

Control of hydrogen sulfide formation during fermentation

Alan T. Bakalinsky, Olga Martin, and Jim Kennedy, Department of Food Science and Technology,
Oregon State University

INTRODUCTION

Hydrogen sulfide and related sulfur compounds produced internally by yeast during fermentation are considered highly undesirable if they are excreted and exceed threshold concentrations in the finished wine, as they cause an unpleasant "reduced character". The threshold for hydrogen sulfide is on the order of parts per billion and that for some of the other reduced sulfur compounds derived from hydrogen sulfide ranges from parts per million to parts per billion. Factors which affect production of these compounds include, but are not limited to, the nutritional status of the grapes, yeast strain, and fermentation conditions (Rauhut, 1993). Formation of excessive reduced character in table wines produced from sound grapes using good winemaking practices is a perplexing problem because it can occur unpredictably, and spoil affected wines. Yeast produces this class of compounds internally as a byproduct of the normal and necessary synthesis of S-adenosyl-methionine and the sulfur-containing amino acids, methionine and cysteine (Thomas and Surdin-Kerjan, 1997). Excessive synthesis leads to excretion of hydrogen sulfide into the wine. Although excess hydrogen sulfide formation has generally been correlated with nitrogen deficiency (Jiranek et al., 1995a, 1995b), deficiencies in vitamin B₆ and pantothenic acid, and residual sulfur on grapes from the application of fungicides have also been implicated. In one study, formation of hydrogen sulfide was found not to correlate with variation in activity of the formative enzyme, sulfite reductase, which reduces sulfite to hydrogen sulfide (Jiranek et al., 1996). This suggests that the amount of extracellular hydrogen sulfide formed is controlled by other rate-limiting steps: its utilization in sulfur amino acid biosynthesis, its active or passive transport from the cell, or consumption through other biochemical pathways. Formation still occurs unexpectedly, and often enough to be considered a persistent problem (Boulton et al. 1996; Park et al. 2000).

One complicating factor in the study of hydrogen sulfide production is that formation during the early stages of fermentation is not always problematic, either because the yeast eventually takes up and reuses the excreted sulfide or because the volatile sulfide is removed by entrainment in the stream of carbon dioxide that evolves during the course of fermentation. At the latter stages of fermentation, when excretion of the hydrogen sulfide is believed to be most troublesome, the less vigorous yeast may no longer take it up, or evolution of carbon dioxide may be so slowed as to no longer be an efficient means of removal. As a consequence, the total amount of hydrogen sulfide formed in a fermentation is likely to be a poorer predictor of eventual reduced character than measurement at the end of fermentation.

Because hydrogen sulfide is produced within the yeast cell directly from sulfite, its immediate biochemical precursor, we are testing the hypothesis that excretion of sulfite by yeast will minimize hydrogen sulfide production. Excretion of sulfite occurs due to the activity of the yeast protein, Ssu1 (Avram and Bakalinsky, 1997; Avram et al. 1999; Park et al. 1999; Park and Bakalinsky, 2000; Goto-Yamamoto et al. 1998), which has also been implicated in selenium efflux (Pinson et al. 2000). If the hypothesis is found to be true, it will provide a simple means of identifying wine yeast strains that tend to produce low levels of hydrogen sulfide during fermentation.

SPECIFIC AIM

The aim of this year's experiments is to determine if increased activity of the yeast sulfite pump (Ssu1) in laboratory strains can minimize hydrogen sulfide formation. If so, experiments will be proposed in the second year to determine Ssu1 activity in wine yeast strains that produce variable amounts of hydrogen sulfide, and to examine ways in which pump activity can be increased.

MATERIALS AND METHODS

Yeast strains, media

Three previously characterized laboratory strains of *Saccharomyces cerevisiae* were used: 3090-9d, *MAT α leu2-3, 112 ura3-52*, 3090-9d-T4-L1, *MAT α leu2-3, 112 ura3-52 ssu1 Δ* , and 3163-1b, *MAT α leu2-3, 112 ura3-52 FZF1-4* (Avram and Bakalinsky, 1997; Park and Bakalinsky, 2000). Coded wine strains 6040, 6494 and 6012 were obtained from Lallemend. Strains were grown in standard rich medium (YEPD) or in a synthetic grape juice (Salmon and Barre, 1998) supplemented with uracil and leucine, and variable amounts of other amino acids and ammonia.

Sulfite efflux pump activity

Activity of the sulfite efflux pump Ssu1 was measured as described (Park and Bakalinsky, 2000). Briefly, yeast cells were harvested in log phase during growth in a rich laboratory medium (YEPD), washed in tartaric acid-based buffer supplemented with glucose, and resuspended in the same buffer at about 3 mg dry weight cells per ml. Sulfite uptake was initiated by addition of 1 mM sulfite at time zero. Cell samples were taken at 30 seconds and at one minute intervals from one through 10 minutes. Total intracellular sulfite was measured based on the pararosaniline colorimetric assay and was expressed as a function of dry weight cells.

Hydrogen sulfide

Hydrogen sulfide was measured at the end of fermentation in synthetic grape as a monobromobimane derivative by HPLC with fluorescence detection (Newton et al., 1981). Samples of the fermented synthetic juice were filtered to remove cells and other insoluble material prior to reaction with monobromobimane. Sodium sulfide was used as a standard.

RESULTS and DISCUSSION

Sulfite accumulation (Ssu1 activity)

Sulfite accumulation was measured in three laboratory strains and three wine strains of the yeast *S. cerevisiae*. This measure reflected activity of the sulfite efflux pump, Ssu1. The three laboratory strains were chosen because they differ widely in pump activity. Strain 3163-1b has a hyperactive pump, strain 3090-9d has a normal pump, and strain 3090-9d-T4-L1 lacks a pump. The maximum level of intracellular total sulfite was found to be about 28 nmoles/mg dry weight cells in 3090-9d-T4-L1, about 18 nmoles/mg dry weight cells in 3090-9d, and about 3 nmoles/mg dry weight cells in 3163-1b (Fig 1). Coded wine strains 6494 and 6012 accumulated about 20 nmoles/mg dry weight cells and wine strain 6040 accumulated about 2 nmoles/mg dry weight cells (Fig 2).

Hydrogen sulfide

A representative HPLC trace of hydrogen sulfide as a bimane adduct is shown in Figure 3. At least 5 other peaks are visible which correspond to breakdown products of monobromobimane. Figure 4 shows excellent correlation between the peak area corresponding to the sulfide-bimane adduct and added sulfide. This method of detection has a sensitivity in the range of picomoles (10^{-12} moles). We have since modified the liquid chromatography gradient so that the sulfide-bimane adduct elutes at about 18 minutes rather than at 50 minutes (data not shown). We have also modified the derivatization reaction to minimize formation of the additional fluorescent bimane breakdown products (data not shown).

Preliminary fermentations

In order to determine if a correlation exists between activity of the sulfite efflux pump, Ssu1, and hydrogen sulfide excretion, trial fermentations were performed. In the first model wines produced by the three laboratory strains, no hydrogen sulfide was detected (data not shown). The model grape juice used had a high nitrogen content (300 mg/L as ammonia and primary amino

acids) and this may have prevented excretion of hydrogen sulfide by the yeast. Additional fermentations are currently being evaluated using model grape juices containing 60 to 100 mg/L nitrogen, and variable amounts of the vitamins biotin and pantothenate. Our rationale is that hydrogen sulfide production will be enhanced if nitrogen or these vitamins are decreased to sub-optimal levels.

LITERATURE CITED

- Avram, D. and Bakalinsky, A.T. 1997. *SSU1* encodes a plasma membrane protein with a central role in a network of proteins conferring sulfite tolerance in *S. cerevisiae*. J. Bact. 179:5971-5974.
- Avram, D., Leid, M., and Bakalinsky, A.T. 1999. Fzf1p of *Saccharomyces cerevisiae* is a positive regulator of *SSU1* transcription and its first zinc finger region is required for DNA binding. Yeast 15:473-480.
- Boulton, R.B., Singleton, V.L., Bisson, L.F., and Kunkee, R.E. 1996. Principles and practices of winemaking. Chapman and Hall, NY.
- Goto-Yamamoto, N., Kitano, K., Shiki, K., Yoshida, Y., Suzuki, T., Iwata, T., Yamane, Y., Hara, S. 1998. *SSU1-R*, a sulfite resistance gene of wine yeast, is an allele of *SSU1* with a different upstream sequence. J. Ferm. Bioengineer. 86:427-433.
- Jiranek, V., Langridge, P., and Henschke, P.A. 1995a. Validation of bismuth-containing indicator media for predicting H₂S-producing potential of *Saccharomyces cerevisiae* wine yeasts under enological conditions. Am. J. Enol. Vitic. 46:269-273.
- Jiranek, V., Langridge, P., and Henschke, P.A. 1995b. Determination of sulphite reductase activity and its response to assimilable nitrogen status in a commercial *Saccharomyces cerevisiae* wine yeast. J. Appl. Bacteriol. 81:329-36.
- Jiranek, V., Langridge, P., and Henschke, P.A. 1995. Regulation of hydrogen sulfide liberation in wine-producing *Saccharomyces cerevisiae* strains by assimilable nitrogen. Appl. Environ. Microbiol. 61:461-7.
- Newton, G.L., Dorian, R., and Fahey, R.C. 1981. Analysis of biological thiols: derivatization with monobromobimane and separation by reversed-phase high-performance liquid chromatography. Analyt. Biochem. 114:383-387.
- Park, H. and Bakalinsky, A.T. 2000. *SSU1* mediates sulfite efflux in *S. cerevisiae*. Yeast 16:881-888.
- Park, H., Lopez, N.I., and Bakalinsky, A.T. 1999. Use of sulfite resistance in *S. cerevisiae* as a dominant selectable marker. Curr. Genet. 36:339-344.
- Park, S.K., Boulton, R.B., and Noble, A.C. 2000. Formation of hydrogen sulfide and glutathione during fermentation of white grape musts. Am. J. Enol. Vitic. 51:91-97.
- Pinson, B., Sagot, I., and Daignan-Fornier, B. 2000. Identification of genes affecting selenite toxicity and resistance in *Saccharomyces cerevisiae*. Mol. Micro. 36:679-687.
- Rauhut, D. 1993. Production of sulfur compounds. In: Wine Microbiology and Biotechnology, ed. G.H. Fleet, p. 183-223, Harwood Academic Publishers. Chur, Switzerland.
- Salmon, J.-M. and Barre, P. 1998. Improvement of nitrogen assimilation and fermentation kinetics under enological conditions by derepression of alternate nitrogen-assimilatory pathways in an industrial *Saccharomyces cerevisiae* strain. Appl. Env. Micro. 64:3831-3837.
- Thomas, D. and Surdin-Kerjan, Y. 1997. Metabolism of sulfur amino acids in *Saccharomyces cerevisiae*. Micro. Mol. Biol. Rev. 61:503-532.

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Figure 1. Sulfite accumulation in three laboratory strains of *Saccharomyces cerevisiae*, 3163-1b, 3090-9d, and 3090-9d-T4-L1. The data are means of two replicates per strain.

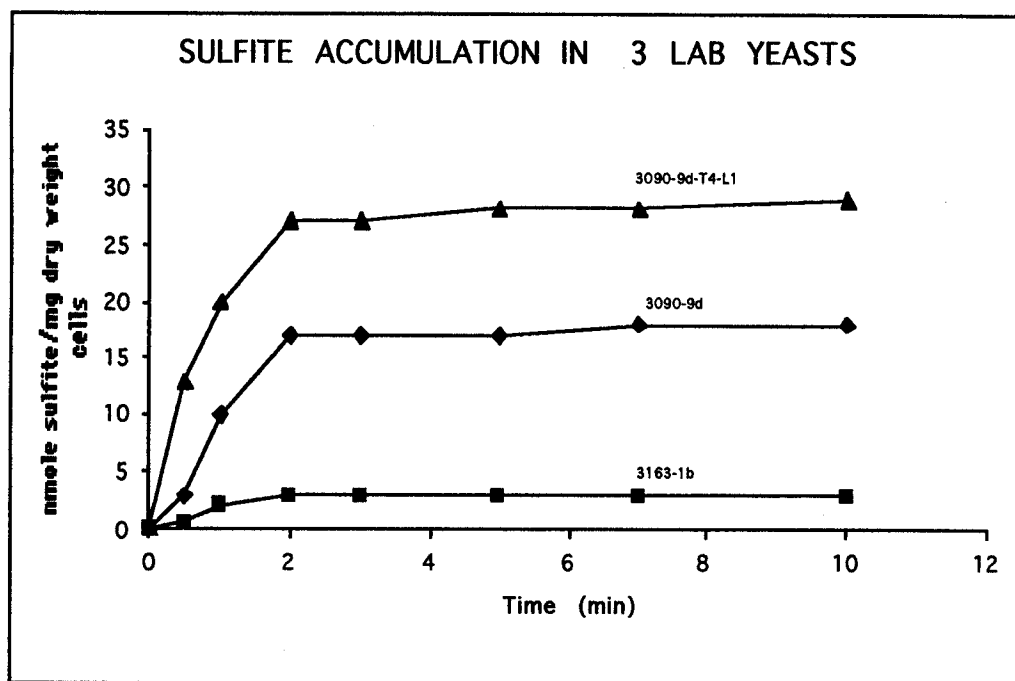


Figure 2. Sulfite accumulation in three wine strains of *Saccharomyces cerevisiae*. The data are means of two replicates, except for strain 6040 which was unreplicated. The data for 6494 overlap that of 6012.

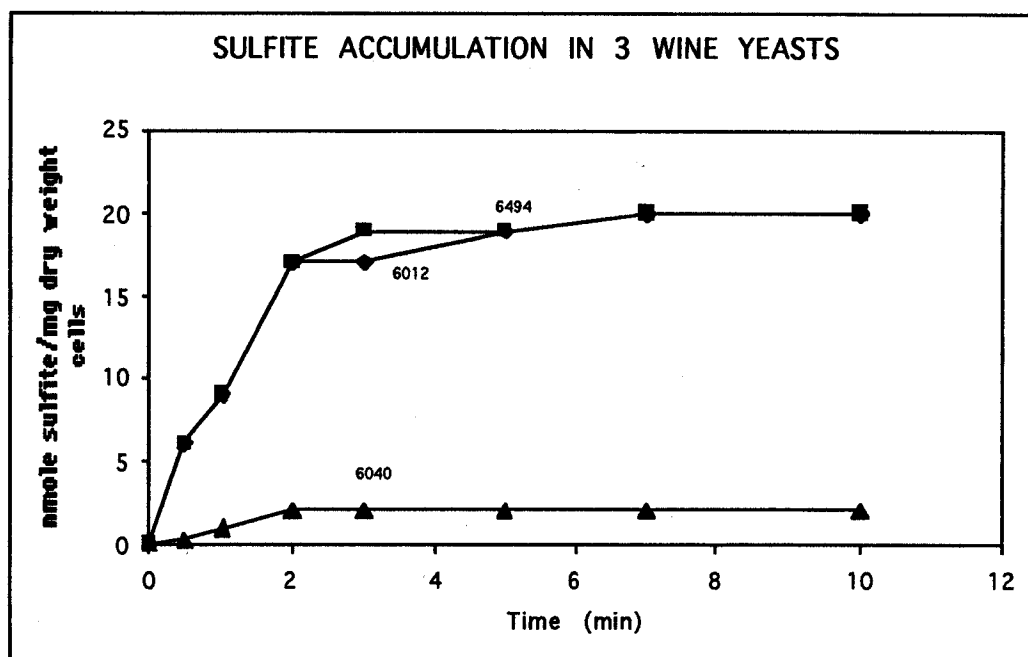


Figure 3. Representative reversed phase HPLC trace of sodium sulfide-bimane adduct eluting at 50.8 minutes. The peak corresponds to 250 picomoles of added sodium sulfide detected by fluorescence (excitation 360 nm, emission 480 nm). The other peaks represent bimane breakdown products.

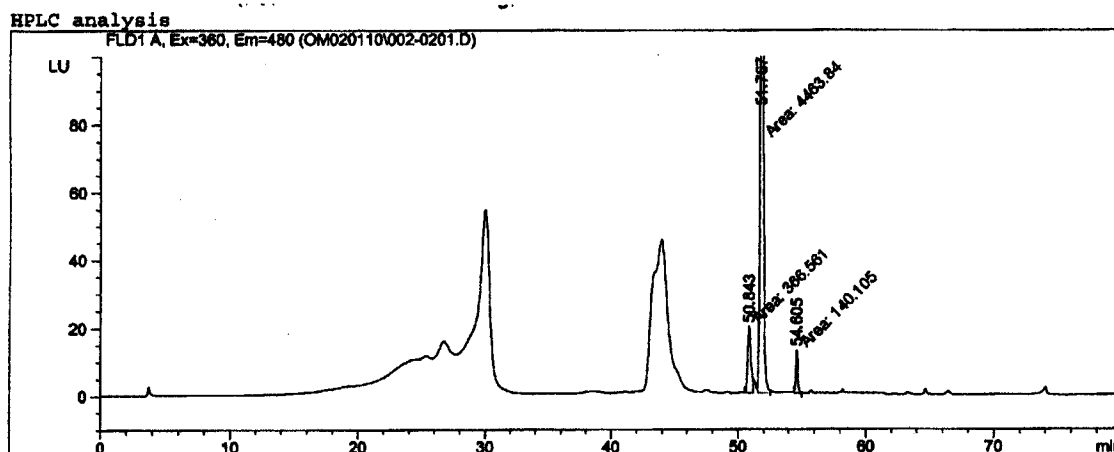


Figure 4. Plot of peak area as a function of increasing amount of sodium sulfide-bimane adduct as detected by fluorescence following separation by reversed phase HPLC.

