Report

AMERICAN VINEYARD FOUNDATION
and
CALIFORNIA COMPETITIVE GRANT PROGRAM FOR RESEARCH IN VITICULTURE AND ENOLOGY
Final Report – January 30, 2004

Project Title: Prediction, Analysis, Prevention and Treatment of Slow and Incomplete Fermentations

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

Objectives and Experiments Conducted to Meet Stated Objectives: This grant was funded as an extension of the Long-Term Research Grant: Analysis of *Saccharomyces* during Normal and Problem Fermentations. Only specific aim 1 of Objective 3 of that grant was funded: *1. Identification and analysis of yeast factors enhancing stress tolerance and promoting fermentation progression.* The specific goals of this aim were to complete the functional genomic analyses of commercial yeast strains under exposure to temperature shock using both transcriptome and proteome technologies. In addition, DNA chip technology was to be used to compare different strains to determine the extent of their genetic relatedness by arraying DNA samples directly. Since individual component of this grant were funded separately, each funded investigator will be submitting their own final report. A summary final report will not be submitted.

Summary of Major Research Accomplishments and Results by Objective: The effect of temperature on the yeast transcriptome was evaluated using two types of experiments. In the first experiment, cells were grown at a single temperature, either 25 or 35°C. In the second experiment, cells growing at 25°C were shifted to 35°C at various time points, with a focus on shifting at 8 and 11% ethanol. Previous work in juice had shown that a shift from 25 to 35°C early in fermentation resulted in arrest of fermentation once 11% ethanol had been attained. However, if the shift occurred later in the fermentation, after attainment of 11% ethanol the fermentation went on to dryness with a final ethanol content of 13%. The purpose of these experiments was to compare the differences between shift to a high temperature at a specific ethanol concentration and growth to that ethanol concentration at a constant temperature. The stains used were *Saccharomyces cerevisiae* race *cerevisiae* and *Saccharomyces cerevisiae* race *bayanus*.

Representative strains of *Saccharomyces cerevisiae cerevisiae* (Cote des Blancs) and *Saccharomyces cerevisiae bayanus* (Premier cuvee) were grown at 25 or 35°C. Cote des Blancs exhibited a greater difference in numbers of genes and gene families expressed at the two different temperatures as compared to Premier cuvee. Premier cuvee was found in earlier studies to be fairly resistant to temperature upshift while Cote des Blancs was not. The strains did display similar responses to elevation in temperature and in transit from active growth to stationary phase metabolism. Premier cuvee seems to have a higher basal level of induction of heat shock or stationary phase genes than Cote des Blancs. Premier cuvee also induced genes at high temperature not induced in Cote des Blancs, such as the *PMA2* gene, which is involved in proton homeostasis. Thus, temperature resistance in Premier cuvee may also be due to the particular genes that are expressed rather than just to the early expression of heat shock genes. Comparing both strains, only 5 genes increased in common upon entry into stationary phase at 35°C that did not increase during stationary phase at 25°C: *DDP1, NPR2, MNR2, OSM1* and *AQY1*. Of these genes, only *AQY1* was found to increase under nitrogen limitation. Thus, the expression of *AQY1* may represent a condition of extreme stress for the yeast.

In addition to these strains, temperature shift experiments were also conducted with two additional commercial strains of *S. cerevisiae cerevisiae*, Montrachet and French Red, and one additional strain of *S. cerevisiae bayanus*, Uvaferm 43. Strains were grown at 25°C then shifted to 35°C at 7-8% and 10-11% ethanol. In contrast to Cote des Blancs, but similarly to Premier cuvee, all three of these strains were able to complete fermentation post-shift. French Red was slightly more sluggish than the other strains, but did go to dryness. Six genes were found to be expressed following temperature up-shift at either ethanol concentration: YPR015C, YOL932W, YBL029W, *BTN2, ZPR1, FES1*. Interestingly, these are not the same genes listed above that consistently appear in strains grown at a higher temperature, but reflect instead a shift to a higher temperature. The differences are not unexpected. In the case of growth at a higher temperature, cells are able to alter the composition of the plasma membrane and other components in the bud. The shift experiment occurs at a high ethanol concentration, after growth has been inhibited. Certain changes, such as insertion of proteins into the plasma membrane, are not possible in the absence of new growth.

The transcriptional response to downshift at 8% ethanol differed from the response at 11%. At 8% all strains decreased expression of several gene families with roles in protein production, growth, secretion, mRNA processing and substrate transport. We did not see an increase in many of the stress response or heat shock genes, although some members of these families did increase in expression level. This is most likely because expression of many of these genes is already elevated during stationary phase, and that by the time the cells reach 8% ethanol they are already under some level of stress. The transcriptional response at 11% was not nearly as great as that observed at 8% for Montrachet and French Red. Uvaferm 43, known to be a very ethanol tolerant strain, showed similar responses at both 8 and 11% ethanol. Thus this stain appears to be fully able of mounting a response to a change in temperature at elevated ethanol levels, confirming the robustness of this strain in restart of arrested fermentations.

We are also evaluating the proteome of strains under identical shift conditions. This work is still in progress as the morphing analysis of the 2D gels is quite time consuming. The initial results appear to support the transcriptome analysis in terms of the magnitude of a response. It is projected that this analysis will be complete by the end of this grant period

S. cerevisiae bayanus had been shortened in the wine literature to *S. bayanus*. However, taxonomists recently changed this convention and decided that *S. bayanus* would be the species name used for a genetically identical group of *Saccharomyces* strains that had other designations and that formed a species distinct from *S. cerevisiae* but which included the original "type strain" of *S. bayanus*. True *S. bayanus* is being sequenced and 98% of the genome is now known (Kellis, et al.). *S. bayanus* is quite different from *S. cerevisiae bayanus*. All strains that we have evaluated are variants of *S. cerevisiae* based on this comparison. Since there was confusion over the nature of the commercial strains used in the last review, following discussion with the advisory committee, we added an objective to the original grant, that of evaluating the relatedness among strains of S. cerevisiae. This is to be done using direct hybridization with DNA to AFFYmetrix DNA chips. Comparison of the transcriptome profiles of these strains strongly suggests that the DNA relatedness will be too high for any differences to be detected on the DNA chips. Nonetheless, this experiment will be conducted in the remaining two months of the grant.

Outside Presentation of Results:

This work or parts thereof, were presented at the ASEV meeting in Reno, by Paula Mara and Stacey Nugent (winner of the best oral enology presentation). Other work from previous grant periods of the stuck fermentation grant was also presented at this meeting, by Vidhya Ramakrishnan. This work has also been presented at various meetings around the state (by L. Bisson), most recently at a meeting in Paso Robles organized by the American Vineyard Foundation, as well as used in short courses for industry members offered on the Davis campus. During this grant period, Jeff Mangahas completed writing of his MS thesis as has Joel Mann. Vidhya Ramakrishnan completed her PhD dissertation and was awarded the PhD degree in Microbiology.

Research Success Statements:

Stuck and sluggish fermentations are a chronic problem for the wine industry. Many causes of arrest and fermentation unpredictability have been determined and fermentation management practices to mitigate these effects have been developed. Stuck fermentations have not been completely eliminated as there still remain some factors, which impact yeast fermentation ability. One of the most common remaining causes of arrest of fermentation is temperature shock. Other causes include the presence of inhibitory microbes (such as certain species of *Lactobacillus*) and juice or grape specific factors that are not nutritional (which are in the process of being identified).

The goal of this proposal was an analysis of the impact of temperature on yeast and developing a greater understanding of when such a temperature effect will lead to arrest and when it will not. Both strain and nutritional issues are important in determining if temperature shock will lead to arrest, but by far the most important variable is the ethanol concentration both at which the shock occurs and which the fermentation will need to attain in order to be considered "dry". Although our work has clearly defined the impact of temperature shock or of elevated temperature on the production of ethanol, obviously we have not been able to prevent the refrigeration failures that lead to the increase in temperature in the first place nor discourage the important practice of long hang times which lead to high ethanol levels. Therefore we have focused on understanding the temperature-ethanol tolerance of those strains that are tolerant, such as Uvaferm 43. This will allow the identification and physiological characterization of even more temperature tolerant strains. We have also identified molecular markers that indicate exposure to extremes of temperature and those that indicate the cells have adapted. If it can be determined early on that the cells have not adapted, then an immediate re-inoculation can occur rather than waiting for full arrest.

Although not fully detailed here, this research has provided a wealth of transcriptome information on the response of yeast cells to elevated temperature. Many of the gene families that change are consistent with what was already known about arrest of growth and of fermentation. Of more interest were the findings of the response during shift of non-growing or stationary phase cells.

Funds Status:

The funds for this proposal will be liened or spent by the termination date of the grant, March 31, 2004.

Literature Cited:

Kellis, M., N. Patterson, M. Endrizzi, B. Birren and E. S. Lander. 2003. Sequencing and comparison of yeast species to identify genes and regulatory elements. Nature 423:241-254.

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Summary:

During this grant period, the transcriptome analyses of the impact of temperature shock at differing ethanol concentrations was completed. A total of five commercial strains were evaluated. In general, strains classified as *S. cerevisiae* race *bayanus* showed higher ethanol and temperature tolerance than strains classified as S. cerevisiae race cerevisiae. Thus we think these distinctions by race are meaningful, even if there is confusion between *Saccharomyces bayanus* and *Saccharomyces cerevisiae bayanus*, two very distantly related species of *Saccharomyces*. Temperature tolerance was correlated with ethanol tolerance. Those stains not inhibited by ethanol were able to mount a transcriptional response to elevated temperature. Strains that were more ethanol sensitive responded to a shift in temperature at 8% ethanol, but not at 11%, under conditions where dryness was attained at 13% ethanol. Thus temperature affects the ethanol tolerance of the strains. Several specific genes were identified that increased or decreased in expression across all strains. These genes are candidates for molecular markers of high temperature exposure. In addition, temperature tolerant strains underwent changes not observed in temperature sensitive strains. The expression of these genes could be used to indicate a successful adaptation to temperature. This work is also characterizing the variability in response to ethanol across commercial and novel isolates of *Saccharomyces*. It will now be possible to screen strains more readily for their commercial potential as high temperature fermenters.

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