AMERICAN VINEYARD FOUNDATION
and
CALIFORNIA COMPETITIVE GRANT PROGRAM FOR RESEARCH IN VITICULTURE AND ENOLOGY
Annual Progress Report, February 1, 2002

Comment:

Project Title: Analysis of *Saccharomyces* during Normal and Problem Fermentations

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Objectives and Experiments Conducted to Meet Stated Objectives: The goal of this proposal is to develop a more sophisticated understanding of the biology of the yeast *Saccharomyces* during both normal and problem enological fermentations. This information will be utilized to generate better methods for the prediction and treatment of problems in yeast progression during fermentation. There are three specific objectives of the proposal. (Funding initiated in October 1998).

1. Gene Expression Pattern and Protein Profile Analysis of Normal and Stuck and Sluggish Fermentations using Microarray and Proteome Analyses
2. Development of Technology for the Prediction of Stuck and Sluggish Fermentations Using Development of Mechanistic Models and Artificial Neural Networks Trained with Historical Data
3. Characterization of Microbial Populations in Normal and Stuck Fermentations

Summary of Major Research Accomplishments and Results by Objective(s):

Objective 1 Gene Expression Pattern and Protein Profile Analysis of Normal and Stuck and Sluggish Fermentations using Microarray and Proteome Analyses. There are four specific goals of this objective: 1) Functional genomic and biophysical analysis of normal and induced-stress fermentations; 2) Functional genomic analysis of arrested fermentation samples from wineries; 3) Functional genomic analysis of addition of nitrogen and oxygen; 4) Analysis of fructose to glucose ration under stress and re-initiation of fermentation. Budget cuts from last year's request resulted in the postponement of the biophysical analysis of goal 1 and most aims of goal 4.

Functional genomic and biophysical analysis of normal and induced-stress fermentations. In the past year, the analysis of normal and nitrogen-limited fermentations by both microarray and proteome comparisons was conducted. The microarray analysis of nitrogen limitation was published (Backhus, et al, 2001). The proteome analysis is finishing and will be submitted for publication soon. Temperature shock and acetic acid challenge assays were performed which has set the stage for microarray and proteome analyses of the physiological impact of these two

critical stress factors. Several yeast strains have been evaluated for their resistance to heat shock in both synthetic juice and natural grape juices and musts (LFB lab). Strains have been divided into those that display high sensitivity to temperature fluctuation and those that do not. Strains are further divided by their ability to maintain fermentation rates at extremes of low or high temperature. So far no strain has been identified that is capable of doing both. Equally good progress to study the effect of acetic acid on fermentation rate and its possible contribution to sluggish and stuck fermentations has been made (HVV lab). Growth curves of the yeast Cotes de blanc have been completed and the effect of different concentrations of acetic acid on the growth and fermentation rate of the yeast are currently being determined. Once these parameters have been established, DNA array analysis will be performed on the yeast cells to determine how acetic acid affects the expression of genes in the yeast cell. Microarray analysis of the impact of temperature on fermentation rates is underway. Messenger RNA has been prepared from replicate samples of strains for roughly 80% of the conditions to be evaluated and the DNA chip analysis will soon commence once all samples are available. This is to avoid any potential lot-to-lot variation with the DNA chips.

Reference gels have been created for the proteome analysis of yeast strains from synthetic grape juice. Over 30 proteins have been unequivocally identified using peptide mass fingerprinting. A sample reference gel is attached. Three gels are presented, one for strain UCD940 from exponential phase and one for the same strain from stationary phase in a standard yeast laboratory medium, YNB with amino acid supplementation. The third figure is of strain UCD932 grown to stationary phase in a synthetic juice medium. This is one gel taken from a time course conducted for this strain. Comparison of the gels indicates that replicate spots can be identified. With knowledge of the identity of only a few spots the gels can be accurately morphed allowing direct comparisons. Proteins listed as identified are unequivocally that protein species. As an experiment to determine if the identity of individual species of proteins could be determined if they were present in a mixture on the gel, protein digests were mixed prior to MALDI-TOF peptide mass fingerprinting. The analysis of the peptide fragments obtained correctly identified the source as a mixture of the two proteins. Thus we are able to identify individual proteins co-migrating on the gel using this analytical tool.

Cluster analysis of protein expression patterns of UCD932 during nutrient sufficient growth in synthetic juice media allowed spots to be divided into several groupings. One class showed high-level expression only during active growth, with proteins not highly represented in gels from stationary phase cells. These proteins decreased during early exponential phase, peaking in the first 10 to 12 hours of growth and reaching a low plateau by 48 hours, or early stationary phase. A second class of proteins peaked at 40 to 48 hours, at the tail end of active growth. Some of these proteins remained at the same level throughout stationary phase (present at end of the experiment, 120 hours) while others dropped within 48 hours of peak expression. A third class of proteins peaked at both 12 and 48 hours. The fourth class of proteins peaked late in stationary (60+ hours), as ethanol has accumulated significantly. Peptide mass fingerprint analysis was used to identify representatives of each class of proteins. Major proteins peaking in early exponential phase are largely enzymes of the glycolytic pathway. Interestingly, at the start of stationary, many of the peptides appearing were degradation products of the glycolytic proteins that appear to be stable, that is, are not degraded to amino acids and small peptides. This may be a significant observation as there was a report in the literature that protein degradation products could afford some protection against environmental stresses such as heat

and ethanol shock. The Met17p protein, involved in methionine biosynthesis also peaks at the end of exponential phase. In other cases, the migration of the glycolytic protein is changing as the culture ages, which suggests specific types of post-translational modifications are occurring that are correlated with the physiological status of the culture.

Progress on the sub-objective of the analysis of the impact of phenolic compounds on yeast physiology has also occurred. During past years of this project we have focused on developing methodologies which will allow us to monitor the antioxidant activity of polyphenols in yeast cells following exposure to oxidative stresses. Preliminary experiments have shown that polyphenols such as resveratrol and catechin may be protective against lipid peroxidation in *Saccharomyces* (Prise de Mousse, UCD594). Quercetin may have protective effects against DNA oxidation at low concentrations in vivo.

Flavonoid-DNA binding is one way in which flavonoids may act at very low levels to prevent DNA oxidation. During the past year we have focused on methods for measuring flavonoid-DNA binding in solution. Factors which affect the solution binding of these compounds such as pH, ionic strength, temperature, solution polarity and solute concentration have been studied using UV spectroscopy. The self- association of certain flavonoids as well as their binding to DNA has been found to be exquisitely sensitive to all of these conditions. Evidence that flavonoids bind to DNA has been presented, but interpretation of the UV spectroscopic data has not been clear-cut due to the many interacting factors that are possible. Therefore, we have used additional tools to characterize flavonoid-DNA binding. One of these assays allows us to study both intercalative binding to DNA and topoisomerase I inhibition. Using this assay the flavonoids quercetin and myricetin have been shown to bind to DNA through intercalation. We have also initiated NMR studies to characterize flavonoid-DNA binding mechanism in more detail and initial results have been very promising.

Results from work will begin to identify specific phenols, which can act as antioxidants *in vivo* as well as providing details about their mechanism of action. Much of the current literature regarding antioxidants is based on in vitro assays; it is therefore essential that the activity of these compounds be confirmed *in vivo*. The *in vivo* analytical assays described, combined with the results from Objectives #1 and #2, will provide important information about the molecular mechanisms by which antioxidants can influence *in vivo* oxidative status. Recent papers appearing in the literature continue to document the role of oxidative stress as exacerbating heat ant ethanol stress. Phenolic compounds may enhance or inhibit some of these effects which may explain the large variability in response to ethanol and heat shock displayed by the same strain in juices of differing phenolic composition.

Functional genomic analysis of arrested fermentation samples from wineries. Samples from the 2001 harvest have not yet been processed. Analysis of earlier samples obtained from commercial wineries indicated that there is considerable mRNA degradation during the processing of the RNA for DNA chip analysis. While it is possible to stabilize the RNA as determined by the Mills laboratory in Objective 3, the persistence of the mRNA of non-Saccharomyces yeast strains and the cross-hybridization of that mRNA to the oligonucleotides on the DNA chips will make microarray analysis of these samples technically quite challenging. Proteome analysis does not suffer from the limitations of cross-hybridization and indeed has been used successfully to identify protein species from two different *Saccharomyces* species in a

mix. Therefore, we will focus our efforts in this sub-objective on a proteomic analysis of the samples from normal and arrested fermentations. The collection of samples will continue in the 2002 harvest, as described under Objective 2.

Functional genomic analysis of addition of nitrogen and oxygen. The effect of timed diammonium phosphate (DAP) additions on the genome–wide expression of genes in wine yeast were investigated (HVV lab). DAP was added to fermenting grape juice after inoculation, and after 30%, 60% and 90% sugars were fermented. Yeast cells were harvested two hours after each time point from fermenting grape juice to which DAP was added, and from the corresponding control fermentation (no DAP). DNA array analyses were performed on yeast cells using the Affymetrix technology. A summary of chip analyses completed is shown in Table 1. The companion analysis of the effects of oxygen addition has not yet been initiated as we are awaiting completion of some preliminary oxygen addition trials (LFB lab).

Table 1: Overview of project progress. The time indicates when the fermentation was split and DAP was added. Total RNA was extracted two hours later.

Experiment	Time (hrs)		DAP added	Chip complete	
	Trial 1	Trial 2		Trial 1	Trial 2
EC	24	24	-	Yes	No
ED			+	Yes	No
30C	69.0	66.4	-	Yes	Yes
30D			+	Yes	Yes
60C	146.7	138.6	-	Yes	No
60D			+	Yes	No
90C	325.6	357.2	-	Yes	No
90D			+	Yes	No

mRNA has successfully been isolated from remaining duplicate fermentations and the array analyses will be completed during 2002.

No problems were encountered with array analyses during later stages of fermentation and we were able to monitor the expression of at least 4500 genes after 90% sugars were fermented.

The data were analyzed and clustered using the Affymetrix DMTv3.0 software. Based on similar expression patterns, genes were clustered into 18 different clusters (Fig. 1).

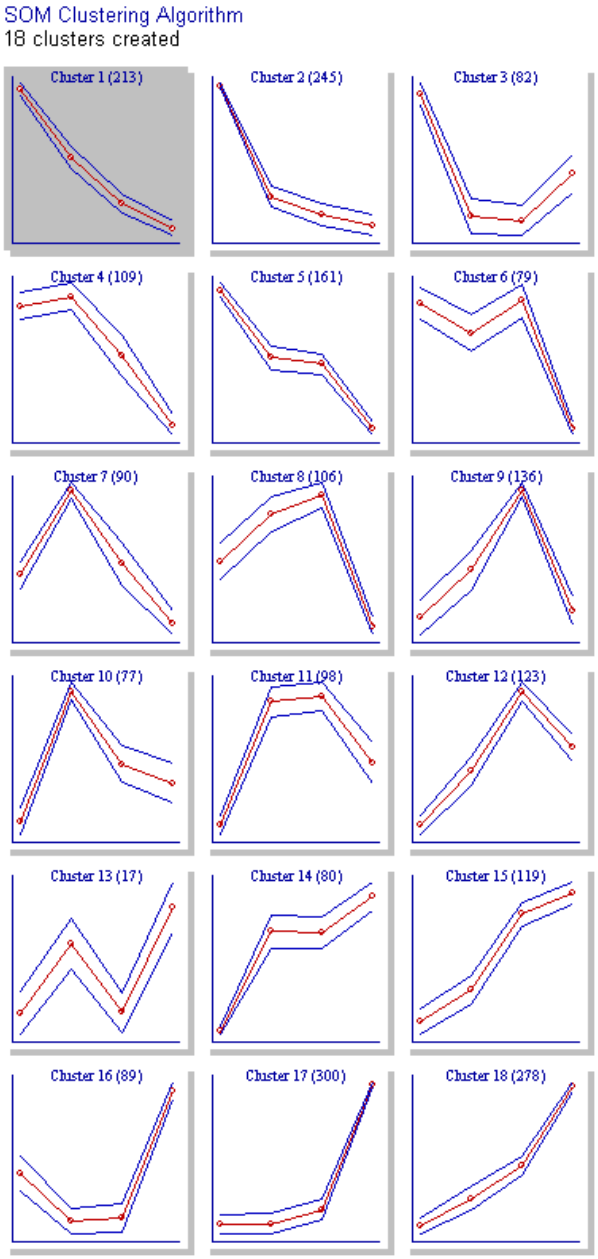


Figure 1: Clusters of probe sets with the same expression patterns, the number in brackets represent the number of probe sets within the cluster. Data represents the signal of the probe set at four stages in the fermentation (<1%, 30%, 60%, and 90% sugars fermented). Clusters were generated with Affymetrix DMT v3.0.

The genes clustered in clusters 1, 2, 17 and 18 were grouped according to cellular function as an example. Data obtained from all clusters will be analyzed (using results from duplicate runs) during 2002 to establish the effect of DAP additions during different time points of fermentation.

Table 2: Categorization of genes found in four clusters, organized by cellular role, as defined by YPD

CELLULAR ROLE	CLUSTER			
	1	2	17	18
aging	0	2	2	2
amino acid metabolism	12	24	7	6
carbohydrate metabolism	4	7	5	14
cell cycle control	4	5	7	4
cell polarity	0	4	3	0
cell stress	4	6	10	15
cell structure	2	3	3	0
cell wall maintenance	3	6	8	9
chromatin/chromosome structure	4	4	7	9
differentiation	3	0	1	7
DNA repair	0	1	2	6
DNA synthesis	2	0	3	3
energy generation	6	1	2	2
lipid, fatty acid and sterol metabolism	6	5	1	9
mating response	6	1	1	6
meiosis	2	2	4	8
membrane fusion	2	1	1	0
mitosis	0	3	6	3
nuclear-cytoplasmic transport	3	2	2	3
other	0	0	2	0
other metabolism	6	7	3	8
Pol II transcription	5	3	12	23
protein degradation	1	0	2	1
protein folding	7	1	8	3
protein modification	7	7	5	5
protein synthesis	76	63	3	4
protein translocation	7	0	5	2
Recombination	0	1	4	2
RNA processing/modification	15	3	10	6
RNA splicing	2	0	4	2
RNA turnover	2	2	1	1
signal transduction	2	2	6	7
small molecule transport	15	14	14	21
unknown	37	42	58	98
vesicular transport	3	8	7	2

Data clearly illustrates that we have overcome the difficulties of doing DNA array analysis on industrial wine yeast strains during later stages of fermentation. This technology can now be applied to study the response of yeast cells to DAP addition during fermentation and its effect on problem fermentations.

Analysis of fructose to glucose ration under stress and re-initiation of fermentation. This goal, which represented a new goal added to the original project, was not funded last round. Therefore no progress was made on the specific aims. This goal is again included in the next round of funding as it is an important aspect of the global study of arrested fermentations. The aim of transcriptome analysis of the sugar kinase mutant strains and of cAMP levels, identified as playing a potential key role in fermentation arrest from the microarray analysis of nitrogen limited cultures, was initiated as the lead student researcher, PhD candidate Viyada Kunathigan, is being supported by the government of Thailand. The Affymetrix DNA chip analysis of these mutant lab strains is in progress.

Objective 2: Development of Technology for the Prediction of Stuck and Sluggish Fermentations Using Mechanistic Models and Artificial Neural Networks Trained with Historical Data.

Use of a mechanistic model to predict methods for resolving problem fermentations. As reported previously, we have developed a mechanistic model to help explain wine fermentation kinetics [Cramer et al., 2002]. In addition to predicting wine fermentation kinetics as a function of initial sugar and nitrogen levels, we demonstrated that this model can also be used to suggest strategies for resolving fermentations destined to be either sluggish or stuck. The predictions that we made and confirmed experimentally fell into two categories. First, we evaluated the use of diammonium phosphate (DAP) additions at various times during fermentations that would otherwise become stuck. The model predicted that DAP additions would be effective at allowing complete sugar utilization any time during the course of the fermentation. Using a series of 400 ml flask fermentations with diluted Chardonnay juice with sugar and nitrogen added back to appropriate levels, we reproduced these simulations experimentally. We found that for addition times from 20 hours to at least 120 hours there was good agreement with model predictions. However, we did find that, unlike the model prediction, addition of DAP right away at 0 hours was not as effective as later addition. In conjunction with Dr. Mills (UC Davis), we are currently evaluating whether this effect is due to the presence and growth of organisms other than *S. bayanus*. In addition, we found experimentally that late additions of DAP were also not effective in causing a complete fermentation. From analysis of the nitrogen utilization kinetics in these experiments, it is evident that, after some point in the fermentation, the yeast are no longer capable of transporting and utilizing the nitrogen. Along with experiments with DAP additions, we also tried supplying nitrogen in the form of yeast extract (Tastone 154) and SuperFood. Yeast extract gave similar results to DAP, while SuperFood was able to resolve the fermentation problem even when added at 0 hours (though late additions remained ineffective).

The second main category of predictions and experiments that we completed was using additions of actively growing cells (about 0.9 g cells/L), again at various times during fermentations destined to become stuck. In this case, the model predicted that cell additions would be effective

at any time during the fermentation. The parallel experiments confirmed these results, including late additions that did not work with DAP additions.

We had previously shown that cells were likely becoming inactive, but not dying, during stuck fermentations. During the last year, we have confirmed this result using plating and colony counts in addition to the methylene blue staining used previously. Along the same lines, we have conducted some preliminary experiments with Dr. Marjorie Longo (UC Davis, Chemical Engineering and Materials Science) to examine the physical nature of this inactivation. In these experiments, we examined the properties of pure lipid bilayers in the presence of ethanol using micropipette aspiration studies. These initial studies indicate that there is, in fact, a significant effect of ethanol on the structure of lipid bilayers.

Portable cell concentration probe for early detection of problem fermentations. Previously, we had demonstrated that the maximum cell concentration in wine fermentations (usually reached within the first two days of the process) is directly proportional to the maximum sugar utilization rate. Experimentally, we found that all fermentations below a certain maximum cell concentration became stuck. Therefore, we proposed designing a portable solids concentration probe that could be taken from tank to tank and from barrel to barrel to check that some minimum cell concentration level was attained. Initially, we worked with Wedgewood Technology in San Carlos, and will continue to do so. However, we have run into scheduling problems as they are in the process of being acquired by a larger company. Therefore, we have also begun to pursue this idea with two other companies, both represented by GusmerCellulo and both with previous experience in the brewing industry.

Assessment of critical inputs in determining fermentations kinetics. We have shown that artificial neural networks should be capable of predicting fermentation kinetics accurately based solely on juice characteristics and intended processing. However, which juice characteristics and processing parameters are actually critical in determining kinetics are not known. Therefore, in the past year, we collected juice (just prior to inoculation) and wine samples from 224 Chardonnay fermentations from 11 wineries in California. We also collected information on processing and the associated Brix curves. We have just started analyzing the Brix curves and have been able to classify between 20 and 70 of these as sluggish or stuck using various definitions. We will take the samples from all of these fermentations and some of the normal fermentations and perform detailed analysis of the composition of the juice. This analysis will include sugars, organic acids, ammonia, alpha-amino nitrogen, individual amino acids, metals, fatty acids, and vitamins. As part of Objective 3, we will also assess the microbial ecology of these samples. We will then use computational techniques such as decision tree analysis to identify the critical parameters that determine fermentation kinetics.

In order to measure vitamin concentration in juice and wine we are currently working on an HPLC-based assay for the water-soluble vitamins. While this method seems to separate the vitamins sufficiently, the detection limits may not be low enough to accurately measure levels in wine. Therefore, we are currently exploring other options for this analysis. We have established methods for the remainder of the analyses.

During the past year, we have further developed our decision tree analysis algorithm for identifying critical input parameters in process databases. Initially, we used a simple Shannon

entropy calculation as a means of quantifying the degree of classification for a given input (this is called a "decision metric") [Subramanian et al., 2001]. However, in the past year, we have also examined other metrics such as a normalized gain and an exact probability metric that reduce some of the biases found with Shannon entropy [Buck et al., submitted]. All of these algorithms will be used on the database when it is complete. As the general performance of these algorithms improves with increasing database size, we will be repeating our sampling in 2002, as well as hopefully collecting samples with a collaborator in Chile (Dr. Eduardo Agosin, Pontificia Universidad Catolica de Chile) in the next few months.

Objective 3: Characterization of Microbial Populations in Normal and Stuck Fermentations.

Optimization of PCR-DGGE for Bacterial Populations (DAM). Numerous primers for amplification of bacterial 16S rDNA have been used in PCR-DGGE studies to characterize bacterial communities. We discovered that often-used primer sets amplify yeast (and fungal) 18S genes, or grape plastid DNA. Since these eukaryotic DNAs are commonly found in wine fermentation samples it was critical to develop specific primer sets to amplify the bacteria found in wine, primarily the lactic acid bacteria (LAB) and acetic acid bacteria (AAB). To accomplish this we systematically evaluated various published primer sets in different combinations for amplification of bacterial, fungal, yeast or grape (Chardonnay) DNA. Unfortunately none of these primers were specific for bacteria. We then designed and analyzed several new primers and demonstrated that two sets, termed WLAB1&2 and WBAC1&2, specifically amplify the bacteria found in wine. One set, WLAB1&2, specifically amplifies the LAB and, to my knowledge, is the only current set that covers the complete range of LAB found in wine. Previously published primers for specific amplification of LAB for DGGE did not amplify Oenococcus oeni, the common ML starter. The second primer set, WBAC1&2 amplifies both the LAB and AAB found in wine. Both WLAB1&2 and WBAC1&2 were shown to specifically amplify bacterial DNA from mixtures of yeast and bacteria in wine. These primers will be implemented in next specific goal (below). This work has been submitted for presentation at the 7th Symposium on Lactic Acid Bacteria in fall of 2002. A manuscript on the development and use of these primers is also in preparation.

Bacterial overgrowth can be a factor in stuck fermentations. One off-shoot of the development of these primers is a new project to characterize the efficacy of lysozyme treatment for lowering bacterial populations in must. During the 2001 harvest, identical trial fermentations were carried out with or without a lysozyme addition. PCR-DGGE and RT-PCR-DGGE (using the WLAB and WBAC primer pairs) will be employed on these samples from these fermentations to discern, by direct analysis, if bacterial populations are differentially impacted by lysozyme treatment.

Analysis of Normal and Stuck Fermentations by PCR-DGGE (DAM, DEB). This goal is to provide initial and final microbial population data on the samples collected in Objective 2. To accomplish this a technical problem needed to be surmounted. In previous DGGE work, we noted that a high level inoculum of *Saccharomyces* can effectively mask lower populations of non-*Saccharomyces* yeasts in DGGE gels. This masking effect is an outcome of a PCR reaction in which a higher concentration template (*Saccharomyces*) is preferentially rapidly amplified over alternate templates at lower concentration. In effort to maximize amplification from these alternate templates, we modified PCR conditions by altering nucleotide, $MgCl_2$ and template

ratios. An outcome of this work is a dual PCR scheme, whereby one set of reaction conditions is used prior to inoculation by *Saccharomyces* and another is used post inoculation. DGGE analysis on samples obtained for Objective 2 (collected during Fall 2001) is currently underway.

Development of Real-Time PCR (RT-PCR) methods for Quantitation of Bacterial and Yeast Populations (DAM). The goal of this objective is to use RT-PCR on the samples collected in Objective 2 in order to quantitate specific microbial populations identified by PCR-DGGE. An additional goal of this approach is to use RNA as a template since RNA is a labile molecule and a better indicator of cell viability (DNA may persist in wine fermentations after cells have died). Collaborators at Gallo (N. Ireland) have developed specific PCR primers and methods for many of the yeasts found in wine. Unfortunately these primers are unsuitable for RNA analysis since one primer anneals within the yeast ITS region. The ITS region is processed out during the formation of 18S and 26S rRNAs and thus not a part of the intact ribosome. Therefore to obtain specific PCR primers that could be used on RNA templates, we designed novel primers based on the yeast 26S D1/D2 region sequence, for which an extensive database exists. By comparing D1/D2 sequence for various wine yeasts we readily designed primers specific for Hanseniaspora uvarum (anamorph Kloeckera apiculata) and Candida stellata. We examined specificity of these primers in mixed culture and, in the case of Candida, employed the PCR amplicons as probes in semi-quantitative slot blots. In taking this approach we demonstrated a large viable but-not-culturable (VBNC) Candida population in a commercial wine fermentation (Mills et al, submitted). Work to design specific 26S-based primers pairs for all the common yeasts found in the wine environment is underway. This will employ numerous isolates from the VEN culture collection to determine genera or species specificity. Specific primers for the bacteria found in wine already exist, such that specific PCR of bacterial populations should be reasonably straightforward. Detection and quantitation of VBNC yeast and bacterial populations is important for potential impact on Saccharomyces growth and physiology as well as possible influence of wine flavor.

At the time the original grant was written (2001) we also asked for a RT-PCR thermocycler to allow quantitation of templates using the specific primers. Since that time another collaborator (Jean VanderGheynst) purchased a RT-PCR thermocycler that she is willing to share with this project. Thus a prominent objective this year will be to test reproducibility and accuracy of RT-PCR using the yeast and bacterial primers obtained (or developed). This will entail quantitation of specific levels of wine-related yeast or bacteria from known microbial mixtures. Additionally we will examine the applicability of our current methods for isolating DNA and RNA from wine samples for RT-PCR of both bacteria and yeast populations (specific primers sets can be differentially sensitive to potential inhibitors from grape juice). Finally, once the initial methods have been developed, we will quantitate different yeast and bacterial populations from the winery fermentation samples collected as part of Objective 2. This analysis will be predicated on the initial DGGE results.

Specific Goal 4. *Characterization of Bacteria with Antifungal Properties (CGE)*

This goal was dropped due to lack of funding.

Publications or Reports Resulting from Project.

Presentations of Research: Results of Objective 1 were presented at two international meetings by LFB. Intervitis Interfructa 2001 (Stuttgart, May 14-16) and at Enoforum 2001 a meeting of the Societa Italiana de Viticoltura ed Enologia also in May of 2001 (Montesilvano). There have been no outside presentations of the results of the phenolic analyses due to their preliminary nature. However, students conducting this research have incorporated their findings into their dissertations and have presented them during their required in-house exit seminars.

Presentations on this work were given at the RAVE meeting at UC Davis in March, at the ASEV meeting in San Diego in June, and in an invited presentation at the Pontificia Universidad Catolica de Chile in November

Mills, D. A., E. Johannsen, and L. Cocolin. Temperature effects on yeast diversity and persistence in sweet white wine fermentations. (submitted to Applied and Environmental Microbiology).

Cocolin, L. and D. A. Mills. A comparative analysis of direct molecular methods for characterizing yeast populations in wine fermentations: evidence of viable but non-culturable yeast populations (manuscript in preparation & submitted for presentation at the 2002 Annual Meeting of ASEV).

I. Lopez, E. Orr L. Cocolin, M. Marshall, F. Ruiz-Larrea, J. VanderGheynst, and D. A. Mills Design and evaluation of PCR primers for direct analysis of bacterial populations in wine (manuscript in preparation & submitted for presentation at the 7th Symposium on Lactic Acid Bacteria, Sept. 2002).

J. Brown, PhD Thesis. In preparation.
C. Cooney. MS Thesis. In preparation.
E. Johannsen. MS Thesis. In preparation.
K. Niznik. MS Thesis, In preparation.
E. Orr. MS Thesis. In preparation.
J. Shreiber MS Thesis. In preparation

Publications:
Backhus, L. E., J. DeRisi, P. O. Brown and L. F. Bisson. (2001) Functional genomic analysis of a commercial wine strain of *Saccharomyces cerevisiae* under differing nitrogen conditions. FEMS Yeast Research 1:111-126.

Bisson, L. F. and D. E. Block. Ethanol tolerance in *Saccharomyces*. Submitted to **"Biodiversity and Biotechnology of Wine Yeast,"** (invited review), Research Signpost Publisher.

Cocolin, L., L. F. Bisson and D. A. Mills (2000). Direct profiling of the yeast dynamics in wine fermentations. FEMS Microbiology Letters 189: 81-87.

Cocolin, L., Heisey A. and D. A. Mills (2001). "Direct identification of the indigenous yeasts in commercial wine fermentations." American Journal of Enology and Viticulture, in press.

Cramer, A. C., S. Vlassides, and D. E. Block (2002). A kinetic model for nitrogen-limited wine fermentations. Biotechnology and Bioengineering. 77:49-60.

Johannsen, E. and D.A. Mills. A comparison of direct and indirect methods for analysis of the yeast ecology in wine fermentations. (MS thesis & manuscript in preparation).

Pallman, C., B. J. B., T. L. Olineka, C. L., D. A. Mills and L. F. Bisson. "Use of WL medium to profile native flora fermentations." American Journal of Enology and Viticulture 52:198-203.

Subramanian, V., K. K. Buck, and D. E. Block (2001). The use of decision tree analysis for determination of critical enological and viticultural processing prarameters in historical databases. American Journal of Enology and Viticulture. 52:175-184.

Research Success Statements: The aims of the first three years of this proposal were to acquire, develop and optimize technologies for the analysis of problem fermentations. The goal of this work is to develop better fermentation management strategies to reduce and hopefully eliminate the incidence of slow and incomplete fermentations. In this first phase of the research we have successfully adapted functional genomic analysis to *Saccharomyces* grown under enological conditions. We have identified several key differences in the physiology of yeast grown under nutrient sufficient versus nitrogen-limited conditions. We have begun identifying molecular markers associated with healthy or robust fermentations and those associated with nutritional or environmental stress. The project is well poised to complete this analysis in the next year and to identify key yeast strain and physiological input factors needed for full optimization of the predictive potential of neural networks. We have developed bacterial-specific primers for direct analysis of bacterial strains in wine. In addition, we have developed and tested several yeast specific primers and employed them on samples obtained from commercial wine fermentations. This approach has resulted in direct identification of viable but non-culturable yeast populations, a potential factor in stuck fermentations. The project is well poised to complete this analysis in the next year and to identify key yeast strain and physiological input factors needed for prediction of fermentation kinetics. In addition to the molecular and physiological work, we are currently completing detailed analysis of samples from over 200 commercial Chardonnay fermentations from the 2001 harvest. Analysis of the juice and wine from these fermentations, which ranged from normal to sluggish and stuck, will allow us to identify juice characteristics and processing choices that are critical in determining fermentation kinetics. We have also developed bacterial-specific primers for direct analysis of bacterial strains in wine. In addition, we have developed and tested several yeast specific primers and employed them on samples obtained from commercial wine fermentations. This approach has resulted in direct identification of viable but non-culturable yeast populations, a potential factor in stuck fermentations. With all yeast physiological and microbial ecology factors, juice characteristics, and processing parameters identified that are critical in determining wine fermentation kinetics, we will be able to predict problem fermentations and their resolution early in the fermentation process.

Funds Status: The following personnel will worked on this project for the 2001-02 academic year. Researchers receiving full or partial support from this grant are indicated in bold. For objective 1, it is anticipated that all funds will be spent or encumbered (salary) by the end of this

grant cycle (March 31, 2001). Some expenditures for salary may occur after that date as some students will likely need to extend their time to the Spring quarter. Also, there is a significant amount of supply monies still available for this objective targeted to the purchase of Affymetrix DNA chips. These chips have a finite shelf life and therefore cannot be purchased until ready to be used. Finally, delays in transfer of funds between UCD and the UBC have resulted in delays in transfer of charges to the AVF funds. All researchers indicated for this project were hired.

Objective 1:

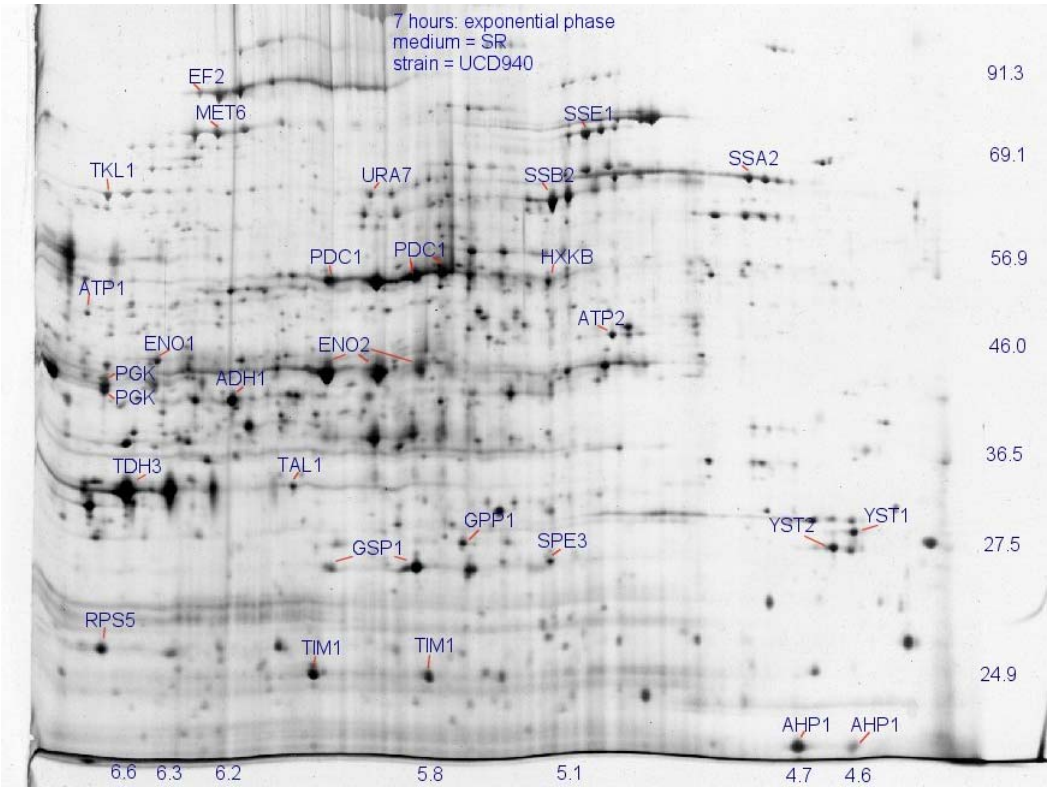
Jim Brown (PhD, UCD)
Chris Cooney (MS, UCD)
John Ebeler (PGR, UCD)
Jeff Mangahas (MS, UCD)
Kurt Niznik (MS, UCD)
Russ Robbins (MS, UCD)
Cherie Sprigs (PhD, UBC)
Alice Sprouse (MS, UCD)
G. K. van der Merwe (Postdoctoral Fellow, UBC)
Michael Webb (PhD, UCD)
Kathryn Weiss (Postdoctoral Fellow, UCD)
Viyada Kunathigan (PhD, UCD)
Vidhya Ramakrishnan (PhD, UCD)
Tammi Olineka (Research Technician, UCD)

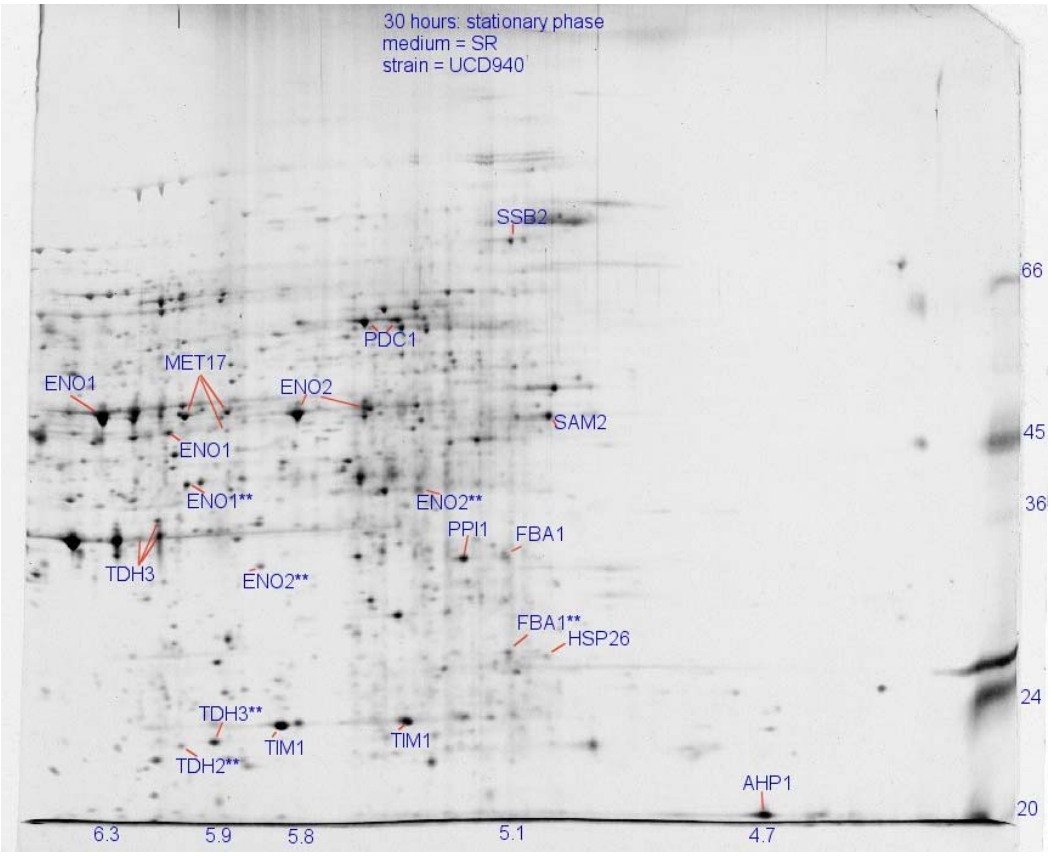
Objective 2:

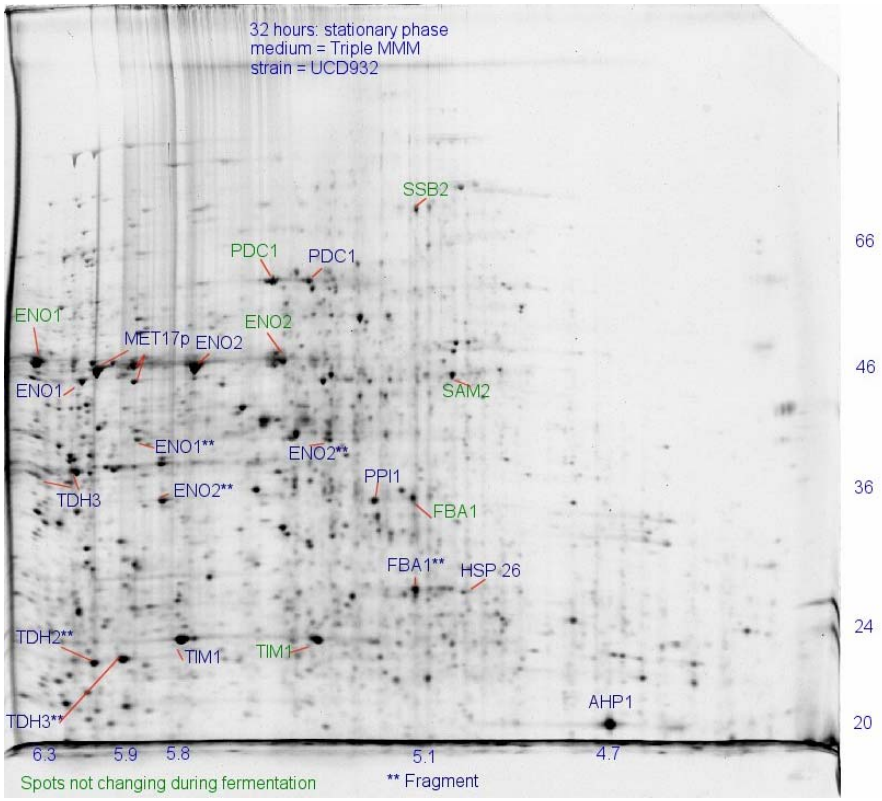
Julie Schreiber (MS, UCD)
Leigh Meyering (MS, UCD)
Kristan Buck (Postdoctoral Fellow, UCD)
Hung Ly (Ph.D, UCD)

Objective 3

Greg Allen (MS, UCD)
Luca Cocolin (Postdoctoral Fellow, UCD)
Lucy Joseph (Culture Curator, UCD)
Isabel Lopez (visiting PhD, UCD)
Erica Orr (MS, UCD)
Trevor Phister (Postdoctoral Fellow)


7 hours: exponential phase
medium = SR
strain = UCD940
EF2
MET6
SSE1
TKL1
URA7
SSB2
SSA2
PDC1
PDC1
HXKB
ATP1
ATP2
ENO1
ENO2
PGK
PGK
ADH1
TDH3
TAL1
GPP1
SPE3
GSP1
YST2
YST1
RPS5
TIM1
TIM1
AHP1
AHP1
91.3
69.1
56.9
46.0
36.5
27.5
24.9
6.6
6.3
6.2
5.8
5.1
4.7
4.6


30 hours: stationary phase
medium = SR
strain = UCD940
SSB2
PDC1
MET17
ENO1
ENO2
SAM2
ENO1
ENO1**
ENO2**
PPI1
FBA1
TDH3
ENO2**
FBA1**
HSP26
TDH3**
TIM1
TIM1
TDH2**
AHP1
66
45
36
24
20
6.3
5.9
5.8
5.1
4.7


32 hours: stationary phase
medium = Triple MMM
strain = UCD932
SSB2
PDC1
PDC1
ENO1
MET17p
ENO2
ENO2
ENO1
SAM2
ENO1**
ENO2**
PPI1
TDH3
ENO2**
FBA1
FBA1**
HSP 26
TDH2**
TIM1
TIM1
AHP1
TDH3**
66
46
36
24
20
6.3
5.9
5.8
5.1
4.7
Spots not changing during fermentation
** Fragment

Analysis of *Saccharomyces* during Normal and Problem Fermentations

Principal Investigator(s): Linda F. Bisson, Christian E. Butzke, David E. Block, S. E. Ebeler, David M. Mills, H. van Vuuren
Department of Viticulture and Enology
University of California, Davis

General Summary:

The goal of this multiyear multi-investigator project is the development of tools enhancing the fermentation management capabilities of commercial wineries with the aim of elimination of slow and incomplete fermentations. In this grant year, functional genomic technologies evaluated in previous years were applied to normal fermentations, to fermentations limited for nitrogen and to fermentations that received addition of nitrogen at various stages of fermentation. The physiological status of the yeasts could be determined from the specific pattern of gene expression. In addition, experiments evaluating the impact of environmental stress, temperature shock and acetate challenge, are in progress. Techniques for assessing the impact of phenolics on protection of yeast from oxidative damage have been developed and continue to be refined. Experiments evaluating the proteome of yeasts from commercial and synthetic juice fermentations were also optimized and analysis of differences in strain proteome profiles was conducted. Some strains display remarkably similar proteomes at all stages of growth (nearly 90% of the protein spots identical) while others are quite diverse (30 to 40% of the spots differing). Studies were also completed examining means of restarting fermentations destined to be stuck, including addition of DAP and complex nitrogen sources and addition of actively growing cells. In order to identify critical factors in juice that determine fermentation kinetics, 224 commercial Chardonnay fermentations were sampled and the analysis of these samples is ongoing. Bacterial-specific primers were developed for the analysis of the microbial profile of wine. Several yeast-specific primers were also evaluated and primers allowing differentiation of wild yeast species during commercial fermentations were identified.