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Metabolism of SO_2 binding compounds by *Oenococcus oeni* during and after malolactic fermentation in white wine

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ABSTRACT

Sulfur dioxide (SO_2) is the key additive for the preservation of wines. Carbonyl and keto compounds in wine can bind to SO_2 and decrease its efficacy, resulting in higher total SO_2 requirements. Increased consumer demand for low sulfite and organic wines pose production challenges if SO_2 binders have not been properly managed during vinification. Malolactic fermentation (MLF) has been known to reduce bound SO_2 levels but detailed time course studies are not available. In this work, the kinetics of major SO_2 binding compounds and malic acid were followed during MLF in wine with 12 commercially available strains of *Oenococcus oeni*. Pyruvic acid, acetaldehyde and α-ketoglutaric acid were degraded to various degrees by *O. oeni*, but galacturonic acid was not. At the time of malic acid depletion, percent degradation of pyruvate, α-ketoglutaric acid and acetaldehyde was 49%, 14% and 30%, respectively. During MLF, the decrease in average bound SO_2 levels, as calculated from carbonyl metabolism, was 22%. The largest reduction in wine carbonyl content occurred in the week after completion of MLF and was 53% (107 mg/L to 34 mg/L) calculated as bound SO_2. Prolonged activity of bacteria in the wines (up to 3 weeks post malic acid depletion) resulted only in reduced additional reductions in bound SO_2 levels.

The results suggest that microbiological wine stabilization one week after malic acid depletion is an effective strategy for maximum removal of SO_2 binders while reducing the risk of possible post-ML spoilage by *O. oeni* leading to the production acetic acid and biogenic amines.

1. Introduction

Sulfur dioxide (SO_2) is an important preservative commonly used in winemaking and the production of other foods (Doyle and Beuchat, 2007). Its anti-microbial (Carreté et al., 2002), anti-oxidant (Danilewicz, 2003) and anti-enzymatic (Main and Morris, 1991; Wedzicha et al., 1991) functionalities at low concentrations make SO_2 an ideal and cost effective food stabilizer. Despite its value and potency, a majority of consumers view sulfite additions to wine as unnatural and unhealthy (Stolz and Schmid, 2008). These apprehensions about SO_2 utilization may stem from historical sulfite abuses in some grocery stores and restaurants (Martin et al., 1986) and sulfite warning labels on wine bottles. The US FDA estimates that 1% of the U.S. population show an increased degree of sensitivity to sulfites (Papazian, 1996). Asthmatics appear to be especially susceptible, with estimates that up to 5% may risk adverse reactions upon sulfite exposure (Snelten and Schaafsma, 1992; Vally et al., 1999). Minimally processed foods and wines with little or no sulfites have been increasingly popular with consumers (Azabagaoglu et al., 2007). In the U.S., sales of certified organic foods have increased from $78 million in 1980 to approximately $6 billion in 2000, with an average annual increase of 24% during the 1990s (Hughner et al., 2007). The organic wine sector has also followed this trend, and in 2005 the world organic wine market grew by 10–15% (Richter and Padel, 2007).

To meet increasing consumer demands, winemakers are challenged to restrict or even eliminate sulfites during vinification, while maintaining high product quality. An important strategy has been to decrease concentrations of carbonyl or keto compounds that bind with SO_2, thus decreasing its preservative activity compared to free SO_2 (Rankine, 1968; Ribéreau-Gayon et al., 1998a). Acetaldehyde is an important SO_2 binding compound found in wine, primarily due to the low dissociation constant of its sulfonate ($K_d = 1.5 \times 10^{-6}$) (Ribéreau-Gayon et al., 1998a). Other SO_2 binding compounds including pyruvate, α-ketoglutaric and galacturonic acids may also have a significant effect on bound SO_2 levels (Ribéreau-Gayon et al., 1998a).

Previous studies have recognized the importance of malolactic fermentation (MLF) to reduce the pool of SO_2 binders in wine (Flamini et al., 2002; Radler, 1986; Zaunmuller et al., 2006), especially acetaldehyde (Osborne et al., 2000). The aim of this work was to provide the comprehensive analysis of the kinetics of acetaldehyde, pyruvate, α-ketoglutaric acid and galacturonic acid concentrations during MLF with 12 commercial strains of heterofermentative *Oenococcus oeni* in wine. This thorough analysis of SO_2 binders during and after

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malic acid metabolism, with concurrent modeling of bound SO_2 levels, offers new insights for sulfite management during vinification.

2. Materials and methods

2.1. Microorganisms and chemicals

Saccharomyces cerevisiae strain CY3079 and 12 commercial *O. oeni* strains were provided by Lallemand Inc. (Montréal, Canada) and stored according to the manufacturer's recommendations. All chemicals, unless cited otherwise, were of analytical grade from Thermo Fisher Scientific (Fairlawn, NJ).

2.2. Grape must and general inoculation procedures

A flash pasteurized German Riesling (Kamil EX-IM, Canada) must was used as medium for all fermentations in this study. The analytical profile of the must and the wine after alcoholic fermentation is summarized in Table 1. Yeast inoculation was conducted in accordance with supplier's recommendations; 250 mg/L of the yeast (CY3079) and yeast hydration nutrient (GoFerm, Lallemand, Canada) were each added to the grape must. Alcoholic fermentation was completed in 2.0 U.S. gallon jugs with air locks to dryness (<5.0 g/L sugars). Bentonite (2.0 g/L Ca-Granulat, Erbslöh Geisenheim, Germany) was added after the completion of alcoholic fermentation to aid in yeast settling and clarification. Wines were then cold stabilized for 5 days at 4 °C and racked in preparation for sterile filtration using nylon membrane filters (0.45 μm, Millipore, Ireland). 200 mL aliquots of Riesling wine were aseptically transferred into previously sterilized 250 mL glass bottles for inoculation with *O. oeni*. *O. oeni* strains were pre-grown in sterile MRS media (BD, Franklin Lakes, NJ) (pH 4.5) until stationary phase, and then centrifuged at 2000 *g* for 10 min to concentrate biomass (~1.0×10^8 CFU/mL). Cells were washed using a sodium hydrogen tartrate buffer containing 7.5 g/L tartaric acid, 495 mg/L $MgSO_4$, 345 mg/L $MnSO_4\cdot5H_2O$ and 0.005% (w/v) Tween 80 adjusted to pH 4.5 with 5 N NaOH. *O. oeni* suspensions thus obtained were then inoculated into each container at a rate of 1% v/v and held at room temperature (20 ± 1 °C) until completion of MLF.

2.3. Sampling

During MLF, samples were taken regularly and immediately frozen (−18 °C) for future analysis. During sampling, food grade nitrogen (Airgas, NY) was used to flush the headspace of fermentation bottles and limit oxygen ingress.

2.4. Analytical methods and statistical analysis

Glucose, fructose and malic acid were measured enzymatically using a commercial test kit (Megazyme, Ireland). Titratable acidity was determined by titration of must with 0.1 M NaOH, using phenolphthalein as an indicator. Total yeast assimilable nitrogen (YAN) was calculated as the sum of primary amino acids and total ammonia. Primary amino acid content was quantified using the NOPA method defined by Dukes and Butzke (1998), while ammonia was quantified using an ion selective electrode (Ammonia ISE electrode, Cole Palmer, Vernon Hills, IL) according to the method of McWilliams and Ough (1974). Alcohol content (%v/v) was quantified by near infrared spectroscopy (Alcolyzer, Anton Paar, Germany).

Table 2
Gradient program for the chromatographic separation of DNPH derivatized SO_2 binding compounds using a Phenomenex Kinetex C18 column (100 × 3.0 mm).

Time (min)	Solvent A	Solvent B
0.00	85	15
4.25	82	18
4.75	75	25
6.50	62	38
12.00	40	60
12.50	10	90
13.25	85	15

2.5. Chemicals and preparation of reagents for SO_2 binder analysis

2,4-Dinitrophenylhydrazine (DNPH) had a purity of 97% (as dry weight) and contained 30–40% water to reduce the explosion hazard. ASTM Class I water was prepared using a water purification system (Arium 611UV, Sartorius, Germany) which was used as a solvent for all reagents and UHPLC solvent A (Table 2).

The derivatizing reagent was prepared by dissolving DNPH in acetonitrile adjusted to pH 1.5 with perchloric acid to obtain an 11 mM DNPH solution. For sample pre-treatments, an aqueous solution of 86 mM EDTA in 1 M sodium hydroxide was prepared and degassed (Aquasonic Model 150D, VWR International, PA) for 20 min. Solutions were stored up to one week at 4 °C.

2.6. Derivatization procedure

Derivatizations were conducted in 2.0 mL glass HPLC vials with Teflon caps (National Scientific, TN). For sulfonate hydrolysis, 200 μL of sodium hydroxide/EDTA solution were added to the vial, followed by 100 μL of sample wine and mixing. After 10 min, 200 μL of 1 M perchloric acid was added followed by 800 μL of the derivatizing reagent. After mixing, the solution was allowed to react for exactly 30 h at 30.0 ± 0.1 °C and then promptly cooled to 4 °C until analysis. Derivatized samples thus prepared were stable for up to five days.

2.7. HPLC Analysis

An ultra high pressure liquid chromatography system (Shimadzu, Japan) consisting of a binary LC-20AD XR pumping unit, a DGU-$20A_3$ degasser, a SIL-20 AC XR autosampler, a CTO-20AC column oven, and a SPD-20A UV/VIS detector were used for separation and analysis of DNPH derivatized wine carbonyls. Data acquisition and analysis were performed with the LCSolution software (1.23). Solvent A consisted of water acidified to pH 2.50 ± 0.01 using perchloric acid. Solvent B was HPLC grade acetonitrile. All solvents were filtered prior to utilization (0.22 μm, nylon, Millipore, Ireland). Samples were held at 4 °C in the autosampler and 5.0 μL of sample injected directly. Separation occurred on a Phenomenex Kinetex C18 stationary phase (100 × 3.0 mm) with 2.6 μm particle size held at 37 °C with a flow rate of 0.75 mL min^{-1}. The analytes were quantified at

Table 1
Analytical profile of Riesling grape must and wine (average of duplicate samples ± SE).

Must parameters				Wine parameters					
Soluble solids (Brix)	pH	Titratable acidity (tartaric acid g/L)	Total YAN (mg/L)	Ethanol (%v/v)	Malic acid (g/L)	Concentration of SO_2 binding compounds before MLF (mg/L)			
						AcHO	Pyr	α-KG	GA
20.6	3.4	7.2	106.2	11 ± 0.1	3.02 ± 0.04	84.6 ± 1.0	11.7 ± 0.5	24.6 ± 0.3	612.4 ± 17.2

AcHO (acetaldehyde), Pyr (pyruvate), α-KG (α-ketoglutaric acid), GA (galacturonic acid).