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Exploring the oenological potential of South African *Saccharomyces cerevisiae* isolates

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ABSTRACT

Over the past fifty years, numerous yeast strains have been selected for the wine industry in a quest to address increasingly specific organoleptic requirements. Indeed, challenges such as the ever-evolving consumer preferences and climate change require the industry to continuously innovate. Interestingly, despite fermentation capabilities and metabolic pathways being mostly conserved across *Saccharomyces cerevisiae* strains, differing intraspecific metabolite production offers never-ending opportunities to meet the industry's demands while enhancing our knowledge of this biodiversity. This study documented selected oenological phenotypic characteristics driven by red wine consumer trends of a collection of *S. cerevisiae* strains collected by the South African Grape and Wine Research Institute. It focussed on the uncharacterised strains' *IRC7* genotype, fermentation kinetics, metabolite production, varietal thiol production, and overall sensory profile of wines. Strain-dependent differences were observed, discovering unique chemical and sensory profiles that contribute to the deeper understanding of the yeast biodiversity within the Stellenbosch region. The distinct strains identified and characterised hold the potential for novel wines that meet the contemporary demands for unique sensorial experiences. This study aids in responding to current consumer trends as well as contributing to the conversation on the importance of natural isolates in sustaining and revitalising the dynamic landscape of the wine industry.

KEYWORDS: wine, *Saccharomyces cerevisiae*, alcoholic fermentation, phenotypic screening, sensory profiles

INTRODUCTION

The use of selected starter cultures to drive alcoholic fermentation is a relatively recent development in the long winemaking history, starting at the very end of the 19th century but becoming a standard practice only in the 1970s. Currently, most industrial starter cultures for winemaking are strains of the species *Saccharomyces cerevisiae*, although other yeast species progressively joined the pool of commercialised yeasts in the past 20 years.

The strain of yeast that ferments the grape juice has a major impact on the final wine style. The combination of genetic diversity within strains of the same species and the environmental conditions occurring during fermentation dictates the yeast metabolic footprint and ultimately the sensory profile of wine (Álvarez *et al.*, 2023). Despite the implementation of various laboratory techniques such as adaptive evolution, breeding, and random mutagenesis to develop new yeast strains exhibiting targeted phenotypes, indigenous yeasts are still acknowledged as a reservoir of untapped diversity (Molinet & Cubillos, 2020). The potential exploitation of such diversity gains popularity when considered alongside the evolving consumer demands for wine, which extend beyond traditional taste preferences to include health and environmental concerns and a desire for sensory novelty (Deroover *et al.*, 2021; Pretorius, 2000). *S. cerevisiae* screening experiments such as those conducted by Bordet *et al.* (2021) and Monnin *et al.* (2024) acknowledge this oenological biodiversity, attributing the phenotypic differences observed to varying specific carbon metabolism-related gene presence. This biodiversity may prove useful for winemakers attempting to address existing challenges faced by the wine industry such as climate change, which impact grape juice composition, an increasingly competitive market, and evolving consumer preferences.

In recent years, consumer preferences have influenced the harvest time, especially in warmer, drier regions. Indeed, winemakers have been extending the grape harvesting period, resulting in grapes that are riper and richer in fruity flavours and exhibit deeper colours amongst other characteristics. These grapes also have higher sugar concentrations resulting in higher alcohol levels, commonly exceeding 13.5 % v/v, with some wines even exceeding 16 % v/v. This shift raises concerns not only related to health but also to economics and wine quality (Goold, 2017). Indeed, wines with higher alcohol content face greater taxation in some countries, increasing costs for consumers. Health professionals and lawmakers often criticise these wines for contributing to alcohol-related societal issues. Additionally, excessive alcohol can disrupt the organoleptic balance of a wine.

In response to these challenges, the wine industry is exploring ways to reduce alcohol content without sacrificing the flavour profiles or increasing the production costs. Strategies include adjusting vineyard practices to control sugar accumulation in grapes, blending different types of grapes, and employing various winemaking techniques to reduce alcohol levels post-fermentation. These efforts aim to maintain the wine's quality while addressing the economic, health, and sensory concerns associated with high alcohol content. The effectiveness and implications of these strategies continue to be a topic of discussion and research in the wine industry.

As a contribution to these global endeavours and in the search for new yeast strains responding to the current challenges face by the wine industry, this study aimed to evaluate and compare oenologically relevant phenotypic data of uncharacterised *S. cerevisiae* yeast strains including hydrogen sulfide ('rotten egg' aroma) production, fermentation performance, metabolite production, potential to release varietal thiols, and sensory attributes. These uncharacterised strains were obtained from the South African Grape and Wine Research Institute's microbial culture collection. Initial experiments were conducted in synthetic grape juice (SGJ), providing a more controlled environment due to the many variables involved in real winemaking. This approach ensured that the strains were evaluated without interference from other microorganisms. Subsequently, fermentations for top-performing strains according to set criteria were carried out using Cabernet Sauvignon and Merlot grape juices to assess the strains in a context more akin to real-world industrial conditions.

MATERIALS AND METHODS

1. Yeast strains used in this study

Forty-two uncharacterised yeast isolates (Table 1) were obtained from the South African Grape and Wine Research Institute's microbial culture collection. These yeast strains were previously identified as being *S. cerevisiae* strains through the amplification and analysis of their ITS1-5.8S rRNA-ITS2 DNA region (data not shown). Additionally, three reference strains (Vivace, WH514, and NT50) were used (Table 1). Isolates Y911 to Y920 were isolated in 2012 from freshly hand-destemmed and crushed Chenin blanc grape must prepared from grapes that were collected aseptically with gloves and ethanol-sterilised sheers, while Y1412 to Y1447 were isolated in 2021 from Pinotage grape must prepared from grapes aseptically collected from different vineyards as part of an investigation of the Pinotage microbiome composition. The Pinotage grapes were machine destemmed and crushed in the experimental cellar of the South African Grape and Wine Research Institute.

2. Storage and preculture conditions

2.1. Yeast cryopreservation

The yeast isolates used in this study were supplied on streak plates containing yeast extract peptone dextrose (YPD) agar from which one colony was inoculated into test tubes containing 5 mL YPD broth consisting of 1 % yeast protein extract, 2 % peptone, and 2 % glucose. These test tubes were incubated on a spinning wheel at 40 rpm at 30 °C for 16 h to allow the yeast isolates to reach the exponential growth phase. The yeast preculture was combined with sterile glycerol to yield 2 mL of 25 % glycerol and the preculture was transferred into cryotubes and stored at –80 °C.

TABLE 1. *Saccharomyces cerevisiae* yeast isolates used in this study. IWBT: Microbial culture collection of the South African Grape and Wine Research Institute (ex-Institute for Wine Biotechnology), Stellenbosch University.

Strain/Isolate	Source (place and/or year of isolation, culture collection, or company commercialising strains)
Vivace	Renaissance Yeast (VIC-23) (Vancouver, VC, Canada)
WH314	Distillery (NRRL Y-567, ARS Culture Collection, Peoria, IL, USA)
NT50	Anchor Oenology (Cape Town, South Africa)
IWBT Y911	Chenin blanc from Riebeek Casteel (2012)
IWBT Y912	Chenin blanc from Riebeek Casteel (2012)
IWBT Y913	Chenin blanc from Riebeek Casteel (2012)
IWBT Y914	Chenin blanc from Riebeek Casteel (2012)
IWBT Y915	Chenin blanc from Riebeek Casteel (2012)
IWBT Y917	Chenin blanc from Riebeek Casteel (2012)
IWBT Y920	Chenin blanc from Riebeek Casteel (2012)
IWBT Y1019	Unspecified South African wine estate (2009)
IWBT Y1020	Unspecified South African wine estate (2009)
IWBT Y1022	Unspecified South African wine estate (2009)
IWBT Y1049	Unspecified South African wine estate (2009)
IWBT Y1167	Unspecified South African wine estate (2009)
IWBT Y1412	Bosman Wines (Haasland)
IWBT Y1452	Kanonkop Wine Estate (Block 411 B)
IWBT Y1453	Beyerskloof Wine Estate (Block I)
IWBT Y1454	Rijks Wine Estate
IWBT Y1417	KWV (Malmesbury)
IWBT Y1418	Groot Constantia Wine Estate
IWBT Y1419	Fairview Estate (Daimant)
IWBT Y1420	Fairview Estate (Spice Route clay soil)
IWBT Y1455	Fairview Estate (Spice Route sandy soil)
IWBT Y1456	Neil Ellis Wine Estate (Jonkershoek)
IWBT Y1457	UniWines Vineyards (Rawsonville)
IWBT Y1458	Delheim Wine Estate
IWBT Y1460	Kaapzicht Wine Estate
IWBT Y1461	De Grendel Wine Estate
IWBT Y1463	Fairview Estate (Spice Route clay soil)
IWBT Y1464	Watervlei Wine Estate
IWBT Y1431	Neil Ellis Wine Estate (Jonkershoek)
IWBT Y1433	Meerlust Wine Estate
IWBT Y1434	Neethlingshof Wine Estate
IWBT Y1435	Kanonkop Wine Estate (Block 512 A)
IWBT Y1436	UniWines Vineyards (Worcester)
IWBT Y1437	Overhex Winery
IWBT Y1439	Overhex Winery
IWBT Y1441	Simonsig Wine Estate
IWBT Y1442	Lanzerac Wine Estate
IWBT Y1443	Robertson Winery (Oppi Klip Koppie)
IWBT Y1444	Unspecified South African vineyard
IWBT Y1445	Robertson Winery
IWBT Y1446	UniWines Vineyard (Rawsonville)
IWBT Y1447	Nuy Winery (Nuwerus)

2.2. Yeast preculture method

All precultures during these experiments were performed in the same manner. Yeast freeze cultures were streaked onto YPD agar plates consisting of 1 % yeast protein extract, 2 % peptone, 2 % glucose, and 2 % agar. These plates were incubated at 30 °C for 48 h after which they were transferred to 4 °C for storage if single colonies were visible. A single colony from the plates was transferred using a sterile loop to a test tube containing 5 mL YPD broth as described above and allowed to incubate spinning at 40 rpm at 30 °C for 16 h. Fifty microliters of inoculated broth were then transferred to a new test tube containing 5 mL YPD broth and again incubated spinning at 40 rpm for 16 h at 30 °C. Yeast growth was then determined using spectrophotometry (optical density at 600 nm) to ascertain the correct volume of YPD to spin down to inoculate the experiment with a standardised concentration of yeast cells (OD_{600nm} = 0.1 corresponding to ca. 10^6 cells/mL).

2.3. Hydrogen sulphide (H_2S) production

The yeast isolates' H_2S production was evaluated using Bismuth Glycine Glucose Yeast (BiGGY)/Nickerson agar (Sigma Aldrich, Saint-Louis, MO, USA) due to its visual discrimination capabilities. The yeast precultures were prepared as previously described and BiGGY agar was prepared according to the manufacturer's instructions. These plates were divided into seven blocks (five isolates and two control strains). Volumes corresponding to 10^6 yeast cells/mL were determined, centrifuged at 16,215 *g* for 5 min, rinsed with 0.9 % NaCl, resuspended in 16 µL 0.9 % NaCl, and spotted onto the BiGGY agar plates. The plates were incubated for five days at 25 °C. Thereafter, images (RAW format) were taken of the respective plates in a controlled light environment. The images were processed using ImageJ-win64 software where they were converted to 8-bit grayscale images. On each image, the negative control (Vivace)'s grayscale value was assigned an arbitrary value of 0 % and the positive control (WH314)'s value that of 100 %. The remaining isolates' grayscale values were then compared to these controls and returned as a percentage. The commercial strain NT50 was arbitrarily used as a reference for acceptable H_2S production where the isolates that produced lower percentages were deemed low H_2S producers, the isolates with higher percentages (up until 75 % of WH314's grayscale value) as moderate producers, and the strains within 25 % of WH314's grayscale value as high producers.

2.4. Fermentations

2.4.1. Synthetic grape juice (SGJ) fermentations

Laboratory fermentations were conducted using SGJ. The medium consisted of 230 g/L total sugar (115 g/L glucose, 115 g/L fructose) and 300 mg/L Yeast Assimilable Nitrogen (YAN). The detailed composition of the medium can be found in Tables S1.1 to S1.5. Precultures were prepared as described above and after 12 h, yeasts were inoculated at 10^6 cells/mL into 50 mL SGJ, aliquoted into 100 mL cylindrical spice jars equipped with a rubber fermentation cap and bubbler. Fermentations occurred at 25 °C on a rotating table (orbital agitation of 125 rpm). All fermentations were performed in triplicate. Fermentation progress was monitored via daily weight loss measurements as a proxy for CO_2 release. Jars containing uninoculated SGJ were included as negative controls to account for water evaporation.

The lag phase was determined as the time taken for each individual strain to release 3 g/L total weight loss, as considered by Zimmer *et al.* (2014). The maximum rate of fermentation (Vmax) was determined as the maximum rate of weight loss. Fermentation duration was defined as the time point at which accumulative weight loss over time of the inoculated samples was no longer significantly different from that of the uninoculated controls. Fifty-millilitre samples were taken at the end of fermentation for chemical analysis. The samples were spun down at 9244 *g* at a temperature of 17 °C for 5 min using a Herolab HiCen SR centrifuge (Wiesloch, Germany) equipped with a SorvallTM SLA600TC rotor. The supernatant was filtered through a 0.22 µm cellulose acetate syringe filter (Sterlitech) into a new sample tube and both the pellet and supernatant were frozen separately at –20 °C until further use.

2.4.2. Grape juice fermentations

Two-hundred-and-fifty kilograms of both Cabernet Sauvignon and Merlot grapes were harvested at Plaisir de Merle wine estate (Simondium, South Africa) on 16 March 2023. The grapes were crushed, de-stemmed, and processed at the Department for Viticulture and Oenology's experimental cellar (Stellenbosch University). For each yeast strain, 3 × 10 kg of crushed grapes from each cultivar were placed in 20 L buckets. To prevent oxidation, 30 ppm of sulphur dioxide (SO_2) was added as potassium metabisulfite and thoroughly mixed.

Parameters	Cabernet Sauvignon	Merlot
Reducing sugar concentration (°Brix)	26.3	23.8
pH	3.57	3.76
Volatile acidity (g/L)	0.08	0.09
Titratable acidity (g tartaric acid/L)	5.22	3.87
Malic acid (g/L)	1.05	1.01
YAN (mg/L)	170	170

TABLE 2. Initial parameters of grape juice after crushing.

Although the aim was to harvest the grapes at a sugar concentration of around 26 °B, the Merlot ripening process stopped at 23.8 °B (Table 2), most likely as a result of the leafroll virus infecting the vineyard. For the purpose of this experiment, the carbon availability needed to be equal between both cultivars, therefore 175 g of sucrose was added to each Merlot bucket to raise the sugar concentration to ± 26 °B. The pH of the Merlot buckets was also lowered to 3.5 using tartaric. Both cultivars showed relatively low YAN concentrations, therefore 2.8 g diammonium phosphate

(DAP) was added to each bucket to raise the YAN level from 170 mg/L to 250 mg/L.

Subsequently, one active dry commercial yeast strain (NT50) was inoculated after rehydration performed according to the supplier's instructions as well as Y1020, Y1022, Y1436, Y1441, and Y1445 strains from liquid cultures, ensuring a temperature difference of less than 10 °C between the must and yeast before inoculation. Fermentation occurred in a controlled environment at 25 °C, with daily monitoring of residual sugar levels. Punch-downs were performed twice a day during this stage. Alcoholic fermentation was monitored until sugar concentrations dropped below 0 °B. Pressing took place when the sugar concentration reached –2 °B and the resulting wine was transferred to 4.5 L bottles for malolactic fermentation (MLF) in a 20 °C fermentation room.

MLF was initiated by inoculation of Solo Select (Anchor Oenology) lactic acid bacteria (LAB) according to the supplier's instructions. To monitor MLF, malic acid was initially determined by enzymatic analyses using an Arena 20XT automated analyzer (Thermo Fisher Scientific, Waltham, MA) weekly until malic acid levels were less than 0.3 g/L. The enzymatic assay used was Enzytec Liquid L-Malic acid (Id-No: E8280, Roche, R-Biopharm). Fifty parts per million SO_2 was added, and the wine was subsequently transferred to a –4 °C environment for stabilisation, where it remained for two weeks. Finally, the wine was racked, free SO_2 adjusted to 35 ppm, and bottled using siphoning. No fining or filtration was employed in the process. One hundred millilitre samples were taken at the end of alcoholic fermentation for chemical analysis, namely, HPLC to quantify sugars, ethanol, glycerol, and organic acids, GC-FID to quantify major volatile compounds, and GC-MS to quantify volatile thiols (3-sulfanylhexanol, 3-sulfanylhexyl acetate, and 4-methyl-4-sulfanyl pentan-2-one) produced by the yeast.

2.5. Genetic screening

2.5.1. DNA extraction

DNA extraction began by preculturing the yeast strains as previously described. Two millilitres of the culture were centrifuged in microcentrifuge tubes at 1841 *g* for 5 min and the supernatant was discarded. The pellet was then treated with 200 µL of lysis buffer, comprising Tris-HCl 10 mM pH 8, EDTA 1 mM pH 8, NaCl 100 mM, TritonX-100 2 % w/v, and SDS 1 % w/v. This mixture was vortexed and transferred to a screw cap microcentrifuge tube containing 200 µL of 0.4 mm glass beads. Then, 200 µL of phenol/chloroform/isoamyl alcohol (25/24/1 v/v) was added, and the mixture was vortexed for 3 min. After adding 200 µL of Tris-EDTA buffer (pH 8), the tubes were centrifuged at 16,215 *g* for 5 min.

The upper phase was then transferred to a new microcentrifuge tube, and the pellet and glass beads were discarded. To the upper phase, 500 µL of chloroform/iso-amyl alcohol (98/2 v/v) was added and the solution was mixed by inversion and centrifuged at 16,215 *g* for 2 min. The aqueous phase was then transferred to a new tube, and the pellet was discarded. Two volumes of absolute ethanol were added to the aqueous phase, and after centrifugation at 16,215 *g* for 5 min, the supernatant was discarded.

The nucleic acid pellet was dissolved in 10 mM TE buffer (pH 8). For every 400 µL, 1 µL RNase was added and the solution was incubated for 5 min at 37 °C, after which 40 µL sodium acetate and 1 mL absolute ethanol were added. After mixing by inversion and incubating at –20 °C for 2 h, the mixture was centrifuged at 16,215 *g* for 3 min. The supernatant was discarded, and the pellet was washed with 70 % ethanol. Following another centrifugation at 16,215 *g* for 3 min and discarding of the supernatant, the pellet was dried and then suspended in milli-Q® water and stored at –20 °C until further use.

2.5.2. Strain genetic fingerprinting

Following the method described by Legras & Karst (2003), PCR amplifications were conducted using the delta12 (5′-TCAACAATGGAATCCCAAC-3′) and delta21 (5′-CATCTTAACACCGTATATGA-3′) primers (Inqaba Biotech, Pretoria, South Africa) in a 25 µL reaction volume comprising 5–20 ng of yeast DNA, Ex Taq™ polymerase (TaKaRa Bio Inc., Japan), 200 µM of each dNTP, 2.5 mM $MgCl_2$, and 1 µM of each primer. Amplifications were performed in a MiniAmp Thermal Cycler model A37028 (Thermo Fisher Scientific) set to an initial denaturation of 4 min at 95 °C, followed by 35 cycles of 30 s at 95 °C for denaturation, 30 s at 46 °C for annealing, and 90 s at 72 °C for extension, with a final elongation step of 10 min at 72 °C. The PCR products were run on 2 % agarose gels containing 0.2 µg/mL ethidium bromide for band visualisation under UV with a 50 bp molecular weight marker (New England Biolabs, Ipswich, MA, USA). Banding patterns were visualised using a Molecular Imager® Gel Doc™ System (Bio-Rad Laboratories) equipped with the Image Lab™ Software v6.0 (Bio-Rad Laboratories).

Banding pattern images were processed using GelJ Software (Heras *et al.*, 2015) and a dendrogram was constructed based on the UPGMA hierarchical clustering method, producing a Newick file which could be visualised in the form of a dendrogram (Figure 1) using the MEGA X software (Tamura *et al.*, 2021). The band similarity was calculated using a Dice coefficient. Two lanes were considered similar above a 90 % match threshold and a 4 % tolerance for band matching was allowed. The dendrogram estimated the degree of relatedness between the strains based on their banding patterns, allowing us to determine if any strain repeats existed within the sample population. To distinguish between genetically distinct and identical strains an arbitrary threshold was implemented in our phylogenetic analysis.

This threshold was established based on the observation that strains to the right of this line exhibited no discernible genetic differences in their banding patterns. Strains falling to the right of the threshold were considered genetically indistinguishable, while those to the left were treated as distinct genotypes. This approach provided a practical method for strain classification, but it is important to note that the threshold is arbitrary and may require adjustment depending on the specific context or more detailed genetic analyses.

2.5.3. *IRC7* genotype screening

To amplify the *IRC7* gene, a PCR was carried out with the primers *IRC7*F, 5′-AGCTGGTCTGGAGAAAATGG-3′ and *IRC7*R, 5′-TCTTCTGCGAGACGTTCAAA-3′ (Inqaba Biotech, Pretoria, South Africa) as described in Roncoroni *et al.* (2011). The PCR reaction mixture consisted of Ex Taq™ polymerase, Ex Taq buffer, 200 µM of each dNTP, 2.5 mM $MgCl_2$, and 1 µM of each primer. The PCR reaction conditions included an initial denaturing step of 2 min at 94 °C followed by 35 cycles of 94 °C for 15 s, 56 °C for 30 s, and 72 °C for 1 min and then a final extension at 72 °C for 5 min. The PCR products were visualised as described above.

2.6. Chemical analyses

2.6.1. Gas chromatography-flame ionisation detector (GC-FID) analysis

GC-FID was performed on synthetic, Merlot, and Cabernet Sauvignon wines (two technical and three biological repeats) at the end of alcoholic fermentation to quantify major esters, higher alcohols, and volatile acids. Samples were spiked with the internal standard 4-methyl-pentan-2-ol (100 µL of 0.5 mg/mL solution in soaking solution) and extracted with diethyl ether. The injection volume was 3 µL and a DB-FFAP, 60 m × 0.32 mm (inner diameter) × 0.5 µm (film thickness) column was used as described in Louw *et al.* (2009).

2.6.2. High-performance liquid chromatography (HPLC) analysis

HPLC was performed on synthetic, Merlot, and Cabernet Sauvignon wines (two technical and three biological repeats) at the end of alcoholic fermentation to determine the concentrations of the major sugars (glucose and fructose), organic acids (citric, tartaric, malic, succinic and acetic acid) as well as glycerol and ethanol. An Agilent 1100 series HPLC system, Chemstation Rev. A10.02 software, an Aminex HPX-87 column (300 mm × 8.8 mm), and a BioRad guard column (30 mm × 4.6 mm) was used, together with the method described by Eyéghé-Bickong *et al.* (2012).

2.6.3. Gas chromatography–mass spectrometry (GC-MS) analysis

GC-MS was performed to quantify volatile thiols at the end of alcoholic fermentation on Merlot and Cabernet Sauvignon wines. It was run at the Central Analytical Facility at Stellenbosch University (Stellenbosch, South Africa).

Briefly, 1 mL sample was added to a 20 mL headspace vial, together with 20 µL of 100 µg/L anisole-d8 internal standard and 9 mL of 20 % NaCl solution. The sample tube was vortexed, sonicated for 3 min, and liquid–liquid extraction (LLE) was then performed. Samples were then centrifuged at 1841 g for 1 min. The diethyl ether layer (top) was transferred into a new vial with sodium sulphate and then transferred again into a 2 mL vial. One microliter was injected with a 5:1 split ratio onto the GC-MS instrument.

Separation was performed on a gas chromatograph (6890N, Agilent technologies network) coupled to Agilent technology inert XL EI/CI Mass Selective Detector (MSD) (5975B, Agilent Technologies Inc., Palo Alto, CA). The GC-MS system was coupled to a CTC Analytics PAL autosampler. Separation of the major wine volatiles was performed on a ZB-FFAP (60 m, 0.32 mm ID, 0.50 µm film thickness) capillary column (Phenomenex, Torrance, California, 33 United States of America). Helium was used as the carrier gas at a flow rate of 1.9 mL/min. The injector temperature was maintained at 240 °C. The oven temperature was programmed as follows: 35 °C for 17 min and ramped at a rate of 12 °C/min until 240 °C and held for 5 min. The MSD was operated in a full scan mode, and the source and quad temperatures were maintained at 230 °C and 150 °C, respectively. The transfer line temperature was maintained at 250 °C. The mass spectrometer was operated under electron impact (EI) mode at an ionisation energy of 70 eV, scanning from 35 to 650 m/z.

2.7. Sensory analysis

Descriptive analysis was performed on both Cabernet Sauvignon and Merlot wines after two months of bottle ageing at 15 °C by a panel of ten members. Prior to the evaluation, institutional ethical clearance was granted (Ref. no: 28416). The panellists were experienced in red wine tasting. The sensory process consisted of three training sessions per cultivar after which a list of sensory descriptors was decided on for each cultivar. The subsequent testing blocks for both cultivars were three days each where panellists were asked to taste in isolated booths. Each treatment was presented to them in clear glasses marked with three-digit codes and a complete Block Design was used to randomise the distribution of the wines presented to the panellists.

2.8. Statistical analysis

Statistical analysis, using XLSTAT software, involved calculating descriptive parameters such as means and standard deviations, conducting a one-way ANOVA, comparing means using Tukey's HSD test with a significance level of $p < 0.05$, and assessing Pearson correlations to elucidate relationships among the analysed parameters. Visualisations were made using XLSTAT and GraphPad (Boston, MA).

The sum-ranked sensory analysis data were analysed using the panel analysis function under the sensory data analysis tab on XLSTAT.

RESULTS

1. Strain differentiation and screening for selected phenotypes of oenological interest

1.1. Genetic fingerprinting

The yeast isolates used in this study had only previously been identified at the species level (*S. cerevisiae*). To ensure that all isolates were indeed different strains, genetic fingerprints were generated by amplifying the inter regions of the different isolates, as a proved method for differentiating strains (Legras & Karst, 2003; Xufre, *et al.*, 2010). The banding patterns were used to generate a dendrogram estimating the degree of relatedness between the isolates (Figure 1, Figure S1).

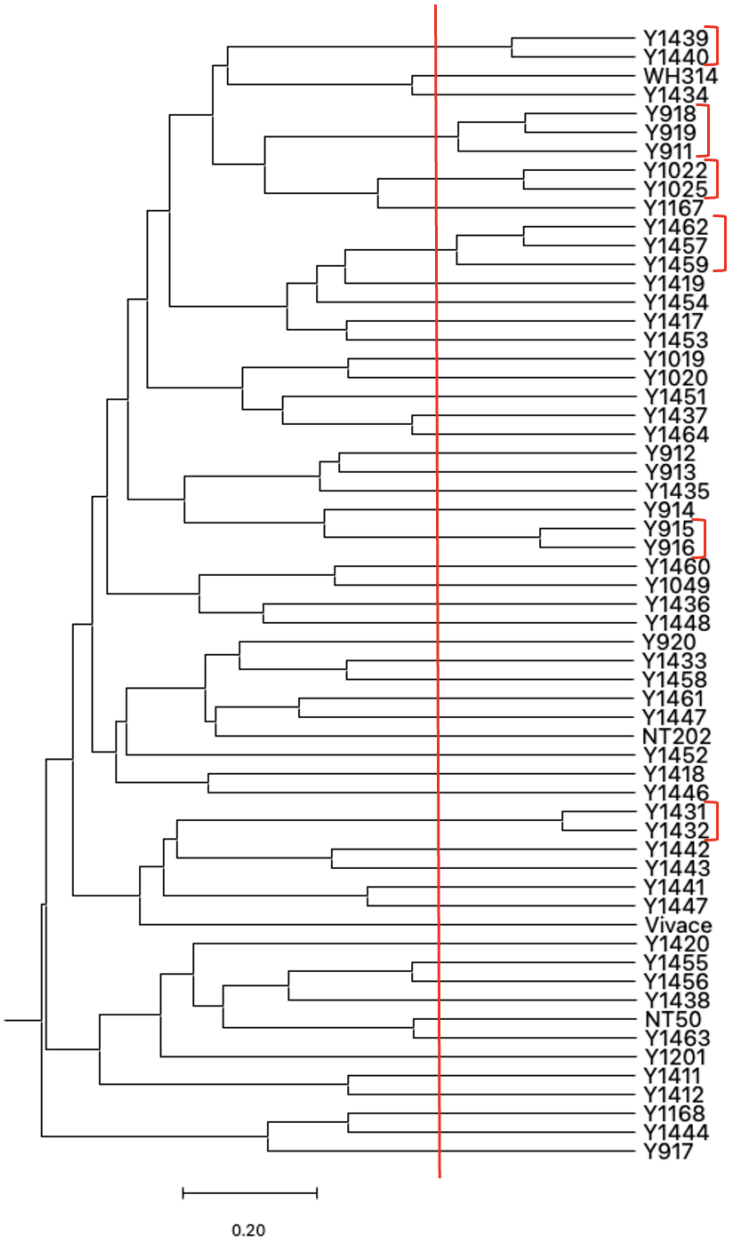


FIGURE 1. Estimated degree of strain relatedness. All isolates sharing nodes to the right of the red line (showing the 35 % difference mark) and sharing a bracket were considered to be identical once cross-referenced with the gel images.

Isolates exhibiting less than 35 % differences were deemed identical or too closely related to be distinguished, as confirmed by visual inspection of the banding patterns. It was observed that Y911 was identical to Y918 and Y919, Y915 identical to Y916, Y1022 identical to Y1025, Y1457 identical to both Y1425 and Y1462, Y1431 identical to Y1432, and Y1439 identical to Y1440. The repeat isolates, which were mostly to be isolated during the same yeast isolation excursions, were subsequently removed from the study.

1.2. *IRC7* genotyping and varietal thiol release

A standard PCR was used to determine the strains' *IRC7* genotype. Indeed, two alleles of the *IRC7* gene have been identified: a full gene and a truncated version resulting from a 38-bp deletion (Roncoroni *et al.*, 2011). 10.4 % of the strains were shown to be homozygous for the long allele, 85.4 % homozygous for the short allele, and 4.2 % were heterozygous. Detailed results per strain are shown in Table S2.

1.3. Hydrogen sulphide (H_2S) production

H_2S production by the various strains was assessed by spot-plating the isolates on BiGGY agar (Table S2 and Figure 2). Commercial strains Vivace and WH314 were used as the lowest and the highest H_2S-producing control strains, respectively, for this experiment as described previously (Erasmus & Divol, 2022).

Vivace's grayscale value was regarded as 0 % production and that of WH314 as 100 %. A wide range of distribution

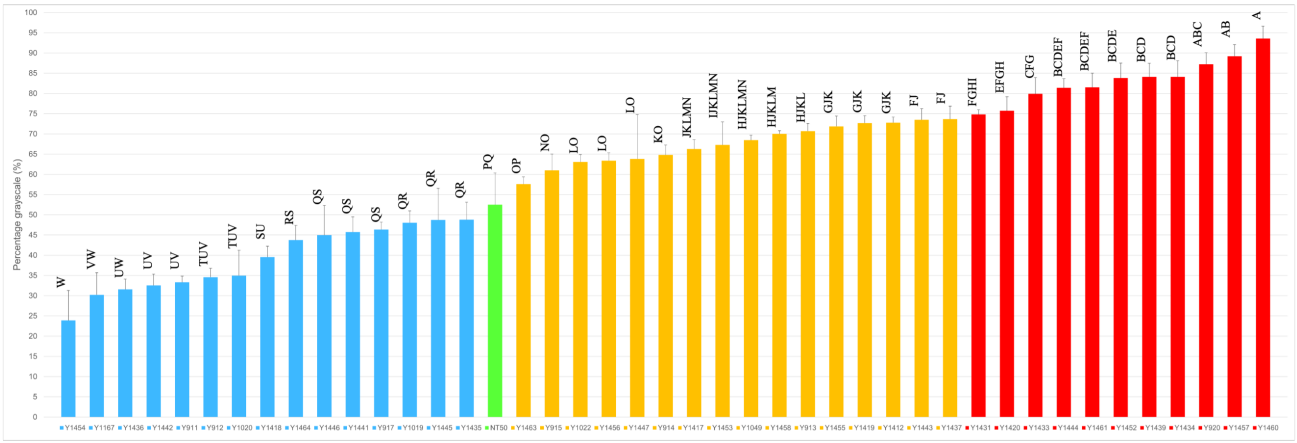


FIGURE 2. Grayscale percentages representing H_2S production of the *S. cerevisiae* strains investigated grown on BiGGY agar. Low producers (< NT50) are shown in blue, NT50 in green, moderate producers (reference < X < 75 % WH314) in yellow and high producers. (75 % < X < 100 % of WH314) in red. Strains sharing the same letter are not significantly different at a $p < 0.05$ threshold.

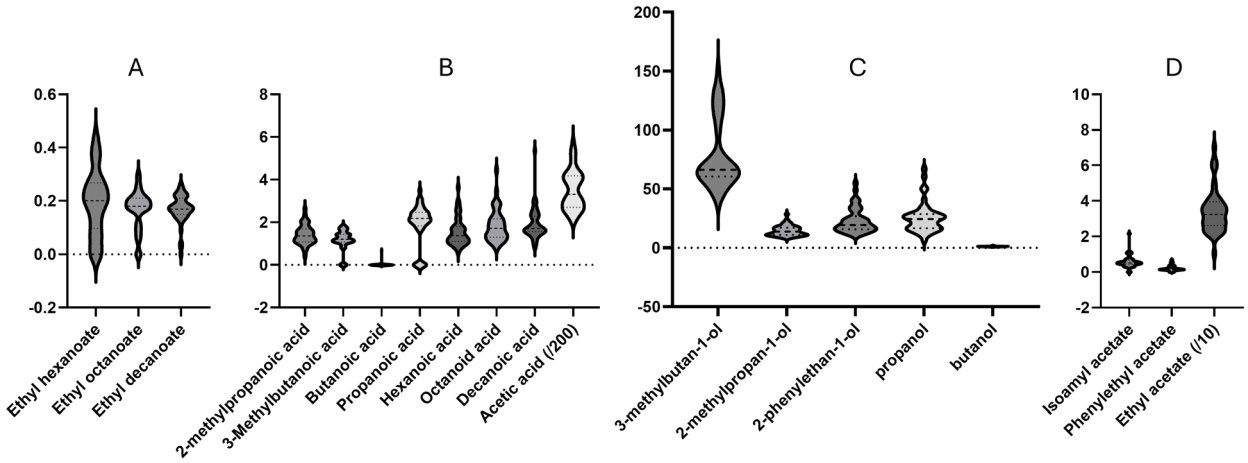


FIGURE 3. Distribution of selected major volatile compounds production (in mg/L) at the end of alcoholic fermentation across investigated strains at the end of fermentation in SGJ. A: medium chain fatty acid ethyl esters, B: volatile fatty acids, C: higher alcohols, D: acetate esters. The concentrations of acetic acid were divided by 200 and those of ethyl acetate by 10 to fit on the graphs.

was observed, with the strains investigated ranging from 24 % to 94 % of WH314's grayscale value. NT50 (green), a strain commercialised by Anchor Oenology for the wine industry, was arbitrarily considered as the 'acceptable' threshold separating low (blue) and moderate (yellow) H_2S producers with the top H_2S producers (within 25 % of WH314's grayscale value) being considered high (red) producers (Figure 2). Amongst the different strains, 32 % were considered low, 54 % moderate, and 10 % high H_2S producers.

1.4. Fermentation kinetics analysis

The 48 strains were inoculated individually into synthetic grape juice and the fermentation kinetics were monitored (Table S2). The data revealed low intraspecific variation. For all strains, lag phase duration ranged from 11 to 13 h, Vmax from 0.71 g/L/h to 1.2 g/L/h, and fermentation duration from 420 to 480 h.

1.5. Relevant metabolite quantification

At the end of fermentation, major volatile compounds were quantified (Figure 3, Table S2), and boxplots were constructed for selected compounds to visualise the diversity of metabolic profiles amongst the strains (Figure 3).

Large variations were observed between strains in the production of most major volatiles, except for butanol and butanoic acid, which remain very low. Apart from 3-methylbutan-1-ol (isoamyl alcohol) which revealed large variability, the other higher alcohols were all produced in a narrow concentration range, with a few outlier strains. Interesting strain variability was observed in ethyl esters. Amongst the fatty acid ethyl esters, ethyl hexanoate production demonstrated the highest variability. The greatest producers of ethyl hexanoate, decanoate, and octanoate were Y1444, Y1442, and Y912, respectively.

Amongst the other ethyl esters, ethyl phenylacetate and ethyl acetate exhibited the most significant variability across different strains. In contrast, the production levels of isoamyl acetate and phenylethyl acetate were generally consistent amongst the strains, with the notable exception of strain Y917. This particular strain produced a markedly higher concentration of isoamyl acetate compared to its counterparts. Maximum producers for ethyl phenylacetate, isoamyl acetate, phenylethyl acetate, and ethyl acetate were strains Y914, Y917, Y1445, and Y1022, respectively.

The concentration of 2-phenylethanol was of importance due to its desirable 'rose-like' aroma contribution to wine and thus was included as a selection criterion for the next set of fermentations in real grape juice. The final SGJ concentrations are shown in Table S3.

1.6. Strain selection for real grape juice fermentations

A comparison table with conditional highlighting was employed to facilitate the selection of five yeast strains with desirable traits for subsequent investigations in real grape juice fermentations (Table S3). This selection process was guided by two primary criteria.

Firstly, the emphasis was placed on secondary metabolites, with a particular focus on 2-phenylethanol, ethyl esters, and acetates. This decision was driven by the current consumer preference for wines with fresh and fruity characteristics (Pretorius, 2019). In this context, the selected strains were compared against the commercial reference strain NT50 to identify notable differences in metabolite production.

Secondly, aspects of fermentation kinetics were considered, specifically the lag time and the maximum rate of fermentation (Vmax). An extended lag time allows for competitive microbes to alter the chemical composition of the grape juice before the dominant strain takes control of the fermentation. This adds a risk factor, especially on an industrial scale seeking consistency, as these effects could be good, bad, or negligible (Albertin *et al.,* 2017). Wine fermentation speed significantly impacts quality, with fast fermentation potentially resulting in the loss of esters in white wines in particular. However, overly long fermentation can delay production and increase the risk of spoilage. Winemakers generally consider managing fermentation kinetics crucial for controlling wine characteristics (Sablayrolles, 2009).

Despite advancements in fermentation techniques, incomplete fermentations remain problematic in some regions. This issue has been exacerbated by a recent trend amongst winemakers to prioritise flavour development in grapes, resulting in higher sugar content in grape must (Martín-García *et al.* 2023). Consequently, stuck or slow fermentations have become more common, potentially causing significant economic losses.

Additionally, the production of hydrogen sulphide (H_2S) on BiGGY agar was evaluated, given the significant variation observed amongst the strains in this experiment (Figure 2). The aim was to identify strains with low H_2S production, a desirable trait for wine yeast. Furthermore, the genotypic analysis of the *IRC7* gene alleles was considered. The presence of the long allele in *S. cerevisiae* strains is rare but although originally associated with the production of certain white wines, varietal thiols can also impart sought-after flavours in red wines (Ferreira *et al.* 2002; Rigou *et al.*, 2014).

Strains Y1020, Y1022, Y1436, Y1441, and Y1445 were chosen for further experiments using real grape juice based on the evaluation of the above factors. These strains exhibited superior production of selected secondary metabolites, surpassing the average level observed in the reference strain NT50. Additionally, these selected strains also demonstrated favourable fermentation kinetics; their lag times did not exceed 14 h, and their maximum rates of fermentation (Vmax) ranked in the fastest two-thirds of the sample set. Furthermore, their H_2S production was relatively low, placing them in the lower two-thirds of production among the tested strains. Y1022 was of special interest due to its heterozygous genotype of the *IRC7* gene.

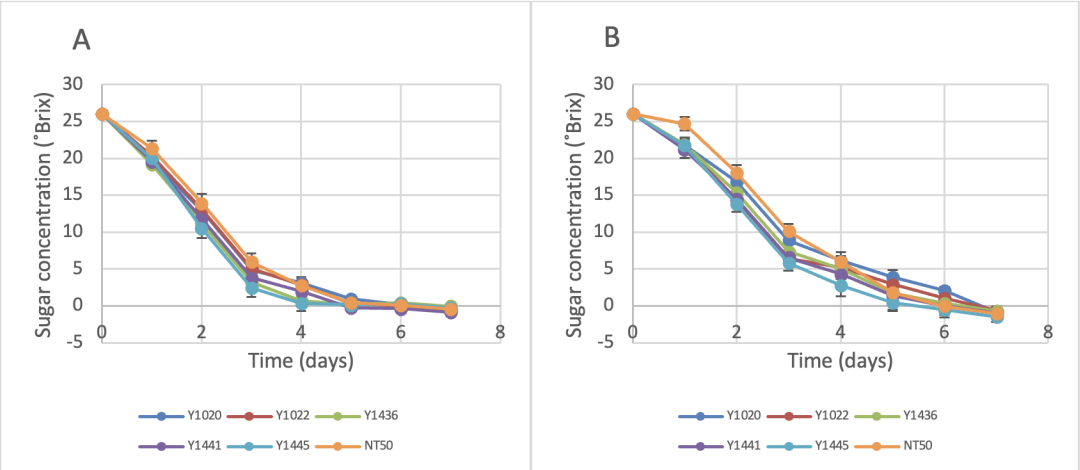


FIGURE 4. Daily hydrometer readings (°Brix) from both Merlot (A) and Cabernet Sauvignon (B) fermentations tracking the progress of alcoholic fermentation.

2. Screening of selected strains using real grape juice

2.1. Merlot and Cabernet Sauvignon fermentation kinetics

Real grape juice fermentations were carried out in both Cabernet Sauvignon and Merlot cultivars. The current environmental pressures driving yeast selection, including climate change, mean that the selected yeast strains should be able to ferment high-sugar musts efficiently, thus the sugar concentration chosen for both cultivars was 260 g/L. Figure 4 shows the kinetics of these fermentations.

All fermentations proceeded till dryness (below 0 °B). In the Cabernet Sauvignon fermentation, NT50 and Y1445 took six days to complete fermentation, and the remaining strains took seven days. In Merlot, Y1441's fermentation took five days whereas that of the rest of the strains took seven days. NT50 recorded a significantly longer lag phase than the other strains.

2.2. Quantification of Sugars and organic acids (HPLC)

Primary metabolites were quantified at the end of alcoholic fermentation. The sugar analysis confirmed that all fermentations proceeded until dryness for both cultivars' fermentations (*i.e.*, residual sugar concentrations < 2 g/L).

The Cabernet Sauvignon wines showed no significant variation in succinic acid, or ethanol production (Table S5). As expected, considering the high initial sugar concentration of the grape must, NT50 produced a high amount of glycerol (15.5 g/L), but remarkably, the other strains produced much less, ranging from 10.7 g/L for the lowest producer Y1436 to 12.2 g/L for Y1445. Interestingly, acetic acid production did not match that of glycerol production with Y1441 producing the highest amount (0.3 g/L) and Y1022 and NT50 producing the least (around 0.13 g/L).

The results obtained from the fermentations of Merlot globally showed similar trends as those of the Cabernet Sauvignon. No significant variation was observed in ethanol production. NT50 again produced the highest amount of glycerol and the lowest amount of acetic acid. Y1436 produced the lowest amount of glycerol and Y1441 was again the highest producer of acetic acid. Significant variation was observed in succinic production with the highest glycerol producer (*i.e.*, NT50) producing the most succinic acid and the lowest glycerol producers (Y1436 and Y1441) producing the least.

S. cerevisiae can both produce and consume malic acid during fermentation (Vion *et al.*, 2023), but does so weakly compared to other yeast species, with production occurring via oxaloacetate reduction and consumption requiring fermentable carbon sources (Saayman & Viljoen-Bloom, 2017). The concentration of malic acid after alcoholic fermentation was compared to the initial concentrations described in Table 2. NT50 and Y1020 were seen to produce significant amounts of malic acid whereas Y1441 and Y1445 consumed malic acid. The remaining strains Y1022 and Y1436 did not appear to significantly impact the malic acid concentration. These results were consistent across both Merlot and Cabernet Sauvignon.

2.3. Quantification of varietal thiol release (GC-MS)

The varietal thiols 3-SH, 3-SHA, and 4-MSP produced by the individual strains were quantified at the end of alcoholic fermentation for the selected strains in the Cabernet Sauvignon and Merlot wines (Figure 5). Concentrations of 3-SHA were below the level of detection in both cultivars.

The thiol quantification results in the Cabernet Sauvignon and Merlot wines showed similar trends. NT50 released the highest amounts of 3-SH and 4-MSP while Y1020 was consistently the lowest releaser for both varietal thiols. The other strains showed overall remarkably consistent intermediate patterns.

2.4. Quantification of major volatiles (GC-FID)

Major volatile compounds were quantified using GC-FID. Principal Component Analysis (PCA) charts were produced to investigate if variation existed amongst strains (Figure 6). Totalled data for the various major volatile groups as well as individual compounds can be seen in Tables S3.1 to S3.3.

The PCA biplots show variation between strains indicated by the distance between the strain's (blue) points. The vectors (red) indicate the drivers of this variation based on length (longer = stronger driver) and direction of the vector where

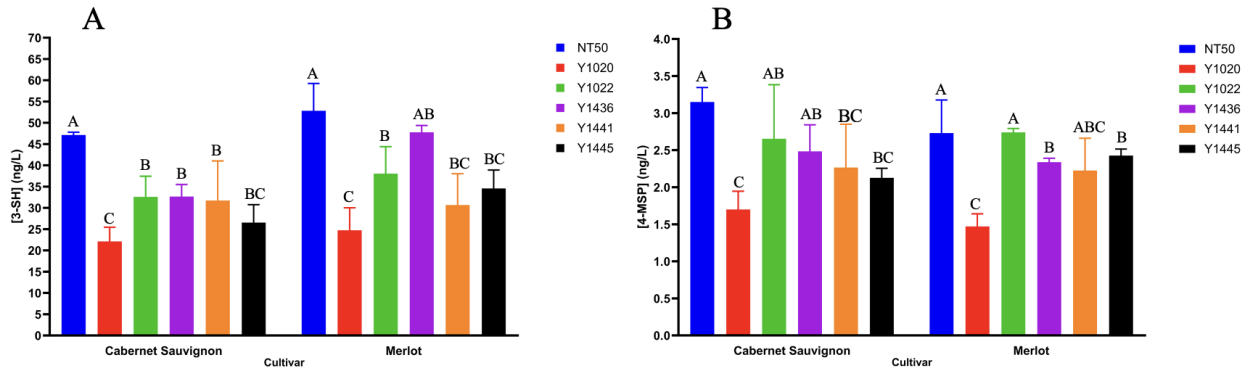


FIGURE 5. 3-SH (A) and 4-MSP (B) concentrations measured in both Cabernet Sauvignon and Merlot at the end of alcoholic fermentation. Strains sharing the same letter are not significantly different at a $p < 0.05$ threshold.

compound vectors pointing towards a strain's point indicate higher production of that compound causing variation and vice versa.

The Cabernet Sauvignon wines displayed distinct metabolic profiles (Figure 6A). NT50 could be differentiated from the other strains by its distinct high production of acetoin with Y1441 being the only other strain appearing within the same quadrant. Y1436 and Y1445 were grouped in the same quadrant with their variation being driven by ethyl acetate production. Y1020 produced a unique metabolic profile most notably driven by a high production of several fermentative flavour compounds such as 2-phenylethyl acetate and isoamyl acetate.

The Merlot wines (Figure 6B) produced did not possess metabolic profiles as distinct as the Cabernet Sauvignon wines. However, some trends were conserved. NT50 was

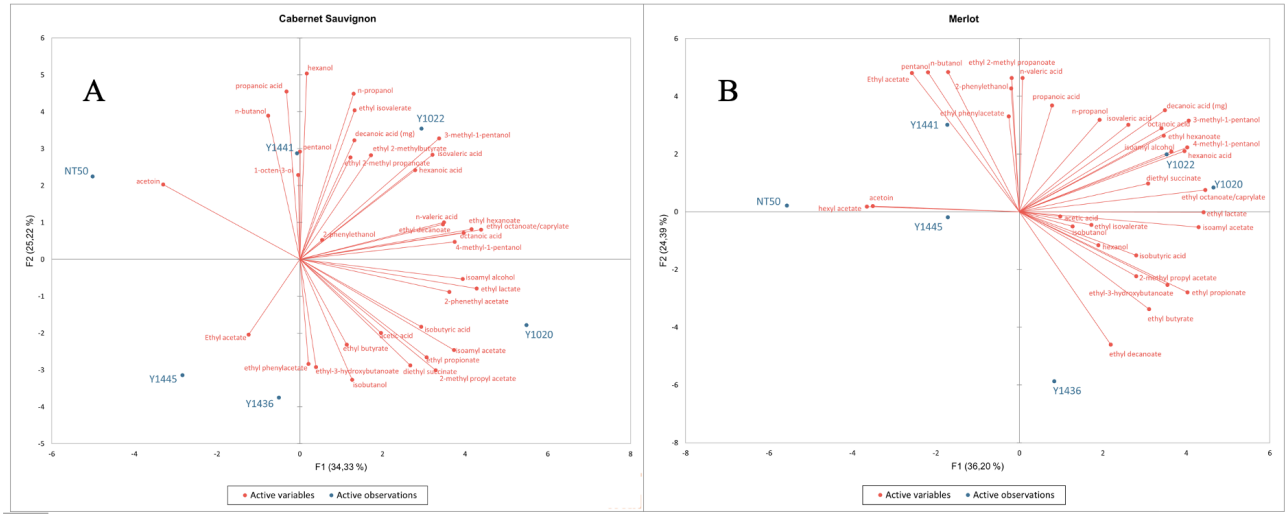


FIGURE 6. Principal component analysis of secondary metabolites driving variation amongst yeast strains after alcoholic fermentation in both Cabernet Sauvignon (A) and Merlot (B).

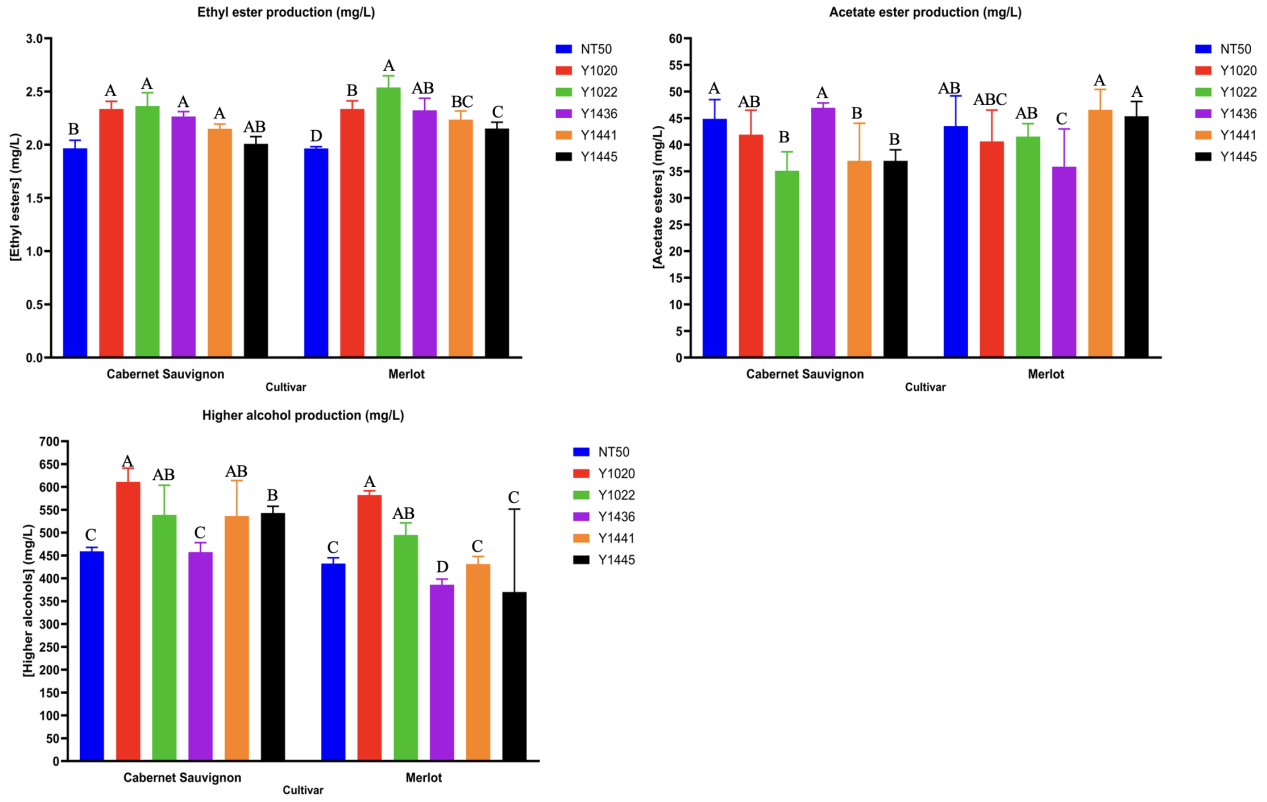


FIGURE 7. Variations shown in ethyl ester, acetate ester, and higher alcohol production in both Cabernet Sauvignon and Merlot after alcoholic fermentation. Strains sharing the same letter within each cultivar are not significantly different at a $p < 0.05$ threshold.

again separated from its counterparts by its higher acetoin production and Y1441 was grouped in the same quadrant. Y1020 was again located in the side section of the graph and was characterised by its high production of a large number of fermentative flavour compounds including isoamyl acetate but grouped more closely to Y1022 than it did in the Cabernet Sauvignon wines. Y1436 was separated from the rest of the strains due to high ethyl decanoate production.

The concentrations of total ethyl esters, acetate esters, and higher alcohols produced by each strain were investigated further (Figure 7). Ethyl ester production trends were conserved between the two cultivars. NT50 produced the lowest concentration and Y1022 the highest. Acetate ester production varied considerably more between the wines made with Y1022 showing the lowest concentration and Y1436 the highest in Cabernet Sauvignon, whereas Y1436 produced the lowest and Y1441 the highest in Merlot. Higher alcohol production patterns remained conserved amongst the media with NT50 and Y1436 consistently within the lowest-producing group and Y1020 in the highest. For exact values refer to Table S4.1.

3. Sensory analysis

Sensory data were collected to investigate the extent to which the different strains affected the sensory profile of the wine. PCA charts (Figure 8) were produced using the rank sum data received from the trained sensory panel.

The Cabernet Sauvignon wine (Figure 8A) produced by Y1020 showed great variation from the other strains, driven mostly by floral characteristics. Y1022 and Y1441 scored average on most descriptors, as indicated by their proximity to the intersection of axes. Y1445's variation was driven mostly by tea/hay/tobacco, NT50 by cooked vegetables, and Y1436 by sweet and jam characteristics.

The Merlot wines (Figure 8B) were slightly less discrete, attributed to the clustering of Y1022, Y1436, and Y1445. NT50 produced a distinct wine driven by jam as well as vanilla and caramel characteristics. Y1441's variation was driven mostly by red berries, and Y1020 by savoury and pepper characteristics. As observed in the PCA charts related to the major volatile compounds' chemical analysis, NT50 and Y1020 were located on opposite sides of the charts. However, the proximity of Y1445 and Y1436 in the chemical PCA charts did not show in the sensory PCA charts of the Cabernet Sauvignon wines and neither did that of Y1022 and Y1020 of the Merlot wines.

DISCUSSION

The search for novel indigenous yeast strains of oenological interest has been a continuous endeavour for several decades. This study confirmed the great variation amongst wild strains of *S. cerevisiae* reported in numerous past studies (Guerra *et al.*, 1999; Romano *et al.*, 2008; Capece *et al.*, 2016). An initial screening phase coupled with fermentations in SGJ allowed for a basic characterisation of these forty-six strains in terms of their metabolite production, fermentation kinetics, H_2S production potential, and *IRC7* genotype under laboratory conditions. These data contributed to the characterisation of these yeast strains within the South African Grape and Wine Research Institute's microbial culture collection as well as identifying strain replicates within the collection. From these data, five strains (i.e., Y1020, Y1022, Y1436, Y1441, and Y1445) were used to conduct real grape juice fermentations using Cabernet Sauvignon and Merlot grape juice.

Very little variation was observed between the strains in terms of their fermentation kinetics except for NT50's longer lag phase duration in real grape juice, which was possibly due to the NT50 inoculum being prepared from an active dry yeast instead of liquid preculture as with all the other strains. The strains which have undergone preculturing, as previously described, are better adapted to growth in liquid media, their cell concentration is calculated, and are at an exponential growth phase at the time of inoculation. The active dry yeast

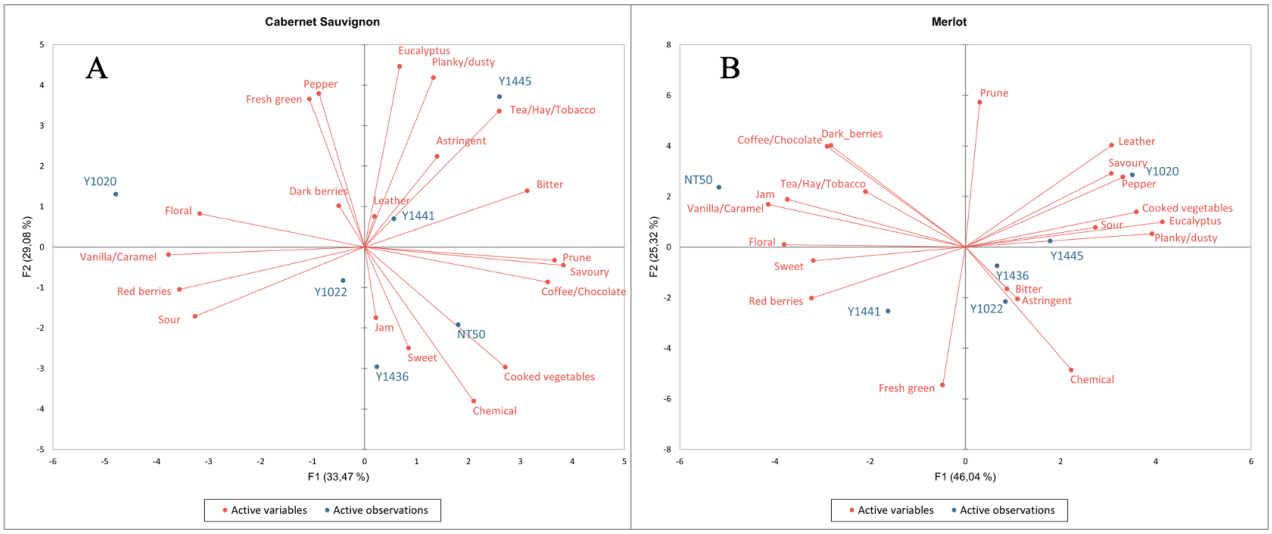


FIGURE 8. Principal component analysis of the sensory profiles for each strain in both Cabernet Sauvignon (A) and Merlot (B).

on the other hand has to undergo rehydration shortly before inoculation, which might result in the cells taking slightly longer to adapt fully to growth in grape juice.

Due to the high sugar concentration in the real grape juices used in this study, the selected strains produced higher amounts of glycerol than in SGJ. Indeed, while under anaerobic conditions, oxygen is not available as the final electron acceptor so both glycerol and ethanol are produced by the yeast to oxidise NADH to NAD^+, thereby regenerating the NAD^+ utilised during glycolysis (Nevoigt & Stahl, 1997), glycerol is also produced as a response to osmotic stress as it increases the osmotic pressure within the cell without disrupting biological processes (Blomberg, 2022). Interestingly, the selected strains all produced significantly less glycerol than the reference strain NT50, which suggested a greater tolerance towards osmotic pressure. NT50 on the other hand, produced the lowest amount of acetic acid and Y1441 the highest. Since NT50 was the highest glycerol producer, it was also expected to produce the highest amount of acetic acid production (Cronwright *et al.*, 2002). This lack of correlation suggests a different redox balancing strategy, possibly reflected in the highest production of succinic acid. Overall, a positive correlation between glycerol and succinic acid production was observed in all strains, particularly in the Merlot fermentations, with a similar trend in Cabernet Sauvignon, attributed to redox balancing (Duncan *et al.*, 2023).

Interestingly, while in synthetic grape juice, the selected strains assimilated 0–25 % of the initial malic acid (with Y1022, Y1020, and Y1436 taking up the most and NT50, Y1441, and Y1445 the least), the strains either assimilated or released malic acid in real grape juice (with NT50, Y1441, and Y1445 releasing the most and Y1022, Y1020, and Y1436 rather taking up the most). *S. cerevisiae* can both produce and consume malic acid, but its capacity for both processes is relatively limited compared to other yeast species (Saayman & Viljoen-Bloom, 2006). As recently reviewed by Vion *et al.* (2024), malic acid uptake and production are both strain-dependent and environmental condition-dependent. In particular, lower initial malic acid concentrations have been reported to result in higher production. The Cabernet Sauvignon and Merlot juices used in this study had much lower malic acid concentrations (around 1 g/L) than the synthetic grape juice (3 g/L), which could at least partially explain the different behaviours observed.

Higher alcohols are compounds derived from yeast amino acid or sugar metabolism via the Ehrlich pathway and, other than 2-phenylethanol's 'rose-like' aroma, can contribute harsh, chemical aromas to the wine, especially at high concentrations (Hazelwood *et al.*, 2008). Y1020 consistently produced a high concentration of higher alcohols whereas NT50 and Y1436 were low producers. This is congruent with a recent report showing that the production of higher alcohols is generally linked to the strains' nitrogen requirements, except for 2-phenylethanol (Gonzalez-Ramirez *et al.*, 2024). On the other hand, acetate ester production varied between Cabernet Sauvignon, Merlot, and the SGJ results, indicating a matrix effect. Acetate esters originate either from sugars or specific amino acids. Although it has been shown that their main origin is from sugar metabolism (Rollero *et al.*, 2017; Rollero *et al.*, 2019), the concentration of individual amino acids may still impact that of the major volatiles produced, together with yeast strain's genetic background and various environmental factors (Saerens *et al.*, 2010). Acetate esters play a significant role in the aroma of alcoholic beverages (Hirst & Richter, 2016). These esters are synthesised from acetyl-CoA and the respective higher alcohol through the action of the enzyme alcohol acetyltransferase (AATase). This process is influenced by numerous variables, such as the presence of unsaturated fatty acids and precursors, the proportion of nitrogen, and the level of oxygen (Kong *et al.*, 2021). Because of this complexity, the production of acetate esters remains difficult to predict. The strains' medium-chain fatty acid ethyl ester production ranks showed consistency across both Cabernet Sauvignon and Merlot (Figure 7). For instance, Y1022 consistently produced the highest amount of ethyl esters and NT50 the lowest. This confirms that strain genetics drive the production of these esters more than the matrix (Sumby *et al.*, 2010) as their production derives from sugar metabolism.

Although not frequently investigated in red grape juice, the release of varietal thiols was quantified in this study, together with the strains' genetic potential. Within the set of Spanish strains investigated by Belda *et al.* (2016), 2.6 % of the strains were $IRC7^{L/L}$, 88 % were $IRC7^{S/S}$ and 9.4 % were $IRC7^{L/S}$. The frequencies observed in this study were remarkably close to those values given the small sample size of only 48 strains where one strain will shift the final percentage by 2.08 %. Moreover, the data confirmed the connection between genotype and varietal thiol production reported previously (Santiago & Gardner, 2015). Indeed, the highest producer of varietal thiols was strain NT50 which was the only strain homozygous for the long allele. Amongst the indigenous strains selected for the real grape juice fermentations, Y1022 appeared to be the second highest producer of 4-MSP, although not always consistently, and was the only heterozygous strain. Cordente *et al.* (2022) demonstrated that thiol release by yeast strains cannot solely be attributed to the presence of an $IRC7^{L}$ allele as most strains possessing $IRC7^{L/S}$ or $IRC7^{L/L}$ genotypes also harbour one or more deleterious single nucleotide polymorphisms (SNPs) which greatly reduce the $IRC7^{L}$ allele's ability to release thiols. The remaining strains are all homozygous for the short allele and produce lower concentrations of varietal thiols.

Overall, the chemical data show Y1022 to be a low acetic acid producer and Y1441 to be a high producer across both cultivars. Y1020 and Y1022 were high higher alcohol producers but Y1441 was the highest 2-phenylethanol producer. All strains produced higher total ethyl esters when compared to commercial strain NT50 and both higher alcohol and ethyl ester production appeared to be highly strain-dependent. Lastly, Y1020 appears to be a low all-round producer of varietal thiols and Y1022 and Y1436's Cabernet Sauvignon wines appear to have the most similar sensory

profiles to NT50's. Interestingly, the PCAs summarising the sensory and the chemical profiles both showed that NT50 and Y1020 yielded the most divergent wines with the other strains in between.

CONCLUSION

Building on the insights of Pretorius (2000), this study's focus on natural isolates from the Stellenbosch region represents an important step in responding to these multifaceted challenges of complex, evolving consumer demands and climate change. By exploring the unique chemical and sensory profiles that these natural isolates can introduce, our study not only contributes to a deeper understanding of the yeast biodiversity in this winemaking region but also paves the way for future innovations in wine production. The distinct strains identified and characterised here hold potential for the development of novel wines that meet the contemporary demands for unique sensorial experiences. Thus, this research not only responds to the current market trends but also anticipates future shifts in consumer preferences, emphasising the crucial role of natural isolates in sustaining and revitalising the dynamic landscape of the wine industry.

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