Directed evolution as an approach to increase fructose utilization in synthetic grape juice by wine yeast AWRI 796

Michelle E. Walker1,4, Tommaso L. Watson1,4, Christopher R. L. Large2,3, Yan Berkovich1, Tom A. Lang1, Maitreya J. Dunham2, Sean Formby4, Vladimir Jiranek1,5,∗

1Department of Wine Science, The University of Adelaide, PMB 1, Glen Osmond, SA 5064, Australia
2Department of Genome Sciences, University of Washington, 3720 15th Ave NE, Seattle, WA 98195, United States
3Molecular and Cellular Biology Program, University of Washington, Seattle, WA 98195, United States
4Bioinformatics Graduate Program, University of British Columbia, Genome Sciences Centre, BCCA, 100-570 West 7th Avenue, Vancouver, BC, V5Z 4S6, Canada
5Australian Research Council Training Centre for Innovative Wine Production, PMB 1, Glen Osmond, SA 5064, Australia

∗Corresponding author: Department of Wine Science, The University of Adelaide, Urrbrae, SA 5064, Australia. Tel: +61 8 313 5561; E-mail: vladimir.jiranek@adelaide.edu.au

One sentence summary: Chemical mutagenesis and directed evolution as an approach to improve fructose utilization during wine fermentation.

Editor: Vivien Measday

‡These authors have contributed equally.

Abstract
In winemaking, slow or stuck alcoholic fermentation can impact processing efficiency and wine quality. Residual fructose in the later stages of fermentation can leave the wine ‘out of specification’ unless removed, which requires reinoculation or use of a more fructophilic yeast. As such, robust, fermentation efficient strains are still highly desirable to reduce this risk. We report on a combined EMS mutagenesis and Directed Evolution (DE) approach as a ‘proof of concept’ to improve fructose utilization and decrease fermentation duration. One evolved isolate, Tee 9, was evaluated against the parent, AWRI 796 in defined medium (CDGJM) and Semillon juice. Interestingly, Tee 9 exhibited improved fermentation in CDGJM at several nitrogen contents, but not in juice. Genomic comparison between AWRI 796 and Tee 9 identified 371 mutations, but no chromosomal copy number variation. A total of 95 noncoding and 276 coding mutations were identified in 297 genes (180 of which encode proteins with one or more substitutions). Whilst introduction of two of these, Gid7 (E726K) or Fba1 (G135S), into AWRI 796 did not lead to the fermentation improvement seen in Tee 9, similar allelic swaps with the other mutations are needed to understand Tee 9’s adaption to CDGJM. Furthermore, the 378 isolates, potentially mutagenized but with the same genetic background, are likely a useful resource for future phenotyping and genome-wide association studies.

Keywords: fructose, directed evolution, Saccharomyces cerevisiae, genomics, fermentation, CRISPR/Cas9

Abbreviations
CNV: copy number variant
EMS: ethyl methane sulfonate
TA: titratable acidity
YAN: yeast assimilable nitrogen

Introduction
Saccharomyces cerevisiae is the principal Saccharomycetes yeast in both spontaneous and inoculated wine fermentations (Fleet et al. 1984, Heard and Fleet 1985). The interaction between the yeast and grape juice influences not only the wine aroma profile, but whether fermentation progresses quickly, slows down or, in the worst-case scenario, arrests. These sluggish and stuck fermentations are problematic as they can affect production time and in the worst-case scenario, arrests. These sluggish and stuck fermentations are problematic as they can affect production time and in some cases, variable (Bisson 1999, Berthels et al. 2004, 2008, Guillaume et al. 2007).

Whilst glucose and fructose are the primary hexose sugars in grape juice (in near equimolar proportions), they are utilized at different rates by the glucophilic S. cerevisiae (Guillaume et al. 2007), with the less preferred fructose predominating in the latter stages of fermentation when nutrients are depleted and alcohol is high. The low ratio of glucose to fructose, itself reported to cause sticking or stuck fermentations (Schütz and Gafner 1993), can make rectifying a problematic or failed fermentation challenging without reinoculation and/or addition of nutrients.

Hexose sugars are transported into the cell and activated through phosphorylation prior to utilization as carbon and energy sources. Key to the preferential utilization of glucose by S. cerevisiae is the substrate affinity of the hexose transporters and sugar phosphorylating enzymes (reviewed in Rodicio and Heinisch 2009). The HXT family of transporters responsible for the facilitated diffusion into the cell, have a higher affinity for glucose compared to fructose (Kruckeberg 1996). This affinity is concentration dependent (Boles and Hollenberg 1997), with Hxt1

Received: July 28, 2021. Revised: March 25, 2022. Accepted: April 22, 2022
© The Author(s) 2022. Published by Oxford University Press on behalf of FEMS. All rights reserved. For permissions, please e-mail: journals.permissions@oup.com
and Hxt3 considered low-affinity transporters, Hxt4 moderately low-affinity, and Hxt2, Hxt6, and Hxt7 high-affinity. Interestingly, improved fructose utilization is associated with allelic variants of Hxt3 (Guillaume et al. 2007, Zuchowska et al. 2015) as well as high affinity fructose/H+ syporter, Fsy1 (de Sousa et al. 2004, Galeote et al. 2010) found in some wine strains considered fermentation efficient e.g. EC1118 and Fermichamp® (Borneman et al. 2016). Whilst the differential expression of HXT1-7, FSY1, and GAL2 (Perez et al. 2005, Nadai et al. 2021) can be correlated with sugar utilization, other important factors include nitrogen utilization and ethanol tolerance, which are also genetically determined (Dequin and Casaregola 2011, Feltier et al. 2019, Kessi-Pérez et al. 2020).

Once taken up, fructose is directly phosphorylated to fructose-6-phosphate, whilst glucose is first phosphorylated to glucose-6-phosphate, before being converted to fructose-6-phosphate by phosphoglucone isomerase (PGI). The initial phosphorylation is undertaken by three sugar kinases, hexokinase isozymes Hxk1 and Hxk2, which phosphorylate glucose and fructose, albeit at different rates, and glucokinase, which is specific to glucose (Serrano and Delafuente 1974, Berthsels et al. 2004, Guillaume et al. 2007). The differences in substrate affinity (Km and Vmax) for glucose and fructose between these enzymes, together with their differential expression (Rossignol et al. 2003), may also contribute towards the differences in glucose and fructose utilization between wine strains (Berthsels et al. 2008). After the isomerization reaction to fructose-6-phosphate, the pathways of metabolism of fructose and glucose do not differ (Boulton et al. 1998). The reaction goes to pyruvate, which then, depending on the conditions, will be metabolized in different ways (Berthsels et al. 2008).

Stuck and sluggish fermentation are commonplace and are difficult to avoid, as there can be many reasons for their occurrence, which makes managing such situations difficult requiring both methods of prevention and correction (Bisson and Butzke 2000, Bisson 2005). Grape juice is not a favourable environment for yeast. Juice pH, yeast assimilable nitrogen (YAN) deficiency, the presence of other microorganisms in the initial stages of fermentation, temperature extremes, and many other parameters influence the final outcome (Alexandre and Charpentier 1998). Furthermore, the accumulation of alcohol limits growth, through disruption of the plasma membrane, intracellular organelles, and proteins including those involved in sugar transport (Berthsels et al. 2004, Stanley et al. 2010). For this reason, the generation of improved strains, which are better able to utilize fructose under such conditions, and so complete fermentation are highly desirable.

To date, recombinant DNA technology has provided an array of proof-of-concept strains with specific genetic modifications, which are well characterized (reviewed in Pretorius 2000). Whilst ML01 (Husnik et al. 2006) and ECM001 (Coulon et al. 2006) are approved in the US (Pérez-Torrado et al., 2015), such yeasts are yet to be fully embraced by the Australian and European wine industries because of consumer rejection and statutory legislation. As such, improvement strategies have relied on nonrecombinant methods, namely clonal selection, mutagenesis, hybridization, and rare mating (Pretorius 2000, Alperstein et al. 2020, Eldarov and Mardanov 2020). With the exception of quantitative trait loci (QTL) assisted breeding (Laffort 2021), these techniques show promise but they do not allow for targeted modifications per se, and may compromise other beneficial attributes. Directed Evolution (DE) represents a complementary paradigm in the development of new wine yeasts, which is being explored (McBryde et al. 2006, Kutyna 2008, López-Malo et al. 2015). The method relies on genome plasticity (Marsit and Dequin 2015), enabling the original population to diverge and evolve over successive generations to a particular environment, with the fittest eventually dominating the population.

The aim of this study was to improve fructose utilization in a commercial wine yeast, AWRI 796, with the view of alleviating poor sugar catabolism (namely of fructose) during the later stages of fermentation. The experiment was a ‘proof of concept’ using a combination of random mutagenesis and experimental evolution to obtain a robust strain. The novelty of the design was the use of limited fructose as a sole carbon source in Chemically Defined Grape Juice Medium (CDGJM), which mimics wine must. An evolved strain, Tee 9, was isolated that showed improved fermentative capacity in this medium but not the juices subsequently tested, and as such may be of limited benefit to industry. Nevertheless, the strain is still of interest in relation to yeast biology, and as such the genomes were sequenced in order to identify non-synonymous single nucleotide polymorphisms (SNPs) in genes related to fermentation. Gid7 (E7Z6K) and Fba1 (G1355) variants were evaluated as allelic swaps (mutant for wild type) as a step towards understanding the improved phenotype.

Materials and methods

Media

Yeast were routinely maintained on YEPD (1% yeast extract, 2% bacteriological peptone, and 2% D-glucose) solidified with 2% agar (Fink 1970). For YEPF, D-fructose replaced D-glucose. CDGJM (Henschke and Jiranek 1993) was prepared according to (McBryde et al. 2006) except that ammonium sulfate was replaced with a mixture of amino acids and ammonium chloride (Table S1, Supporting Information). The total nitrogen concentration (supplied as a 25x amino acid solution) was 600 mg l−1 (551 mg l−1 YAN) for the DE experiment and fermentation trials unless specified. D-glucose and D-fructose concentrations varied depending upon the experiment; the variation denoted as a suffix, e.g. CDGJM_4F contained 4 g l−1 fructose while CDGJM_G_F50 contain 25 g l−1 of each sugar. Solutions were sterilized by filtration (0.22 μm). CDGM starter medium was supplemented with ergosterol (10 mg l−1) and Tween 80® (0.5 ml l−1), and the glucose and fructose concentrations halved. Semillon (2016, Coombe Vineyard, University of Adelaide; 233.91 g l−1 sugar (as glucose and fructose), 96 mg l−1 YAN, TA 4.9 g l−1; pH 3.18) juice was defrosted at 2°C and filter sterilized (0.22 μm). Starter medium was an equal volume of YEPD and Semillon juice.

Yeast strains and maintenance

AWRI 796 (Maurivin, Australia) and Fermichamp® (DSM, Netherlands) were supplied as activated dry wine yeast. Yeast samples were rehydrated in sterile water (20 min), before overnight culturing in YEPD (28°C with shaking) and plating on YEPD agar. Clonal isolates grown in 25 ml YEPD were stored as 1 ml glycerol (26%) stocks at −80°C. A clonal isolate of AWRI 796 (clone 5), provided the genetic background for the DE experiment. Fermichamp® was used as a reference because of its high affinity for fructose. Genetic diversity of the starting population was increased by chemical mutagenesis using ethyl methyl sulfonate (EMS) and Tee 9 was isolated after ~200 generations of DE of the mutated culture (described below).

EMS mutagenesis

AWRI 796 clone 5 was treated with EMS based on Fink (1970) as follows. Cells from a YEPD culture (25 ml; 2 × 10⁸ cells ml−1) were
collected by centrifugation (2236 rcf, 5 min) and washed twice in 15 ml of 0.1 M sodium phosphate buffer pH 7 (2.16 g NaH2PO4 and 6.54 g of Na2HPO4 made up to 200 ml with H2O). Cells were resuspended in fresh buffer to 15 ml (2 x 10^6 cells ml^-1). A volume of 1 ml was untreated (control) and used for cell enumeration and cryostorage. The remainder was treated with 0.63 ml EMS and incubated at 30°C. Samples (1 ml) were collected every 10 min, the EMS inactivated with an equal volume of freshly prepared 5% sodium thiosulphate. Following centrifugation, the cell pellet was washed once before resuspension in YEPD with 26% glycerol for cryostorage and colony enumeration (duplicate samples). Relative survival was calculated from the mean viable cell count. Cells treated with EMS for 50 min (T50; representing 60% survival) provided the starting material for DE.

**DE experiment targeting improved fructose utilization**

The DE experiment used a continuous culture approach (Zeyl 2004) and was pursued in two stages. The first sought to define a fructose concentration in the feedstock and dilution rate that ensured limiting concentrations of fructose in the fermentation vessel and maintained a largely stable cell number (\( \sim 5 \times 10^7 \) ml^-1). The second adopted these conditions and proceeded with the extended evolution experiment.

The experiment was conducted in a BIOSTAT® A plus (Sartorius BBI System GmbH, Germany) equipped with a 1-1 fermentation vessel and controlled using the MFCS/DA A plus 2.1 software (Sartorius BBI System GmbH). EMS treated cells (T50), were streaked from the glycerol stock onto YEPF agar and grown overnight at 30°C. A loopful of yeast (many colonies) was subsequently inoculated into liquid YEPF and grown overnight prior to inoculation at 1 x 10^5 cells ml^-1 in 500 ml CDGJM_F (4 g l^-1 fructose) in the bioreactor. The medium was supplemented with ergosterol (10 mg l^-1) and Tween 80® (0.5 ml l^-1) to supply sterols and fatty acids for cell membrane synthesis under anaerobic conditions (Ribéreau-Gayon et al. 2006). The vessel was maintained at 30°C, continuously agitated (200 rpm) and fitted with a water-filled air lock. The CDGJM_F feedstock initially contained fructose at 20 g l^-1 and was supplied at 50 ml l^-1 (2.13 g l^-1 h^-1 fructose). Inlet and outlet flow rates matched to maintain a constant culture volume of \( \sim 500 \) ml. The fructose content of CDGJM_F was progressively reduced and the dilution rate increased (Fig 1A).

In the subsequent evolution experiment, fermentation was again initiated as a batch culture in CDGJM_F4 using a fresh overnight culture of EMS treated cells (T50), and after 24 h (\( \sim 5 \times 10^7 \) cells ml^-1)), proceeded as a continuous culture, with an initial dilution rate of 0.08 h^-1. The dilution rate was progressively increased (0.12, 0.14, 0.15, and 0.18 h^-1) over 220 h to a final value of 0.2 h^-1 (i.e. 100 ml h^-1), which was held for the duration of the experiment (Fig 1B). Fructose concentration in the exhaust medium was measured using Clinistest® tablets. The culture was sampled every 48 h for cell enumeration, suggesting \( \sim 350 \) generations over the 1278 h (\( \sim 53 \) days) of the experiment. For mutant characterization, 1 ml samples were collected at 50-generation intervals, cells harvested (20 800 rcf, 1 min) and the medium replaced with YEPF and sterile glycerol added (15%) for cryostorage.

**Microscale (0.2 ml) screen for fermentation performance**

Glycerol stocks were streaked onto YEPD agar for single colonies. A total of 54 colonies per time-point were inoculated into deep 96-well plates containing 0.5 ml YEPD, and incubated statically overnight at 30°C. AWRI 796 and the mixed evolved population were included as controls. For each isolate, a 10-ml tube containing 1 ml starter medium with 25 g l^-1 of each of glucose and fructose (CDGJM_G+F50) was inoculated with 20 μl of YEPD culture and incubated (30°C, static) until stationary (\( \sim 10^8 \) cells ml^-1). Each 1 ml culture was then diluted with 4 ml CDGJM_G+F100 (50 g l^-1 glucose and 50 g l^-1 fructose) and 10 μl inoculated into a 96-well plate well containing 190 μl of CDGJM_G+F100 (\( \sim 10^7 \) cells ml^-1). The 54 isolates were arranged between three ‘source’ plates, each being divided into four quadrants (each with 18 isolates plus the parent and MP) and, thus quadruplicates. On column of wells was left uninoculated (sterility control), whilst another remained empty to allow for addition of standards during later analysis of fructose and glucose. A total of six replicates of each source plate were made using a liquid handling robot (Tecan EVO 150), to allow for sacrificial sampling for monitoring of the fermentation (Liccioli et al. 2011b). Plates were sealed with Breath Easy gas permeable membrane (Diversified Biotech, Boston, MA, USA) and incubated at 20°C, 75% humidity, <1% O2 concentration (nitrogen gas). Every 24 h, a single replicate plate was removed and frozen (\( \sim 20°C \)) for later enzymatic determination of glucose and fructose.

**Laboratory scale (250 ml) fermentations of DE isolates**

Fermentation performance of the DE isolates was evaluated against the parent and reference strains according to Walker et al. (2003) with minor modifications. Starter cultures (50 ml; 50 g l^-1 glucose and 50 g l^-1 fructose) were used to inoculate 250 ml (triplicate) fermentations in CDGJM_G+F230 (115 g l^-1 glucose and 115 g l^-1 fructose). A Medecil Explorer bioreactor system (Medicel Oy, Finland) allowed for automatic sampling. Fermentation vessels were fitted with water-filled airlocks and kept at 30°C with constant agitation (magnetic stir bars, 200 rpm). The headspace was continuously flushed with filtered nitrogen (0.45 μm, 5 ml min^-1). Samples (3 ml) were collected and chilled (\( \sim 5°C \)) to prevent metabolic activity, with permanent storage (\( \sim 20°C \)) prior to analysis. Sampling frequency varied from 6 to 8 h during the early and final stages, to 12 hourly mid-fermentation.

**Measurement of sugars, nitrogen, and metabolites**

Clarified supernatant (20 800 rcf, 2 min) was used for metabolite analysis. Residual glucose and fructose were measured enzymatically (Boehringer-Mannheim 1989) with volumes adjusted to 100 μl for microtiter plate analysis (Walker et al., 2014). A liquid handling robot (Tecan EVO 150) collected samples for absorbance readings using an Infinite® 200 PRO microplate reader (Tecan Group Ltd). Samples were diluted 1 in 10, and 1 in 100 for analysis (Walker et al. 2014).

Other analyses included nitrogen by spectrophotometry (Dukes and Butzké 1998) using the Primary amino acid nitrogen (PAN) kit (K- PANOPA; Megazyme), and major metabolites (organic acids, glycerol and ethanol) by HPLC (Lin et al. 2020). Data was organized, analyzed and graphed using GraphPad Prism software (versions 8.0.0 and 9.0.0 for Windows; GraphPad Software, San Diego, www.graphpad.com). Area Under the Curve (AUC) calculations and statistical analysis using One-way Analysis of Variance Analysis (ANOVA) and multiple comparisons testing was also with GraphPad Prism.
Figure 1. DE of AWRI 796 for improved fructose utilization in CDGJM. Continuous culture of AWRI 796 (T50) was undertaken in a CDGJM with fructose (CDGJM_F) in two separate DE experiments to firstly establish the dilution rate and fructose concentration of the feedstock to obtain a stable target cell density of $\sim 5 \times 10^7$ cells ml$^{-1}$ for adaptive evolution (A) and secondly, to generate isolates with increased fructose utilization (B). Residual fructose, flow rate, and cell density were measured throughout the experiments. A total of 54 isolates within each approximately 50-generational sample were evaluated for sugar utilization (glucose and fructose) in CDGJM. The evolving population was monitored over the $\sim$350 generations according to their fructophilicity (C). The GLU/FRU ratio for each individual was calculated from the Area Under the Curve (AUC) of utilization for each sugar and plotted with the mean $\pm$ SD. Each generational sample included the parent AWRI 796 (denoted by ‘P-’), the mixed population (denoted by ‘MP-’) and individual isolates (denoted by ‘I-’). Statistical analysis of the data was via 1-way ANOVA and Tukey’s multiple comparisons tests at $P = 0.01$; see File S1, Supporting Information.)
Evaluation of $[^{14}C]$-fructose uptake

To specifically monitor fructose transport, uptake of $[^{14}C]$-fructose was measured according to Schneider and Wiley (1971). Triplicate cultures of Fermichamp® (fructophilic wine strain), the parent AWRI 796 and evolved strain, Tee 9, were grown in CDGJM_G+F230 with 600 mg l$^{-1}$ N. Cells were collected from the fermenting cultures at 16.91 (AWRI 796), 0.72 (Tee 9) and 0.02 g l$^{-1}$ (Fermichamp®) total sugar, respectively. Dry Cell Weight (DCW) represented the weight of 10 ml of culture on a preweighed 0.22-μm cellulose acetate filter disk (Ø 47 mm, Whatman) when microwave dry (300 W, 5 min). For the experiment, 1.3 ml of culture was washed and resuspended in 0.02 M KH$_2$PO$_4$, to which 2.5 μl of radiolabelled $[^{14}C]$ D-fructose (Bioscientific – 50 μCi, specific activity 9.25–13.3 GBq/nmol) were added. Cultures were constantly agitated and samples (20, 60, 150, and 300 s intervals) washed twice with 0.02 M KH$_2$PO$_4$ on 0.22-μm filters, and placed into scintillation vials containing 4 ml of scintillation fluid (Scintarc, 6013248). The amount of radiolabelled D-fructose retained by harvested cells was determined using a Parkard scintillation counter, counting $^{14}C$ CPM for 2 min. Fructose uptake rate (nmol mg$^{-1}$ DCW) was determined from a calibration curve ($[^{14}C]$ D-fructose spiked filtered fermentation supernatant) and DCW values (data not shown).

Whole-genome characteristics and polymorphic analysis of wine yeast

Genome sequencing

Fermichamp® (fructophilic wine strain), AWRI 796 and the evolved strain, Tee 9 were grown overnight in 10 ml YEPD (30°C; 160 rpm). Cells were pelleted (4500 rcf; 2 min), and sent to the Australian Centre for Ecogenomics at the University of Queensland (Brisbane, Australia) for DNA extraction and genome sequencing. Libraries were prepared using Illumina’s Nextera XT library preparation kit, with NGS sequencing using MiSeq v3 technology producing an average of 5 million pair-end reads per sample (300 bp coverage).

The fastq sequence data (as GZ files) are available upon request. AWRI 796 isolate (MP2_S2_L001_R1_001.fastq.gz, MP2_S2_L001_R2_001.fastq.gz), Tee 9 (MP2_S1_L001_R1_001.fastq.gz, MP2_S1_L001_R2_001.fastq.gz), and Fermichamp® (MP2_S3_L001_R1_001.fastq.gz, MP2_S3_L001_R1_001.fastq.gz).

Alignment and SNP/Indel variant calling

Reads were aligned using the Burrows-Wheeler Aligner (BWA/0.7.15) MEM algorithm (Li 2013) to the SacCer3 reference genome (SGD R64-2-1; http://sgd-archive.yeastgenome.org/sequence/S288C_reference/genome_releases/), then sorted and indexed using SAMTools/1.9 (Li, 2013). Duplicates were marked and removed using Picard tools (picard/2.6.0), sorted and indexed using SAMtools, and the InDels realigned using the GATK/3.7 package. Variant calling analysis used freebayes/1.0.2-6-g3e827d (Garrison and Marth 2012) with modified arguments (-pooled-discrete -pooled-continuous -report-genotype-likelihood-max–allele-balance-priors-off–min-alternate-fraction 0.1) and LoFreq/2.1.2 (Wilm et al. 2012) in a paired mode with their genetic ancestor (aka parent). Called variants were subsequently filtered for uniqueness against their genetic ancestor(s) using bedtools/2.26.0. The variants were filtered for quality using bcftools/19 (Table 1) before being annotated (Pashkova et al., 2013) and manually inspected for validity using the Interactive Genomics Viewer (IGV); Robinson et al. (2011).

Copy number and rearrangement analysis of chromosomes

Two methods were used to analyze the Copy Number Variation (CNV) of the chromosomes.

Method 1 (original analysis)

Using 1000 base pair sliding windows (IGVtools), normalized by the mean total read depth across the genome (GATK/3.7), the copy number across the genome was plotted and manually inspected for changes in copy number with a sample’s genetic ancestor. Copy number change breakpoints were manually inspected to determine the type of rearrangement using split and discordant reads, generated with BWA mem, SAMBLASTER/0.1.24 (Faust and Hall 2014), and SAMtools.

Method 2 (reanalysis of AWRI 796 isolate)

Raw reads for MP2_S2 (AWRI 796 isolate) were de novo assembled using MEGAHIT v1.2.9 with parameters “–no-marcy –prune level 3 –min-count 5” (Li et al., 2015, 2016). The contigs were corrected and scaffolded using the S288C reference genome SGD R64-3-1 (http://sgd-archive.yeastgenome.org/sequence/S288C_reference/genome_releases/) using ragtag v2.01 (Alonge et al., 2019). Contigs less than 25 000 bp were removed from the assembly leaving 17 scaffolds (16 nuclear chromosomes and 1 mitochondrial genome). MP2_S2 raw reads were mapped back to the de novo assembled genome using BWA-mem2 v2.0, SAMBLASTER v0.1.24 and SAMTools v1.7 (Li et al. 2009, Faust and Hall 2014, Md et al. 2019).

The CNVkit batch command was used on the resulting bam with a 10 000-bp target size and in whole genome sequencing (wgs) mode (Talevich et al., 2016). Raw reads for accession number SRR2967854 (AWRI 796) from the SRA (https://trace.ncbi.nlm.nih.gov/Traces/sra/?run=SRR2967854) using SRA toolkit v2.10.9. The CNVkit batch command was used on the resulting bam with a 10 000-bp target size and in whole genome sequencing (wgs) mode (Talevich et al., 2016). Raw reads for accession number SRR2967854 (AWRI 796) from the SRA (https://trace.ncbi.nlm.nih.gov/Traces/sra/?run=SRR2967854) using SRA toolkit v2.10.9. The CNVkit batch command was used on the resulting bam with a 10 000-bp target size and in whole genome sequencing (wgs) mode (Talevich et al., 2016). Raw reads for accession number SRR2967854 (AWRI 796) from the SRA (https://trace.ncbi.nlm.nih.gov/Traces/sra/?run=SRR2967854) using SRA toolkit v2.10.9. The CNVkit batch command was used on the resulting bam with a 10 000-bp target size and in whole genome sequencing (wgs) mode (Talevich et al., 2016). Raw reads for accession number SRR2967854 (AWRI 796) from the SRA (https://trace.ncbi.nlm.nih.gov/Traces/sra/?run=SRR2967854) using SRA toolkit v2.10.9. The CNVkit batch command was used on the resulting bam with a 10 000-bp target size and in whole genome sequencing (wgs) mode (Talevich et al., 2016). Raw reads for accession number SRR2967854 (AWRI 796) from the SRA (https://trace.ncbi.nlm.nih.gov/Traces/sra/?run=SRR2967854) using SRA toolkit v2.10.9.

Allele frequency analysis

The previously processed alignments were used to call variants using GATK’s Haplotype Caller, then filtered using the parameters listed in Table 1. The filtered variant calls were used to gen-
erate allele frequency calls across the genome using an in-house script, which were subsequently plotted. Loss of heterozygosity (LOH) events were manually called using visual inspection.

**Protein prediction software**

Protein prediction SNAP2 software (https://www.rosalab.org/services/SNAP; Hecht et al. 2015), mutfunc (http://mutfunc.com; Wagih et al. 2018), and PROVEAN (http://provean.jcvi.org/seq_submit.php; Choi et al. 2012) were used to predict putative deleterious amino acid substitutions within mutant genes. Homology modelling and sequence comparison between related proteins used Phyre2 software (Protein Homology analogy Recognition Engine version 2; http://www.sbg.bio.ic.ac.uk/Phyre2; Kelley et al. 2015).

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

Two datasets representing genes with nonsynonymous SNPs (nsSNPs) were separately analyzed: the total 180 genes and the 98 predicted to be deleterious to protein function. Hierarchical clustering and over-representation (enrichment) of genes based on shared Gene Ontology (GO) terms was performed SGO GO Slim Mapper (https://www.yeastgenome.org/goSlimMapper) and using GO Finder Version 0.86 software (https://www.yeastgenome.org/goTermFinder) respectively, or together, with ShinyGO v0.741 software (http://bioinformatics.sdstate.edu/go/; Ge et al. 2020).

**Allelic swaps in AWRI 796 using CRISPR/cas9 gene editing**

Single nucleotide changes (C/G>T/A) were introduced via CRISPR/Cas9 to create the amino acid changes Gid7 (E726K) and Fba1 (G135S) as single homozygous mutations in the diploid genome. The two-plasmid system described by Shaw et al. (2019) centered on the guide RNA plasmid, pWS082 and CRISPR/Cas9 expression plasmid, pWS173 and a DNA template with the mutation. Plasmids were bought from Addgene (https://www.addgene.org/guides/crispr/) and propagated in Escherichia coli NEB5-alpha prior to extraction (Wizard Plus SV Minipreps DNA Purification System; Promega).

Guide RNA sequences were designed for the FBA1 (YKL060C) and GID7 (YCL039W) genes in the AWRI 796 genome (taxid:764097) with Benchling (Biology software 2020). Retrieved from https://www.benchling.com/; Lee et al. 2015). These sequences were oligomers (Table 2) that were annealed together and cloned into plasmid pWS082. The recombinant plasmids pWS082_FBA1 and pWS082_GID7 were confirmed by Sanger sequencing (AGRF, Adelaide) using primers pWS082seq (Table 2) and DNA sequence analysis using BLAST software (https://blast.ncbi.nlm.nih.gov/Blast.cgi; Altschul et al. 1990) and/or Clustal Omega (Electronic Source: Clustal Omega; Sievers et al. 2011).

Each double-stranded DNA template (with the single nucleotide mutation, G > A) was amplified using VELOCITY DNA polymerase (Bioline) with 8 μl of each set of overlapping homology-directed repair (HDR) oligomers (100 μM μl⁻¹; Table 2) and gel purified using the Wizard® SV Gel and PCR Clean-Up System (Promega). The mutation was introduced into the yeast genome by HDR. AWRI 796 yeast cells were transformed using the lithium acetate method (Gietz et al. 1992) with a reaction mixture containing the plasmids pWS082_FBA1 or pWS082_GID7, pWS173, and the mutation template. G418 resistant transformants were picked and cured of plasmid by growth on nonselective media (YPED, 3 days at 28°C), with plasmid loss confirmed as growth sensitivity to G418. Genomic DNA extracted using phenol-chloroform/glass beads (Adams et al. 1997) and used to amplify the mutation sequences for GID7 and FBA1 genes with VELOCITY DNA polymerase (Bioline) using gene specific primers (A and D). The gel purified DNA was sequenced using GID7 E726Kseq and FBA1 G135Sseq (Table 2) and compared with the wild type DNA (AWRI 796) using CLUSTAL Omega software.

**Laboratory scale (100 ml) fermentations of Gid7 (E726K) and Fba1 (G135S) mutants**

Triplicate fermentations were performed in 100 ml of CDJM (with variable nitrogen, as described below) or Semillon juice using 250 ml Erlenmeyer flasks fitted with water-filled airlocks and sampling ports.

Single colonies were cultured in 25 ml YEPD (24 h, ambient temperature, 140 rpm). The cultures were inoculated into 50 ml of starter media at 2.5 × 10⁶ cells ml⁻¹ and regrown. For fermentation set up, the starter culture was centrifuged and washed with identical media to that used for the fermentation trial. Fermentations (triplicate) were inoculated at 5 × 10⁶ cells ml⁻¹, incubated at 18°C with shaking (200 rpm), and monitored by refractive index (°Brix). Samples (1 ml) were collected daily and the supernatants stored for later analysis (described above) and considered dry when total sugar was < 2.5 g l⁻¹ as determined by AIMITAB Reducing Sugar Tablets (Rowe Scientific, Adelaide). DCW from 5 ml of culture was determined as described above.

**Fermentation of CDJM with various total N**

CDJM_G+F230 was prepared with various N (90, 250, or 400 mg l⁻¹ total N) using a 25x amino acid stock (Table S1, Supporting Information). These N values equated to 83, 230, and 368 mg l⁻¹ YAN, respectively. CDJM Starter medium contained 50 g l⁻¹ glucose, 50 g l⁻¹ fructose, 450 mg l⁻¹ total N, 10 mg l⁻¹ ergosterol, and 540 mg l⁻¹ Tween 80°.

**Fermentation of Semillon juice**

The juice used was produced from a 2016 Semillon (Coombe Vineyard, University of Adelaide), which was handpicked, processed and immediately frozen in a 5 l plastic container. The juice parameters were 233.91 g l⁻¹ sugar (as glucose and fructose), 96 mg l⁻¹ YAN, TA 4.9 g l⁻¹, and pH 3.18. Prior to use, the juice was thawed at 2°C and filtered (0.45 and 0.22 μm nitrocellulose). Starter medium was an equal volume of YEPD and Semillon juice.

**Cytometry**

Cell number and viability were measured via flow cytometry with propidium iodide (PI) using and a Guava 12HT system and Guava easyCyte™ software (Millipore). Samples were serial diluted in phosphate buffered saline (PBS) with 10% of PI, which was made fresh from a stock solution (1 mg ml⁻¹). Fluorescence attributed to PI (PI positive and negative) was gated on FL3. Total cells, viable cells (PI negative events) and dead cells (PI positive events), and cells per ml were tabulated using Microsoft Excel 2013 software.

**Results**

**Adaptive evolution of a clonal isolate of AWRI 796**

A single clone of AWRI 796 (clone 5) was isolated and evaluated as a physiological representative of the average population from
the commercial package (data not shown). This isolate provided the genetic background for selective improvement of fructose utilization, a reference for the characterization of the evolving population, and was the ancestral strain (parent) in genomic analysis. As genetic variation is key to adaptive evolution (Chambers et al. 2009), random mutations were introduced into the genome of clone 5 using EMS, to increase genetic heterogeneity, and the probability of isolating beneficial mutants. The T50 culture (EMS, 50 min, 60% survival) was used for the study.

A preliminary bioreactor experiment was undertaken to establish the conditions for continuous culture to establish a cell density to allow for adaptive evolution (Fig. 1A). This was followed by a second bioreactor experiment to generate isolates with increased fructose utilization (Fig. 1B).

In the first experiment, the bioreactor contained 500 ml of CDGJM_F4 (4 g l\(^{-1}\) fructose) and \(\sim 1.5 \times 10^9\) cells ml\(^{-1}\) before fructose was supplied at 2.13 g l\(^{-1}\) h\(^{-1}\) and a dilution rate of 0.1 h\(^{-1}\) (i.e. 20 g l\(^{-1}\) feedstock, 50 ml h\(^{-1}\) flow rate). Over the first 420 h, the fructose concentration was incrementally decreased in the feedstock (i.e. 20 g l\(^{-1}\) feedstock, 50 ml h\(^{-1}\) flow rate) whilst the dilution rate increased to 0.2 h\(^{-1}\) up to a target of \(\sim 5 \times 10^7\) cells ml\(^{-1}\) before fructose was undetectable in the fermentation vessel (Fig. 1B), indicating rapid consumption of supplied fructose.

Given an estimate (Zeyl 2004) of the rate of adaptive mutation (1 per 10\(^{11}\) cell divisions; \(\sim 40\) generations) and allowing for additional divisions to achieve significance in a population, the culture was sampled seven times at \(\sim 50\) generation intervals (Fig. 1B), to allow for emergence and identification of a beneficial mutant. Previously, we reported that beneficial phenotypes were observed after \(\sim 250\) generations (\(\sim 270\) days) under a sequential batch culture scenario (McBryde et al. 2006). By comparison, continuous culture (with an exponential culture) used here, reduced the evolutionary experiment timeframe to only 53 days to achieve 350 generations at a dilution rate of 0.2 h\(^{-1}\) and average population of \(\sim 5 \times 10^7\) cells ml\(^{-1}\).

### Screening of the evolved population in microscale fermentations

The evolving population was sampled every 50 generations and evaluated as single colony isolates for fermentation improvement using a high-throughput screening approach (Liccioli et al. 2011b). A total of 378 isolates (54 per population; 7 populations) were evaluated in 21 microfermentations (0.2 ml, 96-well plate) screens, with each fermentation performed in quadruplicate with 18 isolates per plate. AWRI 796 and the corresponding ‘mixed population’ (MP) were included in each plate as controls. The isolates were evaluated in CDGJM\(_G\)+F100 (50 g l\(^{-1}\) each of glucose and fructose) with 600 mg N l\(^{-1}\) (551 mg YAN l\(^{-1}\) rather than that of typical fermentation screens (\(\geq 200\) g l\(^{-1}\) sugar, \(\leq 450\) mg N l\(^{-1}\)), which mimic wine fermentation (Walker et al. 2014, Peter et al. 2018b). Whilst N was kept constant, the sugar had been increased from 20 g l\(^{-1}\) feedstock to 40 g l\(^{-1}\) feedstock.

#### Table 2. Primers used in this study.

| Oligomer primers | Sequence 5’–3’ |
|------------------|----------------|
| sgFBA1 F         | g acctttGAAGCTTACTCTCAAGGAACA |
| sgFBA1 R         | aaacTGTTCCTTGAGTAACCTTCC |
| sgGID7 F         | gacctttAAAGAAATTTTGTCTCTG |
| sgGID7 R         | aaacGCGAAGCGAAGCACCTT |
| HDR FBA1 forward | CATGTTGGATGTGTGGAGCTGATGTTACTTCAAGGAACACACAGTAACACATTT |
| HDR FBA1 reverse | ATCGTTTTCTTCAGACAAATCCAAACATGTGGGAGGAG |
| HDR GID7 forward | AACCATGACTACCTGTCAGCAGACG |
| HDR GID7 reverse | GGTTCAGATCTATGCTGAGACACG |
| FBA1             | CCATTTGCTATGGATATGCCA |
| YKL060C, A\(^+\) | GCTCATCTGGAGTCTCTGG |
| YKL060C, D\(^+\) | TGGACAGAAGCGAAGAC |
| YCL039W, A\(^+\) | GCTTACCTGCTGCTG |
| YCL039W, D\(^+\) | GTAATCAGATCAGATCAGAT |
| pWS082 seq\(^a\) | GTAATCAGATCAGATCAGAT |
| GID7 E726 seq\(^b\) | GTAATCAGATCAGATCAGAT |
| FBA1 G1555 Seq\(^c\) | GTAATCAGATCAGATCAGAT |

Primers were sourced from Sigma Aldrich (Australia) as 100 μM μl\(^{-1}\) stocks (0.05 μM scale, desalted). *Primers for amplification of genes for sequencing. \(^a\)Primers also used for sequence confirmation of CRISPR/Cas9-derived constructs. \(^b\)Primer sequences were from the Saccharomyces Genome Deletion Project (http://www.yeastgenome.org/). HDR, Homology Directed Repair; sg, single stranded guide. Bold font represents nucleotides to reconstruct BsmBI site in the CRISPR/Cas9 sg RNA in the plasmid pWS082 (Lee et al. 2015).
Fermentation performance varied between the isolates. Figure S1 (Supporting Information) shows the results from a typical batch, with a few isolates fermenting faster than the parent, while others were slower. Although the former suggest a possible improvement in fructose utilization and overall fermentation in some isolates, the resolution of the fermentation curves was insufficient, thus differences were assessed using Area Under the Fermentation Curve (AUC) values, which bidimensionally represent residual sugar vs. time. The ratio between the AUC values for glucose vs. fructose utilization, i.e. the GLU/FRU ratio, was then calculated as a measure of an isolate’s ability to use fructose compared to glucose independent of overall fermentation duration. The closer the GLU/FRU ratio to 1, the smaller the difference between the kinetics of glucose vs fructose utilization and hence the more fructophilic the strain.

The GLU/FRU ratios of the isolates were plotted along with the parent and MP for each generational sampling (Fig. 1C). The marked variation between plates in the GLU/FRU ratio of the parent most likely arose due to the screen being conducted over several weeks and not as a single experiment. Also, false positives/negatives and outliers were inevitable given the fermentation scale (0.2 ml) and small inoculum volume, together with variations in microenvironmental conditions (plate position, humidity, temperature, and oxygen; Liccioli et al. 2011b). While the outliers in the parental data obscured the significance of differences in early samples, a trend towards higher GLU/FRU ratios (average value, and proportion of isolates per generation) was clear at 200 generations and beyond, and alluded to the population becoming more fructophilic as the DE progressed (Fig. 1C; File S1, Supporting Information).

Using the GLU/FRU ratio, the 50 best performing isolates from across those evaluated were readily identified (File S1, Supporting Information). It was reasoned that this number of candidates for further evaluation could be reduced by excluding those with poor overall fermentation performance (OFP), despite showing increased fructophilicity. Thus, for each isolate a measure of OFP was determined as a ratio of the AUC for its total sugar (glucose and fructose) catabolism compared to that of the parent in the corresponding plate. OFP ratios of 1 or less, would indicate isolates able to complete fermentation at least as rapidly if not more so than the parent. Using these two criteria, 19 isolates were selected from the 50 shortlisted, with representatives from the following generations: 50th (1 clone), 200th (3 clones), 250th (2 clones), 300th (2 clones), and 350th (11 clones). The GLU/FRU ratios of these isolates (0.79–0.84 [Ave. = 0.80 ± 0.01]) compared to the parent (0.73 ± 0.05), together with low OFP ratios (0.85–0.95 of the parent value) were indicative of overall improvement (File S1, Supporting Information).

When assessed in a second microscale screen aimed at eliminating false positives and further narrowing the number of promising candidates for evaluation, a range in performance relative to the parent was evident (Fig. 2). Of 19 isolates previously shown to have higher GLU/FRU ratios and improved OFP, five (6, 10, 15, 16, and 19) performed worse in the second screen, having OFP ratios greater than 1. The GLU/FRU ratios of the parent was 0.63, while most isolates ranged between 0.63 and 0.71, and isolate 10 was 0.94 (Table S2, Supporting Information). These differences between the screens reiterated that while convenient, the microfermentation screen has a degree of variability. Notwithstanding this, three out of 19 isolates (3, 9, and 11) were selected as having both superior OFP compared to the parent (i.e. OFP ratios of 90%–95%) and a GLU/FRU ratio greater than 0.63. Isolate 7 was also retained because of this higher fructophilicity (0.68 vs. 0.63), despite having a similar OFP to the parent (0.99 vs. 1.0; Table S2, Supporting Information).

**Fermentation performance of evolved isolates in CDGJM**

Selected isolates were evaluated in two experiments in CDGJM+G+F230 at a larger scale (250 ml), which allowed greater precision in inoculation rates and control of environmental conditions. Isolates 3, 7, 9, and 11 were initially compared against the parent (Fig. 3), where a clear differentiation in the second half of the fermentation was observed. Isolate 9 had the quickest fermentation, depleting all sugars within 117 h compared to parent AWRI 796 (153 h), whilst isolate 11 took 135 h. Isolates 3 and 7 failed to complete, leaving ~30 g 1⁻¹ residual sugar (mainly as fructose). Glucose consumption was essentially the same between the parent and all four isolates (Fig. 3A) with differences noted in fructose consumption (Fig. 3B). The selective loss of fructose utilizing ability in isolates 3 and 7 was unexpected, but may be linked to the higher ethanol yield (11.75% by volume) of these high sugar (230 g 1⁻¹ total) fermentations compared to the adaptive experiment (trace ethanol). The data implies not only the adaptive evolution of isolates to improved fructose utilization (e.g. isolates 9 and 11), but also the potential, inadvertent decrease in ethanol tolerance in some isolates (e.g. 3 and 7) when ethanol is not one of the selective pressures.

Isolates 9 and 11 were next compared with AWRI 796 and two commercial strains, EC1118 and Fermichamp® under the same conditions (Figure S2, Supporting Information), revealing different extents of fermentation. AWRI 796 (3.7 ± 1.5 g 1⁻¹ residual sugar) and isolate 11 (3.8 ± 0.9 g 1⁻¹) performed similarly, whilst isolate 9 was the best performer, having only 1.2 ± 0.3 g 1⁻¹ residual sugar in the terminal sample, below what is considered dry (2.5 g 1⁻¹). Interestingly, EC1118 and Fermichamp® failed to complete fermentation, with 16.6 ± 0.8 g 1⁻¹ and 9.7 ± 1.4 g 1⁻¹ sugar, respectively. Based on this data, isolate 9 was chosen for detailed characterization.

**Increased uptake of ¹⁴C fructose in cells of DE isolate 9 and Fermichamp®**

Isolate 9 (Tee 9) was compared to the parent strain, AWRI 796, and Fermichamp® (Oenobrand), a fructophilic S. cerevisiae wine strain typically used to restart high alcohol fermentations that
Figure 3. Fermentation performance of four evolved yeast isolates compared to parent strain AWRI 796. Sugar consumption curves are shown for four isolates and the parent, AWRI 796. (A) Residual glucose; (B) residual fructose, and (C) total residual sugars. Fermentations were conducted in 250 ml of CDGJM_G+F230 (115 g l⁻¹ glucose, 115 g l⁻¹ fructose, and 600 mg l⁻¹ YAN). DCW of 10 ml of culture was determined as was uptake of [¹⁴C] D-fructose (50 μCi, specific activity 9.25–13.3 GBq/nmol) by the culture (1.3 ml of fermenting culture resuspended in 0.02 M KH₂PO₄). Washed samples (on filters) placed in 4 ml scintillation fluid were counted as ¹⁴C CPM for 2 min. Fructose uptake rate (nmol mg⁻¹ DCW) was determined from a calibration curve ([¹⁴C] D-fructose spiked filtered fermentation supernatant) and DCW values (data not shown).

Figure 4. Uptake of ¹⁴C fructose by yeast cells from fermenting cultures in CDGJM. Triplicate cultures of AWRI 796, Tee 9 and Fermichamp® were grown in CDGJM_G+F230 (115 g l⁻¹ glucose, 115 g l⁻¹ fructose, and 600 mg l⁻¹ YAN). DCW of 10 ml of culture was determined as was uptake of [¹⁴C] D-fructose (50 μCi, specific activity 9.25–13.3 GBq/nmol) by the culture (1.3 ml of fermenting culture resuspended in 0.02 M KH₂PO₄). Washed samples (on filters) placed in 4 ml scintillation fluid were counted as ¹⁴C CPM for 2 min. Fructose uptake rate (nmol mg⁻¹ DCW) was determined from a calibration curve ([¹⁴C] D-fructose spiked filtered fermentation supernatant) and DCW values (data not shown).

Whole genome sequence analysis to identify mutations introduced via mutagenesis and DE

The genomes of DE isolate Tee 9 and the parental strain, AWRI 796, together with Fermichamp® were sequenced using Illumina MiSeq v3 technology. Sequence comparison of our isolate of Fermichamp® to the SacCer3 reference genome (lab strain S288c) confirmed the presence of a variant HXT3 allele reported to be responsible for enhanced fructose fermentation (Guillaume et al. 2007). Zuchowska et al. (2015) found that strains with the S288c allele took up glucose faster than fructose, whereas strains with the Fermichamp® allele utilized fructose to a greater extent. AWRI 796 and Tee 9 lacked all 10 nonsynonymous mutations seen in the Fermichamp® Hxt3p (data not shown; Zuchowska et al., 2015). The FSY1 gene encoding the high affinity fructose H⁺ symporter (data not shown; Galeote et al. 2010, Borneman et al. 2016) was also evident in the Fermichamp® sequence but not AWRI 796. Furthermore, no similarities were found between Fermichamp® and Tee 9 that were not in AWRI 796 that could explain the improved fermentation phenotype of Tee 9 (data not shown).

For the purpose of this paper, we report only on the bioinformatics analysis of the genomes of Tee 9 and the ancestral strain, AWRI 796, which was previously sequenced to 20-fold coverage with a combination of shotgun and paired-end methods using Roche 454 (GS FLX Titanium) chemistry (Borneman et al. 2011). The authors reported AWRI 796 as largely diploid with amplification of chromosome I and a 200 kb segment of chromosome XIV detected through CNV analysis. Furthermore, AWRI 796 was mostly homozygous with 8996 homozygous SNPs and 1041 heterozygous SNPs. The clonal isolate used in this experiment differed in that copy number variants were not found (File S2, Supporting Information), although the high similarity scores using Sourmash (Pierce et al. 2019); MP2_S2 (99.7%) vs. SRR2967854 (99.2%) in comparison to S288c (86.8%) suggested that the isolates were the same genotype.

Tee 9 was confirmed by allele frequency data as mostly homozygous and diploid, with no evidence of extensive LOH. However, 11 homozygous de novo SNPs were present in the Tee 9 genome, with one region on chromosome XIII containing six of these SNPs (highlighted in File S3, Supporting Information), that may be the result of small mitotic recombination or gene conversion events (all homozygous SNPs noted in File S5, Supporting Information). These results are interesting given that low heterozygosity and extensive LOH in natural wine isolates (Peter et al. 2018a) are reportedly associated with phenotypic variation (Sam-
variants in Tee 9 (File S5, Supporting Information). Interestingly, none of the hex-SNPs (83 mutations), which did not affect the protein sequence, were synonymous. In total, 297 genes were mutated, with 180 encoding proteins with one or more amino acid substitution (represented as 93 nonsynonymous SNPs). A total of 82 genes had synonymous mutations (83 mutations), which did not affect the protein sequence (File S5, Supporting Information). Interestingly, none of the hexo-transporters (HXT1-17 and GAL2) nor glucose sensors (SNF3 and RGT2; Krueckberg 1996) were affected.

One gene (LUC7) had a heterozygous single nucleotide insertion (CTTCTTTTG>CCTTCTTTTG) resulting in a frame-shift (File S5, Supporting Information). The encoded protein, Luc7, is a component of the U1 snRNP complex responsible for the correct 5’ splicing of some 280 intron-containing genes (Parenteau et al. 2019). From that study of intron deletion (ΔI) in strains grown at stationary phase or under starvation, it was proposed that introns acted to repress ribosomal protein genes (RPGs) in response to the TORC1–PKA pathway. The effect of such potentially ineffective splicing is unclear especially given that AR2, BAF1, RPL7A, and URA2, which all have introns (Parenteau et al., 2019), also encode for protein function (File S5, Supporting Information). Fba1 (G135S) was predicted to be deleterious (PROVEAN and PhD) using three different software (Kelley et al. 2015), where G135 could be substituted with Arg, Asp, Gln, or Pro, consistent with the predictions from the work of Boer and coworkers (2008) in which Fba1 (G135S) behaves similarly to the parent, AWRI 796, under all three nitrogen conditions (Fig. 5). The ratio between glucose and fructose consumption, a measure of fructophilicity, is similar for the parent and single gene mutants, with Tee 9 having a slightly larger ratio, indicative of increased fructophilicity (Table S3, Supporting Information). Under low nitrogen (83 mg l⁻¹ YAN), the parent, AWRI 796, and single gene mutants failed to complete fermentation by the time the experiment was terminated at 384 h. The residual sugar was 23.7 ± 3.2 g l⁻¹ (AWRI 796), 20.78 ± 2.6 g l⁻¹ (AWRI 796 Fba1 (G135S)), and 20.4 g ± 3.3 g l⁻¹ (AWRI796 Gid7 (E726K)). Fructose represented 93.3%, 93.4%, and 94.5% of the respective residual sugar at this point. Acetic, malic, and succinic acid levels (and glycerol) were reduced in many cases with Tee 9, and whilst statistically significant are likely to only have an impact on wine sensory properties in the case of acetic acid (Fig. 6). The effect of the Fba1 (G135S) and Gid7 (E726K) mutations on these metabolites was inconsistent.

Under all conditions, Tee 9 had increased cell mortality at stationary phase compared to the other strains, which was reflected in the decreased biomass (mg/10⁶ total cells; Figure S4, Supporting Information). The work of Boer and coworkers (2008) suggests that Tee 9 may have underlying auxotrophic requirements such that when the specific nutrient runs out, the strain fails to undergo cell cycle arrest at G0/G1 and enter a resting state required for survival. Failure to regulate growth control networks such as TOR and PKA regulatory networks results in wastage of cellular glucose and ultimately in death (Boer et al. 2008).

The fermentation outcomes for AWRI 796 Fba1 (G135S) and AWRI796 Gid7 (E726K) support the predictions made from comparisons of Fba1 with other aldolase proteins using Physre2 software (Kelley et al. 2015), where G135 could be substituted with several structurally unrelated amino acids, substantiating that it is not critical for activity. Similarly, analysis of Gid7 with other WD40 motif proteins showed E726K to be a common variant (Phyre 2; data not shown), which is consistent with the protein predictions made by the three algorithms. We conclude that whilst protein predictions may provide useful guidelines for protein structure–function relationships, they are not always reliable.
Figure 5. Sugar utilization curves of yeast grown in CDGJM of different nitrogen content and Semillon juice. AWRI796, Tee 9 and CRISPR mutants AWRI 796 Gid7 (E726K) and AWRI 7896 Fba1 (G135S) were evaluated through triplicate fermentations of conducted in CDGJM+G230 (115 g l–1 glucose and 115 g l–1 fructose) with various N contents (83, 230, and 368 mg l–1 YAN) or Semillon juice (113 g l–1 glucose, 120 g l–1 fructose, and 96 mg l–1 YAN) at 18 °C. Residual glucose (*), fructose (abcd), and total sugar (▲) are shown.

and open to interpretation. From the data, it is clear that the two mutations chosen, Fba1 (G135S) and Gid7 (E726K), do not affect protein function nor fermentation outcome, and that other mutations identified through SNP analysis need further investigation in a similar manner.

Idenfication of potential genes associated with adaption and improved fermentation in CDGJM using GO analysis

GO analysis was undertaken to focus on other processes that could be relevant to Tee 9’s adaption and improved fermentation performance in CDGJM. The 180 genes having nonsynonymous mutations (together with the 98 predicted to be deleterious) were classified accordingly using GO Slim Mapper (SGD) and ShinyGo (Ge et al. 2020; File S5, Supporting Information). GO term enrichment analysis (GO Term Finder; SGD) highlighted genes over-represented (enriched) for GO terms related to function (P < 0.05). 42 out of the 180 genes were associated with ‘small molecular binding’ (SGD); 25 were predicted to have deleterious mutations, and were also associated with ‘nucleotide binding’ and ‘nucleoside phosphate binding’ (File S5, Supporting Information). Glycogen metabolism is a possible focal point for future analysis, given that four genes involved in synthesis and degradation were over-represented amongst the 42 genes.

Discussion

Random mutagenesis in combination with DE was used to target the differential consumption of glucose and fructose during alcoholic fermentation, with the goal of increasing fructophilicity and OFP. Whilst strains such as EC1118 and Fermichamp® are already efficient at fructose utilization, especially under conditions leading to high ethanol content wines, the aim was not to see whether such strains could be further enhanced. Instead, the goal was to determine whether a commonly used strain with otherwise desirable winemaking properties could be improved specifically in its fermentation dynamics. AWRI 796 met the criteria, based on its fermentation performance (Liccioli et al. 2011a), and prominence in red wine production in Australia (Nordestgaard 2019). This sector alone, represents 58% of export market valued at $2.8 b (https://www.wineaustralia.com/market-insights/australian-wine-sector-at-a-glance).
Figure 6. Metabolite production by yeasts grown in CDGJM with variable nitrogen and Semillon juice. AWRI796, Tee 9 and CRISPR mutants AWRI 796 Gid7 (E726K) and AWRI 796 Fba1 (G135S) were evaluated as triplicate fermentations in CDGJM_G+F230 (115 g l⁻¹ glucose and 115 g l⁻¹ fructose) with various nitrogen contents (83, 230, and 368 mg l⁻¹ YAN) at 18 °C. Fermentations in Semillon juice (113 g l⁻¹ glucose, 120 g l⁻¹ fructose, and 96 mg l⁻¹ YAN) were undertaken separately. All strains completed fermentation under the different conditions except for CDGJM_G+F230 with 83 mg l⁻¹ YAN, where only Tee 9 finished. Acetic acid, succinic acid, glycerol, and ethanol were measured by HPLC on thawed terminal samples. One-way ANOVA with Dunnett’s multiple comparison testing was undertaken against AWRI 796 (P < .05). One asterisk (*) identifies adjusted P-values between .01 and .05, two asterisks (**) identify adjusted P-values between 0.01 and 0.001, and so on.
Prior work demonstrated AWRI 796 to be a medium-fast fermenting strain in CDGJM containing equimolar glucose and fructose, or fructose alone (Liccioli et al. 2011a). However, it still favoured glucose consumption, having a glucose to fructose (GLU/FRU) ratio of 0.64 compared to the fructophilic wine strain, Fermichamp® (0.76; Liccioli et al. 2011a). The latter is commonly used to restart fermentations especially in high ethanol wines. The two strains differ in genetic backgrounds in terms of hexose transporters. AWRI 796 lacks the high affinity fructose transporter gene, FSY1 (Galeote et al. 2010) present in the genomes of EC1118 and Fermichamp® (Borneman et al. 2016), as well as the variant HXT3 allele thought responsible for the strain’s superior ability to consume fructose (Guillaume et al. 2007).

The approach was a proof of concept whereby random mutagenesis was used together with DE, the originality of the experiment being the use of fructose alone as the selective condition for adaptation. The genetic heterogeneity of the starting population was artificially increased by EMS treatment to enhance the likelihood of adaptive mutations sooner in continuous culture. For the DE experiment, the culture was grown in a defined medium reminiscent of grape juice in terms of phosphate, sulfate, vitamins, and minerals (Henschke and Jiranek 1993). Fructose was limiting, with ergosterol and Tween 80 as a source of steroids and fatty acids (Ribéreau-Gayon et al. 2006), and excess nitrogen (as amino acids and ammonium) added to promote cellular metabolism (Bisson, 2005). Fructose supply was regulated such that the overall concentration in the bioreactor was sufficient to maintain an appropriate cell density (Lane et al. 1999) required for DE (Paquin and Adams 1983, Wahl and Krakauer 2000, Wick et al. 2002). It was hypothesized that the most efficient fructose utilizers would be the fittest under these conditions, thus cell density was reduced and only a low concentration of fructose supplied such that it remained undetectable in the bioreactor. These conditions were sufficient to select pressure to generate adaptations to improve the efficiency of fructose utilization.

Sacharomyces cerevisiae (as an asexual population) is known to evolve as a mixture of distinct genotypes, which either coexist or undergo a temporal and dynamic succession within the population (Kao and Sherlock 2008). Given that the estimated rate of adaptive mutation is in the order of 1–10¹¹ cell divisions (Zeyl 1983, W icketal. 2002). It was hypothesized that the most efficient fructose utilizers would be the fittest under these conditions, thus cell density was reduced and only a low concentration of fructose supplied such that it remained undetectable in the bioreactor. These conditions were sufficient to select pressure to generate adaptations to improve the efficiency of fructose utilization.

Saccharomyces cerevisiae (as an asexual population) is known to evolve as a mixture of distinct genotypes, which either coexist or undergo a temporal and dynamic succession within the population (Kao and Sherlock 2008). Given that the estimated rate of adaptive mutation is in the order of 1–10¹¹ cell divisions (Zeyl 1983, W icketal. 2002). It was hypothesized that the most efficient fructose utilizers would be the fittest under these conditions, thus cell density was reduced and only a low concentration of fructose supplied such that it remained undetectable in the bioreactor. These conditions were sufficient to select pressure to generate adaptations to improve the efficiency of fructose utilization.

Saccharomyces cerevisiae (as an asexual population) is known to evolve as a mixture of distinct genotypes, which either coexist or undergo a temporal and dynamic succession within the population (Kao and Sherlock 2008). Given that the estimated rate of adaptive mutation is in the order of 1–10¹¹ cell divisions (Zeyl 1983, W icketal. 2002). It was hypothesized that the most efficient fructose utilizers would be the fittest under these conditions, thus cell density was reduced and only a low concentration of fructose supplied such that it remained undetectable in the bioreactor. These conditions were sufficient to select pressure to generate adaptations to improve the efficiency of fructose utilization.

Saccharomyces cerevisiae (as an asexual population) is known to evolve as a mixture of distinct genotypes, which either coexist or undergo a temporal and dynamic succession within the population (Kao and Sherlock 2008). Given that the estimated rate of adaptive mutation is in the order of 1–10¹¹ cell divisions (Zeyl 1983, W icketal. 2002). It was hypothesized that the most efficient fructose utilizers would be the fittest under these conditions, thus cell density was reduced and only a low concentration of fructose supplied such that it remained undetectable in the bioreactor. These conditions were sufficient to select pressure to generate adaptations to improve the efficiency of fructose utilization.

Saccharomyces cerevisiae (as an asexual population) is known to evolve as a mixture of distinct genotypes, which either coexist or undergo a temporal and dynamic succession within the population (Kao and Sherlock 2008). Given that the estimated rate of adaptive mutation is in the order of 1–10¹¹ cell divisions (Zeyl 1983, W icketal. 2002). It was hypothesized that the most efficient fructose utilizers would be the fittest under these conditions, thus cell density was reduced and only a low concentration of fructose supplied such that it remained undetectable in the bioreactor. These conditions were sufficient to select pressure to generate adaptations to improve the efficiency of fructose utilization.
indeed necessary, with the construction of a double mutant or reciprocal construction, i.e. the introduction of the AWRI 796 alleles into Tee 9, providing better insight into this.

High coverage illumina genome sequencing together with variant calling analysis was undertaken to compare the genomes of Tee 9, its parent, AWRI 796, and Fermichamp®. We were interested to see the mutational landscape (i.e. transitions, transversions, indels, and so on; Cano and Payne 2020) in Tee 9 arising from mutagenesis and adaption to low fructose-containing CDGJM. Moreover, how the few differences between the two strains (Tee 9 vs AWRI796) could result in very different fermentation phenotypes, given the genetic diversity available in wine strains (Borneman et al. 2016) was also of interest. In total, 371 mostly heterozygous mutations (given the usage of a mutagen) were identified by variant calling analysis (File S5, Supporting Information). Not all are relevant to the improved phenotype since most mutations do not have a phenotype when heterozygous. Furthermore, whilst some of the mutations were beneficial, other mutations present might also cause undesirable traits unrelated to fermentation performance. One such example is Aus1(K788A) and (H935A). Aus1 is the major transporter for cholesterol and plant sterols with uptake of yeast ergosterol being low in a mutant (Papay et al. 2020). Expression is triggered under anaerobic growth when ergosterol synthesis is arrested and cells utilize exogenous sterol. The K788A mutation located within the nucleotide binding domain (782–789) abolishes ATPase activity and thus reduces cholesterol, plant sterol, and ergosterol uptake by 86%, 91%, and 50%, respectively, leading to cell death under anaerobic conditions (Papay et al. 2020). H935A, a ‘H-loop’ mutation, effects ATPase activity but not viability. The T1267I mutation identified in this study is also considered a ‘H-loop’ mutation and may have a similar effect on ATPase activity and substrate specificities. These mutations may have contributed to the lower viability of Tee 9 (Figure S4, Supporting Information).

GO analysis of genes having nonsynonymous SNPs giving rise to protein variants (File S5, Supporting Information) not surprisingly highlighted a diversity of processes and major gene/pathway interactions (Costanzo et al. 2010) RNAseq (Rossignol et al. 2003, Marks et al. 2008) could corroborate in silico predictions made by homology modelling and GO analysis, such as genes related to glycan synthesis (UPC1 and GLG1) and mobilization (GPH1 and DBI). Furthermore, YDR277C encoding Mth1, a negative regulator of the glucose-sensing signal transduction pathway (Lafuente et al. 2000) and YGR014W encoding Msb2, an osmosensor in the Sho1p-mediated HOG pathway (Hohmann, 2009), have yet to be tested in relation to fermentation. FAT3 (required for fatty acid uptake) and TPK1 (a CAMP-dependent protein kinase involved in Ras-CAMP signalling) are also likely candidates, as deletion of their respective paralogs, INA1 and TPK2, is associated with shortened fermentation duration (Peter et al. 2018b).

The difficulty in predicting the outcome of the DE strategy, in this instance improved sugar utilization (which inadvertently is specific to CDGJM), highlights the complexity behind genome plasticity during adaption to different environmental niches. However, given the relatively small number of mutations compared to what is represented in the overall diversity of wine, industrial and wild Saccharomyces (Borneman, et al. 2016, Peter et al. 2018a, Duan et al. 2018), Tee 9 is still useful in genome-wide association studies whilst the collection of isolates evolved from AWRI 796 could be used in screening for other phenotypes. Ideally, the identification of one or two SNPs as causative mutations, which could be evaluated in other genotypes and used in QTL breeding programs, would allow for the transfer of a beneficial trait to produce tailored wine strains (Peltier et al. 2019). Evaluation of such variants would also better our understanding of the metabolic processes behind adaptation to fermentation stress.

**Data availability**

Materials and data are within the article and supplementary data.

**Supplementary data**

Supplementary data are available at FEMSYR online.

**Authors’ contributions**

M.E.W. wrote the the manuscript. T.L.W. and V.J. designed the DE experiments. T.L.W. performed the DE and evaluation of evolved isolates and genome sequencing. C.R.L.L. and M.E.W. undertook the genome analysis. S.F. reanalyzed the CNV data. M.E.W., T.A.L., and Y.B. designed and constructed yeast FBA1 and GIT7 mutants via CRISPR, and did the fermentations. M.E.W., V.J., C.R.L.L., M.J.D., and S.F. contributed to the paper revision. V.J. is the corresponding author. All authors read and approved the manuscript.

**Authorship declaration**

All authors agree that this manuscript is original, has not been published before, and is not currently being considered for publication elsewhere. We confirm that the manuscript has been read and approved by all named authors, and all have contributed significantly to the paper. The order of authors listed in the manuscript has been approved by all of us. We understand that the corresponding author is the sole contact for the editorial process and is responsible for communicating with the co-authors on the progress of the submission.

**Acknowledgements**

Special thanks to Dr Cristian Varela and Dr Simon Schmidt for their technical help with the continuous culture experiments and high-throughput sugar analysis. Dr Paul Chambers is also acknowledged for his guidance as a PhD supervisor to T.L.W. Thank you also to Nick van Holst Pellekaan for assistance with flow cytometry analysis and HPLC analysis of metabolites. We acknowledge the help of Dr Federico Tondini with the preliminary ‘gene targeted’ bioinformatics analysis of Tee 9 vs. AWRI 796 in relation to hexose transport. Thanks also to Dr Jennie Gardner for the final read-through and commentary on the paper.

**Funding**

This project was supported by funding from Wine Australia (UA0505, UA1101, and UA1302) and The Australian Research Council Training Centre for Innovative Wine Production (www.ARCwinecentre.org.au; project number 1C170100008), which is funded by the Australian Government with additional support from Wine Australia and industry partners. Wine Australia invests in and manages research, development, and extension on behalf of Australia’s winegrowers and winemakers and the Australian Government. The University of Adelaide is a member of the Wine Innovation Cluster (http://www.thewaite.org/waite-partner/s/wine-innovation-cluster/). T.A.L. is a recipient of a University of Adelaide Faculty of Sciences Divisional Scholarship and Wine Australia Supplementary Scholarship (AGW Ph1603). C.R.L.L. was
supported by NIH grant T32 HG00035. M.J.D. and C.R.L.L. were supported by the National Science Foundation under grant 1516330. The research of M.D. was supported in part by a Faculty Scholar grant from the Howard Hughes Medical Institute.

Conflict of interest statement. The authors declare there are no conflicts of interest regarding the study.

References

Adams A, Gottschling DE, Kaiser CA et al. Methods in Yeast Genetics. Cold Spring Harbor: Cold Spring Harbor Laboratory Press, 1997.

Alexandre H, Charpentier C. Biochemical aspects of stuck and sluggish fermentation in grape must. J Ind Microbiol Biotechnol 1998; 20: 20–7.

Alonge M, Suyk S, Ramakrishnan S et al. Fast and accurate reference-guided scaffolding of draft genomes. Genome Biol 2019; 20: 1–17.

Alperstein L, Gardner JM, Sundstrom JP et al. Yeast bioprospecting versus synthetic biology—which is better for innovative beverage fermentation? Appl Microbiol Biotechnol 2020; 104: 1999–53.

Altschul SF, Gish W, Miller W et al. Basic local alignment search tool. J Mol Biol 1990; 215: 403–10.

Anjos J, de Sousa RH, Roca C et al. Fsy1, the sole hexose-proton transporter characterized in Saccharomyces yeasts, exhibits a variable fructose: H+ stoichiometry. Biochim Biophys Acta Acta Biomemb 2013; 1828: 201–7.

Berthels NJ, Cordero-Otero R, Bauer F et al. Correlation between glucose/fructose discrepancy and hexokinase kinetic properties in different Saccharomyces cerevisiae wine yeast strains. Appl Microbiol Biotechnol 2008; 77: 1083–91.

Berthels NJ, Otero C, Bauer FF et al. Correlation between glucose/fructose discrepancy and hexokinase kinetic properties in different Saccharomyces cerevisiae wine yeast strains. FEMS Yeast Res 2004; 4: 683–9.

Bisson LF, Butzke CE. Diagnosis and rectification of stuck and sluggish fermentations. Am J Enol Viticult 2000; 51: 168–77.

Bisson LF. Stuck and sluggish fermentations. Am J Enol Viticult 1999; 50: 107–19.

Bisson LF. Stuck and sluggish fermentations. Internet J Viticult Enol 2005; 9: 1–14.

Boehringer-Mannheim. Methods of biochemical analysis and food analysis using test-combinations. D-glucose/D-fructose. UV method. Boehringer-Mannheim GmbH Biochemicals. Mannheim: 1989, 50–5.

Boer VM, Amini S, Botstein D Influence of genotype and nutrition on survival and metabolism of starving yeast. Proc Natl Acad Sci 2008; 105: 6990–5.

Boles E, Hollenberg CP. The molecular genetics of hexose transport in yeasts. FEMS Microbiol Rev 1997; 21: 85–111.

Borneman AR, Desany BA, Riches D et al. Whole-genome comparison reveals novel genetic elements that characterize the genome of industrial strains of Saccharomyces cerevisiae. PLoS Genet 2011; 7: e1001287.

Borneman AR, Forgan AH, Kolouchova R et al. Whole genome comparison reveals high levels of inbreeding and strain redundancy across the spectrum of commercial wine strains of Saccharomyces cerevisiae. G3 Genes (Genomes) (Genetics) 2016; 6: 957–71.

Boulton RB, Singleton VL, Bisson LF, et al. Principles and Practises of Winemaking. Berlin, Heidelberg: Springer Science & Business Media, 1998.

Braun B, Pffrmann T, Menssen R et al. Gid9, a second RING finger protein contributes to the ubiquitin ligase activity of the Gid complex required for catabolite degradation. FEBS Lett 2011; 585: 3856–61.

Cano AV, Payne JL. Mutation bias interacts with composition bias to influence adaptive evolution. PLoS Comput Biol 2020; 16: e1008296.

Chambers PJ, Bellon JR, Schmidt SA et al. Non-genetic engineering approaches for isolating and generating novel yeasts for industrial applications. In: Satyanarayana T, Kunze G (eds.), Yeast Biotechnology: Diversity and Applications. Dordrecht: Springer, 2009.

Choi Y, Sims GE, Murphy S et al. Predicting the functional effect of amino acid substitutions and indels. PLoS ONE 2012; 7: e46688.

Cieśla M, Mierzejew ska J, Adamczyk M et al. Fructose bisphosphate aldolase is involved in the control of RNA polymerase III-directed transcription. Biochim Biophys Acta Mol Cell Res 2014; 1843: 1103–10.

Costanzo M, Baryshnikova A, Bellay J et al. The genetic landscape of a cell. Science 2010; 327: 425.

Coulon J, Husnik JL, Inglis DL et al. Metabolic engineering of Saccharomyces cerevisiae to minimize the production of ethyl carbamate in wine. Am J Enol Viticult 2006; 57: 113–24.

de Sousa RH, Spencer-Martins I, Gonçalves P. Differential regulation by glucose and fructose of a gene encoding a specific fructose/H+ symporter in Saccharomyces sensu stricto yeasts. Yeast 2004; 21: 519–30.

Dequin S, Casaregola S. The genomes of fermentative Saccharomyces. C R Biol 2011; 334: 687–93.

Duan SF, Han PJ, Wang QM et al. The origin and adaptive evolution of domesticated populations of yeast from far east Asia. Nat Commun 2018; 9: 2690.

Dukes B, Butzke C. Rapid determination of primary amino acids in grape juice using an o-phthaldialdehyde/N-acetyl-L-cysteine spectrophotometric assay. Am J Enol Viticult 1998; 49: 125–34. https://www.ajevonline.org/content/49/2/125.

Eldarov MA, Mardanov AV. Metabolic engineering of wine strains of Saccharomyces cerevisiae. Genes 2020; 11: 964.

Faust GG, Hall IM. SAMBLASTER: fast duplicate marking and structural variant read extraction. Bioinformatics 2014; 30: 2503–05.

Fink GR. The biochemical genetics of yeast. In: Tabor H, White Tabor (eds.). Methods Enzymol. Vol. 17, 1970, 59–78.

Fleet GH, Lafon-Lafourcade S, Ribéreau-Gayon P. Evolution of yeasts and lactic acid bacteria during fermentation and storage of Bordeaux wines. Appl Environ Microbiol 1984; 48: 1034–8.

Galeote V, Novo M, Salema-Oom M et al. FSY1, a horizontally transferred gene in the Saccharomyces cerevisiae EC1118 wine yeast strain, encodes a high-affinity fructose/H+ symporter. Microbiology 2010; 156: 3754–61.

Gardner JM, McBryde C, Vystavelova A et al. Identification of genes affecting glucose catabolism in nitrogen-limited fermentation. FEMS Yeast Res 2005; 5: 791–800.

Garrison E, Marth G. Haplotype-based variant detection from short-read sequencing. arXiv 2012. https://arxiv.org/abs/1207.3907.

Ge SX, Jung D, Yao R. ShinyGO: a graphical gene-set enrichment tool for animals and plants. Bioinformatics 2020; 36: 6268–9.

Gietz D, St Jean A, Woods RA et al. Improved method for high efficiency transformation of intact yeast cells. Nucleic Acids Res 1992; 20: 1425.

Gonçalves P, de Sousa RH, Spencer-Martins I. FSY1, a novel gene encoding a specific fructose/H+ symporter in the type strain of Saccharomyces carlsbergensis. J Bacteriol 2000; 182: 5628–30.

Guillaume C, Delobel P, Sablayrolles J-M et al. Molecular basis of fructose utilization by the wine yeast Saccharomyces cerevisiae: a mutated HXT3 allele enhances fructose fermentation. Appl Environ Microbiol 2007; 73: 2432–9.

Heard GM, Fleet GH. Growth of natural yeast flora during the fermentation of inoculated wines. Appl Environ Microbiol 1985; 50: 727–8.

Hecht M, Bromberg Y, Rost B. Better prediction of functional effects for sequence variants. BMC Genomics 2015; 16: S1.
Henschke P, Jiranek V. Yeasts: metabolism of nitrogen compounds. In: Fleet GH, (ed.), Wine Microbiology and Biotechnology. Chur: Harwood Academic Publishers, 1993, 77–163.

Hohmann S. Control of high osmolarity signalling in the yeast Saccharomyces cerevisiae. FEBS Lett 2009;583:4025–9.

Husnik JI, Volschenk H, Bauer J et al. Metabolic engineering of malolactic wine yeast. Metab Eng 2006;8:315–23.

Kao KC, Sherlock G. Molecular characterization of clonal interference during adaptive evolution in asexual populations of Saccharomyces cerevisiae. Nat Genet 2008;40:1499–504.

Kelley LA, Mezulis S, Yates CM et al. The Phyre2 web portal for protein modeling, prediction and analysis. Nat Protoc 2015;10:845–58.

Kessi-Pérez E I, Molinet J, Martínez C. Disentangling the genetic bases of Saccharomyces cerevisiae nitrogen consumption and adaptation to low nitrogen environments in wine fermentation. Biol. Rev 2020;53:2.

Krueckeberg AL. The hexose transporter family of Saccharomyces cerevisiae. Arch Microbiol 1996;166:283–92.

Kutyna DR. Isolation of low ethanol producing yeast strains using adaptive evolution. Ph.D. Thesis, Victoria University, 2008. https://vuir.vu.edu.au/15484/ (17 May 2022, date last accessed).

Laffort. Focus_EN_Breeding. 2021. https://laffort.com/wp-content/uploads/Focus/Focus_EN_Breeding.pdf (17 May 2022, date last accessed).

Lafuente MJ, Gancedo C, Jauniaux J-C et al. Adaptation of a continuous-culture technique for the selection of mutants tolerant to extreme environmental stress. Biotechnol Bioeng 2009;53:351–6.

Lee ME, DeLoache WC, Cervantes B et al. A highly characterized yeast toolkit for modular, multipart assembly. ACS Synth Biol 2015;4:975–86.

Li D, Liu C-M, Luo R et al. MEGAHT: an ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph. Bioinformatics 2015;31:1674–6.

Li D, Luo R, Liu C-M et al. MEGAHT v1.0: A fast and scalable metagenome assembler driven by advanced methodologies and community practices. Methods 2016;102:3–11.

Li H, Handsaker B, Wysoker A. Genome Project Data Processing Subgroup et al. Genome Project Data Processing Subgroup. The sequence alignment/map format and SAM tools. Bioinformatics 2009;25:2078–9.

Li H. Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. arXiv 2013. https://arxiv.org/abs/1303.3997.

Liccioni T, Chambers PJ, Jiranek V. A novel methodology independent of fermentation rate for assessment of the fructophilic character of wine yeast strains. J Ind Microbiol Biotechnol 2011a;38:833–43.

Liccioni T, Tran TMT, Cossolino D et al. Microvinification—how small can we go?. Appl Microbiol Biotechnol 2011b;89:1621–8.

Lin MM-H, Boss PK, Walker ME et al. Evaluation of indigenous non-Saccharomyces yeasts isolated from a South Australian vineyard for their potential as wine starter cultures. Int J Food Microbiol 2020;312:108373.

López-Malo M, García-Rios E, Melgar B et al. Evolutionary engineering of a wine yeast strain revealed a key role of inositol and manno-
a genome-wide screen identifies eight novel GID genes and indicates the existence of two degradation pathways. Mol Biol Cell 2003;14:1652–63.

Ribéreau-Gayon P, Dubourdieu D, Donèche B et al. Handbook of Enology. Hoboken: John Wiley and Sons Ltd., 2006.

Robinson JT, Thorvaldsdóttir H, Winckler W et al. Integrative genomics viewer. Nat Biotechnol 2011;29:24–6.

Rodicio R, Heinisch JJ. Sugar metabolism by Saccharomyces and non-Saccharomyces yeasts. In: König H, Unden G, Fröhlich J (eds.), Biology of Microorganisms on Grapes in Must and in Wine, Berlin, Heidelberg: Springer, 2009, 113–34.

Rossignol T, Dulau L, Julien A et al. Genome-wide monitoring of wine yeast gene expression during alcoholic fermentation. Yeast 2003;20:1369–85.

Sampaio NMV, Watson RA, Argueso JL. Controlled reduction of genomic heterozygosity in an industrial yeast strain reveals wide cryptic phenotypic variation. Front Genet 2019;10:782.

Schneider RP, Wiley WR. Kinetic characteristics of the two glucose transport systems in Neurospora crassa. J Bacteriol 1971;106:479–86.

Schütz M, Gafner J. Sluggish alcoholic fermentation in relation to alterations of the glucose-fructose ratio. Chemie, Mikrobiologie, Technologie der Lebensmittel 1993;15:73–8.

Sega GA. A review of the genetic effects of ethyl methanesulfonate. Mut Res Rev Genet Toxicol 1984;134:113–42.

Serrano R, Delafuente G. Regulatory properties of the constitutive hexose transport in Saccharomyces cerevisiae. Mol Cell Biochem 1974;5:161–71.

Shaw WM, Yamauchi H, Mead J et al. Engineering a model cell for rational tuning of GPCR signaling. Cell 2019;177:782–96.

Sievers F, Wilm A, Dineen D et al. Fast, scalable generation of high-quality protein multiple sequence alignments using clustal omega. Mol Syst Biol 2011;7:539.

Stanley D, Bandara A, Fraser S et al. The ethanol stress response and ethanol tolerance of Saccharomyces cerevisiae. J Appl Microbiol 2010;109:13–24.

Talevich E, Shain AH, Botton T et al. CNVkit: genome-wide copy number detection and visualization from targeted DNA sequencing. PloS Comput Biol 2016;12:e1004873.

van den Brink J, Canelas AB, van Gulik WM et al. Dynamics of glycolytic regulation during adaptation of Saccharomyces cerevisiae to fermentative metabolism. Appl Environ Microbiol 2008;74:5710.

Wagih O, Galardini M, Busby BP et al. A resource of variant effect predictions of single nucleotide variants in model organisms. Mol Syst Biol 2018;14:e8430.

Wahl LM, Krakauer DC. Models of experimental evolution: the role of genetic chance and selective necessity. Genetics 2000;156:1437–48.

Walker ME, Gardner JM, Vystavelova A et al. Genome-wide identification of the fermentome, genes required for successful and timely completion of wine-like fermentation by Saccharomyces cerevisiae. FEMS Yeast Res 2003;4:339–47.

Walker ME, Nguyen TD, Licioci T et al. Genome-wide identification of the fermentome, genes required for successful and timely completion of wine-like fermentation by Saccharomyces cerevisiae. BMC Genomics 2014;15:552.

Wick LM, Weilenmann H, Egli T. The apparent clock-like evolution of Escherichia coli in glucose-limited chemostats is reproducible at large but not at small population sizes and can be explained with Monod kinetics. Microbiology 2002;148:2889–902.

Wilm A, Aw PPK, Bertrand D et al. LoFreq: a sequence-quality aware, ultra-sensitive variant caller for uncovering cell-population heterogeneity from high-throughput sequencing datasets. Nucleic Acids Res 2012;40:11189–201.

Zeyl C. Capturing the adaptive mutation in yeast. Res Microbiol 2004;155:217–23.

Zuchowska M, Jænicke E, König H et al. Allelic variants of hexose transporter Hxt3p and hexokinases Hxk1p/Hxk2p in strains of Saccharomyces cerevisiae and interspecies hybrids. Yeast 2015;32:657–69.