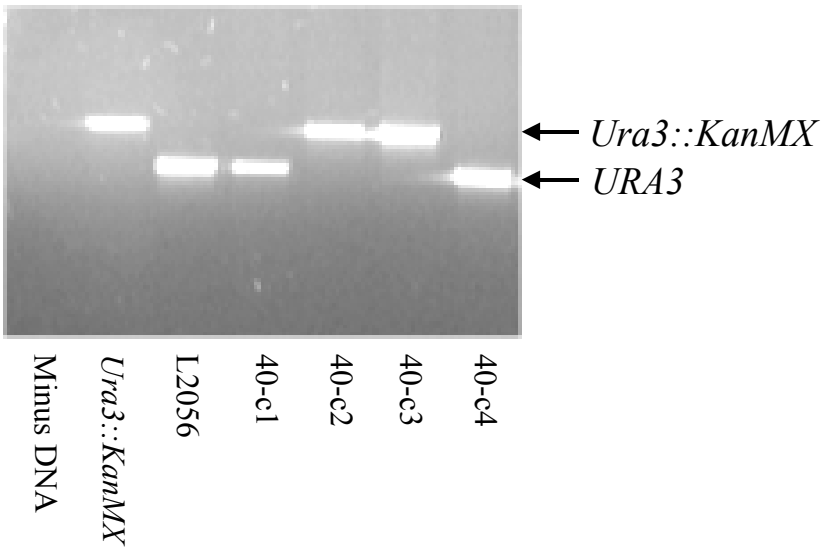


Figure 13. Segregation pattern of *URA3* and *ura3::KanMX* genes in spores derived from the sporulation of geneticin-resistant transformant of L2056, #40. L2056 had been transformed with the *ura3::KanMX* fragment as described in the text. Note the 2:2 pattern of segregation.

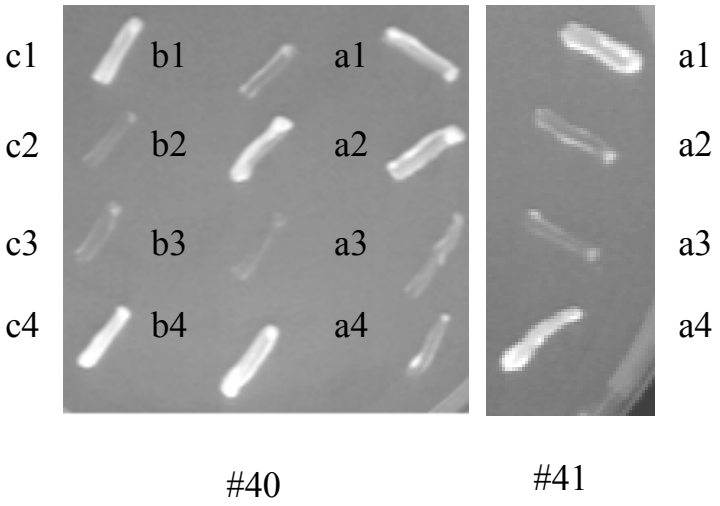


Figure 14. Segregation of the uracil-requirement phenotype in spores derived from the sporulation of geneticin-resistant transformants of L2056. L2056 had been transformed with the *ura3::KanMX* fragment as described in the text to produce transformants #40 and #41. A varying number of tetrads were dissected from each before plating of spore colonies onto the uracil-minus SD media. Spores are numbered 1 – 4. For each transformant, those spores derived

from the same tetrad are designated with same alphabetic prefix. Note the 2:2 pattern of segregation.

Studies by previous groups have shown that the generation of haploids from wine yeast at times highlights the complexity of the parental genome. In some cases the parental strains are not precisely diploid (i.e. 2n) but may contain entire or partial additional sets of chromosomes. Thus when the yeast are sporulated, additional copies of individual genes segregate non-uniformly between the haploid progeny. Discussions with researchers at INRA, Montpellier, revealed that some wine yeast strains do appear to retain extraneous genetic material after being reduced to haploid cells by repeated cycles of sporulation. Specifically, the V5 strain referred to in some reports by this group (Salmon and Barre, 1998) appears to carry an additional copy of at least the *URA3* gene (J.-M. Salmon, *pers. comm*.). This finding is only made apparent during classical genetic studies wherein aberrant segregation of this marker is observed.

We were therefore keen to confirm the situation with respect to *URA3* in haploids developed throughout this study. The classical segregation of the *URA3* gene in the diploid *URA3* disruptants derived from L2056 suggested there was only one gene. However to confirm this we examined all the strains we had used in the study; the three wine strains, their haploid derivatives, and the diploid *ura3* strains, by PFGE-CHEF with Southern blotting. The *URA3* gene was identified by using a DIG-labelled *URA3* probe generated with the primers URA3F and URA3R (Appendix table 1b). Yeast chromosomes were prepared according to Jespersen *et al*. (1999) with minor modifications. Yeast were grown from a single colony in YPD for 48 h at 30^{o}C, prior to dilution into fresh medium (50 mL) and incubation for a further 24 h. Cells were centrifuged, washed in water, and resuspended in 8 mL spheroplasting buffer (1.2 M sorbitol, 10 mM Tris HCl, 10 mM $CaCl_2$, pH 7.5). Cells were again collected by centrifugation and resuspended in 6 mL spheroplasting buffer containing 100 μg Lyticase (Sigma), and incubated at 37^{o}C for 2 h to form spheroplasts. The spheroplast suspension was then cast in agarose. Pulsed-field gel electrophoresis was carried out using a BioRad CHEF apparatus. The agarose plugs were embedded in a 1% agarose gel made with 0.5 x TBE (Tris-borate) buffer, and the gel was run in the same buffer for 24 hr. The pulse time was as follows: initial time set to 60, final time to 90, limit at 0.3 and voltage at 200V. After electrophoresis the gel was stained with ethidium bromide prior to photography and Southern blot analysis (Maniatis *et al.* 1983). The probe was generated using a DIG-labelling PCR kit (Boehringer Mannheim) and used un-purified. Southern blot and chemiluminescent detection conditions were the same as for the *HO*-disruption analysis.

Figure 15 depicts the stained CHEF gel and corresponding Southern blot. A single band detectable by the *URA3* probe was seen in all the strains except the diploid *ura3/ura3* mutants #40-c2 and #41-a2, where the coding sequence and some of the untranslated sequences were replaced by the *ura3::KanMX* disruption construct. This band corresponds to chromosome V, the location of the *URA3* gene as determined in the *Saccharomyces* Genome Database (genome-www.Stanford.edu/Saccharomyces/). The faint signal shown in the lanes corresponding to the *ura3* mutants is attributable to weak binding of the probe given the presence of a residual 111 bp of *URA3* sequence at the 3' end of the *ura3::KanMX* disruption construct which shares homology to the *URA3* probe used. PCR analysis of gene segregation and Southern blot analysis of the CHEF gel, strongly indicated the presence of only one *URA3* gene in the three wine strains L2056, O16 and CY3079.

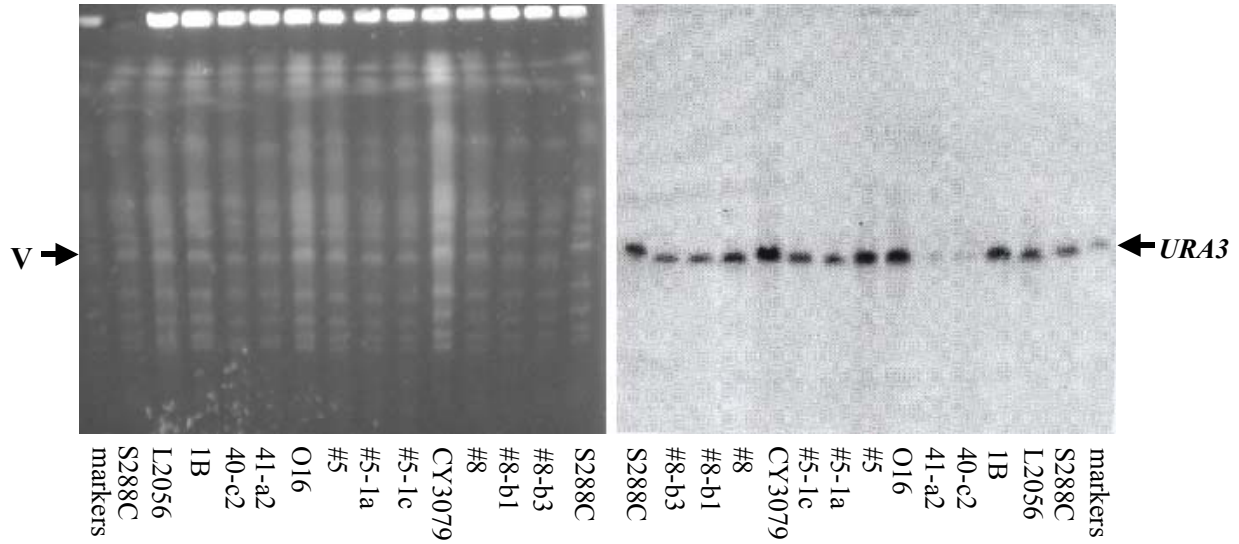


Figure 15. Pulsed field gel electrophoresis and Southern blot analysis for *URA3*-homologous sequences of chromosomal preparations from wine yeasts and derivatives. The ethidium bromide stained gel showing the position of Chromosome V is depicted in the left panel. The corresponding Southern blot is depicted in the right panel (as a mirror image).

Construction of a haploid (*ho*) *ura3* mutant: Strategy 2

Having successfully produced diploid strains that were uracil-requiring (i.e. *ura3::KanMX/ura3::KanMX*), work progressed to combining this attribute with the stable haploid (*ho::KanMX*) background described above. Initial efforts centred on cell-spore mating whereby spores from the sporulated diploid were mixed with cells of the haploid strains 1B (*ho::KanMX, MAT*α) or 1D (*ho::KanMX, MAT*a) on a YEPD plate and incubated overnight. It was expected that germinated spores from the diploid *ura3* strain would mate with a *ho* strain before the former switched mating type and self-diploidised. Thus after sporulation of the diploid cells that did develop, the desired spores were sought by PCR and Southern blot analysis. Early indications did not suggest that the desired mating occurred in significant numbers. Thus to simplify the process of identifying spores from the desired cell-spore mating, a selection strategy was applied. Using ethidium bromide mutagenesis, a *mit*$^-$ derivative of 1B was generated which was devoid of mitochondria and hence unable to grow on non-fermentable carbon sources (2% ethanol and 3% glycerol; J. Gardner, *pers. comm*.). The selection of diploids derived from cell-spore matings relied on their ability to grow on minimal medium (i.e. absence of uracil) containing 2% ethanol and 3% glycerol. Diploids have been isolated and very recently confirmed by PCR to be heterozygous for the appropriate marker (i.e. *ura3::KanMX*/*URA*; data not shown).

We need only sporulate this diploid and dissect the tetrad to isolate strains of each mating type. Once complete we will have succeeded in meeting this key objective of producing a matched set

of haploid wine yeasts with a *ura3* marker i.e. *ura3::KanMX, ho::KanMX, MAT*α and *ura3::KanMX, ho::KanMX, MAT*a.

In parallel to the above effort, which are now likely to have succeeded, we initiated a back-up strategy for disrupting specific genes in a *ho::KanMX* background (to produce research-only strains). This strategy involves the use of an alternate dominant selectable marker, thereby circumventing the limitation of not being able to loop-out the *KanMX* gene. During the course of this study the use of the hygromycin resistance gene (*HPH*) was reported (Goldstein and McCusker, 1999) and so is being incorporated into a new *URA3* disruption construct (i.e. *ura3::HPH*). Thus, plasmid *HPH* from pAG34 will be used to construct a tailored version of pBS418-HODR2, wherein *KanMX* is replaced by *Hph*. We therefore anticipate the alternate generation of a haploid wine strain with both *ho* and *ura3* mutations by December 2001 or shortly after.

Whatever the strategy used to ultimately yield a *ho*, *ura3* yeast, such strains will then be utilised (as part of UA 01/04) to generate optimised yeasts for small scale trials in defined media and juices.

Fermentation profiles of diploid *ura3::KanMX* strains

Prior to the reduction of the isolated diploid *ura3* strains to the haploid state, fermentations were carried out in investigate the impact of this disruption on the basic fermentative performance of these strains. Duplicate fermentations were carried out as described for the *HO*-disruptants. Thus CDGJM medium with and without uracil supplements was inoculated and incubated with a fermentation lock. Two mL of 2 mg/mL uracil (4 mg) were added to 50 mL of starter culture, and 4 mL of 2 mg/mL uracil (8 mg) was added to100 mL CDGJM prior to inoculation. Uracil supplements (4 mg) were further added at 21.5 h, 49.5 h and 70 h, to ensure this nutrient was not depleted. Fermentation was monitored as the cumulative mass change of the cultures. Cells and media were harvested and frozen once glucose had become depleted. HPLC analysis was performed as previously described.

The fermentation profiles of the diploid *ura3/ura3* strains were compared with L2056 (Figure 16). An absolute requirement for uracil was shown, by the lack of fermentation and growth of the mutants introduced into CDGJM in the absence of uracil. The profiles were comparable with the control (CDGJM minus yeast) in which any losses in weight were attributable to evaporation (data not shown). Growth and fermentation were restored upon uracil supplementation, thereby making the fermentative properties essentially the same as those of L2056. Analysis of key metabolites in the supernatant at the completion of the fermentations further supported the conclusion that the diploid *ura3* strains were not dissimilar to the parental L2056 despite the genetic modifications they carried (Table 3).

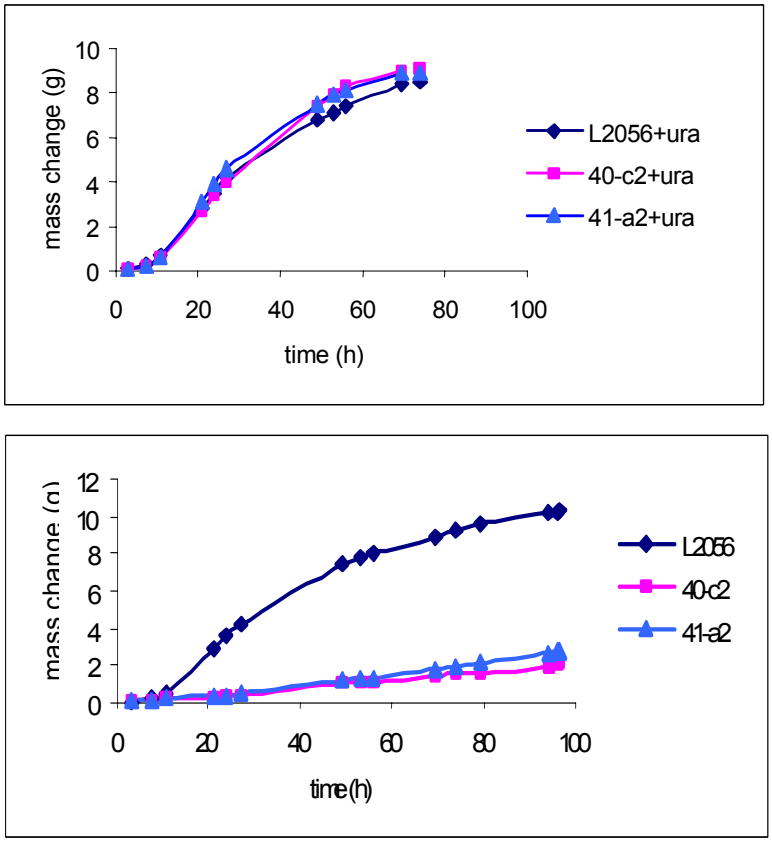


Figure 16. Fermentation profiles of L2056 and *URA3*-disruptants, #40-c2 and #41-a2, grown in the presence (top panel) or absence (bottom panel) of uracil. Plots are the average of duplicate fermentations.

Table 3. HPLC analysis of supernatants from fermentations conducted by strain L2056 and *URA3*-disruptants #40-c2 and #41-a2.

	Strain		
Metabolite (g/L)	**L2056**	**#40-c2**	**#41-a2**
Glucose	0.02	0	0
Succinic acid	0.15	0.21	0.18
Lactic acid	0.17	0.18	0.15
Glycerol	8.02	8.33	8.13
Acetic acid	1.17	1.17	1.30
Ethanol	92.0	99.3	92.6
Total SO_2 (mg/L)	38.0	58.4	42.1

Fermentations were carried out in CDGJM with uracil supplementation as described in the text. Values are the average of single determinations from duplicate fermentations.

Conclusions

The key objectives of this project were to: i) generate a haploid wine yeast strain from a suitable commercial strain, ii) confirm the utility of such a strain as a vehicle for genetic investigations, iii) produce a complement of molecular tools for performing genetic modifications in such a haploid strain, and iv) demonstrate the efficacy of this suite of tools by utilising them to produce a modified wine yeast.

Wine yeast strains are not selected because of their amenability to classical and molecular genetic manipulation. Instead, industrial considerations form the primary selection criteria. We therefore surveyed commercial wine yeast strains to identify those, which despite these criteria, retain satisfactory rates of sporulation, forming primarily 4-spore asci with a high proportion of 4-spore viability. We successfully identified three such strains, L2056, O16 and CY3079.

Since polyploid strains such as commercial strains bear multiple copies of individual genes, they also have the potential to possess different versions or alleles of such genes. Recent work suggests that some of the properties of industrial strains may be the result of such genetic heterogeneity (Mortimer, 2000). If correct, such a notion predicts that as a polyploid strain is sporulated and allowed to spontaneously re-diploidise, thereby duplicating its reduced complement of genetic information, it will become more homogeneous. In so doing, it would loose properties seen in the parental strain whose expression is dependent on a mixture of alleles. To obtain an indication of the degree of heterogeneity of these yeasts, and particularly the importance of such genomic variability on fermentation performance, preliminary fermentation trials were carried out.

Parental wine yeasts were therefore compared with their derivatives, which had been produced through multiple cycles of sporulation, dissection and spontaneous re-diploidisation. As stated in *Results*, such yeasts did not differ greatly from the parental strain in terms of basic attributes such as fermentation kinetics and the formation of key metabolites and biomass. Similar findings were made for more detailed fermentation trials of *ura3* diploid strains and *ho* haploids elsewhere in the project. The latter therefore also argue against the dependence of the phenotypes observed on higher gene copy number. Thus while a more comprehensive comparison of parental strains and haploid or diploids of greater homogeneity is clearly required, our early indications are that the reduced haploid cell is representative of the more complex, originating wine yeast. This conclusion therefore validates the approach to strain construction used here. That is, the introduction of genetic modifications into a haploid strain followed by mating of such haploids to effect the reconstruction of the higher ploidy state. Comparative microarray, proteome and metabolome studies would however be warranted in the future to more fully explore this issue.

Work next centred on developing a construct for the one-step integrative disruption of the *HO* gene in three commercial yeasts. Disruption of *HO* was successfully produced in L2056, O16 and CY3079. The construct was based on a previously reported system and was selected because of the reported ability of the selectable marker to be removed (and hence reused) when no longer required. Southern and PCR analysis confirmed the presence of the disruption cassette in the target strains along with the simultaneous loss of the native *HO* gene. Sporulation of the

transformed strains and isolation of stable (non-switching) *ho* haploids was successfully completed. Haploids were found to have become more flocculent than the parental strains, a finding in line with previous reports (Kron 1997). The determination of mating type of the haploid strains was achieved by classical genetic techniques and PCR. In being easy to achieve practically, the former also demonstrated the desired compatibility of these haploids with the use of classical genetic techniques.

To further evaluate the suitability of the *ho* haploids for genetic studies, we chose as a test case the reduction of the haploid-specific flocculation in *ho* strains by chemical mutagenesis followed by a screening procedure. The successful reduction of flocculation was a straight forward accomplishment. This outcome now enables the accurate quantitation of cell numbers by microscopic count and viable plating as well as permitting meaningful culture optical density measurements to be made. Most importantly though, this outcome further reinforces the equivalence of the *ho* haploids to laboratory strains in terms of their amenability to standard classical genetic manipulation techniques. As such, this key project objective has been achieved.

PCR was next used to confirm the choice of strains for inclusion in congenic pairs of *ho* haploids of each mating type. Mating types were confirmed. In addition, this experiment also elegantly demonstrated the effectiveness of the *HO*-disruption as well as the switching efficiency of the wild type *HO* spores. Data in Figure 4 clearly shows the presence of two MAT-related bands in all spores with a functional *HO* gene, thereby demonstrating these spores having switched and re-diploidised. Only the *ho* strains possessed a single MAT band, indicative of the haploid nature of these strains.

Small scale fermentation trials of the *ho* strains revealed a close match with the parental strains in terms of fermentation kinetics and metabolite formation. Again, such findings demonstrate that the reduced genetic makeup of the haploid is sufficient to produce the same fermentation behaviour as the parental strain, at least in terms of the selected parameters measured here. Also, for these parameters, there appears to be no adverse effects stemming from the disruption of the *HO* gene. The latter is somewhat surprising, as previous reports have hinted at an indirect effect of *HO* disruption (Salmon, 1997). Specifically, genetically reduced and hence physically smaller cells such as haploids are able to effect fermentation more quickly than a larger diploid cell. The advantage of haploid cells is their larger surface area to volume ratio and hence improved trans-membrane movement of nutrients and wastes (Salmon, 1997). No such benefit was apparent here however.

Despite the fulfillment of all objectives to this point of the project, a critical block to subsequent progress was uncovered at this stage. Namely, the *KanMX* selectable marker failed to loop-out of the recipient cell genome under non-selective conditions. Acting on the advice of the originating group (Wach *et al*. 1994) and others, extensive efforts were made to encourage loop-out, however, to no avail. The continuing appeal of an ability to loop-out the *KanMX* marker relates to the possibility of rationalizing the design of gene-disruption vectors and transformant screening. Essentially, marker loop-out allows the same selectable marker cassette to be utilised for the disruption of different genes. The only changes needed in disruption vectors would be to the flanking sequences whose homology with the target gene correctly directs the integration event. The selection of transformants would also be simplified since alternate antibiotics would

not be needed and neither would the added work involved in determining the sensitivity of individual strains to each new antibiotic.

For these reasons, the project objectives were modified with the aim of overcoming the inadequacies of the *KanMX* system as reported. Accordingly, we undertook to construct a new *HO*-disruption vector capable of looping-out. This project has guided by the desire to facilitate the obtainment of release approval for optimised strains by minimising foreign sequences in such yeast. Thus during redesign of the *HO*-disruption vector we sought also to replace the foreign sequences of the direct repeats with native sequences. This way the single direct repeat which ordinarily remains in the genome after loop-out (Wach *et al.* 1994), would be indistinguishable from native sequences. A novel "one-PCR/two-cloning" procedure for preparing tailored disruption vectors was used successfully and will be prepared for publication elsewhere.

The improved *ho::KanMX* disruption vectors have been shown to be successful in the generation of *ho* haploid wine yeasts. Unfortunately, delays incurred with the design and construction of an alternate disruption system have prevented the completion of a characterisation of these strains or a determination of the efficacy of loop-out of the *KanMX* sequences at this time. This information is expected by early 2002.

Failure of *KanMX* loop-out in the original *HO*-disruption system also prevented reuse of this approach to demonstrate the feasibility of the *KanMX* system for the disruption of further specific genes in a *ho* background. We sought to disrupt the *URA3* gene, which conveniently would also introduce a new selectable marker, *ura3*, manifested as a uracil auxotrophy. As such is was still possible to proceed with a *KanMX*-based disruption system (Replogle *et al.* 1999) since an alternate selection for a uracil auxotrophy was possible. However, all putative transformants isolated in this way (i.e. via resistance to the uracil analogue, FOA) were found to be spontaneous uracil auxotrophs. Clearly then the selection strategy would need to utilise a dominant selectable marker to avoid such spontaneous mutants in the target gene. Therefore, focus returned to the parental wine yeast strain as the background in which geneticin resistance could be used to identify a *ura3* strain.

Geneticin resistance transformants were successfully isolated and the homozygous diploids produced through sporulation, tetrad dissection and spore rediploidisation. The appropriate number of *ura3*/*URA3* genes was confirmed by PFGE-CHEF/Southern analysis. We next sought to introduce the *ura3* mutation into a *ho* strain. This was attempted by cell-spore mating. Thus spores from the sporlated *ura3* diploid were mixed with cells of the haploid *ho::KanMX* strains, however with no success. While the mating event might have occurred, the correct products could not be found amongst the mix of undesired cells. A selection strategy was therefore utilized. The *ura3* spores clearly could already be selected against due to their uracil auxotrophy. Ethidium bromide was used to produce mitochondria-deficient (*mit*$^-$) strains of 1B (*ho::KanMX*) which could in turn be selected against by growth on non-fermentable carbon sources, glycerol and ethanol. Putative *ho::KanMX*, *ura3* colonies able to survive this two-way selection have been produced and have been confirmed by PCR analysis.

Fermentation trials will then be performed to compare the behaviour of the double (*ho*, *ura3*) mutants to the parental L2056 strain. However significant deviation from the fermentative

attributes of the individual mutants which have already been defined is not expected. Specifically, the *ho* haploid has been shown to perform similarly to L2056 as do the diploid *ura3* mutants. While not a haploid, the latter, is a rediploidised haploid, and thus largely of homogeneous genotype, and hence is anticipated to be representative of the fermentative behaviour of haploid *ura3* strains.

In conclusion, we have succeeded in producing *ho* haploids of 3 wine yeast strains as well as wine yeast with the versatile *ura3* marker. Strains bearing both mutations have also been produced and confirmed by PCR. Such strains, strains will prove highly useful as research tools to dissect yeast physiological behaviour as it relates to influencing wine composition. As a prerequisite for such laboratory based studies, these strains have been shown to be highly amenable to numerous classical and recombinant genetic techniques. Further exploitation of these strains will continue with the commencement of project UA 01/04 – *Microbial enhancement of wine complexity and quality*. Prime candidate genes for manipulation will be those conferring efficient use of assimilable nitrogen, allowing proline utilisation under winemaking conditions or influencing the formation of flavour/mouthfeel compounds.

Following closely behind the development of the above research strains, is the development of strains specifically intended as vehicles of the development of optimised commercial yeasts. Such stains demand the presence of minimal or no foreign sequences. The tailoring of gene disruption/manipulation vectors is well advanced with the initial phase expected to be completed shortly. Such a process though must remain an ongoing exercise not only as new target genes for manipulation are identified but also as new, more efficient and less intrusive manipulation strategies are developed. Notwithstanding this, however, much sequence and metabolic information has already been accumulated to support any application for permission to release for pilot scale trials modified yeasts constructed using the methods and vectors developed in this study. Further work is required and is continuing.

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Appendix

Table 1a: Yeast strains used in study.

Strains	Source	Ploidy	Relevant Genotype
Premier Cuvee	Red Star		HO^+
Pasteur Red	Red Star		HO^+
L2056	Lallemand	diploid	HO^+
Flor Sherry	Red Star		HO^+
R2	Lallemand		HO^+
EC1118	Lallemand		HO^+
CY3079	Lallemand	diploid	HO^+
O16	Lallemand	diploid	HO^+
CEG	Lallemand		HO^+
Cote des blanc	Red Star		HO^+
#5 (O16)	this study	diploid	HO^+ (*HO/ho::KanMX*)
#5-1a	this study	haploid	ho^- (*ho::KanMX), MAT*α
#5-1b	this study	diploid	HO^+ (*HO)*
#5-1c	this study	haploid	ho^- (*ho::KanMX), MATa*
#5-1d	this study	diploid	HO^+ (*HO)*
#6 (O16)	this study	diploid	HO^+ (*HO/ho::KanMX*)
#6-1a	this study	haploid	ho^- (*ho::KanMX,) MATa*
#6-1b	this study	haploid	ho^- (*ho::KanMX), MAT*α
#6-1c	this study	diploid	HO^+ (*HO)*
#6-1d	this study	diploid	HO^+ (*HO)*
#8 (CY3079)	this study	diploid	HO^+ (*HO/ho::KanMX*)
#8-b1	this study	haploid	ho^- (*ho::KanMX), MATa*
#8-b2	this study	diploid	HO^+ (*HO)*
#8-b3	this study	haploid	ho^- (*ho::KanMX), MAT*α
#8-b4	this study	diploid	HO^+ (*HO)*
1B (L2056)	this study	haploid	ho^- (*ho::KanMX), MAT*a
1D (L2056)	this study	haploid	ho^- (*ho::KanMX), MAT*α
#40 (L2056)	this study	diploid	ura^- (*URA3/ura3::KanMX*)
#41(L2056)	this study	diploid	ura^- (*URA3/ura3::KanMX*)
#40-c2 (L2056)	this study	diploid	ura^- (*ura3/ura3::KanMX*)
#40-c3 (L2056)	this study	diploid	ura^- (*ura3/ura3::KanMX*)
#41-a2 (L2056)	this study	diploid	ura^- (*ura3/ura3::KanMX*)
#41-a3 (L2056)	this study	diploid	ura^- (*ura3/ura3::KanMX*)
KP2 (W303)	Kate Poole	haploid	*MAT*α
KP3 (W303)	Kate Poole	haploid	*MAT*a

Table 1b: Primers used in this study.

Primers	
HOFga	5'-TGAGCTGTTGCTTACGGTGC-3'
HORga	5'-CAAACCTAATGTGACCGTCGCT-3'
MAT-specific	5'-AGTCACATCAAGATCGTTTATGG-3'
α-specific	5'-GCACGGAATATGGGACTACTTCG-3'
a-specific	5'-ACTCCACTTCAAGTAAGAGTTTG-3'
G418 Fwd	5'-AAAAGACTCACGTTTCGAGGC-3'
G418 Rev	5'-CGAGCATCAAATGAAACTGC-3'
HOP1	5'-TCATGAGCTCTGACAACATGGTAGGCGCGCCTGTCTCATGGAAATTGATGCAG-3'
HOP2	5'-TTGGGGTACCACAATTAAAATATTTTTCTTTCGCGTAAATATTTAGCTT GATAACTGTTACTGATAGTTAACGTGGACATGACATGAATTTTCGC-3'
M13-20mer	5'-TGACCGGCAGCAAAATG-3'
URAR	5'-TTGGTTCTGGCGAGGTATTG-3'
URA3F	5'-AGGGAAGACAAGCAACGAAA-3'

Table 1c: Plasmids used in this study.

Plasmid	**Source**
pGEMT	Promega
pGEMT-HO	Jennie Gardner (this study)
pGEMTho::KanMX3	Jennie Gardner (this study)
pFA6-lacKanMX3	Wach *et al*., (1994)
pBS418	Jeff Eglinton, AWRI
pBS418-HODR1	Michelle Walker (this study)
pBS418-DR2	Michelle Walker (this study)
pBS418-DR21	Michelle Walker (this study)
pDR1123	Replogle *et al*. (1999)

Implications and Recommendations

The reported findings demonstrate the immediate availability of a haploid wine yeast suitable for research use. Such strains will be used by this group in several projects aimed at expanding our knowledge of the biochemical and genetic basis for several important properties of wine yeasts. In particular we intend to use the *ho* haploid strains to; 1) determine the importance of multiple gene copy and genetic heterozygosity for key oenological attributes, 2) as a background for identification of genes influencing the efficiency with which assimilable nitrogen is used by yeast or 3) which influence the formation of flavour compounds. In other projects undertaken by this group, we have developed laboratory yeasts with novel metabolic capabilities. We plan to incorporate such changes into the haploid and, in turn, diploid wine yeast background for fuller evaluation of their benefits. We will be publishing the findings from this work, in addition to the preliminary reports already made at conferences. As such it is intended that these strains be made available to interested research groups.

Accordingly, the benefits for the Australian wine industry will stem from; the greater knowledge about wine yeast biochemistry, as it influences wine composition, as well as the generation of optimised yeasts for commercial use.

Intellectual Property

The key items of intellectual property arising from this project include; 1) three isogenic pairs of haploid (*ho*) wine yeasts as well as their *ura3* derivatives, 2) a set of *ho*, *ura3* strains, 3) a series of *ho* and *ura3* disruption vectors, including 4) those designed to loop-out and leave no foreign sequences in the recipient genome.

Technical Summary

In addition to the strains developed and described above, the project has yielded information about the following.

The significance of genetic make-up on fermentation properties. Haploids appear to be representative of the diploids from which they are derived in that despite the reduced genetic content they share similar fermentation kinetics and generate analogous amounts of key metabolites.

The feasibility of performing classical genetic studies with *ho* haploids.
The haploids generated from this project have to date shown excellent amenability to standard genetic techniques, despite being derived from parents of unknown ploidy. Based on the behaviour of these strains during cycles of mating, sporulation and dissection, many of the wine yeasts examined appear to be diploids.

The practically of existing marker gene loop-out systems. Contrary to reports in the literature, loop-out of the *KanMX* selectable markers does not appear to occur at the reported frequency. After screening > 6,000 colonies of cells passaged through non-selective media for up to 18 times, no loop-outs were found. Favourable conditions/constructs for loop-out are being defined.

Development of a novel "one-PCR/two-cloning" system for construction of disruption vectors. A strategy for the construction of new gene disruption vectors has been developed. The strategy involves the PCR amplification of sequences (~300 bp) flanking the target gene in a single PCR reaction. Two cloning steps introduce the flanking sequences around a selectable marker thereby targeting the vector to the desired gene as well as providing repeat sequences to allow loop-out of the selectable marker region under non-selective conditions. The approach can therefore use either a standard or an alternate selectable marker and has the benefit of increasing the likelihood of leaving only native sequences at the target site after loop-out.