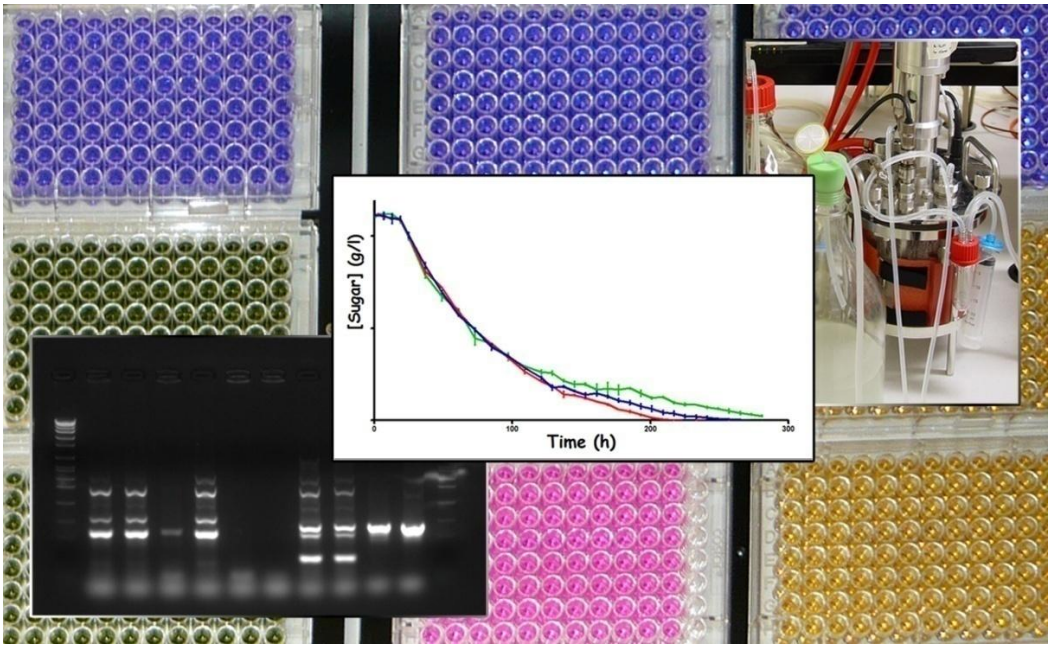
Overcoming Inefficient Utilisation of Fructose as a Cause of Problem Fermentations



FINAL REPORT to

GRAPE AND WINE RESEARCH & DEVELOPMENT CORPORATION

Project Number: **UA 05/05**

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1. ABSTRACT

In this report are detailed the outcomes of a PhD project, aimed to improve the ability of wine yeast to consume fructose. This objective was sought using adaptive evolution as the improvement strategy, thereby also providing an evaluation of the use of adaptive evolution to improve wine yeast for commercial application.

In the first part of the project, AWRI 796 was identified as the most suitable candidate strain from a list of 20 commercially available strains. Then, a mutagenised population of this strain was used as initial genetic background for long term exposure to selective pressure in continuous fermentation. The final part focussed on the identification of phenotypes isolated from the continuous fermentation that showed a faster rate of fructose utilisation. New methods for comparing the fructophilicity of strains and for high throughput screening of isolates have been developed to achieve the planned outcomes.

2. EXECUTIVE SUMMARY

- The glucophilic character of the yeast *Saccharomyces cerevisiae* has been reported in the literature (Berthels *et al.*, 2004; Guillaume *et al.*, 2007). By this attribute glucose and fructose (the two main sugars present in grape juice) are depleted at different rates during the alcoholic fermentation, with fructose predominating in the latter and more difficult stages. At this time when nutrients are low and alcohol concentration is high, the greater ratio of fructose compared to glucose can make attempts to reinvigorate sluggish fermentations or restart stuck fermentations very challenging. Lower ratios of glucose compare to fructose (e.g. ~1:10) have themselves been reported as a cause of arrested fermentation (Schutz and Gafner, 1993).
- Problem fermentations delay grape processing, tie up valuable and limited fermentation space, demand greater resources to manage and rescue, and frequently result in a sub-optimal product due to oxidation and spoilage. Ironically, the frequency of such fermentations means that they are widely accepted as an unavoidable downside of winemaking. Improved strains, however, are likely to offer a solution.
- In seeking to produce strains which are better able to complete fermentation and specifically exhibit a greater ability to utilise fructose, a range of improvement strategies can be used. These can be broadly classed as either recombinant or non-recombinant. Both have disadvantages: on one hand in terms of the degree of knowledge of the genetic basis of the sought property and the acceptability of recombinant products to consumers, and on the other, the limited extent of improvement that is possible or likely.
- A non-recombinant technique that warrants greater exploration in improving microorganism is adaptive evolution. This method has already shown much promise through early work with this technique as part of GWRDC project UA 01/04 and ongoing work in project UA 05/01. Strains generated in this way (McBryde *et al.*, 2006) show an extent of improvement comparable to genetic engineering techniques and are now undergoing extensive evaluation.
- Demonstration of the ability of adaptive evolution to improve fructose utilization in wine yeast was a key objective of this study.
- The first step of this process was to identify a suitable candidate strain for the adaptive evolution strategy. For this reason the fermentation performance of 20 commercially available wine yeast strains was tested with particular attention to their relative ability to utilize fructose in relation to glucose. To describe and compare the fructophilicity of the strains, the ratio between the area under the fermentation curves of glucose and fructose was used, an approach recently published (Liccioli *et al.*, 2010).
- AWRI 796 was identified as the most suitable strain as it showed a low rate of consumption of fructose compared to glucose. Chemical mutagenesis using ethyl methanesulfonate was used to increase the genetic variability of the parental strain. This was intended to reduce the running time of the adaptive evolution strategy. Then, a specific selective pressure was applied to the mutagenised population and maintained under continuous fermentation conditions for 350 generations to isolate desired phenotypes with increased fructophilicity. Culture samples were collected every ~50 generations.

- A method for screening a large number of isolates from the adaptive evolution cultures was optimised for use in 96 well microtiter plates. This method and the relationship of results obtained therein to those from traditional lab scale fermentations has been submitted for publication.
- From a screening of 378 isolates, two showed increased fructophilicity compared to the parent in defined media representative of grape juice. Accordingly the ratio of the area under the glucose compared to the fructose curves (fructophilicity index) was increased to 0.588 for evolved strain 9 compared with 0.538 for the parental strain, whilst fermentation duration was reduced to 117 h from 153 h. The isolates were confirmed by micro-satellite PCR to be closely related to (and presumably derived from) the parental strain. Physiological properties, such as the production of key metabolites, were also unchanged in the evolved strains.
- In the last stage of the project, the fermentation performance of the identified evolved strains was assessed in more wine-like conditions. Preliminary fermentations in grape juice were carried out on two scales: 250 ml of white juice or 20 kg of red grapes). In white juice (250 ml) the evolved strain retained an improved fermentation capability (235 h *vs* >259 h) and improved fructophilicity index compared to parental strain (0.56 vs 0.49). When fermenting red grapes (20 kg) the fermentation rate was again increased, albeit modestly (i.e. 287 vs >293 hr), however the increased fructophilicity index was not longer apparent under these conditions. More extensive trials are warranted to more fully define the beneficial properties of the evolved strains.
- The targets for dissemination of project findings (i.e. number of publications and presentations) were met.
- The PhD student completed his thesis and submitted it for examination in August 2010.
- Discussions have been initiated with parties with potential interest in commercialising the isolated strains.

3. BACKGROUND

Incomplete or slow, and hence unpredictable, fermentation performance by yeast is an ongoing problem for the industry. This reality demands greater monitoring of fermentation progress, increased use of additives and can result in quality erosion and inefficient use of winery fermentation infrastructure - in other words, a serious loss of industry efficiency and wine value. Ironically, the frequency of such problem fermentations means that they are widely accepted as an unavoidable downside of winemaking.

While many causes of problem fermentations have been defined and appropriate remedies proposed, one cause cannot be easily overcome by careful propagation of the inoculum or mere addition of yeast nutrients. This cause, which is to be targeted through this project, is the inherently poor ability of yeast to catabolise fructose late in fermentation when glucose is almost exhausted. Slow or stuck ferments arising as a result of this can only be cured by inoculation with a fresh or alternate yeast culture. In targeting this problem, the use of a non-recombinant approaches such as adaptive evolution, was the key to generate wine yeast strains with a greater capacity to ferment residual fructose in the later stages of fermentation. Such strains will be applicable to all fermentations since all juices naturally contain roughly equal amounts of glucose and fructose. The benefits of efficient „fructose-utilising' strains will range from the elimination of many problematic fermentations through to more rapid completion of fermentations.

4. PROJECT AIMS AND PERFORMANCE TARGETS

As per the approval from GWRDC date 20 July 2007 all of the Project Outputs (see below) were deferred by two years and the Project End Date was extended until 30 June 2010.

Outputs and Performance Targets 2005-06

Outputs	Performance Targets
1. Information on the identity of genes critical to fructose utilisation.	A literature review identifying the critical processes involved in fructose utilisation. The relevant genes sourced from other labs, or by cloning.
2. A system for modelling stuck, fructose-rich ferments.	A lab protocol for modelling ferments that are delayed or stuck because of the high fructose content of the medium. Definition of the conditions for adaptive evolution of superior strains.
3. Work commenced on an optimised fructose-utilising strain.	Construction of a recombinant strain that displays enhanced fructose utilisation capabilities initiated.
4. Adaptive evolution screen initiated	A selective screen for isolation of non-recombinant strains with improved fructose utilisation capabilities initiated.
5. Completion of the PhD *Structured Component.*	Attendance at formal sessions for commencing PhD students, a literature review and research proposal submitted and one seminar presentation given.

Outputs and Performance Targets 2006-07

Outputs	Performance Targets
1. Adaptively evolved strains.	1 – 3 strains better able to use fructose, especially towards the end of fermentation, produced and evaluated in lab fermentations.
2. Adaptively evolved strains characterised.	Evolved strains characterised in terms of physiological and/or other properties.
3. Findings disseminated.	1 – 3 manuscripts published or presentations which describe key findings from the project to date.

Outputs and Performance Targets 2007-08

Outputs	Performance Targets
1. Information on strain performance under wine-like conditions.	Assessment of most promising strain(s) in grape juice fermentations (ideally at pilot-scale) initiated. Standard wine analysis data available.
2. Discussions with yeast suppliers on strain commercialisation initiated.	Meetings held with industry supplier(s) to progress commercialisation of optimised strains.
3. Findings disseminated.	1 – 3 manuscripts published or presentations (e.g. Aust Wine Ind Tech Conf) describing key findings from the project to date.
4. Submitted PhD thesis.	Thesis prepared by the PhD candidate submitted for examination.

The original targets of the project, as reported in the Final Project Application, anticipated the use of yeast mutant libraries to identify genes involved in fructose utilization mechanisms. Then, genetic engineering techniques were to be used to construct wine yeast to express genes to maximise efficient fructose transport and/or catabolism, and demonstrate the benefits for fermentation efficiency. The subsequent step was to define optimal conditions for adaptive evolution to generate superior lab and wine yeast strains. The latter stages of the project included the genetic and physiological characterization of generated strains to identify the basis of improved fructose utilization. Pilot scale or industry trials of the new strains were also to be initiated.

These original targets were in part changed: the necessity to identify a suitable candidate strain to provide the appropriate genotype for the success of the project, replaced the initial genetic characterization of the genes involved in fructose utilization. Thus, instead of screening the yeast mutant libraries and constructing a fructophilic strain by genetic engineering, 20 commercially available strains were screened and their fermentative ability (with particular attention to fructose consumption in relation to glucose) was measured in order to identify a pre-existing reference strain.

5. METHODS

For each experiment, the corresponding methods, results and discussion will be analysed together for clarity in the following section.

6. RESULTS AND DISCUSSION

This section will be divided in four parts:

6.1 – Identification of a suitable candidate strain for adaptive evolution.

6.2 – Establishing an adaptive evolution strategy using continuous culture to generate fructophilic genotypes.

6.3 – Screening of isolates from continuous culture to identify fructophilic genotypes.

6.4 – Physiological and genetic characterization of the identified isolates.

6.1 – IDENTIFICATION OF A SUITABLE CANDIDATE STRAIN FOR ADAPTIVE EVOLUTION

This work was aimed at identifying a candidate strain suitable for improvement of its fructose utilization capabilities. The genetic background used for the adaptive evolution experiment was that of a commercially available strain. To identify the candidate strain, the phenotype of 20 commercially available strains was characterized in terms of their fermentative properties, particularly their ability to ferment fructose, either in the presence or absence of glucose. A description of this screening exercise is given in the manuscript attached in Appendix 1a.

From the results of this screening AWRI 796 was identified as the most suitable candidate strain for the adaptive evolution strategy. This strain showed a large difference between fructose and glucose fermentation, although a medium to fast overall fermentation rate. The first characteristic increases the possibility of observing significative improvements in the fructophilicity of the improved mutants derived from AWRI 796, since the possible margin of improvement is greater and more appreciable than for a strain with a smaller difference between glucose and fructose fermentation. The second characteristic reduces the duration of the adaptive evolution experiment compared to that of a slower fermenter. Otherwise, the total duration of the evolution experiment could be excessively extended.

6.2 – ESTABLISHING AN ADAPTIVE EVOLUTION STRATEGY USING CONTINUOUS CULTURE TO GENERATE FRUCTOPHILIC GENOTYPES

Before starting an adaptive evolution strategy, it was important to prepare the initial population. Despite claims of suppliers, commercial packages of yeast are not pure cultures. Thus, for the purpose of this study it was important to isolate a single clone of AWRI 796, physiologically representative of the average of the population. This isolate provided the genetic background for selective improvement and was a reference for characterization of adaptively evolved isolates.

Genetic variation is the raw material for adaptive evolution (Chambers et al., 2007). Thus, the more genetic diversity that exists in a population, the more rapidly a selection pressure will isolate adaptively evolved novel phenotypes. Starting an adaptive evolution

experiment with a clonal population will therefore greatly reduce the chance of isolating evolved strains with desirable phenotypes in a reasonable time. Thus, chemical mutagenesis was used to generate genetic variation in the starting population, to be used in the adaptive evolution experiment described later.

6.2.1 – Isolation of a representative single clone of AWRI 796

To isolate a representative clone of AWRI 796, the strain was plated onto YEPD agar (10 g/l yeast extract, 20 g/l bacto-peptone, 20 g/l D-glucose) from -80°C glycerol stock. From this plate, six individual colonies and a mixed population derived from a patch from a densely growing region of the plate, were transferred into separate 250 ml conical flasks containing 50 ml YEPD broth. The seven flasks were incubated overnight at 28°C, shaking at 160 rpm. Each was then inoculated at a rate of 1 x 10^6 cells/ml into 50 ml Chemically Defined Grape Juice Media starter (CDGJM with 50 g/l glucose, 50 g/l fructose – as described in Henschke and Jiranek, 1993) in 250 ml conical flasks (air/liquid ratio > 66%) and incubated overnight at 28°C, shaking at 180 rpm. Each of these cultures was then used to inoculate 3 replicates of 100 ml CDGJM (with 115 g/l glucose, 115 g/l fructose) at a rate of 5 x 10^6 cells/ml in 250 ml conical flasks fitted with water filled air locks and sampling ports. Fermentation progress was monitored by CO_2 loss, weighing flasks every hour using the Multi-Scale Fermentation Facility (The University of Adelaide – http://www.sciences.adelaide.edu.au/wine/msff/), which controls the fermentation temperature (30°C), agitates the culture (with magnetic stir bars) and monitors weight loss of the flasks, robotically, on high sensitivity balances.

CO_2 losses (Figure 1A) were expressed in g of CO_2 lost per 100 g of medium fermented and fitted with a non-linear, third order polynomial fit using GraphPad Prism (GraphPad Software Inc., La Jolla, C.A., USA). For the last third of the fermentation °Brix of clarified (20,800 rcf, 2 min) culture samples was also monitored. Fermentation completion (< 2.5 g/l of sugar) was determined using Clinitest® tablets (Bayer). Supernatants were stored at -20°C for subsequent determination of residual sugar content (Figure 1B and C) as described in (Boehringer-Mannheim, 1989) with final volumes adjusted to 300 µl for analysis in 48 well microtiter plates.

Fermentation progress of six clonal isolates and the mixed population was monitored. All fermentation curves show a similar initial profile, but some differences become evident, especially in the last third of the fermentation (Figure 1A). However, clone 5 showed a CO_2 weight loss profile almost identical to the mixed population. This data was confirmed by the sugar utilization profile, where clone 5 was able to catabolise all of the sugar in 113.5 hours, the same time as required by the mixed population (Figure 1 B and C).

Due to its similarity in fermentation profile to the mixed population sample of AWRI 796, clone 5 was chosen as the parent strain for subsequent adaptive evolution experiments.

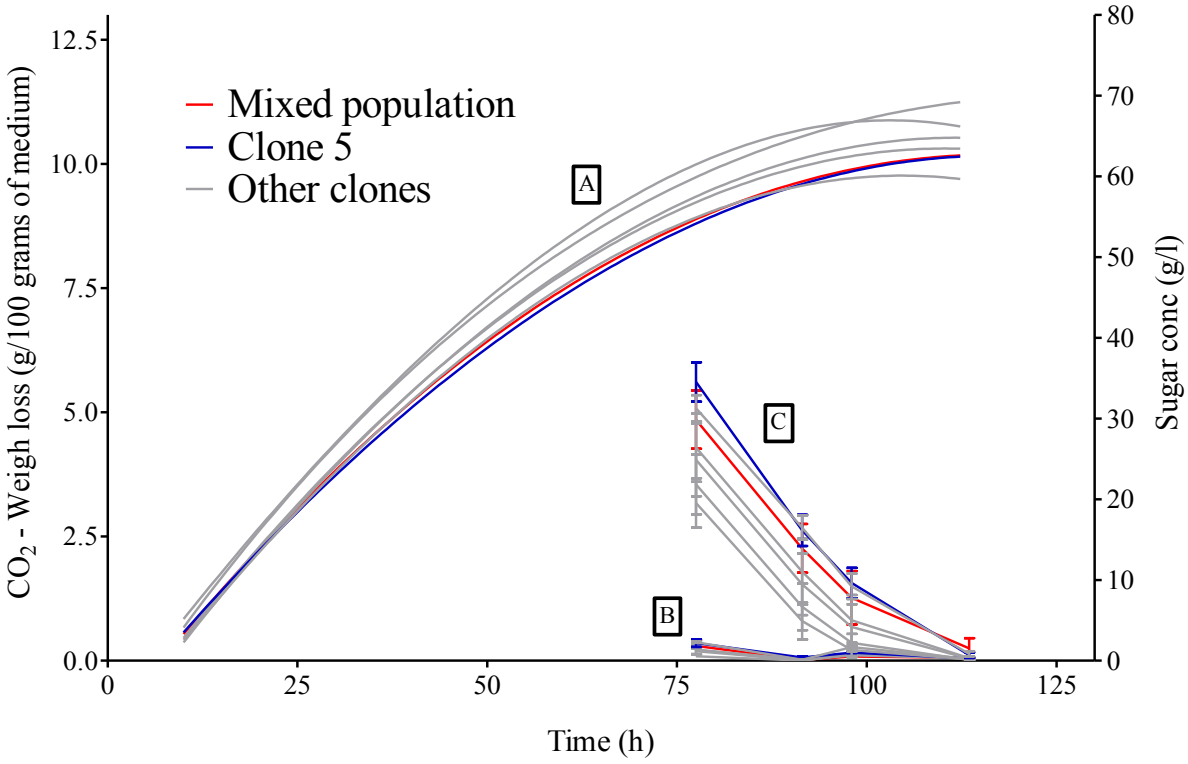


Figure 1 – A - Non linear (third order polynomial – confidence interval 95%, $R^2 > 0.98$ for the fit of all curves) fit for a mixed population and six clones of AWRI 796 (left axis). B - Glucose concentration (plotted on right axis). C - Fructose concentration (right axis). Each curve is plotted from data from the average of three replicate fermentations. Standard deviations are shown as error bars.

6.2.2 – Induced mutagenesis of AWRI 796.

Mutagenesis was induced artificially in clone 5 of AWRI 796 using ethyl methanesulfonate (EMS) using a method adapted from Fink (1970). Thirteen samples were collected at 10 minute intervals following exposure to 45 µl/ml of EMS and the mutagenesis process arrested with sodium phosphate buffer. Samples were appropriately diluted and plated onto YEPD plates, while the remainder of each sample was stored in glycerol at -80°C. From YEPD plates viable cell counts were determined. As can be seen (Figure 2) residual viability decreased progressively with time of exposure to EMS, such that no viable cells were evident after 120 minutes.

The population treated with EMS for 50 minutes (EMS5), which generated ~40% death (60% survival), was chosen as the starting population for an adaptive evolution experiment. Based on past experiences in the laboratory, this population was anticipated to have a substantial amount of genetic variation.

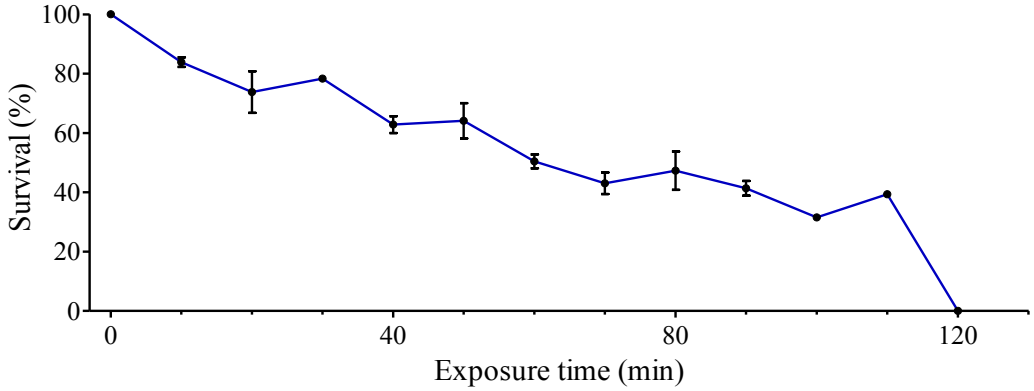


Figure 2 – Survival in a population of clone 5 after varying times of exposure to EMS.

6.2.3 – Defining experimental conditions for adaptive evolution.

There are essentially two different approaches for adaptive evolution of microorganisms in the laboratory: sequential batch or continuous culture. Continuous fermentation was the strategy chosen for this work as it considerably reduced the running time of the experiment. In fact, maintaining a population in the exponential phase eliminates the time necessary for the complete depletion of sugar from the media and the sequential inoculation into a fresh batch fermentation. In previous adaptive evolution experiments (McBryde *et al.*, 2006), using similar conditions to those required for the experiment described here, sequential batch proliferation over 250 generations of a population of wine strain of *S. cerevisiae* required 198 days. In a chemostat, the same number of generations (with a dilution rate of 0.2 h^{-1}) would require an estimated 35 days. While it was recognised that the possibility of affecting the oenological properties of the evolved strains may exist, a continuous fermentation allows for shorter experiments. This decrease in running time would allow completion of several adaptive evolution experiments in the same amount of time required for a single sequential batch approach. This also allowed trialling of several strategies to generate strains with improved phenotypes.

Before commencing a long term adaptive evolution experiment it is essential to establish the optimal conditions for selection of the desired phonotype. This includes: determining the „strength' of the selective pressure, optimal cell density, dilution rate and composition of feed medium.

Determination of the optimal level of stress for adaptive evolution is critical to drive selection of the desired phenotype (Butler *et al.*, 1996). It was reasoned that having fructose as the only sugar in the medium, and maintaining it at a limiting concentration would be an appropriate selective pressure to generate adaptations to improve the efficiency of fructose utilization. Using a chemostat which maintained a continuous low level of fructose provided an effective means of achieving this. An absence of fructose in the waste medium was taken as indication that the fructose supplied to the bioreactor was limiting. In this environment, the most efficient fructose utilisers should be fittest.

Maintaining an appropriate cell density is crucial to this approach (Paquin and Adams, 1983; Wahl and Krakauer, 2000; Wick *et al.*, 2002) and this can be achieved by varying the amount of carbon supplied to the chemostat (Lane *et al.*, 1999). In this context, an

appropriate level of selective pressure (i.e. limiting sugar concentration) will impose sufficient stress to reduce growth rate without compromising cell density. For this reason it was important to understand the yeast's physiological response and tolerance to the intended stress conditions. Thus, information on the maximum fructose consumption rate and associated growth rate for EMS5 was sought.

Preliminary experiments were performed to estimate the conditions for a continuous culture experiment in which fructose was limiting (data not shown). From these initial experiments it was possible to establish the appropriate initial conditions for the adaptive evolution experiment. To maintain a cell density of ~5 x 10^7 cells/ml in a 500 ml bioreactor at a dilution rate of 0.2 h^{-1} it was necessary to maintain a fructose concentration in the feed medium of 4 g/l. These experimental conditions were thought to be appropriate to generate adaptively evolved phenotypes showing improved fructophilic characteristics.

6.2.4 – Adaptive evolution to generate fructophilic wine yeast

An aliquot of frozen cells of EMS5 from a -80°C glycerol stock, was streaked onto YEPF (10 g/l yeast extract, 20 g/l bacto-peptone, 20 g/l fructose) and grown overnight at 30°C. A patch from a densely growing region of the plate was then inoculated into liquid YEPF and incubated overnight at 30°C with agitation on a shaker at 160 rpm. A continuous culture was then set up by inoculating from the overnight culture 10^5 cells/ml into CDGJM (500 ml with 4 g/l fructose). All continuous culture experiments were conducted in a BIOSTAT® A plus (Sartorius BBI System GmbH) equipped with a 1.0 litre fermentation vessel and controlled using the MFCS/DA A plus 2.1 software (Sartorius BBI System GmbH). The medium was supplemented with ergosterol and Tween80® to supply sterols and fatty acids, essentially for cell membrane synthesis under anaerobic conditions (Ribereau-Gayon *et al*., 2005). CDGJM with the same composition was also used as the feed during the continuous culture experiments. Fermentation temperature was kept at 30°C and cells were suspended with agitation at 200 rpm. Head space in the fermentation vessel was saturated with the CO_2 produced during the fermentation and pressure released using a water filled air lock.

Fermentation was started as a batch culture and after 24 hours (cell density ~5 x 10^7 cells/ml) the feed pump was started, with an initial dilution rate of 0.08 h^{-1}. At the same time the waste pump was started (same flow rate as feed pump), ensuring a constant culture volume at ~500 ml. Over the following 220 h of the experiment, the dilution rate was increased progressively from 0.08 h^{-1} to 0.12, 0.14, 0.15, 0.18 h^{-1}, until reaching a final value of 0.2 h^{-1}. This dilution rate was maintained until the end of the experiment. The duration of the experiment was 1278 hours (~53 days), the time necessary to allow the multiplication of approximately 350 generations. The bioreactor was sampled every 48 hours. For each sampling point cell density was measured (cell count by haemocytometer) and fructose concentration in the exhaust media was monitored using Clinitest®. Figure 3 shows the parameters measured during the progress of the experiment. It is possible to observe the variation in the flow rate, maintained constant after 220 hours and the moderate fluctuation of cell density (between 4.05 x 10^7 and 6.75 x 10^7 cells/ml). Fructose was not detectable in the fermentation vessel (data not shown). For characterization of

mutants, 1 ml samples of the population were collected every 50 generations and centrifuged 20,800 rcf for 1 min. The supernatant was removed and cells resuspended in 1 ml of fresh YEPF medium and 0.5 ml of sterile 80% (v/v) glycerol to enable long term storage at -80°C.

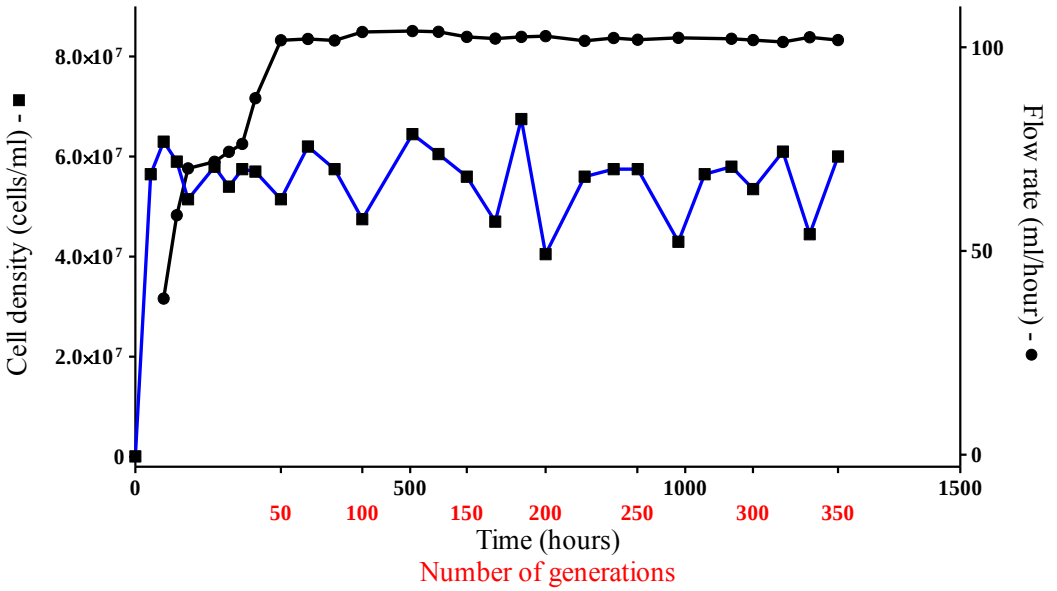


Figure 3 – Parameters measured during continuous fermentation with limiting sugar (fructose). Flow rate (•) is on the right axis; cell density (▪) is on the left axis.

6.2.5 – Conclusions

To generate fructophilic phenotypes with an adaptive evolution strategy, a mutagenised population of AWRI 796, EMS5, was maintained in continuous culture for 350 generations with limiting fructose as the only source of sugar. Based on the information reported by other authors (Paquin and Adams, 1983; Zeyl, 2004) the observed frequency of the occurrence of an adaptive mutation is somewhere between 10^{11} and 10^{12} cell divisions for non-mutagenised starting populations. Considering that in the experimental conditions used in this study the initial population was artificially mutagenised (with the effect of an increase of the genetic diversity), the population size (around 2.5 x 10^{10} cells) and the time necessary for a mutation to become fixed, it was decided to sample the culture every 50 generations. These samples were screened for fructophilic phenotypes as described in the following part of this report.

6.3 – SCREENING OF ISOLATES FROM CONTINUOUS CULTURE TO IDENTIFY FRUCTOPHILIC GENOTYPES

6.3.1 – Screening for isolates showing improved fructophilic ability

A population of microorganisms forced to live in submerged culture under a specific stress will eventually acquire isolates that show characteristics of adaptation to that particular environment. However, the resulting population is not a monoclonal population derived from the one preceding it, as stipulated in the classical model of adaptive evolution of an asexual population (Muller, 1932). Other studies have shown that an evolving population of asexual yeast follow more complex dynamics, resulting in a mixture of different genotypes, ultimately contributing to different extents to the total population during the progress of fermentation (Kao and Sherlock, 2008). Thus, it appears clear that the selection of an adapted clone is not a simple process. As such, a heterogeneous mixture of different sub-populations will require a reliable methodology capable of screening several clones to identify those that have adapted to the selective pressure.

It was decided to use laboratory scale fermentations to compare the fermentative performance of isolates. However, to screen hundreds of isolates in replicate necessitates more cultures than would be logistically possible. The solution was to reduce the volume of such cultures to 200 µl and take advantage of 96 well microtiter plates as the fermentation vessel. These plates also permit the use of automated robotic systems to simplify handling and thereby increase the number of isolates that could be examined. The use of such small volumes required validation. The relationship between the performance of strains in 96 microtiter plates compared to large cultures (100 ml) was assessed as part of a collaborative project with researchers at AWRI, resulting in a manuscript submitted for publication (Liccioli, T.; Tran, T.M.T.; Cozzolino, D.; Jiranek, V.; Chambers; P.J., Schmidt, S.A. 2010 – "Microvinifications – How small can we go?". Submitted to Journal of Applied Microbiology and Biotechnology). The key findings were:

1. No difference was observed in the maximum biomass yield and rate of growth between wine yeast cultures propagated in self-induced anaerobic 100 ml conical flasks and 96 well microtiter plates;
2. Comparable fermentation rates were seen between wine yeast cultures performed in 100 ml self-induced anaerobic conical flasks and 96 well microtiter plates. Even though fermentations in microtiter plates were typically faster, the relative sugar utilization profiles of the strains examined were unchanged compared to fermentations in flasks.

Thus, the use of microtiter plates became a valid tool to screen large numbers of isolates during this study. Using this approach, clones isolated every 50 generations during the continuous culture experiment, were investigated to identify the presence of phenotypes showing improved fructophilic characteristics. Thus, isolates from 7 sequential sampling times were tested. For each point, the fermentative performance of 54 isolates was compared against the parental strain and a representation of the mixed population at the same 50 generation sampling point. The screening method used 96 well microtiter plates with a sacrificial sampling approach. Essentially, several copies of the same plate were

prepared such that a fresh plate was used at each time point to allowing monitoring of sugar depletion profiles, without influencing fermentation performances in the small volume of the microtiter plates cultures.

Specifically, from -80°C glycerol stocks, cells were streaked onto YEPD plates and incubated overnight at 30°C. The cells used had been collected previously from the continuous fermentation experiment at 50 generation intervals. From the YEPD plates, 54 single colonies were picked and inoculated into 500 µl of YEPD broth, incubated without shaking overnight at 30°C. The parental strain and a mixed population derived from a patch of a densely growing region of the culture sample streak plate were grown similarly. For each culture, 1 ml of CDGJM starter medium (25 g/l glucose, 25 g/l fructose) was inoculated with 20 µl of YEDP culture and incubated at 30°C in 10 ml Falcon tubes without agitation. Once the cells reached saturation (overnight incubation), each culture was supplemented with 4 ml of CDGJM containing 50 g/l glucose, 50 g/l fructose (i.e. a 1 in 5 dilution). Ten micro litres of each suspension were used to inoculate 96 well microtiter plates containing 190 µl of CDGJM (50 g/l glucose, 50 g/l fructose) in each well. The 54 isolates were distributed over 3 plates and each plate also contained the parental strain and the mixed population. Each plate was then replicated six times, in an operation performed with an automated plate and liquid handling machine (Tecan EVO 150). The dilutions, gave a dilution factor of 100 for the starter culture. Thus, the average density of the starter culture was therefore diluted from ~10^8 cells/ml to around 10^6 cells/ml. For each culture, four replicates of the same isolate were inoculated on each 96 well microtiter plate. Some unutilised wells in the plate contained uninoculated CDGJM as a control for sterility, while others were used to hold standards when sub-sequentially performing enzymatic sugar assays described in Boehringer-Mannheim (1989) with final volumes adjusted to 100 µl for analysis in 96 well microtiter plates. Typical plate layout is shown in Figure 4.

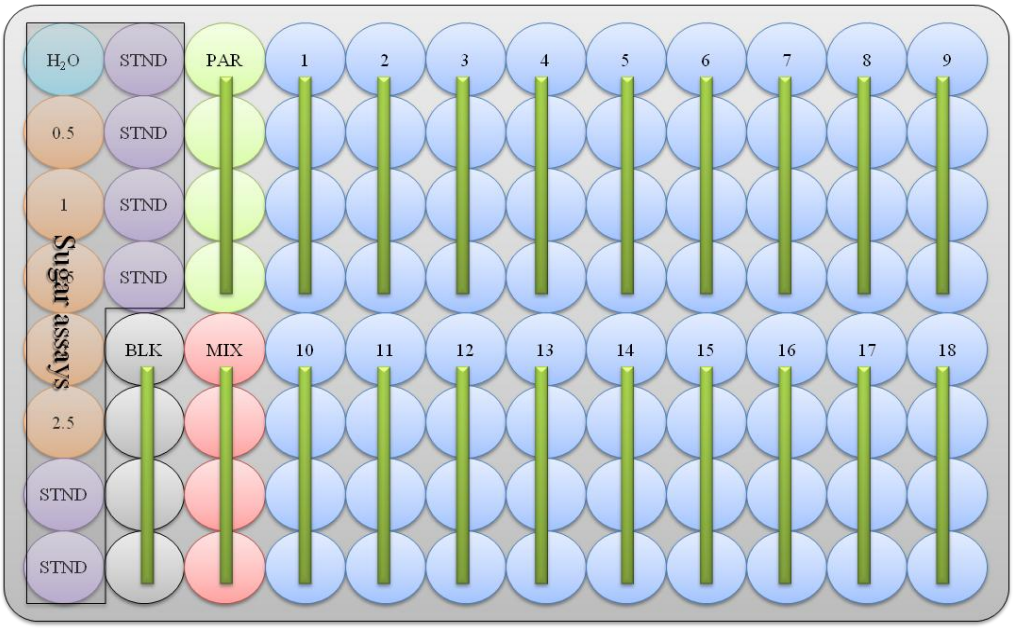


Figure 4 – Example of plate layout used for screening isolates from continuous culture every 50 generations. Each plate contained 18 isolates in four replicates, as well of the parental strain (PAR), mixed population of the corresponding 50-generational point (MIX), uninoculated wells for checking for contaminations (BLK) and empty wells used subsequently for sugar determinations (boxed area). Each plate was replicated six times for analysis of fermentation performance using a sacrificial sampling approach.

Each of the 6 replicate plates, were sealed with breathable membrane (*Breathe-easy*, Diversified Biotech) and placed in an incubator at a controlled temperature (20°C), humidity (75%) and atmosphere saturated with nitrogen gas (O_2 concentration < 1%). The elevate humidity was intended to limit evaporation from the fermentations, while the nitrogen atmosphere reduced the influence on fermentation due to oxygen. One of each of the 6 sacrificial plates was frozen at -20°C (for samples time see Figure 5) every 24 hours, for subsequent enzymatic determination of glucose and fructose. Sugar determination was performed semi-automatically, preparing the enzymatic reactions using the same liquid handling robot (Tecan EVO 150). An integrated spectrophotometric plate reader (Tecan M200 Infinite) was used for colorimetric measurements.

Examples of the high throughput analysis of fermentation performance are shown in Figure 5, where the sugars utilization of 18 isolates, the parental strain and the mixed population are represented. In addition to the curve of total sugar depletion (Figure 5A), utilization of glucose (Figure 5B) and fructose (Figure 5C) are shown individually.

A clear differentiation between many of the isolates, the parental strain and the mixed population is evident (Figure 5). Several of the isolates show an improvement in overall fermentation performance (Figure 5A), depleting all sugars in a shorter period of time compared to the parent. Comparing Figure 5A with 5B and 5C it is possible to see that the greater contribution to such improvement can principally be attributed to an improvement of the utilization of fructose. Such an observation suggested a possible improvement in the fructophilicity of at least some of the isolates, as per the objective of this study.

The spread in fermentation curves seen across the isolates was typical for each batch investigated during screening. Thus, in each case, the fermentation kinetics of a few isolates were faster than the parental strain, while others were slower. However, such simplistic graphical evaluation of fermentation curves did not provide a detailed enough comparison of the relative fructophilicity of the isolates. For this reason differences in fermentation rate were quantified paying particular attention to ability of isolates to utilize fructose compared to glucose. Once again, determination of the value of the area under the fermentation curve (described in the manuscript included in Appendix 1a) appeared the most appropriate for this purpose. To eliminate possible external influence on the fermentative performance due to differences in experimental or environmental conditions between microtiter plates, the fermentation rate of every isolate was standardized relative to the parental strain included in each plate. Thus, the value of the area under the fermentation curve was expressed as a percentage of the parental strain within every plate. With this approach and in turn the calculation of the ratio between areas, it was possible to identify and quantify isolates showing improved overall fermentative performance and to rank them according to fructophilicity.

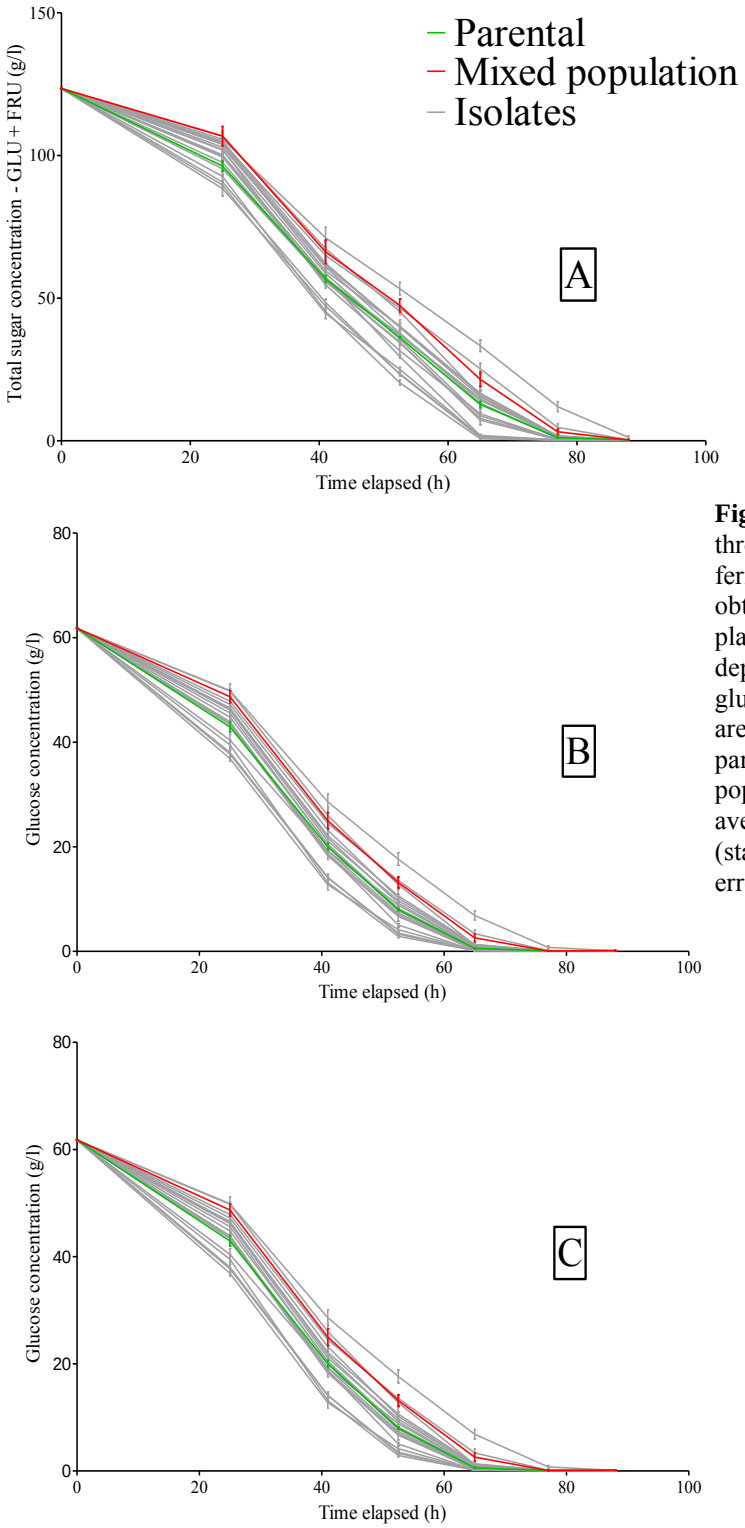


Figure 5 – Example of high throughput analysis of fermentation performance obtained using the sacrificial plate approach. Curves of depletion of total sugar (A), glucose (B) and fructose (C) are shown for 18 isolates, the parental strain and the mixed population. Results are the average of four replicates (standard deviation shown as error bars).

The ratio between the area under the fermentation curve of glucose and fructose was calculated (as described in Appendix 1a) for each of 378 isolates and for each of the parental strains (one for each set of plates = 21 fermentations). The distribution of these

values was examined across the seven groups of 50-generation isolates (Figure 6). An apparent trend was observed: a progressive increase in the value of the ratio between the areas under the glucose and fructose curves with an increasing number of generations of exposure to the selective pressure. By comparison the performance of the parental strains, whilst broadly distributed much as the isolates were, nonetheless tended to remain in the lower range of ratio values (i.e. 0.630 – 0.765). However, this parameter alone was not sufficient to assure the identification of isolates showing improved characteristics. Clones showing greater fructophilicity but slower overall fermentation compared to the parental strain were not considered suitable for further studies. To identify candidate suitable for further characterization, the value of the ratio of the areas under the fermentation curves of glucose and fructose was combined with the overall fermentation performance. In this way it was possible to identify 4 isolates showing improved fructophilic characteristics and a faster overall fermentation performance. For these 4 clones specifically, the ratio of the area under the fermentation curves of glucose and fructose was seen to have increased compared to the parental strains (indicating an increased fructophilicity). In every case these clones also showed values of the area under the overall fermentation smaller than the parental value, thereby indicating faster fermentations. These 4 isolates were chosen for further comparison of fermentation performances and physiological and genetic characterization as described in Section 6.4.

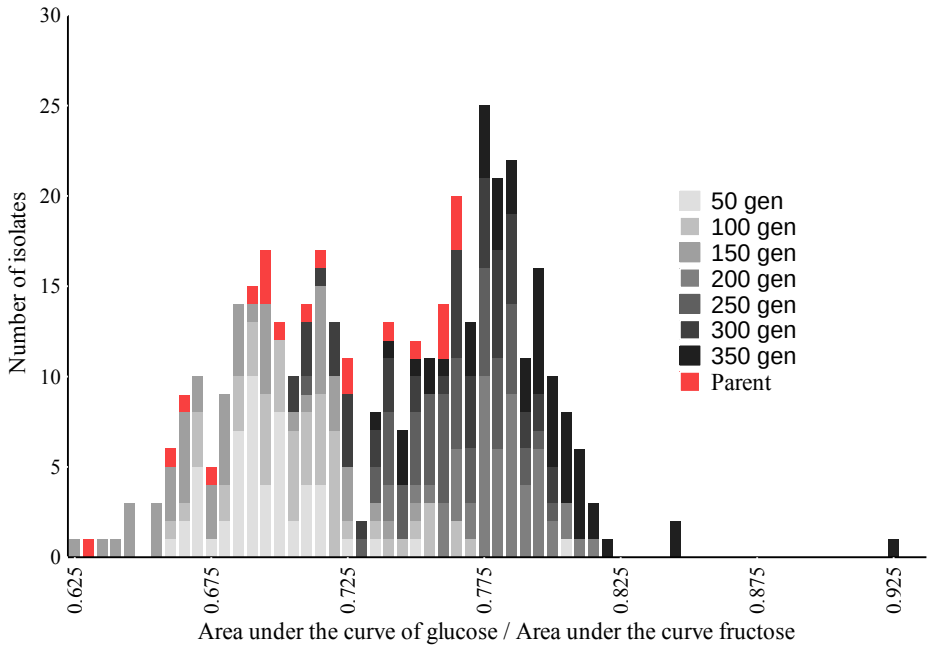


Figure 6 – Absolute value of the area under the fermentation curve of glucose divided by the value of the area under the curve of fructose as an indication of the fructophilicity of individual isolates. Isolates are grouped (refer to shading) according to the 50-generational sample from which were obtained. The distribution in the performance of replicates of the parental strain is indicated by the red bars.

A final point to be made in regard to the fermentation rate of the parental strain is related to the proportion of improved isolates. For each of the 50-generational samples screened, the number of isolates showing a faster fermentation rate compared to the parental strain was shown to change with the progress of the adaptive evolution experiment (Figure 7). As can be seen, while not statistically significant, there appeared to be a trend to an increasing proportion of improved isolates toward 250-300 generations. Beyond this number of generations, the proportion of isolates improved relative to the parent appeared to decline.

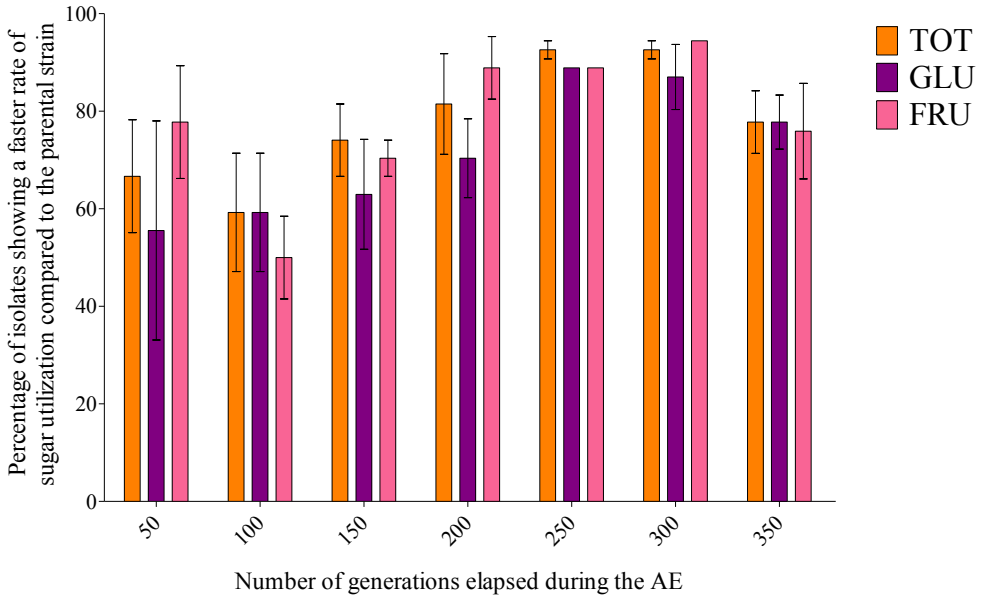


Figure 7 – Number of isolates (expressed as a percentage of the 54 isolates screened from each 50-generational sample) showing faster fermentation rate (smallest area under the fermentation curve) compared to the parental strain for total sugar or glucose and fructose individually. The seven generational points are shown separately. Standard deviation is shown as error bars.

6.3.2 – Conclusions

The identification of a methodology suited to the screening of a large number of isolates and able to describe their fermentation kinetics, especially the ability to utilise fructose compared to glucose, enabled identification of four candidates for further investigation. These candidates, proliferated during continuous fermentation under selective pressure, showed improved fructophilic characteristics and a faster fermentation rate compared to the parental strain during the screening experiments. However, to confirm and better characterize the improved ability of these clones to consume fructose, especially in larger scale fermentations and in media including grape juice, the appropriate strain evaluation was undertaken and is discussed in the next section.

A final comment on this section has to be made about the distributions of isolates showing improved fructophilicity and improved fermentation abilities compared to the parental

strain. Even though the data analysed are only derived from a screening experiment and further characterisation is required before it could be considered a comprehensive study of evolution in an evolving population, the trends exhibited in Figure 6 and 7 appear to suggest a progressive adaptation of the mutants to the imposed stress. Thus the trend shown in Figure 6 highlights the higher frequency of improved fructophilicity with the progress of the adaptive evolution experiment. The literature reports that adaptation continues with exposure to a selective pressure (Helling *et al*., 1987; Zeyl, 2005). Whilst more work is required, s similar trend would appear to have developed through thus this study.

Previous studies, although on a much larger scale (Helling *et al*., 1987; Kao and Sherlock, 2008), showed that improvement is not a linear trend, but that adaptation of a population to selective pressure follows oscillatory patterns. Again a similar pattern would appear to be developing in this study, with a decline in the number of isolates with improved fermentative ability compared to the parental at around 250-300 generations (Figure 7).

6.4 – PHYSIOLOGICAL AND GENETIC CHARACTERIZATION OF THE IDENTIFIED ISOLATES

Four isolates showing improved fructophilic and overall fermentation performances were used in further experiments aimed to more fully evaluate the fermentation of these. Approaches and techniques with increased sensitivity were therefore used and represented the initial part of the transition between the laboratory scale to more wine-like environments. In particular, the screening of the isolates to this point had been performed in CDGJM containing 50 g/l each of glucose and fructose. In grape juice the sugar concentration is at least double this and so too the concentration of alcohol produced.

In addition to fermentation performances it was also important to understand if isolates differed from the parental strain in terms for other oenological traits and genetic attributes. The concentrations of organic acids, alcohols and hydrogen sulfide after the complete catabolism of sugars were measured. Molecular typing of the strains permitted determination of the extent of the genetic relatedness of isolates to the parental strain.

The last investigations were aimed at evaluating the fermentative performance of the mutants in grape juices, to understand if the mutation(s) isolated in artificial (laboratory) conditions conferred faster fructose fermentation in a more wine-like environment.

6.4.1 – Fermentation performances of 4 isolates in a higher sugar concentration medium

Four isolates (named 3, 7, 9 and 11) highlighted from the previous screening experiment (Section 6.3.1) were streaked onto YEPD plates from -80°C glycerol stocks, incubated overnight at 30°C and checked for purity. The parental strain was prepared similarly. A colony representative of each isolate and multiple colonies of the parental strain were then inoculated into YEPD broth and grown overnight at 30°C with agitation at 160 rpm on a

shaker. Cells of each culture were inoculated into 50 ml aliquots of CDGJM starter medium (50 g/l glucose, 50 g/l fructose) to an inoculation density of 2.5 x 10^6 cells/ml. The starter cultures were grown as before and triplicate 250 ml aliquots of CDGJM (115 g/l of glucose, 115 g/l of fructose) inoculated to 5 x 10^6 cells/ml. Flasks were sealed with lids provided with water filled air locks, placed into an automatic fermenter (*Medicel Explorer*) with continuous flushing of the head space with filtered nitrogen (0.45 µm, 5 ml/min), constant agitation (magnetic stir bars, 200 rpm) and kept at 30°C (in a water bath). Three ml samples were collected automatically and held at -5°C until transferred to -20°C prior to enzymatic determination of glucose and fructose (Boehringer-Mannheim, 1989) with final assay volumes adjusted to 100 µl for analysis in 96 well microtiter plates. The frequency of sampling was generally 6-8 hours at the beginning and final stages of fermentation, but decreased to 12 hours in the middle of the fermentation.

The strains showed a clear differentiation in the second half of the fermentation (Figure 8). The parental strain completed the fermentation in 153 hours. By comparison, isolate 9 showed a marked reduction in fermentation duration depleting all sugars in 117 hours. Isolate 11 showed an intermediate fermentation duration of 135 hours. Isolates 3 and 7 failed to complete the fermentation and became stuck at about 30 g/l, which incidentally was composed mainly of fructose. The improvement in the fermentation kinetic of isolates 9 and 11 was reflected in the area under the fermentation curves (Table 1). These isolates showed a reduction in this value of ~19% and ~12%, respectively, for the total area.

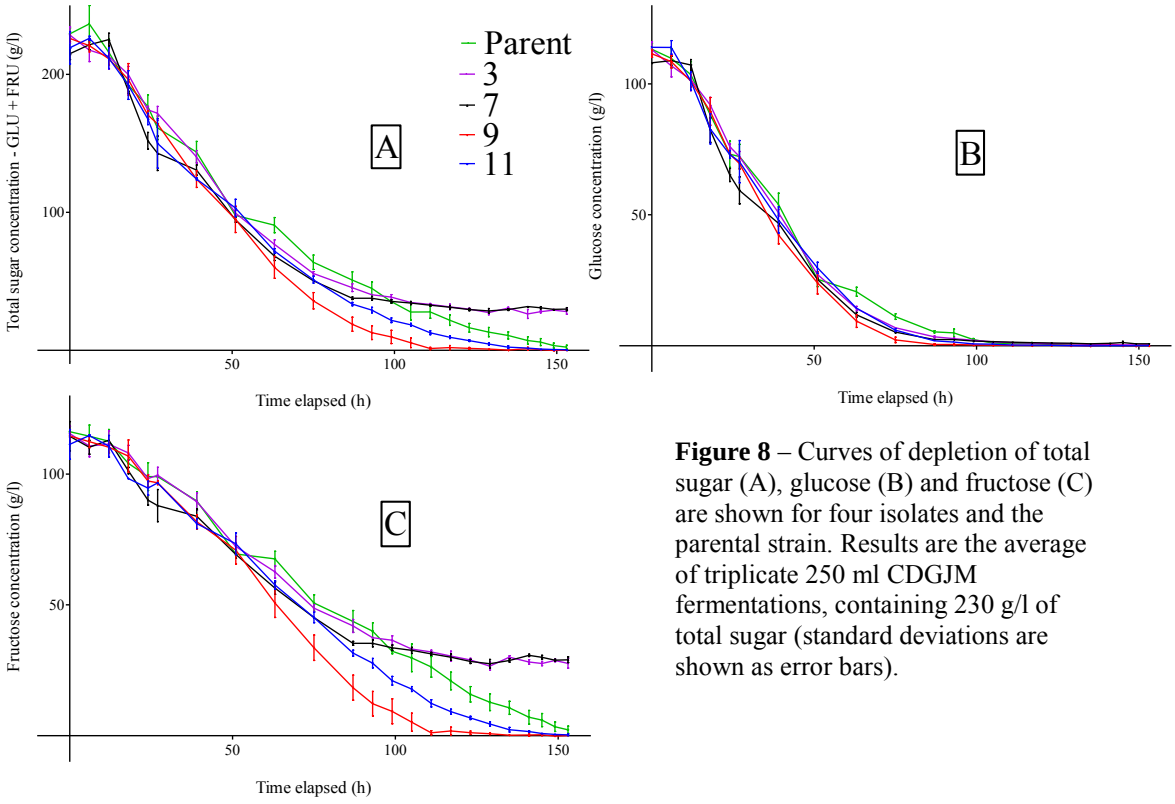


Figure 8 – Curves of depletion of total sugar (A), glucose (B) and fructose (C) are shown for four isolates and the parental strain. Results are the average of triplicate 250 ml CDGJM fermentations, containing 230 g/l of total sugar (standard deviations are shown as error bars).

Parameter	Strain		
	PAR	9	11
Area TOT %	100	81.37	87.99
Area GLU %	100	86.40	92.31
Area FRU %	100	79.09	87.77
Ratio Areas (GLU/FRU)	0.538	0.588	0.566

Table 1 – Percentage of the area under the fermentation curves for isolates 9 and 11 compared to the parental strain for total sugar or glucose and fructose individually. The fructophilicity of the strains is expressed as a ratio between the area under the fermentation curves of glucose and fructose. Isolates 3 and 7 were not considered further as they were unable to complete fermentation.

By considering the utilization of the individual sugars, it is possible to see that the improvement in fermentation was mainly due to an improved fructophilicity of the two isolates. The increased ability to consume fructose compared to glucose was confirmed by the calculation of the ratio between the area under the curve of glucose and fructose, which increased from 0.538 of the parental strain to 0.588 and 0.566 for isolates 9 and 11, respectively.

As stated, isolates 3 and 7 failed to complete fermentation, leaving mainly residual fructose. Interestingly the utilisation of glucose by both isolates 9 and 11 was essentially the same as seen for the parental strain. The basis of the selective loss of fructose utilising ability is unknown, but maybe linked to the higher ethanol yield of these high sugar fermentations (230 g/l total)

6.4.2 – Evaluation of the fermentation kinetics of isolates 9 and 11, the parental strain and two commercially available strains.

EC1118 and Fermichamp, two commercially available strains, already used in this study (see manuscript in Appendix 1a), were included as a reference for the performance of isolates 9 and 11. EC1118 is one of the most widely used strains in winemaking and wine research. Fermichamp has been described as a strain showing high fructophilicity, due to a mutation in the hexose transporter encoded by *HXT3* (Guillaume *et al.*, 2007). The preparation and execution of the experiment was the same as that described for the previous experiment (Section 6.4.1). The only difference was the replacement of mutants 3 and 7 with the two reference strains, EC1118 and Fermichamp.

EC1118 and Fermichamp performed similarly to the parental strain, but they did not complete fermentation in the time frame of the experiment, leaving approximately 10 g/l of sugar (mainly fructose) in the medium (Figure 9). By comparison, isolate 9 and 11 performed strongly and completed the fermentation in 160 and 168 hours, respectively, while the parental strain required 186 hours. Superior performance of the parental strain compared to EC1118 and Fermichamp was attributable to more rapid utilization of glucose. Isolates 9 and 11 exhibit more rapid utilisation of both glucose and fructose compared to the other strains. Unlike the previous experiment where the improved performance of these isolates was largely attributable to fructose utilization, in this case a similar, proportional improvement was seen for both sugars. As a result, there was no change in the fructophilicity of the isolates as demonstrated by the fact that the ratio between the areas under the fermentation curves of glucose and fructose was unchanged compared to the parent (Table 2). Despite this the superior fermentation capability of

isolates 9 and 11 warranted their being further characterized in terms of their physiological and genetic properties.

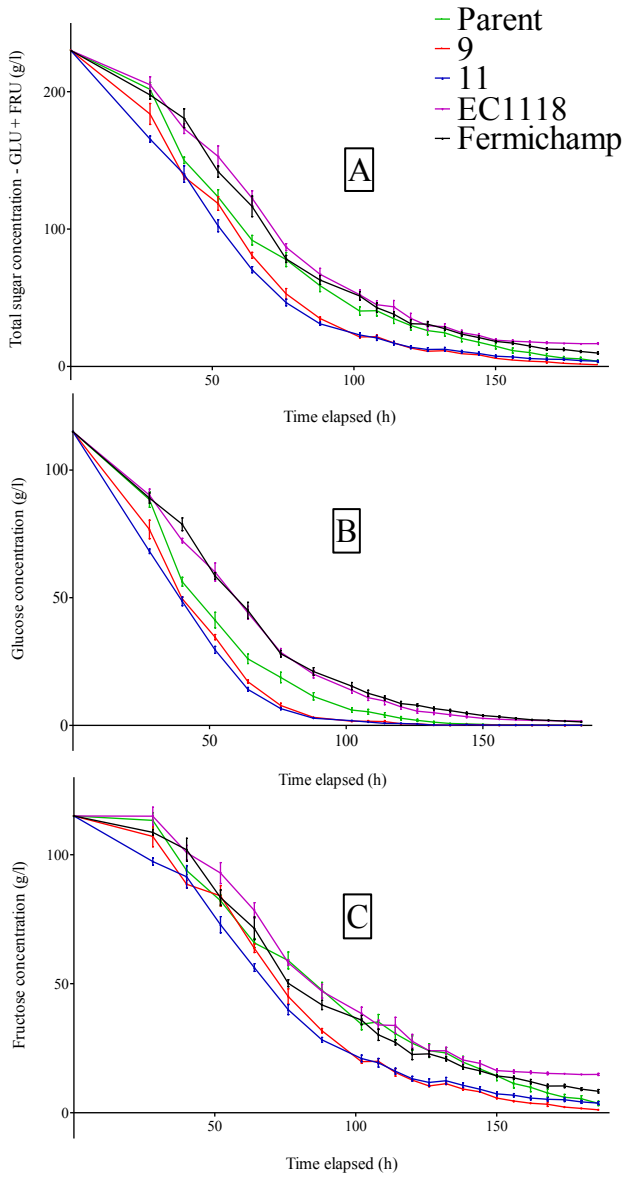


Figure 9 – Curves of depletion of total sugar (A), glucose (B) and fructose (C) are shown for isolates 9 and 11, the parental strain and references EC1118 and Fermichamp. Results are the average of triplicate 250 ml CDGJM fermentations, containing 230 g/l of total sugar (standard deviations are shown as error bars).

Parameter	Strain				
	PAR	9	11	EC1118	Fermichamp
Area TOT %	100	84.84	80.49	112.82	108.50
Area GLU %	100	84.13	78.52	122.77	125.59
Area FRU %	100	85.24	81.58	107.34	99.12
Ratio Areas (GLU/FRU)	0.549	0.541	0.528	0.627	0.695

Table 2 – Percentage of the area under the fermentation curves for isolates 9 and 11 compared to the parental strain, EC1118 and Fermichamp for total sugar or glucose and fructose individually. The fructophilicity of the strains is expressed as a ratio between the areas under the fermentation curves for glucose and fructose.

6.4.3 – Metabolite production during fermentation

The concentration of the organoleptically relevant organic acids (citric, tartaric, malic, succinic, lactic, acetic) and alcohols (ethanol and glycerol) was measured by HPLC (Walker *et al.*, 2003) for isolates 9 and 11 and the parental strain in samples from the fermentations described in Section 6.4.2. Frozen ferment samples were thawed and centrifuged for 5 minutes at 20,800 rcf. Samples were diluted 1:10 and resolved on either a Benson Carbohydrate SS H^+ column (300 mm × 7.8 mm; Alltech, Deerfield, IL, USA) or an Aminex HPX-87H column (300 mm × 7.8 mm; Bio-Rad, Hercules, CA, USA). Elution was performed at 60°C with 2.5 mM H_2SO_4 at a flow rate of 0.5 ml/min. Detection was achieved using an RID-10A refractive index detector (Shimadzu, Kyoto, Japan). Quantification was achieved by comparison with prepared standards in CDGJM using Delta integration software (Deltaware, Charlottetown, Canada – Figure 10).

Isolates 9 and 11 showed no large variation in the amount of metabolites produced during fermentation. The only significant differences observed were the lower concentration of citric acid and the higher production of acetic acid for the parental strain compared to the isolates. The amounts of acetic acid produced were of the order of the legal limit for this acid in commercial wines (Hornsey, 2007) and while somewhat high, were typical for fermentations of CDGJM (e.g. McBryde *et al.*, 2006). Determination of the sensory significance of these differences in acetic acid yield was held over for fermentations of genuine grape juice.

Hydrogen sulfide production by isolates 9 and 11 compared to the parental strain, EC1118 and Fermichamp was determined by a different set of fermentations. H_2S production was quantified using lead acetate detector tubes (*Katagawa* Precision Gas Detector Tubes, model 120SB-Hydrogen Sulphide, range 0.75-300 ppm, Kawasaki-City, Japan) as described elsewhere (Ugliano and Henschke, 2010). Starter cultures were prepared as detailed in Section 6.4.1 and fermentations were conducted in triplicate in 90 ml of CDGJM (with 115 g/l glucose, 115 g/l fructose) in 250 ml conical flasks with shaking (160 rpm). Fermentations were complete (total sugar < 2.5 g/l) after 282 hours at 30°C. The quantity of H_2S produced from each flask (read on the graduated scale of the detector tubes) was converted into micrograms per litre using a previously determined calibration curve (data not shown).

Although some strain differences were evident, the quantities of H_2S produced during these fermentations (Figure 11) were lower than those found in another study and deemed sensorially perceptible. Ugliano and co-workers (2009) reported values between 9 and several hundred micrograms per litre of grape must. The higher values were mainly associated with fermentations deficient in assimilable nitrogen. In the present study the CDGJM was prepared with a high nitrogen content (600 μg/litre as mixed amino acids), thereby likely explaining the limited production of H_2S.

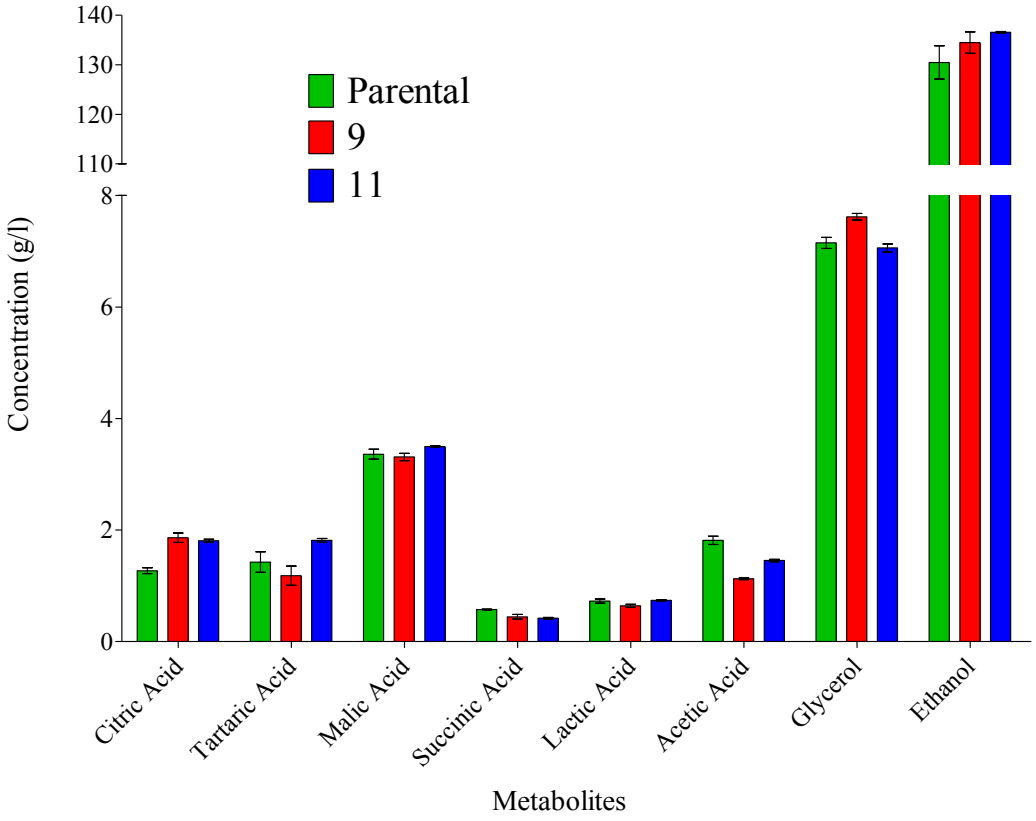


Figure 10 – Metabolite concentration in end of fermentation (< 2.5 g/l sugar) samples for isolates 9 and 11 and the parental strain. The values are the average of single determinations from triplicate 250 ml CDGJM fermentations, containing 230 g/l of total sugar (standard deviations indicated as error bars).

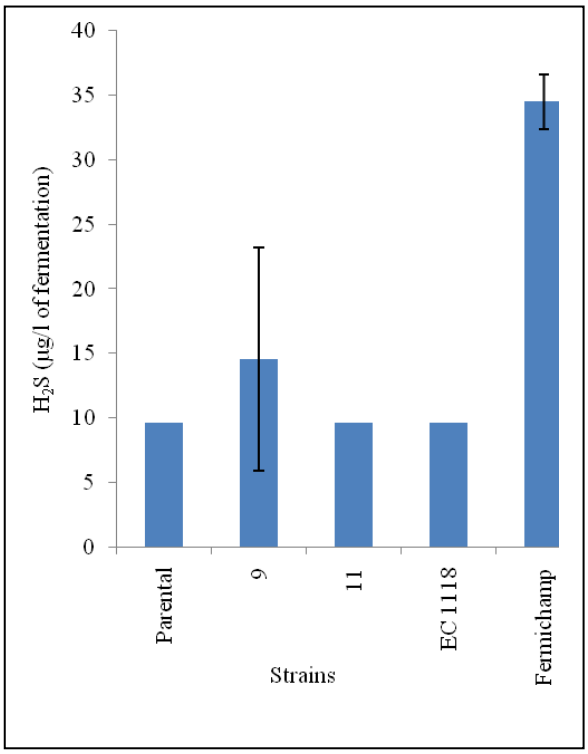


Figure 11 – Quantity of H_2S produced per litre of fermentation by mutants 9 and 11, the parental strain and references EC1118 and Fermichamp. Values are the mean of triplicate 90 ml fermentations in CDGJM with 230 g/l of total sugar (standard deviations are indicated as error bars).

6.4.4 – DNA fingerprinting of evolved strains

Any change in the physiological characteristics of a strain, most likely reflects changes at the DNA level (Giudici *et al.*, 2005). It would ultimately be desirable to determine the genome sequence of the isolates and the parental strain in order to define the precise nature of any mutation(s) in the former. However, before committing to such an undertaking, the use of less detailed genetic comparative techniques was thought appropriate to confirm the close relatedness of isolates and the parental strain and confirm that the isolates were not in fact contaminants. The DNA fingerprinting method of Ness and co-worker (1993) was chosen since this is a commonly used protocol for industrial strains of *S. cerevisiae*. In brief, the method involves amplification of the repeated elements that flank the Ty1 retrotransposon. Genomic DNA was purified from duplicate clonal cell cultures as described in (Adams *et al.*, 1997) and its integrity checked by resolution on a 2% agarose gel in 1 x TAE buffer (Tris-acetate-EDTA) by electrophoresis.

A distinct banding pattern was observed for each strain set (Figure 12). In particular the genotypic profile of the parent strain (lanes 2 and 3) was discernibly different from EC1118 (lanes 8 and 9) and Fermichamp (lanes 10 and 11). Importantly, the genotypic profile of the 2 clonal cultures of isolates 9 (lanes 4 and 5) and 11 (lanes 6 and 7) was identical to the parent, but different from EC1118 and Fermichamp.

These results suggest that it is unlikely that isolates 9 and 11 are contaminants and instead appear to be mutants derived from the parental strain.

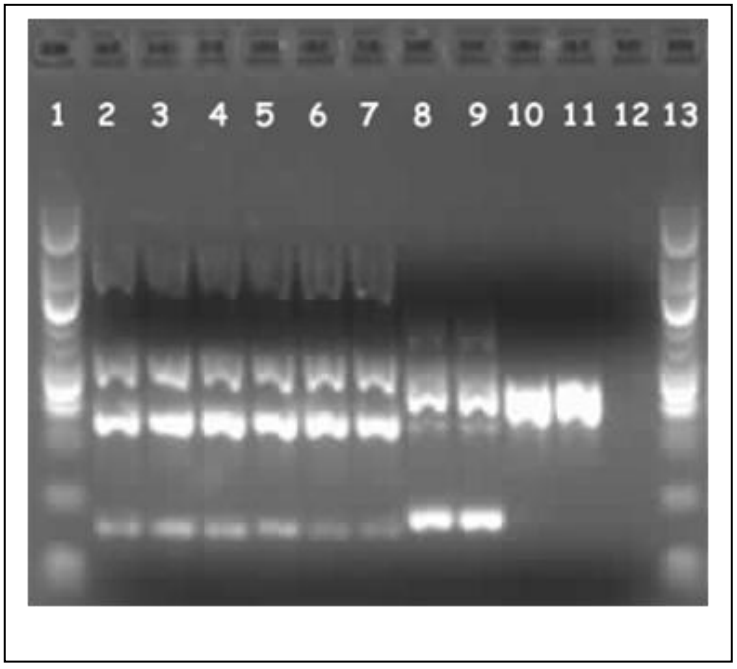


Figure 12 – Genomic fingerprinting obtained with the Polymerase Chain Reaction (PCR) targeting the Ty1 elements in the genome of the parental strain (lanes 2, 3), mutants 9 (lanes 4, 5) and 11 (lanes 6, 7) and references strains EC1118 (lanes 8, 9) and Fermichamp (lanes 10, 11). Lanes 1, 13 include molecular weight markers (100 base pair ladder) and a PCR product without DNA (blank) is shown in lane 12.

6.4.5 – Phenotype stability

In order to observe the long term stability of the phenotype, mutants were propagated under low stress conditions for over 60 generations. This number was considered sufficient considering that 50 generations was the threshold interval at which it was possible to observe occurrence of new mutants (Figures 6 and 7). In this way any mutation that was not fixed in the population would be lost and the efficient fermentation phenotype lost with it. Similarly such a passaging exercise would eliminate the possibility that the observed phenotype was mainly due to adaptation or pre-conditioning (transient expression of e.g. stress response genes).

Mutants (isolates) 9 and 11 were streaked onto YEPD agar from a -80°C glycerol stock and grown overnight at 30°C. A single colony from each plate was transferred into 10 ml of liquid YEPD in a 50 ml Falcon tube and grown overnight at 30°C. Ten µl of each of these cultures were transferred into 10 ml of fresh YEPD and growth repeated. This operation was repeated 5 more times. It was estimated that around 10 generations would elapse for each passage. An aliquot of the resulting populations was streaked onto YEPD plates and grown as before. Similarly fresh YEPD plates of the parental strain, a reference strain (Fermichamp) and mutants 9 and 11 prior to passaging („9 original' and „11 original'), were prepared. Sixteen colonies from each passaged population as well as a colony from the parental strain, the reference strain and mutants 9 and 11 (original populations) were transferred into 1 ml of YEPD and grown overnight at 30°C. From this point the experiment was the same as that described in Section 6.3.1, utilising duplicate sacrificial plates (200 µl per well). See Figure 13 for the plate layout.

There were no differences between fermentation kinetics of the passaged mutants (16 isolates) 9 and 11 compared to the corresponding mixed population and mutants pre-passaging (Figure 14, panels A to F). Moreover all of the mutants showed faster fermentation compared to the parent and Fermichamp. In particular and as has been shown previously for these experimental conditions (Section 6.4.2), the improvements in fermentation kinetics were largely attributable to the improvement in fructose utilization. These data indicate that the changes that have occurred in the mutants and conferred superior fermentation properties are stable to passaging under low stress conditions for at least 60 generations.

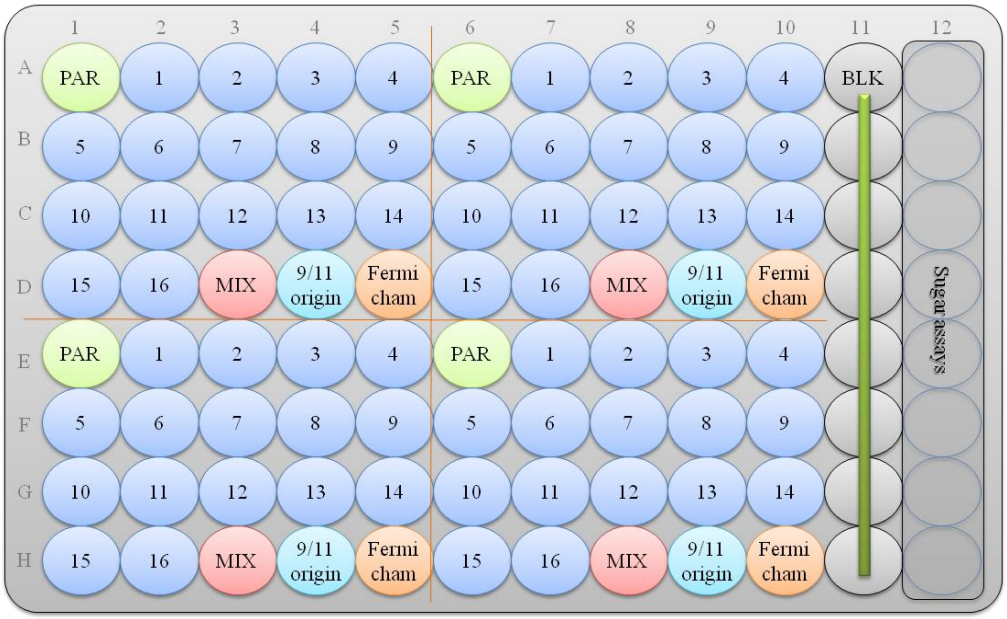


Figure 13 – Layout used for the evaluation of sixteen isolates from the passaging of mutants 9 or 11 as well as the mixed population of the passaged cultures (MIX), the parental strain (PAR), a culture of the appropriate original (pre-passaging) mutants 9 and 11 (9 origin or 11 origin) and a reference strain (Fermichamp). Wells in column 11 of the plate were filled with uninoculated media as a sterility check. The wells in column 12 were used during subsequent quantification of sugar concentration.

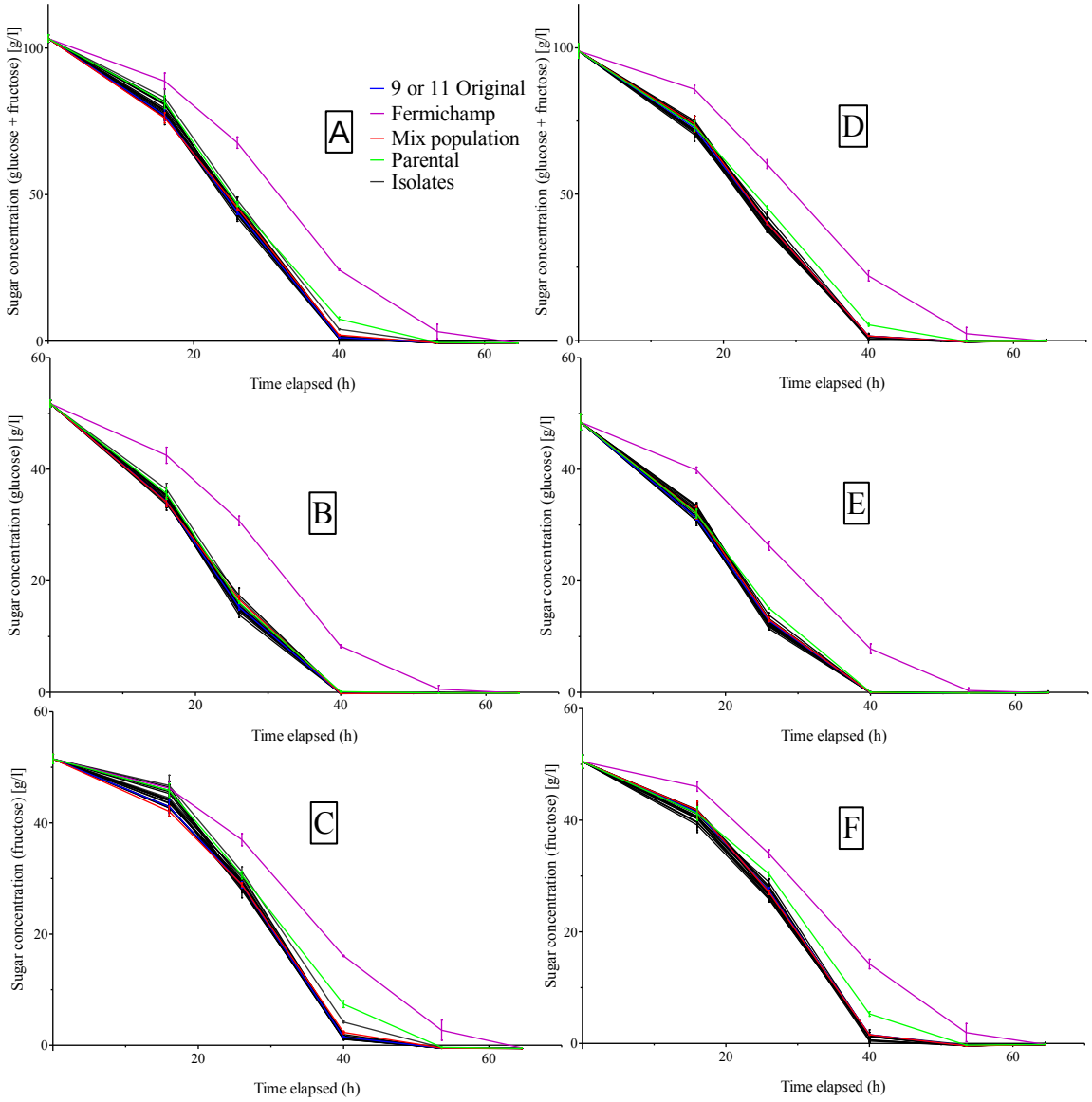


Figure 14 – Curves of depletion of total sugar (A, D), glucose (B, E) and fructose (C, F) are shown for 16 isolates from passaged cultures of mutants 9 (A, B and C) and 11 (D, E and F) along with the corresponding mixed and pre-passaged (original) population of each and the parental strain. Fermichamp was included as reference. Results are the average of four replicates performed in 200 µl in microtiter plates (standard deviations are shown as error bars)**.**

6.4.6 – Grape juice fermentations

The conditions used in this study to isolate improved mutants are ultimately artificial and do not match a grape juice matrix. In addition the continuous fermentation strategy used does not capture the dynamic compositional phases of fermentation. In order to begin to evaluate these strains in more industrially relevant conditions, fermentations were conducted in grape juice and must on a 250 ml and a 20 kg scale, respectively.

Evaluation of fermentation attributes in white grape juice

Fermentations were performed in sterile (0.2 µm) juice obtained from 2007 Chardonnay grapes grown in the Eden Valley (South Australia). Basic compositional features are shown in Table 3. The preparation and execution of the experiment was largely as described in Section 6.4.1. Key differences were the incubation temperature (20°C) and the fact that the starter culture was prepared in white grape juice diluted 1:1 with water. The fermentation performance of mutants 9 and 11 was compared against the parental strain and reference strains EC1118 and Fermichamp. The experiment was repeated with similar results, thus only one set of results is shown.

Fermentations of the five strains fell into three different profiles (Figure 15). Fermichamp required 144 hours to deplete all of the sugar from the juice, EC1118 and mutants 9 and 11 required 235 hours, while the parental strain had not finished fermentation even after 259 hours (9 g/l residual fructose). Rates of glucose consumption were similar across all strains, whereas much of the observed differentiation was attributable to differences in fructose fermentation rates. As a result, the ratio of the area under the glucose and fructose fermentation curves was highest for Fermichamp (0.75) followed by EC1118 (0.63) and mutants 9 and 11 (0.56 and 0.53; Table 4). Once again this experiment demonstrated the extraordinary ability of Fermichamp to consume fructose (Guillaume *et al.*, 2007).

Table 3 – Key compositional features of 2007 Eden Valley Chardonnay juice used to evaluate mutant fermentation performance.

PARAMETER	VALUE	
Glucose + fructose	213.5	g/l
Malic acid	2.92	g/l
Sulphur dioxide (total)	21	mg/l
Sulphur dioxide (free)	<5	mg/l
pH	3.34	
Titratable acid (pH 7.0)	5.4	g/l
Titratable acid (pH 8.2)	5.7	g/l
Yeast assimilable nitrogen	381	mg/l
Ammonia	109	mg/l
Alpha amino nitrogen	291	mg/l

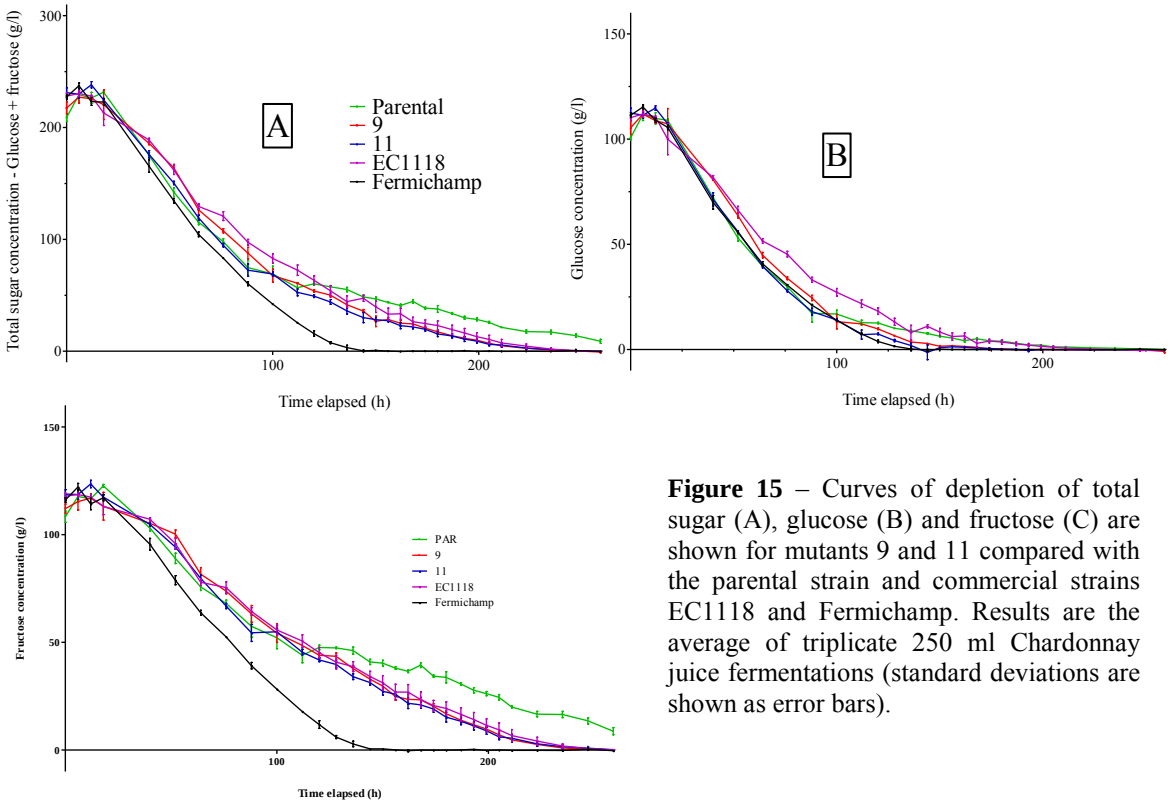


Figure 15 – Curves of depletion of total sugar (A), glucose (B) and fructose (C) are shown for mutants 9 and 11 compared with the parental strain and commercial strains EC1118 and Fermichamp. Results are the average of triplicate 250 ml Chardonnay juice fermentations (standard deviations are shown as error bars).

Parameter **Strain**

	PAR	9	11	EC1118	Fermichamp
Area TOT %	100.00	93.06	89.03	98.81	71.43
Area GLU %	100.00	100.93	93.31	114.91	92.19
Area FRU %	100.00	89.37	87.14	90.90	61.12
Ratio Areas (GLU/FRU)	0.49	0.56	0.53	0.63	0.75

Table 4 – Percentage of the area under the fermentation curves for isolates 9 and 11 compared to the parental strain, EC1118 and Fermichamp for total sugar or glucose and fructose individually. The fructophilicity of the strains is expressed as a ratio between the areas under the fermentation curves for glucose and fructose. Results are derived from triplicate 250 ml Chardonnay juice fermentations.

Evaluation of fermentation attributes in red grape must

As for the previous experiment, the complete set of fermentations was repeated, however, in this case using two different batches of Merlot grapes (Table 5). Given the similarity of the results across the two musts, only data from the Lobethal Merlot fermentations are reported here.

The grapes were handpicked into 20 kg crates and kept at 4°C until crushed. For each strain, three replicate fermentations of 20 kg of grapes were prepared using fruit randomly taken from different crates. Fermentations were conducted in 30 litre plastic containers, provided with a 7 mm hole for release of CO_2. The preparation of the inocula followed the same procedure as described for the Chardonnay fermentations.

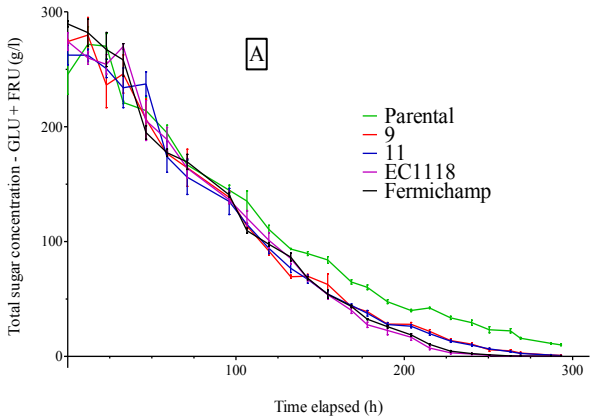
PARAMETER	VALUES Merlot "Waite"	Merlot "Lobethal"
Glucose + fructose (g/l)	250	265
pH	4.02	3.87
Total acidity (g/l)	3.3	5.0
Alpha Amino Nitrogen (mg/l)	96	93
Ammonia (mg/l)	34	22
Yeast Assimilable Nitrogen (mg/l)	124	111
ADDITIONS		
SO_2 (mg/l)	25	25
Tartaric acid (g/l)	2.3	1
Diammonium phosphate (mg/l)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

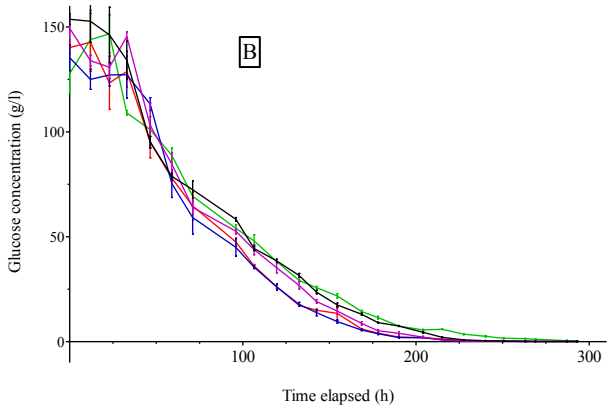
Table 5 – Key compositional features of 2010 Merlot grapes used to evaluate mutant fermentation performance. Grapes were harvested from the Waite Institute experimental vineyards (Adelaide, South Australia) or at Lobethal (South Australia).

In this experiment, due to the presence of solids, the must was not sterilised. Fermentations were conducted at room temperature (~25°C) and plunged and sampled at 12 hours intervals. Samples of 1 ml were stored at -20°C for future enzymatic determination of glucose and fructose concentration (Boehringer-Mannheim, 1989) with final assay volumes adjusted to 100 µl for analysis in 96 well microtiter plates. Other parameters measured at the end of the fermentations were relevant organic acids (malic, succinic, lactic, acetic) and alcohols (ethanol and glycerol; HPLC method adopted is described in Section 6.4.3). Colour measurement (CIELab) of cell free supernatants (20,800 rcf, 1 min) was determined at the end of fermentation. Calculation of CIELab was obtained measuring the visible transmission spectrum from 380 nm to 780 nm in 5 nm increments (75 µL in a 96 well microtiter plate, µQuant Microplate Spectrophotometer, Bio-Tek Instruments). The spectral data was then converted to L^* (lightness, 0 – black to 100 – white), a^* (-265, green to 265, magenta) and b^* (-265, blue to 265, yellow) using the conversion equations and tables developed by the Commission Internationale d'Eclairage. Results are expressed using a D65 illuminant at 10° standard observer. Hue (H, 0° - 360°) and chroma (C, 0 - 100) values were calculated using the following equations:

$$C = (a^{*2} + b^{*2})^{0.5} \qquad H = arctan\left(\frac{b^*}{a^*}\right) \times \frac{360°}{2 \times 3.142}$$

As for the Chardonnay juice, fermentations in red must allowed the five strains to be allocated according to three profiles (Figure 16). EC1118 and Fermichamp depleted the sugars in 240 hours, mutants 9 and 11 required 287 hours, while the parent had not finished fermentation even after 293 hours (~10 g/l residual fructose). Fructose fermentation rate mainly contributed to differences observed in the overall fermentation performance. However, in this case, the ratio of the area under the glucose and fructose fermentation curves did not changed for mutants 9 and 11 compared to the parental strain (~0.65), while EC1118 and Fermichamp showed higher values (0.77 and 0.84, respectively; Table 6). Once again, these reference strains showed higher fructophilicity.





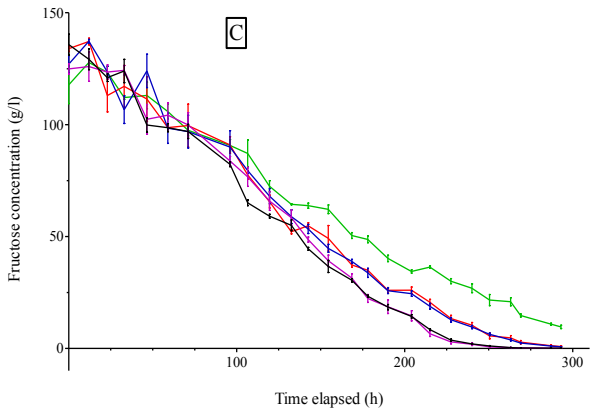


Figure 16 – Curves of depletion of total sugar (A), glucose (B) and fructose (C) are shown for mutants 9 and 11 compared with the parental strain and commercial strains EC1118 and Fermichamp. Results are the average of triplicate 20 kg Merlot grape fermentations (standard deviations are shown as error bars).

Parameter	Strain				
	PAR	9	11	EC 1118	FERM
Area TOT %	100.00	88.01	87.38	86.93	87.93
Area GLU %	100.00	88.17	86.27	95.59	101.50
Area FRU %	100.00	87.90	88.11	81.26	79.06
Ratio Areas (GLU/FRU)	0.65	0.66	0.64	0.77	0.84

Table 6 – Percentage of the area under the fermentation curves for isolates 9 and 11 compared to the parental strain, EC1118 and Fermichamp for total sugar or glucose and fructose individually. The fructophilicity of the strains is expressed as a ratio between the areas under the fermentation curves for glucose and fructose. Results are derived from triplicate 20 kg Merlot grape fermentations.

From the analysis of the principal metabolites (Figure 17), Fermichamp produced a larger amount of succinic acid (~5 g/l *vs* ~3 g/l). Mutants 9 and 11 and Fermichamp showed a greater concentration of glycerol (~13 g/l) than EC1118 and the parental strain (~11 g/l).

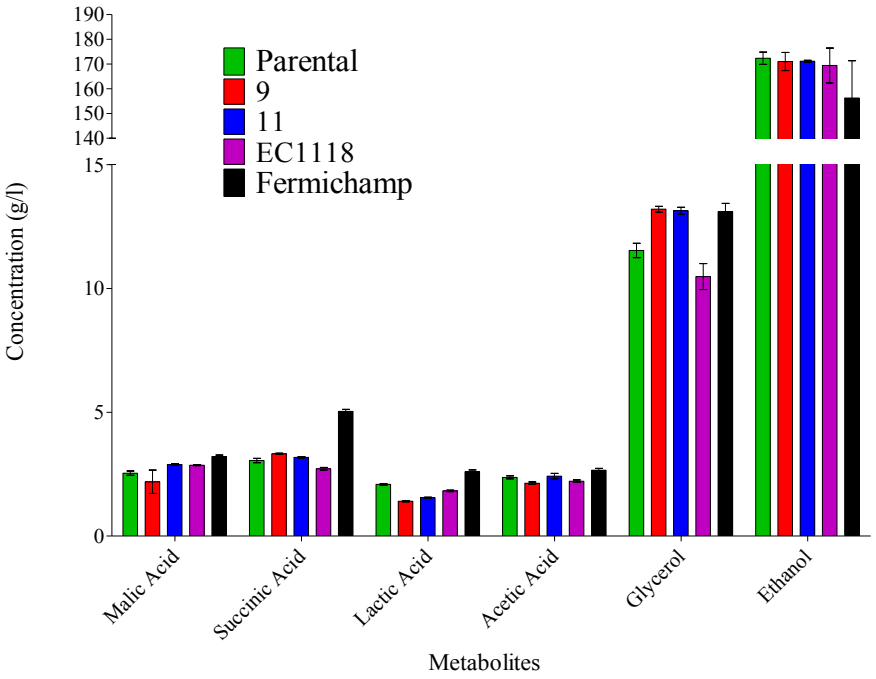


Figure 17 – Metabolite concentration in end of fermentation (< 2.5 g/l sugar) samples for isolates 9 and 11, the parental strain and references EC1118 and Fermichamp. The values are the average of single determinations from triplicate 20 kg Merlot grape fermentations (standard deviations indicated as error bars).

When expressing red wine colour as a CIELab measurement, differences of 3.0 or more of the calculated *DeltaE* ($\Delta E = (L_1 - L_2)^2 + (a_1 - a_2)^2 + (b_1 - b_2)^2$), are considered to exceed the threshold for detectability by the human eye (Martinez *et al.*, 2001). The values of ΔE calculated in this experiment, were generally higher than 3.0 CIELab units (Figure 18) suggesting that the differences produced by strains in colour would be detectable. Unfortunately no sensory panel was available to confirm this.

In conclusion, these final experiments confirmed the improved fitness of two mutants isolated using an adaptive evolution strategy. Thus mutants 9 and 11 showed a faster rate of utilization of both glucose and fructose compared to the parental strain (AWRI 796). This was observed in CDGJM (250 ml, 230 g/l of sugars), Chardonnay juice (250 ml, 213 g/l of sugar) and Merlot must (20 kg, 265 g/l of sugar). In most cases fructose was utilised at a faster rate than the parental strain, thereby confirming the effectiveness of the targeted adaptive evolution strategy adopted in this study.

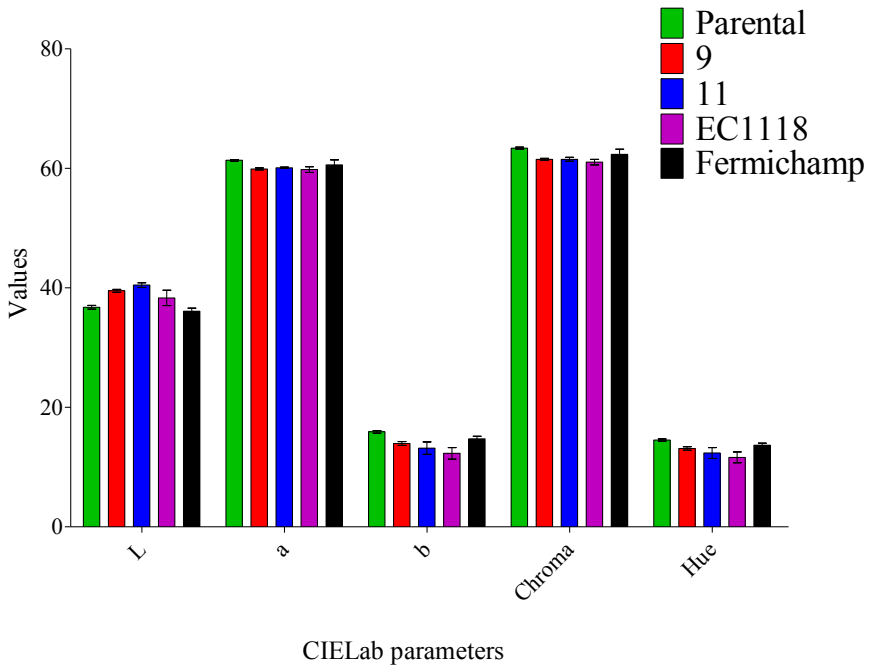


Figure 18 – CIELab parameters measured at end of fermentation (< 2.5 g/l sugar) samples for isolates 9 and 11, the parental strain and references EC1118 and Fermichamp. The values are the average of single determinations from triplicate 20 kg Merlot grape fermentations (standard deviations indicated as error bars).

7. CONCLUSION

This project aimed to generate wine yeast strains with improved fructose utilization, and the strategy employed was adaptive evolution. The objectives of the study were:

1. To test adaptive evolution as a strategy to generate improved phenotypes in wine yeast for the wine industry;
2. Specifically to improve the fructophilicity of a commercially available wine yeast strain with the aim of increasing fermentation reliability or rate and/or decreasing residual fructose in fermentations.

Both of these objectives were achieved. Fructophilic variants of AWRI 796 were generated using adaptive evolution. Fermentation rates of fructose were greater in the mutants compared to the parental strain and the fructophilic index[*] for the mutants also increased when they were grown in CDGJM. Interestingly, however, whilst overall fermentation rate was still higher for the mutants than the parent in grape juice, the fructophilic index was unchanged, suggesting the increased fructophilicity, specifically, was not the basis improved fermentation under these conditions. Further characterisation of these mutants is warranted.

[*] Defined in this study as the ratio between areas under the fermentation curves of glucose and fructose

8. RECOMMENDATIONS

Experimental adaptive evolution has been used several times in the past to generate yeast with improved phenotypes (e.g. Brown and Oliver, 1982; Hall, 1992; McBryde *et al.*, 2006). The work described in this project built on this past work and successfully generated a novel yeast strain with improved fructose utilization. However the results highlighted an important limitation of adaptive evolution strategies: evolution is not driven solely by the selective pressure, but may also be shaped by the matrix of the medium. This may be the reason why fructophilic mutants showed clear increases in fructophilic index for the medium used in the adaptive evolution experiment (CDGJM), but for grape juice the index was unchanged from the parent. Clearly, from a wine industry perspective, it will be important in future studies to use a medium that reflects as closely as possible the matrix of the grape juice where the mutants will be used in industry.

There are numerous ways in which this project could be developed to improve on the adaptive evolution strategy that was used. For example:

- EMS mutagenesis increases genetic variation, however, the probability of generating specific advantageous mutations is limited when performed on a single strain. Other techniques for generating genetic variation that could be used include mass mating (or genome shuffling) of many wine yeast. Where genetic material is exchanged by these approaches, the result may be the generation of unique gene combinations in a range of wine-relevant genetic backgrounds, thereby increasing the probability of generating highly novel genotypes and phenotypes for adaptive evolution to work on.
- The use of other selective pressures to select for fructophilic mutants should be trialled. For example, 2-deoxy-D-glucose, a glucose homologue, is toxic to *S. cerevisiae* and has been used in studies of hexose uptake (Novak *et al.*, 1990). If used in a medium with fructose as the principal source of sugar it should select for mutants with superior fructose utilization at the expense of glucose uptake.
- Other approaches to adaptive evolution have been described and used in the past. BOICS (Brown and Oliver Interactive Continuous Selection) is a strategy consisting of an interactive chemostat selection, the intensity of the selective pressure being automatically adjusted via a feedback control circuit (Brown and Oliver, 1982). Thus, by monitoring parameters such as physiological changes in the bioreactor (e.g. variation in cell density) or fermentation products (e.g. CO_2 release), it is possible to increase the selective pressure without the risk of washing out the population. The originators state, *"the technique of continuous selection with feedback should be generally applicable to the isolation of mutants of any microorganism to improved tolerance to any inhibitory condition of either its physical or chemical environment"* (Brown and Oliver, 1982). Thus an inhibitor such as 2-deoxy-D-glucose could be introduced in this type of approach as well. Moreover, the BOICS strategy could also be used in a situation where limited fructose concentration determines the selective pressure. Fructose could be added to the bioreactor, in ever decreasing concentrations, in the absence of other carbon sources. When the cell density drops below a given threshold (before wash out), the fructose level would be increased to rescue survivors, before being decreased again to reintroduce the selective pressure. This could be performed over many cycles to select for improved mutants.

Beyond isolating further evolved strains, those successfully generated through this study, should characterised further. It has already been shown that they have a faster fermentation rate compared to the parent, and, from preliminary experiments (metabolite concentration using HCPL, CIELab colour measurements), there were no large differences variation the final composition of the wine. However, more detailed investigations including larger scale winery fermentations and evaluation of the resulting wine through a sensory panel, are required to confirm the ability of adaptive evolution to generate specifically improved strains which maintain key oenological traits of the parent.

A final area of great interest is the determination of the nature of the mutation(s) that confer increased fructophilicity in the mutants generated in this study. The absence of definitive clues as to the nature of these mutations combined with the ever decreasing cost of sequencing, mean that determination of the whole genome sequence may be the simplest means of pursuing this area. Once identified any genes of gene modifications contributing to enhanced fructophilicity, could be exploited in a number of ways. Thus they could be utilised in the generation of recombinant strains, they could be introduced into other strains through classical genetics, or they could provide novel insights into the basis for inefficient fructose utilisation by wine yeast during fermentation. Genetic manipulations represent an important modern approach in the generation of optimised strains for the wine and food industry. Despite some debates around the use of genetically modified organisms, given their marked benefits, it is likely that they will be accepted in the near future for the production of food and beverages.

9. APPENDIX 1: Communication

Other outcomes derived from the project include:

1. Presentation of Posters:
   a. School of Agriculture, Food and Wine Research Day 2007
   b. School of Agriculture, Food and Wine Research Day 2008
   c. School of Agriculture, Food and Wine Research Day 2009
   d. Australian Wine Industry Technical Conference (AWITC) 2010

2. Talks:
   a. School of Agriculture, Food and Wine Research Day 2009
   b. School of Agriculture, Food and Wine Postgraduate symposium 2009
   c. 3 minutes thesis competition 2010
   d. Student forum "In the wine light", AWITC 2010
   e. AWITC 2010

3. Publications
   a. Liccioli, T.; Chambers, P.J.; Jiranek, V. (2010). A novel methodology independent of fermentation rate for assessment of the fructophilic character of wine yeast strains. Journal of Industrial Microbiology and Biotechnology (in press, manuscript attached).
   b. Liccioli, T.; Tran, T.M.T.; Cozzolino, D.; Jiranek, V.; Chambers; P.J., Schmidt, S.A. (2010). Microvinification – How small can we go? Journal of Applied Microbiology and Biotechnology (submitted).
   c. Liccioli, T. Schmidt, S.A.: Schmid, F.; Chambers, P.J.; Jiranek, V. (2010c). Improving fructose utilization in wine yeast. Proceedings of the 14th AWITC conference (in press).
   d. Liccioli, T.; Chambers, P.J.; Jiranek V. (2010). Improving fructose utilization in wine yeast using adaptive evolution. Adelaide, SA: The University of Adelaide. 189 p. PhD thesis (under examination).

A novel methodology independent of fermentation rate for assessment of the fructophilic character of wine yeast strains

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Abstract

The yeast *Saccharomyces cerevisiae* has a fundamental role in fermenting grape juice to wine. During alcoholic fermentation its catabolic activity converts sugars (which in grape juice are a near equal ratio of glucose and fructose) and other grape compounds into ethanol, carbon dioxide and sensorily important metabolites. However, *S. cerevisiae* typically utilises glucose and fructose with different efficiency: glucose is preferred and is consumed at a higher rate than fructose. This results in an increasing difference between the concentrations of glucose and fructose during fermentation. In this study 20 commercially available strains were investigated to determine their relative abilities to utilise glucose and fructose. Parameters measured included fermentation duration and the kinetics of utilisation of fructose when supplied as sole carbon source or in an equimolar mix with glucose. The data were then analysed using mathematical calculations in an effort to identify fermentation attributes which were indicative of overall fructose utilisation and fermentation performance. Fermentation durations ranged from 74.6 to over 150 hours with clear differences in the degree to which glucose utilisation was preferential. Given this variability we sought to gain a more holistic indication of strain performance that was independent of fermentation rate and therefore utilize the Area Under the Curve (AUC) of fermentation of individual or combined sugars. In this way it was possible to rank the 20 strains for their ability to consume fructose relatively to glucose. Moreover, it was shown that fermentations performed in media containing fructose as sole carbon source did not predict the fructophilicity of strains in wine like conditions (equimolar mixture of glucose and fructose). This work provides important information for programs which seek to generate strains that are faster or more reliable fermenters.

Keywords: glucose, fructose, fermentation progress, strain comparison, composite trapezoid rule.

Abbreviation: Area Under the Curve, AUC.

Introduction

When exposed to mixtures of glucose and fructose, as occurs during the fermentation of grape juice into wine, *Saccharomyces cerevisiae* utilises these sugars at different rates [2,18]. As a result the ratio between the concentration of glucose and fructose changes, so that late in fermentation fructose becomes the predominant sugar. This is reported to be one of the causes of arrested or so-called stuck fermentation [13]. Attempts to restart the fermentation through re-inoculation with fresh but nonetheless glucophilic cultures are challenging [7]. Regardless of the cause of stuck fermentation, excess residual sugar is undesirable as it puts the palate of the wine out of balance. Furthermore, the fact that fructose predominates compounds the problem since fructose is sweeter than glucose [20].

In seeking the precise basis for differences in the rate of glucose *vs* fructose utilisation, steps in the metabolism of hexoses prior to the formation of fructose 1,6 bisphosphate are implicated. In particular this includes the systems for sensing of extracellular sugars, their transport across the plasma membrane and phosphorylation as part of the first steps of glycolysis. Plasma membrane sensor-proteins have been identified which show affinity for glucose, with at least one exhibiting a different affinity for glucose and fructose [30,31]. The presence of sensors specific for fructose has not been reported [2]. Phosphorylation of internalised glucose and fructose occurs via the hexokinases Hxk1 and Hxk2, albeit at different efficiencies, with glucose additionally being acted upon by glucokinase Glk1 [2,15,34]. Since the phosphorylation capacity of these enzymes exceeds the amount of sugar transported into the cell, it would appear that this enzymatic process is not the basis for differences in the utilisation of glucose compared to fructose. However, a recent study [3] demonstrate that over-expression of hexokinases could in fact alter the rate of fermentation. On the other hand, the hexose transport system has been clearly shown by many authors to be a critical point in determining fermentation rate.

In *S. cerevisiae*, hexose uptake is largely mediated by facilitated diffusion [5], where 20 genes (*HXT1* to *HXT17*, *GAL2*, *SFN3* and *RGT2*) encode the related proteins. Despite such plurality, only those transporters encoded by *HXT1* to *HXT7* appear to be of importance for glucose and fructose utilisation under fermentation conditions [9,10,12,25,26,29]. Some of these latter HXT transporters have relatively low-affinity (high K_m) for hexose, whilst others are considered high-affinity (low K_m), and all have the ability to transport both glucose and fructose. Importantly, whether they are high- or low-affinity systems, all have a greater affinity for glucose than for fructose. The expression of each HXT gene is regulated by environmental factors, especially the extracellular hexose concentration [25]. The high-affinity transporters are induced when the glucose concentration is low (~1-4 mM or 0.18-0.72 g/l) and are repressed when the concentration of glucose is higher [8]. Conversely low-affinity carriers are induced by high glucose concentrations (~50-100 mM or 9-18 g/l) if not constitutively expressed. HXT3, a low-affinity transporter, is considered key to determining glucose transport and, thereby, could play a fundamental role in the different rates of glucose and fructose utilization [15]. Further work is required to define this role.

Beyond affinity differences, other factors such as nitrogen availability and response to ethanol may be of importance. Ethanol is known to have protein denaturing properties and disrupts plasma membrane components [4,35]. Due to increases in membrane permeability and passive proton flux upon ethanol exposure, damage to intracellular enzymes and structures might also occur. Berthels and co-authors [2] observed that a high ethanol concentration inhibited sugar utilization, but to different extents for glucose and fructose. This led them to hypothesise that the glucose utilization capability was more robust than fructose utilization. Moreover ethanol is able to shift the tautomeric equilibrium of fructose from the readily transported pyranose form to the furanose form. Thus unlike glucose which is entirely in the transportable pyranose form, typically only 70% of fructose takes the

pyranose form, and less in the presence of ethanol. Thus not only is there a discrepancy between glucose and fructose transport, it also changes during the progress of fermentation [2] and may be part cause and part effect of different ethanol tolerance of various strains.

A further possibility is linked with nitrogen utilisation and availability. Sugar transporters have been shown to be turned over quickly ($t_{½}$ ~5 hours) compared to other proteins [33] thereby creating a demand for active protein synthesis and assimilable nitrogen. In every case of nitrogen supplementation to nitrogen starved cultures, fructose consumption was enhanced to a greater extent than glucose [2]. Different strains have been shown to utilise nitrogen to different extents [17]. Perhaps therefore as fermentation progresses strains with higher nitrogen demands experience greater or earlier restrictions on assimilable nitrogen availability and thus their ability to maintain fructose transport, thereby resulting in a higher rate of glucose transport.

Available evidence affirms that hexose utilisation depends on numerous environmental and biological parameters. Thus determining the precise basis for the glucose/fructose discrepancy in order to target efforts to reduce such differences and presumably improve strain performance and wine composition are difficult. In this study 20 commercially available wine strains were chosen for characterization of their fructose utilisation proprieties. Such data was sought so as to begin to determine the relationship between abilities around utilisation of individual sugars and relative fermentative performance. Fermentations were conducted in a chemically defined grape juice medium (CDGJM) [16] with two different sugar compositions. Either an equimolar mix of glucose and fructose or else a medium containing fructose as the only sugar were used. Various parameters related to total fermentation duration and kinetics of fructose and/or glucose utilisation were measured and the resulting values compared though mathematical calculations to identify relationships.

Materials and methods

Strains and maintenance

A total of 20 commercial strains of *S. cerevisiae* were selected as representative of commonly used wine yeast and, where possible, chosen with consideration of published information about their ability to consume fructose compared to glucose. [2,15]. The strains used where: B, UCD522, CRU-BLANC, PRIMEUR, AWRI 350, AWRI 796, AWRI 1503, ELEGANCE (AB Mauri, Sydney, Australia); EC1118, V1116, D254, W27, BM45, SYRAH, BORDEAUX RED, S6U, UVAFERM 43 (Lallemand, Montreal, Canada); VIN13, NT202 (Anchor Yeast, Cape Town, South Africa), FERMICHAMP (DSM, Netherland). Strains were collected aseptically from active dried commercial preparations, re-hydrated in sterile water (20 min) and inoculated into YEPD medium (20 g/l D-glucose, 10 g/l yeast extract, 20 g/l Bacto peptone) in a flask (liquid/air ratio > 66%) before overnight incubation at 28°C with shaking at 180 rpm. Cultures were then streaked onto YEPD agar plates and grown overnight at 28°C to check for purity. Multiple representative colonies were inoculated into 25 ml of YEPD broth and grown as above. These served as starter cultures for the fermentation experiments detailed below or, with the combination of 1 ml of culture with 0.5 ml of sterile 80% (v/v) glycerol, enabled long term storage at -80°C.

Fermentation experiments

Starter cultures were used to generate pre-cultures, which in turn were used to inoculate fermentation experiments as detailed elsewhere [22]. For each of the 20 strains, fermentations were performed in order to define their sugar utilisation and fermentation kinetics. To do this,

two different formulations of a CDGJM were used. The first was representative of a typical grape juice in that glucose and fructose were supplied in equimolar amounts to a combined total of 230 g/L. For the second condition an equivalent amount of sugar was supplied but as fructose only. In each case 600 mg N/l (as amino acids; [15]) were used and the triplicate fermentations were incubated at 28°C with shaking at 160 rpm.

Fermentation progress was estimated from the Brix value of clarified (14,000 rpm, 2 min) culture samples and fermentation completion (< 2.5 g/l) determined using Clinitest® tablets (Bayer). Supernatants were stored at -20°C for subsequent determination of residual sugar content by an enzymatic method [6] adapted for 48 well plates.

Analyses

For every strain, the values obtained from the determination of individual sugars in each medium where considered separately. Four datasets with regard to sugar concentration during the progress of fermentation were obtained, i.e. for glucose, fructose and glucose + fructose in the mixed sugar condition as well as fructose from the fructose-only media. These datasets were used to plot the respective sugar utilization curves for each strain. In this way, 20 charts were obtained each containing four areas delimited by the fermentation curves for the four sugars or combinations (Fig. 1). The value of these areas was calculated using the composite trapezoid rule (GraphPad Prism 5 - GraphPad Software Inc., La Jolla, C.A., U.S.A.), a numerical integration method for approximating an integral or AUC where the function of the curve is unknown. The value of the AUC is returned in units of the X axis times the units of the Y axis. Statistical analysis of the data (one-way ANOVA with Dunnet and Tukey's multiple comparison post-test analysis) was performed with the same software.

Results

Fermentation duration

The times required for complete utilisation of glucose and/or fructose were defined for 20 commercial strains of yeast and are summarised in Table 1. For most strains, fermentation of each medium was completed (i.e. < 2.5 g/l residual sugar) in less than 150 h. The only exception was Maurivin strain B, for which the mixed sugar fermentation required 151 hrs and the fructose-only condition failed to complete within this preset maximum duration. In every case the utilisation of fructose lagged behind that of glucose. Similarly fermentation of fructose took longer than the fermentation of an equivalent amount of mixed sugars. Glucose depletion from the mixed sugar condition occurred in between 60 h (UCD 522) and 115 h (B) whereas fructose depletion occurred within 75 h (UCD 522) and 151 h (B). Since fructose depletion always took longer than that of glucose, the time for the former to occur also equated to the total duration of the mixed sugar fermentations. By comparison, where complete utilisation of 230 g/l of fructose was seen, a total of between 94 h (UCD 522) and 134 h (BM 45) was required for this eventuality. Thus the complete fermentation of the fructose-only medium took longer than the equivalent mixed sugar fermentation. These observations confirm the apparent glucophilicity of *Saccharomyces* yeast, and suggest that the utilisation of fructose is the rate-limiting step and that the time taken for this defines the total duration of fermentation.

The inclusion of a medium containing fructose as the only sugar provided an opportunity to determine whether this condition was predictive of fructose utilisation capabilities in the mixed sugar medium. The duration of a fructose-only fermentation was not strongly correlated ($R^2 = 0.524$) with the time required for fructose depletion from the mixed sugar

189 medium. This indicates that the duration of utilisation of fructose as sole carbon source is not
190 necessarily a good predictor of the duration of fructose utilisation in the presence of glucose.

191 Since fermentation duration alone did not fully describe the performance of individual
192 strains, other features of the pattern of sugar utilisation were sought by plotting the utilisation
193 data (Fig. 1). Several points are evident when the data were presented graphically. Firstly, all
194 fermentations showed some degree of an initial lag, followed by an extended period of rapid
195 fermentation before a progressive slowing toward the complete catabolism of sugars. Thus
196 these periods would collectively and to different degrees contribute to the overall times
197 required for catabolism of individual sugars. Secondly, differences existed between strains in
198 terms of the extent to which the pattern of utilisation of a given sugar(s) reflected that of
199 another sugar(s). Such differences are highlighted by shading of the area below each curve
200 which distinguishes it from the adjacent curves (Fig. 1). Using this approach and ignoring
201 total fermentation duration, it is evident that strains differ in the extent to which they
202 preferentially utilised glucose compared to fructose in the mixed sugar condition.
203 Consequently, FERMICHAMP has the smallest discrepancy between the two curves (Fig. 1,
204 light gray area), whereas strains such as PRIMEUR, AWRI 796 and BORDEAUX RED
205 appear to have the largest discrepancy. The other key observation is that the difference
206 between the profile for the combined catabolism of glucose and fructose compared with the
207 pattern of fructose utilisation from the single sugar medium, also highlights differences
208 between strains (Fig. 1, hatched area). Accordingly, strains such as AWRI 796, SYRAH and
209 BM45 have a large discrepancy, while FERMICHAMP, CRU-BLANC and UVAFERM 43
210 do not (Fig. 1).
211
212 *Application of the composite trapezoid rule*

213 Casual observation suggests that there are no examples of particularly anomalous behaviour
214 amongst the strains. That is, there are no instances where a given fermentation duration is in
215 fact not the result of a relatively consistent progression of fermentation, but instead the result
216 of a protracted commencement followed by a very rapid completion. However, the validity of
217 this notion was best assessed through a closer, more quantitative, examination of the data. For
218 this reason much of the subsequent analysis and strain comparison considers the AUC of
219 utilisation of each sugar as determined using the composite trapezoid rule (Table 2).

220 If, in fact, the rate of fermentation was consistent, then there should be a relationship between
221 the AUC of utilisation for that particular sugar and the time taken for the sugar to be depleted.
222 This was not always the case (N.B. Maurivin strain B was excluded from this comparison as
223 it was a clear outlier). Thus a comparison of the AUC of glucose (Table 2) was somewhat
224 correlated to the time to glucose depletion (R^2 = 0.715). However, the correlation for the
225 analogous values for fructose in the mixed sugar condition yielded an R^2 value of 0.645.
226 These findings are explained by the fact that in the case of the latter, there are examples of
227 strains (e.g. BORDEAUX RED and NT202) with similar AUC (i.e. 5833 and 5918) but
228 markedly different times for fructose catabolism (i.e. 115 and 94 h). The reverse was also
229 evident, that is similar durations of fructose utilisation (i.e. 101 and 99 h) but different areas
230 under the corresponding fructose utilisation curve (6016 and 5404) for AWRI 350 and MB45,
231 respectively. These examples highlight the complexity of fermentation phenotypes and the
232 difficulty of comparing strains. When considering the combined catabolism of glucose and
233 fructose from the mixed sugar fermentation, a poor correlation (R^2 = 0.461) between the
234 value for the AUC and the total duration of catabolism was again observed, as was the case
235 for the fructose only condition (R^2 = 0.482). Given these obvious complexities, we therefore
236 suggest that compared to duration alone, the area under the sugar utilisation curve provides a

more complete account of sugar utilisation, as it incorporates trends occurring during fermentation.

Utilisation of fructose compared with glucose

Of key interest in this study was the manner in which the kinetics of fructose utilisation influenced the overall fermentation performance of individual strains. For this reason we sought to identify a basis for comparison of strains which was independent of overall fermentation duration. As such we compared each strain in terms of the ratio between the areas under each of the glucose and fructose utilisation curves (Table 2). All strains exhibited a glucose:fructose ratio which was markedly less than 1, clearly demonstrating the ability of these strains to preferentially utilise glucose over fructose. The strains ranged in terms of this ratio, such that BORDEAUX RED exhibited a value of 0.57 compared to 0.76 for FERMICHAMP.

The ratio of AUC of the utilisation of glucose compared with fructose in the mixed sugar condition, is proposed to give an indication of the relative glucophilicity (increased fructose or poor glucose consumption) of individual strains. Based on this concept, we averaged the values of the ratio between glucose and fructose areas for the 19 strains (B excluded) and used the resulting average as the reference ratio (0.66). Thus, it was possible to rank all the strains based on their individual glucose:fructose ratio deviation from the mean of all ratios (Figure 2). Two groups of strains can be identified, the first represents the more fructose efficient strains (negative values), and the second represents the less fructose efficient strains (positive values). In this way FERMICHAMP appears to be either more fructophilic or less efficient at utilising glucose. Additionally, all strains were compared against each other to

260 determine any significant differences (Table 3). Most strikingly FERMICHAMP and
261 BORDEAUX RED were the two strains with most significant differences to the other strains.

262 As was shown for the duration of fermentation, further evidence for the poor relationship
263 between performances in one condition (medium) compared to another is provided by
264 comparison of the ratio of the AUC of utilisation of both glucose and fructose compared to
265 fructose alone (Table 2). Such a comparison yielded a correlation of $R^2 = 0.449$. Further
266 consideration of strain performance was therefore limited to a comparison of the relative
267 utilisation of glucose and fructose in the mixed sugar condition.
268

269 ## Discussion

270 Developing a valid method to assess glucose and fructose utilization during alcoholic
271 fermentation is not a straightforward matter. Several authors have proposed criteria for this,
272 but what is clear, and supported by our data, is that the method must be independent of the
273 overall rate or duration of the fermentation. Also, to simplify its application, especially for
274 comparison of many strains, the approach must minimise the need for real-time analysis and,
275 given the dynamic nature of fermentation by different strains, must incorporate trends from as
276 many stages of the fermentation as possible. Thus, even if the second half of the fermentation
277 is the most critical, it is not appropriate to ignore overall fermentation performance up to this
278 point. The method suggested from findings of the present study takes into account all these
279 considerations.

280 The analysis of the fermentation performance of the 20 strains revealed differences between
281 some strains to be as much as 2-fold, which equates to about 3 days under our conditions.
282 Such a difference is likely to have dramatic impacts on winery throughput and juice
283 processing, particularly at the height of vintage, and no doubt forms an important criterion in

284 the selection of strains by winemakers. In terms of the profiles of sugar utilisation, some
285 common trends were seen. Thus, after an initial lag, sugar utilisation increased markedly
286 before this phase was followed by one of a slowing of utilisation. In addition an ordered
287 utilisation was evident such that glucose was removed more rapidly than fructose. Similar
288 reports of glucophilicity in industrial strains have been made by several authors
289 [18,23,24,36,39]. Whilst the fermentation of a fructose medium was slower compared to an
290 equivalent amount of mixed sugars, as reported recently [18], it is interesting that the relative
291 strain performance in the former was not an effective predictor of performance in the mixed
292 sugar condition.

293 A recent study of the ability of yeast to grow in a fructose-only medium [1], compared the
294 area under growth curves to estimate preference or tolerance of different *Saccharomyces*
295 yeast for fructose. This method was suggested as a possible tool for initial screening of yeast.
296 However, with the caveat that our study examined sugar utilisation rather than growth, the
297 mismatch we observed between a glucose and fructose mixture and that of fructose alone (R^2
298 = 0.083) suggests it unlikely that a fructose-only medium will be useful to screen for
299 performance in an extended mixed sugar fermentation. The presence of glucose is highly
300 influential on fructose metabolism [14,18,19,21,27,28,32,37,38]. At this point it is not
301 possible to state how glucose influenced fructose consumption. The effect may be elicited at
302 the level of the membrane (transport and sensing) or phosphorylation during the first steps of
303 glycolysis, through a higher K_m for fructose compared to glucose. Thus, further work is
304 necessary to better explain the complex interaction between yeast and the simultaneous
305 presence of two or more sugars. For the moment, however, it is possible to affirm that this
306 phenomenon is highly strain dependent and that fermentation performance in presence or
307 absence of glucose can vary considerably between strains.

In attempting to define fermentation curves of different yeast strains in a mixed sugar medium, others have developed equations to fit fermentation profiles [39]. We chose not to do this, given the high frequency of sampling and good agreement between replicates in our study. Instead, we used the composite trapezoid rule to determine the AUC describing the fermentation of each sugar in either medium. The result is a bi-dimensional measurement of fermentation, which relates residual sugar concentration and duration of fermentation. This would appear to be the most inclusive approach to defining fermentation performance used to date. The subsequent comparison of areas under each of the fructose and glucose curves therefore provides an overall indication of the sugar affinity of each strain, taking into account all stages of fermentation. Importantly, the ratio of values derived using this approach also provides a convenient means for normalising data for strains that require different times to complete fermentations.

Other approaches to describe different utilisation rates of glucose and fructose whilst normalising data for different fermentation durations have been described. Berthels and colleagues [2] calculated the ratio between glucose and fructose at four points in a fermentation when 20, 30, 40 and 50% of the glucose had been consumed. Over this part of the fermentation they observed a linear increase in the glucose:fructose discrepancy. Moreover they were able to sort strains according to the slope of the increase in glucose:fructose discrepancy. The reason for selecting four specific residual glucose concentrations as the points for comparing all strains is not made clear, but presumably other points within this window would suffice. If not, following such a prescriptive approach would require a possibly unmanageable high degree of frequent and real-time glucose quantitation.

As an alternative to such real-time analysis, Guillaume et al. [15] plotted fermentation according to g/l of residual sugar normalised against g/l of CO_2 released, the latter being easily determine by weight loss measurements. With this approach they were able to graphically rank two strains based on their different pattern of fructose and glucose utilization and thereby differentiate the strains. A shortcoming of the work arises from the fact that the curves were mathematically fitted and therefore clearly approximations. In a similar approach Dumont and co-workers [11] introduced a fructophilic index as a criterion by which to describe the abilities of yeast to consume fructose compared to glucose. These workers focussed on the area between the fermentation curves for glucose *vs* fructose, in the latter $⅔^{rd}$ of the fermentation curve (also expressed as g/l of residual sugars *vs* g/l of CO_2 released). Accordingly, strains showing the lowest value, due to smallest area of differences between the glucose and fructose curves were said to be fructophilic and were presumed to perform better in situations with high fructose concentrations. Finally, Tronchoni and colleagues [36] fitted sugar consumption curves with various mathematical equations and achieved R^2 values of 0.95 and higher. This enabled them to confidentially estimate the time necessary for different strains to consume 50% and 100% of glucose and fructose, and, in turn, the residual fructose concentration at these points. Thus ultimately the comparison between strains was at one or two time points and did not consider the characteristics of the fermentation beyond these.

From our results, it was encouraging to observe that FERMICHAMP showed the highest ratio between glucose and fructose AUC (0.76). This result agrees with the finding described by Guillaume and collaborators [15] about the exceptional ability of this strain to consume fructose. Similarly, Berthels and colleagues [2] described the discrepancy in glucose and fructose for several strains, including EC1118, VIN13 and BORDEAUX RED. Similar results for these three strains have been found in our study where BOREAUX RED was the

slowest fructose fermenter, VIN13 medium and EC1118 one of the fastest. These similarities with other studies increase the confidence of considering our glucose/fructose comparative approach as a valid method in describing relative sugar consumption profile during fermentation. Moreover it was possible to rank 19 commercial wine strains according to their ability to consume fructose in relation to glucose and identify which strain was significantly different from the others in terms of fructophilicity (Table 3, Figure 2). Therefore FERMICHAMP and BORDEAUX RED are located at the opposite ends of the chart and they are the only two strains significantly different from the mean. Other strains such as S6U and EC1118 are significantly different from most of the strains belonging to the less fructophilic group, while strains such as D254 and PRIMEUR were significantly different from most of the strains grouped as the most fructophilic.

In this study, the sampling frequency produced comprehensive fermentation curves and obviated the need for curve fitting and limitations arising out of this means of approximation. The post fermentation determination of residual sugars reduced the analytical burden during the fermentation and the calculation of the AUC is the most comprehensive encapsulation of all aspects of the fermentation performance of each strain. Finally, the latter benefit can be said to apply to the ratio of the areas of the glucose and fructose curves, whilst such ratios also have the advantage of normalising performance of strains with regard to fermentation duration.

Conclusion

The study proposes a novel approach (AUC) to determine fermentation performances in wine yeast strains, with particular attention in identifying a methodology to describe the fructophilicity of the strains. Although one of the goals of the study was to use a medium

containing fructose as only carbon source to predict relative preference for fructose by strains and to relate this to fermentation performance, the complexity of glucose and fructose utilization for individual strains remains too great. Further work is warranted to ascertain the significance of fructose utilising capability to overall fermentation behaviour and wine quality, and thereby to optimize strain development programs.

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Table and figure legends

Table 1. Duration of sugar catabolism and fermentation for 20 commercial yeast strains during growth in media containing glucose and fructose or only fructose to a total concentration of 230 g/L. Values are the average of triplicate fermentations.

Table 2. AUC of glucose and fructose for fermentations by 20 commercial yeast strains of media containing mixed sugars (glucose and fructose) or only fructose to a total concentration of 230 g/L. Values are the average of triplicate fermentations.

Table 3. One-way ANOVA with Tukey's multiple comparison post-test analysis of 19 commercial yeast strains (and average of all strains) in terms of their ratio of the AUC of glucose utilisation and fructose utilisation from media containing both sugars at 115 g/L (as reported in Table 2).

Figure 1. Composite plots showing sugar utilisation profiles of 20 commercial wine strains from two growth media. Yeast were grown in CDGJM containing either 230 g/l of fructose only (top curve in each plot) or 115 g/l each of glucose and fructose (1:1; bottom 3 curves in each plot). Curves are derived from the mean of triplicate fermentations with error bars indicating standard deviation. Moving from top to bottom, the curves correspond to 1) fructose fermentation in a fructose-only medium, 2) combined glucose and fructose fermentation from the mixed sugar medium, 3) fructose fermentation from the mixed sugar medium and 4) glucose fermentation from the mixed sugar medium. The hatched area between the top curve (fructose fermentation in fructose-only medium) and the curve below (combined glucose and fructose fermentation from the mixed sugar medium) highlights the difference in total fermentation profile in the two media. The area corresponding to glucose

573 fermentation from the mixed sugar medium is highlighted in dark grey, while the extent to
574 which fructose utilisation from the mixed sugar condition was delayed compared to glucose
575 is highlighted in light grey.
576
577 **Figure 2**. Differences from the mean (baseline) of ratios between AUC of glucose and
578 fructose in the mixed media conditions (as reported in table 2). The values were calculated
579 with one-way ANOVA Dunnett's multiple comparison post-test at 95% confidence interval.
580 FERMICHAMP and BORDEAUX RED (in dark) were the only two strains showing a
581 significant differences from the average.

Figure 1

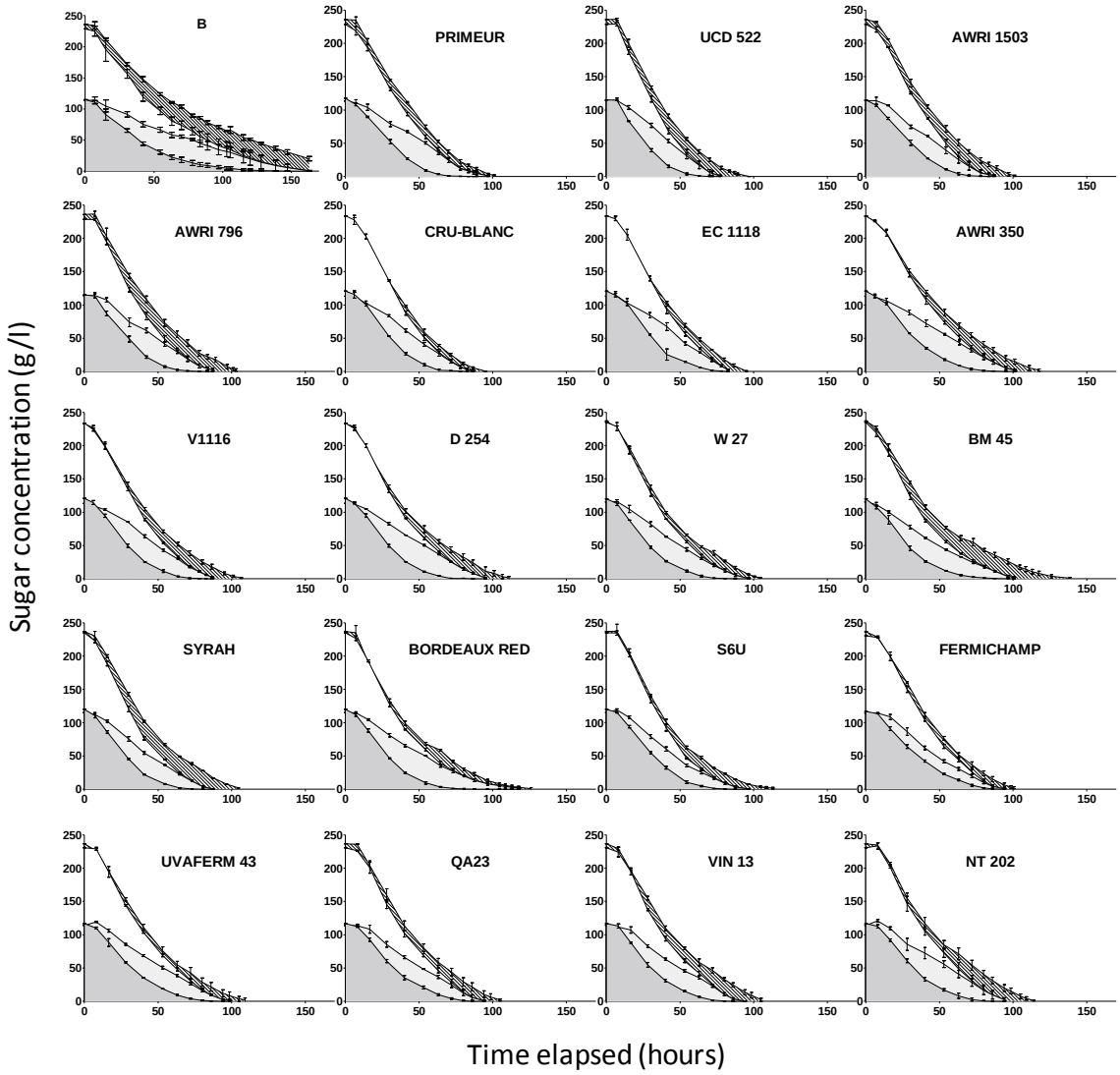


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Figure 2

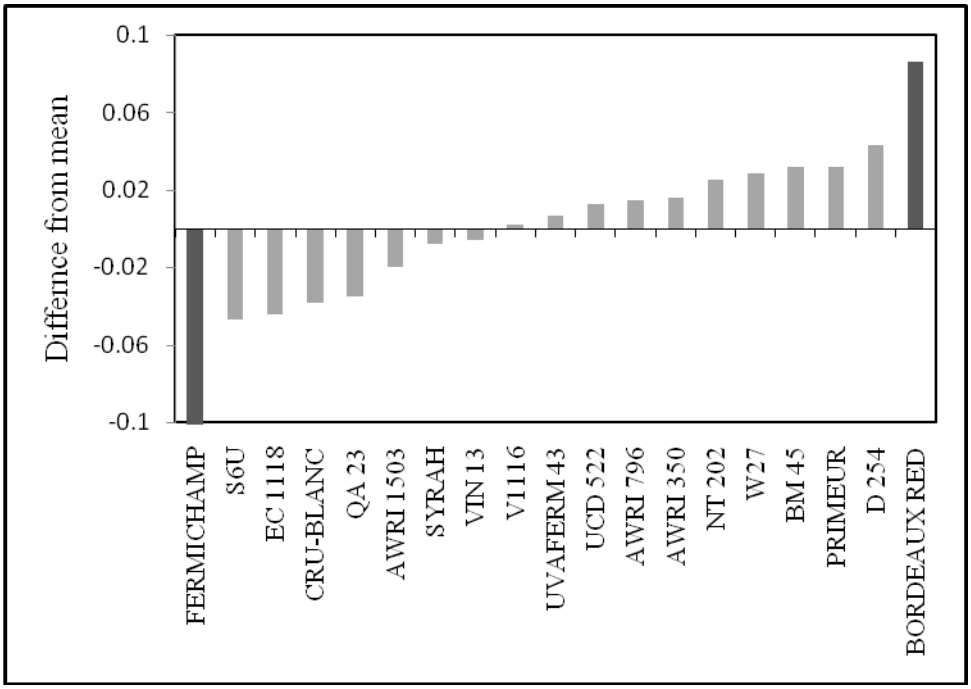


Figure 2. Differences from the mean (baseline) of ratios between glucose and fructose areas in the mixed media conditions (as reported in table 3). The values were calculated with one-way ANOVA Dunnett's multiple comparison post-test at 95% confidence interval. FERMICHAMP and BORDEAUX RED (in dark) were the only two strains showing a significant differences from the average.

Table 1. Duration of sugar catabolism and fermentation for 20 commercial yeast strains during growth in media containing glucose and fructose or only fructose to a total concentration of 230 g/L. Values are the average of triplicate fermentations.

Strain	Duration (h)					
	Glucose (mixed sugars)		Fructose or Total sugars (mixed sugars)		Fructose (fructose only)	
	Average	SD	Average	SD	Average	SD
B	115.3	12.5	151.3	20.2	DNC[1]	DNC
BORDEAUX RED	73.0	0.0	114.5	5.2	120.3	4.9
AWRI 350	77.2	4.9	101.0	0.0	115.3	3.7
BM 45	75.5	4.3	99.2	2.3	134.3	7.2
UVAFERM 43	80.0	0.0	97.0	2.6	106.3	2.3
W 27	73.0	0.0	96.5	0.0	104.5	0.0
D254	71.5	0.0	95.0	0.0	109.5	2.6
PRIMEUR	70.0	0.0	94.3	4.6	101.0	0.0
FERMICHAMP	80.0	0.0	94.0	0.0	99.8	1.1
QA23	80.0	0.0	94.0	0.0	102.8	3.7
VIN 13	72.0	0.0	94.0	0.0	105.0	0.0
NT 202	77.3	4.6	94.0	0.0	112.3	2.9
S6U	73.0	0.0	93.5	5.2	110.0	2.6
SYRAH	64.5	0.0	87.5	0.0	104.5	0.0
V1116	71.5	0.0	86.5	0.0	104.7	3.2
AWRI 796	67.7	4.0	86.0	1.7	102.3	1.1
CRU-BLANC	66.2	4.6	85.2	2.3	95.0	0.0
AWRI 1503	67.7	4.0	84.0	5.2	99.7	2.3
EC1118	71.5	0.0	82.5	0.0	95.0	0.0
UCD 522	60.3	4.6	74.6	4.0	94.3	4.6

[1] DNC, Fermentations did not complete.

Table 2. Area under the glucose and fructose utilisation curves for fermentations by 20 commercial yeast strains of media containing mixed sugars (glucose and fructose) or only fructose to a total concentration of 230 g/L. Values are the average of triplicate fermentations

Strains	Area under the fermentation curve (trapezoid rule)									
	Glucose (mixed sugars)		Fructose (mixed sugars)		Glucose area: Fructose area (mixed sugars)	Total sugars (mixed sugars)		Fructose (fructose only)		Total sugars area (mixed sugars): Fructose area (fructose only)
	Average	SD	Average	SD		Average	SD	Average	SD	
B	4677	384	8186	828	0.57	12864	1211	DNC[1]	-	-
AWRI 350	3861	77	6015	250	0.64	9876	324	11123	252	0.89
NT 202	3743	180	5918	303	0.63	9662	481	11123	572	0.87
UVAFERM 43	3732	77	5731	88	0.65	9463	159	10201	468	0.93
QA 23	3844	111	5548	261	0.69	9392	371	10550	565	0.89
FERMICHAMP	4047	116	5317	91	0.76	9364	184	10108	184	0.93
BORDEAUX RED	3336	65	5833	64	0.57	9169	129	10026	122	0.91
D 254	3404	52	5535	64	0.61	8939	109	9937	352	0.90
VIN 13	3560	77	5363	68	0.66	8923	140	10311	180	0.87
PRIMEUR	3426	45	5476	157	0.63	8902	159	10082	136	0.88
EC 1118	3646	73	5201	216	0.70	8847	146	9456	111	0.94
S6U	3650	16	5180	123	0.70	8830	110	9857	53	0.90
W27	3400	55	5403	84	0.63	8804	139	9709	105	0.91
BM 45	3385	113	5405	76	0.63	8790	127	11065	330	0.79
V1116	3410	95	5201	46	0.66	8611	98	9789	289	0.88
CRU-BLANC	3516	92	5052	107	0.70	8568	178	8946	119	0.96
AWRI 796	3297	7	5135	267	0.64	8432	272	10227	495	0.82
AWRI 1503	3394	8	5017	252	0.68	8411	244	9944	224	0.85
SYRAH	3216	25	4832	20	0.67	8048	25	9915	27	0.81
UCD 522	3001	70	4650	191	0.65	7651	258	9067	179	0.84

[1]DNC, Fermentations did not complete

Table 3. One-way ANOVA with Tukey's multiple comparison post-test analysis of 19 commercial yeast strains (and average of all strains) in terms of their ratio of the area under the curve of glucose utilisation and fructose utilisation from media containing both sugars at 115 g/L (as reported in Table 2).

Strains	S6U	EC1118	CRU-BLANC	QA 23	AWRI 1503	SYRAH	VIN 13	V1116	UVAFERM 43	UCD 522	AWRI 796	AWRI 350	NT 202	W 27	BM 45	PRIMEUR	D 254	BORDEUX RED	ALL
FERMICHAMP	-	-	•	•	••	•••	•••	•••	•••	•••	•••	•••	•••	•••	•••	•••	•••	•••	•••
S6U		-	-	-	-	-	-	-	-	-	•	•	••	••	••	••	•••	•••	-
EC1118			-	-	-	-	-	-	-	-	-	•	••	••	••	••	•••	•••	-
CRU-BLANC				-	-	-	-	-	-	-	-	-	•	•	••	••	••	•••	-
QA 23					-	-	-	-	-	-	-	-	•	•	•	•	••	•••	-
AWRI 1503						-	-	-	-	-	-	-	-	-	-	-	•	•••	-
SYRAH							-	-	-	-	-	-	-	-	-	-	-	•••	-
VIN 13								-	-	-	-	-	-	-	-	-	-	•••	-
V1116									-	-	-	-	-	-	-	-	-	•••	-
UVAFERM 43										-	-	-	-	-	-	-	-	••	-
UCD 522											-	-	-	-	-	-	-	••	-
AWRI 796												-	-	-	-	-	-	••	-
AWRI 350													-	-	-	-	-	••	-
NT 202														-	-	-	-	•	-
W 27															-	-	-	-	-
BM 45																-	-	-	-
PRIMEUR																	-	-	-
D 254																		-	-
BORDEAUX RED																			•••

Significant differences were calculated at p value of < 0.05 (•), 0.01 (••) or 0.001 (•••). Non significant differences were represented by (-).

10. APPENDIX 2: Intellectual Property

Two mutants of *Saccharomyces cerevisiae* AWRI 796 (designated in this study as 9 and 11) showing commercial potential.

11. APPENDIX 3: References

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12. APPENDIX 4: Staff

The following staff contributed to this project.

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