Self-produced hydrogen sulfide improves ethanol fermentation by *Saccharomyces cerevisiae* and other yeast species

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Abstract

Hydrogen sulfide (H₂S) is a gas produced endogenously in organisms from the three domains of life. In mammals, it is involved in diverse physiological processes, including the regulation of blood pressure, and its effects on memory. In contrast, in unicellular organisms the physiological role of H₂S has not been studied in detail. In yeast, for example, in the winemaking industry H₂S is an undesirable byproduct because of its rotten egg smell; however, its biological
relevance during fermentation is not well understood. The effect of H$_2$S in cells is linked to a posttranslational modification in cysteine residues known as S-persulfidation. We evaluated S-persulfidation in the *Saccharomyces cerevisiae* proteome. We screened S-persulfidated proteins from cells growing in fermentable carbon sources and we identified several glycolytic enzymes as S-persulfidation targets. Pyruvate kinase, catalyzing the last irreversible step of glycolysis, increased its activity in the presence of a H$_2$S donor. Yeast cells treated with H$_2$S increased ethanol production; moreover, mutant cells that endogenously accumulated H$_2$S produced more ethanol and ATP during the exponential growth phase. This mechanism of the regulation of the metabolism seems to be evolutionarily conserved in other yeast species, because H$_2$S induces ethanol production in the pre-Whole Genome Duplication species *Kluyveromyces marxianus* and *Meyerozyma guilliermondii*. Our results suggest a new role of H$_2$S in the regulation of the metabolism during fermentation.

**Keywords:** H$_2$S; S-persulfidation; fermentation; yeast; metabolism; posttranslational modification; hydrogen sulfide.

**Introduction**

Hydrogen sulfide (H$_2$S) is a gasotransmitter produced endogenously in cells. It has been associated with diverse physiological processes such as vasodilation [1], pain [2] and longevity in animals [3], plant growth and development [4], bacterial antibiotic resistance [5], and as a byproduct of alcoholic fermentation in yeast [6]. Surprisingly, the biological function of H$_2$S in yeast is not fully understood [7]; the majority of reports describe how it is produced or how to prevent its production during fermentation [8–10]. In yeast, H$_2$S is involved in heavy metal detoxification [11], population synchrony [12], and chronological...
aging [3]; however the molecular mechanisms behind these phenomena have not been fully elucidated.

In yeast, the main metabolic pathway that produces H$_2$S is the sulfate assimilation pathway [13], where inorganic sulfate is transformed to H$_2$S and used in the synthesis of methionine and cysteine. This pathway is highly active in the exponential growth phase, as the principal H$_2$S producer, sulfite reductase (encoded by MET5 and MET10) [6], is highly active. The synthesis of H$_2$S takes place in the first hours of fermentation and decreases at the final stages when cells reach the stationary phase [14]. The sulfur transferase Tum1p is another protein involved in H$_2$S production during fermentation when high concentrations of cysteine are present in the media [15]. H$_2$S is metabolized by Met17p a sulfhydrylase that catalyze the incorporation of sulfide for the biosynthesis of sulfur-containing amino acids [16].

The molecular effect of hydrogen sulfide depends on a posttranslational modification named S-persulfidation (originally termed sulfhydration) [17]. S-persulfidation involves addition of a thiol group to the cysteine residues (-S-SH) in proteins. This posttranslational modification has been associated with the activation and inhibition of protein activity [17,18].

In this work, for the first time, we evaluated the S-persulfidation of yeast proteins. We report that hydrogen sulfide is a regulator of glycolysis that increases ethanol production in S. cerevisiae. This was observed using an exogenous donor of hydrogen sulfide or mutant strains that accumulate or produce less H$_2$S. This mechanism of regulation was conserved in pre-Whole Genome Duplication (WGD) species, such as the thermotolerant Kluyveromyces marxianus from the KLE clade and the oleaginous yeast Meyerozyma guilliermondii from the CUG-Ser1 clade. This work provides an insight into how H$_2$S regulates glucose metabolism through an evolutionarily conserved mechanism, constituting an important role of H$_2$S in fermentation.
Materials and Methods

Yeast strains, media, and growth conditions

Saccharomyces cerevisiae strains used in this study were S288C-derived laboratory strains BY4742 (MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0) referred as wt, BY4741 (MATa his3Δ1 leu2Δ0 met17Δ0 ura3Δ0). Kluyveromyces marxianus and Meyerozyma guilliermondii were isolated from mezcal producers in Michoacán, México [19]. Deletion strains derived from BY4742 were constructed by PCR-based gene replacement [20] using synthetic oligonucleotides and the kanMx and natMx disruption modules contained in plasmids pUG6 and pAG25. Gene deletions were confirmed by PCR using A and D oligos. Strains and oligonucleotides are listed in supplementary table 1. Strains were cultured at 30°C in liquid YPD medium (1% yeast extract, 2% dextrose, 2% peptone) or YPG medium (1% yeast extract, 2% galactose, 2% peptone) until reaching the exponential growth phase (optical density at 660nm [OD660] =0.5-0.6) and cells were collected then for protein extraction.

Reagents

Sodium hydrosulfide (NaHS), Methyl methanethiosulfonate (MMTS), dithiothreitol (DTT), antibiotin antibody, neocuproine, deferoxamine and others chemicals were purchased from Sigma-Aldrich, St Louis MO, rabbit polyclonal anti-GAPDH (GTX100118, Genetex) were purchased from Genetex, Irvine, CA and N-(6-(biotinamido)hexyl)-3'-(2'-pyridyldithio)-propionamide (HPDP-biotin) (sc-207359), mouse monoclonal anti-enolase (sc-21738), goat polyclonal anti-CBS (sc-46830) and rabbit polyclonal anti-TIM (FL-249) were purchased from Santa Cruz Biotech, Dallas Tx.
Modified biotin switch assay

The modified biotin switch assay was performed as described previously [17,21]. Briefly, after yeast cultures reached exponential phase, cells were collected, and intracellular proteins were extracted with chilled glass-beads in HEN buffer (250 mM HEPES-NaOH pH 7.7, 1 mM EDTA) supplemented with 1% triton X-100, 0.1 mM neocuproine, 0.1 mM deferoxamine and 1X protease cocktail inhibitor (Roche, Switzerland). Cell lysates were centrifuged at 16900 x g for 1 hr at 4ºC, total extracts (1-2 mg) were blocked in HEN buffer with 2.5% SDS and 20 mM MMTS at 50ºC for 20 min. The MMTS was removed by acetone precipitation and the protein pellet was resuspended in HEN buffer with 1% SDS. Protein labeling was performed with 0.8 mM HPDP-biotin for 3 h at room temperature in the dark. The biotinylated proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and subjected to immunoblot analysis.

Purification of biotinylated proteins

After biotin switch assay, labeled extracts were subjected to streptavidin-based affinity precipitation with magnetic beads. Labeled extracts were incubated with 3X volumes of neutralization buffer (20 mM HEPES-NaOH pH 7.7, 100 mM NaCl, 1 mM EDTA, 0.5% triton) and 25 µl of streptavidin magnetic beads (Pierce) with agitation, overnight at 4ºC. Magnetic beads were collected and washed with wash buffer as indicated by manufacturer's instructions, biotinylated proteins were eluted with IP-MS elution buffer and analyzed using LS-MS or SDS-PAGE.

Immunoblot analysis

Protein extracts were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore-Merck, Germany). Membranes were
blocked with 5% non-fat milk and incubated with a specific anti-biotin antibody overnight at 4°C. Proteins were detected with chemiluminescence using horse-radish peroxidase conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA). Before to immunoblot, membranes were stained using Ponceau red (Millipore-Merck, Germany) as protein loading control.

Quantification of intracellular ATP concentration

NaHS was added to cells cultures at specific timepoints, then cells were centrifuged, and intracellular ATP was measured using the ATP Bioluminescent Assay Kit HS II (Roche, Switzerland). Cell samples were prepared by diluting treated cells to a final concentration of 3.7x10^9 cells·mL^{-1} in 500 μl with a buffer containing 100 mM Tris-HCl pH 7.8 and 4 mM EDTA. After 2 min incubation, samples were immersed in boiling water for 2 minutes, and the resulting cell extracts were incubated for 5 minutes at 4°C, cell debris were removed by centrifugation at 16900 x g for 5 min and supernatants were used to measure the amount of intracellular ATP using an ATP calibration curve prepared each time, as indicated by the manufacturer. Bioluminescence was detected in a POLARstar Omega luminometer (BGM LABTECH, Offenburg, Germany). Three independent experiments with three replicas were performed, and values are represented as mean ± standard error.

Detection of H₂S production

H₂S production by yeast strains colonies was detected through the generation of a visible black precipitate indicating that the hydrogen sulfide gas has reacted with lead nitrate [22]. Yeast strains were diluted, and cell density normalized to 3x10^7 cells·mL^{-1}. Cells were spotted in solid media (3.2% dextrose, 0.4% yeast extract, 0.24% peptone, 0.016% ammonium sulfate, 0.08% lead nitrate, 1.6%
agar) and plates were kept at 30°C for 5-7 days. Also, H₂S production was measured as reported previously [23] with some modifications. BY4742 wt strain were precultured at 30°C with constant shaking for 2 days in fresh YPD media. The assay was performed on a 96 well plate (COSTAR). Each well had 185 μL of YPD media, 5 μL of methylene blue (1 mg·mL⁻¹) diluted in citrate buffer (100 mM, pH 4.5) and 10 μL of cells, for a final OD₆₀₀ of 0.2. Growth was measured in an Infinite 200 (TECAN, Life Sciences) at 600 nm and 663 nm during 15 hours with intervals of 15 minutes between readings. During measures cells were incubated at 30°C with occasionally shaking. Three experimental replicates were made, with six different biological replicates in each experiment. Data for the hydrogen sulfide production were analyzed with the following formula:

\[
\frac{(OD_{600nm\ t0} - OD_{663nm\ t0}) - (OD_{600nm\ tx} - OD_{663nm\ tx})}{OD_{600nm\ tx\ from\ no\ reaction\ mix}}
\]

Fermentation assays

BY4742 wt and derived mutants were precultured in liquid YPD medium for 24 h at 30°C, under agitation at 90 rpm in an Excella E24 incubator Shaker (New Brunswick Scientific, USA), then were inoculated in a 2L flask containing 500 mL of fresh YPD with an initial OD₆₀₀=0.2 and incubated in the same conditions. When cells reached DO₆₆₀=0.5, a pulse of NaHS was added. After 7h of NaHS addition, aliquots of 2 ml were obtained, DO₆₆₀ was measured, and cells centrifuged at 16900 x g for 1 min. Supernatants were stored at -20°C for subsequent ethanol quantification. For mutant and wt strains, aliquots were taken every hour after cells were inoculated, supernatants were stored at -20°C. Ethanol production was evaluated through enzymatic assay coupled to NAD⁺ reduction. Briefly, supernatants were incubated in buffer (114 mM K₂HPO₄ pH 7.6), 1.8 mM NAD⁺ and 39μg·mL⁻¹ alcohol dehydrogenase (ADH) for 30 min at 30°C with vigorous agitation [24]. Produced NADH was monitored by the increase in absorbance at 340 nm. The results are reported as mM ethanol per 1x10⁷ cells.
Three independent experiments with three replicas were performed, and values are represented as mean ± standard error. *K. marxianus* fermentation assay was performed as in *S. cerevisiae* strains, when cells reached DO$_{660}$=0.5, a pulse of NaHS 0.1mM was added. After 7h of NaHS addition, aliquots of 2 ml were obtained, and ethanol was quantified. For *Meyerozyma guilliermondii* when cells reached DO$_{660}$=0.5, a pulse of NaHS 0.1mM was added, 24h later another pulse of same concentration was added and 7h later ethanol was quantified.

**Enzymes activity assays**

Glyceraldehyde 3 phosphate dehydrogenase (GAPDH) [17], pyruvate kinase (PK) [25] and alcohol dehydrogenase (ADH) [26] activities were measured by specific reaction assays and monitored spectrophotometrically at 340 nm, recording the rate of NAD to NADH reduction. Cells cultures were exposed to NaHS at different times, protein extracts were quantified, and 10 µg of protein were incubated in assay buffer as follows: for GAPDH (20 mM Tris-HCl pH 7.8, 100 mM NaCl, 0.1 mg·mL$^{-1}$ Bovine serum albumin, 2 mM NAD$^+$, 10 mM sodium pyrophosphate, 20 mM sodium arsenate, 500 mM DTT buffer, phosphate buffered saline (PBS) 1X, 27.3 mM glyceraldehyde 3-phosphate [G3P]). For PK (50 mM Imidazole·HCl, 120 mM KCl, 62 mM MgSO$_4$ pH 7.6, 45 mM ADP, 6.6 mM NADH, 45 mM phosphoenolpyruvate [PEP], 1.3 KU·mL$^{-1}$ lactate dehydrogenase). For ADH (114 mM K$_2$HPO$_4$ pH 7.6), 1.8 mM NAD+, and 16.4 mM ethanol). Three independent experiments with three replicas were performed, and values are represented as mean ± standard error.

**Oxygen consumption rate assay**

BY4742 wt and derived mutant cells were precultured in liquid YPD medium for 48 hrs at 30°C, then were cultured in YPD medium with an initial DO$_{600}$=0.2 under
agitation in an Excella E24 incubator (New Brunswick Scientific, USA) for 7h at 30°C. Basal oxygen consumption was measured in resting cells with a Clark electrode (Oximeter model 782, Warner/Strathkelvin Instruments, North Lanarkshire, Scotland) in a water-jacketed chamber. Temperature was kept at 30°C using a water bath (PolyScience 7 L, IL). Oxygen consumption reaction mixture was MES 10 mM pH 6 and 500 mg (wet weight) of cells were added at chamber [24]. To evaluate role of NaHS addition, when BY4742 cells reached OD$_600$=0.5, a pulse of 0.1 mM NaHS was added. Seven hours later, basal oxygen consumption was measured as abovementioned. Results are reported as natgO/wet weight g/ mn and values are represented as mean ± standard error. (wet weight) of cells were added at chamber.

Sample preparation and LC-MALDI-MS/MS

Biotinylated proteins were digested with 250 ng of trypsin mass spectrometry grade (Sigma-Aldrich, St. Louis, MO) in 50 mM of ammonium bicarbonate (ABC). Resulting tryptic peptides were desalted using ZipTip C18 (Millipore) and concentrated to an approximated volume of 10 μL. Afterward, 9 μL were loaded into ChromXP Trap Column C18-CL precolumn (Eksigent, Redwood City CA); 350 μm X 0.5 mm, 120 Å pore size, 3 μm particle size and desalted with 0.1% trifluoroacetic acid (TFA) in H2O at a flow of 5 μL min$^{-1}$ for 10 min. Then, peptides were loaded and separated on a 3C18-CL-120 column (Eksigent, Redwood City CA); 75 μm X 150 mm, 120 Å pore size, 3 μm particle size, in a HPLC Ekspert nanoLC 425 (Eksigent, Redwood City CA) using as a mobile phase A, 0.1% TFA in H2O and mobile phase B 0.1% TFA in acetonitrile (ACN) under the following lineal gradient: 0-3 min 10% B, 60 min 60% B, 61-64 min 90 % B, 65 to 90 min 10% B at a flow of 250 nL min$^{-1}$. Eluted fractions were automatically mixed with a solution of 2 mg·mL$^{-1}$ of alfa-cyano-4-hydroxycinnamic acid (CHCA) in 0.1% TFA and 50% ACN as a matrix, spotted in an Opti-TOF plate of 384 spots using a MALDI Ekspot (Eksigent, Redwood City CA) with a spotting velocity of 20 s per spot at a matrix flow of 1.6 μL min$^{-1}$. The generated spots were analyzed by a
MALDI-TOF/TOF 4800 Plus mass spectrometer (ABSciex, Framingham MA).

Each MS Spectrum was acquired by an accumulation of 1000 shots in a mass range of 850-4000 Th with a laser intensity of 3800. The 100 more intense ions with a minimum signal-noise (S/N) of 20 were programmed to fragmenting. The MS/MS spectra were obtained by fragmentation of selected precursor ions using Collision-Induced Dissociation (CID) and acquired by 3000 shots with a laser intensity of 4300. Generated MS/MS spectrums were compared using Protein Pilot software v. 2.0.1 (ABSciex, Framingham MA) against Saccharomyces cerevisiae, strain ATCC 204508/S288c database (downloaded from Uniprot, 6049 protein sequences) using Paragon algorithm. Search parameters were: Not constant modifications in cysteines, trypsin as a cutter enzyme, all the biological modifications and amino acids substitution set by the algorithm (including carbamidomethylated cysteine as a variable modification); as well as phosphorylation emphasis and Gel-based ID as special factors. The detection threshold was considered in 1.3 to acquire 95% of confidence; additionally, the identified proteins showed a local FDR of 5% or less. Since a peptide derived from a given fragmentation spectra may be shared among redundant proteins during database search, it is necessary group all competing proteins and report only the protein with more spectrometric evidence; for this reason, identified proteins were grouped by ProGroup algorithm contained in the software Protein Pilot to minimize redundancy.

Results

S-Persulfidation of yeast proteins growing on a fermentable carbon source

Protein S-persulfdation was detected using the modified biotin switch method [17]. In order to validate the method in yeast, we performed the assay in either a poor producer (met5Δmet10Δ) or an accumulator (met17Δ) strain of H2S and compared them to the wt (BY4742) (Figure 1A). Cells were grown using glucose as the carbon source, and at the exponential phase, when H2S is produced [6],
the protein was extracted. S-Persulfidated proteins were accumulated in met17Δ in comparison to the met5Δmet10Δ strain and wt as expected (Figure 1B).

In yeast, H₂S is produced during fermentation, however, the S-persulfidation target proteins are not known. We used mass spectrometry to analyze the S-persulfidated proteins in cells growing at the exponential phase in two different fermentable carbon sources: glucose and galactose. Glucose is the preferred fermentable carbon source of yeast, while galactose needs to be isomerized to enter the glycolytic pathway. We found 42 S-persulfidated proteins; 21 were specific to glucose-grown cells, 4 were specific to galactose-grown cells, and 17 proteins were found in both conditions (Supplementary Table 2). Among the generally expressed 17 proteins, 15 were reported before as proteins with a redox-regulated cysteine [27], which is a feature of cysteines susceptible to posttranslational modifications [28]. Cytoplasmic translation (seven proteins) and glycolysis (seven proteins) were the most represented biological processes in the cells growing in either condition. Interestingly, pyruvate decarboxylase 1 (Pdc1), a key enzyme in alcoholic fermentation, and Adh1, the major enzyme responsible for ethanol synthesis, were also found, suggesting a possible role of S-persulfidation in fermentation. The identities of some glycolytic enzymes, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), enolase, and triosephosphate isomerase (Tdh3, Eno2, and Tpi1, respectively) were confirmed using specific antibodies (Figure 2). We also tested cystathionine beta-synthase (Cys4), which was described before as a possible S-persulfidated protein [17, 29], although it must be considered that in our mass spectrometry analysis it did not pass the threshold (unused score of 1.04, coverage of 43.98%), we did find that Cys4 was a target of S-persulfidation.

H₂S production during yeast growth

In order to evaluate H₂S production during yeast growth, we determined H₂S in the wt. The H₂S reached a maximal amount during the log phase and dropped its
production (Figure 3). Suggesting that the H$_2$S concentration is not constant and drops when the culture stops growing.

$H_2S$ increases glycolytic enzymes activities

Among the first effects of S-persulfidation described was the increase in GAPDH activity [17]. Considering that Tdh3 (GAPDH) was one of the glycolytic enzymes targeted for S-persulfidation, we decided to test the effect of NaHS (a donor of H$_2$S) on GAPDH activity. Cells were stimulated with 0.1 mM or 0.25 mM NaHS for two and seven hours. Then, the protein was extracted, and the GAPDH activity was measured. We found that after two hours of NaHS stimulation, both at 0.1 mM and at 0.25 mM, increased GAPDH activity 1.4 times (Figure 4A), and the effect was lost at seven hours (Figure 4B). Another protein identified by mass spectrometry was pyruvate kinase (PK) that catalyzes the last irreversible step of glycolysis. We measured the activity of the pyruvate kinase at two hours of treatment with 0.1 mM or 0.25 mM NaHS finding that NaHS increased PK activity 2.39 times (Figure 4C, Supplementary Table 3), and lost its effect at seven hours (Figure 4D). Finally, we subjected alcohol dehydrogenase (ADH) to the same treatment, and we did not find any significant difference between the treated and untreated cells, i.e., at these concentrations NaHS did not affect ADH activity (Supplemental Figure 1).

$H_2S$ stimulates fermentation

The glycolytic enzymes GAPDH and PK increased their activity in response to H2S. In addition, mass spectrometry data indicated that these and other enzymes from glycolysis were S-persulfidated. Thus, we decided to test whether H$_2$S influenced the synthesis of ethanol. Exponential-phase-grown cells were treated with 0.1 mM NaHS and after seven hours, the supernatant was collected. It was
observed that the treated cells had increased ethanol production as compared to the control (Figure 5A).

In order to test the effect of endogenous H$_2$S in fermentation, we decided to compare ethanol production in the two isogenic lab strains BY4741 and BY4742. The only difference between these two strains is that BY4741 has a deletion of $MET17$ and strain BY4742 has a deletion of $LYS2$. As mentioned before, the $met17\Delta$ strain endogenously accumulates H$_2$S, because $MET17$ codifies for the enzyme using H$_2$S and O-acetyl homoserine to synthetize homocysteine. An overnight preculture of each strain was diluted to an OD$_{600}$=0.2, the supernatants were collected after 24 h, and the ethanol was quantified. The strain ac-cumulating H$_2$S endogenously, BY4741 $met17\Delta$, produced more ethanol than the strain BY4742 $MET17$ (Figure 5B).

Ethanol is the main product of fermentation. Additionally, during glycolytic fermentation two ATP molecules are synthesized. Thus, we decided to quantify the ATP after treatment with NaHS. Exponential-phase cells were treated with the same quantities of NaHS used before, and the ATP was quantified two and four hours after treatment. After two hours, the treated cells produced more ATP than the untreated cells; this effect was lost four hours after treatment (Figure 5C). These results suggest that H$_2$S stimulates glycolysis, and that fermentation is enhanced to produce both ATP and ethanol.

Based on these results we decided to compare whether the endogenous H$_2$S had an influence on the onset of ethanol synthesis. We measured the ethanol production of the poor H$_2$S producer strain $met5\Delta met10\Delta$, the H$_2$S accumulator $met17\Delta$ strain, and the wt. The cells from 48 hours preculture were resuspended in fresh media and aliquots from the supernatant were collected every hour. The $met17\Delta$ strain initiated ethanol production at five hours, the wt strain initiated production at six hours, and the $met5\Delta met10\Delta$ initiated production after seven hours (Figure 6A). This result showed that the cells with high endogenously accumulated H$_2$S began ethanol production before the cells with a lower H$_2$S concentration.
Finally, in each of these strains we quantified the ATP at the exponential or stationary phase. We found that the endogenously $\text{H}_2\text{S}$ accumulator strain produced the most ATP during the exponential phase, while there were no differences in the ATP concentration at the stationary phase between the wt and mutant strains (Figure 6B). These results support our proposal that $\text{H}_2\text{S}$ stimulates ethanol and ATP production.

Endogenous $\text{H}_2\text{S}$ accumulation promotes basal oxygen consumption

ATP could be synthesized as product of the glycolysis and the oxidative phosphorylation. In order to elucidate if the ATP produced by $\text{H}_2\text{S}$ stimulation was from oxidative phosphorylation, we measured oxygen consumption from wt strain and mutants. A 48 hours preculture of each strain was diluted to an $\text{OD}_{600}=0.2$, and oxygen consumption was measured. Then, after seven hours (when cells were at exponential phase) oxygen was measured again (Figure 7). We found, in the diluted cells, that the $\text{met}5\Delta\text{met}10\Delta$, and the $\text{met}17\Delta$ consumed more oxygen than the $\text{wt}$ strain. On the other hand, after seven hours of growing, we found that the $\text{met}17\Delta$ strain maintained the elevated rate of oxygen consumption. This result suggest that endogenously accumulated $\text{H}_2\text{S}$ promotes oxygen consumption.

$\text{H}_2\text{S}$ stimulates ethanol production in Meyerozyma guilliermondii and Kluyveromyces marxianus

Ethanol synthesis is more robust in Crabtree positive yeast species; this phenomenon is associated with the WGD [30]. Evidence suggests that the WGD event arose from an interspecies hybridization between a strain from the KLE clade (genera Kluyveromyces, Lachancea and Eremothecium) and a strain from the ZT clade (Zygosaccharomyces and Torulaspora) [31]. The CUG-Ser1 clade
first appeared approximately 117 million years before the WGD event; the CUG-
Ser1 clade is characterized by a change in codon usage [32]. Considering this,
we decided to test the effect of H₂S during ethanol synthesis on the KLE clade
strain, *K. marxianus* and in *M. guilliermondii* from the CUG-Ser1 clade. *K.
marxianus* exponential-phase cells were stimulated with NaHS. Treatment with
the H₂S donor in *K. marxianus* increased ethanol synthesis as in *S. cerevisiae*
(Figure 8a). In *M. guilliermondii*, it was noted that ethanol synthesis took longer
than 24 h when glucose was the carbon source [33]; for this reason, the
exponential-phase cells were treated with NaHS and treated again 24 h later. As
observed in *K. marxianus*, ethanol synthesis increased after the H₂S donor
treatment on *M. guilliermondii* (Figure 8b) confirming that there is an effect of H₂S
on the fermentation activity of these two species of yeast.

**Discussion**

Hydrogen sulfide is produced endogenously in yeast, and it is considered a fer-
mentation byproduct; however its biological role is unknown. The biological
effects of H₂S are linked to a cysteine posttranslational modification termed S-
persulfidation [17]. In this work, we analyzed S-persulfidated proteins on the yeast
proteome. We identified several glycolytic enzymes as S-persulfidation targets,
as reported previously in tissues such as the brain, heart and liver [29], in
hepatocytes [17], a pancreatic beta cell line [34], HEK293 cells [29], plant [35,36]
and bacteria [37]. Interestingly, Fu and collaborators reported six S-persulfidated
glycolytic enzymes (ALDOA, GAPDH, PGK1, ENO1, PKM and LDHA) when they
evaluated S-persulfidated proteins in cells overexpressing the H₂S-producer
enzyme cystathionine gamma-lyase (CSE) [29]. In a previous report, in
pancreatic beta cells, metabolites were measured, and H₂S was associated with
an increased glycolytic metabolic flux of cells under chronic stress. All these
reports agreed that several glycolytic enzymes were S-persulfidated, even when
the activity of GAPDH was the only one measured [17]. Cysteine’s
posttranslational modifications of glycolytic enzymes regulate the subcellular
localization and oligomerization, which can impact its activity [38–40]. In a cell culture, H$_2$S production is not constant, it starts to decline when the cells are at the middle of the logarithmic phase, suggesting that H$_2$S synthesis could be regulated by the metabolic conditions. In S. cerevisiae, we found that GAPDH increased its activity with the H$_2$S donor NaHS two hours after the treatment, and the effect was lost at seven hours. It is important to note that H$_2$S is released from NaHS just a few seconds after the sodium salt is dissolved [41]; hence, the effect of NaHS two hours after treatment may be attributed to a chemical modification of the enzymes, such as S-persulfidation. The thioredoxin system eliminates this posttranslational modification [42,43], which is consistent with the idea that cellular mechanisms maintain protein S-persulfidation homeostasis [44]. Seven hours after stimulation, there was no effect on GAPDH activity probably due to the loss of S-persulfidation by the protein. Furthermore, the cells were no longer at an exponential phase seven hours after the OD$_{600}$ reached 0.5, and the yeast metabolism changed to aerobic at the diauxic shift. The enzymes catalyzing the irreversible steps regulate the glycolytic pathway [45]; and we found that the enzyme involved in the last irreversible step, pyruvate kinase, increased its activity when stimulated with NaHS. The increase in pyruvate kinase activity may have at least two important consequences: feeding the Krebs cycle and/or stimulating the synthesis of ethanol. Considering that yeast synthesizes H$_2$S during fermentation, we decided to test whether NaHS increased ethanol production. We found that cells treated with the H$_2$S donor produced more ethanol. In order to confirm our observations, we measured the ethanol production in the isogenic strains BY4741 and BY4742. These strains have almost the same selection markers, and they differ only in one of them, BY4741 accumulates H$_2$S because it is met17$\Delta$; BY4742 is lys2$\Delta$, and thus, it does not accumulate H$_2$S. We observed that BY4741 produced more ethanol than BY4742, suggesting that endogenous H$_2$S levels increase ethanol synthesis. Previously, it was reported that BY4742 fermenting activity was slower than in BY4741; it would be interesting to analyze the role of H$_2$S in this system [46]. Considering these results, we proposed that if H$_2$S stimulates fermentation, then mutants accumulating H$_2$S would begin ethanol production before strains producing less H$_2$S. We tested this hypothesis by comparing ethanol production between a lower producer of H$_2$S, the strain met5$\Delta$met10$\Delta$, the accumulator
strain $met17\Delta$, and the $wt$. We found that the $met17\Delta$ strain started to produce ethanol before the $wt$ strain; in turn, the $met5\Delta met10\Delta$ strain production of ethanol was delayed even longer. The results confirmed that endogenous concentrations of $H_2S$ affects ethanol synthesis. Finally, we measured ATP production in all strains, and we found that at the logarithmic phase the $met17\Delta$ strain produced more ATP than the others. This result supports the idea that in addition to increasing an early synthesis of ethanol, $H_2S$ and also enhances ATP production at the exponential phase of growth.

The fermentation and the oxidative phosphorylation could yield ATP. We found that at exponential phase the $met17\Delta$ strain produced more ATP than the $wt$ and $met5\Delta met10\Delta$ strains. In order to test if the ATP was produced from the oxidative phosphorylation, we measured basal oxygen consumption on these strains. We found that the $met17\Delta$ strain has an elevated rate of oxygen consumption, and this is sustained when cells were at exponential phase of growth; suggesting that endogenously accumulated $H_2S$ induces oxygen consumption. This would be contradictory to a report where described that exogenous $H_2S$ inhibit respiration [47], however, at physiological concentrations $H_2S$ could induce the S-persulfidation of the ATP synthase from mammals and increases its activity [48]. The S-persulfidation takes place at cysteines 244 and 294 of human ATP synthase. The yeast ATP synthase (Atp1) conserved the cysteine 244 (Supplementary figure 2) in lineal sequence and has similar orientation on protein structure; therefore, the S-persulfidation could also be carried out in Atp1. Our results suggest that endogenous $H_2S$ has an effect on glycolysis and oxygen consumption. The effect of endogenous $H_2S$ on metabolism may explain the advantage of the $met17\Delta$ strain growing on fermentable carbon sources (glucose and galactose), over the $wt$ and $met5\Delta met10\Delta$ strains (Supplementary figure 3).

It is accepted that the origin of $S. cerevisiae$ comes from a WGD event, probably by the interspecies hybridization between a strain from the KLE clade and a strain from the ZT clade [31]. WGD species have a more pronounced Crabtree effect than non-WGD species [30], so we decided to test whether H2S influenced yeast from the parental KLE clade that originated S. cerevisiae and a Crabtree-negative
species from the CUG-Ser1 clade. The origin of this clade is estimated to be between 178 and 248 million years ago (mya), and this event occurred before WGD, estimated to be between 82 and 105 mya [32]. We found that both species increase ethanol production after the NaHS treatment suggesting that i) H2S is a positive regulator of fermentation and ii) this effect is evolutionarily conserved.

Overall, H2S is considered as a fermentation byproduct on yeast even when its biological effect is unknown. Here, we proposed a very different picture that will change our vision of how H2S regulates cell metabolism.

5. Conclusions

In conclusion, our data demonstrated that H2S is a regulator of energetic metabolism. These results fill a major gap in the understanding of H2S and its control of ethanol production, which is evolutionarily conserved among yeast species. Finally, our work provides the foundation for a mechanistic understanding of the effects of H2S.

Supplementary Materials: Figure S1: Alcohol dehydrogenase activity; Figure S2: ATP synthase alignment; Figure S3: wt, met5Δmet10Δ, and met17Δ strains growth curves; Table S1: Strains and oligonucleotides primers; Table S2: Mass spectrometry results; Table S3: Protein activity data

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**Figure 1.** H$_2$S productivity correlates with S-persulfidated proteins levels. (A) H$_2$S productivity by *wt, met5Δmet10Δ* and *met17Δ*. Cells were incubated at 30°C on YPDL plates for 4 days. (B) S-persulfidated proteins in *wt, met5Δmet10Δ* and *met17Δ* strains. Whole cell extracts from exponential phase cultures were subjected to the modified biotin switch assay with antibody against biotin (α-Biotin Ab) to detect S-persulfidation.

**Figure 2.** Confirmation of S-persulfidated proteins by streptavidin beads precipitation. A. Whole cell extracts from exponential phase cultures were subject to the modified biotin switch assay, precipitated with streptavidin beads and detected with antibodies specific to each protein; enolase (Eno2), glyceraldehyde 3 phosphate dehydrogenase (Tdh3), cystathionine beta synthase (Cys4) and triose phosphate isomerase (Tpi1). HPDP-B: (N-[6-(biotinamido)hexyl]-3'-(2'-pyridyldithio)propionamide). B. Whole cell extract from exponential phase cultures after modified biotin switch assay was used as input control. (-) line shows the proteins that reacts with anti-biotin antibody. (+) line shows biotinylated proteins after modified biotin switch assay. TCL: Total cell lysate, PP STREP: Streptavidin precipitation.

**Figure 3.** H$_2$S production reach a maximal during log phase. Yeast cell were cultured in 96 wells microplate at 30°C and growth was measured at 600 nm. H$_2$S was detected measuring methylene blue reduction at 663 nm.

**Figure 4.** H2S increase the activity of GAPDH and Pyruvate Kinase two hours after stimulation. (A) Yeast cell cultures at exponential phase were treated with NaHS 0.1 and 0.25 mM. Two hours later whole cell extracts were used to measure GAPDH activity in vitro at 37°C. One-way ANOVA ** P<0.0001. (B) Yeast cell cultures at exponential phase were treated with NaHS 0.1 and 0.25 mM. Seven hours later whole cell extracts were used to measure GAPDH activity in vitro at 37°C. (C) Yeast cell cultures at exponential phase were treated with NaHS 0.1 and 0.25 mM. Two hours later whole cell extracts were used to measure Pyruvate Kinase activity in vitro at 37°C. (D) Yeast cell cultures at exponential phase were treated with NaHS 0.1 and 0.25 mM. Seven hours later
whole cell extracts were used to measure Pyruvate Kinase activity in vitro at 37°C. One-way ANOVA * P<0.01. Closed circles, untreated cells; closed squares, NaHS 0.1 mM; closed triangles, NaHS 0.25.

Figure 5. Exogenous and endogenous H₂S on yeast cells induce ethanol production and ATP synthesis. (A) BY4742 yeast cell cultures were treated with NaHS 0.1 mM and seven hour later supernatants were collected. Ethanol production was measured in vitro at 37°C. Closed circles, untreated cells; closed squares, NaHS 0.1 mM. Unpaired t * P=0.04. (B) Yeast cell cultures of the strains BY4741 and BY4742 supernatants were collected at 24 h. Ethanol production was measured in vitro at 37°C. Closed squares, BY4741; open squares, BY4742. Unpaired t * P=0.02 (C) Yeast cell cultures at exponential phase were treated with NaHS 0.1 and 0.25 mM. Two and four hours later whole cell extracts were lysated and ATP was quantified. ATP production was measured in vitro at 37°C. Closed circles, untreated cells; closed squares, NaHS 0.1 mM; closed triangles, NaHS 0.25 mM. One-way ANOVA ** P<0.001.

Figure 6. Yeast mutants that accumulate H₂S synthesize ethanol faster and more ATP than lower endogenously accumulated H₂S. (A) 48 hours precultures of yeast cell of the strains wt, met5Δmet10Δ and met17Δ were resuspended in fresh media and supernatants were collected every hour. Ethanol production was measured in vitro at 37°C. (B) Yeast cell cultures at exponential and stationary phase of the strains wt, met5Δmet10Δ and met17Δ were lysated and ATP was quantified. ATP production was measured in vitro at 37°C. One-way ANOVA * P<0.05, ** P<0.01. Open circles, wt; closed squares, met5Δmet10Δ; closed diamonds, met17Δ.

Figure 7. Endogenous H₂S promotes basal oxygen consumption. 48 hours precultures of the wt and the mutants was diluted to an OD₆₀₀= 0.2, and oxygen consumption was measured. The basal oxygen consumption was measured in resting cells in a Clark electrode at 30°C. Seven hours after dilution oxygen consumption was measured again. One-way ANOVA * P<0.05, ** P<0.01. Open circles, wt; closed squares, met5Δmet10Δ; closed diamonds, met17Δ.
Figure 8. Exogenous H$_2$S induce ethanol production in *K. marxianus* and *M. guilliermondii*. (A) *K. marxianus* yeast cell cultures were treated with NaHS 0.1 mM and seven hour later supernatants were collected. Ethanol production was measured in vitro at 37°C. Unpaired t ** P=0.002. (B) *M. guilliermondii* yeast cell cultures were treated with NaHS 0.1 mM and 24 h later cells were treated again with same concentration of NaHS. Seven hour later supernatants were collected. Ethanol production was measured in vitro at 37°C. Unpaired t * P=0.04. Closed circles, untreated cells; closed squares, NaHS 0.1 mM
