Screening of Oenological Yeasts for Volatile Phenol Release in Smoke-exposed Red Wine

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The frequency of wildfires has increased over the past few years, particularly in winemaking countries. This is partly because of climate change, thereby elevating the risk of producing smoke-tainted wines. The off-flavours associated with smoke taint originate from volatile phenols, which are progressively liberated from their glycosylated precursors during the winemaking process. Current strategies to prevent and/or mitigate the negative impact of smoke taint in wine production are not fully effective because it is difficult to remove glycosylated volatile phenols without affecting wine quality. One ill-explored avenue could be to use wine yeasts with extracellular glycosidase activities in combination with other downstream remediation processes. For this study, grape juice was obtained from smoke-exposed Pinotage grapes. Thirty yeast strains were screened for their potential to release volatile phenols. Partial fermentations were conducted on laboratory scale for 96 h, after which volatile phenols were quantified using GC-MS. A large variation was observed between yeast strains in their ability to liberate volatile phenols, particularly guaiacol, which serves as a key indicator of smoke taint in wine. Most strains released guaiacol at levels above its detection threshold in wine (23 µg/L). Nevertheless, *Lachancea thermotolerans* IWBT Y940, *Starmerella bacillaris* IWBT Y550 and *Wickerhamomyces anomalus* IWBT Y541 displayed the highest total volatile phenol release in smoked Pinotage, close to that observed with a commercial glycosylase formulation.

INTRODUCTION

The occurrence of wildfires in winemaking countries such as South Africa, Australia, France, Greece, Spain, Portugal and the USA has increased in the last few decades due to climate change (Flannigan *et al*., 2009; Turco *et al*., 2014; Di Virgilio *et al*., 2019; Dupuy *et al*., 2020). Indeed, most vineyards are situated in regions with hot, dry summers, which leads to high chances of bushfires. Beside the risk of direct damage from such fires, the smoke that is generated can negatively affect grapevines in close proximity (Summerson *et al*., 2021). The chemical composition of grape berries is altered after exposure to bushfire smoke and the wines produced from smoke-tainted grapes can exhibit faulty aromas described as "ash", "burnt rubber" and "smoked meat" (Kennison *et al*., 2009; De Vries *et al*., 2016).

Various volatile phenol (VP) compounds have been detected in smoke-tainted wines, including guaiacol, 4-methylguaiacol, 4-ethylguaiacol, 4-ethylphenol and eugenol (Kennison *et al*., 2008). These compounds are produced from the combustion of lignin, but can also originate from oak barrels during wine maturation (Del Alamo Sanza *et al*., 2004). In addition, spoilage yeasts from the genus *Brettanomyces/Dekkera* and a few other non-*Saccharomyces* species are capable of producing 4-ethylguaiacol and 4-ethylphenol from precursor phenolic acids in red cultivars (Kheir *et al*., 2013). The exact mechanism for the uptake of smoke-derived VPs in grapevine is not fully known; however, it has been confirmed that the free volatiles are glycosylated to various sugars within the berry skins, pulp and leaves (Hayasaka *et al*., 2010; Dungey *et al*., 2011). The phenol glycoconjugates are non-volatile and therefore sensorially imperceptible in grape juice and wine. Nevertheless, the hydrolysis of phenol glycoconjugates into their free volatile forms occurs during alcoholic and malolactic fermentations via the glycosidase enzymatic activity of yeasts and bacteria (Krstic *et al*., 2015). Red wine is especially vulnerable to the negative effects of smoke taint due to the extended grape berry skin maceration occurring during alcoholic fermentation, which enhances the concentration of phenol glycoconjugates in the fermenting grape must. The acidic pH of wine also induces a slow hydrolysis of bound VPs during wine ageing. Finally, during wine tasting/consumption, human saliva contains enzymes that are capable of releasing VPs (Mayr *et al*., 2014).

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The fact that VPs can be released after bottling, while remaining imperceivable in grape juice/wine presents a serious problem for wineries. Elevated VP concentrations result in rejection of the wines by consumers, resulting in economic losses. It has therefore become imperative to find methods that (i) reduce the glycosidically bound smoke-associated volatile and then (ii) remove – or at least drastically reduce – all the free VPs from the affected grape juices/wines before bottling. This could prevent the occurrence of smoke taints after bottling or during consumption due to the hydrolysis of their corresponding glycosides.

The impact of commercial *Saccharomyces cerevisiae* wine yeast strains on VP release and sensory properties of smoke-tainted red wine has been studied under industrial winemaking conditions (Caffrey *et al*., 2019; Mirabelli-Montan *et al*., 2021; Summerson *et al*., 2021; Whitmore *et al*., 2021; Oberholster *et al*., 2022). Glycosidase enzymes, such as β-glucosidase, can be produced extracellularly by yeasts and hydrolyse bound VPs during fermentation (Krstic *et al*., 2015). Commercial *S. cerevisiae* strains generally exhibit low glycosidase activity, while non-*Saccharomyces* yeasts are capable of expressing relatively high activity, as previously demonstrated when they were investigated for enhancing terpene release in wine fermentations (Cordero Otero *et al*., 2003; González-Pombo *et al*., 2011; De Ovalle *et al*., 2018; Salgado *et al*., 2018; Zhang *et al*., 2020; Han *et al*., 2021). Yeasts from the following genera have been reported to display significant β-glucosidase activity: *Candida*, *Brettanomyces*, *Debaryomyces*, *Hanseniaspora*, *Lachancea*, *Metschnikowia*, *Pichia*, *Rhodotorula*, *Torulaspora*, *Wickerhamomyces* and *Zygosaccharomyces*, as reviewed by Zhang *et al*. (2021). The differences in VP release between commercial *S. cerevisiae* strains seems to be negligible compared to the effect of grape cultivar, while sensory masking of the smoke taint character by the production of fruity aroma compounds plays a more prominent role (Ristic *et al*., 2017). It has also recently been reported that the downstream removal of free VPs from smoke-tainted wine is possible after alcoholic fermentation using treatments such as activated charcoal or molecularly imprinted polymers, but glycosylated VPs remain unaffected (Wilkinson *et al*., 2022; Huo *et al*., 2024). The ability of non-*Saccharomyces* wine yeasts to liberate bound VPs from smoke-tainted grape juice fermentations has not been investigated and may provide an alternative solution for enhancing the mitigation of affected wines via downstream remediation techniques.

This study aimed to screen a collection of 30 non-*Saccharomyces* yeasts isolated from various wine environments in South Africa for VP release when inoculated in smoke-exposed grape juice. These yeasts were selected based on previous literature indicating genera and species most likely to exhibit high glycosidase activity (Han *et al*., 2021; Zhang *et al*., 2021). The detection of VPs was performed using an optimised GC-MS method based on previous studies conducted in our research institution (De Vries *et al*., 2016; McKay *et al*., 2019), while Pinotage grapes were exposed to smoke using a vineyard-based strategy adapted from Australian researchers (Kelly *et al*., 2012).

MATERIALS AND METHODS

Yeast selection

Yeast strains from various wine-related non-*Saccharomyces* species were obtained from the culture collection of the South African Grape and Wine Research Institute, Stellenbosch University, South Africa (Table 1). Strains were streaked from freeze cultures stored at -80°C in yeast extract 10 g/L, peptone 20 g/L, glucose 20 g/L and glycerol 15% v/v onto Wallerstein laboratory nutrient (WLN) agar (Merck-Millipore, Burlington, MA) and incubated at 30°C for 48 h to obtain single colonies.

Smoke treatment of grapes

Pinotage grapes were exposed to smoke and harvested from Neethlingshof wine estate, Stellenbosch, South Africa (-33.94878, 18.80805). A single 9 m-long section of grapevines was covered with a greenhouse-grade plastic sheet to ensure smoke did not drift throughout the vineyard and affect surrounding vines. Flora was collected from the surrounding area of the vineyard and used as a fuel source to represent a scenario in which local wildfires occur. This consisted mainly of wild oats (*Avena fatua*) and ryegrass (*Lolium* spp.), which are ubiquitous across agricultural crop and pasture systems in the Western Cape province of South Africa. A commercial bee smoker was filled with the dry vegetation and ignited, while smoke was pumped continuously into the tented vines for one hour. The plastic sheet was left to cover the vines for 24 h before removal. One week after the grape bunches were exposed to smoke, they were harvested by hand at a reducing sugar level of 240 g/L. Immediately after picking the grapes, they were transported to Stellenbosch University's experimental cellar, crushed and destemmed into food-grade plastic buckets. Then, 30 mg/L SO_2 and 2 g/hL Rapidase Xtra Fruit (Oenobrands, Montpellier, France) were added directly after crushing. The crushed grapes were stored at 4°C overnight for cold soaking to take place. The juice was then pressed with a basket press, clarified and frozen in 20 L aliquots at -20°C until required. The grape juice was thawed at 4°C and filter-sterilised through 0.22 µm PVDS membranes (Merck-Millipore, Burlington, MA) when needed for subsequent experiments. Negative controls included samples from uninoculated smoked juice, and the treatment of the same juice with Rapidase Revelation Aroma (Oenobrands, France) glycosidase enzyme mixture served as a positive control.

Fermentation conditions

Yeast strains were streaked onto fresh WLN agar from freeze cultures, as described previously. Single colonies were transferred to 5 mL sterile YPD broth (yeast extract 10 g/L, peptone 20 g/L, glucose 20 g/L, pH 6.5) in test tubes and placed on a rotating test tube wheel (40 rpm) for 16 h at 30°C. Each overnight culture was inoculated in triplicate into 50 mL filter-sterilised smoke-exposed grape juice in 100 mL glass spice jars, fitted with rubber stoppers and S-type airlocks. The inoculations were normalised by diluting pre-cultures to achieve an OD_{600nm} of 0.1. Yeast cells were centrifuged (9 000 x *g*) and rinsed with 0.9% w/v NaCl prior to inoculation. After inoculation, the flasks were incubated for 96 h at 25°C without agitation. The

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TABLE 1
Yeast strains used in this study. All strains belong to the microbial culture collection of the South African Grape and Wine Research Institute, Stellenbosch University.

Species	Strain	Source and year of isolation
Hanseniaspora uvarum	Y537	Grenache grape juice, Swartland (2018)
Hanseniaspora uvarum	Y545	Cabernet Sauvignon grape juice (2014)
Hanseniaspora uvarum	Y968	Cabernet Sauvignon grape juice (2014)
Hanseniaspora uvarum	Y969	Cabernet Sauvignon grape juice (2014)
Hanseniaspora vineae	Y971	Cabernet Sauvignon grape juice (2014)
Hanseniaspora vineae	Y520	Cabernet Sauvignon grape juice, Stellenbosch (2015)
Lachancea thermotolerans	Y1017	Cabernet Sauvignon grape juice, Stellenbosch (2015)
Lachancea thermotolerans	Y1038	Cabernet Sauvignon grape juice, Stellenbosch (2015)
Lachancea thermotolerans	Y1109	Cabernet Sauvignon grape juice, Stellenbosch (2015)
Lachancea thermotolerans	Y1147	Cabernet Sauvignon grape juice, Stellenbosch (2015)
Lachancea thermotolerans	Y1202	Cabernet Sauvignon grape juice, Stellenbosch (2015)
Lachancea thermotolerans	Y1206	Cabernet Sauvignon grape juice, Stellenbosch (2015)
Lachancea thermotolerans	Y1240	Cabernet Sauvignon grape juice, Stellenbosch (2015)
Lachancea thermotolerans	Y513	Sauvignon blanc grape juice, Stellenbosch (2015)
Lachancea thermotolerans	Y519	Cabernet Sauvignon grape juice, Stellenbosch (2015)
Lachancea thermotolerans	Y540	Cabernet Sauvignon grape juice, Stellenbosch (2020)
Lachancea thermotolerans	Y548	Cabernet Sauvignon grape juice, Stellenbosch (2020)
Lachancea thermotolerans	Y905	Chenin blanc grape juice, Swartland (2012)
Lachancea thermotolerans	Y940	Blend, Nietvoorbij (2010)
Lachancea thermotolerans	Y973	Cabernet Sauvignon juice (2014)
Lachancea thermotolerans	Y976	Cabernet Sauvignon juice (2014)
Metschnikowia bicuspidata	Y549	Grenache grape juice, Swartland (2018)
Metschnikowia pulcherrima	Y1123	Sauvignon blanc grape juice, Somerset West (2009)
Pichia guilliermondii	Y993	Sauvignon blanc grape juice, Elgin (2015)
Pichia terricola	Y553	Cabernet Sauvignon grape juice, Stellenbosch (2020)
Starmerella bacillaris	Y550	Cabernet Sauvignon grape juice, Stellenbosch (2020)
Suhomyces pyralidae	Y1140	Cabernet Sauvignon grape juice (2009)
Torulaspora delbrueckii	Y551	Cabernet Sauvignon grape juice, Stellenbosch (2020)
Wickerhamomyces anomalus	Y541	Grenache grape juice, Swartland (2020)
Zygosaccharomyces parabailii	Y554	Cabernet Sauvignon grape juice, Stellenbosch (2020)

supernatant was filtered through 0.22 µm membrane filters (Merck-Millipore, Burlington, MA) and stored at -20°C for GC-MS analysis.

Quantification of volatile phenols by gas chromatography–mass spectrometry (GC-MS)

Sample preparation followed the method described by McKay *et al*. (2019). Stock solutions of 1 mg/L of reference compounds, obtained from Sigma-Aldrich/Merck (KGaA, Darmstadt, Germany), were diluted, in triplicate, to create an eight-point calibration series ranging from 0.05 to 200 µg/L. Two 10 mL aliquots of each wine sample were transferred into 20 mL SPME glass vials. An internal standard, 3-octanol, was added to each vial at a concentration of 10 µg/L. In addition, 2 mL of 30% w/v NaCl solution were added to each vial. The vials were sealed and mixed vigorously for 30 s before being placed on the auto-sampler (Thermo Scientific TriPlus RSH, Waltham, MA). The vials were incubated in the autosampler for 5 min at 50°C, after which a 65 µm polydimethylsiloxane/divinylbenzene (PDMS/DVB)

'Stableflex' SPME fibre (Supelco, Bellefonte, PA, USA) was exposed to the headspace for 15 min at the same temperature. Following exposure, the fibre was injected and left for 10 min to allow for the desorption of volatiles. The injector operated in splitless mode. The analysis of VPs was performed using a Thermo Scientific trace 1300 gas chromatograph (Anatech Instruments (Pty) Ltd, Bellville, RSA). The MS detector was set for acquisition in selected ion monitoring (SIM) mode. Chromatographic separation of the VPs was performed on a polar Zebron ZB-FFAP (30 m, 0.25 mm ID, 0.25 µm film thickness capillary column. The initial oven temperature was 50°C, held for 3 min. It was then increased to a final temperature of 250°C at a rate of 15°C/min and a final hold time of 3 min. The injector, ionisation source and transfer line temperatures were maintained at 250°C. Helium at 1 mL/min flow rate was used as carrier gas.

Statistical analyses

Data was analysed by performing a one-way ANOVA with Tukey's test and multiple comparisons of means ($p < 0.05$) for significant differences. GraphPad Prism software was used to create all figures and carry out statistical tests.

RESULTS

GC-MS optimisation

The detection of 12 target VPs was tested by adapting sample preparation and instrument parameters from previous studies conducted in the same research institute (De Vries *et al*., 2016; McKay *et al*., 2019). Adjustments were required due to the implementation of the method on a different GC-MS instrument, and the analysis of a sample matrix, originating from the above-mentioned fermentation conditions, which had not been calibrated or tested under the initial GC-MS parameters before. The 12 analytical standards for each VP were diluted for an eight-point calibration (0.5 µg/L to 200 µg/L) and analysed on a non-polar column with full-scan mode. The retention times were determined and post-analysis filtering with m/z ions was used to identify the compounds (data not shown).

The raw data for the initial linear calibration was integrated to determine the limit of detection (LOD) and limit of quantification (LOQ) for each compound (data not shown). The lower point of the linear range was not accurate enough to detect concentrations of the compounds below 20 µg/L, or to quantify them below 30 µg/L, with the LOQ of phenol, *m*-cresol and 4-ethylphenol exceeding 100 µg/L. It was thus decided to change to a new polar column and run the analysis in selected ion monitoring (SIM) mode to improve the detection of the VPs.

The 12 VP analytical standards were diluted to a lower starting point for a new calibration series (0.05 µg/L to 200 µg/L) and qualifier/quantifier ions were selected (Table 2) to complete the analysis via SIM mode to improve the sensitivity of the method. The second linear calibration dataset (Table 3) from the analysis with adjusted parameters yielded improved LOD and LOQ values compared to the initial calibration for all compounds, while retaining a satisfactory level of linearity. The reduction in LOD was slightly less for 2,3-dimethylphenol, eugenol and 3,4-dimethylphenol (~11 µg/L) compared to the other VPs (~5 µg/L), while their respective LOQ concentrations followed a similar trend (~36 µg/L compared to ~15 µg/L).

A stock solution containing a mixture of all 12 VPs (1 mg/mL per standard) was spiked into non-smoked Pinotage grape juice at a final concentration of 100 µg/L and the samples were tested on the polar column, operated in SIM mode as described above. The grape juice without spiked VPs was analysed as a negative control and displayed no peaks for any of the 12 VP target compounds (data not shown). Preliminary fermentation trials were conducted by inoculating a randomly selected strain (Y940) into smoked Pinotage juice to test the detection of VPs with the updated GC-MS method (Fig. 1).

All 12 VPs were detected in the spiked juice samples and quantified accurately, while only six of the compounds were quantified in the fermentation sample from Y940 (viz. guaiacol, 4-methylguaiacol, phenol, o-cresol, *m*-cresol and 4-ethylphenol). The other six compounds were not detected due to concentrations below the respective LODs, while *p*-cresol was not resolved from *m*-cresol due to similar retention time (Rt) and ion (m/z) spectra.

Fermentations in smoke-exposed Pinotage juice

Thirty yeast strains (Table 1) were inoculated individually into the smoked grape juice to evaluate their potential release of free VPs.

The variation for each detected VP across 30 tested strains in smoked Pinotage fermentations is shown in Fig. 2 – the odour detection threshold (ODT) of guaiacol of 23 µg/L (Table 2) is indicated with a red arrow. Six VPs were detected at the end of the incubation period of all yeast strains. The six missing VP compounds (Fig. 1) were not detected at concentrations above the LODs across all 30

TABLE 2

Retention times (Rt) and quantifier/qualifier ions (m/z) of 12 volatile phenol analytical standards analysed with selected ion monitoring (SIM) mode.

Compound	Rt (min)	Quantifier ion	Qualifier ion
3-octanol (IS)	8.135	-	-
Guaiacol	12.174	109	124
2,6-dimethylphenol	12.524	122	107
4-methylguaiacol	12.847	137	123
o-cresol	13.15	108	107
Phenol	13.193	94	152
4-ethylguaiacol	13.334	137	152
p-cresol	13.685	107	108
m-cresol	13.738	108	107
2,3-dimethylphenol	14.103	107	122
Eugenol	14.228	164	149
4-ethylphenol	14.278	107	122
3,4-dimethylphenol	14.576	107	122

strains, either due to their not being liberated at all or being liberated at concentrations below the sensitivity threshold of the method. Amongst the six detected VPs, guaiacol and phenol displayed the greatest variation amongst yeast strains, with the average concentration of guaiacol significantly ($p < 0.05$) above the ODT (data not shown). However, while the average phenol concentration detected in these samples was close to 100 µg/L, it remained well below its ODT in wine (5 900 µg/L). Similarly, the average concentrations for 4-MG, 4-EP, *m*-cresol and *o*-cresol were all below their respective ODT values.

Guaiacol concentrations for each yeast strain or

TABLE 3
Calibration data for the 12 volatile phenol standards in concentration range (0.5 µg/L to 200 µg/L) analysed on a polar column, including standard error (STE) of the covariance XY, limit of detection (LOD) and limit of quantification (LOQ).

Compound	R^2	Slope	Y-intercept	STE(XY)	LOD (µg/L)	LOQ (µg/L)
Guaiacol	0.9954	0.0004	0.0028	0.0014	5.15	13.79
2,6-dimethylphenol	0.9979	0.0024	0.0072	0.0039	5.35	16.26
4-methylguaiacol	0.9996	0.00007	0.0001	0.0001	5.62	17.03
o-cresol	0.9984	0.0018	0.0042	0.0029	5.36	16.25
Phenol	0.9973	0.0003	0.0028	0.0006	5.92	17.94
4-ethylguaiacol	0.9992	0.0031	0.0087	0.0066	6.07	18.40
m-cresol	0.9957	0.0003	0.0008	0.0005	5.46	16.56
p-cresol	0.9995	0.0009	0.0020	0.0015	5.52	16.74
2,3-dimethylphenol	0.9991	0.0008	0.0049	0.0031	11.98	36.32
Eugenol	0.9995	0.0009	0.0055	0.0034	11.96	36.24
4-ethylphenol	0.9992	0.0010	0.0032	0.0019	6.07	18.42
3,4-dimethylphenol	0.9982	0.0006	0.0040	0.0024	11.84	35.88

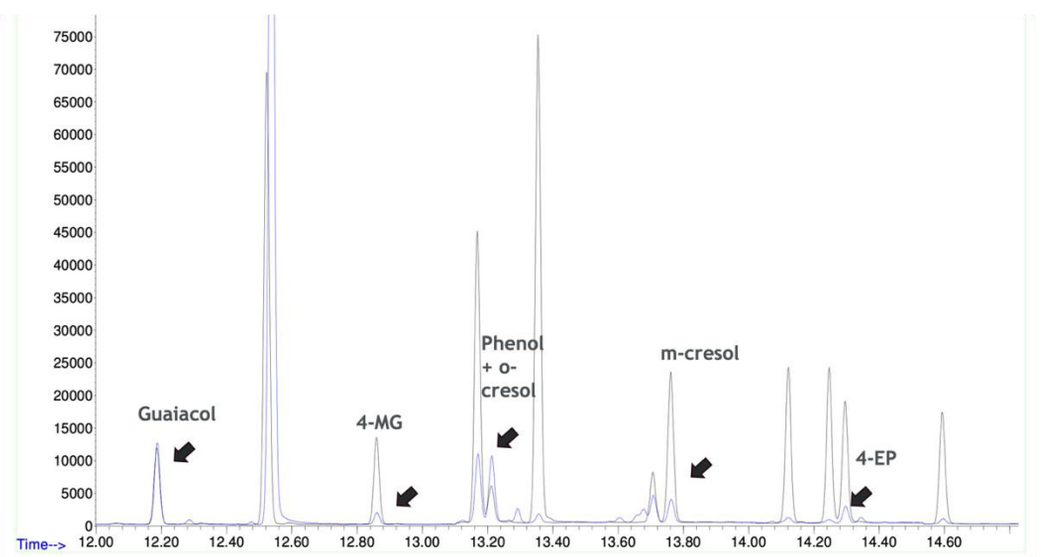

FIGURE 1
Chromatogram indicating detection of target volatile phenols (VPs) by GC-MS operated in selected ion monitoring (SIM) mode. Black peaks indicate pure analytical standards of 12 respective VPs chosen for quantification and spiked into non-smoked Pinotage juice, while blue peaks are overlayed from the Y940 fermentation samples in smoked Pinotage juice after 96 h. Arrows indicate compounds detected in the linear calibration range. Signal intensity is shown on the y-axis, while time on the x-axis is in minutes.

treatment are shown in Figure 3 – the concentration in the negative (uninoculated juice) control (11 µg/L) was lower than the ODT of 23 µg/L, while the enzyme-treated juice had a concentration of 62 µg/L. All strains increased the concentrations of guaiacol to varying degrees, while Y554 was the only strain that did not exceed the ODT. The *L. thermotolerans* species displayed an overall relatively high release of guaiacol for all strains tested (range 37 µg/L to 56 µg/L), while strains in the *Hanseniaspora* genus released slightly lower amounts (range 36 µg/L to 45 µg/L). The other genera varied widely, with two of the highest releasing yeasts (*Wickerhamomyces anomalus* Y541 and *Starmerella bacillaris* Y550) contrasting with some of the lowest guaiacol-rereleasing yeasts (*Zygosaccharomyces parabailii* Y554 and *Pichia/Metschnikowia* strains). The guaiacol:4-MG ratios are shown in Figure 3, and ranged from 3.5 to 6.5 for all strains.

The combined concentrations of the six detected VPs are shown in Fig. 4. The same trends observed for guaiacol release (Fig. 3) applied to the combined VP concentrations for each strain. The hydrolysis of bound VPs by the commercial enzyme treatment increased the free concentrations of the total VPs by almost double that of the most effective yeast strains and resulted in levels elevated more than 10 x when compared to the uninoculated smoked juice.

DISCUSSION

Various sample preparations and techniques have been developed for the GC-MS analysis of VPs in smoke-tainted wines (Mayr *et al*., 2014; Noestheden *et al*., 2017; Liu *et al*., 2020). The most widely used technique is the stable isotope dilution assay, but due to the unavailability of deuterated standards, it was decided to use 3-octanol as an internal standard for the analysis in this study (De Vries *et al*., 2016). The complexity of the wine matrix and low concentrations of VPs typically present in smoke-tainted wine require an extraction step before GC-MS analysis. Solid-phase microextraction (SPME) was successful during the setup of the method in this study. Although liquid-liquid extractions have been used previously, SPME is more convenient to carry out (Pollnitz *et al*., 2004), despite its reported limited capacity for volatile compound extraction and competition from other volatile compounds in the sample matrix for active sites (Zhou *et al*., 2015). Resolution was not achieved between *m*- and *p*-cresol due to the structural similarity and identical ion selection. The improved separation and resolution of compounds with highly similar Rt values can be achieved by triple quadrupole MS detectors (Dang *et al*., 2019; Liu *et al*., 2020). One study used qualifying ions 107.79 and 107.77, respectively, for *m*-and *p*-cresol, while utilising ethylene glycol/polydimethylsiloxane stir bar sorptive extraction GC-MS (Yang *et al*., 2021). Due to the fact that *m*- and *o*-cresol were released by the strains at low levels during fermentation, coupled with information in the existing literature that is inconclusive regarding the effect of these compounds in smoke-tainted wines, it was not necessary to utilise equipment and methodology to improve the detection of these compounds (Favell *et al*., 2022). The GC-MS workflow that was established effectively quantified important smoke taint marker compounds, namely guaiacol

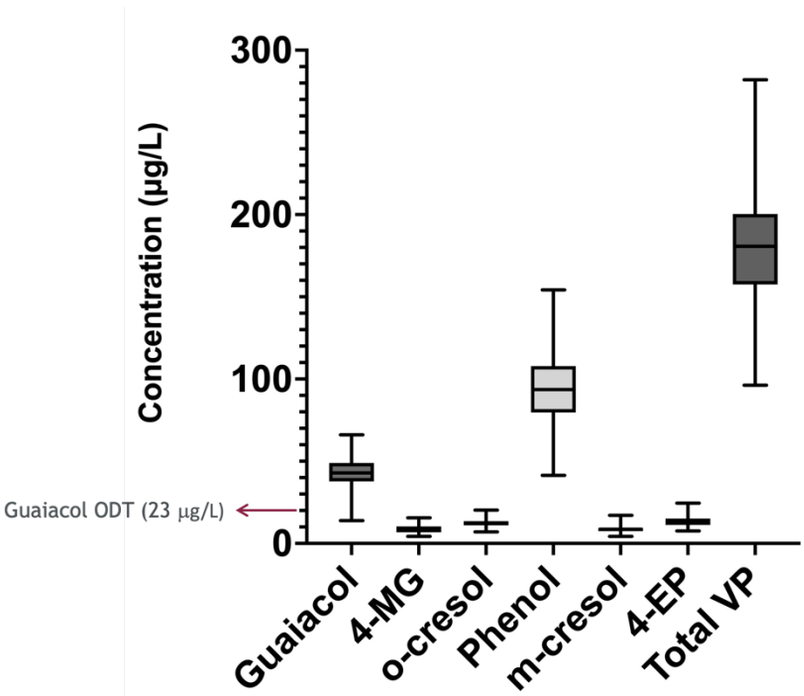

FIGURE 2

Box and whisker plots showing the variations in the amounts of volatile phenol released within the group of 30 tested wine yeast strains after six days of incubation in smoke-exposed Pinotage juice at 25°C.

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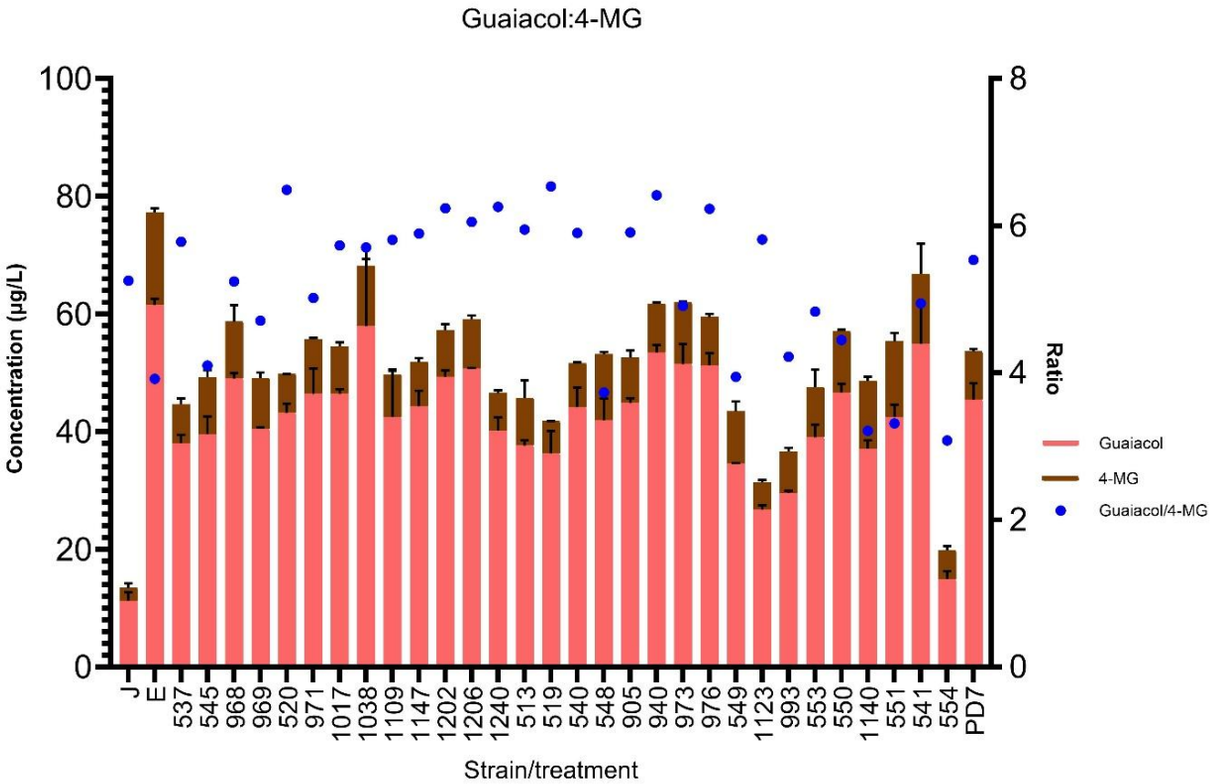

FIGURE 3

Concentrations of guaiacol detected in fermentation samples after 96 h in smoke-exposed Pinotage juice (left y-axis), ratio of guaiacol:4-methylguaiacol indicated on right y-axis. Uninoculated smoke-exposed juice was also measured before and after treatment with a commercial glycosidase enzyme mixture (Rapidase Revelation Aroma). Strain numbers are indicated on the x-axis, while smoked juice (J) and enzyme (E) treatments are on the far left.

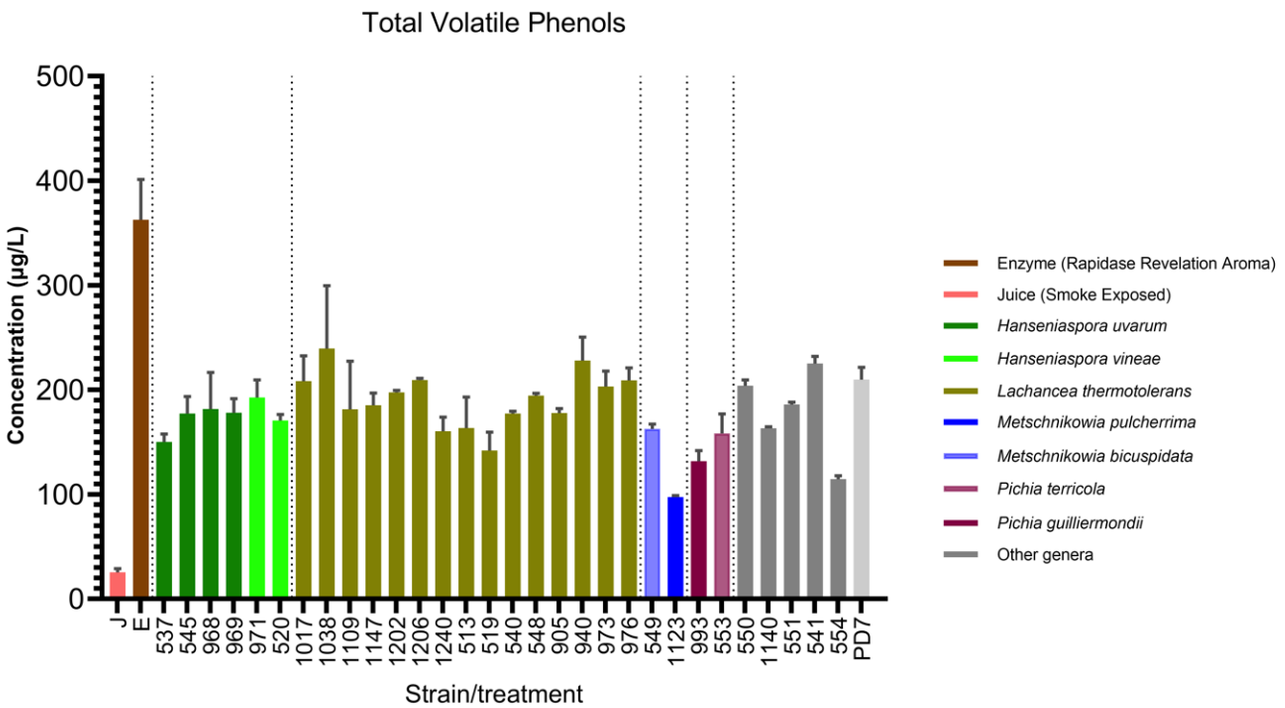

FIGURE 4

Concentrations of total VPs detected in fermentation samples after 96 h in smoke-exposed Pinotage juice. Uninoculated smoke-exposed juice, treated with a commercial glycosidase enzyme, was also measured before and after treatment.

and 4-methylguaiacol, which were prioritised for the purposes of this study.

Six of the 12 initially targeted VPs were detected in samples of smoked Pinotage inoculated with selected yeast strains (Table 1). Guaiacol and phenol were detected at low levels in the smoked juice, while 4-MG, *m*-cresol, *o*-cresol and 4-EP were not detected. The relatively low concentration of free VPs in the smoke-exposed grapes and juice prior to fermentation and bottling has been reported extensively (Kennison *et al*., 2008; Ristic *et al*., 2011; Fudge *et al*., 2012; Kelly *et al*., 2012; Cain *et al*., 2013; Mayr *et al*., 2014; Ristic *et al*., 2015, 2017; Van der Hulst *et al*., 2019). The only two VPs detected in the smoked juice were also the highest released compounds after treatment with enzyme or yeast. An elevated abundance of glycosylated precursors of guaiacol and phenol compared to the other glycosylated VPs has been reported previously, which is consistent with our results (Noestheden *et al*., 2017; Whitmore *et al*., 2021). Guaiacol was one of the first VPs linked to smoke taint in wine, and this compound has been reported widely as a key marker compound (Kennison *et al.*, 2008, 2009; Ristic *et al*., 2011; Fudge *et al*., 2012; Ristic *et al*., 2015). Although the presence of other VPs can exhibit a synergistic, additive effect on the overall smoke taint sensory character in wine, guaiacol seems to drive this phenomenon (De Vries *et al*., 2016).

Many studies have utilised harsh acid hydrolysis to estimate the total bound and free VPs in smoked-tainted juice and wine, yet it has recently been found that glycosidase enzyme cocktails can exhibit similar efficacy (Cui *et al*., 2024). The treatment of smoked Pinotage juice with a glycosidase enzyme mixture led to a guaiacol release only slightly higher than the best-performing yeast strains, yet roughly double the release of combined VPs in contrast with the yeast treatments. The substrate specificity and diversity of yeast glycosidase enzymes may explain these differences. In addition, the composition of bound VPs in the smoke-exposed juice matrix may also contribute to this variation (Hu *et al*., 2016; Li *et al*., 2020; Qin *et al*., 2021). The two most significant indicator compounds that have been reported for smoke-tainted grapes and wine are guaiacol and 4-MG, which constitute roughly 20% of total VPs present in wildfire smoke, regardless of fuel source (Kelly *et al*., 2012). Thus, it is no surprise that guaiacol was detected at such elevated levels in the yeast screening, with most strains releasing levels above that of the ODT. The ratio of guaiacol to 4-MG in the smoke-tainted juice fermentations was between 3.5 and 6.5. A study conducted in Oregon, USA profiled 376 smoke-tainted wines and reported a shift in the frequency of guaiacol:4-MG that increased to an average of 4.5 compared to non-smoked wines (Yang *et al*., 2023). The same study also found the highest correlation for smoke-tainted wines with both guaiacol and 4-MG, while the cresols (*m*-, *p*- and *o*-) were the lowest contributors.

A large number of *Hanseniaspora uvarum* and *Lachancea thermotolerans* strains were selected due to previously reported ß-glucosidase activity for multiple strains within these species (Porter *et al*., 2019; Fan *et al*., 2022). Overall, eight genera were selected for the screening to increase the diversity of enzymatic activity. The diversity of wine yeast metabolism and extracellular glycosidase production, specifically β-glucosidase, has been reported (Manzanares *et al*., 2000; Cordero Otero *et al*., 2003; González-Pombo *et al*., 2011; Zhang *et al*., 2021). To our knowledge, the variation observed for guaiacol release between genera and within species of non-*Saccharomyces* yeasts in smoke-tainted wine has not been reported before. A study by Whitmore *et al*. (2021) investigated the potential impact of *S. cerevisiae* commercial strain selection on VP release in smoke-exposed grape juice fermentations. The authors did not observe any significant differences between the six strains selected for VP release, while phenol and guaiacol were the most abundant free VPs detected in the final wines. However, there are some cases where *S. cerevisiae* strains have been observed to differ more significantly with VP release in smoke-tainted wines (Kennison *et al*., 2008; Ristic *et al*., 2011). Nevertheless, the variation between selected non-*Saccharomyces* strains in this study was very pronounced (> twofold difference between highest and lowest strains). The *L. thermotolerans* species displayed a relatively high release of VPs overall, but still exhibited some variation between strains. *L. thermotolerans* Y940, one of the highest producers in this screening, was previously investigated for displaying ß-glucosidase activity in Muscat fermentations in a study aimed at identifying yeasts from the *Lachancea* genus for potential terpene release to improve wine aroma (Porter *et al*., 2019). The strain was reported to exhibit strong extracellular β-glucosidase activity in both synthetic grape must and real grape juice, but the enzyme characterisation has yet to be carried out.

The strains from *Hanseniaspora* displayed a slightly greater variation in VP release, and it has been reported that the β-glucosidase activity of *H. uvarum* is strain specific, where certain strains display high activity, while others display none at all (Arévalo Villena *et al*., 2005; Fan *et al*., 2022). *W. anomalus* Y541 was also amongst the bigger releasers of VPs in our study, in accordance with the literature, which reports the diverse extracellular enzyme activity of this species as being suitable for winemaking, including ß-glucosidase activity (Padilla *et al*., 2018; Han *et al*., 2021). Another strain of interest was *S. bacillaris* Y550, which may be suitable for oenological application due to certain strains from this species being reported to display strong fructophilic tendencies and extracellular enzyme activity (Englezos *et al*., 2017, 2018).

CONCLUSIONS

This study served as a preliminary investigation of selected non-*Saccharomyces* yeasts and their ability to release VPs in smoke-tainted grape must. The best performing strains could potentially be utilised in wine fermentations as part of a two-step mitigation strategy: First inoculating them into grape must alongside a commercial *S. cerevisiae* strain to release a large fraction of bound VPs, followed by the removal of free VPs by a downstream remediation technique such as the addition of activated charcoal. Nevertheless, it would be important to ensure that such selected strains do not produce spoilage compounds or interfere negatively with the fermentation. Furthermore, the glycosidase enzyme activity of the best-performing strains should be

characterised further to unravel the impact of parameters such as pH, temperature, ethanol concentration and glucose concentration on the respective enzyme activities. This would allow the identification of the best-suited inoculation scenario for the most effective exploitation of these yeasts' glycosidase activities.

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