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

**II. Principal Investigator:** Linda F. Bisson
Department of Viticulture and Enology
University of California, Davis
Davis, CA 95616-8749
Phone: (530) 752-3835
FAX: (530) 752-0382
Email: lfbisson@ucdavis.edu

**Cooperators:** None

**III. Summary:** In this current grant year, the analysis of the impact of over-expression of two genes involved in consumption of reduced sulfur, *CYS4* and *MET17*, on $H_2S$ formation in commercial and natural wine strains of *Saccharomyces* was completed. Interestingly, increasing the level of expression of the *CYS4* gene completely eliminated hydrogen sulfide production in four strains, had no effect in others, and in a few resulted in an increase in $H_2S$. Similar results were obtained for *MET17*. So far, strains that showed reduced volatile sulfur formation with *CYS4* did not show any effect with *MET17* and those showing an effect with *MET17* showed no or increased $H_2S$ formation with over-expression of *CYS4*. Strains that were high produces of $H_2S$ tended to decrease sulfide release when *CYS4* was present, while the moderate producers showed a stronger response with *MET17*. Thus, there are multiple underlying genetic causes for the production of hydrogen sulfide. This analysis does indicate that once the cause of $H_2S$ release is known for a given strain, it can be corrected genetically. It will also be possible to screen for strains naturally possessing alleles leading to reduced sulfide production to be used in conventional breeding programs.

This research has further clarified the basis for the two phases of hydrogen sulfide release observed during fermentation. The early phase of hydrogen sulfide production occurs shortly after maximal cell biomass is attained, within the first few days of active fermentation, and is related to the relative activities of the enzymes generating and consuming reduced sulfur. The later stage, which occurs at the end of fermentation, is related to the nitrogen recycling behavior of the culture. Genomic data indicates that at this point in time numerous pathways have been induced that shunt nitrogen between amino acid components. When this occurs, there is a net shift of nitrogen from the sulfur containing amino acids to the non-sulfur containing amino acids. If nitrogen levels are in

ample supply, this is prevented from occurring. Interestingly, analysis of the pattern of production of hydrogen sulfide of the 12 strains used in this study revealed that many of the strains produce hydrogen sulfide continuously during fermentation. Over-expression of *MET17* and *CYS4* has the highest impact on the continual producers versus the transient producers.

**IV. Objective(s) and Experiments Conducted to Meet Stated Objectives:** The overall goal of this work is to determine the physiological and genetic basis of the high degree of variability in hydrogen sulfide production exhibited by different wine yeast strains. The ultimate aim of this study is to identify those genetic factors reducing or resulting in minimal to no volatile sulfur production so that yeast strains may be constructed that will not produce these compounds under enological conditions. An aligned goal is to identify the environmental factors that lead to formation of high levels of sulfur volatiles during wine production in order to determine if the appearance of these compounds may be controlled by must additions.

This research program has two broad objectives:

Objective 1: Comparison of protein expression patterns of genetically related and genetically distinct yeast strains displaying varying degrees of hydrogen sulfide formation under the same conditions of growth.

Objective 2: Analysis of factors regulating the sulfate reduction pathway in wine strains of *Saccharomyces*.

**V. Summary of Major Research Accomplishments and Results by Objective:**
*Objective 1*: In grant year 2000-01, the specific goals for Objective 1 were to use the proteome analysis developed in previous years to compare protein expression profiles of the same strain under conditions of high and low hydrogen sulfide production. However, this analysis showed numerous changes occurred in these cultures and it is difficult, if not impossible, to determine which changes are correlated with sulfide production and release. A global effect on gene expression caused by nitrogen limitation was not unexpected. This analysis also indicated that more optimization research needed to be done for the proteome analysis. We discovered some flaws or errors in the published techniques for 2D SDS PAGE that seem to be responsible for lack of reproducibility. We have also compared several gel staining protocols to determine which one yields the best representation of spot patterns from commercial and native strains. We are currently re-running the analysis with a focus on comparing different strains that differ in $H_2S$ production patterns under identical nitrogen conditions.

*Objective 2*: With respect to objective 2, a set of 12 yeast strains were transformed with either the *MET17* (*O*-acetylserine – *O*-acetylhomoserine sulfhydrylase) or *CYS4* (homocysteine methyl transferase) 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. The

analysis of the *CYS4* transformants was completed. Investigation of the impact of *MET17* gene is in progress for several strains, but has been completed for two commercial strains, French White and Montrachet. The *MET17* gene virtually eliminated hydrogen sulfide production in the French White strain, but had no impact on Montrachet with $H_2S$ levels remaining high in this strain impacts sulfate reduction. The *MET17* transformants were difficult to obtain. We discovered that over-expression of *MET17* is toxic in some genetic backgrounds. This result was not expected from the activity of the enzyme as neither homocysteine nor methionine has been reported to be toxic. Toxicity may indirectly arise from redirection of cellular resources towards homocysteine. Alternately, imbalances in homocysteine or methionine pools may be inhibitory under enological conditions.

Of the 12 strains transformed with *CYS4,* five showed no change in $H_2S$ production, four showed a very dramatic decrease and three showed a slight increase under high nitrogen conditions. 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 an increase in production under low nitrogen. 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. Analysis of internal 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. 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.

The data clearly indicates that in some strains modification of the steps leading to methionine/S-adenosyl methionine has a striking impact on reduction of sulfide production while in others amplification of the pathway leading to cysteine is important. This suggests that both pathways of regulation that have been described in the literature, methionine/SAM repression at the level of transcription or gene expression and cysteine inhibition of enzymatic activity and blockage of inducer production are both important in the regulation of sulfate reduction. Some natural isolates appear to be more responsive to changes in one regulatory pathway versus the other. Still other strains, most notably UCD522 (Montrachet), which has been characterized as a high $H_2S$ producer, has high pool levels of all sulfur metabolites yet still releases sulfur volatiles. It may be that this particular strain carries mutations affecting the normal regulatory circuits or that sulfur amino acid recycling is elevated. The conclusion from this work is that the tendency toward release of elevated hydrogen sulfide arises from differing genetic factors. There is no single gene that can be modified or evaluated in all commercial strains that will eliminate the production of sulfur volatiles. However, individual strategies for given strains may work well. It is unclear how such genetic diversity has arisen in the yeast world. This is a critical pathway that produces toxic intermediates and end products. What is obvious is that variations in regulation of the pathway still result in viability.

**VI. Outside Presentation of Research:** Two publications have appeared on this work and more are in preparation.

Spiropoulos, A. and L. F. Bisson. 2000. *MET17* and hydrogen sulfide formation in *Saccharomyces cerevisiae*. Applied and Environmental Microbiology 66:4421-426.

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

Copies of the publications are available upon request. This research program has been presented in short courses hosted by the Department of Viticulture and Enology and University Extension.

**V II. Research Success Statement:** Production of the hydrogen sulfide off-odor is a persistent problem in wine making. While this compound can be removed by copper treatments, such treatments are not neutral having other impacts on wine quality. Yeast strains not producing hydrogen sulfide under enological conditions are therefore highly desirable. Such strains have been reported in the past, but none have been found to be low hydrogen sulfide producers under all enological conditions and many have apparently lost this property during winery cultivation. This study has clarified of our understanding of the variables and factors impacting hydrogen sulfide formation by yeast under enological conditions and underscores the complexity of regulation of this pathway. More importantly, this study demonstrates that by replacement of one gene type (allele) with another, the tendency of a given strain to produce hydrogen sulfide can be diminished if not eliminated. Thus it will be possible to select for or construct low hydrogen sulfide producers using conventional yeast breeding techniques.

**VIII. Funds Status:** Funds requested for student salary were used to support two MS students, Kurt Niznik and John Skiadas, who are conducting objectives 1 and 2 respectively. Both students are expected to complete thesis requirements and their respective roles on this grant by the end of the year.