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Hydrogen sulfide production during yeast fermentation causes the accumulation of ethanethiol, *S*-ethyl thioacetate and diethyl disulfide

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ABSTRACT

Hydrogen sulfide (H_2S) is produced by yeast during winemaking and possesses off-flavors reminiscent of rotten eggs. The production of H_2S during fermentation has also been associated in the finished wine with the rise of additional volatile sulfur compounds (VSCs) with strong aromas of cooked onions and vegetables. To characterize these more complex VSCs produced from H_2S, we performed fermentations in synthetic grape juice. H_2S production was manipulated experimentally by feeding increasing concentrations of sulfate to mutant strains that are unable to incorporate H_2S efficiently as part of the sulfur assimilation pathway. In finished wines from these mutants, three VSCs – ethanethiol, *S*-ethyl thioacetate and diethyl disulfide – increased proportionally to H_2S. ^{34}S-labeled sulfate fed to the *MET17*-deleted strain was incorporated into same three VSCs, demonstrating that they are formed directly from H_2S.

1. Introduction

Volatile sulfur compounds (VSCs) comprise a large family of molecules commonly found in fermented foods and beverages, where they play an important role in conditioning the quality of the product (Boelens & van Gemert, 1993; Mestres, Busto, & Guasch, 2000). In wines, they contribute to aroma complexity and uniqueness (Dittrich, 1987; Fedrizzi, Magno, Badocco, Nicolini, & Versini, 2007; Mestres et al., 2000). Wine VSCs can arise from the grape juice composition, during microbial fermentation, and from chemical reactions occurring both before and after bottling (Maga, 1976). Many have low sensory perception thresholds (Maga, 1976; Mestres et al., 2000) and most have primary aroma notes that are considered to have a negative impact on the finished wine (Dittrich, 1987). However, several varietal thiols contribute to fruity aromas in many wines (Darriet, Lavigne-Cruège, & Tominaga, 1999) and some of the negatively perceived VSCs, like dimethyl sulfide (DMS), can add to the overall bouquet by synergistically aiding the perception of red fruit aromas (Darriet et al., 1999; Landaud, Helinck, & Bonnarme, 2008; Shankaranarayana, Raghavanb, Abrahamc, Natarajand, & Brodnitze, 1974).

Hydrogen sulfide (**1**) (Fig. 1), with its strong "rotten egg" aroma, is arguably the most studied and most undesirable VSC formed by yeast during winemaking. Recent detailed genetic analyses of yeast strains have revealed that **1** biogenesis is primarily linked to the sulfur assimilation pathway (SAP) (Fig. 2), where it is produced as an intermediate step in the reduction of sulfate and it is subsequently incorporated into the two sulfur-containing amino acids, methionine and cysteine. In particular, the enzyme sulfite reductase (encoded as two subunits by the *MET5* and *MET10* genes) converts sulfur dioxide to **1** (Masselot & De Robichon-Szulmajster, 1975). The enzyme O-acetyl homoserine sulfhydrylase (OAHS, encoded by the *MET17* gene) subsequently incorporates this **1** into O-acetyl homoserine to produce homocysteine (Masselot & De Robichon-Szulmajster, 1975). Inactive mutants of *MET17* are known to overproduce **1** at high concentrations (Linderholm, Findleton, Kumar, Hong, & Bisson, 2008). Natural and artificial variants of the *MET5* and *MET10* genes also affect the rate of **1** production by yeast by varying the amount of this substrate available for

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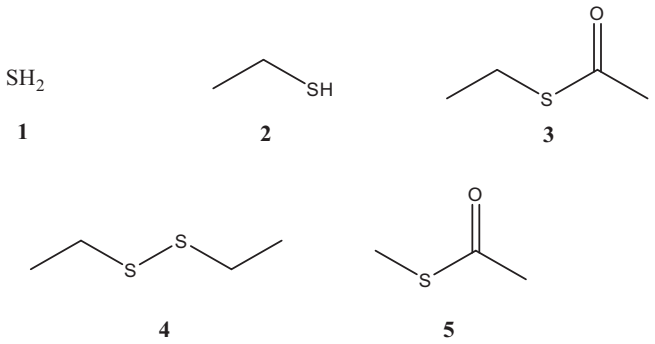

Fig. 1. Structures of VSCs studied in this work. H_2S (**1**), ethanethiol (**2**), *S*-ethyl thioacetate (**3**), diethyl disulfide (**4**), *S*-methyl thioacetate (**5**).

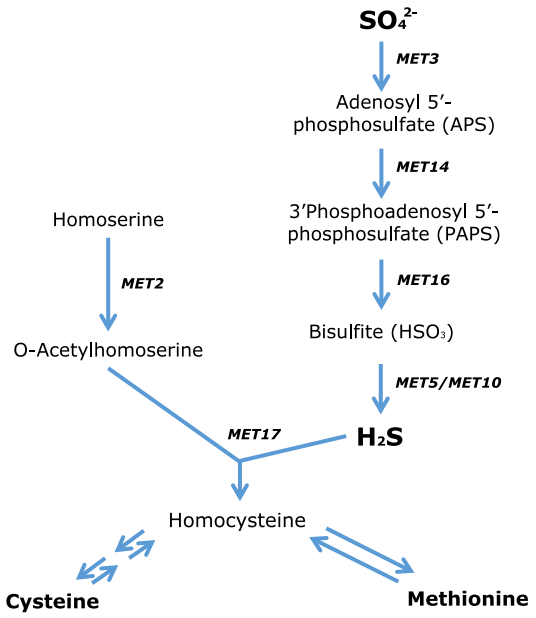

Fig. 2. The sulfate assimilation pathway (SAP) in *Saccharomyces cerevisiae*.

OAHS (Cordente, Heinrich, Pretorius, & Swiegers, 2009; Linderholm et al., 2008), as does the *SKP2* gene via a more general regulatory role (Noble, Sanchez, & Blondin, 2015). Finally, a natural mutant in *MET2* that affects the supply of O-acetyl homoserine for the MET17 enzyme also alters **1** production during fermentation (Huang, Roncoroni, & Gardner, 2014; Noble et al., 2015). Mutants in all of these four genes have been utilized to develop novel commercial yeast strains with reduced production of **1** during fermentation (Berlese-Noble et al., 2014; Scerri & Silvano, 2009; www.renaissanceyeast.com).

In addition to its inherent off-flavor, **1** is highly reactive (Carballal et al., 2011) and when it is produced during fermentation it is believed to combine with other compounds to form more complex and undesired VSCs associated with cooked onion or vegetable aromas (Vermeulen, Gijs, & Collin, 2005). These higher-MW, less reactive VSCs are considered to be more problematic in the final wine, since, in contrast to **1**, they cannot be removed by copper treatment (Landaud et al., 2008). However, these compounds have not been well characterized, and the pathway to their formation remains largely speculative. *In vitro*, **1** can react with ethanol or acetaldehyde to give ethanethiol (**2**) (Fig. 1), for example, but it is not clear if this reaction can occur in wine (Rauhut & Kurbel, 1994). Furthermore, it was observed that **2** can dimerize to form diethyl disulfide (**4**) (Fig. 1) in a wine-like solution, through a steady-state equilibrium with bisulfite that was pH-dependent (Bobet, Noble, & Boulton, 1990).

S-ethyl thioacetate (**3**) and *S*-methyl thioacetate (**5**) (Fig. 1) are two other VSCs identified in beer and wine (Leppanen, Denslow, & Ronkainen, 1980) that are also attributed rotten vegetable aromas (Landaud et al., 2008). At low pH it was observed that both **3** and **5** can be hydrolyzed to give free thiols, potentially acting as an additional source of off-odors (Leppanen et al., 1980).

The objective of this work was to identify those VSCs originating in wine due to the accumulation of **1** using hyphenated mass spectrometry techniques (Kinzurik, Herbst-Johnstone, Gardner, & Fedrizzi, 2015; Nguyen, Nicolau, & Kilmartin, 2012). We utilized yeast genetic mutants in chemically defined media to vary **1** production quantitatively during fermentation. In particular, we used yeast deletion mutants of *MET17*, completely lacking OAHS activity, and of *GLO1*, which we have recently found is partly deficient in the same enzyme activity. Both strains produce **1** when supplied with sulfate during fermentation. A suite of 12 VSCs was analyzed in the final wine to identify those that responded to changes in the concentration of **1**, and isotopic labeling with ^{34}S was used to identify their origin.

2. Materials and methods

2.1. Chemicals

VSCs studied included hydrogen sulfide (**1**), ethanethiol (**2**), diethyl disulfide (**3**), *S*-ethyl thioacetate (**4**) and *S*-methyl thioacetate (**5**). Dimethyl-d_6 sulfide (d_6-DMS), dipropyl disulfide (DPDS) and 3-(methylthio)-1-hexanol (MTH) were used as internal standards (IS). All of the purchased analytes had a purity of 98% and were supplied by Sigma-Aldrich (Sigma-Aldrich, Germany), except for ETA (Alfa Aesa, USA). Absolute ethanol was of analytical grade and purchased from Ajax Finechem (Mt. Wellington, Auckland, New Zealand). Tartaric acid was obtained from Sigma-Aldrich (Sigma-Aldrich, Germany).

2.2. Yeast strains and culture

This study used the diploid auxotrophic lab strain BY4743 (*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 met15Δ0/MET15 ura3Δ0/ura3Δ0*), and two deletion mutants in the same genetic background: *MET17* (YLR303W) and *GLO1* (YML004C). Strains were obtained from Euroscarf.

Yeast pre-cultures were grown in YPD media (10 g L^{-1} yeast extract, 20 g L^{-1} casein peptone and 20 g L^{-1} glucose) at 28 °C. Once saturation was reached, the cell pellet was washed twice with sterile water and 2×10^6 cells mL^{-1} were inoculated into 100-mL cultures of synthetic grape media (SGM) (Kinzurik et al., 2015) [pH 3.2, 210 g/L total sugar, 301.5 mg N L^{-1} total YAN]. We note that we added a 10-fold excess of supplements required for auxotrophy (leu, his, ura), following our findings that this is needed for complete fermentation (Harsch, Lee, Goddard, & Gardner 2010).

For the experiment in which increasing amount of sulfate was added to induce H_2S production, in the cases of those strains unable to assimilate sulfate (*met17* and *glo1*), 0.075 mM methionine was included to allow for growth. This concentration is the minimum that allows growth to normal OD, and is less than the typical content of methionine in grape juice. Fermentation rate was not affected by sulfate supplementation.

2.3. Fermentation and H_2S quantitation

Conical flasks (250 mL) containing 100 mL of SGM were used in triplicate for each fermentation. Rubber stoppers fitted with H_2S