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Development of a New Assay for Measuring H_2S Production during Alcoholic Fermentation: Application to the Evaluation of the Main Factors Impacting H_2S Production by Three *Saccharomyces cerevisiae* Wine Strains

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Abstract: Hydrogen sulfide (H_2S) is the main volatile sulfur compound produced by *Saccharomyces cerevisiae* during alcoholic fermentation and its overproduction leads to poor wine sensory profiles. Several factors modulate H_2S production and winemakers and researchers require an easy quantitative tool to quantify their impact. In this work, we developed a new sensitive method for the evaluation of total H_2S production during alcoholic fermentation using a metal trap and a fluorescent probe. With this method, we evaluated the combined impact of three major factors influencing sulfide production by wine yeast during alcoholic fermentation: assimilable nitrogen, sulfur dioxide and strain, using a full factorial experimental design. All three factors significantly impacted H_2S production, with variations according to strains. This method enables large experimental designs for the better understanding of sulfide production by yeasts during fermentation.

Keywords: hydrogen sulfide; wine; *Saccharomyces cerevisiae*; alcoholic fermentation; nitrogen; sulfur dioxide; assay; fluorescence

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1. Introduction

Saccharomyces cerevisiae yeast is one of the most important microorganisms in wine-making, mainly because it has an unrivalled ability to ferment sugars, producing heat, carbon dioxide and ethanol. It can easily colonize its environment, coping successfully with adverse conditions prevailing in grape juice, such as low pH, high initial sugar content and increasing concentrations of alcohol.

Alcoholic fermentation and yeast multiplication in grape must are accompanied by the production of numerous metabolites necessary for yeast growth. These metabolites can be excreted from the cell (i.e., into the must) and may have an impact on wine aroma, color or taste. Among these metabolites, volatile sulfur compounds (VSCs) play a key role on wine complexity, shifting the balance towards positive notes or outright undesirable aromas, depending on the amount and nature of the molecules involved. Hydrogen sulfide is one the VSCs with the highest impact of wine sensory profile, being both associated with reductive aromas due to its rotten egg odor, and able to overwhelm fruity and floral wine attributes [1]. In addition, the low threshold of H_2S perception in wine, in the µg/L range (reviewed by Mestres and colleagues [2]) and the high volatility of this molecule, make the monitoring of its content even more critical. Furthermore, hydrogen sulfide is highly reactive: its combination with either must or wine molecules can lead to the synthesis of other volatile sulfur compounds such as ethanethiol [3], dimethyl disulfide, dimethyl trisulfide, and dimethyl tetrasulfide, also known to be detrimental to wine quality (reviewed by Waterhouse, Sacks and Jeffery [4]).

Yeast's major metabolic pathway for H_2S synthesis is the sulfur assimilation pathway (SAP) [5] (Figure 1). The main sulfur source in grape must is sulfate [6] and, so far, three transporters (Sul1, Sul2 and Soa1) have been characterized for their ability to uptake extracellular sulfate [7,8]. In the cell, sulfates are reduced to sulfides through a series of activation, phosphorylation and reduction reactions catalyzed by ATP sulfurylase (Met3), 5′-adenylyl sulfate (APS) kinase (Met14 regulated by Skp2), 3′-phospho-5′-adenylylsulfate (PAPS) reductase (Met16) and sulfite reductase (heterotetramer Met5–Met10). The incorporation of sulfide into the carbon chain of o-acetyl homoserine (OAH), carried out by the o-acetyl homoserine-o-acetyl serine sulfhydrylase Met17, leads to the biosynthesis of the non-proteinogenic α-amino acid homocysteine, precursor of the sulfur-containing amino acids methionine and cysteine. The genes of the whole biosynthetic pathway are regulated through a feed-back mechanisms by cysteine allowing an adjustment of sulfur flux to biosynthetic needs [9]. In addition, the sulfur assimilation pathway is tightly connected to the yeast nitrogen metabolism since the sulfide acceptor OAH is derived from the serine amino acid. Indeed, a disequilibrium between sulfide formation and OAH flux can lead to H_2S release.

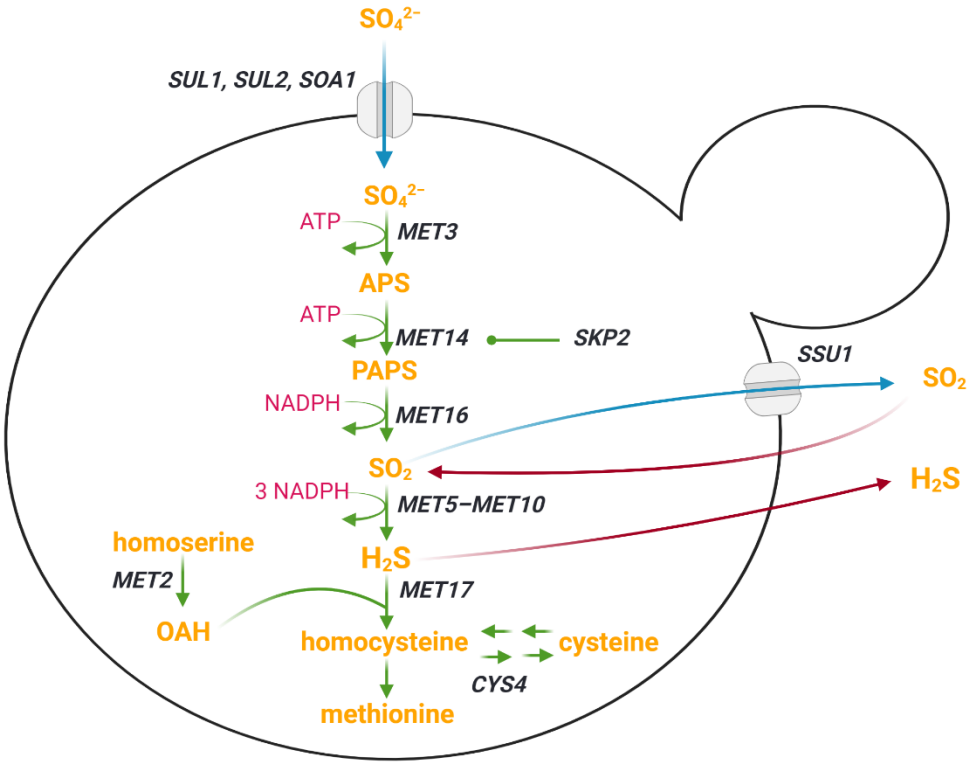

Figure 1. Sulfur assimilation pathway (SAP) in *Saccharomyces cerevisiae*. Blue arrows: transporter mediated flux. Red arrows: diffusion. Green arrows: enzymatic reactions. Dot line: protein regulator. Text in orange: compounds. Text in pink: cellular energetic cost. Text in black: genes.

Although absolutely necessary for the biosynthesis of sulfur-containing amino acids in alcoholic fermentation conditions, the overproduction of sulfide by wine yeasts is a serious issue in winemaking.

Multiples factors have an impact on the sulfur metabolism and thus sulfide excretion. Among them, the first is the availability of different substrates and co factors required for the pathway. However, in addition, specific mechanisms regulate this pathway.

Sulfur available in grape must, either in the form of elemental sulfur, sulfate or SO_2 used in winemaking as antimicrobial and antioxidant is directly related to sulfide production (reviewed by Huang et al. [10]).

How sulfur dioxide (exogenous or derived from the reduction of sulfate) affects hydrogen sulfide production strongly depends on the efficiency of sulfide reductase, the

key enzyme in the sulfur assimilation pathway. A huge variability in its activity has been described, both in terms of overall efficiency and timing of H_2S release [11]. The partial or full inactivation of the gene encoding one of the two subunits of the enzyme (*MET10*) [12] or the presence of spontaneous or chemically induced mutations in the *MET10* and *MET5* genes modify sulfide production [13,14].

In addition, the availability of intermediate metabolites of the SAP such as methionine, S-adenosylmethionine and cysteine [9], can result in a pathway shutdown, when the cell does not require the neo-synthesis of these metabolites. In addition, this SAP activity and thus the potential for hydrogen sulfide production is also linked to the availability of nitrogen in the must or the ability of the yeast itself to utilize it. Indeed, the presence of sufficient quantities of nitrogen ensures the biosynthesis of precursors for the sequestration of the sulfide ion, thus avoiding an over-release of H_2S.

Besides substrates and intermediary metabolites, the availability of thiamine, pyridoxine, biotin, or pantothenic acid [15,16] alters yeast capability to produce H_2S. Pantothenic acid, for example, is a component of Acetyl-CoA: its deficiency leads to metabolic depletion of the sulfide acceptor OAH and thus an accumulation of H_2S [17].

The main transcription factor regulating the sulfur assimilation pathway is the transcriptional activator Met4. In the active form, Met4 interacts with Met31 and Met32, or alternatively with Cbf1, and the complex is stabilized by the cofactor Met28 [18].

Last, it should be pointed out that besides this metabolic regulation, a specific SCF ubiquitin protease complex regulates SAP enzymes and thus specific mutations can lead to variable modulation of its activity [19].

The multiple metabolites and regulation mechanisms may explain the high variability that has been observed among strains for sulfide production [20–26].

Given this complexity, different studies tried to evaluate the impact of several factors alone or in combination on the production of sulfide such as assimilable nitrogen and strain [23], sulfur dioxide and strain [25], assimilable nitrogen and pantothenic acid [17], vitamins [15,16].

Nevertheless, the volatile nature of hydrogen sulfide makes its accurate quantification difficult, and a major issue. Nowadays, the main available methods, listed in Table 1, are either semi quantitative and inexpensive or quantitative but require a more expensive equipment and are low throughput methods.

Table 1. Available H_2S measurement methods.

Method	Characteristics	References
Lead Acetate Paper Strips	Sensitive, high throughput, qualitative	[22]
Electrochemical: H_2S Sensors	Rapid, highly sensitive, simple, real-time, quantitative, one-by-one measurement *	[26]
GC-MS or GC Coupled with Sulfur Detectors (FPD, PFPD, SCD)	Real-time, quantitative, only headspace H_2S content, one-by-one measurement *	[2,27–29]
Fluorescent Probes	Sensitive, easy to use, suitable for high throughput analyses, cannot be used directly on a gas (as H_2S)	[30]

* Not suitable for high throughput analyses.

Each method relies on a different principle and can be suitable for a specific application. Unfortunately, none of them is at the same time quantitative, high throughput, non-hazardous, and inexpensive, while allowing the measurement of the total amount of sulfide produced during alcoholic fermentation.

As a consequence, the primary goal of this study has been to develop a low-cost, rapid and sensitive method for the evaluation of cumulated H_2S production during fermentation, while being also compatible with large scale experiments. We used it to evaluate simultaneously the importance of three major factors affecting H_2S production: assimilable nitrogen

content, strain genetic background, and sulfite that is being added almost systematically during winemaking.

2. Materials and Methods

2.1. Strains

Three commonly used *Saccharomyces cerevisiae* wine starter strains, isolated from wine in France, were obtained from Lallemand SAS, namely: EC1118 [31], a spore derived from LMD1 (MTF1832) and LMD17 [32]. All strains were maintained on YEPD agar plates (2% glucose, 1% yeast extract, 2% peptone, and 2% agar) at 4 °C.

2.2. Fermentation Condition

Fermentations were conducted in synthetic musts (SM) mimicking a natural must, with some adjustment compared to what was described by Bely et al. (1990) [33]: a total amount of 200 g/L of a 1:1 mix of glucose and fructose served as carbon source and the anaerobic factors were reduced to $\frac{1}{4}$ of the amount described in order to limit the excess of lipids. According to the experimental plan, three yeast assimilable nitrogen (YAN) and three sulfur dioxide (SO_2) contents were used: for nitrogen: (i) 100 mg/L, (ii) 200 mg/L, (iii) 300 mg/L; for SO_2: (i) 0 mg/L, (ii) 30 mg/L, (iii) 60 mg/L. SO_2 was added as potassium metabisulfite ($K_2S_2O_5$) 24 h before fermentation onset.

For each strain, 6 mL of YEPD liquid medium were inoculated with a single colony and grown for 24 h in an Infors HT incubation shaker at 28 °C and 180 rpm. A 1:100 dilution of the first preculture was transferred to 6 mL of fresh sterile-filtered synthetic must containing the same YAN content as the corresponding final fermentation, but without any sulfite addition, and incubated for 24 h in the same conditions as the first preculture.

250-mL screw-cap bioreactors were inoculated at 10^6 cells/mL, determining cell number with an electronic particle counter (Multisizer 3 Coulter Counter, Beckman Coulter France SAS, Villepinte, France). Fermentations were carried out at 28 °C under permanent stirring until 95% of total sugar was consumed (i.e., 87.4 g/L of CO_2 released). CO_2 produced was monitored by weight loss, since there is a linear relationship between the glucose consumed, the ethanol produced and the CO_2 released [34].

2.3. Hydrogen Sulfide Measurement

In order to measure the hydrogen sulfide cumulate production of each strain during fermentation, a fluorocolorimetric method relying on a metal trap was developed.

Specific glassware was designed to convey the gas from the bioreactor into 15 mL Falcon tubes filled with 6 mL of 1% zinc acetate trapping solution and sealed with SeptaSecure Uncut Caps (Syringa Lab Supplies, Boise, ID, USA); a needle in the septum ensured CO_2 escape.

The sparging of the fermentation gas into the tube containing the zinc solution ensured simultaneously the trapping of produced H_2S, as a zinc sulfide precipitate (ZnS), and the maintenance of anaerobiosis inside the bioreactor. ZnS was collected by centrifugation (30′, 4700× *g*), diluted in 1mL 1X pH 7 phosphate-buffered saline (PBS) and transferred to a glass vial. For hydrogen sulfide quantification, a sulfide-specific fluorescent probe (6-(2, 4-dinitrophenoxy)-2-naphthonitrile) prepared in DMSO was used at the final concentration of 10 µM; it was synthetized according to Wang et al. (2018) [35], with some adjustments, as described below. The zinc sulfide-PBS-probe suspension was treated with 0.1 M pH 7 Ethylenediaminetetraacetic acid (EDTA) to release the sulfide and let it react with the fluorescent probe. After 40 min of incubation at 37 °C, samples fluorescence was read with a spectrofluorometer (Shimadzu RF-5301PC; 309 nm excitation and a 441 nm emission). A visual summary of the method is shown in Figure 2.

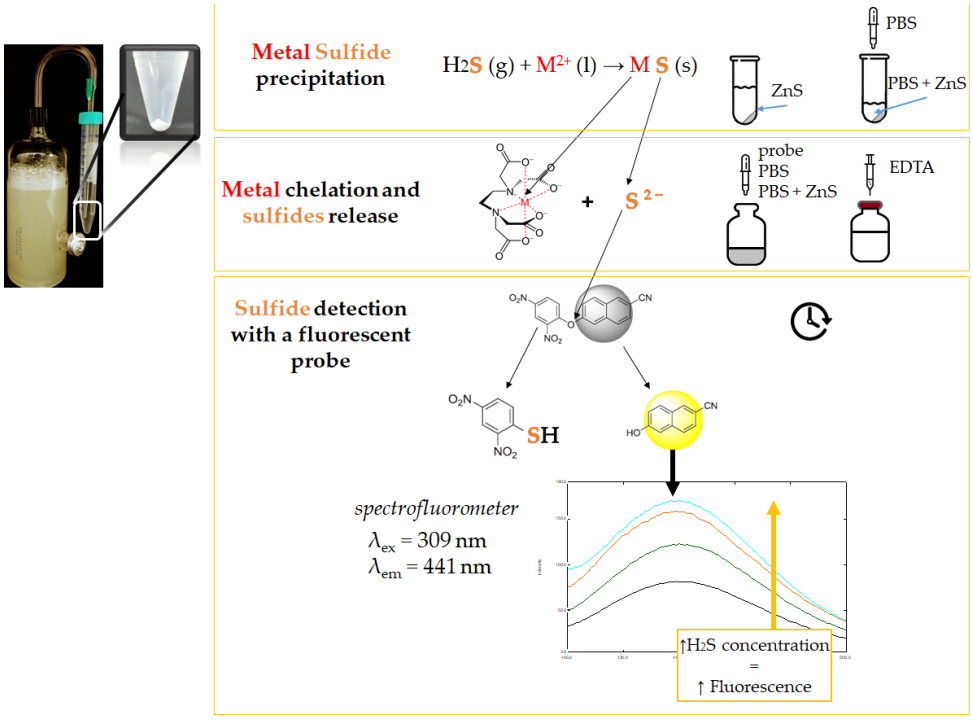

Figure 2. Visual summary of the analytical workflow.

For all the preliminary tests and the calibration curve, whenever it was necessary to work with a known amount of H_2S, sulfide production during alcoholic fermentation was mimicked by injecting a Na_2S solution, at a given concentration, into the zinc trap. A linear model enabled predicting the concentration of total H_2S produced during alcoholic fermentation by each strain under different conditions from the measured fluorescence.

The limit of detection (LOD) and limit of quantification (LOQ) of the method were estimated based on the standard deviation of the response and the slope [36]:

LOD = 3.3 × SD OF BLANK/SLOPE OF THE CALIBRATION LINE

LOQ = 10 × SD OF BLANK/SLOPE OF THE CALIBRATION LINE

A new calibration curve was constructed for each experiment, to ensure maximum quantification accuracy and to normalize variations due to environmental factors.

2.4. Preparation of Fluorescent Probe

A fluorescent probe was prepared by modifying the synthesis proposed previously [35] (Figure 3).

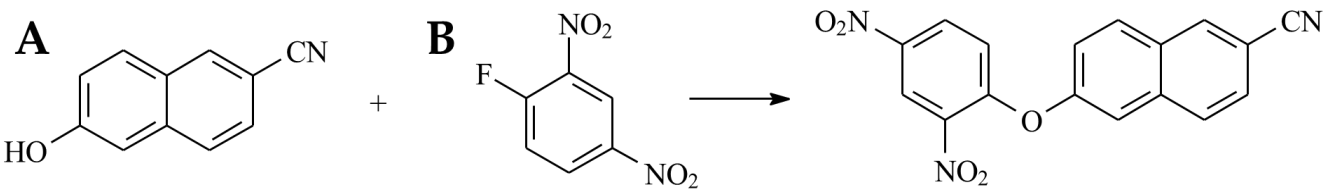

Figure 3. Fluorescent probe synthesis from (**A**) 6-hydroxy-2-naphthonitrile and (**B**) 1-fluoro-2,4-dinitrobenzene.

Samples of 6-hydroxy-2-naphthonitrile (**A**) (1.075 g, 6.4 mmol) and 1-fluoro-2,4-dinitrobenzene (**B**) (1.13 g, 6 mmol) were dissolved in chloroform (25 mL). The mixture was stirred for 10 min and then triethylamine (0.850 mL) was slowly added. The reaction mixture was subsequently heated and reaction progress was periodically followed by thin-layer chromatography with a mixture of 60/40 hexane/ethyl acetate as migration solvent. Retention factors for **A**, **B** and probe were 0.6, 0.8 and 0.7 respectively. The

complete reaction was achieved in 6 h. The precipitate was collected by evaporation, and then recrystallized from ethanol to yield the probe as a light yellow solid (1.87 g).

The synthesized product (16.3 mg), dissolved in DMSO-*d6* (500 µL), was analyzed at 25 °C by nuclear magnetic resonance (NMR) using a 500 MHz DD2 NMR spectrometer (Agilent Technologies, Santa Clara, CA, USA) equipped with a 5 mm indirect detection Z-gradient probe. NMR spectra were processed using both VNMRJ4.2 and MestReNova 14.2.1 (Mestrelab Research, Spain) softwares. ^{1}H, and ^{13}C chemical shifts were reported to that of internal DMSO at 2.5 ppm and 39.5 ppm respectively. Both chemical shifts and coupling constant JHH values (Supplementary data S1) confirmed the chemical structure of the fluorescent probe. Accurate concentration determination of the compound present in the sample were performed thanks to an absolute intensity qNMR method using external calibration [37]. The concentrations, measured from 1D 1H surface signal integrations, were found to be about 98.6 mM for the synthesized product and 12 mM for residual water, i.e., respectively about 16.5 mg and 0.1 mg in the analyzed sample. Altogether, the NMR results show that the synthesized product was very clean with a purity close to 100%.

2.5. Hydrogen Sulfide Detection

To confirm the presence or absence of the gaseous form of hydrogen sulfide during the preliminary tests, lead acetate test strips (Sigma-Aldrich, Buchs, Switzerland) were used. H_2S produces a black precipitate upon contact with lead acetate, therefore, a visible black-colored reaction on the paper strip indicated H_2S presence in the gas phase.

2.6. Experimental Design

A three-level full factorial experiment (3^k) was designed with the Rpackage AlgDesign version 1.2.0 [38] and analyses were performed in R environment (R version 4.0.2 [39]), considering assimilable nitrogen (100, 200 and 300 mg/L), and sulfur dioxide (0, 30 and 60 mg/L) contents and strains as variables. The plan was applied to each strain (given the impossibility of hierarchizing yeast strains and considering them as a factor), and the fermentations were carried out in duplicate.

3. Results

3.1. Development of a Colorimetric Method for the Quantification of Total Hydrogen Sulfide Production during Fermentation

As H_2S is produced all along fermentation, it is necessary to trap this gas to assess the total production of one alcoholic fermentation. We used the capacity of metal salts to trap H_2S during alcoholic fermentation of a sulfide producing yeast MTF1832 in SM200 medium. We tested two metal salts, Cu(II) and Zn(II), as trapping solutions, because of their high sulfide binding capacities (100% and 94% respectively [40]). We used lead acetate paper strips to check the absence of H_2S in the vapor phase of the system.

The absence of a dark precipitate on a lead acetate paper test strip (Figure 4A) shows that both metal solutions are able to trap all H_2S produced during fermentation. The reaction of the sulfide-containing fermentation gas with the trap leads to the formation of a black or white precipitate for the copper and zinc trapping solutions respectively (Figure 4B).

We then used ethylenediaminetetraacetic acid (EDTA) to release H_2S from the precipitate, through the chelation of the divalent metal ions. As shown in Figure 4C, hydrogen sulfide release was only obtained from zinc sulfide, as EDTA permitted a complete dissolution of the zinc sulfide precipitate (Figure 4D). Therefore, a 1% zinc acetate solution was chosen as sulfide trap.

The trapping of H_2S produced during alcoholic fermentation by *S. cerevisiae*, was also simulated using an aqueous H_2S stock solution from Na_2S. The linearity by injecting was tested by injecting aliquots of different concentrations in the zinc trapping solution.

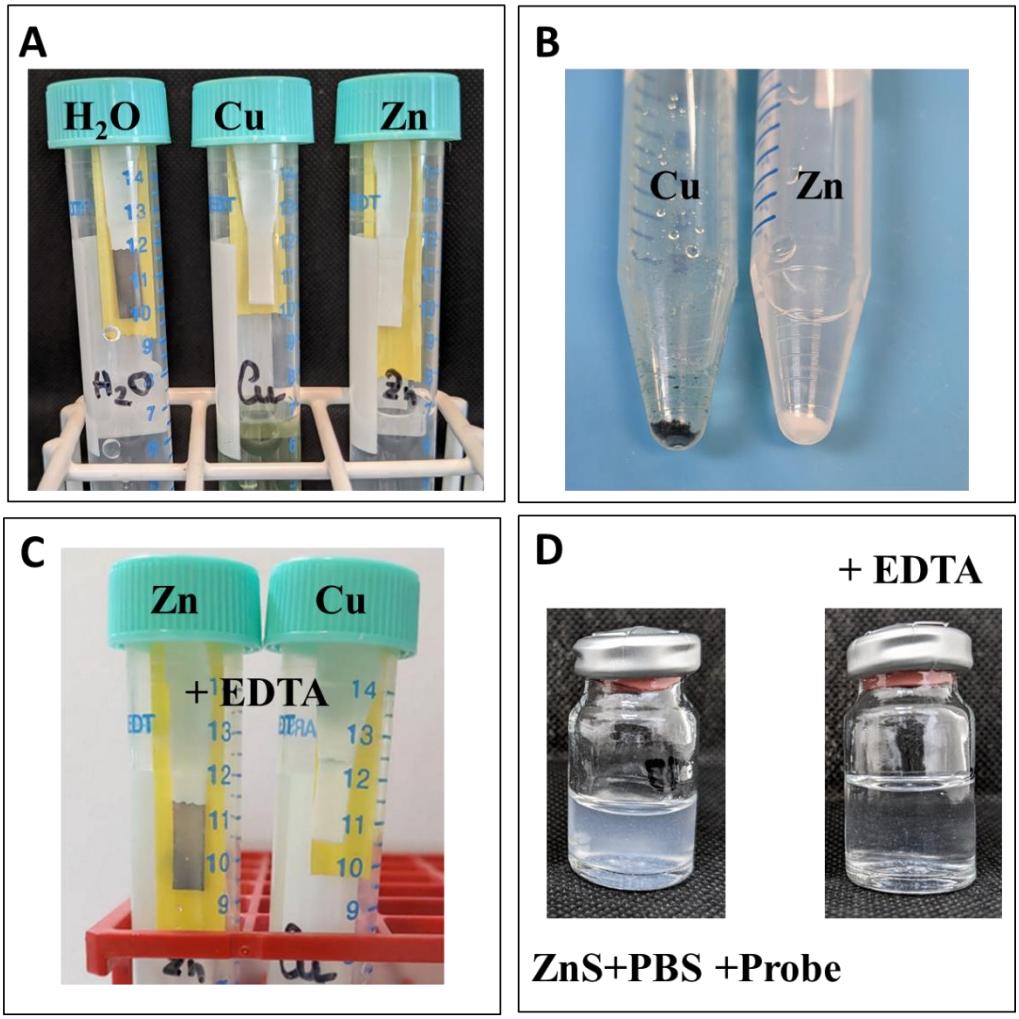

Figure 4. Efficiency of different trap solutions. (**A**) Detection of residual H_2S from fermentation gas after bubbling through a solution containing H_2O (control), $CuCl_2$, or $ZnC_4H_6O_4$. (**B**) Aspects of copper sulfide and zinc sulfide precipitates. (**C**) H_2S release from the zinc sulfide (but not copper sulfide) complex, after EDTA addition. (**D**) dissolution of the zinc sulfide precipitate after EDTA addition. White/black strips respectively indicate the absence/presence of H_2S.

Three EDTA concentrations (0.01 M, 0.05 M and 0.1 M, pH 7) were tested, in order to check their compatibility with the fluorescent probe 6-(2,4-dinitrophenoxy)-2-naphthonitrile. For all three concentrations, the fluorescent probe was able to detect the H_2S released by EDTA (Figure 5, solid lines). The specificity of the fluorescence towards EDTA was confirmed by the overlay of the spectra obtained for the three concentrations in the absence of H_2S (Figure 5, dashed lines). As the highest fluorescence was obtained with EDTA 0.1 M, this concentration was chosen for all further experiments. As a slight fluorescence can be observed for EDTA alone (Figure 5), we avoided to use higher EDTA concentration to limit basal fluorescence.

As some residual fluorescence exists in the blank, the differences in fluorescence between the reference and that obtained for each samples were used to draw a calibration curve. The calibration curve presented in Figure 6 shows a clear linearity in the range 0 to 150 µg of H_2S collected in the trap. For higher concentrations, appropriate dilutions enabled to widen the range of quantifiable H_2S. The precipitate was simply resuspended in a two, three, or five-time higher volume of PBS, and then H_2S was released from the diluted precipitate and quantified. This enabled to maintain the linearity of the assay.

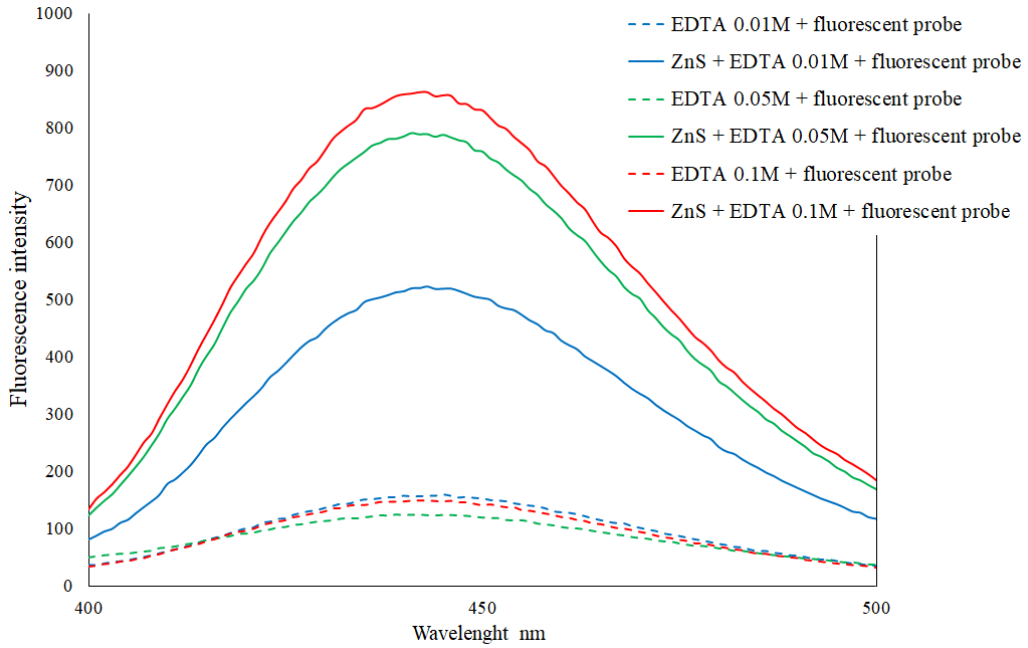

Figure 5. Fluorescence spectra.

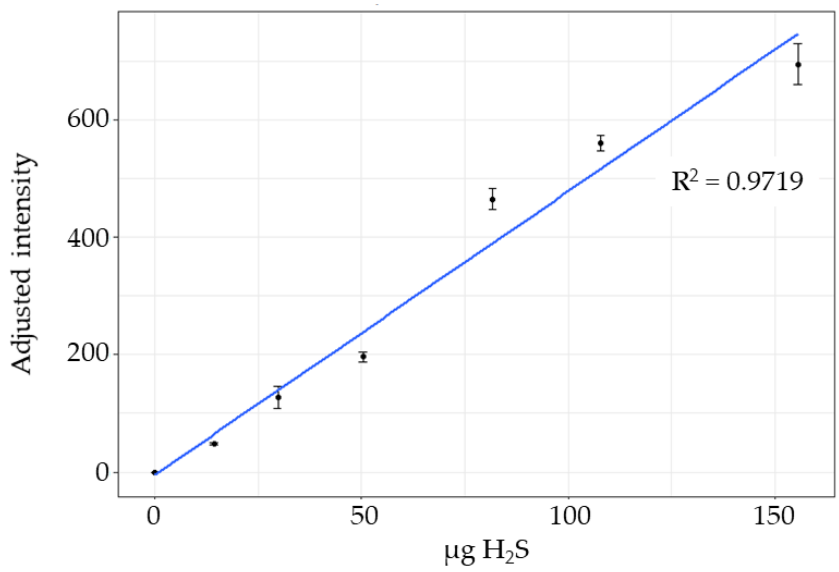

Figure 6. Calibration curve.

The lowest experimentally measured quantity was of 14 µg of H_2S, obtained by the injection of 500 µL of a 10 mg/50mL Na_2S stock solution in 6 mL of 1% zinc trapping solution, even though the estimated LOD, calculated as three times the standard deviation of the blank divided by the slope of the calibration curve, was 1.0 µg and the LOQ, calculated as ten times the standard deviation of the blank divided by the slope of the calibration curve was 3.0 µg (estimation based on the standard deviation of the response and the slope [36]).

3.2. Application of the Colorimetric Assay for the Assessment of Strains Response to Factors Impacting H_2S Production during Alcoholic Fermentation

Strain genetic background, sulfite and nitrogen amounts are factors that can influence H_2S production in alcoholic fermentation. In order to evaluate the impact of each factor individually and any possible interaction, we analyzed the production of H_2S by three strains in response to three different concentrations of YAN and SO_2. As expected, the

increase of the nitrogen content was correlated with an increased fermentation rate for all strains. However, a slight inhibition of fermentation by SO_2 was observed for strain EC1118 at the highest concentration (Figure S1).

The total amount of hydrogen sulfide produced during alcoholic fermentation by the three yeast strains tested ranged from undetectable to more than 1700 µg H_2S/250 mL (Figure 7).

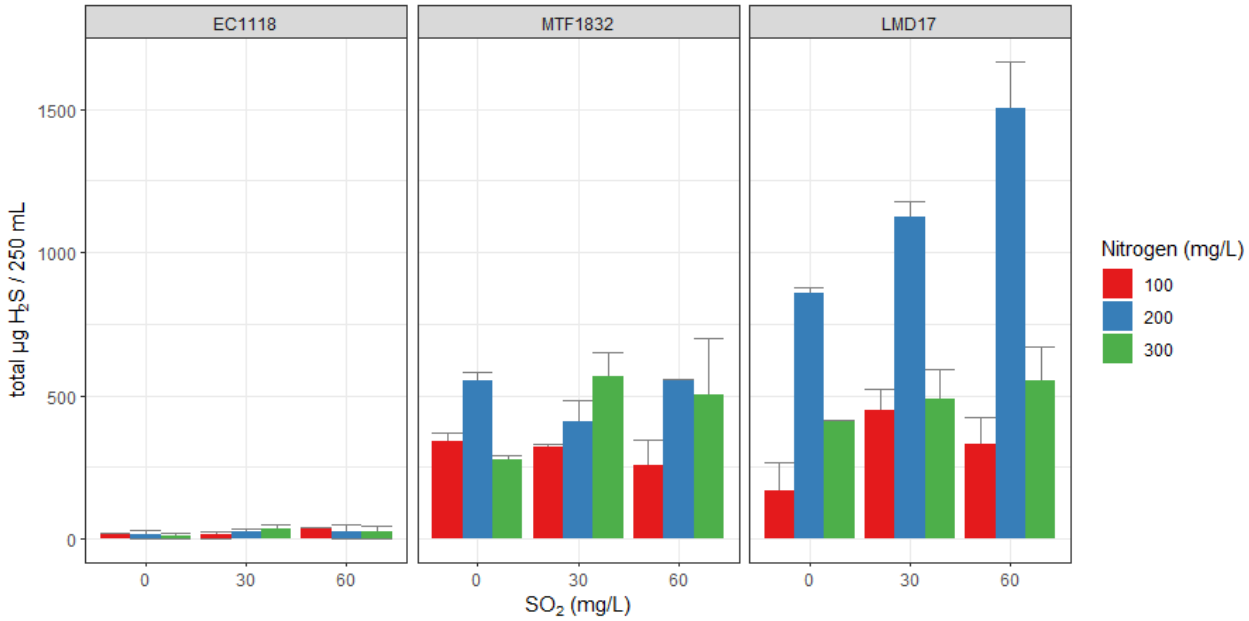

Figure 7. Total H_2S production in presence of different assimilable nitrogen (colors) and sulfur dioxide (*x* axis) content for each strain.

The three factors tested in our experimental conditions were shown to have an impact on hydrogen sulfite production (Table 2). The ability to produce H_2S during alcoholic fermentation appeared as extremely strain dependent (p-value < 2.2×10^{-16}), with strain LMD17 producing up to 1700 µg H_2S/250 mL while strain EC1118 produced almost no H_2S, independently of the nitrogen or sulfur dioxide amounts in the fermentation medium. As expected, YAN content of the media had a highly significant effect on H_2S production, as low nitrogen content led to low H_2S production while the maximum production was observed for a condition of that is considered as medium YAN constraint (200 mg/L). The sulfite content of the fermentation must significantly affected H_2S production, but with a lower magnitude.

Table 2. Analysis of variance table.

Response: H_2S	**Df**	**Sum Sq**	**Mean Sq**	**F value**	**Pr (>F)**	
strain	2	4,829,394	2,414,697	108.4715	$< 2.2 \times 10^{-16}$	***
N	2	1,610,762	805,381	36.1788	2.24×10^{-10}	***
SO_2	2	293,462	146,731	6.5914	0.002917	**
strain:N	4	1,873,705	468,426	21.0424	3.79×10^{-10}	***
strain:SO_2	4	252,459	63,115	2.8352	0.03415	*
Residuals	49	1,090,795	22,261			

Significance codes: *** <0.001, ** <0.01, * <0.05.

Moreover, it is noteworthy to highlight the high variations observed in the response of the strains to the YAN content of the media and the strain specificity with regards to sulfite addition, as indicated by the interactions (p-values = 3.79×10^{-10} and 0.034 respectively).

4. Discussion

4.1. Development of a New Quantitative Fluorometric Method for High throughput Evaluation of H_2S Production

H_2S is a key volatile compound that is produced by yeast during alcoholic fermentation and has a high impact on wine sensory profiles. Former methods proposed for the H_2S assay in wine fermentation were either inexpensive semi-quantitative ones, raising however health hazard issues such as those methods relying on heavy metals, or highly efficient techniques such as electrochemical probes or gas chromatographs, yet difficult to apply to a large sample set.

We propose here a simple procedure for the measurement of cumulated H_2S during fermentation. H_2S is combined in a metal ion trap, released with EDTA and eventually measured with a fluorescent dye. We showed that this method is sensitive (minimum quantified amount 14 µg, estimated LOD = 1.0 µg, estimated LOQ = 3.0 µg), and gives a linear response in a large range of H_2S amounts, up to 150 µg; it can nonetheless be adjusted to quantify higher H_2S concentrations by a simple dilution of the zinc sulfide precipitate in the resuspension step.

The first strength of the proposed method is that it is quantitative, not affected by subjective interpretation errors, as can happen with colorimetric systems, which can only be semi-quantitative. Moreover, compared to lead acetate strips or tubes methods, it can measure accurately whatever H_2S quantity is produced during fermentation without the risk of saturating the strip or tube.

The method is both inexpensive and easy to set up at laboratory scale volumes, allowing a quantitative measure of the total sulfide amount produced, which is not the case for other methods; however, it does require a lab fluorimeter. On-line gas-chromatography systems, for example, allow real-time quantification of H_2S in the head space (useful for H_2S production kinetics, but less so for cumulate production evaluation) and, generally, imply experiments with few bioreactors, with a much larger volume of medium and with expensive equipment. In addition, the trap system does not disturb the gas–liquid partition of hydrogen sulfide as it does not involve any sampling during fermentation (which may cause a depression in the bioreactor and a change in H_2S (g) solubility in the must (l)).

Trapping H_2S as zinc sulfide also has the advantage of allowing precipitate storage and thus delayed analyses at the end of the experiment. The method was initially set up to assess the capacity of each strain to produce H_2S along fermentation, but it can be adapted to investigate H_2S production at specific fermentation phases through the fractioning of H_2S precipitates collection during different fermentation intervals. The method was developed on wine fermentation but can also be applied to other fermentation conditions, such as beer or cider brewing.

4.2. Evaluation of the Different Factors Influencing H_2S Production

Hydrogen sulfide is required by *S. cerevisiae* to synthetize sulfur-containing amino acids and the incorporation of sulfide ions is coupled with the consumption of carbon chains, provided by the aspartate pathway; hence, it is logical to consider the sulfur assimilation pathway as part of YAN metabolism. It is thus foreseeable to observe an effect of must YAN content on the production of H_2S by *S. cerevisiae* during alcoholic fermentation as we detected in our experiment. Different research groups observed contrasting sulfide production in response to YAN content. In general, YAN deficiency goes hand-in-hand with high levels of hydrogen sulfide. This widely accepted knowledge is mainly based on the work of Vos and Gray (1979) [41], who observed a negative, but not strong, correlation between the free amino acid nitrogen content and hydrogen sulfide production. Similarly, Jiranek et al. (1995) [42] assessed that the maximum H_2S production during alcoholic fermentation occurred when the yeast had consumed almost all the nitrogen in the medium. It is important to note that these works took in account either the free amino nitrogen or a diammonium phosphate (DAP) supplementation as nitrogen source, respectively, not a combination of both.

We observed that assimilable nitrogen can indeed modulate hydrogen sulfide production, but that each strain reacts specifically. Strain EC1118 never produces sulfides, whatever the nitrogen limitation, as it has been observed a few times for other strains [20,43]. Regarding strain MTF1832, H_2S production seems to be constant in all conditions, with slight, not significant, differences in the three YAN levels. The scenario changes with strain LMD17: sulfide production is strongly modulated by nitrogen content, with yield peaking at the intermediate nitrogen level (i.e., 200 mg/L YAN). Our results are in agreement with more recent observations according to which an intermediate YAN level (provided as a mix of amino acids and ammonium) leads to the higher H_2S production [20,23,43,44].

It is not unrealistic to say that it is difficult to reach a unanimous conclusion on the relationship between nitrogen and H_2S production, because all experiments conducted in the previous decades tested different strains, different YAN sources and ratios (free amino nitrogen, ammonia, DAP), in synthetic or natural must, managing (or not) other variables such as sulfites, vitamins, lipids or oxygen. Notwithstanding, we can agree that monitoring the initial assimilable nitrogen level is necessary to have an idea of the total H_2S production trend for a given yeast strain.

Sulfur dioxide is largely used in winemaking for its antioxidant and antimicrobial activity and, as it is an SAP intermediary metabolite, it is also necessary to take this addition into account. When added to the must, SO_2 diffuses into the cells and thus enters the intracellular sulfite ion pool, produced from the reduction of extracellular sulfate. Little is known about the impact of SO_2 addition on hydrogen sulfide production by *S. cerevisiae* during alcoholic fermentation. Recently, Morgan et al. (2019) [25] noticed that, for strains BRL97 and QA23, the addition of SO_2 to the must was necessary for H_2S production during fermentation. Our results provide further clues about how sulfite addition leads to an increase in H_2S production, and highlight the specificity of the response of each strain. Interestingly, *S. cerevisiae* has developed different strategies to cope with the high sulfite concentrations encountered during wine fermentation, mainly through different translocations permitting a higher expression of the sulfite efflux pump SSU1 [45–48]. Surprisingly, we have no information on the variability in wine yeast strains ability to reduce sulfite and how this metabolic reaction could be used for sulfite detoxification.

Yeast strains have been shown to be one of the most important factor impacting hydrogen sulfide production during alcoholic fermentation, [11,14,20–24] mainly due to the variability of sulfide reductase activity within the *S. cerevisiae* wine clade. In addition to genetic variation in sulfide reductase, the allelic variations of several genes (i.e., MET2, SKP2, CYS4) have been characterized for their participation to the variation in the production of H_2S in yeasts [19,49,50]. Our results highlight that, although belonging to the same species, different yeast strains are able to produce considerably different amounts of sulfides. Moreover, our results not only show the extreme strain-dependency of H_2S production ability, but also clearly emphasize that this production is depending of two important interactions under oenological conditions: between the strain and the YAN content of the must, and between the strain and the variable amount of SO_2 in the must. Even testing only three genetic backgrounds, we found different behaviors for each strain. For strain EC1118, H_2S total production is very low and not influenced either by nitrogen content variation, or by sulfur dioxide addition. On the other hand, strains MTF1832 and LMD17 produce more H_2S and are more affected by changes in fermentation conditions, but not in the same manner: for LMD17, an intermediate level of YAN (200 mg/L) coupled with the higher sulfur dioxide content used in this study (60 mg/L) triggers a much larger H_2S production compared to MTF1832.

Nowadays, we know that diversity inside the species *Saccharomyces cerevisiae* is huge [51–54] and this genetic complexity explains the phenotypic differences observed between strains. Nevertheless, despite the large amount of work done, we are not yet able to link the difference in H_2S production to these specific genomic variations.

5. Conclusions

In conclusion, we propose here a straightforward method to detect differences in hydrogen sulfide production among *Saccharomyces cerevisiae* wine strains. Its first application to the evaluation of the phenotypic variability of H_2S production under nitrogen constraint, and in the presence of sulfite addition confirmed the role of these factors, and enabled classifying their relative significance. This method provides a unique tool for the testing of more combinations of macro or micro nutrient limitations or excesses. Additionally, it offers an opportunity for genetic deciphering of the sulfur metabolism and, in turn, prospects for the improvement of starter yeasts for winemaking or ecological and evolutionary studies.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/fermentation7040213/s1, Supplementary data S1: NMR spectra ^{1}H and ^{13}C, Figure S1: Time for consumption of 95% of the sugar depending on nitrogen content (*x*-axis) and sulfur dioxide addition (colors).

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