AMERICAN VINEYARD FOUNDATION
and
CALIFORNIA COMPETITIVE GRANT PROGRAM FOR RESEARCH IN VITICULTURE AND ENOLOGY
Final Report
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I. Project Title: Identification of Yeast Strain Genetic Factors in the Formation of Volatile Sulfur Compounds

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III. Objective(s) and Experiments Conducted to Meet Stated Objectives: This grant was initially funded in June of 1997 and has received continuous funding through March of 2002, a period of five years. This grant was an extension of a previous grant to Drs. Noble, Boulton and Bisson: "Volatile Sulfur Compounds: Incidence and Factors Affecting Their Formation in California Wines" which terminated in June of 1997. The goal of that grant was to develop the technology to identify and quantify sulfur containing volatiles and determine their incidence in commercial production (Noble), to use multivariate statistical techniques to analyze industry sulfur volatile production and juice and must composition data to define the factors most impacting the appearance of sulfur volatiles in the California industry (Boulton) and to understand the role of the yeast strain in the appearance of sulfur volatiles (Bisson). This first grant was successfully completed, and a final report submitted in June of 1997. The statistical analysis confirmed the importance of a subset of known factors in the formation of sulfur volatiles during production. This work also demonstrated that one of the most critical variables in sulfur volatile formation was the yeast strain. Yeast strain genetic background was found to be a confounding variable meaning that it compromises the analysis of the role of other factors if that analysis is done across a population of different strains. Since multiple yeast strains are used commercially, the effect of other important factors such as nitrogen level or fermentation practices are obscured. This finding was the impetus for the current grant and the focus on understanding the genetic basis of strain differences in the formation of volatile sulfur compounds.

The original objectives of the grant were:

1. Define synthetic grape juice media conditions maximizing and minimizing the differences between strains with respect to hydrogen sulfide formation.

2. Compare gene expression patterns of genetically related and genetically distinct yeast strains displaying varying degrees of hydrogen sulfide formation in media and in natural juices.

Objective 1 was completed in the first three years. The specific approaches taken in objective 2 changed over the years as new technologies for the functional genomic analysis of *Saccharomyces* were developed and successfully applied to wine yeast strains and as new information was obtained.

IV. Summary of Research by Objective:

Objective 1: Define synthetic grape juice media conditions maximizing and minimizing the differences between strains with respect to hydrogen sulfide formation. Most previous studies of hydrogen sulfide production in *Saccharomyces* focused on one or a couple of strains and obtained somewhat conflicting results with respect to the principle factors, such as nitrogen, fermentation rate or presence of sulfur dioxide, that were most important in predicting strain behavior. This objective was undertaken in order to compare the effects of the different media used in these studies on yeast production of H_2S across a set of strains selected for native variability in volatile sulfur formation, and to examine the impact of composite factors on sulfur production. Several synthetic juice media had been described in the literature when we initiated this research program. All media were systematically evaluated across an initial set of 33 strains: single colony isolates from 6 commercial strains (Montrachet, Premier Cuvee, French White, Pasteur Champagne, Pasteur Red and Cote des Blancs) and 27 natural isolates obtained from Dr. Robert Mortimer that had been cultured from various regions of Italy and all of which were "normal" homothallic diploids (that is, containing only two copies of each of the yeast 16 chromosomes, and that were self-fertile, capable of switching mating type spontaneously and diploidizing). The commercial isolates contain variable numbers of individual chromosomes (from 1 to 3) and are therefore subject to greater effects of aberrant gene dosage. Initial analysis of these strains revealed a broad range of hydrogen sulfide production (20 fold differences among the natural isolates, 10 fold differences among the commercial strains) when strains were cultivated under identical conditions of fermentation. Strain behavior was not consistent across the various media with some strains displaying high levels of production under specific nutrient conditions but low to no production under others. Thus this set of strains is representative of the variability observed during commercial production.

Various parameters believed to impact hydrogen sulfide production were assessed across the majority of strains. All strains displayed an increase in volatile sulfur formation with decreasing

nitrogen content in the medium, but the level of nitrogen at which this response was triggered varied across the strains. With respect to vitamin limitation, only one strain showed a clear increase in hydrogen sulfide production. The others arrested fermentation or never initiated fermentation if vitamin content was limited. In general, glutathione supplementation was ineffective in reducing volatile sulfur formation. It increased volatile sulfur compounds in some strains or had little to no effect in others. Methionine and cysteine have been reported to be important in repressing the activity and expression of genes involved in sulfur reduction. Accordingly, the effect of supplementation with each of these compounds was evaluated. Cysteine supplementation did not affect hydrogen sulfide production in any of the strains tested. However, this compound is poorly transported into yeast cells and it is not clear that external supplementation would be effective. Off-character degradation products of cysteine were noted, so use of this compound is not recommended. Methionine addition has been reported in the literature to lead to the repression of the sulfate reduction pathway and therefore reduce H_2S production and release. The effect of methionine supplementation varied across the strains with some strains showing little to no effect on sulfate reduction while others such as Montrachet showed a dramatic effect. We did not see a clear correlation between the effect of methionine and the level of hydrogen sulfide produced. Some high producers showed reduced H_2S with elevated methionine and some showed no response. It did appear that methionine was more effective at reducing hydrogen sulfide production when that production occurred early in fermentation as opposed to late in fermentation, but the set did not contain enough early high producing strains to reach this conclusion definitively.

Threonine is known to inhibit biosynthesis of homoserine a precursor of methionine. In the presence of excess threonine sulfate reduction is activated due to the decrease in methionine content. Threonine supplementation was found to increase hydrogen sulfide formation in all strains tested at both high and low nitrogen contents in the medium. The magnitude of this effect varied with some strains doubling the amount of hydrogen sulfide produced and others only increasing it by less than 50%. This observation does indicate however that the sulfate reduction pathway in wine strains responds to the same control mechanisms that have been described for laboratory strains and that these control mechanisms are relevant to the production of hydrogen sulfide under commercial conditions.

There was no correlation observed between relative fermentation rate and production of hydrogen sulfide across the strains. Some individual strains appeared to produce higher levels of sulfur at faster fermentation rates, but this appeared to be related to differences in total biomass not necessarily in production behavior by individual cells. Temperature effects were also variable, with some strains producing more hydrogen sulfide at lower temperatures, some producing more at higher temperatures and others unaffected by differences in temperature. Again, this appeared to be more tightly correlated with differences in cell numbers and relative biomass rather than a specific effect on sulfate reduction or incorporation.

We had noted that there were differences in H_2S production between yeast strains grown in natural juices and those grown in synthetic juice media, and that the most consistent variable

impacting behavior was nitrogen content. We therefore examined the impact of differing amino acid ratios on hydrogen sulfide formation. This was accomplished by performing amino acid analyses of natural Pinot noir juices and creating a synthetic juice medium (based on the triple M medium of Ralph Kunkee) with the identical amino acid content to the Pinot noir juices. Hydrogen sulfide production was always greater in the Pinot noir juice for those strains producing H_2S than in the medium with the identical amino acid composition. This indicates that although nitrogen is an important factor, it is not the only factor. Specific phenolic compounds gallic acid and catechin, the predominant cinnamate and flavan-3-ol found in wines, were evaluated in triple M medium for an impact on H_2S production to determine if the presence of phenolic compounds influences volatile sulfur formation. Phenolic addition had no effect on the formation of hydrogen sulfide. Thus the differences in media and juice levels of H_2S are not due to the impact of these phenolic compounds on cellular redox status and glutathione recycling.

The goals of Objective 1, to define media exacerbating strain differences in the production of sulfur volatiles were completed in year 3 of the grant. Our overall conclusions were: nitrogen limitation leads to increased levels of H_2S in all strains tested, but the level at which this occurs varies across strain types. Methionine supplementation reduces H_2S in several strains, but not in all. The effect of methionine may be countered by the presence of other amino acids such as threonine. Therefore not only total nitrogen but also its distribution will affect volatile sulfur formation. Finally, there was no consistent trend across all the strains explaining their level of hydrogen sulfide formation. Instead the data was most consistent with individual strains bearing mutations in genes affecting various aspects of sulfide production and consumption.

Further analysis utilized the triple M medium of Ralph Kunkee with modifications of the nitrogen content. This medium contains ample supply of other nutrients in known concentrations and supported the growth of all of the strains of our study. Fermentation and growth rates of the strains in this medium were similar which is an important factor for the use of the global gene expression analyses for objective 2. For these techniques to be effective, it is important that the overall physiological behavior of the strains be similar, if not identical, and the only variable biological property be the one under investigation.

Objective 2: Compare gene expression patterns of genetically related and genetically distinct yeast strains displaying varying degrees of hydrogen sulfide formation in media and in natural juices. The initial aim of this objective was to use microarray analysis to determine the mRNA expression profiles of yeast strains under conditions of nitrogen limitation and sufficiency in order to comparatively evaluate high and low hydrogen sulfide producing strains. A subset of 12 strains displaying variable hydrogen sulfide production behavior but well matched in growth and fermentation parameters were selected. Early on in the course of this analysis it became clear that subtle differences in level of activity of the sulfate reduction pathway were likely directing the amount of H_2S released and that these differences may be undetectable in a microarray analysis. Microarray analyses have subsequently been shown to accurately reflect differences only above a magnitude of a three-fold or greater effect (increase or decrease) on the level of

expression. We did not see these types of effects except on some of the genes downstream of the sulfate reduction pathway, those involved in the formation of S-adenosylmethionine. These differences were correlated with nitrogen content and completion of fermentation but not with hydrogen sulfide production when compared across strains. It was therefore necessary to employ a different technique that would allow us to detect more subtle differences in level of protein activity. Toward that end, we elected to use a proteome rather than transcriptome analysis. Proteome analysis allows detection of moderate changes in protein levels and of key changes in protein modification that impact migration. Proteins of the sulfate reduction pathway and immediately downstream in reduced sulfate consumption were identified by peptide mass fingerprinting. Most spots were similar in density suggesting similar levels of expression. The protein encoded by the *MET17* gene, O-acetylserine/O-acetylhomoserine sulfhydrylase, Met17p, showed some variability in level of expression across the strains examined but the variation did not seem to correlate with the production of hydrogen sulfide.

Sulfate reductase activity has been evaluated by use of a medium known as BiGGY agar. This medium contains bismuth, which in the presence of reduced sulfur will form a brown pigment. Basal level of activity of sulfite reductase can be determined using this medium. Strains vary in colony color from nearly white to almost black on this medium. There was no correlation between relative levels of sulfite reductase and hydrogen sulfide production behavior by the strains. One strain in our collection carried a mutation in the *MET5* (component of sulfite reductase) gene. The sulfite and sulfide production behavior of this strain was characterized (by J. Tanaka) as part of the earlier grant to Profs. Noble, Boulton and Bisson. It was found that this strain produces no hydrogen sulfide but makes high levels of sulfite and higher levels of some volatile sulfur degradation components of methionine and cysteine. Since this strain is not able to synthesize sulfur containing amino acids they must be supplemented to the cells. It was quite difficult to find a concentration of sulfur containing amino acids that meet the biosynthetic needs of the cell without the production of off-characters. Genetic analysis of another strain (UCD934) indicated that it segregated spores with widely varying volatile sulfur production capacities upon sporulation and self-diploidization. This suggests that there is considerable allelic variation at the loci encoding genes specifying or regulating sulfate reduction or incorporation into biomolecules.

Genes in the sulfate reduction pathway are regulated primarily by transcriptional controls, but there are important posttranslational controls regulating uptake of sulfate and of the enzymes involved in incorporation of reduced sulfur into amino acids. We decided to take a direct approach to determine if changes in the level of expression of sulfate reduction or of the enzymes that are regulatory targets of sulfate incorporation varied across the strains under investigation. This was accomplished by direct assay of enzymatic activity and by alteration of enzymatic activity by transformation of the strains with plasmids carrying specific genes. The presence of multiple copies of the gene for a specific protein would be expected to lead to increased levels of that protein due to a gene dosage effect. Cytoplasmic pool levels of cysteine and methionine were also evaluated.

The set of 12 yeast strains were transformed with either the *MET17* (*O*-acetylserine – *O*-acetylhomoserine sulfhydrylase) or *CYS4* (cystathionine β-synthase) as these two enzymatic activities lead to the consumption of reduced sulfur and it has been shown in brewing strains that over-expression of either one of these genes will reduce release of hydrogen sulfide. Investigation of the impact of *MET17* gene has not yet been completed, due to immigration problems in hiring of the postdoctoral fellow that was to conduct this objective. She is now in the lab and the project is underway. To date, the *MET17* gene virtually eliminated hydrogen sulfide production in the French White strain, but had no impact on the other strains evaluated. Analysis of the *MET17* allele in French White revealed the presence of a large insert leading to the production of a defective gene in one of the alleles. The other allele is apparently normal. This suggests that a less than 2N dosage of the *MET17* gene may result in increased expression of hydrogen sulfide. None of the other strains examined to date displayed a mutant allele, consistent with the failure to observe an effect of over-expression of *MET17* on hydrogen sulfide production. We analyzed the *MET17* gene for allele variation across the set of 12 strains. All wine strains from which the gene was isolated had an identical amino acid sequence for Met17p. Across the population there were two neutral (no change in encoded amino acid) base pair substitutions. We have also recently found that the *MET17* gene that was cloned from the brewing strain that we used in the over-expression studies contains a mutation changing an isoleucine to a valine in the first third of the protein. The impact of this alteration on gene function is unknown. None of the wine strains of *Saccharomyces* carry this mutation. Further, over expression of *MET17* is toxic in several strains. Therefore the strains are intolerant of abnormal expression levels of this protein.

The *CYS4* gene encodes cystathionine β-synthase, Cys4p. This enzyme is regulated post-translationally by oligomerization. The homodimer is twice as active as the tetramer and octamer forms of the protein. Enzymatic activity is rapidly down-regulated by oligomerization of the enzyme. Our analysis of the relationship of internal methionine and cysteine pool levels on hydrogen sulfide production indicated that it is correlated with internal cysteine. Cys4p is required for the production of cysteine. Therefore by controlling oligomerization the cells regulate the level of cysteine and therefore of the entire sulfate reduction pathway. Other regulatory mechanisms also exist to regulate the expression of sulfate reduction but are not as rapid as the post-translational control by oligomerization. Of the 12 strains transformed with *CYS4,* five showed no change in H_2S production, four showed a very dramatic decrease and three showed an increase under high nitrogen conditions. One of the strains showing an increase was UCD934. The strains behaved the same under low nitrogen conditions, with the exception of the French White strain, UCD713, which changed from no effect to showing a very slight increase in production under low nitrogen (Table 1). Of the four strains showing a reduction in H_2S, two were classified as high producers, one as a moderate producer and one as a low producer. Thus, the impact of *CYS4* over-expression appears unrelated to initial strain behavior, as was seen before. Analysis of cystathionine β-synthase activity and intracellular cysteine revealed a strong correlation of high levels of enzymatic activity, increased cysteine levels, and reduction in volatile sulfur production. In untransformed strains, there likewise appeared to be a correlation between high levels of cysteine and low production of hydrogen sulfide (Table 2). Intracellular

cysteine levels seemed to be associated with higher levels of *CYS4* activity, but this did not hold true for all stains. However, analysis of cysteine levels should provide a good indication of the tendency of the strain to be a high, moderate or low sulfide producer.

This mechanism of regulation of sulfate reduction, by oligomerization of Cys4p makes sound physiological sense. Cysteine in not only required for protein synthesis but also needed for the formation of cystathionine, and important regulator of cell redox potential that may be involved in ethanol tolerance. Cells may therefore need higher levels of cysteine to deal with the appearance of stress in the medium. Cys4p activity must elevate quickly in response to oxidative stress, this mechanism allows a fast response, as the oligomers merely have to be re-converted to active dimers. Indeed, under hydrogen peroxide imposed oxidative stress Cys4p activity increases without an accompanying increase in protein levels or transcription of the *CYS4* gene. The C-terminal domain of Cys4p has been shown to mediate oligomerization and regulation of protein activity while the N-terminal domain specifies catalytic activity. Thus, mutation of the regulatory domain preventing or inhibiting oligomerization would be predicted to result in higher basal levels of Cys4p.

Therefore we also undertook an analysis of the *CYS4* allele in the 12 strains of this study. One of these strains, UCD932 (Ba2), is consistently a low H_2S producer in both juice and synthetic juice media. It only becomes a high producer of volatile sulfur when starved for pantothenic acid, which blocks synthesis of cysteine. As indicated in Tables 1 and 2, this strain displays 2 to 5-fold higher levels of Cys4p activity and has higher internal pools of cysteine than most of the other strains analyzed. Preliminary DNA sequence analysis revealed that the UCD932 *CYS4* allele carries a specific mutation converting a serine residue in the C-terminal oligomerization domain to an asparagine. Oligomerization is largely dependent upon the formation of hydrogen bonds, which requires that hydroxyl groups be in specific positions. The loss of a hydroxyl group could have a dramatic impact on oligomerization and activity of the protein. This mechanism of regulation can also explain a decrease in cysteine production with elevation of Cys4p activity. Increased levels of enzyme may accelerate the process of oligomer formation resulting in an overall reduction in activity in spite of an increase in the protein level.

The main conclusion from this objective is that it is indeed possible to determine the basis for hydrogen sulfide production behavior in individual strains following a battery of genetic and biochemical tests. In general, low sulfide producing strains have high cysteine levels indicating the important role of cysteine in repression of the sulfate reduction pathway. Other strains respond to methionine supplementation suggesting defects in methionine-mediated regulation (or that of its product, S-adenosylmethionine). Other strains display elevated hydrogen sulfide production due to mutative loss of one allele encoding proteins involved in consumption of reduced sulfate. Finally, while this did not appear in any of our strains, other researchers have reported low hydrogen sulfide activity resulting from loss of sulfite reductase activity. Thus, the hydrogen sulfide producing behavior of any given individual strain can be predicted once the specifics of relative enzyme activities and their regulation are known in that strain.

Unfortunately, there is not one factor that singly explains the variation in sulfur production, consistent with our previous genetic analysis of this trait in UCD934.

Impact of this Work for the California Wine Industry: What are the practical benefits of this work? First, we have a better understanding of strain variability under production conditions. If the nitrogen requirements and biochemical characteristics of a strain are known, then nitrogen supplementation can be used effectively to reduce the incidence of volatile sulfur. However, this information is not trivially gotten. Second, supplementation with specific amino acids (methionine and cysteine) will not be generally effective and are strain specific. High threonine content relative to methionine should be avoided. Further, imbalances in sulfur containing amino acids in the juice could lead to the formation of complex volatile sulfur compounds that are the degradation products of these amino acids. We recommend against indiscriminant supplementation with sulfur containing amino acids. Strain response to increases in fermentation rate, changes in temperature, and stress with respect to H_2S formation are variable. Analysis of each strain must occur, as general principles predicting the behavior of all strains do not exist unless the genetic background of the strain has been fully characterized. Finally, one of our additional goals was to determine the best method for the construction of strains with reduced H_2S production. Mutational decreases in specific enzymes of the sulfate reduction pathway, such as via the elimination of sulfite reductase, led to more problems than they solved. Dramatically higher sulfite production occurred. Further, increases in levels of enzymes that had been shown to reduce hydrogen sulfide in brewing strains were of limited effect. Over-expression of some is toxic and therefore not a viable means to generate commercial strains. Analysis of the one strain that was a low producer under all conditions tested indicated that it carries a specific mutation in an important regulatory region of the *CYS4* protein allowing high Cys4p activity and maintenance of high cysteine pools under conditions of stress. Our next grant in this area will focus on this gene and the possible use of this naturally occurring allele to reduce sulfide production across a wide spectrum of strains.

V. Outside Presentation of Research: The five-year period of this grant has resulted in three student theses (one PhD dissertation and two MS thesis). Two publications have appeared on this work and more are in preparation. Copies of the two publications are attached. A fourth student is completing his MS thesis based entirely on research conducted for this grant. He anticipates finishing the thesis in spring of 2002. Other current and former students have received partial support from this grant for performing specific objectives: James Brown, Vidhya Ramakrishnan, Kathryn Weiss. Undergraduate and rotation graduate students were also employed on this grant as needed.

Theses:

Ioannis Flerianos. Factors Affecting H_2S Production During Fermentation, 1999. MS Thesis

Apostolos Spiropoulos. *Saccharomyces* Strain Variability in Hydrogen Sulfide Formation Under Enological Conditions, 2000. PhD Thesis

John Skiadis. The Effect of Over-Expression of *CYS4* (Cystathionine-β-synthase) in Hydrogen Sulfide Formation in Commercial and Natural Wine Yeast Strains, 2001. MS Thesis

Kurt Niznik, MS Thesis, In preparation

Publications:

Spiropoulos, A. and L. F. Bisson. 2000. *MET17* and hydrogen sulfide formation in *Saccharomyces cerevisiae*. Appl. Environ. Microbiol. 66:4421-4426.

Spiropoulos, A., J. Tanaka, I. Flerianos and L. F. Bisson. 2000. Characterization of hydrogen sulfide formation in commercial and natural wine isolates of *Saccharomyces* Am. J. Enol. Vitic. 51:233-248.

Over the five years of this project, numerous presentations to industry groups and at national and international meetings of this work have occurred. These presentations have been made by the students supported by the grant or by myself. In each case the source of support of the project has been indicated to the audience.

VIII. Funds Status: Funds were used to support postdoctoral fellow Dr. Yeun Hong. She is currently finishing the studies of the allele analysis of the *MET17* strain and conducting the transformation experiments for the remaining strains, she is being assisted by laboratory research technician Tammi Olineka. Funds were used for partial support of three graduate students, Kurt Niznik, Troy Laudette and Joel Mann who are working on aspects of this proposal. Kurt is expected to complete degree requirements in the spring of 2002. Troy will be obtaining his MS degree by exam and therefore will not be writing a thesis. Joel's thesis will largely focus on arrest of fermentation due to temperature and acetic acid effects. He is working on aspects of both this research program and our other AVF grant on the analysis of normal and problematic fermentations. Permission to carryover unspent funds after the March 31st termination deadline of the grant to continue to support these researchers and complete objectives was requested and granted. It is anticipated that all pending work will be completed by the end of June.

Table 1. Effect of transformation with the *CYS4* gene on hydrogen sulfide production under high and low nitrogen conditions.

Strain	Plasmid Status	Total Hydrogen Sulfide Production (ng)	
		High Nitrogen (433 mg N equiv/L)	Low Nitrogen (208 mg N equiv./L)
UCD932	None	708	1,033
	Vector	923	1,057
	CYS4	553	352
UCD934	None	547	7,890
	Vector	1,223	8,023
	CYS4	3,957	15,272
UCD935	None	5,357	13,507
	Vector	5,123	13,657
	CYS4	6,457	10,443
UCD937	None	1,135	5,355
	Vector	1,353	6,490
	CYS4	150	585
UCD939	None	6,557	22,205
	Vector	6,490	18,788
	CYS4	587	532
UCD940	None	7,257	23,507
	Vector	7,353	20,173
	CYS4	7,090	18,405
UCD941	None	1,288	15,873
	Vector	967	14,640
	CYS4	2,135	17,440
UCD949	None	743	6,238
	Vector	753	4,420
	CYS4	1,553	3,350
UCD952	None	1,217	15,188
	Vector	1,352	15,752
	CYS4	1,748	16,123
UCD957	None	13,040	21,407
	Vector	10,790	17,837
	CYS4	283	313
UCD713	None	1,203	7,723
	Vector	1,190	6,102
	CYS4	1,157	10,788
UCD522	None	3,237	23,290
	Vector	3,453	20,357
	CYS4	4,220	23,790

Table 2. Cystathionine β-synthase activity, cysteine levels and hydrogen sulfide production in wine strains of *Saccharomyces*.

Strain	Nitrogen Level (mg N equiv/ L)	Cys4p Activity at 48 hours	Cysteine Levels (nmole/mg dry wt)	Hydrogen Sulfide (ng at 48 hrs)
UCD932	433	6.24	1.75	5
	208	6.73	1.98	12
UCD939	433	2.29	0.83	100
	208	2.50	0.55	220
UCD957	433	3.22	0.86	220
	208	2.63	0.60	350
UCD713	433	1.95	1.24	25
	208	2.37	1.33	80
UCD552	433	3.57	2.74	80
	208	1.82	2.11	300

Identification of Yeast Strain Genetic Factors in the Formation of Volatile Sulfur Compounds

Linda F. Bisson, PI

General Summary of the Five-Year Grant Period:

The goal of this proposal was the determination of the genetic basis of the variation in hydrogen sulfide formation observed in commercial and native isolates of *Saccharomyces cerevisiae*. An initial goal was the characterization of the variability in strain behavior using different synthetic media and natural juices. No correlation was found between the basal level of sulfite reductase activity and hydrogen sulfide production. While strains lacking sulfite reductase did not produce hydrogen sulfide, they did produce copious amounts of sulfite. Methionine and cysteine supplementation had variable effects on volatile sulfur formation. In general these compounds were only of limited effectiveness or lead to the formation of higher sulfur volatiles that are the degradation products of these amino acids. All yeast strains evaluated show an effect of limiting nitrogen in the increased production of hydrogen sulfide, but the level of nitrogen at which this occurs is variable and impacted by medium composition, likely due to relative differences in demand for nitrogen.

There are several major conclusions of this work. First, cytoplasmic cysteine levels are correlated with activity of the sulfate reduction pathway and the production of hydrogen sulfide. Second, cytoplasmic cysteine levels vary up to five fold in different genetic backgrounds in strains grown under identical conditions. Those strains maintaining high internal concentrations of cysteine produce lower amounts of sulfide. Third, methionine and glutathione levels were not found to be correlated with sulfate reduction and H_2S production. Fourth, cytoplasmic cysteine levels were a function of the activity of cystathionine β-synthase (*CYS4*) in most genetic backgrounds. In one genetic background, UCD713, a defective allele of *MET17*, the step immediately preceding *CYS4*, carried a mutation reducing cysteine production. Elevation of the activity of this protein restored cysteine levels and reduced sulfide production. Fifth, elevation of *CYS4* activity by transformation of strains with a plasmid leading to over-expression of the protein increased cysteine pools and reduced hydrogen sulfide formation in some strains. In other strains over-production of the enzymatic activity did not occur, and in others it was reduced, suggesting that high enzyme levels can catalyze oligomer formation. Sixth, *CYS4* allele analysis revealed that one strain that consistently produces little to no H_2S carries a mutation in an important regulatory domain. Thus, the differences in hydrogen sulfide formation of different enological strains of *Saccharomyces* are due to differences in the pool levels of cysteine, which is a function of the relative activity of the enzymes directly involved in cysteine biosynthesis. Finally, alterations of the activity and regulation of Cys4p are associated with greatly diminished hydrogen sulfide production. Allele swap technology should be useful for the generation of low sulfide producing variants of any commercial strain.