AN ABSTRACT OF THE THESIS OF

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Reductive sulfate assimilation, the biological process by which sulfur-containing amino acids and key derivatives are synthesized from sulfate, is broadly shared among bacteria, fungi, and plants. It is the major, if not sole source of methionine and cysteine for *Saccharomyces cerevisiae* during wine fermentation. Two obligate intermediates formed in the process, sulfite and hydrogen sulfide, are important in winemaking because both compounds can be excreted during fermentation and influence wine quality. Hydrogen sulfide is highly undesirable if it exceeds threshold concentrations and is not reabsorbed by the yeast or lost by evaporation. Winemakers commonly add and monitor levels of sulfite for use as a mild antioxidant and antimicrobial agent because

most wine yeasts do not excrete more than 10-30 mg/L. However, too much sulfite, whether excreted or added, can inhibit the *Oenococcus oeni*-mediated malolactic fermentation (MLF) that typically follows the alcoholic fermentation in the production of red and some white wines. Deliberate exploitation of the natural ability of yeast to excrete high amounts of sulfite could potentially replace the need for additions made by winemakers for the production of white wines that are neither aged nor undergo the MLF. This is relevant to organic winemaking because unlike the sulfite additions that are disallowed by current USDA regulations, sulfite produced by yeast is permissible. While the causes of sulfite excretion by yeast during fermentation are not well understood, cultural conditions and genotype are key factors. This project investigated the nutritional and genetic basis for excretion of high levels of sulfite during lab-scale Pinot gris fermentations. The nutritional study examined the question of how nitrogen and pantothenic acid availability affected sulfite excretion by two high-sulfite-excreting and two low-sulfite-excreting commercial strains of *Saccharomyces cerevisiae*. While nitrogen supplementation generally stimulated sulfite excretion in the low-sulfite producers, a uniform response by the high-sulfite producers was not observed. The response to the form of added nitrogen--ammonia, alanine or yeast extract--was also non-uniform. The addition of pantothenic acid in the presence of low or high nitrogen levels had no effect on excretion by any strain. The genetic analysis uncovered the basis for sulfite excretion in one of the high-sulfite-excreting wine strains. Mating-competent derivatives of one “high” and one “low” sulfite-excreting strain were crossed to

generate a hybrid. Meiotic progeny of the hybrid were scored for their ability to excrete sulfite. DNA from “high” and “low” sulfite-excreting progeny was isolated, pooled, sequenced, and subjected to bulk segregant analysis. Three genes were identified as responsible for the high-sulfite excretion phenotype: *MET10*, *ADH2*, and *SKP2*. A new allele of *MET10,* encoding sulfite reductase, was identified as the most significant factor. Relative to two low sulfite-producing wine strains, eight single nucleotide polymorphisms were observed, of which five resulted in amino acid changes. Four of these five mutations are not present in *MET10-932*, an allele previously reported to cause a reduction in hydrogen sulfide excretion. *ADH2*, which encodes alcohol dehydrogenase, and *SKP2*, that plays a role in the stability of the enzyme that generates the immediate biosynthetic precursor of sulfite, were also implicated. These findings suggest that the high-sulfite excretion phenotype can be introduced into other wine strains of *S. cerevisiae* by a traditional breeding program.

Nutritional and Genetic Aspects of Sulfite Excretion During Fermentation by Wine Strains of *Saccharomyces cerevisiae*

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

María del Pilar Alessandri, Author

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CONTRIBUTION OF AUTHORS

Dr. James Osborne assisted with election of strains and fermentation conditions for Chapter 2.

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TABLE OF CONTENTS

TABLE OF CONTENTS (CONTINUED)

TABLE OF CONTENTS (CONTINUED)

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1 – Introduction

Although there is evidence for wine making dating over eight thousand years (McGovern, et al. 2017), it was not until the nineteenth century that Louis Pasteur described yeast as responsible for the alcoholic fermentation of grape must, isolating several yeast species from the surface of grapes (Pasteur 1866). Alcoholic fermentation is generally dominated by *Saccharomyces cerevisiae*, but many other organisms, yeast and bacteria, are part of the grape microflora and are present during the wine making process (Ribéreau-Gayon, et al. 2000). Even today, unlike the process of making beer or sake, winemaking does not include any sterilization step; organisms found on the grapes or winery equipment can potentially find their way into the wine and participate. These organisms can have a major impact on the organoleptic properties of the finished wine, as they alter chemical composition and produce volatile compounds that can exceed threshold concentrations (Herraiz, et al. 1990). Early reports of the antimicrobial effect of sulfite date to Pliny the Elder's *Natural History*, written in the first century AD (Healey 1991). From the early twentieth century, reports show that the use of sulfur dioxide as a sanitizer and as an antimicrobial agent could prevent growth of mold and undesirable yeast and bacteria, and ensure a pure fermentation resulting in wines with lower volatile acidity and better storage quality (Bioletti 1911, Cruess 1912). This practice relies on the ability of wine strains of *S. cerevisiae* to tolerate higher concentrations of sulfite than other organisms.

Today, sulfite is added to grape must and wine throughout the vinification process. Sulfite has four main properties beneficial to wine. It is an antiseptic, since it inhibits growth of most microorganisms, it acts as an antioxidant, binding dissolved oxygen, it inhibits the action of certain oxidative enzymes, and it binds acetaldehyde and other carbonyl-containing compounds (Ribéreau-Gayon, et al. 2000). Once it has been added to wine, sulfur dioxide dissolves as sulfurous acid and establishes an equilibrium between protonated and deprotonated forms; the pH of the wine will determine the proportion of each. In addition, bisulfite can bind carbonyl-containing compounds, such as acetaldehyde, to form bound forms of sulfite. In general, only free sulfite in the form of sulfurous acid, known as the molecular form, has significant antimicrobial activity. At wine pH, this species constitutes a minor fraction of the total sulfite present.

Excessive additions of sulfite to wine can be detrimental to wine aroma and flavor, and can interfere with the malolactic fermentation. However, even more concern has arisen from health-related issues. Thorough research has been made on the toxicity of sulfite, both in humans and animals, and signs of acute toxicity associated with consumption of large quantities of sulfite have been observed (Taylor, Higley and Bush 1986). Although there are no secondary effects known to the exposure to lower amounts, like those found in wine, severe allergic reactions to low concentrations are a recognized health hazard for steroid-dependent asthmatics (4%-10% of the asthmatic population in the U. S.) (Stevenson and Simon 1981, Simon 1989). Documented cases of

life-threatening exposures in the 1980s eventually led the Food and Drug Administration (FDA) to require labeling of sulfite if concentrations exceeded 10 mg/L, and spurred a movement among winemakers to reduce or eliminate sulfite additions. Further, the United States Department of Agriculture (USDA) forbids the addition of sulfite at any stage in the making of organic wines. This puts organic winemakers in the United States at a disadvantage because European winemakers are allowed to use sulfites, and thus have better control over the quality of their wines.

Even when no sulfite additions are made, low levels of sulfite are always present in wine, since it is excreted by yeast during the fermentation process. The generation of naturally-occurring sulfites by yeast represents a potential opportunity to reduce or completely replace sulfite additions, if the levels generated are sufficiently high.

Use of sulfite in winemaking

Sulfite, or sulfur dioxide, is a gas under normal conditions, but is tipically added to wine in the form of potassium salts. Once dissolved, it hydrates and acts as a weak acid, with protonated and ionized forms in equilibrium. The effectiveness of its use as an antimicrobial and antioxidant agent depends on its concentration and the proportion of each of the forms present.

Chemistry of sulfur dioxide

Hydrated sulfur dioxide is a diprotic weak acid, with pK_a values of 1.77 and 7.20 (King, et al. 1981). The presence of ethanol and other chemical

species in wine can alter the equilibria (Usseglio-Tomasset and Bosia 1984). The relative concentrations of the various forms of sulfurous acid, H_2SO_3, bisulfite ion, HSO_3^-, and sulfite ion, SO_2^{2-}, are depenedent on pH. At wine pH, usually between 3 and 4, the predominant form is bisulfite. Each of these species behaves differently and has an impact in wine**.** The molecular form, or sulfurous acid, has antimicrobial activity (Rahn and Conn 1944, Macris and Markakis 1974) and is probably the most important from a winemaking point of view, the bisulfite ion can form adducts with carbonyl-containing compounds that are inactive (Burroughs and Sparks 1973) and the sulfite ion has antioxidant activity and consumes dissolved oxygen (Poulton 1970).

One aspect that is of particular interest in winemaking is the formation of sulfite adducts, or hydroxysulfonates, between sulfite and the carbonyl group of various compounds. The most abundant and stable of these adducts in wine is 1-hydroxyethane sulfonate that results from the reaction with acetaldehyde and which has a $K_{dissociation} = 1.5 \times 10^{-6}$ at pH 3 (Burroughs and Sparks 1973). It is worth noting that acetaldehyde is produced by yeast as a normal intermediate during alcoholic fermentation, but can also be produced in higher concentration by spoilage yeast and bacteria (Liu and Pilone 2000). Other adducts commonly found in wine are formed with malvidin-3-glucoside, pyruvate, 2-ketoglutarate, galacturonate and glucose, with $K_{dissociation}$ values ranging from 6.0×10^{-5} to 6.4×10^{-1} (Burroughs and Sparks 1973, Beech, et al. 1979). Sulfite-carbonyl adducts are commonly known as bound forms of sulfite.

The pH of the wine will largely determine how effective any sulfite addition will be since at higher pH the molecular form will be present at very low concentrations, and greater additions will be necessary; 6% of the free sulfur is in the molecular form at pH 3, but at pH 4 it only represents 0.6%. In some cases, a small pH adjustment can have a significant impact on sulfite effectiveness and is preferable over greater sulfite additions. On the other hand, from a practical point of view, winemakers typically monitor the concentration of the free form of sulfite in order to assess the risk for spoilage. Since bound and free forms are in equilibrium can and is regenerated by dissociation of the bound species over time. Eventually, residual free sulfite in wine is oxidized irreversibly to sulfate.

Antimicrobial properties of sulfite

Some studies have shown that sulfite kills microbes while others indicate inhibition of growth or a prolonged lag phase. The antimicrobial effect of sulfite is most effective against gram-negative bacteria, followed by gram-positive bacteria, molds and yeast (Ough 1993). The exact mechanism of toxicity is not clear; there is evidence of depletion of ATP in yeast (Schimz and Holzer 1979) and lactic acid bacteria (Hinze, Maier and Holzer 1981), but it has also been suggested that formation of various sulfur-containing free radicals might inhibit lactic acid bacteria during fermentation (Chang, Kim and Shin 1997). The effect of sulfite as an antimicrobial agent is pH dependent (Müller-Thurgau and Osterwalder 1915) and limited over time, since this activity is progressively lost as sulfite is oxidized irreversibly to sulfate. There have been reports of

temperature and ethanol concentration affecting the antimicrobial effect of sulfite (Britz and Tracey 1990). There is debate on the inhibitory effect of the bound forms of sulfite, particularly towards lactic acid bacteria (Wells and Osborne 2011).

Common practices in winemaking

The most widely-used form of sulfite in wineries is potassium metabisulfite, prepared as a concentrated solution. The amount added depends on the condition of the grapes; early studies recommended additions from 75 mg/L for underripe clean grapes to 270 mg/L for overripe moldy grapes (Amerine and Joslyn 1951).

Sulfite is added at different times during the winemaking process, commonly starting during or immediately after crushing of the grapes. Additions during fermentation are avoided, due to the active production of acetaldehyde rapidly consuming the added sulfite. Throughout the process, levels of free sulfite are monitored, and additions are made to maintain sufficient levels. The last addition is usually made just before bottling.

Sulfur metabolism in yeast

Sulfite produced by yeast during fermentation is a normal intermediate in sulfur metabolism. Sulfur is an essential element for all living organisms, necessary for the formation of sulfur-containing amino acids among other biomolecules. Sulfur can be obtained from organic sources, by the direct

assimilation of methionine and cysteine, or as inorganic sulfur which is incorporated into organic form (Lafaye, et al. 2006).

Reductive sulfate assimilation

In *Saccharomyces cerevisiae*, the main source of sulfur is inorganic sulfate. As shown in Figure 1, sulfate enters the cells via sulfate permeases and is then reduced to synthesize methionine, cysteine and S-adenosylmethionine (AdoMet). The biological reduction of sulfate (SO_4^{2-}), requires energy-dependent activation into adenylate compounds; adenylation of sulfate lowers its electropotential and allows its reduction by NADPH, via thioredoxin. Activation of sulfate is followed by reduction in two successive steps, resulting in sulfite and hydrogen sulfide (H_2S). This last intermediate is then condensed with *O*-acetyl homoserine to form homocysteine, that is converted into other sulfur-containing metabolites (Thomas and Surdin-Kerjan 1997). A number of enzymes and genes have been identified that play significant roles in this pathway, those of particular importance for this project are discussed in depth below.

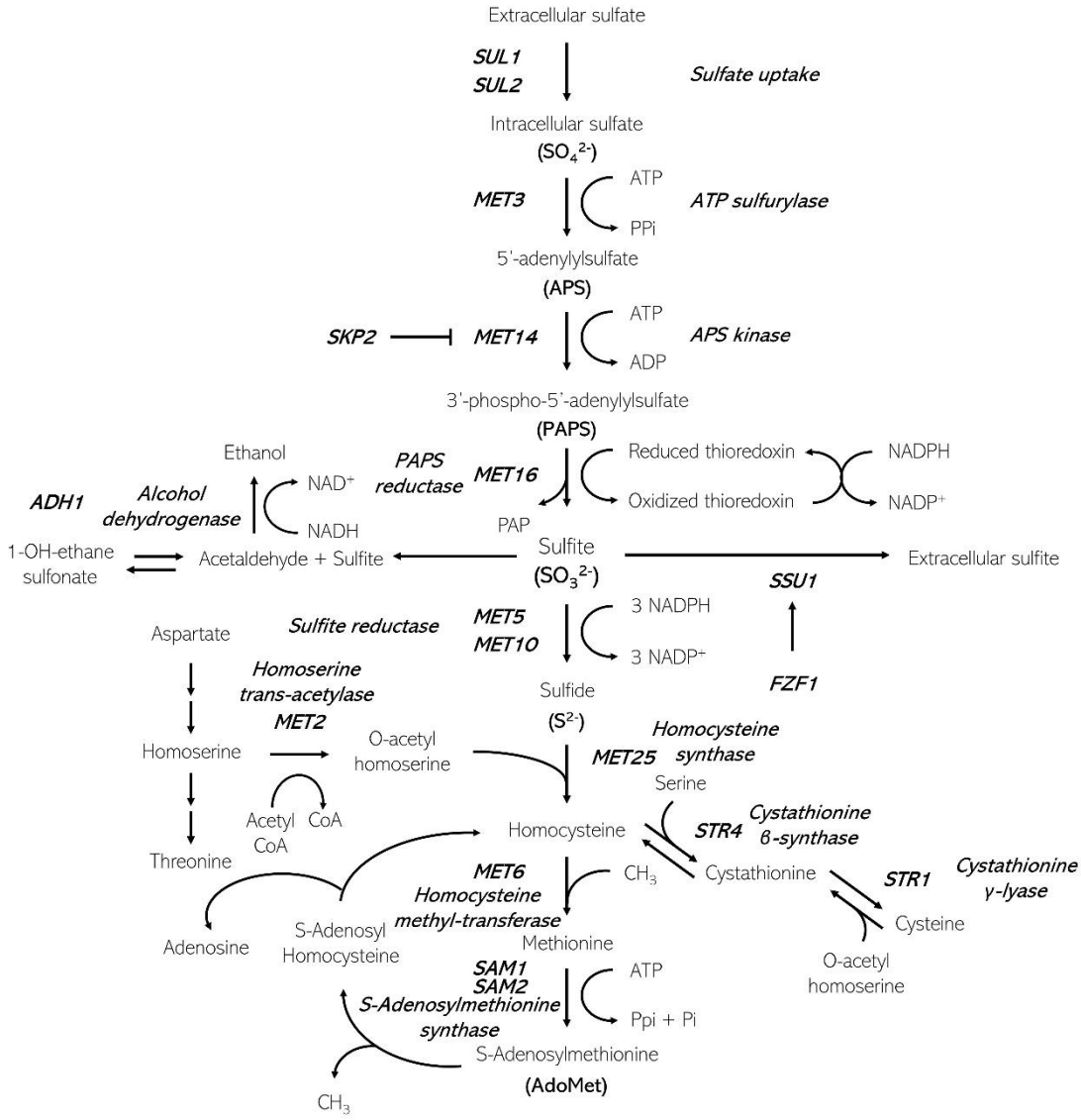

Figure 1: Reductive sulfate assimilation in yeast. Adapted from Thomas and Surdin-Kerjan (1997).

Genetic control of sulfur metabolism

The genes involved in sulfur metabolism in yeast have been described and are shown in Figure 1.

Three genes encode enzymes that participate directly in the formation of sulfite, by activation and reduction of sulfate; *MET3*, *MET14* and *MET16*. When studying gene expression levels in brewer's yeast strains, by mRNA levels, it was observed that *MET3* was weakly expressed in all strains studied, while levels of *MET14* and *MET16* seemed to correlate with higher levels of sulfite being excreted. In addition, the overexpression of these two genes, separately or together, caused an increase in sulfite excretion by yeast. The highest increase was caused by overexpression of *MET14* and *SSU1* (Donalies and Stahl 2002).

MET4 and *MET28* are important transcriptional activators and are responsive to changes in the concentration of S-adenosylmethionine. The disruption of *MET4* abolished transcription of all structural genes in the pathway, while *MET28* only appears to affect some, including *MET3, MET10, MET14* and *MET16* (Kuras, et al. 1996). In addition to higher resistance, it has been observed that overexpression of *SSU1* can lead to an elevated accumulation of sulfite during fermentation (Donalies and Stahl 2002).

MET10, encoding the α subunit of the enzyme sulfite reductase, has been shown to modulate sulfite excretion in a brewer's yeast, where partial or complete elimination of its activity resulted in increased sulfite accumulation

(Hansen and Kielland-Brandt 1996a). This same gene has been studied in wine strains and a specific allele, *MET10-932*, was found to greatly reduce hydrogen sulfide excretion, though its effect on sulfite excretion was not evaluated (Linderholm, Dietzel, et al. 2010). In a different study, strains generated by random mutagenesis from a wine strain showed that certain *MET10* mutants excreted higher levels of sulfite than the parent, and significantly lower levels of hydrogen sulfide. Mutations in *MET5*, encoding the β subunit of sulfite reductase, resulted in similar behavior (Cordente, et al. 2009).

SKP2 encodes an F-box type protein involved in protein degradation via ubiquitination. It is directly related to stability of APS kinase (encoded by *MET14*), which has been observed to be more stable in a *skp2*Δ background (Yoshida, et al. 2011). One study identified a particular allele of *SKP2* that resulted in lower stability of APS kinase and lower sulfite excretion by a wine strain. In the same study, it was observed that a hyperactive *MET2* allele, led to more efficient conversion of homoserine to *O*-acetyl homoserine, which in turn acted synergistically with the mutation in *SKP2* to decrease sulfite and hydrogen sulfide. However, this *MET2* allele on its own did not have a significant effect on sulfite levels (Noble, Sánchez and Blondin 2015).

Sulfite resistance in yeast

In *Saccharomyces cerevisiae*, sulfite causes ATP depletion by inactivating glyceraldehyde-3-phosphate dehydrogenase and alcohol dehydrogenase, in addition to inhibiting oxygen consumption (Maier, Hinze and

Leuschel 1986). The overproduction of carbonyl-containing molecules, such as acetaldehyde, has been proposed as a resistance mechanism (Thomas and Surdin-Kerjan 1997). However, it appears that sulfite efflux through a transporter encoded by *SSU1* explains the high sulfite resistance exhibited by wine yeast strains (Avram and Bakalinsky 1997). *SSU1* encodes a plasma membrane protein involved in export of sulfite and has been implicated in sulfite resistance. Mutations in *SSU1* cause sulfite sensitivity while overexpression results in higher resistance. Increased resistance has also been observed in strains containing particular dominant alleles or overexpression of *FZF1*, a transcriptional activator of *SSU1*. *FZF1* is able to increase sulfite resistance though hyperactivation of *SSU1* (Park and Bakalinsky 2000).

Nutrition: Relationship between nitrogen and sulfur metabolism

Nitrogen content in grape must can have a major impact on how the alcoholic fermentation proceeds, to the extent that nitrogen deficiency is one of the most common causes of sluggish or stuck fermentations (Agenbach 1977, Bisson and Butzke 2000, Henschke and Jiranek 1993, Blateyron, Ortiz-Julien and Sablayrolles 2003). Moreover, insufficient or excessive nitrogen levels have been associated with the production of undesirable volatile compounds, including volatile acidity (Bely, Rinaldi and Dubourdieu 2003), H_2S (Eschenbruch, Bonish and Fisher 1978, Park, Boulton and Noble 2000) and other volatile sulfur compounds (Kinzurik, et al. 2015). Agenbach first defined the minimum nitrogen requirements for ensuring a complete fermentation at

25°C as 130 mg/L for a low-sugar must, or 140 mg/L for a high-sugar must, by measuring the amount of residual nitrogen after complete consumption of sugar. Additional nitrogen simply resulted in increased yeast biomass generated during fermentation (Agenbach 1977). Since then, many studies have tried to answer the same question using different approaches. Great variability due to yeast strain and other fermentation conditions has been observed. A range from 70 to 270 mg/L nitrogen has been defined as the minimum necessary depending on various factors (Reed and Peppler 1973, Bezenger and Navarro 1988, Bely, Sablayrolles and Barre 1990, Cantarelli 1957, Mendes-Ferreira, Mendes-Faia and Leao 2004). Winemakers can choose to supplement with inorganic or organic forms of nitrogen such as ammonia, commonly in the form of a phosphate salt, or complex nutrient supplements derived from yeast, with a high amino acid content.

Yeast assimilable nitrogen (YAN) correspond**s** to the specific forms of nitrogen that can be utilized by yeast during fermentation and include**s** ammonia and primary amines, found in the common amino acids, except proline (Cooper 1982). In grape must, the main sources of YAN are typically arginine and ammonia (Ough and Bell 1980, Ough and Kriel 1985). Yeast nitrogen metabolism is complex. The phenomenon known as nitrogen catabolite repression (NCR) ensures that yeast takes up preferred forms of nitrogen preferentially even when non-preferred forms (e.g., proline) are available (Beltran, et al. 2004). However, under low YAN conditions, all assimilable

nitrogen sources are utilized. When YAN levels are sufficiently high, yeast takes up ammonia first, followed by glutamate, aspartate and glutamine if present (Schure, Riel and Verrips 2000, Magasanik and Kaiser 2002). NCR strongly influences yeast growth and fermentation kinetics and is a direct consequence of the nitrogen forms present in grape must (Deed, Van Vuuren and Gardner 2011). NCR can also have an impact on the organoleptic properties of wine, such as the content of volatile thiols (Thibon, et al. 2008).

The relationship between low YAN, problematic fermentations and sulfur metabolism has been studied mostly in relation to excretion of excessive H_2S. The use of diammonium phosphate (DAP) to prevent hydrogen sulfide formation is a widely used practice. *In vitro* assays have shown that ammonia salts can inhibit sulfite reductase, the enzyme that reduces sulfite into H_2S (Yoshimoto and Sato 1970). More recent studies have suggested that ammonia can activate the expression of *MET10* gene, that codes for this enzyme (Jiménez-Martí and Olmo 2008, Mendes-Ferreira, Barbosa, et al. 2010). It has been proposed that excess H_2S is excreted under low YAN conditions due to exhaustion of *O*-acetylhomoserine (OAH), which normally reacts with H_2S during the formation of homocysteine, allowing its consumption. Limiting levels of OAH would result in accumulation of H_2S, produced by sulfite reductase (Jiranek, Langridge and Henschke 1995, Jiranek, Langridge and Henschke 1996). Studies have suggested that a similar scenario can occur when pantothenic acid is limiting. This vitamin acts as a precursor for coenzyme A (CoA), needed to generate

OAH (Wang, Bohlscheid and Edwards 2003). Pantothenic acid is not a common limiting factor in wine fermentation (Hagen, Keller and Edwards 2008), although its deficiency has been related to problematic growth and undesirable volatile compounds produced by yeast (Wainwright 1970, Shimada, Kuraishi and Aida 1972).

The relationship between nitrogen content in grape must and sulfite excretion during wine fermentation has been noted but, to our knowledge, not extensively studied. Eschenbruch noted that methionine or cysteine can regulate the uptake of sulfate and formation of sulfite (Eschenbruch 1972), and similar observations have been made since (Duan, et al. 2004, Dufour, et al. 1989). Giudici observed that the addition of ammonium salts increased sulfite excretion by two different yeast strains during wine fermentation, but thet methionine could be inhibitory (Giudici and Kunkee 1994). Similarly, brewer's yeast grown in synthetic media has been shown to excrete higher levels of sulfite with ammonium salt additions in the presence or absence of cysteine (Duan, et al. 2004).

The present study was undertaken to determine nutritional and genetic influences on sulfite excretion during wine fermentation. The major motivation for the work was to answer the question of whether commercial strains of wine yeast can excrete a sufficient amount of sulfite to substitute for the additions made by winemakers. This is relevant to the making of certain organic wines where "naturally-produced" sulfite excreted by yeast is allowed, but additions

made by winemakers are not. Clearly, only wines that do not undergo the malolactic fermentation (e.g., Pinot gris) would be likely candidates for use of a wine yeast that excretes high amounts of sulfite because residual sulfite can inhibit the malolactic fermentation that is desirable in red and in some white wines.

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CHAPTER 2: The effect of yeast assimilable nitrogen on sulfite excretion by *Saccharomyces cerevisiae* during wine fermentation

Abstract: Sulfite is commonly used during winemaking as an antimicrobial agent and antioxidant. Apart from the additions made by winemakers, naturally-occurring sulfites are produced by yeast as normal metabolic byproducts. This is relevant to organic winemaking because unlike the sulfite additions disallowed by current USDA regulations, sulfite produced by yeast is permissible. Previous studies have found that production is affected by both environmental and genetic factors. In some cases, production appears to be high enough to potentially replace or significantly reduce additions. However, the sources of variability in sulfite excretion by wine yeast are not sufficiently well understood to allow accurate quantitative predictions. The present study examined the question of how nitrogen availability during laboratory-scale fermentation of Pinot Gris affected sulfite excretion by commercial wine strains of *S. cerevisiae*: two high-sulfite-producing strains, P7Y9 and NT112, and two low producers, CY3079™ and OKAY®. The addition of yeast extract was found to increase production in all strains except NT112. The addition of diammonium phosphate increased sulfite production in OKAY® and CY3079™, but had no effect on the high-sulfite producers P7Y9 or NT112. The addition of alanine increased sulfite produced by CY3079™, decreased sulfite produced by P7Y9 and had no effect on OKAY or NT112. The addition of pantothenic acid, in the presence of low or high nitrogen, had no effect on production by any strain.

Key words: wine, sulfite, sulfur, yeast, nitrogen, *Saccharomyces cerevisiae*, methionine.

Introduction

Sulfites refer to the various chemical forms of sulfurous acid, e.g., SO_2, H_2SO_3, HSO_3^- $K_2S_2O_5$. Their deliberate use in winemaking as antioxidants and antimicrobial agents is ancient. Winemakers typically add the minimum amount necessary which is dependent on the quality of grapes at harvest and subsequent processing through bottling. Sulfite is also a natural product of yeast metabolism, produced as an intermediate during methionine and cysteine biosynthesis. Sulfite is formed from 3'-phospho-5'-adenylylsulfate (PAPS) by PAPS reductase and is converted into hydrogen sulfide by sulfite reductase. Hydrogen sulfide in turn, is condensed with *O*-acetylhomoserine by homocysteine synthase to form homocysteine, a shared precursor of both methionine and cysteine (Thomas, Barbey and Surdin-Kerjan 1990). Because the concentration of sulfur-containing amino acids in grape juice is typically low (Huang and Ough 1991), sulfite formation via internal biosynthesis is required during fermentation. In healthy cells, the rate of sulfite formation is expected to keep pace with the need for methionine and cysteine for which demand would be highest during exponential growth. Excretion of sulfite is likely a response to overproduction relative to consumption which could plausibly result from faulty regulation, cell stress, or both. In winemaking, excretion of 10-20 mg/L sulfite by commercial yeasts is common and non-problematic as sulfite has a relatively high sensory threshold (Blesic, et al. 2014, Amerine and Joslyn 1951). Excretion of higher levels however, can inhibit the subsequent malolactic fermentation (Carreté, et al. 2002). The fact that naturally-occurring sulfites produced by yeast

during fermentation are allowed by U.S. organic regulations creates a potential avenue for deliberate use of this characteristic to reduce the risk of spoilage in making organic wines that neither undergo the malolactic fermentation nor are aged, e.g., Pinot Gris.

The question of what factors control sulfite excretion by *S. cerevisiae* has not been systematically evaluated, but growth conditions and genotype have been found to be major influences (Eschenbruch 1974). In addition to observed natural variation in sulfite excretion among wine strains, deliberate genetic interventions have been introduced that both increase and decrease excreted sulfite (Korch, Mountain and Byström 1991, Hansen and Kielland-Brandt 1996a, Hansen and Kielland-Brandt 1996b, Park and Bakalinsky 2000, Avram and Bakalinsky 1997, Donalies and Stahl 2002, Yoshida, Imoto, et al. 2008, Noble, Sánchez and Blondin 2015). With respect to the influence of growth conditions on sulfite excretion, Eschenbruch (1972) reported that two high-sulfite producing wine yeasts took up about twice as much sulfate as two low sulfite-producers, but that increasing additions of either methionine or cysteine reduced production by all four strains. In contrast, in some brewer's strains, the addition of either methionine or cysteine was found to increase sulfite excretion (Duan, et al. 2004). Giudici and Kunkee (1994) reported that moderate additions of ammonia and non-sulfur-containing amino acids increased sulfite levels in two different wine strains but that both methionine and cysteine reduced extraction. In general, winemakers have taken far greater interest in the relationship between nitrogen availability and both the problem of stuck or sluggish fermentations

(Agenbach 1977, Bisson and Butzke 2000, Henschke and Jiranek 1993, Blateyron, Ortiz-Julien and Sablayrolles 2003), and the production of undesirable volatile compounds including hydrogen sulfide, which has a sensory threshold about 1,000 times lower than that of sulfite (Eschenbruch, Bonish and Fisher 1978, Park, Boulton and Noble 2000, Amerine and Joslyn 1951). Studies have also suggested that undesirable levels of hydrogen sulfide can be excreted when pantothenic acid is limiting. This vitamin is a precursor of acetyl coenzyme A which is a required cofactor for many reactions, but also for the specific reaction that converts hydrogen sulfide into homocysteine (Wang, Bohlscheid and Edwards 2003). Winemakers typically pay close attention to the nutritional status of must and add supplements as needed (various forms of nitrogen, vitamins and other components) to avoid both stuck fermentations and excessive hydrogen sulfide excretion. In the case of organic winemaking, the choice of permitted nutritional additives is limited. The present study evaluated sulfite excretion by two high sulfite- and two low sulfite-producing wine strains of *S. cerevisiae* as a function of a common winemaking practice, addition of various forms of nitrogen.

Materials and Methods

Yeast strains. The strains used are listed in Table 1. The indicated sulfite excretion phenotypes were determined in the present study. High and low sulfite excretors were defined as strains that excreted >40 or <25 mg/L total sulfite, respectively, during fermentation of unsupplemented Pinot Gris as described below in “Small-scale fermentations.” The P7Y9 strain of *S. cerevisiae* contains *MET10-932* (Laurie, Calderon and Agosin 2012), a functional allele of the alpha subunit of sulfite reductase that has been linked to a no hydrogen sulfide excretion phenotype (Linderholm, Dietzel, et al. 2010). Anecdotally, this allele has also been associated with a high-sulfite excretion phenotype (Osborne, J., personal communication, 2015).

Table 1 Strains of *S. cerevisiae*

Strain	**Source**	**Relevant phenotype**
P7Y9[a]	Phyterra Yeast	High sulfite excretor
NT112	Anchor Wine Yeast, through Scott Laboratories	High sulfite excretor
Lalvin CY3079™	Lallemand	Low sulfite excretor
Lalvin ICV OKAY®	Lallemand	Low sulfite excretor
BY4742	ThermoFisher Scientific	Laboratory strain

[a]No longer available commercially.

Media and growth conditions. Strains of *Saccharomyces cerevisiae* were routinely grown in liquid YEPD (10 g/L Bacto yeast extract, 20 g/L Bacto peptone, 20 g/L D-glucose) at 30°C and 200 rpm, or statically at 30°C on YEPD plates (liquid YEPD + 18 g/L agar), unless specified otherwise.

Must. Fermentations were performed using Pinot Gris must (22.8°B, pH 3.22) obtained from grapes that had been harvested from the Oregon State University Woodhall Vineyard in 2015, crushed, pressed, and subsequently held frozen at -20°C. The must was thawed at 4°C overnight and racked to reduce suspended solids, and then distributed into one-liter aliquots which were frozen. During the course of one year, aliquots were thawed at 4°C for individual experiments. After thawing, but 24 hours before fermentation, 0.01% dimethyl dicarbonate (DMDC) was added to sterilize the must which was held at 4°C until inoculation.

Small-scale fermentations. Inocula were prepared by growing yeast cultures for 12-16 h in liquid YEPD after which titers were determined by counting cells under 400X magnification using a hemocytometer. Cells were washed twice by centrifugation with sterile distilled water and then used to inoculate 30 ml fermentations of DMDC-sterilized must to an initial titer of about 10^6 cells/mL in 50 mL sterile polypropylene tubes fitted with fermentation locks. In cases where must was supplemented with yeast extract, diammonium phosphate or pantothenic acid, the additions are indicated. Fermentations were performed in duplicate at 13°C for 15 days. Samples were withdrawn aseptically over the time course to determine sulfite and sugar concentrations. All fermentations were

completed, with residual reducing sugar concentrations ≤0.6%. Sulfite excretion by strain was observed to vary modestly between experiments in unsupplemented must. Variation was close to 10% for P7Y9, OKAY® and NT112 10%, and about 25% for CY3079™. This variation could have been due to differences in settling of residual suspended solids between the frozen aliquots of must that were held sampled over >12 months during which the experiments were performed. For this reason, treatments were only compared with the unsupplemented control performed at the same time using the same aliquot of must.

Reagents. All chemicals used were of reagent grade. Reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless specified otherwise.

Sulfite determination. Total sulfite was measured by the Ripper assay (Ripper, 1898) as described (Illand, 2000). Briefly, to 5 mL fermentation samples, 2 mL of 1 N NaOH were added. After a 10-min incubation at room temperature, 200 µL of 1% soluble starch and 1 mL of 50% H_2SO_4 were added. Samples were then immediately titrated with 1 mM iodine to a blue endpoint.

Yeast assimilable nitrogen. Yeast assimilable nitrogen refers to the forms of nitrogen that are typically found in grape juice and that serve as nitrogen sources: ammonia and primary amino acids. Ammonia was measured using an enzymatic kit (Boehringer Mannheim/r-biopharm UV-method) as described by the manufacturer, except that volumes were reduced to 1/3 of the original. Primary amine nitrogen was measured according to the N-OPA assay, as described (Dukes, 1998), except that volumes were reduced to 1/3 of the

original. Briefly, samples--diluted as necessary--were mixed with the *O*-phthaldialdehyde/N-acetyl-L-cysteine (OPA/NAC) reagent in a boric acid/sodium hydroxide buffer and were incubated for 10 minutes before measuring absorbance of isoindole derivatives at 335 nm. YAN measurements were performed in triplicate.

Sugar and pH. Sugar content in the must, expressed as soluble solids (°B), was measured using a DMA™ 35 Anton Paar Portable Density Meter. Residual sugar after fermentation was determined using Clinitest® as described by the distributor (All World Scientific, Monroe, WA). pH was measured using a Thermo Orion 350 pH Meter.

Statistical analysis. The significance of differences was determined by Student's t-test or ANOVA, followed by Tukey's *post hoc* tests, using Minitab 16 statistical software, $p<0.05$. Data were graphed using Microsoft Office Excel 16.

Results and Discussion

Yeast assimilable nitrogen (YAN) in grape juice includes ammonia and primary amines. The Pinot Gris must used for this study contained less than 140 ppm YAN, below the recommended levels for a healthy white wine fermentation (Agenbach 1977). The must was supplemented with nitrogen in the form of yeast extract, diammonium phosphate (DAP), or alanine to order to distinguish the effects of the different forms and amounts on sulfite excretion by the two high-sulfite and two low-sulfite-excreting wine yeasts (Table 2). Separately, the must was supplemented with a single addition of 400 µg/L pantothenic acid to determine a potential effect on sulfite production independent of YAN because yeast extract contains both pantothenic acid and YAN.

Table 2 YAN content of Pinot gris must with and without added nitrogen.

	Ammonia (N, mg/L)	Primary Amines (N, mg/L)	Total N (mg/L)
Pinot Gris Must	29.1 ± 2.5	109.1 ± 12.9	138.2
Pinot Gris Must + Yeast Extract 2.1 g/L	40.2 ± 4.1	200.6 ± 18.7	240.8
Pinot Gris Must + Yeast Extract 4.2 g/L	39.3 ± 0.9	276.7 ± 16.1	316.0
Pinot Gris Must + Yeast Extract 6.3 g/L	39.7 ± 1.2	363.0 ± 19.7	402.7
Pinot Gris Must + DAP 0.4 g/L	113.0 ± 6.3	104.8 ± 16.3	217.7
Pinot Gris Must + DAP 0.8 g/L	185.6 ± 5.6	121.4 ± 23.9	307.0
Pinot Gris Must + DAP 1.2 g/L	253.9 ± 1.6	116.7 ± 18.1	370.5
Pinot Gris Must + Alanine 0.43 g/L	31.9 ± 2.6	221.6 ± 1.0	253.5
Pinot Gris Must + Alanine 0.86 g/L	30.3 ± 0.5	306.0 ± 11.5	336.3
Pinot Gris Must + Alanine 1.28 g/L	33.2 ± 1.4	404.8 ± 9.7	438.0

Values are means ± standard deviations, n=3. Yeast extract was found to contain 42 ± 3 mg/g primary amine nitrogen and 1.4 ± 0.1 mg/g nitrogen as ammonia.

Winemakers often adjust YAN in must before and/or during alcoholic fermentation using a variety of commercial products including DAP and yeast extracts, the latter being a source of small amount of ammonia and primary amines. In the case of organic winemaking, nutrient additions are restricted to approved organic formulations. In this study, yeast extract served as a proxy for an approved organic nitrogen source.

In order to determine whether nitrogen addition could alter sulfite excretion by the chosen yeast strains, sulfite was assayed every 3 days in small-scale fermentations performed in duplicate. The maximum total sulfite concentrations attained within the first fifteen days of fermentation are presented in Figures 1-3. Addition of YAN in the form of yeast extract resulted in an increase in sulfite excreted by three of the four strains (Figure 1A). The high sulfite producer P7Y9 excreted more sulfite relative to the unsupplemented must at the two highest YAN additions, 316 and 403 mg/L. At the latter addition, an increase of >40% sulfite was observed. In the case of OKAY®, a low sulfite excretor, a significant increase was only observed at the highest YAN addition. Nonetheless, the increase in sulfite was still below 20 mg/L. The high sulfite excretor NT112 appeared to be insensitive to the addition of yeast extract. That is, no significant differences in excretion were observed at any level of added YAN. Interestingly, sulfite excreted by the low sulfite producer CY3079™ doubled in the presence of 316 and 403 mg/L YAN. The behavior of CY3079™ is consistent with previous observations of higher nitrogen levels increasing sulfite excretion by other strains of *S. cerevisiae* (Giudici and Kunkee 1994, Duan, et al. 2004). This

increase could simply be a consequence of more cell growth as added nitrogen stimulates yeast growth (Agenbach, 1977).

The response to similar quantitative additions of YAN in the form of DAP were similar for 3 of the strains, but not for the high sulfite excretor P7Y9 (Figure 1B). In the case of P7Y9, the addition of DAP to yield 307 mg/L YAN led to a reduction in excreted sulfite, whereas addition of more or less YAN had no effect relative to the control. However, the magnitude of the reduction in sulfite was relatively small, about 10%. Just as was observed for the yeast extract additions for the low sulfite excretor OKAY®, only the highest addition of DAP resulted in more sulfite, which still did not exceed 20 mg/L. No change in levels of excreted sulfite were observed for NT112. While an increase in sulfite was observed for the 307 and 371 mg/L YAN levels for CY3079™, the increase was lower, 22-25 mg/L sulfite, than what was observed for similar additions of YAN in the form of yeast extract, where 45-47 mg/L total sulfite was observed.

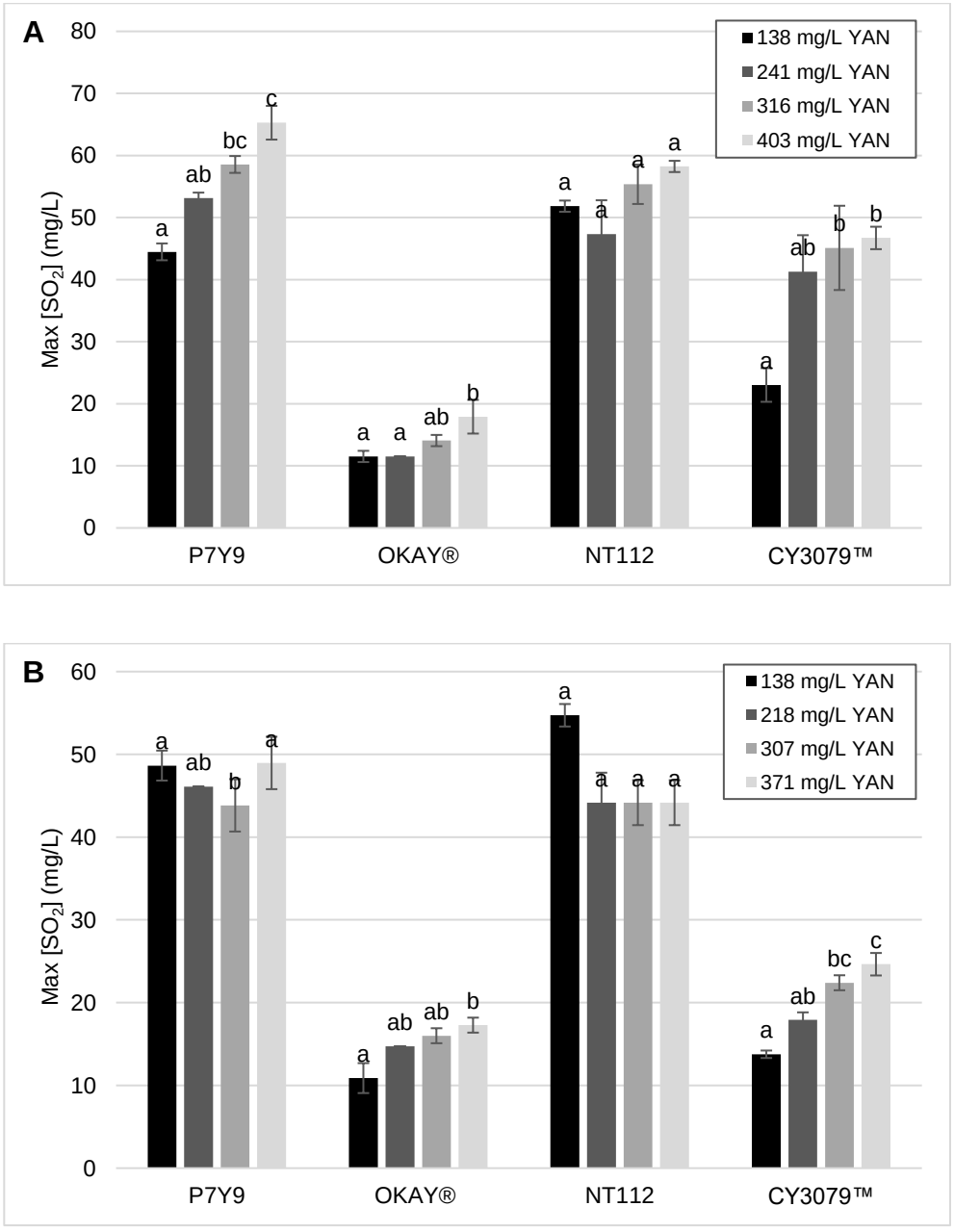

Figure 1. Sulfite excretion by wine yeast in must supplemented with yeast extract or DAP. **A.** Yeast assimilable nitrogen adjusted by supplementing grape must with yeast extract. **B.** Yeast assimilable nitrogen adjested by supplementing grape must with diammonium phosphate. Maximum sulfite reached during fermentation, n=2. Bars that do not share a letter are significantly different according to ANOVA and Tukey's test.

Because yeast extract is a complex nutritional source containing low levels of ammonia, primary amines, vitamins, and other components that could potentially affect sulfite excretion, the must was also supplemented with alanine alone as a source of primary amines only (Figure 2). The high sulfite excretor P7Y9 was found to excrete about 15% less sulfite at the lowest and highest alanine additions, relative to the unsupplemented must. Sulfite excretion by OKAY® and NT112 was not affected by any addition of alanine. In contrast, sulfite excretion by CY3079™ roughly doubled at all alanine additions relative to the unsupplemented must. How does this pattern compare with what was observed in must supplemented with yeast extract? The responses of NT112 (no effect) and of CY3079™ (stimulatory) were about the same. The trend for OKAY® was similar but the slight increase in sulfite excretion observed upon addition of yeast extract was not significant in the case of alanine supplementation. The reduction in sulfite excreted by P7Y9 upon alanine addition contrasted with the increase observed upon addition of yeast extract, suggesting that the stimulatory effect of yeast extract was due to some component or components other than primary amine nitrogen. Because the yeast extract was found to contain only 0.14% (w/w) ammonia (Table 2 footnote), this effect was likely probably due to some other component.

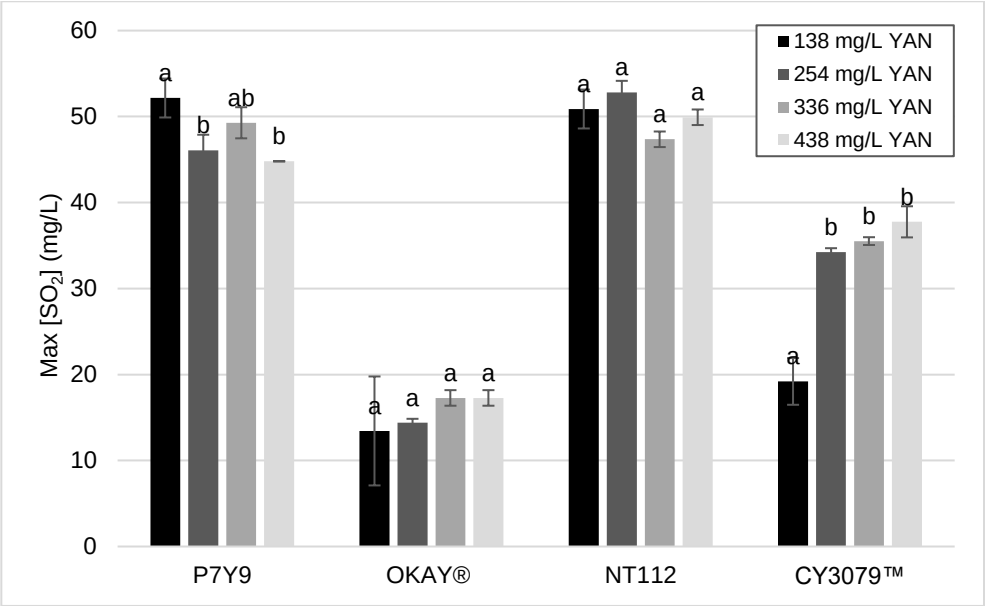

Figure 2. Sulfite excretion by wine yeast in must supplemented with alanine. Maximum sulfite reached during fermentation, n=2. Bars that do not share a letter are significantly different according to ANOVA and Tukey's test.

As noted earlier, it has been suggested that the inhibitory effect of added nitrogen on H_2S production could be due to greater availability of *O*-acetyl homoserine (OAH), allowing for more consumption of H_2S. The availability of OAH is dependent on acetyl-CoA for which pantothenic acid is a precursor. It has been reported that pantothenic acid levels relative to YAN can have an impact on H_2S production during fermentation (Wang, Bohlscheid and Edwards 2003). To test the possibility that pantothenic acid alone affected sulfite excretion and possibly contributed to the effect of added yeast extract, which contains this vitamin, sulfite was also measured in Pinot Gris must supplemented with 400 µg/L pantothenic acid (Figure 3). This addition was not found to alter sulfite excretion by any of the strains. The same pantothenic acid

addition was also tested in the presence of a high level of added nitrogen (438 mg/L total YAN, adjusted with alanine). No significant differences in excreted sulfite were observed (data not shown).

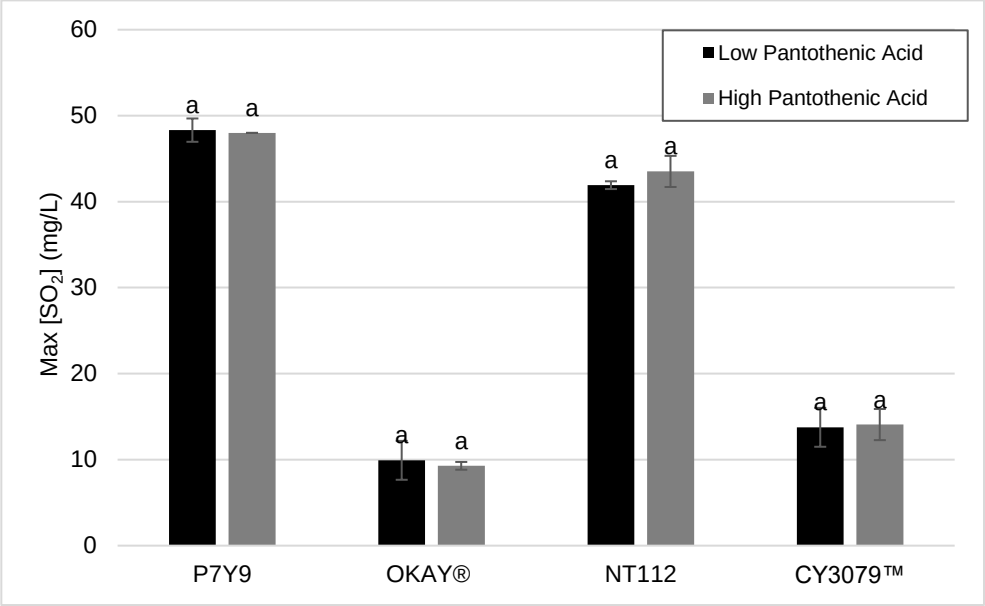

Figure 3. Sulfite excretion by wine yeast in must supplemented with Pantothenic acid. Maximum sulfite reached during fermentation, n=2. Bars that do not share a letter are significantly different according to ANOVA and Tukey's test.

Conclusion

This study found that supplementation of YAN altered sulfite excretion by two high- and two low-sulfite-excreting wine yeasts during fermentation of Pinot gris. Addition of pantothenic acid had no effect. The lack of a uniform stimulatory response by the high excretors suggests that nitrogen supplementation of must will not necessarily serve as a general tool for increasing levels of "natural" sulfite that could have value in the making of certain organic wines in the U.S.

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CHAPTER 3: Genetic basis for sulfite excretion during fermentation by a wine strain of *Saccharomyces cerevisiae*

Abstract: Reductive sulfate assimilation, the biological process by which sulfur-containing amino acids and key derivatives are synthesized, is broadly shared among bacteria, fungi, and plants. It is the major, if not sole source of methionine and cysteine for *Saccharomyces cerevisiae* during wine fermentation. Two obligate intermediates formed in the process, sulfite and hydrogen sulfide, are important in winemaking because both compounds can be excreted during fermentation and influence wine quality. Winemakers commonly add and monitor levels of sulfite for use as a mild antioxidant and antimicrobial agent because most wine yeasts do not excrete more than 10-30 mg/L. Deliberate exploitation of the natural ability of yeast to excrete high amounts of sulfite could potentially replace the need for additions made by winemakers. While the factors that control sulfite excretion in yeast during fermentation are not well understood, genotype and cultural conditions are key parameters. This study investigated the genetic basis for excretion of high levels of sulfite during lab-scale wine fermentation. Mating-competent progeny of “high” and “low” sulfite-excreting wine strains were crossed to generate a hybrid. Over 200 random spore progeny were scored for their ability to excrete sulfite. DNA from “high” and “low” sulfite-excreting spores was isolated, pooled, sequenced, and subjected to bulk segregant analysis (BSA). A new allele of *MET10* was identified as a highly significant factor in the high sulfite excretion phenotype.

Relative to two low sulfite-producing wine strains, eight single nucleotide polymorphisms were observed, of which five resulted in amino acid changes. Four of these five mutations are not present in *MET10-932*, an allele previously reported to cause a reduction in hydrogen sulfide excretion. Alleles of *SKP2* and *ADH2* were also found to play contributing roles.

Key words: wine, sulfite, sulfur, yeast, *MET10*, sulfite reductase, *SKP2*, *ADH2*, *Saccharomyces cerevisiae*, BSA.

Introduction

Sulfite is the most widely added antioxidant and antimicrobial agent used in winemaking. However, deliberate addition by winemakers is not the only source because sulfite is also a normal product of yeast metabolism, produced as an intermediate in the reductive sulfate assimilation pathway that generates methionine, cysteine, S-adenosyl methionine, glutathione and other sulfur metabolites (Thomas and Surdin-Kerjan 1997). Because grape juice contains low levels of sulfur-containing amino acids (Huang and Ough 1991, Huang and Ough 1989, Spayd, et al. 1994, Sponholz 1991, Hernandez-Orte, Guitart and Cacho 1999), operation of the reductive assimilation pathway is essential during fermentation. For reasons that are not well understood, wine yeasts also excrete variable amounts of sulfite, typically less than 30 mg/L. The excreted amount presumably represents some fraction of excess production relative to need and may reflect impaired regulation of the pathway, but also increased stress (e.g., proton influx) induced directly and indirectly by increasing ethanol concentration during fermentation. High levels of excreted sulfite have been reported among a limited number of wine strains of *S. cerevisiae* (Weeks 1969, Rankine and Pocock 1969, Patrignani, et al. 2016). This has practical implications because current USDA regulations do not allow sulfite additions in organic wine (United States Department of Agriculture 2015) in contrast to the regulations that govern organic wine production in the EU. Of key importance, sulfite produced naturally by yeast during fermentation is permitted by the USDA regulations. Deliberate

exploitation of the natural ability of yeast to excrete high amounts of sulfite could potentially replace additions for the production of organic white wines that are neither aged nor undergo the MLF.

The question of what factors control sulfite excretion in S. cerevisiae has not been systematically evaluated, but genotype and growth conditions have been found to be major influences (Eschenbruch 1974, Yoshida, Imoto, et al. 2011, Wells and Osborne 2011). With respect to genetic control, inactivation of either *MET2*, encoding homoserine O-acetyl transferase (Hansen and Kielland-Brandt 1996b) or *MET10* (Hansen and Kielland-Brandt 1996b) encoding sulfite reductase (Hansen, Cherest and Kielland-Brandt 1994) have been found to increase sulfite production in brewer's yeast. *MET10-932*, a functional allele of sulfite reductase isolated from a wine strain, was found to cause a significant reduction in H_2S excretion, although its effect on sulfite was not evaluated (Linderholm, Dietzel, et al. 2010). Variants of *MET10* generated by random mutagenesis of a wine strain resulted in higher excretion of sulfite and lower levels of H_2S. Similarly, mutations in *MET5* that encodes the beta subunit of sulfite reductase also resulted in higher levels of sulfite excretion (Cordente, et al. 2009). Simultaneous overexpression of *MET14* encoding 5'-adenylylsulfate (Korch et al., 1991) and *SSU1*, encoding a sulfite efflux pump (Avram and Bakalinsky, 1997; Park and Bakalinsky, 2000) was found to greatly increase sulfite excretion in a laboratory strain (Donalies and Stahl, 2002). Yoshida et al., (2008) reported that sulfite excretion could be increased without a concomitant increase in hydrogen sulfide by lager yeast by increasing flux from aspartate to

O-acetyl homoserine and from sulfate to sulfite. Overexpression of *MET16* that encodes PAPS reductase which forms sulfite has also been shown to increase sulfite excretion (Donalies and Stahl 2002). Overexpression of *FZF1*, a transcriptional activator of *SSU1* has also been shown to cause a similar phenotype (Park and Bakalinsky 2000). *SKP2* encodes an F-box type protein involved in mediating the stability of APS kinase, encoded by *MET14* (Yoshida, et al. 2011). A particular allele of *SKP2*, associated with increased stability of APS kinase was found to increase levels of excreted sulfite. This effect was even greater in the presence of a hyperactive allele of *MET2* which catalyzes formation of *O*-acetyl homoserine (Noble, Sánchez and Blondin 2015). Inactivation of *MET2* in brewer's yeast was also found to increase sulfite excretion (Hansen and Kielland-Brandt 1996b).

The present study undertook a genetic analysis of sulfite excretion by a wine yeast during fermentation based on scoring this phenotype among the progeny of a hybrid generated by crossing "high" and "low" sulfite-excreting wine strain parents. Subsequent bulk segregant analysis (BSA) coupled with DNA sequencing identified an allele of *MET10*, encoding the alpha subunit of sulfite reductase as a major contributor to high levels of sulfite excretion. Specific alleles of *ADH2*, encoding alcohol dehydrogenase, and *SKP2*, were also identified as significant factors in the excretion phenotype.

Materials and Methods

Yeast strains. The strains used in this study are listed in Table 1. Relevant genotypic and phenotypic features are indicated. The indicated sulfite production phenotypes were determined in the present study. High and low sulfite producers were defined as strains that produced >40 or <25 mg/L total sulfite, respectively, during fermentation of unsupplemented Chardonnay as described below in the "Small-scale fermentations."

Table 1 Strains of *S. cerevisiae*

Strain	Genotype	Phenotype	Source
BY4742 hoΔ::KanMX	$MAT\alpha\ his3\Delta1\ leu2\Delta0\ lys2\Delta0\ ura3\Delta0\ ho\Delta::KanMX$	Laboratory strain, low sulfite producer	ThermoFisher Scientific
P7Y9[a]	$\frac{MAT\alpha\ \ HO\ \ MET10-932}{MAT\alpha\ \ HO\ \ MET10-932}$	High sulfite producer	Phyterra Yeast
NT112[b]	$\frac{MAT\alpha\ \ ho}{MAT\alpha\ \ ho}$	High sulfite producer	Anchor Wine Yeast, through Scott Laboratories
NT112-SP1	$MATa\ ho$	High sulfite producer	This study
Lalvin ICV OKAY® [c]	$\frac{MAT\alpha\ \ HO}{\frac{MAT\alpha}{MATa}\ \ HO}$	Low sulfite producer	Lallemand
OKAY-SP2 *hoΔ*[d]	$MAT\alpha\ ho\Delta::KanMX$	Low sulfite producer	This study
NBH[e]	$\frac{MAT\alpha\ \ ho\Delta::KanMX}{MATa\ \ \ \ \ ho}$	Low sulfite producer	This study
NOH[f]	$\frac{MAT\alpha\ \ ho\Delta::KanMX}{MAT\alpha\ \ \ \ \ ho}$	Intermediate sulfite producer	This study

[a] Ploidy uncertain based on sequence data analysis.
[b] Heterothallic diploid.
[c] Presumptive diploid, trisomic for chromosome III based on DNA sequence and segregation data.
[d] Could also be disomic for chromosome III and homozygous at *MAT*: *MATα/MATα*.
[e] Hybrid obtained by crossing BY4742 *hoΔ::KanMX* with NT112-SP1.
[f] Hybrid obtained by crossing OKAY-SP2 *hoΔ* with NT112-SP1. The hybrid could be trisomic for chromosome III and heterozygous at *MAT*: *MATa/MATα/MATα*.

Media and growth conditions. Strains of *Saccharomyces cerevisiae* were grown in liquid YEPD (10 g/L Bacto yeast extract, 20 g/L Bacto peptone, 20 g/L D-glucose) at 30°C and 200 rpm, or were grown statically at 30°C on YEPD plates (liquid YEPD + 18 g/L agar), unless specified otherwise. Selective media included YEPD supplemented with 200 µg/mL G418 (Sigma) and/or 300 µg/mL Hygromycin B (Sigma). Strains were sporulated on potassium acetate plates (10 g/L potassium acetate and 18 g/L agar). Spores were germinated on petite plates (20 g/L Bacto peptone, 10 g/L Bacto yeast extract, 0.25 g/L D-glucose, 30 g/L glycerol and 18 g/L agar). Selection of prototrophic strains was done on M plates (6.7 g/L bacto yeast nitrogen base without amino acids, 20 g/L D-glucose and 18 g/L agar). Fermentations were performed using Chardonnay must, 23.6°B, pH 3.57. The must was obtained from grapes that had been harvested from the Oregon State University Woodhall Vineyard in 2016, crushed and subsequently held frozen at -20°C. The must was thawed at 4°C overnight and racked to remove suspended solids. After thawing and 24 hours before fermentation, the sterilant dimethyl dicarbonate (DMDC) was added to a final concentration of 0.01%. The DMDC-treated must was supplemented with a sterile solution of yeast extract (5 g/L) and held at 4°C until inoculation.

Reagents. All chemicals used were reagent grade. Reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless specified otherwise.

Sulfite assay. Free and total sulfite was measured by the Ripper assay (Ripper, 1892) as described (Illand 2000). Briefly, to measure free sulfite, 5 mL

fermentation samples were taken and 200 µL of 1% soluble starch and 1 mL of 50% H_2SO_4 were added. Samples were immediately titrated with 1 mM iodine to a blue endpoint. To measure total sulfite, 2 mL of 1 N NaOH were added to the sample which was held for 10 min at room temperature before the addition of starch and H_2SO_4. A micro-version of the Ripper assay was also performed on samples taken from fermentations carried out in 96-deep-well microtiter plates (See “Small-scale and micro-fermentations” below). Total sulfite in micro-fermentations was measured after 5-7 days by transferring 100 µL aliquots from each well to a new standard 96-well plate. To each well, 40 µL of 1 N NaOH were added and the plate was held at room temperature for 10 minutes. Then, 4 µL of 1% soluble starch and 20 µL of 50% H_2SO_4 were added, and samples were immediately titrated by successive addition of 5 µL aliquots of 1 mM iodine until a blue color change occurred.

Crosses. After 12-16 h of growth on YEPD plates at 30°C, cells of opposite mating type were mixed and incubated on the same plates for about 24 h. Zygote formation was monitored microscopically during the first 5-7 h after mixing. The hybrid formed by crossing NT112-SP1 to BY4742 hoΔ::KanMX was selected on M + G418. The hybrid formed by crossing NT112-SP1 to OKAY-SP2 ho was identified by streaking on a YEPD plate 24 h post-mating. The hybrid was presumed to form large colonies relative to colonies formed by the unmated parents in the mating mixture. A putative hybrid was confirmed by assessing sporulation proficiency and observing segregation for sulfite excretion by random spore progeny.

Sporulation. Strains were grown on YEPD plates at 30°C for 12-16 h and then transferred to KAc plates, which were incubated at room temperature for 3-12 days. Spore formation was monitored by microscopic inspection.

Generation of random spores. Random spores were generated by ether treatment (Dawes and Hardie 1974) as described (Bakalinsky and Snow 1990). Briefly, sporulated cells from a KAc plate were suspended in 500 µL sterile water. An aliquot of 500 µL of ethyl ether was added and the mixture was incubated for 20-30 minutes at room temperature and 200 rpm. The lower spore-containing aqueous phase (400 µL) was then collected and centrifuged for 2 min. The supernatant was removed, and spores were washed twice by centrifugation in 1 mL aliquots of sterile water. The washed spore pellet was suspended in 1 mL sterile water and held at 4°C. A number of dilutions were plated on petite plates which were incubated at 30°C for 48-96 h to yield <100 colonies/plate. To avoid inadvertent bias, all the colonies on a plate were patched into individual wells of 96-well microtiter plates containing 200 µL YEPD/well to constitute a population of random spores. Microtiter plates were incubated statically at 30°C for 24 hrs.

Genetic Analysis. Segregation of phenotypes among random spore progeny of hybrids was assessed as follows. Drug resistance and auxotrophies were analyzed by replica-plating to selective plates and scoring for growth after 24-48 h at 30°C. Sulfite excretion during fermentation was assessed by the micro-version of the Ripper method (Illand 2000) as described above (“Sulfite assay”).

The significance of segregation frequencies was assessed by the χ^2 test. Both the parents and hybrids were included as controls on all plates used to score phenotypes.

Small-scale and micro-scale fermentations. Inocula were prepared by growing cultures for 12-16 h at 30°C in YEPD after which cell titers were determined using a hemocytometer. Cells were washed twice by centrifugation with sterile water and fermentations of DMDC-sterilized Chardonnay supplemented with 5 g/L yeast extract, (15 or 30 or 100 mL) were then inoculated at a titer of about 10^6 cells/mL. The fermentations were performed at 14±1°C for 5-15 days. Samples were withdrawn aseptically for sulfite and sugar analyses. Micro-scale fermentations were performed at 14±1°C for 5-7 days in 96-deep-well microtiter plates (Falcon®, Corning) containing 500 µL/well of Chardonnay must supplemented with 5 g/L yeast extract. The micro-fermentations were inoculated with segregants pre-grown in YEPD for 16-24 h using a sterile 48-pin replicator. Plates were covered with sealing tape (Thermo Scientific) to minimize evaporation.

Sugar measurement. Sugar content in grape must was measured as soluble solids using a refractometer (Bellingham + Stanley Inc. RFM 81 Multiscale Automatic Refractometer). Sugar consumption during fermentation was measured as soluble solids with a portable density meter (DMA™ 35 Anton Paar).

Transformations. *S. cerevisiae* strains were transformed using the LiAc/SS-DNA/PEG method as described (Gietz and Woods 2002). Cultures in 1 mL 2X YEPD were grown overnight at 30°C and 200 rpm. After 12 h, cell titers were determined with a hemocytometer and 20 mL 2X YEPD cultures were inoculated at 5 x 10^6 cells/mL and incubated at 30°C and 200 rpm. After 4-5 h, when the cell titer reached at least 2 x 10^7 cells/mL, cells were harvested by centrifugation (5 minutes at about 1,000 x *g*) and washed twice with 25 mL sterile distilled water. Cells were resuspended in 1 mL sterile distilled water from which 100 µL aliquots were removed for each transformation. The 100 µL aliquots were centrifuged and the supernatants were removed. The pelleted cells were resuspended by vortexing after sequential addition of 240 µL of 50% (w/v) PEG 3500, 36 µL of 1 M lithium acetate, 50 µL of 2 mg/mL boiled SS-DNA (sodium salt of DNA from salmon testes, Sigma) and 100-500 ng of plasmid or genomic DNA. The transformation mixtures were then incubated at 42°C for 40 min. Cells were centrifuged for 30 seconds at 22,000 x *g*, resuspended in 1 mL YEPD and plated on selective plates that were incubated statically at 30°C for 48-96 h. DNA insertions or replacements in yeast transformants were confirmed by PCR using Promega GoTaq® Green Master Mix according to the manufacturer's instructions.

DNA extraction. Genomic DNA was extracted from yeast as described (Schwartz, 2016) Briefly, yeast strains were grown in 20 mL YEPD overnight at 30°C and 200 rpm. Cells were harvested by centrifugation at 1,000 x *g* for 5 minutes and washed with 10 mL sorbitol solution (0.9 M sorbitol, 0.1 M EDTA

disodium salt, 0.1 M Tris-HCl, pH 7.5). Cells were resuspended in 5 mL sorbitol solution containing 2.5 uL Zymolyase-100T (ZymoResearch) and 5 µL of β-mercaptoethanol (Sigma) and were incubated at 37°C for 30 min. Spheroplasts were centrifuged at 500 x *g* for 5 minutes and resuspended in 5 mL Tris/EDTA solution (20 mM EDTA disodium salt, 50 mM Tris-HCl pH 7.5), 500 µL of 10% SDS and 2 mL of 5 M potassium acetate before incubating on ice for 30 minutes. Samples were then centrifuged at 1,000 x *g* for 10 min to remove the pellet. To the supernatant, 15 mL of 100% ethanol were added before incubating at room temperature for 10 min. Samples were centrifugated at 1,000 x *g* for 10 minutes to allow removal of the supernatant. The DNA pellet was air dried before resuspension in 500 µL of TE (10 mM EDTA, 100 mM Tris-HCl, pH 8.0). Chloroform (500 µL) and 500 µL of phenol were added and the mixture was vortexed before centrifugation at 22,000 x *g* for 5 minutes. The upper phase (400 µL) was recovered to which 40 µL of 3 M sodium acetate and 1 mL of 100% ethanol were added. The solution was then held at -70°C for 1 h. Samples were centrifuged at 22,000 x *g* for 30 minutes, the pellet was washed once with 75% ethanol and dried, after which the DNA was dissolved in water.

PCR. DNA amplicons for yeast transformation were obtained by PCR using a DNA polymerase according to the manufacturer's instructions (New England Biolabs® Inc. Phusion® High-Fidelity DNA polymerase). DNA primers are listed in Table 2.

Table 2 DNA primers

Name	Sequence (5'→3')	Use
HO-Kan Fw	CTAAACGCACTATTCATCATTA	Generate a 1613 bp amplicon. *KanMX* flanked by *HO* upstream and downstream regions.
HO-Kan Rv	GTATTTCTACTCCAGCATTCTAG	

DNA sequencing. After extraction from yeast, DNA was quantified using a NanoDrop ND-1000 UV-Vis spectrophotometer (Thermo Fisher Scientific). Separate pools of DNA were created from "high sulfite- and from "low-sulfite-" excreting segregants such that each segregant contributed an equal amount of DNA to its respective pool. DNA libraries were prepared using an Illumina® Nextera™ XT DNA Library Preparation kit, according to the manufacturer's instructions. High-throughput sequencing (Illumina® MiSeq) was performed with single-end 150-bp length reads. Both library preparation and DNA sequencing were done at the Oregon State University Center for Genome Research and Biocomputing (http://cgrb.oregonstate.edu/core).

Bioinformatic and statistical analysis. Sequencing data were aligned to the *Saccharomyces cerevisiae* reference genome using BWA (Li and Durbin 2009). Single nucleotide polymorphisms (SNPs) were identified with the GATK tool HaplotypeCaller (Poplin, et al. 2018). Allele frequencies for each pool were calculated for biallelic SNPs and LOD scores were generated using Multipool (Edwards and Gifford 2012). LOD threshold for statistical significance was set at 3, corresponding to $\alpha = 0.001$. All graphs were generated using R 3.4.4 (R

Core Team 2018). Bioinformatic and statistical analyses were performed at the Oregon State University CGRB.

Results and Discussion

Approach and choice of yeast strains

We chose to couple bulk segregant analysis (BSA) (Michelmore, Paran and Kesseli 1991) with high-throughput genomic sequencing to determine the genetic basis for the high sulfite excretion phenotype. This involved mating high- and low-sulfite-excreting strains of *S. cerevisiae*, isolating and pooling DNA exclusively from progeny that exhibited the two parental phenotypes, generating single nucleotide polymorphism (SNP) maps between the two pools, and identifying SNPs with significant linkage to the pool derived from high-sulfite-excreting progeny. Initially, it was essential to identify true-breeding (homozygous) high-sulfite-excreting parental strains. To this end, putative high excretors were obtained from commercial sources and screened in lab-scale fermentations. Strains that excreted a low amount of sulfite were also screened. Two wine strains were identified as high excretors: NT112 (67 ± 4 mg/L), and P7Y9 (59 ± 4 mg/L), and two strains were identified as low excretors: the laboratory strain BY4742 *ho*Δ*::KanMX* (7 ± 0.7 mg/L), and the wine strain OKAY® (12 ± 1 mg/L).

The high-sulfite-excreting heterothallic diploid NT112 was chosen for the analysis. NT112 was sporulated and haploid progeny were scored for sulfite excretion. A high-excreting *MATa* haploid derived from NT-112 was designated NT112-SP1 and was subsequently hybridized with appropriate low-excreting parents. Two low-excreting parents were used. The laboratory strain BY4742 *ho*Δ*::KanMX*, and OKAY-SP2 *ho*Δ, a derivative of the homothallic wine strain

OKAY® which was specifically bred to produce low levels of both sulfite and hydrogen sulfide (Berlese-Noble, et al. 2014). OKAY-SP2 *ho*Δ was derived from OKAY® in the following manner. OKAY® was sporulated, random spores were generated, low-excreting spore progeny were identified, and resporulated. A low-excreting spore segregant following the second round of sporulation was selected and transformed with a PCR-generated DNA construct to disrupt *HO*, *ho*Δ*::KanMX*. An *HO* disruptant was selected on a YEPD + G418 plate and subsequently sporulated. As expected, resistance to G418 was found to segregate 1+:1- among random spore progeny (data not shown) and stable haploids of both mating types were obtained. One was designated OKAY-SP2 *ho*Δ, which was confirmed to be of the alpha mating type, to harbor the KanMX construct at the *HO* locus, and to be a low sulfite excretor. The high excretor NT112-SP1 was mated separately with BY4742 *ho*Δ*::KanMX* and with OKAY-SP2 *ho*Δ to generate “high” x “low” hybrids.

Hybrid from wine and laboratory strain cross is not informative

The hybrid NBH generated by crossing NT112-SP1 with BY4742 *ho*Δ*::KanMX* was sporulated and a total of 144 random spore progeny together with the parents and the hybrid were scored for sulfite excretion after a 7-day micro-fermentation in 96-deep-well microtiter plates. While the parents exhibited their respective high and low sulfite excretion phenotypes, the hybrid was found to be a low sulfite-excretor, 16 mg/L. Over 50% of the progeny were also found to be low excretors. Unexpectedly, high sulfite-excretors were virtually absent. By visual inspection of cell turbidity in the deep-well microtiter plates, it was

noted that progeny grew unevenly as though some failed to complete the fermentation. We speculated that the slow growth interfered with sulfite excretion and that the laboratory parent BY4742 likely introduced alleles that caused slow growth in grape juice containing about 24% (w/w) fermentable sugar. If true, this would prevent progeny that inherited alleles linked to high sulfite excretion from being scored as high excretors simply because they failed to grow rapidly enough. To test this possibility, the ability of BY4742 *ho*Δ*::KanMX* to complete a grape juice fermentation was assessed in parallel with NT-112 and OKAY®. Figure 1 shows sugar consumption (drop in soluble solids as measured in °Brix) in Chardonnay must supplemented with 5 g/L yeast extract as a function of time. BY4742 was found to grow significantly more slowly than the wine strains, consistent with slow growth interfering with the sulfite excretion phenotype among the progeny of NBH.

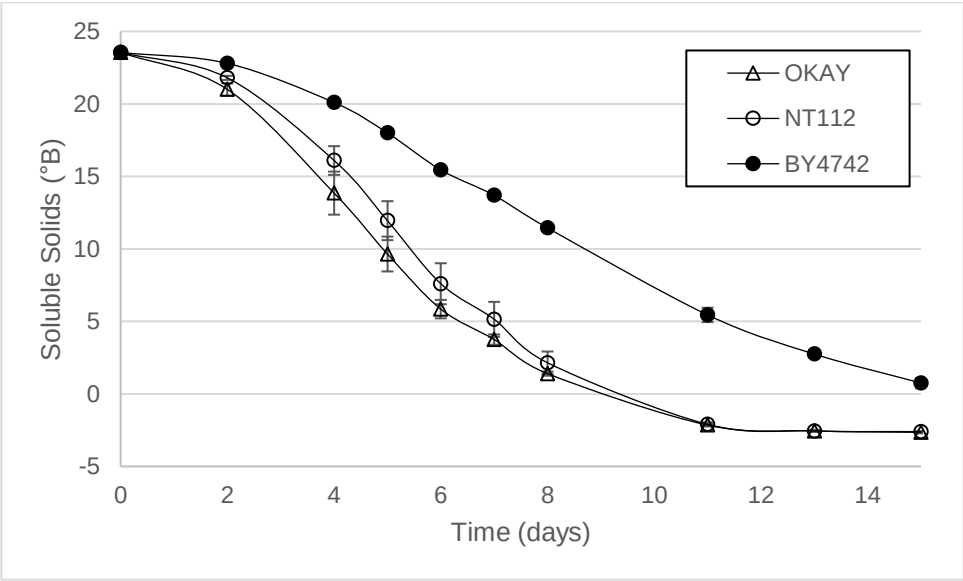


Figure 1: Reducing sugar, measured as soluble solids, by OKAY®, NT112, and BY4742.

Progeny from high x low sulfite-excreting wine strain hybrid is informative

To avoid growth-related interference with the genetic assessment of sulfite excretion, the hybrid NOH generated by crossing NT112-SP1 with OKAY-SP2 *ho*Δ was chosen for analysis. NOH was sporulated and 217 random spore segregants were scored for sulfite excretion. Segregants were grouped into classes based on levels of excretion (Figure 2). Designating upper and lower limits was somewhat arbitrary because sulfite excretion is a quantitative trait. The parents were scored in parallel. As expected, NT112-SP1 was found to excrete ≥60 mg/L sulfite and OKAY-SP2 *ho*Δ excreted ≤20 mg/L. The hybrid itself was found to excrete 42 mg/L sulfite. Among progeny, both parental classes were observed, <20 and >60 mg/L sulfite, as well as intermediate phenotypes. Although six classes are shown, one could also group progeny into four classes: <30, >30 but <40, >40 but <50, and >50 mg/L. The expected segregation pattern for 2 or 3 major genes controlling sulfite excretion during fermentation would generate 4 or 8 genotypic classes, respectively. The observed segregation suggests that at least two genes modulate excretion and that the recombinant genotypes likely correspond to the intermediate classes.

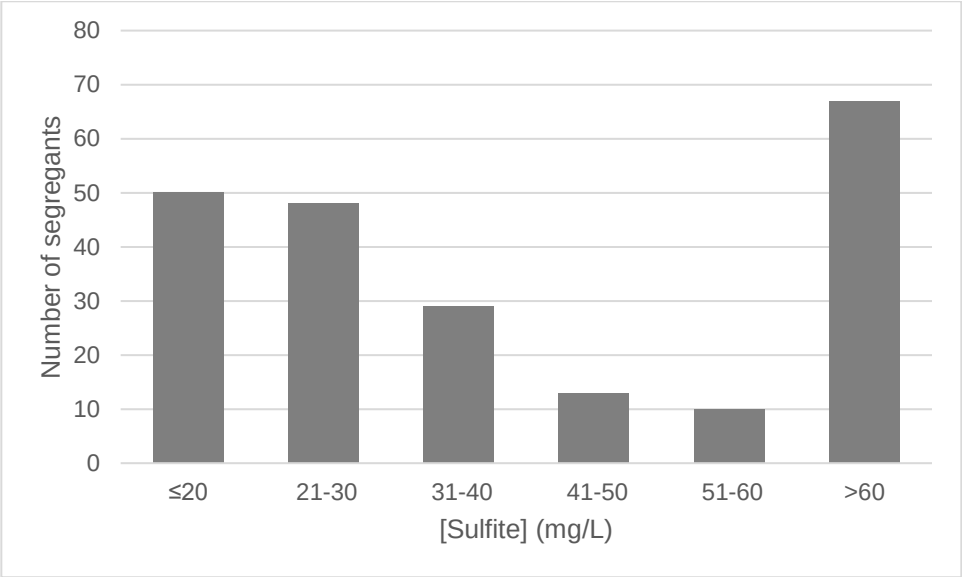


Figure 2: Segregation of sulfite excretion among random spore progeny obtained from a cross between derivatives of NT112 and OKAY®. Screening of 217 segregants after fermentation of Chardonnay must + 5 g/L yeast extract at 14±1 °C for 7 days.

BSA coupled with high throughput genomic sequencing

In order to map regions of the genome linked to the high excretion phenotype, DNA was isolated individually from 29 of the lowest sulfite excretors and from 12 of the highest sulfite excretors. “High” and “low” pools were generated by mixing equal amounts of DNA from each of the 29 or 12 segregants, to constitute the “high” and “low” pools, respectively. The two pools of DNA were sequenced and subjected to quantitative trait locus (QTL) analysis based on allele frequency for each SNP, relative to the yeast reference genome (www.yeastgenome.org). LOD scores were calculated to assess the significance of potential linkage (Figure 3). The most significant linkage was found on chromosome VI, which had a LOD score of 4.2, corresponding to $p<0.001$. Three more loci, on chromosomes VII, XIII and XIV, had LOD drops

>2.5, suggesting possible involvement with the high sulfite-excretion phenotype as well. A LOD drop refers to the difference between the LOD score associated with the locus and the baseline region which in this study = -1.2.

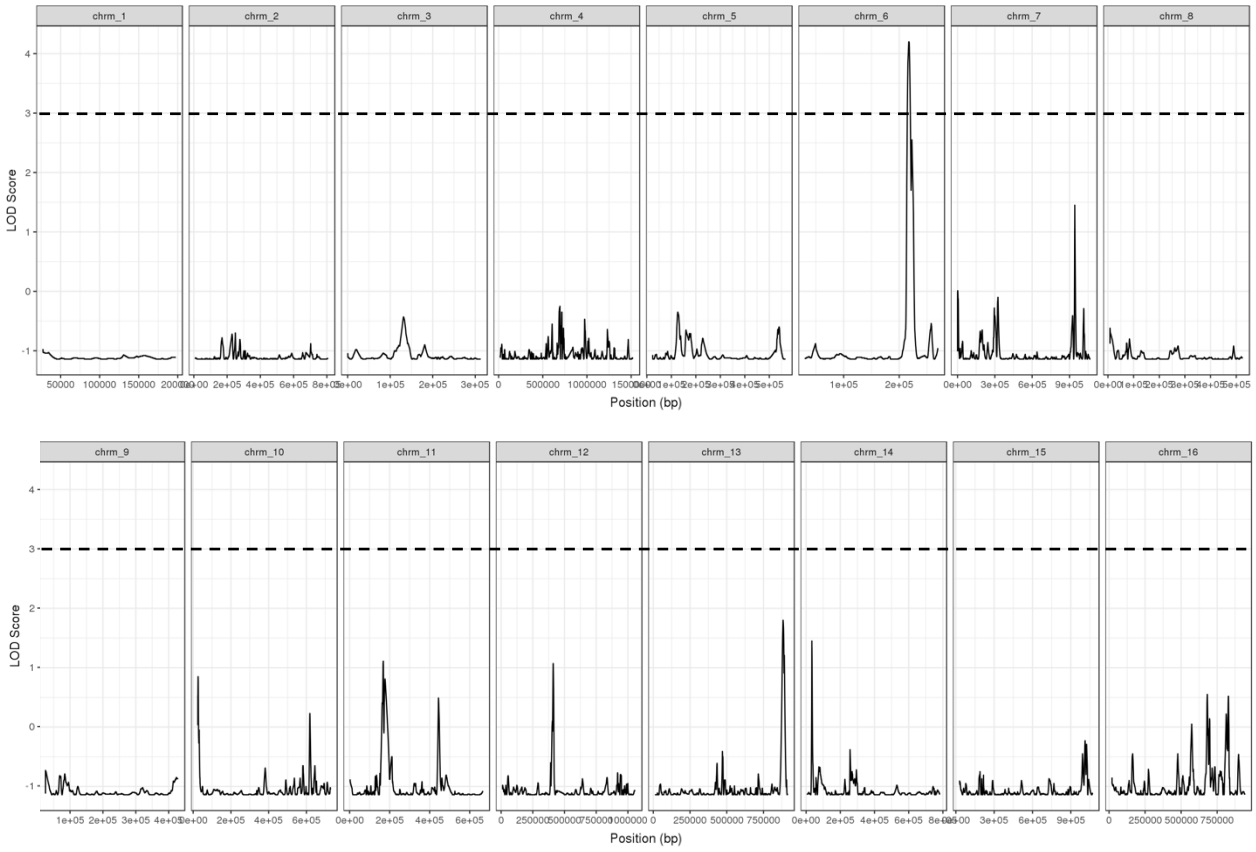


Figure 3: LOD scores generated from bulk segregant analysis for the progeny of NT112-SP1 x OKAY-SP2 hoΔ. A LOD score is the logarithm of the ratio of the probability of linkage to the probability of no linkage and was calculated for each SNP in the genome. The dotted line indicates the statistical threshold value of 3.

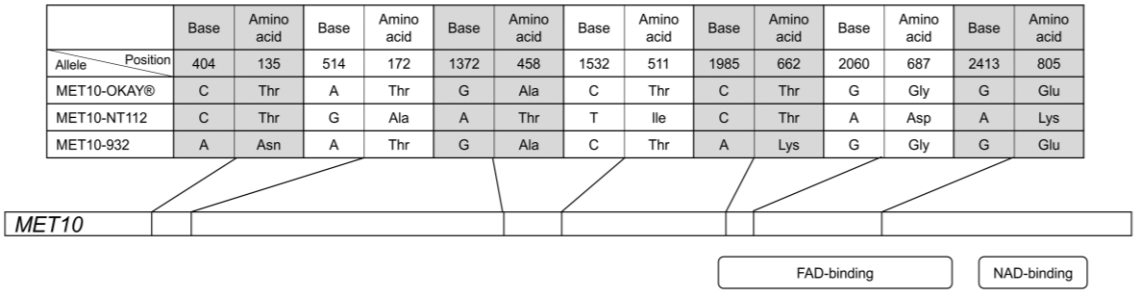
The LOD peak on chromosome VI was mapped to the reference *S. cerevisiae* genome and found to correspond to the *MET10* gene (Figure 4). Because *MET10* encodes the alpha subunit of sulfite reductase which catalyzes

sulfite formation, these results strongly suggest that the allele present in NT112 contributes to the high sulfite excretion phenotype.

Figure 4: Quantitative trait loci linked to high sulfite production in NT112. A. Allele frequencies for each SNP in the low sulfite-producing and high sulfite-producing pools. Dots represent frequency for individual SNPs, line represents average frequency in a 6,000 bp sliding window. Region found to be significantly linked by LOD scores marked between dotted lines. B. Genes annotated in the linked region of chromosome 6, coordinates 209,238-225,796, corresponding to region between dotted lines in A. From www.yeastgenome.org.

The sequence of the *MET10* allele present in NT112 was analyzed and compared to the allele present in OKAY®. Eight positions in the nucleotidic sequence were found to be different, five of which correspond to synonymous mutations. The positions that resulted in changes in amino acid sequence are shown in Figure 5. The *MET10* sequence was also compared with a previously described allele, *MET10-932*, that alters sulfite and H_2S formation. Four of the amino acid changes described in the present study are not shared between

MET10-NT112 and *MET10-932*. However, the low H_2S phenotype caused by *MET10-932* was previously shown to require a single amino acid change, Thr662Lys. Introduction of a threonine or a serine at position 662 was sufficient to restore normal levels of H_2S (Linderholm, Dietzel, et al. 2010). Although this change is not present in the *MET10-NT112* allele, two other amino acid changes in the same FAD-binding domain were found.



	Base	Amino acid	Base	Amino acid	Base	Amino acid	Base	Amino acid	Base	Amino acid	Base	Amino acid	Base	Amino acid
Allele \ Position	404	135	514	172	1372	458	1532	511	1985	662	2060	687	2413	805
MET10-OKAY®	C	Thr	A	Thr	G	Ala	C	Thr	C	Thr	G	Gly	G	Glu
MET10-NT112	C	Thr	G	Ala	A	Thr	T	Ile	C	Thr	A	Asp	A	Lys
MET10-932	A	Asn	A	Thr	G	Ala	C	Thr	A	Lys	G	Gly	G	Glu

Figure 5: Amino acid differences in three alleles of Met10. Only SNPs resulting in amino acid changes are shown. Relative positions in the MET10 ORF are indicated and known functional domains are shown, from Pfam (http://pfam.xfam.org/).

Genes that map to the less significant LOD peaks on chromosomes VII, XIII and XIV are listed in Table 3. Although none of the genes in the implicated region on chromosome VII can currently be easily rationalized to play a role in sulfite metabolism, *ADH2* on chromosome XIII and *SKP2* on chromosome XIV have metabolic relevance. *SKP2* encodes an F-box protein involved in degrading Met14 that encodes APS kinase. The function of Skp2 has previously been correlated with levels of sulfite and H_2S production in wine strains (Noble, Sánchez and Blondin 2015). The specific *SKP2* allele found in OKAY® has been

shown to contribute to reduced secretion of both H_2S and sulfite (Berlese-Noble, et al. 2014). Therefore, linkage of the *SKP2* allele in NT112 with the high sulfite-production phenotype by BSA is likely due to the presence of this previously described allele in the low sulfite-producing pool. Comparison of the *SKP2* nucleotide sequence in NT112 and OKAY® revealed two differences. Only one resulted in an amino acid change. The amino acid present at residue 357 is threonine in NT112 but isoleucine in OKAY®. Noble (2015) noted that the *SKP2* allele in OKAY® had two amino acid changes relative to a high H_2S and sulfite-excreter at positions 350 and 357 that could potentially influence both sulfite and H_2S production. Our results indicate that this effect is exclusively due to the isoleucine substitution at residue 357.

Table 3: Genes of known function anotated in the significantly linked regions according to LOD scores.

Chromosome	Coordinates (bp)[1]	Genes[2]	
VI	209,238-225,796	*CDC14* *PTR3* ***MET10*** *SMC2*	*RPL2A* *RPL29* *QCR6* *PHO4*
VII	916,389-962,723	*ZPR1* *SL1* *RTA1* *RPSOA* *RSM27* *GPI1* *CCH1* *CRM1* *MRPL9* *TOS2* *PET54* *HSV2*	*AZR1* *AMA1* *DIE2* *SMI1* *BNS1* *PHB2* *NAS6* *PHO81* *YHB1* *MIC26* *SPG1*
XIII	867,387-915,129	*ATM1* *YME2* ***ADH2*** *UBP15* *SCW10* *FKS3* *GAS1* *PSE1* *NIP1*	*GLC8* *ELP6* *TGL3* *PRE5* *YMR315W* *DIA1* *ADH6* *FET4*
XIV	28,322-61,721	*FIG4* *LEM3* *KRE1* *VNX1* *HXT14* *PFS2* *PHA2* *ATP11* *DAL82* *EMW1*	*RFA2* ***SKP2*** *ZIM17* *STB1* *KRI1* *MCK1* *MRPS18* *BXI1* *YPT11*

[1] Coordinates of the regions with significant linkage to the high sulfite-excretion phenotype.
[2] For each chromosomal region, genes are listed in physical order (centromere to telomere) reading the left column top to bottom, and then the right column, top to bottom.

ADH2 encodes glucose-repressible alcohol dehydrogenase II that is normally active only when glucose is depleted and cells are growing on ethanol as a carbon source (Willis and Martin 1984). This enzyme would be expected to generate acetaldehyde from ethanol only when glucose is depleted. *ADH1*

encodes the constitutive form of alcohol dehydrogenase that generates ethanol from acetaldehyde and is active during fermentation (Ciriacy 1975). To our knowledge, *ADH2* has not been previously associated with sulfite excretion, although its activity is relevant because acetaldehyde, like other carbonyl-containing compounds, can form adducts with sulfite non-enzymatically. It forms a particularly stable adduct with acetaldehyde, 1-hydroxyethane sulfonate, that has a $K_{dissociation}$ = 1.5 x 10^{-6} at pH 3 (Burroughs and Sparks 1973). 1-hydroxyethane sulfonate is not a substrate for either sulfite reductase or alcohol dehydrogenase. Thus, higher levels of acetaldehyde in the cell could reduce free sulfite concentrations by causing more 1-hydroxyethane sulfonate to form, which in turn could stimulate more sulfite formation by sulfite reductase. The differences between the *ADH2* alleles in NT112 and OKAY® are in the region of a shared ~100 bp deletion near the N-terminus of the protein. Because an insufficient number of reads were obtained in this region, it was not possible to determine the precise differences. The promoter regions were found to be identical.

Proposed model for high sulfite excretion

The highly significant linkage detected between the *MET10-NT112* allele and the high sulfite excretion phenotype suggests that this gene alone is likely the most important contributor to the excretion phenotype, at least in the OKAY® genetic background. If true, this would greatly simplify the breeding effort needed to constuct high-sulfite-excreting wine yeast strains by traditional hybridization.

Assuming that the alleles of *MET10*, *SKP2*, and *ADH2* in NT112 all make important contributions to the sulfite excretion phenotype as suggested by the BSA, eight possible genotypes should have been generated among the progeny of the NT112-SP1 x OKAY-SP2 *ho*Δ hybrid. The observation of 4-6 phenotypic classes suggests that not all of the genotypes are distinguishable phenotypically. The model proposed in Fig 6 shows the relationship between the known biochemical activities of Met10, Skp2, and Adh2 and sulfite formation or consumption in yeast. We presume that high-sulfite-excreting NT112 has a *MET10* allele that encodes a less active form of the enzyme such that the rate of H_2S formation from sulfite is slow. We also presume that it carries an allele of *SKP2* that slows normal proteolytic degradation of Met14 such that PAPS formation does not limit sulfite biosynthesis. Finally, we presume that towards the end of fermentation when glucose is nearly depleted, the *ADH2* allele carried by NT112 more readily oxidizes ethanol back to acetaldehyde than the allele in OKAY®. *ADH2* in laboratory strains has been described as repressible by glucose, becoming active only upon glucose depletion (Willis and Martin 1984)**.** A less glucose-repressible *ADH2* allele or one that has greater affinity for ethanol in NT112 would be expected to be more active during fermentation. Clearly, this is a speculative model that must be confirmed experimentally.

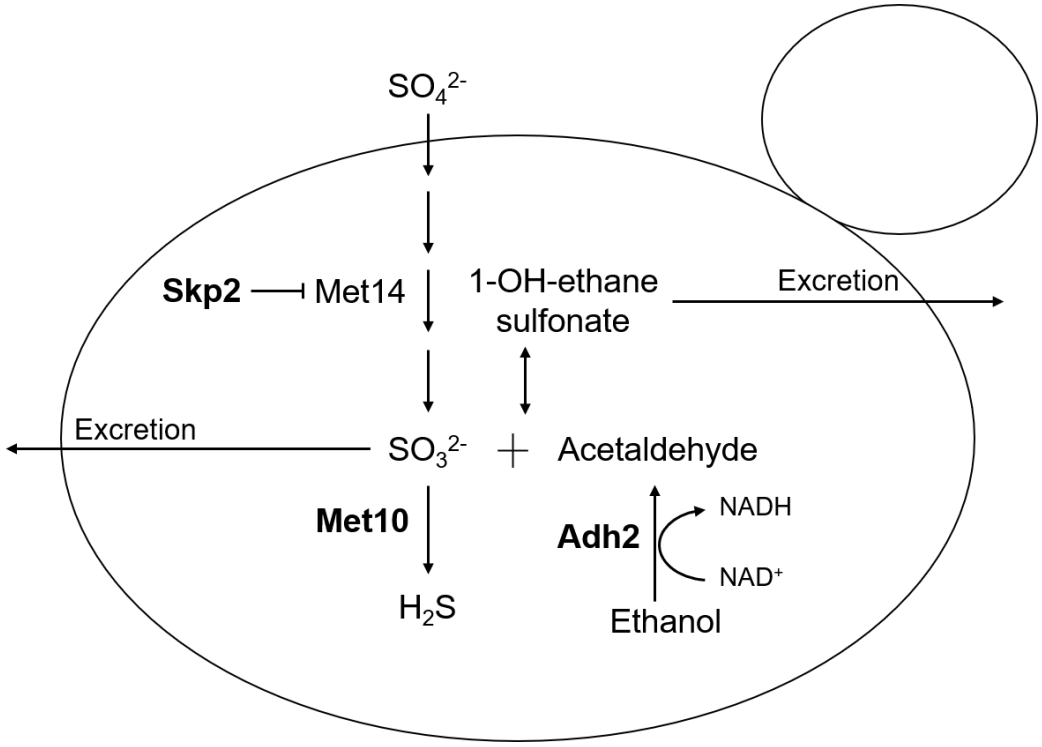


Figure 6: Proposed model for the high-sulfite excretion phenotype. Alleles from the high-sulfite excretor NT112 are indicated. The MET10 allele encodes a less active form of the alpha subunit of sulfite reductase leading to a build-up of sulfite. The SKP2 allele mediates slower than normal proteolytic degradation of Met14 leading to greater formation of the sulfite precursor PAPS. The glucose-repressible ADH2 allele oxidizes more alcohol than normal to generate acetaldehyde which in turn reacts with sulfite to generate 1-hydroxyethane sulfonate. Formation of 1-hydroxyethane sulfonate stimulates further production of both acetaldehyde and sulfite. Sulfite efflux is mediated by Ssu1. It is not known what transporters, if any, mediate efflux of 1-hydroxyethane sulfonate.

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CHAPTER 4: Conclusion

Restrictions in sulfite use represent a limitation for winemakers entering the organic market. Although the excretion of moderate levels of sulfite by yeast during fermentation has been known for a long time, its use as a tool to replace additions has not been exploded. Sulfite excretion by yeast is highly variable and is regulated both by growth conditions and genetic traits inherent to each yeast strain; the thorough understanding of these regulation mechanisms will allow winemakers to take advantage of this excretion.

Our results indicate that the effect of nutrient supplementation in must has variable and unpredictable effects on sulfite production, and that it depends on yeast strain. It is unlikely that, under these conditions, nitrogen additions can be used to modulate sulfite excretion and obtain high levels during wine fermentation.

We have identified new alleles of three genes potentially linked to high sulfite excretion by NT112, *MET10*, *SKP2* and *ADH2*. A metabolic model for these genes in NT112 has been proposed and *MET10* is thought to be the major contributor to this phenotype. This information can be utilized to generate a breeding program to produce high sulfite-excretor yeast strains.

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