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ORIGINAL PAPER

Occurrence of hydrogen sulfide in wine and in fermentation: influence of yeast strain and supplementation of yeast available nitrogen

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Abstract Hydrogen sulfide (H_2S) is a powerful aroma compound largely produced by yeast during fermentation. Its occurrence in wines and other fermented beverages has been associated with off-odors described as rotten egg and/or sewage. While the formation of hydrogen sulfide (H_2S) during fermentation has been extensively studied, it is the final H_2S content of wine that is actually linked to potential off-odors. Nevertheless, factors determining final H_2S content of wine have received little attention, and it is commonly assumed that high H_2S-forming fermentations will result in high final concentrations of H_2S. However, a clear relationship has never been established. In this report, we investigated the contribution of yeast strain and nitrogen addition to H_2S formation during fermentation and its consequent occurrence the resulting wines. Five commercial *Saccharomyces cerevisiae* wine yeast strains were used to ferment a Chardonnay juice containing 110 mg/l of YAN (yeast assimilable nitrogen), supplemented with di-ammonium phosphate (DAP) to increase YAN concentration to moderate (260 mg/l) and high (410 mg/l) levels. In contrast to the widely reported decrease in H_2S production in response to DAP addition, a non-linear relationship was found such that moderate DAP supplementation resulted in a remarkable increase in H_2S formation by each of the five wine yeasts. H_2S content of the finished wine was affected by yeast strain, YAN, and fermentation vigor. However, we did not observe a correlation between concentration of H_2S in the finished wines and H_2S produced during fermentation, with low-forming fermentations often having relatively high final H_2S and vice versa. Management of H_2S in wine through nitrogen supplementation requires knowledge of initial YAN and yeast H_2S characteristics.

Keywords Hydrogen sulfide · *S. cerevisiae* · Nitrogen · Vitamins · Fermentation

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Introduction

Off-flavors due to volatile compounds derived from yeast metabolism during alcoholic fermentation are of major concern in the production of quality fermented beverages, such as beer, cider, and wine. Hydrogen sulfide (H_2S) is an important contributor to the so-called 'reductive' off-flavor present in some wines, with an odor threshold of only 1.6 μg/l in white wine and an odor often described as 'rotten eggs' and 'putrefaction' [22]. In wine fermentations, H_2S is largely formed by reduction of exogenous sulfate during the biosynthesis of the sulfur-containing amino acids cysteine and methionine [7, 10, 24]. Sulfite, which is commonly added to grape musts as an antioxidant and antimicrobial agent, can also enter the cell and become a source of H_2S, after reduction by sulfite reductase [7, 9, 24]. Based on the observation that a negative correlation exists between naturally occurring nitrogen in the juice and total H_2S formed during fermentation [28], one commonly adopted strategy to limit formation of H_2S in wine fermentations is to provide adequate yeast assimilable nitrogen (YAN) to ensure sufficient availability of the amino acid precursors [reviewed in 1]. However, recent data have suggested that the addition of YAN to wine fermentations not only does not reduce H_2S formation, but in some cases even appears to exacerbate the problem [14, 25, 26, 29]. The observation, reported by the same authors, that this effect is

also strain-dependent, suggests that interactions between YAN and the genetic background of individual yeasts are a key factor in determining the amount of H_2S formed during fermentation. Regulation of H_2S metabolism has been shown to be genetically complex and highly variable between strains [12]. However, the intrinsic ability of most commercial yeast strains to produce H_2S has only been studied in model systems, often under conditions largely different from those commonly found in wine fermentations [4, 8, 15, 19, 20, 23].

H_2S is formed in concentrations up to hundreds of μg/l during fermentation [18, 27]. However, only a minor component of this is left in the wine at the end of fermentation (typically 1–20 μg/l), due to H_2S high volatility and the purging action of CO_2. However, while in the vast majority of the studies, factors controlling H_2S formation during fermentation have been studied, there is still a significant gap in understanding the relation between production of H_2S during fermentation and concentration of this metabolite in finished wines. Indeed, in many published studies it is implied that the total amount of H_2S formed during fermentation is important to wine quality due to its unpleasant aroma properties. It appears however, that the occurrence of H_2S in finished wines is ultimately the quality parameter that needs to be managed.

The aim of this study was to investigate the relationship between H_2S production during fermentation and the H_2S present in finished wine, which can be linked to reductive off-flavors. Fermentation conditions known to strongly affect fermentation H_2S production, that is varying initial juice nitrogen over a wide range of concentrations in conjunction with different commercial yeast, were used. These conditions were achieved by supplementing a low YAN Chardonnay juice with two increasing concentrations of DAP and fermenting with five different *Saccharomyces cerevisiae* wine strains with different H_2S production characteristics. H_2S was monitored throughout fermentation and then measured in the finished wines along with other major wine compositional parameters, in order to investigate the contribution of fermentation and technological variables responsible for residual H_2S concentration in wine.

Materials and methods

Yeast strain

The five yeasts used in this study were *Saccharomyces cerevisiae* strains AWRI 796, AWRI 1483 (Lalvin D254, Lallemand Inc., Montreal, Canada), AWRI 838 (isolated from Lalvin EC1118, Lallemand Inc.), AWRI 1493 (Lalvin 71B, Lallemand Inc.), AWRI 1537 (VIN 13, Anchor Foods, South Africa). Yeasts were maintained by bimonthly serial propagation on yeast-malt extract (YM) medium (Amyl Media, Dandenong, Australia) supplemented with 1.5% agar with storage at 4°C. All strains were obtained from The Australian Wine Research Institute Culture Collection (Adelaide, Australia). These yeasts were selected to cover a broad range of H_2S formation capabilities. This intrinsic ability was assessed by means of a bismuth-containing indicator media (BiGGY agar, Oxoid, England) [8]. Cultures were grown in YPD (Amyl Media, Dandenong, Australia) overnight, then 20 μl of the culture was deposited on the plate, which was incubated at 27°C for 48 h. Colonies were then assessed for the intensity of the brown color, which is directly correlated with H_2S production. A H_2S-negative strain (AWRI 1640) and a known high producer (AWRI 1813) were used as reference strains. Figure 1 shows that the H_2S production by the five strains used in this study ranged from potentially low producers such as AWRI 838 and 796 to high producers such as AWRI 1493, with the other yeasts exhibiting intermediate degrees of H_2S production.

Fermentations

A Chardonnay juice having a total YAN of 110 mg/l (FAN = 83 mg/l; NH_3 = 31 mg/l) was used for this study. Other analytical parameters were as follows: total soluble solids, 21.5° Brix; titratable acidity, 7.1 g/l as tartaric acid; pH, 3.5. The juice was filter sterilized with a 0.2-μm membrane filter (Millipore, Australia). For the preparation of the starter cultures, a loopful of yeast cells was incubated in 10 ml of YM medium (Amyl Media, Australia) with shaking for 24–48 h at 28°C. Preadaptation of the cells to the fermentation medium was carried out by inoculating 100 μl of the cultures into 20 ml of filter sterilized 50% (v/v)

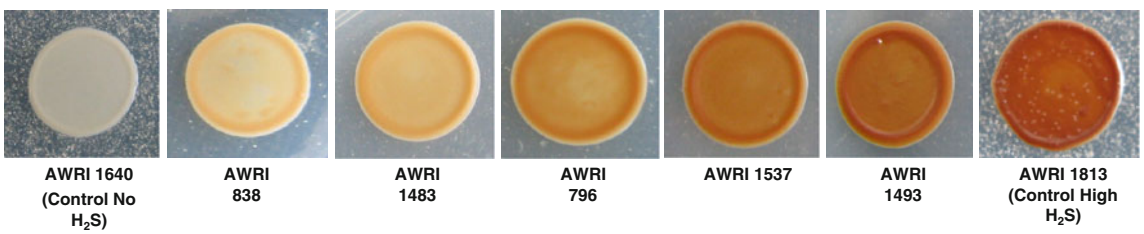

Fig. 1 Production of H_2S by the five strains studied on BiGGY agar

Chardonnay juice in sterile, deionized water. These subcultures were grown at 28°C until a biomass of 1×10^8 cells/ml was obtained, after which they were inoculated in the Chardonnay juice at a final concentration of 1×10^6 cells/ml. Fermentations were carried out at 18°C in 250-ml Erlenmeyer flasks containing 200 ml of sterile juice, under constant shaking. Biomass growth was monitored by hemocytometer count. All flasks were fitted with silver nitrate detector tubes for the quantification of H_2S formed in fermentation. Prior to inoculation, the addition of DAP was performed where required, according to an experimental design consisting of three YAN concentrations, each one fermented in triplicate, for a total of nine fermentations for each yeast. A control that did not receive any DAP addition represented the lowest nitrogen concentration (110 mg/l YAN), while in the two other treatments DAP was added to a final YAN concentration of 260 mg/l YAN and 410 mg/l YAN. The pH of the fermentation medium was readjusted to 3.5 with 1 N HCl following DAP additions.

Chemical and microbiological analyses

YAN was calculated as the sum of ammonia-derived nitrogen and FAN, and therefore did not include proline [25]. The fermentation progress was monitored by daily analysis of residual sugar by means of an enzymatic kit. Cell population at different stages of fermentation was determined by microscope count using a hemocytometer. Monitoring of H_2S development during fermentation was carried out by means of silver nitrate selective gas detector tubes (Komyo Kitagawa, Japan), as described by Ugliano and Henschke [27]. H_2S in the finished wines was measured by gas chromatography, as described by Ugliano et al. [25]. For this analysis, wines were cold-settled for 6 days at 4°C under N_2 headspace to remove yeast cells, then 10 ml of clear wine was sampled and used for the analysis. Each analysis was performed in duplicate.

Statistical analyses

Analysis of variance (ANOVA) was carried out using JMP 5.0.1 (SAS, Cary, NC). Multiple linear regression (MLR) analysis was carried out using Unscrambler 9.5 (CAMO, Oslo, Norway).

Results and discussion

Effect of nitrogen supplementation on H_2S production during fermentation

Fermentation performance of the five yeast strains under the different nutritional conditions are summarized in

Table 1 Fermentation performances of the five *S. cerevisiae* strains studied

	AWRI 796			AWRI 838			AWRI 1483			AWRI 1537			AWRI 1493		
	110[a]	260	410	110	260	410	110	260	410	110	260	410	110	260	410
Time to completion (days)	21 ± 0.5	13 ± 1	11 ± 0.5	15 ± 0.5	14 ± 0.5	12 ± 0.5	19 ± 0.5	14 ± 0	12 ± 0.5	15 ± 0	14 ± 0.5	12 ± 0.5	24 ± 0.5	16 ± 0.5	14 ± 0.5
Average fermentation rate (g/day)[b]	10 ± 2	15 ± 1	18 ± 2	13 ± 1	14 ± 2	17 ± 1	11 ± 2	14 ± 1	17 ± 1	13 ± 1	14 ± 2	17 ± 2	8 ± 1	13 ± 1	14 ± 1
Max. population (1×10^8 cells/ml)	1.4 ± 0.2	2.0 ± 0.2	2.3 ± 0.1	1.1 ± 0.1	2.1 ± 0.2	2.4 ± 0.1	1.1 ± 0.1	1.8 ± 0.1	2.3 ± 0.2	1.3 ± 0.2	2.1 ± 0.2	2.3 ± 0.1	1.3 ± 0.3	1.9 ± 0.2	2.0 ± 0.2
Time to Max population (days)	9 ± 0.5	8 ± 0.5	6 ± 0.5	9 ± 0	7 ± 0.5	6 ± 0	9 ± 0	8 ± 0.5	6 ± 0.5	10 ± 1	6 ± 0.5	6 ± 0.5	14 ± 0.5	10 ± 1	8 ± 0.5
Nitrogen depletion[c]	22% ± 5	48% ± 3	80% ± 5	22% ± 4	50% ± 2	75% ± 3	20% ± 2	30% ± 2	62% ± 6	23% ± 4	43% ± 5	80% ± 2	40% ± 3	60% ± 5	82% ± 4

Means of triplicate fermentation ± SD

[a] Yeast assimilable nitrogen (YAN), expressed in mg/l

[b] Total sugars consumed (g)/total fermentation time (days)

[c] Values indicate the stage of fermentation at which YAN became not detectable (expressed as percentage of sugar consumed)

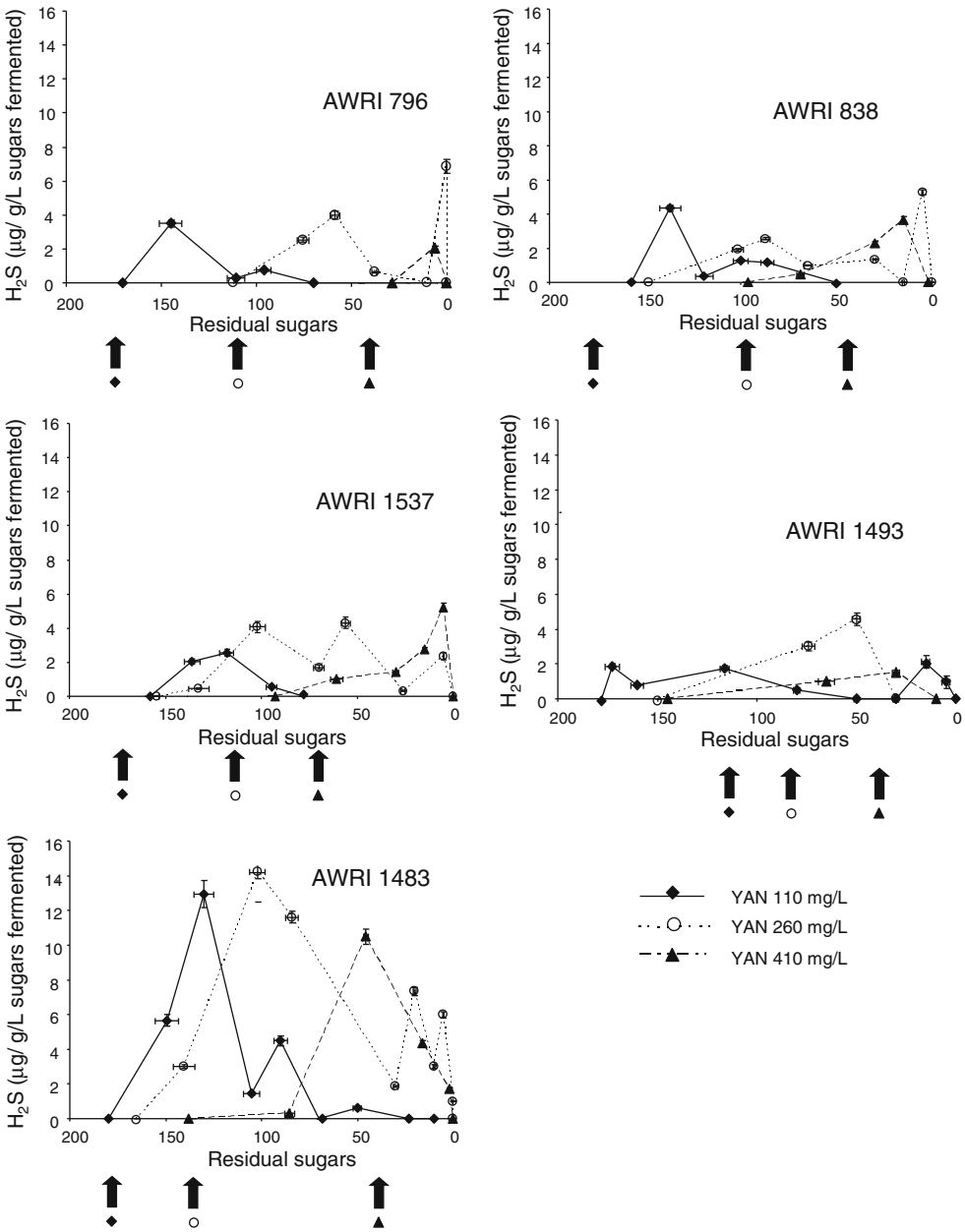

Fig. 2 Profiles of H_2S production in the head space during fermentation with five yeast strains of a low-nitrogen Chardonnay juice supplemented with DAP to give three YAN concentrations. For each YAN concentration, *arrows* indicate the stage of alcoholic fermentation at which YAN was depleted

Table 1. All treatments reached dryness (residual sugars <1 g/l), indicating that, for all the yeast tested, available nitrogen did not compromise the ability of yeasts to achieve dryness in a filter-clarified grape juice. Initial YAN increased the fermentation rate and maximum cell density and population, resulting in reduced fermentation duration, and shorter time to reach maximum cell density, which was in agreement with previous findings [2].

The initial nitrogen content of the Chardonnay juice had a strong effect on the profile of H_2S production during fermentation (Fig. 2). In the non-supplemented fermentations (110 mg/l YAN at inoculation), evolution of H_2S was observed to commence upon consumption of approximately 25% of the available sugars, whereas a longer lag was observed between the beginning of alcoholic fermentation and the onset of H_2S production in nitrogen-supplemented treatments, consistent with previous findings [5, 7, 9]. In general, when H_2S was formed during the cell-growth phase, a very high rate of formation commenced upon depletion of YAN, as observed in the non-supplemented fermentations (Fig. 2). However, exceptions to the general trend were observed. For example, yeast AWRI 1483 showed similar maximum rates of H_2S production irrespective of the yeast growth phase (exponential or stationary) or initial nitrogen content of the medium. The data on total H_2S production (Fig. 3) shows the differences in total H_2S produced by the different fermentations. ANOVA analysis indicated that yeast was the major source

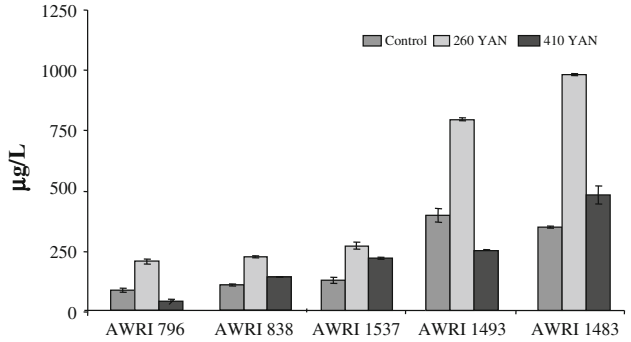

Fig. 3 Effect of yeast strain and initial nitrogen on total H_2S produced during fermentation of Chardonnay juice

of these differences across the whole data set ($F = 12.49$; $p < 0.001$) followed by YAN concentration ($F = 4.41$; $p < 0.05$). Yeast × YAN interactions were also highly significant ($F = 89.1$; $p < 0.001$), suggesting that the amount of H_2S produced by yeast in response to nitrogen supplementation is highly dependent on the genetic background of different strains. Moderate nitrogen supplementation, that is to 260 mg/l YAN, generally resulted in a 2–3 fold increase in total H_2S formed, whereas higher supplementation, to 410 mg/l YAN, resulted in total H_2S production similar to or lower than the control, non-supplemented fermentations. Overall, these results appear in contrast with the generally accepted paradigm that increased YAN availability reduces formation of H_2S. In particular, the early results of Vos and Gray [28] established the existence of a negative correlation between YAN and H_2S production. However, in that study, the average YAN of the various grape juices tested was 265 mg/l, suggesting that the study was based around a panel of juices with YAN values generally much higher than the Chardonnay juice studied here. Our findings made with a low nitrogen juice (110 mg/l) indicated that DAP supplementation can result in increased H_2S production, particularly for moderate DAP additions, in agreement with recent observations [13, 25]. Mendes-Ferreira et al. [14] reported that, in a model grape juice, *MET* genes involved in the formation of H_2S were specifically down-regulated under conditions of nitrogen starvation (YAN 66 mg/l), while supplementation of nitrogen to an initial YAN of 267 mg/l resulted in upregulation of these genes and maximum H_2S formation. Moreover, under our conditions, moderate nitrogen fermentations had generally increased cell population, and entered stationary phase between day six and day seven of fermentation, while nitrogen was depleted between day three and day four (Table 1). Therefore, larger biomass (and therefore sulfite reductase activity) was exposed to nitrogen starvation during the growth phase in these fermentations. The observation that DAP addition to a low-YAN juice determines a non-linear dose–response for H_2S production is particularly relevant in light of the data indicating the very frequent occurrence of low nitrogen grapes (YAN < 140 mg/l) in different wine-making countries [3, 16, 25, 30].

Based on a rapid screening method that employs a bismuth-containing growth medium, the yeast strains used in this study show a wide range of H_2S production capabilities (Fig. 1). Comparison of Figs. 1 and 3 suggests a general correlation in the trend of total H_2S produced during fermentation, irrespective of the initial YAN of the Chardonnay juice. This result is consistent with the observations of Jiranek et al. [8] from which they proposed that the agar plate method provides an indication of the maximum potential for a strain to produce H_2S. However, AWRI 1483 is an anomalous strain in this regard, which showed low production in the plate assay and maximum production in the fermentation experiment. Nevertheless, these results generally support the validity of the agar plate method as a rapid screening tool to assess the maximum potential for a strain to produce H_2S [8], but reinforces the widely reported observation that H_2S production in fermentation, although being strain-dependent, is significantly affected by the composition of the medium [6, 7, 23]. Therefore, plate assays such as the one used here cannot be used to systematically predict H_2S formation during fermentation but provide a good indication of the potential of yeast strains to produce H_2S.

Residual H_2S concentration of finished wines: effect of yeast strain and initial juice nitrogen

Analysis of H_2S was performed on the wines following fermentation to assess the influence of yeast and nitrogen on wine H_2S concentration after clarification by cold-settling of the yeast. The results are shown in Fig. 4. H_2S concentrations were generally below 1 µg/l, whereas other authors have reported much higher H_2S concentrations at the end of fermentation [11, 21]. These low concentrations might reflect the fact that solids were completely removed from the must in the current study [11]. Moreover, different analytical methods (gas-chromatography versus colorimetric reaction) were used for these studies, which might have accounted for some of the differences observed, as recently discussed [27]. ANOVA analysis indicated that, generally, YAN concentration was a greater source of variation than yeast strain ($F = 8.2$, $p < 0.001$ and $F = 3.1$, $p < 0.05$, respectively). Once again, yeast × YAN interactions had a much bigger influence than yeast or YAN alone, highlighting the complex response of individual yeast strains to nitrogen supplementation. Based on ANOVA analysis, low and moderate nitrogen fermentations gave wines with significantly higher residual H_2S compared to wines from high nitrogen fermentations.

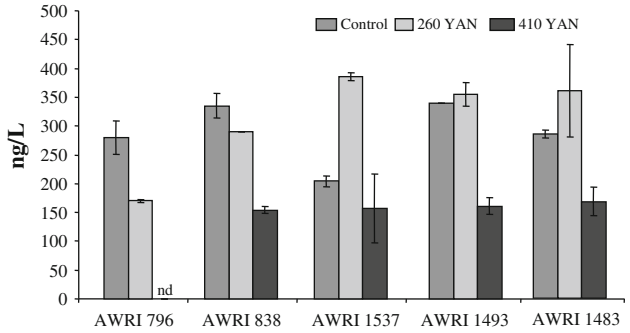

Fig. 4 Concentration of residual H_2S in the final wines. *nd* indicates not detected

When comparing this with the data on total H_2S formed during fermentation (Fig. 3), it appeared clear that the amount of H_2S generated during fermentation is not directly responsible for H_2S concentration in the finished wines (Fig. 4). Despite each yeast producing significantly increased total H_2S during fermentation of the moderate N (260 mg/l) juice, the difference between residual H_2S in low and moderate nitrogen fermentations was not significant. Therefore, for a given yeast, the highest H_2S-producing fermentations did not systematically generate wines with maximum residual H_2S, and, in general, high H_2S-producing yeasts, for example AWRI 1483, did not give wines with increased residual H_2S.

Unexpectedly, in spite of the large strain difference in fermentation-related H_2S, the maximal residual H_2S in wines ranged 300–400 µg/l irrespective of yeast strain, which suggested that this concentration might represent an equilibrium level for these wines under the conditions of the experiment. In a recent study we suggested that H_2S formed late in fermentation could be responsible for increased residual H_2S, possibly due to the reduced purging effect of CO_2 in the last stages of fermentation [25]. However, in this study, relatively little H_2S was produced during the final stages of fermentation, which might explain why such behavior was not observed. Furthermore, differences in size of the fermentors (200 ml compared to 30 l) and nature of the matrix (white juice compared to red must) could have affected retention of the highly volatile H_2S. Interestingly, ANOVA analysis also indicated that final H_2S in high nitrogen wines was significantly lower than in low and moderate nitrogen fermentation ($p < 0.01$), although these fermentations did not form less H_2S (Fig. 3). Considering the high volatility of H_2S, fermentation vigor, that is, the rate of CO_2 evolution and its purging effect, might have played a role, as high nitrogen fermentations exhibited maximum fermentation rate (14–18 g sugars consumed/days of fermentation). However, moderate nitrogen fermentations had residual H_2S similar to or higher than low nitrogen fermentations, in spite of their fermentation rates being generally higher, suggesting that fermentation vigor is only partially correlated to final H_2S. The role of certain reactive species, including phenolics and quinones, which can trap –SH compounds, remains to be established. H_2S can also be uptaken by the yeast form the fermentation environment towards the end of fermentation [17].

In conclusion, by using a panel of yeast strains with different H_2S production characteristics combined with fermentation conditions that modify H_2S production over a wide range, this study suggests the residual content of H_2S in finished wine is not always determined by the total amount of H_2S produced during fermentation. Furthermore, at least under the conditions of this experiment, residual H_2S in wine could not be linked to kinetic aspects of H_2S production. However, the highest nitrogen fermentations always produced wines with the lowest residual H_2S, irrespective of yeast strain or H_2S produced during fermentation. It remains to establish whether H_2S in wines at the end of fermentation might also be affected by chemical reactions between H_2S and wine constituents, for example phenolics and quinones.

Although DAP supplementation can strongly affect H_2S production during fermentation, the ability of this common wine-making practice to systematically reduce formation of H_2S is questionable. Moderate nitrogen supplementation of a low nitrogen juice was generally associated with increased production of H_2S. Also, for some strains, moderate nitrogen supplementation resulted in increased final concentration of H_2S compared to non-supplemented controls. Given the importance of H_2S management in the modern wine industry, these results indicate the need for further research on the mechanisms determining H_2S content of wine.

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