

Acetaldehyde metabolism in industrial strains of *Saccharomyces cerevisiae* inhibited by SO₂ and cooling during alcoholic fermentation

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ABSTRACT

Aim: The addition of SO₂ is a common technique for stopping alcoholic fermentation by *Saccharomyces cerevisiae* and producing beverages with residual sugar. However, SO₂ causes a metabolic shift in active yeast leading to the formation of acetaldehyde and resulting in higher preservative SO₂ requirements in the final product. The current work investigated the effects of stopping alcoholic fermentation using two industrial strains of *Saccharomyces cerevisiae*, by means of cooling and/or addition of SO₂, on the kinetics of hexoses and acetaldehyde.

Methods and results: Alcoholic fermentation was conducted by inoculating natural Chardonnay grape must with two commonly used strains of *Saccharomyces cerevisiae* (CY3079 and EC1118). Ten days after inoculation, cooling (to 4 °C) and/or addition of SO₂ (50-350 mg/L) were applied to stop fermentations at approximately 70-90 g/L of residual sugar. Incubations were carried out in an anaerobic chamber to prevent the formation of acetaldehyde resulting from chemical oxidation. Samples were taken regularly and analysed for glucose, fructose and acetaldehyde levels.

In this work, addition of SO₂ to 150 mg/L or more were effective in inhibiting further and practically relevant degradation of hexoses even in non-cooled control treatments. With concurrent cooling, an addition to 50 mg/L was sufficient. Addition of SO₂ always led to a slow increase in yeast acetaldehyde formation over time, regardless of cooling or the apparent inhibition of yeast sugar metabolism. Acetaldehyde increases were reduced with larger SO₂ additions.

Conclusions: When using SO₂ to stop alcoholic fermentations, large doses should be used and wines separated from the sedimented biomass soon thereafter. Nevertheless, rapid cooling remains preferable to SO₂ addition and can prevent further microbial formation of acetaldehyde.

Significance and impact of the study: Results from the current work show that acetaldehyde, and therefore bound SO₂ formation, can be reduced when alcoholic fermentation is halted to obtain wines with residual sweetness.

KEYWORDS

yeast, alcoholic fermentation, *Saccharomyces cerevisiae*, acetaldehyde, wine, SO₂, residual sweetness

INTRODUCTION

During alcoholic fermentation in grape wine production, it may be desirable to conserve residual sugar in order to balance high acidity, which is more common in cool climates, or achieve sweet wine styles that are appreciated by some consumers (Fischer and Wilke, 2000). Residual sugar can be conserved by stopping alcoholic fermentation (AF) before the natural sugar is depleted by yeast metabolism (Boulton *et al.*, 1996). This can be achieved by removing the yeast biomass (by filtration or centrifugation) or by inhibiting its metabolism by adding distillates (in the production of special fortified wines), cooling or adding SO_2 (Bird, 2010). The filtration or centrifugation of wines undergoing AF requires suitable equipment that is not widely available, while cooling involves high energy costs. Accordingly, the addition of SO_2 remains a cheap, rapid and common technique for stopping alcoholic fermentation. SO_2 is an ideal and cost-effective wine preservative because of its antimicrobial (Delfini and Formica, 2001; Doyle and Beuchat, 2007), antioxidant (Danilewicz, 2003) and anti-enzymatic properties (Wedzicha *et al.*, 1991). SO_2 also causes a significant metabolic shift in yeast that has been described early on (Neuberg and Reinthur, 1918). By strongly binding acetaldehyde (the terminal electron acceptor of alcoholic fermentation) (Gottschalk, 1986), the biosynthesis of glycerol intensifies as an alternative pathway for the regeneration of reduced dinucleotides (Remize *et al.*, 2000) and, concurrently, yeast acetaldehyde formation increases (Jackowetz *et al.*, 2011).

Wines with high acetaldehyde residues require larger SO_2 additions in order to maintain sufficient concentrations of free SO_2 for anti-microbial and anti-oxidant activity (Jackowetz and Mira de Orduña, 2013). Given the recent reduction of legal SO_2 limits in some markets and consumer concerns regarding its concentration in wines (Stolz and Schmid, 2008; Guerrero and Cantos-Villar, 2015), controlling the microbial formation of acetaldehyde is desirable. Recent work has shown that the SO_2 -induced increase in residual acetaldehyde levels in fermentations with *Saccharomyces* and non-*Saccharomyces* yeast ranged from 217 to 530 μg acetaldehyde per mg of SO_2 added to the must before fermentation (Li and Mira de Orduña, 2017). However, the effects on acetaldehyde formation of adding SO_2 during an active AF has not yet been quantified.

The purpose of this work was to study the effects of SO_2 addition and/or cooling for interrupting alcoholic fermentation using two commercial *Saccharomyces cerevisiae* strains on the time course of hexose and acetaldehyde concentrations. Incubations were conducted in an anaerobic chamber to avoid chemical acetaldehyde formation from ethanol oxidation (Danilewicz, 2012).

MATERIALS AND METHODS

1. Yeast strains and grape must

The commercial active dry yeast strains, *Saccharomyces cerevisiae* CY3079 and EC1118, were obtained from Lallemand Inc. (Montreal, Quebec, Canada) and used according to manufacturer recommendations at an inoculation

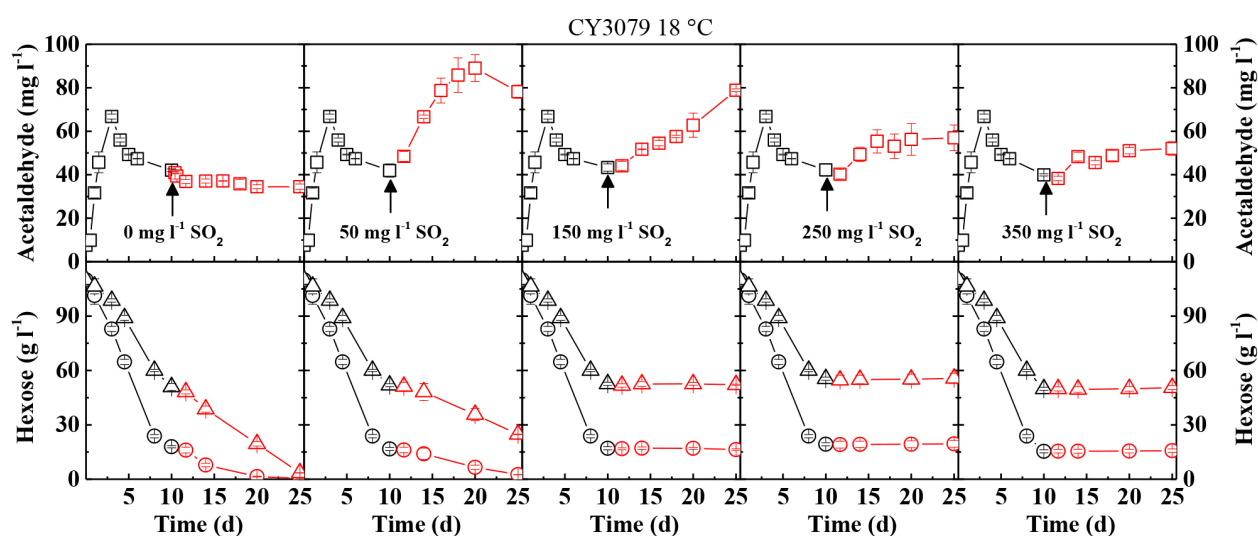


FIGURE 1. Time course of acetaldehyde (□), glucose (○) and fructose (Δ) concentration in Chardonnay fermentation of *Saccharomyces cerevisiae* CY3079, with different amounts of SO_2 added at day 10. Fermentations were settled at 18 °C after SO_2 addition. Average values of duplicate treatments \pm SD shown.

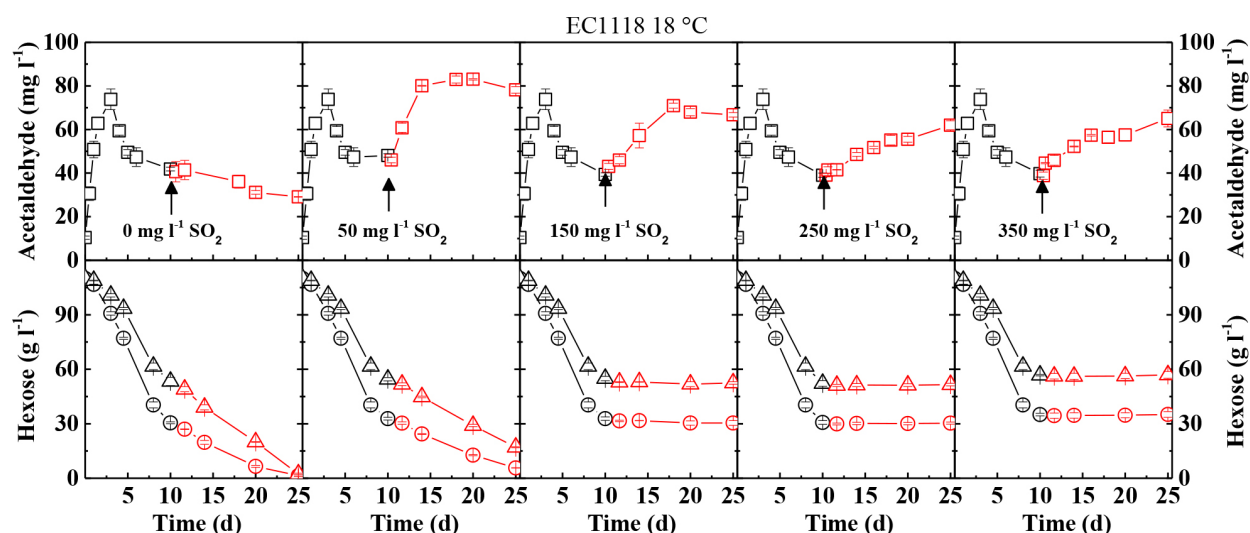


FIGURE 2. Time course of acetaldehyde (□), glucose (○) and fructose (Δ) concentration in Chardonnay fermentation of *Saccharomyces cerevisiae* EC1118 with different amounts of SO₂ added at day 10. Fermentations were settled at 18 °C after SO₂ addition. Average values of duplicate treatments ± SD shown.

rate of 0.25 g/L. A flash-pasteurised grape must (Chardonnay) from the Languedoc region (Kamil Juices, Canada) was filter-sterilised (0.45 µm nitrocellulose membrane, Millipore, MA, USA) and used as fermentation medium. The must had 21.4 °Brix, the pH was 3.2 and the titratable acidity was 8.4 g/L, expressed as tartaric acid. The initial yeast assimilable nitrogen (YAN) concentration was 126 mg/L. The must was supplemented with a complex yeast derived nutrient (0.25 g/L Fermaid K, Lallemand Inc., Montreal, Canada) before inoculation.

All fermentations were conducted statically with 800 mL of grape must in 1000 mL glass bottles in an anaerobic chamber (Coy Laboratory Products Inc., MI, USA) to prevent the formation of acetaldehyde from chemical oxidation reactions. The oxygen concentration in the chamber was monitored with a fluorescence lifetime quenching trace level oxygen meter (Fibox3 LCD trace, PreSens, Regensburg, Germany) and remained below 1 µg/l dissolved oxygen (reference H₂O) throughout the experiment. After an incubation period of 10 d at 18 °C, SO₂ was added to 0–350 mg/L (sterile filtered water was added to all treatments <350 mg/L SO₂ in order to normalise the volume increase). The fermentations were then either kept at 18 °C or cooled to and kept at 4 °C for another 15 d (Table 1). Cooling to 4 °C was achieved within 1.5 hours using a cooled water bath. SO₂ additions were made by adding appropriate volumes of a 50 g/L SO₂ stock solution, which was freshly prepared by dissolving 8.675 g of potassium metabisulfite in water and

adjusting the volume to 100 mL. All fermentations were carried out in duplicate. Samples were taken periodically during fermentations and stored at -20 °C for subsequent analysis.

2. Analytical methods and statistical analysis

Total acetaldehyde was measured enzymatically with a commercial test kit (Megazyme, UK). Glucose and fructose were measured by HPLC using a Shimadzu Prominence System (Columbia, MD, USA). Following filtration (0.22 µm, nylon membrane, Whatman, NJ, USA), a 5 µL sample was injected and separated using a sulfonated styrene-divinylbenzene stationary phase (RHM Monosaccharide H+8 %, 300×7.8 mm, Phenomenex, Torrance, CA, USA) at 85 °C. Hexoses were quantified by refractive index detection (RID-10A, Shimadzu Tokyo, Japan). The mobile phase consisted of ASTM Class I water and the flow rate was 0.5 ml/min. Data representation and rate fittings were carried out using OriginLab Origin v9.0 (OriginLab, Northampton, MA, USA) and the statistical analysis was carried out using SPSS v.16 (Chicago, IL, USA).

RESULTS

From an initial hexose concentration of 230 g/L combined glucose and fructose, an average of 69 g/L (30 %) and 86 g/L (38 %) remained after 10 d of alcoholic fermentation in treatments with *S. cerevisiae* strains CY3079 and EC1118 respectively (Table 1), resulting in 7.5–8.5 % (vol.) alcohol. Figures 1 and 2 provide a graphical

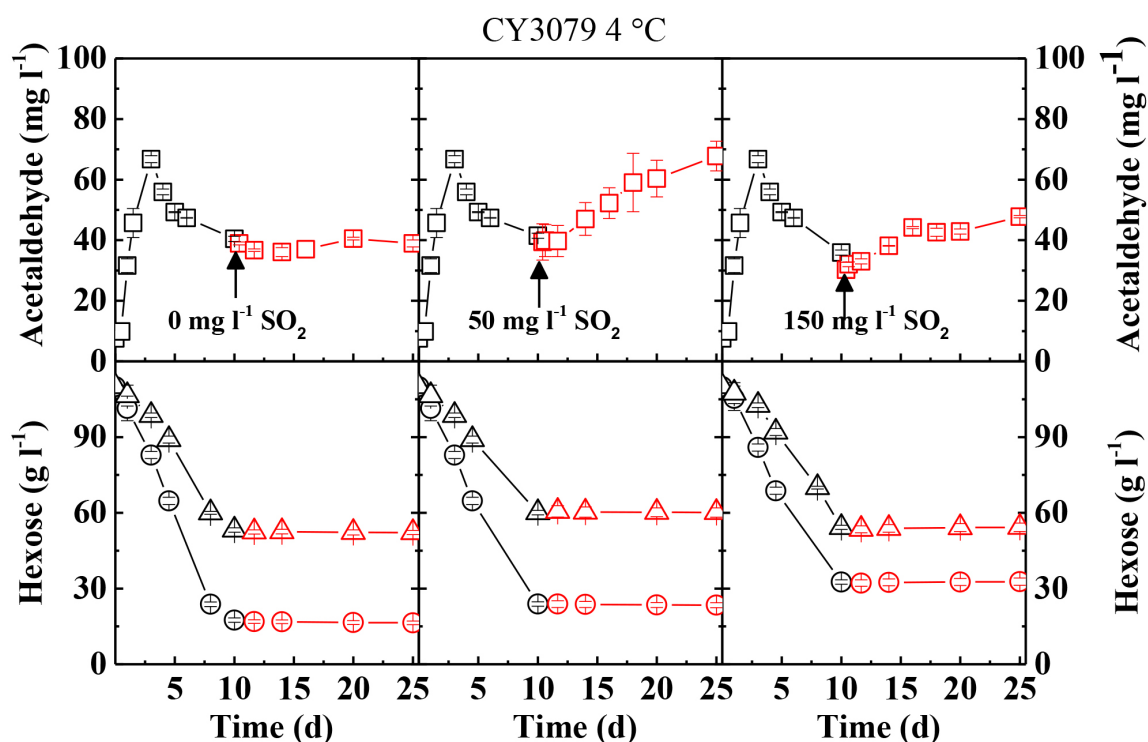


FIGURE 3. Time course of acetaldehyde (□), glucose (○) and fructose (Δ) concentration in Chardonnay fermentation of *Saccharomyces cerevisiae* CY3079 with different amounts of SO₂ added at day 10. Fermentations were cooled down to 4 °C after SO₂ addition. Average values of duplicate treatments ± SD shown.

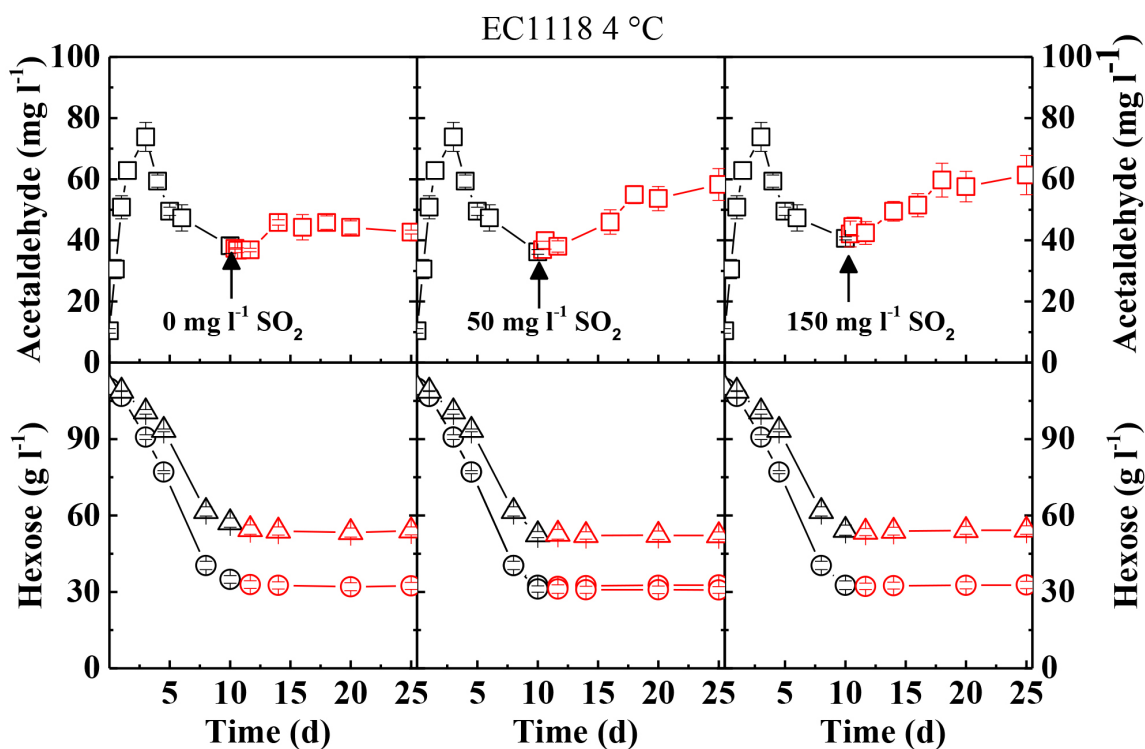


FIGURE 4. Time course of acetaldehyde (□), glucose (○) and fructose (Δ) concentration in Chardonnay wine fermentation of EC1118 with different amounts of SO₂ added at day 10. Fermentations were cooled down to 4 °C after SO₂ addition. Average values of duplicate treatments ± SD shown.

representation of the course of hexose and acetaldehyde concentrations in treatments in which alcoholic fermentation was to be interrupted after 10 days by adding SO_2 .

Degradation of hexoses continued beyond 10 d in the control treatments without added SO_2 , and the wines almost reached dryness (<4 g/L) during the experimental observation period of 25 d. A single SO_2 dose increasing the SO_2 concentration by 50 mg/L reduced the sugar degradation rate, but it was insufficient to halt fermentations. Accordingly, after 25 d an average of only 40 % (CY3079) and 26 % (EC1118) of the residual sugar content at d=10 remained in the wines. On the contrary, single doses increasing the SO_2 concentration by 150, 250 or 350 mg/L after 10 d halted AF in treatments with both yeast strains, thus retaining fermentable sugars.

Acetaldehyde concentrations were further reduced by 18 % (CY3079) and 30 % (EC1118) between 10 and 25 d in the control treatments without added SO_2 . In contrast, in all treatments with added SO_2 , acetaldehyde concentration resurged. The re-increase was particularly marked in treatments where yeast sugar degradation was only partially inhibited; i.e., at 50 mg/L added SO_2 (Table 1), when acetaldehyde concentrations increased by up to 36 mg/L after addition of SO_2 .

Figures 3 and 4 represent data from treatments where cooling was applied. In cooling treatments without SO_2 addition, small amounts of fermentable sugars were degraded during the cooling phase, but concentrations remained stable afterwards. Combined cooling and SO_2 additions immediately prevented further sugar degradation, even at the lowest dose of SO_2 (50 mg/L).

Compared with the control treatment at 18 °C, the degradation of acetaldehyde was inhibited by cooling even without addition of SO_2 . A small increase (+5.5 mg/L) was recorded in the cooled treatment with strain EC1118 (Table 1). In treatments with combined cooling and SO_2 addition, acetaldehyde concentrations re-increased significantly, such as for treatments at 18 °C. The observed increases occurred throughout the observation period (10-25 d) and not merely during the cooling phase.

Figure 5 provides graphical summaries of the data and allows comparing the effect of all treatments on the degradation or formation of acetaldehyde by the two yeast strains investigated.

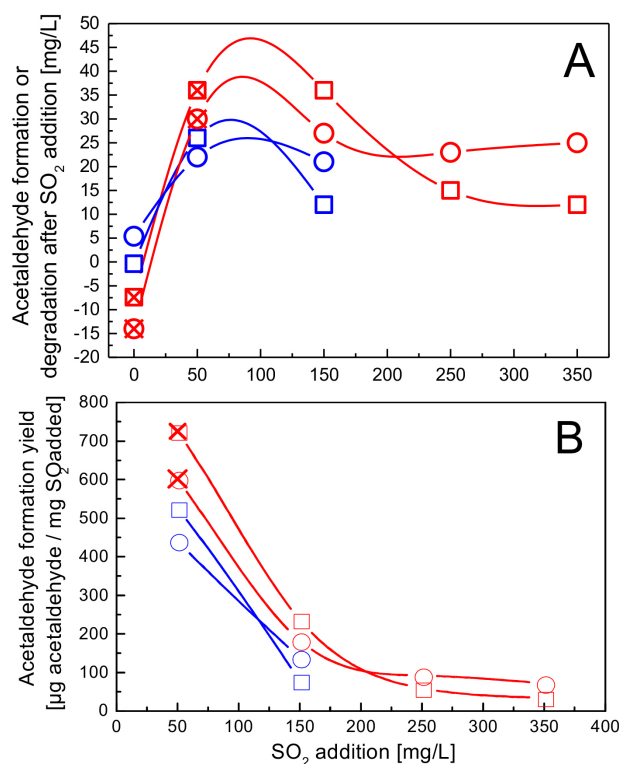


FIGURE 5. Effect of storage temperature and SO_2 addition during AF (10 d) on (A) the degradation or formation of acetaldehyde in the second fermentation phase (10-25 d), and (B) the resulting acetaldehyde formation yields. Red, 18 °C; Blue, 4 °C; (□) CY3079; (○) EC1118. Crossed out symbols highlight treatments where AF was not fully halted (degradation >5 g/L) until the end of the observation period (25 d).

Interestingly, SO_2 additions always led to acetaldehyde concentration increases regardless of any apparent degradation of sugar. However, large SO_2 additions (250, 350 mg/L) caused more modest acetaldehyde formations than lower (50, 150 mg/L) additions (Figure 5A). This relationship is also visible from the SO_2 -based acetaldehyde production yields (Table 1): at 50 and 150 mg/L added SO_2 , the formation yields ranged between 180-720 μg acetaldehyde per mg added SO_2 , while for 250 and 350 mg/L added SO_2 the values remained at <100 μg acetaldehyde per mg added SO_2 (Figure 5B). Concurrent cooling always resulted in a reduction of acetaldehyde formation when compared to the same SO_2 addition without cooling.

DISCUSSION

So-called «off-dry» wines that contain a certain amount of residual sugar, or semi-sweet and sweet wines, play an important role among commercial grape wines (Robinson, 1999). In certain markets,

TABLE 1. Effects of different amounts of SO₂ addition during alcoholic fermentation on hexose and acetaldehyde kinetics of *S. cerevisiae* CY3079 and EC1118 in Chardonnay must.

Yeast strain	SO ₂ addition (mg/l)	Temp. °C	Sugar(g/l)		Sugar degraded after SO ₂ addition (g/l)	Acetaldehyde (mg/l)		Acetaldehyde degraded/formed after SO ₂ addition (mg/l)	SO ₂ -based acetaldehyde formation yield (µg mg ⁻¹)
			10 th d	25 th d		10 th d	25 th d		
CY3079	0	18	68.9±1.4	3.9±0.1	64.9±1.5	42.1±0.6	34.5±1.2	-7.6±1.2 ^d	NA
CY3079	50	18	68.6±2.9	27.3±0.1	41.3±2.8	41.9±3.1	78.2±3.0	36.2±6.1 ^a	725±122 ^a
CY3079	150	18	69.7±1.9	68.4±0.7	NS	43.3±1.7	78.8±0.7	35.6±2.4 ^a	237±16 ^{ab}
CY3079	250	18	74.7±3.9	75.1±4.7	NS	42.2±2.8	56.9±5.9	14.7±3.2 ^b	59±13 ^c
CY3079	350	18	65.3±2.0	66.3±2.0	NS	39.9±0.5	52.1±3.1	12.1±3.6 ^b	35±10 ^c
CY3079	0	4	70.7±1.3	68.6±1.6	2.1±0.2	40.4±0.3	38.9±1.1	-1.5±0.9 ^c	NA
CY3079	50	4	84.0±3.4	83.5±2.8	NS	41.5±3.4	67.8±4.9	26.3±1.6 ^{ab}	526±31 ^{ab}
CY3079	150	4	86.8±3.7	87.0±3.2	NS	35.9±0.4	47.8±0.4	11.9±0.7 ^b	79±5 ^c
EC1118	0	18	83.8±2.9	3.7±1.0	80.1±3.8	41.9±0.9	29.1±0.1	-12.8±2.1 ^c	NA
EC1118	50	18	87.3±1.6	22.7±0.4	64.6±1.3	48.1±0.8	78.2±1.5	30.1±0.7 ^a	602±14 ^a
EC1118	150	18	85.6±1.2	84.8±0.9	NS	39.4±0.8	66.8±1.0	27.4±1.8 ^a	183±12 ^b
EC1118	250	18	82.9±2.6	81.8±1.3	NS	39.0±1.0	61.9±2.1	22.9±3.1 ^a	92±12 ^b
EC1118	350	18	91.8±1.1	92.0±3.5	NS	39.7±1.6	65.1±3.9	25.3±2.3 ^a	72±7 ^b
EC1118	0	4	91.1±3.4	86.3±2.9	4.8±0.5	38.2±2.5	42.7±0.7	5.5±1.9 ^c	NA
EC1118	50	4	83.6±2.4	82.9±2.8	NS	36.2±0.8	58.3±5.2	22.1±4.4 ^a	441±88 ^a
EC1118	150	4	86.8±3.7	87.0±3.2	NS	40.6±0.5	61.4±6.4	20.7±5.9 ^a	138±39 ^b

Letters display statistically significant differences in a column (p<0.05).

it is possible to produce such wines by adding sugar (typically sucrose) to «dry» wines in which sugar has been depleted after alcoholic fermentation (AF). Where this may not be legal, natural grape juice, which is set aside and conserved after grape pressing (so-called «sweet reserve»), may be used to back-sweeten dry wines (Boulton *et al.*, 1996). Completing AF, however, leads to wines that are also higher in alcohol levels. If wine styles with residual sugars and reduced alcohol concentrations are to be achieved, AF needs to be stopped before the naturally contained sugar is completely transformed to alcohol. Stopping vigorous AF by technological means (i.e., cross-flow filtration or centrifugation) is possible (Bird, 2010), but equipment purchasing costs are significant. Most wineries have cooling

equipment that may be used if appropriate heat exchangers are available, but cooling has high energy requirements. Accordingly, SO₂ addition is widely applied, especially in small wineries (Ough, 1992). The current work investigated the effect of cooling and/or addition of SO₂ on the course of sugar and acetaldehyde concentrations in wines fermented by two commercial yeast strains. The study was carried out in an anaerobic environment thus preventing interference from chemical oxidation and allowing changes in acetaldehyde concentrations to be attributed to yeast metabolism.

Saccharomyces cerevisiae strains naturally produce some acetaldehyde as a fermentation by-product (Cheraiti *et al.*, 2010). It is well

established that adding SO₂ to metabolically active yeast will shift yeast metabolism, causing increased acetaldehyde production to compensate for losses caused by SO₂-binding of the principal electron acceptor of AF (Jackowetz *et al.*, 2011). Commonly, some SO₂ (30-50 mg/L) is added to must during or after grape pressing to control indigenous yeast and bacteria. A recent study (Li and Mira de Orduña, 2017) on the effect of adding SO₂ to must on the metabolism of *Saccharomyces* and non-*Saccharomyces* yeast has revealed average SO₂-based acetaldehyde production yields of 325 µg acetaldehyde/mg added SO₂. An average of 400 µg acetaldehyde/mg added SO₂ has been found in another study (Jackowetz *et al.*, 2011) on two oenological yeasts under different winemaking conditions. The present work has revealed values of <100 to >700 µg acetaldehyde/mg added SO₂, depending on the amount of SO₂ introduced to stop AF and whether cooling was applied or not. Large SO₂ additions and cooling reduced the formation yields. If powerful cooling equipment is not available, high SO₂ doses (within legal constraints) would thus be preferable for stopping AF.

The data from our study also showed that an apparent lack of sugar degradation, as quantified within the limits of the analytical technique applied and relevant in practical terms, did not preclude yeast acetaldehyde formation. This suggests that residual yeast metabolic activity persisted and caused small, but steady, acetaldehyde concentration increases, even at 4 °C. A low level or absence of sugar degradation results in the loss of yeast CO₂ production and thus yeast buoyancy (König *et al.*, 2009). Reducing SO₂-mediated acetaldehyde increases may therefore be possible by decanting wines off the sedimented yeast soon after SO₂ addition. However, the current work strongly suggests that cooling alone should be the preferred method for avoiding yeast acetaldehyde formation. Applying a faster cooling rate can be achieved by using a heat exchange system, thus preventing small reductions in sugar concentrations as observed in our study.

CONCLUSIONS

The present work investigated the effects of cooling and/or addition of SO₂ during alcoholic fermentation using commercial *S. cerevisiae* strains on the time course of concentrations of sugars and acetaldehyde. It was shown that the addition of SO₂ always led to a slow increase in yeast acetaldehyde formation over time, regardless of cooling or the apparent inhibition of yeast sugar

metabolism. The work suggests that rapid cooling should be used to stop AF and prevent increases in acetaldehyde concentrations which lead to increased bound and total-SO₂ in finished wines. If SO₂ addition is to be used alone, high doses should be considered and wines decanted (racked off) the yeast lees in a timely fashion.

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