Hydrogen sulfide production by yeast during alcoholic fermentation: mechanisms and mitigation

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Advances in the understanding of the yeast genetic mechanisms of hydrogen sulfide production have led to the recent development of a number of non-GMO yeasts that prevent the rotten egg gas from forming. These strains enable winemakers to fully prevent H_2S rather than rely on remediation methods.

Hydrogen sulfide (H_2S) is a chemically reactive, volatile, organosulfur compound common to fermented alcoholic beverages, including wine, cider, beer, sake, distilled spirits and others[1]. Collectively, H_2S and its derivatives—mainly ethyl mercaptan (ethanethiol) and diethyl disulfide[2]—impart a 'reductive' characteristic to wine, most notably described as aromas of rotten eggs, garlic or burnt rubber[3]. Although the effects of H_2S can be mitigated by various means, its high aromatic potency (detection at ~2ppb)[4] suggests that, even at very low concentrations, H_2S can impact the sensory profiles of wines. When left untreated, or when present in sufficient concentrations beyond the capacity of remediation methods, H_2S contamination can result in complete spoilage[3,5].

Conventional practices for the post-fermentation removal of H_2S include aeration, inert gas stripping, precipitation by copper (II) sulfate, and blending[6]. Although these methods can be effective, they can also be subject to secondary cost, quality and efficiency problems[7]. For example, post-fermentation H_2S removal techniques are non-specific in their mechanism. That is, while they remove H_2S, other compounds—many of which may be desirable, e.g., volatile aroma compounds such as esters, thiols and terepenes—can also be removed. In addition, conventional remediation methods remove H_2S subsequent to its formation; thus, problems associated with H_2S-derived compounds (mercaptans and disulfides) can still occur[2,8]. In contrast to H_2S, these chemicals have lower sensory thresholds and are not easily removed[9]. ▸

Renaissance Yeast's hydrogen sulfide-preventing wine yeasts are available commercially in five red and white strains and one USDA-certified organic for red, white, cider and fruit organic wine production.

Until recently, the only (semi)-preventative method to mitigate H_2S formation by yeast (*Saccharomyces cerevisiae*) during fermentation has been nutrient supplementation. Indeed, it is well-established that nitrogen limitation (especially the amino acids serine, aspartic acid, cysteine and methionine) and vitamin limitation (specifically, pantothenic acid) potentiate yeast's ability to form H_2S[1,10,11]. However, other factors also play a role in determining the volume of H_2S a given yeast strain will produce. These include high levels of elemental sulfur in the grape juice and/or vineyard[12], high levels of sulfur dioxide (SO_2) during fermentation[13-15], and the presence of organic sulfur-containing precursor compounds[16]. In addition to these environmental variables affecting H_2S production, genetic differences among yeast strains dictate each strain's response to these variables. Indeed, surveys of various yeast strains indicate that, while all yeast strains produce some level of H_2S, there is a high degree of variability in the amount of H_2S produced. Moreover, even in well-fed fermentations, i.e., those with sufficient nitrogen and vitamins, many yeast strains still produce H_2S[16-19].

Broadly speaking, H_2S is formed as a byproduct of yeast metabolism during primary alcoholic fermentation[3,5]. More specifically, the sulfide (S^{2-}) ion—which forms H_2S upon leakage out of the cell into the acidic wine environment[20]—is formed as an intermediary molecule in the sulfate-reduction sequence pathway (Figure 1). This pathway is responsible for the yeast's ability to utilise sulfur so as to produce the sulfur-containing amino acids (cysteine and methionine) needed for growth[1,12]. Briefly, exogenous sulfate (SO_4^{2-}) from the juice is imported into the cell, where it is reduced through a series of enzymatic steps to form sulfite (SO_3^{2-}), and then

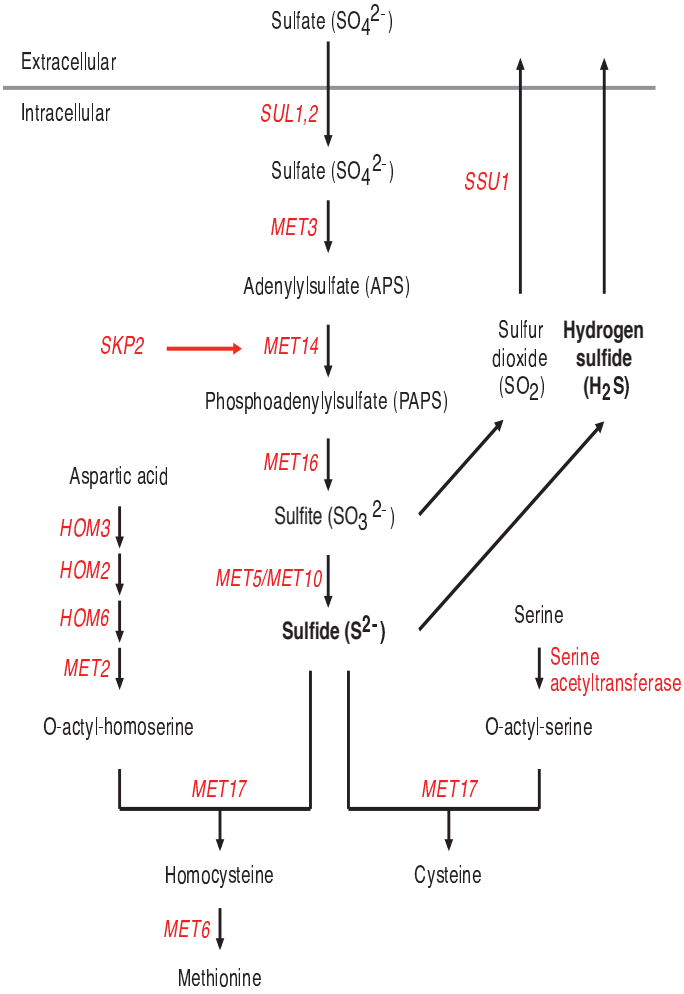

Figure 1. Sulfate reduction sequence (SRS) pathway in *S. cerevisiae*. Adapted from Wang *et al*[10]. Metabolic compounds are shown in black; genes encoding the relevant enzymes are show in red.

further reduced to sulfide (S^{2-}). This sulfide is then used by the cell as a sulfur donor for the production of homocysteine and cysteine, as well as an assortment of downstream sulfur-containing compounds, including methionine, Met-tRNA, S-adenosyl-methionine (SAM), glutathione, etc[19]. However, when environmental conditions are such that sulfide production is out of equilibrium with its utilisation (as discussed above), excess sulfide diffuses out of the cell and into the wine, where it forms H_2S and results in spoilage.

An understanding of the genetic underpinnings of H_2S formation, as well as the observation that certain yeast strains produce little to no H_2S during fermentation, have led to the idea that strains of yeast—both natural and engineered—may be used as a tool to prevent H_2S contamination of wines. Indeed, a number of non-GMO H_2S-reducing/preventing commercial winemaking strains have recently been developed, each of which is based on a different core genetic technology.

TECHNOLOGY 1: MET2/SKP2 – JN17

Numerous genes involved in sulfur metabolism have been implicated in yeast's propensity to produce H_2S. The most notable of these are genes directly involved in the enzymatic conversion of sulfate into sulfide (MET2, MET3, MET5, MET6, MET10, MET14, MET17, MET16, and CYS4)22-27. However,

other genes, such as those involved in the regulation and stability of the SRS pathway enzymes (MET4 and SKP2), have also been implicated in H_2S production[28]. Collectively, these data suggest that this trait is, in fact, controlled by multiple distinct genetic mechanisms that may interact in a complex way.

To begin to resolve this interplay, Nobel *et al.* recently performed a quantitative trait loci (QTL) analysis of two wine yeast strains: a high sulfite-producing strain (JN10) and a low sulfite-producing strain (JN17)[29]. QTL is a genome-wide, linkage analysis tool that allows researchers to dissect the contribution and interaction of different genes to complex multi-genic traits, such as sulfide production. In this way, Nobel *et al.* were able to genetically map versions of the MET2 and SKP2 genes that drive H_2S production in the high-sulfite strain JN10. To develop a commercial product, the authors then used genetic breeding and selection to transfer the lowsulfite versions of MET2 and SKP2 from JN17 to the high-sulfite yeast JN10. In this way, the authors were able to replace the MET2 and SKP2 genes of JN10, thereby reducing its propensity to form H_2S, SO_2 and acetaldehyde. This novel yeast strain is currently commercially available, and is marketed as a general strain for "young, fresh and aromatic rose, white, and red wines."

TECHNOLOGY 2: MET5/MET10 – PDM

As previously mentioned, the SRS pathway converts sulfate into sulfite and then sulfide. Sulfite reductase, a heterodimer enzyme encoded by MET5 and MET10, catalyses the key enzymatic step that forms H_2S[21], and a positive correlation has been observed between H_2S production and sulfite reductase activity[30].

Given the relationship between sulfite reductase and H_2S, Cordente *et al.* were able to use chemical mutagenesis and selection methods to isolate lowH_2S variants of the wine yeast PDM31. Genetic characterisation of these variants confirmed that a variety of single amino acid mutations within both MET5 and MET10 were causative of the stains' reduced propensity to form H_2S. Analysis of basic chemical parameters in the finished wine, including residual sugar, glycerol, acetic acid and SO_2, indicated that the mutagenised strains were similar to the parent PDM—except for SO_2, which was much higher in some of the mutant strains[31]. A number of these novel strains—all derivatives of PDM—are currently commercially available. They are marketed as general strains "ideal for all varietals and wine styles" and recommended for the production of "fruit-driven wines with only a small contribution from the yeast."

TECHNOLOGY 3: MET10 – UCD932

Several lines of evidence have established the importance of the MET10 sub-unit of sulfite reductase in determining how much H_2S a given yeast strain produces. Indeed, numerous mutations in MET10 have been shown to modify its activity, thereby lowering H_2S levels[31,32]. Moreover, a genome-wide screen of yeast identified MET10 as one of four genes that, when deleted, eliminate H_2S production[27].

In an attempt to identify strains producing low H_2S, two independent screens identified a Italian vineyard isolate, UCD932, that produces no detectable H_2S[17,19]. Genetic analysis of UCD932 identified a single amino acid mutation in MET10 that results in the strain's characteristic inability to produce H_2S during fermentation[7]. Further characterisation of the strain indicated that the version of MET10 in UCD932 does not affect the strain's ability to synthesise methionine, nor does

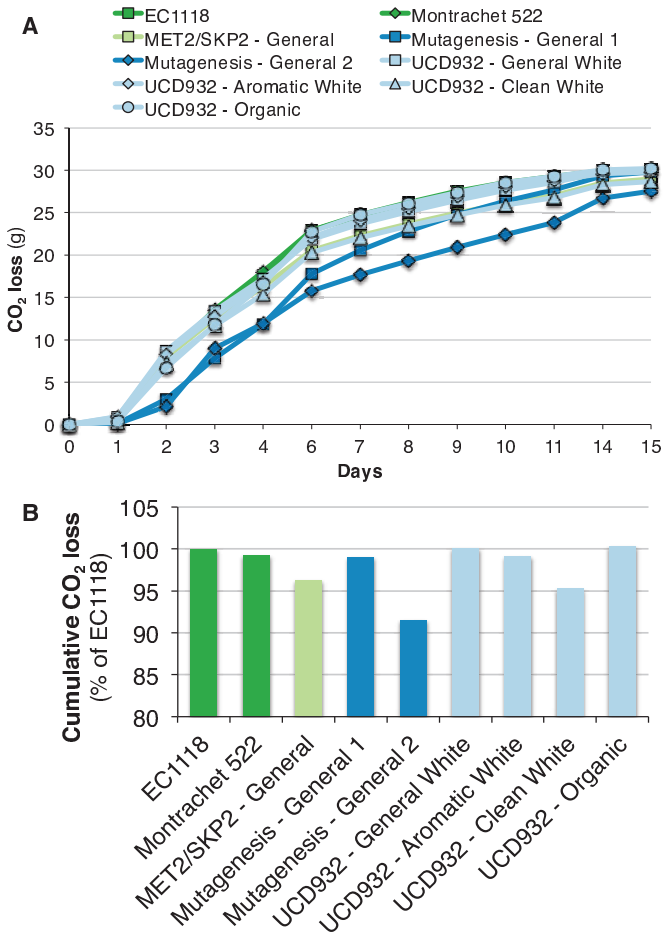

Figure 2. Fermentation kinetics analysis. Laboratory-scale fermentations (300mL) of Chardonnay grape juice (Brix 19.9°, YAN 182g/L, total SO_2 28ppm, free SO_2 5.76ppm) were conducted at 21.5°C for 15 days. Fermentation kinetics were measured by CO_2 weight loss. A) Fermentation kinetics throughout fermentation. B) Cumulative CO_2 loss relative to EC1118 at the end of fermentation.

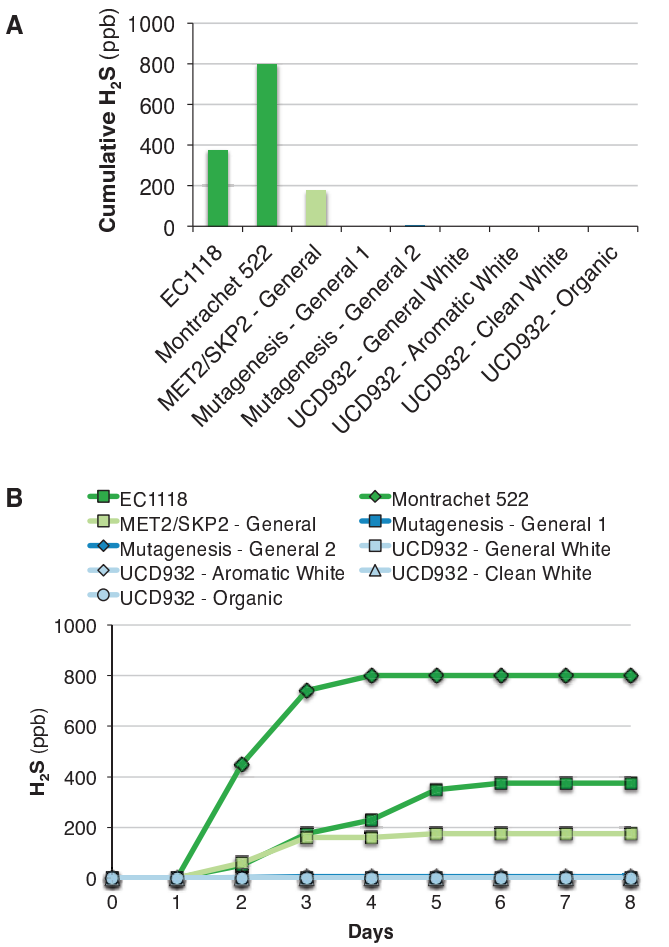

Figure 3. H_2S production analysis. Laboratory-scale fermentations (300mL) of Chardonnay grape juice (Brix 19.9°, YAN 182g/L, total SO_2 28ppm, free SO_2 5.76ppm) were conducted at 21.5°C for 15 days. H_2S production was measured by Sensidyne H_2S Precision Gas Detection tubes fitted to the fermentation vessels. A) Cumulative H_2S production as measured at the end of fermentation. B) H_2S production kinetics in the first eight days of fermentation.

it alter the strain's fermentation rate and fitness[7]. To develop a commercial product, researchers have used breeding and selection to transfer the H_2S-preventing version of MET10 from UCD932 to various winemaking yeast strains. By replacing the version of MET10 in any particular parent strains with that of UCD932, a novel H_2S-preventing strain is created. To date, a variety of H_2S-preventing yeast strains, all incorporating the MET10–UCD932 technology, are currently commercially available for white and red varietals. Each one is developed and marketed for a specific application, e.g., general white, aromatic white, clean white, general red, full-bodied red, Pinot Noir-specific and organic.

OBJECTIVE

Recent advancements in the understanding of yeast genetics and sulfur metabolism have led to the development of a variety of low or no-H_2S winemaking yeast strains. To compare the performance of these strains, we conducted fermentations of Chardonnay grape juice and evaluated the strains in terms of fermentation kinetics, H_2S production, SO_2 production and volatile acidity (acetic acid). In all of these tests, we compared the low or no-H_2S strains to EC1118 (a popular generalist winemaking strain) and Montrachet 522 (a common strain known to produce high levels of H_2S).

EXPERIMENTAL METHODS

Commercially available active dry yeast suitable for white wine production (i.e., no red wine-specific strains were evaluated in this study) were rehydrated and inoculated into 300mL fermentations of flash pasteurised Australian Chardonnay juice (Brix 21.1°, YAN 182g/L, total SO_2 28ppm, free SO_2 5.76ppm) according to manufacturers' instructions. Fermentations were incubated at 21.5°C for 15 days and kinetics were monitored by CO_2 loss. H_2S was measured by Sensidyne H_2S Precision Gas Detection tubes (0.75-300ppm Tube No. 120SB, 25-2000ppm Tube No. 120SF) fitted to the fermentation vessels. Total SO_2 was measured by iodometry (A17 revised by 377/2009). Method: OIV-MA-AS323-04B. Acetic acid was measured by rapid, manual end-point AK/PTA enzymatic method (Megazyme International, Ireland 2015, K-ACETRM 06/15).

RESULTS

As shown in Figure 2, the majority of the low or no-H_2S strains were able to complete the fermentation within 15 days, with the 'UCD932-General White', 'UCD932-Aromatic' and 'UCD932-Organic' strains having comparable kinetics

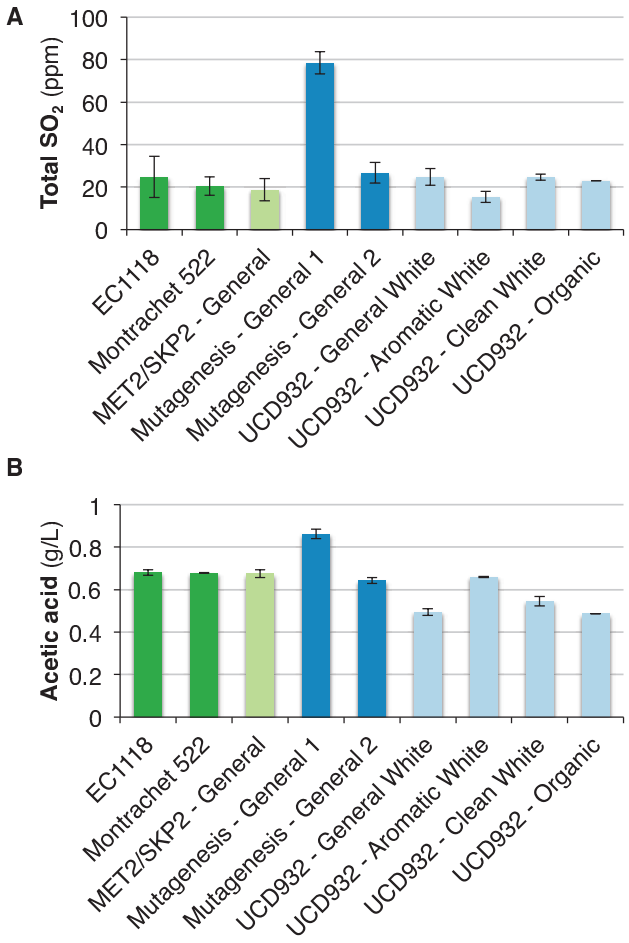

Figure 4. SO_2 and acetic acid analysis. Yeast strains do not produce excess SO_2. Laboratory-scale fermentations (300mL) of Chardonnay grape juice (Brix 19.9°, YAN 182 g/L, total SO_2 28ppm, free SO_2 5.76ppm) were conducted at 21.5°C for 15 days. A) Total SO_2 was measured at the end of fermentation by iodometry (A17 revised by 377/2009). Method: OIV-MA-AS323-04B. B) Acetic acid was measured at the end of fermentation by rapid, manual end-point AK/PTA enzymatic method (Megazyme International, Ireland 2015, K-ACETRM 06/15). Error bars represent one standard deviation in triplicate experiments.

to the conventional strains EC1118 and Montrachet 522. Interestingly, the two mutagenesis-derived strains (General 1 and General 2) were approximately one to two days slower than the rest of the strains, with General 2 achieving only 92% of the CO_2 loss of the control EC1118 within the 15-day fermentation (Figures 2A and 2B). In addition, the 'MET2/SKP2–General' strain and the 'UCD932–Clean White' strain were able to achieve only 96% and 95%, respectively, of the CO_2 loss of EC1118 (Figure 2B).

We next examined H_2S production by the yeast during the fermentations (Figure 3). As expected, the conventional strains EC1118 and Montrachet 522 produced significant amounts of H_2S: 375 and 800ppb, respectively (Figure 3A). Moreover, these yeast produced H_2S early in the fermentation: between two and six days after inoculation (Figure 3B). In contrast, the majority of the low or no-H_2S yeast did not produce any detectable H_2S throughout the fermentation (Figures 3A and 3B). Surprisingly, however, the 'MET2/SKP2–General' did produce 175ppb of H_2S between day two and day five of fermentation (Figures 3A and 3B).

Given the interplay between H_2S and sulfur metabolism, some low or no-H_2S strains have been noted to result in high SO_2 production[22-24,31]. While SO_2 production by yeast can be helpful in terms of microbial stability of the wine, excess SO_2 is detrimental, as high levels of SO_2 strongly inhibit malolactic fermentation, impart sensory defects, and can exceed legal limits in various markets. To determine if any of the low or no-H_2S strains produce excess SO_2, we measured total SO_2 levels at the end of fermentation (Figure 4A). Compared with the conventional strains EC1118 and Montrachet 522, all of the low or no-H_2S strains, with the exception of 'Mutagenesis–General 1', produced comparable volumes of SO_2 (approximately 20ppm). In contrast, the 'Mutagenesis–General 1' strain produced significant amounts of SO_2 (79ppm) (Figure 4A).

In addition to H_2S and SO_2, yeast also produce acetic acid in response to nutrient imbalance and stress. Excess acetic acid (>0.8g/L) can result in high levels of volatile acidity that negatively impacts wine quality and can exceed legal limits in various markets[33]. To determine if any of the low or no-H_2S strains produce excess volatile acidity, we measured acetic acid levels at the end of fermentation (Figure 4B). Compared with the conventional strains EC1118 and Montrachet 522, a number of the strains—namely, 'MET2/SKP2–General', 'Mutagenesis–General 2' and 'UCD932–Aromatic White' - produced equivalent amounts of acetic acid (approximately 0.65g/L) (Figure 4B). In contrast, three of the UCD932-based strains ('UCD932–General White', 'UCD932–Clean White' and 'UCD932–Organic') produced less acetic acid, approximately 0.5g/L (Figure 4B). However, one strain—'Mutagenesis–General 1'—had elevated levels of acetic acid (0.86g/L) (Figure 4B).

DISCUSSION AND CONCLUSION

The recent development of low or no-H_2S yeast strains is an exciting new tool for winemakers. Born of a more thorough understanding of the genetic mechanisms underlying yeast sulfur metabolism, these yeast strains can limit or prevent the production of H_2S during fermentation. In doing so, these strains enable winemakers to fully prevent H_2S rather than simply focus on remediation. However, to be useful to winemakers, low or no-H_2S yeast strains must perform comparably to conventional strains in a number of key attributes.

In this study, we evaluated the performance of a range of low or no-H_2S strains derived from three distinct core genetic technologies: 1) genetic mapping and breeding of MET2/SKP2 variants; 2) mutagenesis and selection of MET5/MET10 variants; and 3) breeding and selection of MET10-UCD932. In doing so, we measured a number of key functional attributes of winemaking yeast, including fermentation kinetics, SO_2 production, volatile acidity and H_2S production.

Our data indicate that low or no-H_2S yeast strains are not equal with respect to some of these key parameters. For example, we observed that the 'MET2/SKP2–General' actually produced notable amounts of H_2S under the conditions tested—none of the other low or no-H_2S strains produced any detectable H_2S (Figure 3). In terms of fermentation kinetics, the 'Mutagenesis–General 1' and 'Mutagenesis–General 2' strains were slower than conventional yeast and the rest of the low or no-H_2S strains (Figure 2). Moreover, the 'MET2/SKP2–General' and 'UCD932–Clean White' strains were able to achieve only 92%–96% CO_2 loss, relative to the conventional strain EC1118. In terms of SO_2 production, we noted that the 'Mutagenesis–General 1' strain produced significant amounts of SO_2 during fermentation—approximately four-fold more than the conventional yeast and the rest of the low or no-H_2S strains (Figure 4A). Finally, when we examined volatile acidity, we noted that the 'Mutagenesis–General 1' strain produced elevated levels of acetic acid, approximately 1.5-fold more than the other strains (Figure 4B).

In conclusion, our data highlight the variability in winemaking attributes between low or no-H_2S strains, and especially between different H_2S-preventing core genetic technologies. As such, it would be prudent for winemakers to critically evaluate their options in selecting low or no-H_2S yeast strains, so as to choose strains that provide optimal performance and results in any given application.

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