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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 Mechanistic Models and Artificial Neural Networks Trained with Historical Data
3. Analysis of the Microbial Flora in Grape Must Fermentations:
   Profiling of Fermentations by:
   A. Temperature Gradient Gel Electrophoresis
   B. Cellular Fatty Acid Analysis

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: One of the goals of this objective is to define the physiological characteristics of a normal fermentation. Toward that end, it has been necessary to determine what factors actually lead to fermentation arrest under enological conditions as well as to identify the spectrum of yeast responses to changes in environmental conditions that encompass the term “normal”. We have evaluated the impact of temperature stress as well as fatty acid limitation on a set of commercial strains. We have also under more limited conditions, evaluated the tendency of strains to arrest in the presence of a simultaneous malolactic fermentation. Not surprisingly given its reputation, the commercial strain Premier Cuvee was the most stress-resistant strain. It displays greater temperature tolerance and resistance to inhibition by non-*Saccharomyces* flora. It also maintained a higher rate of fermentation under fatty acid limitation, just as it does under nitrogen

limitation. However, it did display sensitivity to inhibition by seed phenolic compounds. The phenolics study revealed that commercial preparations of Premier Cuvee are comprised of more than one strain or biotype. One biotype was far more sensitive to phenolic inhibition (and stimulation) than the other. Both biotypes are have now been isolated in pure culture and can be evaluated separately in the future.

The French White strain generally displayed behavior closest to the mean values of the strains tested, and the Cote des Blancs strain appeared to be the most sensitive to environmental stress. In general, if a strain was sensitive to one stress factor it also appeared to be sensitive to others. This suggests a commonality of factors involved in general stress response of yeast under commercial conditions. This is in excellent agreement with the literature on laboratory strains. A generalized stress response exists that is induced under conditions of nutrient limitation or the imposition of environmental stress.

The temperature shift experiments showed that strains shifted to high temperature at a low ethanol level (3°Brix fermented) did not arrest until 11% ethanol had been attained. Parallel cultures not receiving a temperature shock completed fermentation to dryness (13% ethanol). While Cotes des Blancs was the most sensitive, all strains tended to show this same pattern of arrest. Shift at 11% ethanol resulted in immediate arrest of this strain, while Premier cuvee seemed better able to complete fermentation if the upshift in temperature occurred at 11 versus 3 or 8% ethanol. Therefore, it appears that temperature shifts impact the ethanol tolerance of the culture or strain, even if the initial shock occurred days before attainment of the high ethanol concentration. This suggests fundamental differences in the physiology of the cells as a function of temperature. Cote des Blancs was sensitive to the magnitude of the change, arresting if the upshift was 10°C or greater. In contrast, arrest in Premier cuvee occurred at 35°C, independently of the magnitude of the shift. These two strains show fundamental differences in their ability to handle high fermentation temperatures and shifts from one temperature to another.

Fatty acid release was monitored across temperature shifted and un-shifted control cultures. Numerous fatty acid species were released. While no one species was present in a high enough concentration to be considered inhibitory, if the fatty acid species act in concert, being either additive or synergistic, arrest may be due to the presence and accumulation of inhibitory fatty acids. This hypothesis can be tested using transcriptome and proteome analysis to profile mRNA and protein expression patterns.

Because French White appeared most frequently to match the norm of the response of cells to environmental and nutritional stress, a microarray analysis of this strain was performed in year two of the grant. This analysis generates a tidal wave of data, which was largely processed in the current grant year. A clear view of the relative physiological status of nutrient sufficient and nitrogen-limited populations has emerged from this analysis. Nutrient sufficient cultures that enter a stationary phase based upon attainment of maximal cell density display higher levels of expression of genes involved in biosynthesis of precursors of macromolecules, and appear to retain competence for cell division. In contrast, energy-sufficient cultures entering stationary phase due to nitrogen limitation display greater expression of genes involved in translation and in oxidative carbon metabolism, suggesting that respiration is more conserving of nitrogen resources than fermentation. The low nitrogen culture also displayed higher mRNA levels for genes involved in nitrogen compound recycling. Expression of the stress genes examined in

general indicated higher levels of expression upon entry into stationary phase or was higher in the high nitrogen time course, which may reflect the added stress of high ethanol concentrations. Several genes were identified that appeared to increase in expression only during nitrogen-limited stationary phase. One of these genes encodes the *RGS2* protein, which inhibits the cAMP/PKA signal transduction cascade thus restricting responses to glucose [29]. This observation explains the characteristics of arrested fermentations during wine production. Such arrested cultures do not respond to simple nutrient addition, as would be expected from inhibition of the cAMP/PKA cascade. However analysis of other genes repressed by the cAMP/PKA cascade did not reveal any changes in pattern of expression (to be reported) suggesting that only a subset of PKA-regulated genes is being derepressed under these conditions.

Several genes are induced by environmental and nutritional stress in S. cerevisiae [reviewed in 6]. Previous studies of the expression of stress related genes under the control of the STRE element during wine production conditions identified several heat shock proteins that are expressed upon entry into stationary phase [20]. Expression of these and other stress and heat shock proteins [6] was therefore evaluated. *HSP12* and *HSP26* encode small proteins of unknown function that were found to be induced upon entry into stationary phase, consistent with the earlier study [20]. *HSP12* and *HSP26* are more highly expressed at later time points in the high nitrogen culture. *HSP12* mRNA levels were elevated in the early time point for the low nitrogen time course while *HSP26* shows no further induction. The mRNAs for both *HSP12* and *HSP26* were present in higher concentration in the early low nitrogen time point as compared to the early high nitrogen time point, confirming that a greater proportion of this population has entered stationary phase. *HSP12* is also more highly expressed in the late time point of the low nitrogen culture as compared to the late time point of the nitrogen sufficient culture. Thus, expression of these two genes is consistent with the expected replicative status of the cultures.

For the Hsp70 family (*SSA1* to *SSA4*, *SSB1*, *SSB2*, *SSC1*), *SSA1* and *SSB1* show little to no change and *SSA2* is higher in the early time points as compared to both late time points regardless of the nitrogen status of the culture. *SSA3* and *SSA4* are more highly expressed at the latest time point in the high nitrogen culture, and in the early time point of the nitrogen deficient culture versus the same point of the early culture. Both also tend to show higher expression at the early time point of the low nitrogen culture as opposed to later time points. *SSA3* is higher in the nitrogen-rich late time point, H85, as compared to the late time point from the nitrogen deficient culture. Thus, expression of these two genes seems to be associated with a nutritionally sufficient stationary phase. They are high in H85 and in L33, two samples that are likely in an equivalent stationary phase.

Similar results, higher expression in a nutrient sufficient stationary phase, are seen for *HSP60* and *HSC82*. *HSP42* expression also appears to be higher in the more replicatively active cultures, H33 and L33 and in H85 versus L85. The *HSP30* mRNA, also reported to be higher in stationary phase during grape juice fermentation, was elevated in the low nitrogen time course as compared to high nitrogen, but this trend was not consistent in other samples suggesting that expression of this gene might be restricted to a particular stage of stationary phase. Other heat shock genes did not show a clear trend.

Several other genes are also associated with entry into stationary phase. The *GAC1* gene which is involved in activation of glycogen synthesis [10] was expressed at both the mid and late time point of the high nitrogen sample as compared to the early time point. This gene was also expressed more strongly in the early time point of the low nitrogen culture, in agreement with the literature [26]. Expression of *GSY2*, which encodes the major isozyme of glycogen synthetase, did not vary much across the samples. On the other hand, *GSY1* showed greater expression in the high nitrogen time course as compared to low nitrogen. Several genes, *SOD2, TPS1, TPS2* and *UBI4*, also associated with stress response [6] showed higher expression in the low nitrogen late time point as compared to the high nitrogen sample, and the *TPS* genes also show greater expression at the earlier time point of the high nitrogen time course. This pattern of expression is similar to that observed for the glycolytic genes.

The stress and heat shock genes examined seemed to be indicators of stress or entry into stationary phase, but either did not distinguish between the nutritional status of the cultures or were more highly expressed under nutrient-sufficient situations where entry into stationary phase can be considered to be caused by attainment of maximal cell density as opposed to nutrient limitation. The YER150W gene (*SPI1*) has been reported to be expressed in stationary phase in wine fermentations [20]. This gene was found to be more highly expressed in the L33 sample, as compared to all other conditions. Expression did not appear to increase at later time points or under nutrient sufficient conditions. We therefore wanted to search for genes that would be more highly expressed in nutritionally poor stationary phase. Eight genes showed a high level induction in the late time point of the low nitrogen culture as compared to the high nitrogen culture. One, YJL221C (*FSP2*, homology to α-D-glucosidase) was highest in both late time points, but was higher in the low nitrogen condition. This gene is induced in limiting conditions and displays high identity to the *MAL62* protein [28]. YML128C, YNL200C and YPR195C, encoding proteins of unknown function, were more highly expressed in the low nitrogen culture than the high nitrogen condition, but showed no difference in expression in the high nitrogen condition. YML128C and YNL200C were previously identified as genes expressed under stationary phase conditions during wine fermentation [20]. Thus these proteins might be an indicator of nutritional stress. YOR107W expression was also high in the lower nitrogen condition and in the late time point of the high nitrogen samples. This gene has been designated *RGS2* and is a negative regulator of glucose-induced cAMP signaling pathway [29], and a higher level of expression may indicate that a larger percentage of the population is in a true G0 state. Rgs2p plays a role in regulation of STRE gene expression [29]. The fact that ribosomal protein synthesis increases at this time point suggests that the cAMP/PKA pathway is functional as the increase in expression of the *RPL* and *RPS* genes under nitrogen limiting conditions is mediated by PKA [16]. The role of the Rgs2p may be to limit response of the cAMP/PKA pathway to external nutrients but allow response to internal signals. Thus the appearance of this protein might be an excellent indicator of the nutritional status of the culture.

YPR192W (*AQY1*), which has homology to plant and animal aquaporins [2], is more highly expressed in the low nitrogen time course and in both the mid and late time points of the high nitrogen condition. This gene is expressed more highly during nitrogen limitation [2], consistent with our data. Interestingly, it is believed to be non-functional in laboratory strains of *Saccharomyces*. The role of the *AQY1* protein in yeast is unknown but it has been shown to induce water uptake in *Xenopus* oocytes [2].

The *GRE1* gene was originally identified as a gene expressed under hyperosmotic conditions [11]. Gre1p resembles the late embryogenesis abundant protein of higher plants. The *GRE1* gene is negatively regulated by the cAMP-*PKA* pathway and is induced upon nitrogen limitation [11]. The appearance of *GRE1* mRNA is consistent with inhibition of the cAMP signal transduction pathway by Rgs2p. YFL030W encodes an alanine:glyoxylate aminotransferase which is strongly glucose repressed but expressed during gluconeogenic growth on acetate and ethanol [27]. The observation of expression under these conditions suggests it is not required for gluconeogenesis so much as for amino acid biosynthesis. Its presence may be indicative of more versatile amino acid recycling.

Analysis of this set of genes has revealed several that may be excellent indices of the physiological status of fermenting cultures. Expression of *RGS2* would indicate exit from permissive growth conditions, while several of the *HSP* genes would indicate entry into stationary phase. The presence of both would indicate nutrient deficiency of stationary phase. *GRE,* which is induced by nitrogen limitation, may be a reporter specific for nitrogen starvation along with YFL030W. Further studies will define the role of these genes in nutrient-limited energy sufficient stationary phase as well as characterize the status of the cAMP/PKA signal transduction cascade.

Some cautionary notes in interpretation of this data are warranted. First, it is not clear if mRNA levels of non-growing cells will reflect differences in protein levels. That is, transcriptome analysis might not reflect the proteome. However, the information on transcriptional activity is clearly important and reflects a need for the gene product. The absence of a transcriptional signal does not imply the converse, that the gene product is not required under the conditions analyzed. Higher levels of expression may simply be needed to maintain a "status quo" level of protein, especially under conditions of accelerated mRNA and protein turnover. Further, it is important to bear in mind that the data is being generated across a population, and certain expression levels may be due to only a percentage of the population being in a specific physiological state. Alternately, mRNA levels might indeed reflect the status of the entire population. Finally, the mRNAs of genes with a significant homology can cross-hybridize, impacting assessment of true changes in the level of expression of individual members of gene families. The DNA Chip analysis, which is based upon homology to oligonucleotides rather than to PCR fragments of the microarray, has undergone significant improvement since it was evaluated in this grant two years ago. The DNA Chips now contain single base pair miss-match controls for each oligonucleotide increasing the specificity of the analysis. The chips are being produced differently, which has eliminated much of the lot-to-lot variation of the earlier products. Finally, improvements to the quantification of the fluorescent staining of the cDNA have been made allowing quantitation over a wider range of signals. For this reason, future transcriptome analysis will utilize the chip rather than the microarray technology.

A second aspect of this work is to use also analyze the protein profiles of yeast strains from various growth conditions. There is not a good correlation between the transcriptome and the proteome of yeast cells except during active growth or under conditions of energy limitation. In metabolically active non-growing cells mRNA levels serve to sustain protein levels and are therefore impacted by protein turnover and stability as well as by other post-transcriptional controls. However, important physiological information can be obtained from the analysis of the mRNA profile of these cells. The current consensus in the field is that both proteome and

transcriptome analysis must be conducted to determine the true physiological status of the cells. Our data would agree with this assessment. Therefore we have dedicated a significant amount of time to the optimization of the proteome analysis. This technique is based upon running large format 2D SDS PAGE gels, followed by comparison of the protein patterns and identification of individual spots by peptide mass fingerprinting. Proteome analysis is still considered to be more of an art than a science due to the extreme attention to experimental detail that must be paid. We have sought to understand the steps in the procedure that are the major causes of irreproducibility. One of the main problems arises from the methods used to assess protein concentration of replicate extracts. In spite of advertisements to the contrary we have found that the Bradford assay, which is the most commonly used protein assay for proteome analysis, is not linear under the conditions being used. The Amido Black assay does appear to be linear and leads to greater reproducibility of the gels. Another problem area concerns gel staining and destaining. Most of the protocols are vague and recommend staining until dark enough. The staining methodology used clearly impacts the number of spots detected and the ability to do comparative analyses. This year we systematically evaluated the five most popular staining techniques to determine which one is least susceptible to experimenter bias in determination of the timing of staining. We are currently comparing the two "chemistries" used for the 2D gels as well (glycine and tricine). This is still underway. In addition to optimization of the 2D gels, we are also adapting the technique used for peptide mass fingerprinting to our conditions.

Use of peptide mass fingerprinting to identify proteins separated by 2 dimensional electrophoresis (2DE) requires detection methods that do not interfere with subsequent protein cleavage and extraction of peptides. If the limit of detection for visualization of protein spots is lower than the amount of protein needed for successful identification of peptide fragments, less abundant proteins cannot be reliably identified. Initial 2DE methods utilized Coomassie Blue staining [22,23,24]. Coomassie blue dye is easily removed from proteins by destaining, and this method does not irreversibly alter the protein, permitting enzymatic digestion of the protein. The limit of detection is relatively high (100 ng), so only the most abundant proteins will be visualized when standard protein amounts are loaded (<50 μg). Silver staining methods provide greater sensitivity (1-10 ng), which accounts for their increasing popularity. Initial staining protocols involved protein fixation and sensitization with glutaraldehyde or formaldehyde [21]. These practices increase the sensitivity, but reduce the efficiency of protein digestion and peptide extraction. Recent modifications, including those devised in our laboratory, have reduced the amount of formaldehyde used while maintaining sensitivity in the nanogram range [1,25]. The limit of visualization is less than 10 nanograms for known proteins separated by 1 DE. This staining method is compatible with in-gel tryptic digestion.

The method used for in-gel digestion was adapted from that used by the Molecular Structure Facility, University of California, Davis. Excised protein spots are destained to remove silver [12]. The proteins are reduced, alkylated, and digested with trypsin while in the polyacrylamide gel pieces. The peptides are extracted, concentrated, and desalted prior to mass spectrometric analysis. The mass spectrometric method used, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, was performed using a Bruker Biflex III instrument. The peptides masses thus obtained were subjected to database comparison using Protein Prospector search engine (University of California, San Francisco). In most cases, a mass tolerance of 50 ppm was selected; no restrictions such as organism, molecular weight, or pI were placed on the database.

At this level, the number of false-positive matches is reduced [3]. The mass accuracy of identified peptides was calculated to be between 10 and 50 ppm.

The reproducibility of digestion method and limit of detection were assessed using three known proteins (bovine serum albumin, bovine casein, and chicken egg white lysozyme). After separation by 1DE, these proteins could be visualized at 100, 10, and 1 ng levels (100 ng is approximately 1.5 pmole albumin, 4.3 pmole casein, or 7.0 pmole lysozyme). Albumin and lysozyme were identified from peptide mass fingerprints resulting from digestion of the 100 and 10 ng spots. A number of the peptides matching masses in the database were observed in all digests of that protein. Both the number of peptides observed in the MALDI spectra, and the number matching the theoretical digestion decreased with amount of protein in the spot. Masses of the limited number of peptides detected in digestions of the 1 ng spots were consistent with the protein digested, but the number of peptides was insufficient for identification except in the case of albumin. Identification of casein at all amounts was difficult due to the limited number of peptides between 1000 and 4000 Da produced by tryptic digestion. No differences in peptide profiles or detection limit were observed between digestions of dried and wet gels. The majority of peptides resulted from complete cleavage of the protein, with few missed cleavages observed.

Comparisons of protein maps from total yeast cellular proteins have also been undertaken. To date, two proteins have been identified on two or more separate 2D gels. Enolase 2 (*ENO2*) was identified from gels prepared by three different experimenters. Five identified peptides were common to all three tryptic digestions. Peptide coverage of the protein sequence ranged from 21 to 33%. Peroxisomal alkyl hydroperoxide reductase (*APH1*) was identified in spots from two different gels. In this case, two peptides were common to both gels, and total sequence coverage was 21 and 40%, respectively. These results are comparable to those observed with known proteins, where sequence coverage ranged from 32 to 8% for 100 and 1 ng bovine serum albumin, and from 65 to 20% for 100 and 10 ng lysozyme. Analysis of additional spots is continuing to identify protein "landmarks" that will facilitate comparisons between gels. Creation of reference gels are essential, since Gygi *et al.* [14] reported that only 14 proteins of the 58 predicted to be found in a narrow molecular mass and pI region of a 2DE gel could be identified.

Objective 2: Development of Technology for the Prediction of Stuck and Sluggish Fermentations. The ultimate goal of this objective is the development of methodology for prediction of the kinetics of both normal and problem fermentations, based upon juice characteristics and intended processing. There are two components to this objective, generating data on the impact of various parameters on fermentation kinetics from defined studies that will lead to a mechanistic model of cell growth and sugar utilization and use of that information for the training of neural networks that can predict fermentation behavior.

Development of a Mechanistic Model for Wine Fermentation Kinetics

Initially, a mechanistic model was developed for yeast fermentation kinetics based on a nitrogen-limited juice. In this model, maximum cell concentration is determined by initial nitrogen concentration, and ethanol production is completely non-growth associated (i.e. most production

occurs after exponential growth has ceased). Cell death or inactivation is proportional to the ethanol concentration. This model is able to predict the transition from normal to sluggish to stuck fermentation with decreasing initial nitrogen. In addition, this model also predicts the necessity of high initial nitrogen for completion of high sugar fermentations. We have confirmed that the model qualitatively agrees well with data from small-scale experiments in our laboratory [7].

During the course of finding the death constant for the model experimentally, it became clear that cell viability may not be determining factor in stuck fermentations. Instead, cells may become inactive (in the conversion of sugar to ethanol) while remaining viable. This hypothesis is consistent with data that we have collected that shows good viability in fermentations destined to become sluggish or stuck. While there may be biological explanations for this inactivation (to be explored in Objective I), physical changes to the yeast cell membrane due to ethanol contact could also explain some of the observed behavior. This has led us to collaborate with Prof. M. Longo in the Department of Chemical Engineering and Materials Science at UC Davis in order to investigate the effects of ethanol on lipid bilayers in a more fundamental and controlled manner. This work will now be included as part of Objective I.

After developing the model, a series of small-scale fermentations were completed using Chardonnay juice that examined the effects of various juice and processing parameters on fermentation kinetics in order to find the most important factors. Variations in initial sugar, initial nitrogen, juice variety, initial pH, initial dissolved oxygen concentration, and yeast variety were used. From these studies, it was confirmed that the sugar utilization rate is a function of initial nitrogen concentration. However, the impact of nitrogen on fermentation rate is dependent on yeast strain. It was also found that nitrogen was used less efficiently for cell growth when oxygen was not present in the juice initially. These data indicate that oxygen may play a role in determining fermentation kinetics, in addition to the established role of oxygen as necessary for the formation of certain "survival factors." All of these results indicated that maximum viable cell concentration would be a good diagnostic tool in predicting the likely sugar utilization pattern. In fact, we have also found an inverse relationship between the maximum cell concentration at approximately 24 hours into the fermentation and the amount of time needed for the fermentation to reach 10 g/L residual sugar (an indication of fermentation kinetics). In our experiments, all fermentations have stuck when the maximum cell concentration has not reached 3 g biomass/L. This type of relationship should be useful in the early prediction of problem fermentations and only requires a simple optical density measurement after one or two days of the fermentation.

In the past year, we have focused on using the model developed to predict ways of completing fermentations destined to be sluggish or stuck [Schreiber, M.S. Thesis]. We have investigated DAP additions at various concentrations and times, viable cell additions at various concentrations and times, and combined additions of nitrogen and cells. For each set of conditions, the kinetics were predicted and found experimentally using flask-scale experiments. Good qualitative agreement was found, with these results indicating the validity of using the model for these purposes. The model prediction consistently deviated from the experimental results at two points during the fermentations. First, it was found that nutrient additions were more effective after 20 hours than at inoculation (time=0 hr). We are currently investigating the role of the pre-inoculation microbial ecology in this phenomenon (in conjunction with D. Mills

and Objective III) since early nutrient additions may be used by indigenous yeasts or bacteria. Second, all cell and nitrogen additions become ineffective at fixing problem fermentations after some point in the fermentation (>360 hr in our laboratory experiments). We have also observed an increase in measured α-amino nitrogen during later stages of these fermentations. We are currently investigating whether there is some relationship between these two phenomena by examining specific amino acids. This work will also continue in conjunction with L. Bisson to study cell behavior at this point in the fermentation. If a relationship can be found, it may be possible to use a simple assay to determine when a fermentation is no longer easily correctable.

In addition, we have evaluated the use of Tastone 154 (yeast extract) and SuperFood (yeast extract, yeast hulls, and DAP) additions in parallel with DAP additions for fixing problem fermentations. SuperFood has proven to be more effective, though it is not yet clear why this is the case. However, addition of cells and juice used for the cell growth was also more effective at avoiding problem fermentations than addition of cells alone. Therefore, minor nutrient components found in the juice or complex additives may prove important in determining fermentation kinetics under certain situations. Analysis of these components will be the main focus in the coming year.

Development of Neural Network Technology for Prediction of Fermentation Kinetics

Artificial neural network technology is being developed to predict wine fermentation kinetics as a function of juice characteristics and intended processing. The development of neural network training methods has been completed and used to predict fermentation kinetics in 5 gal Sauvignon blanc fermentations [30]. Neural networks were able to interpolate fairly well among data they had previously seen, though the ability to extrapolate was variable depending on the linearity of the relationships between inputs and model outputs (kinetics). The key, therefore, will be to identify all key factors affecting fermentation kinetics and the ranges over which they vary. We have initiated collecting juice samples from industrial wine fermentations that have demonstrated normal or problem fermentation kinetics. By analyzing these samples and associated wine samples, we should be able to build a database of most potentially critical factors. We have recently begun to use methods such as Decision Tree Analysis in our laboratory in order to identify critical process inputs in large historical databases of information [Subramanian, M.S. Thesis]. Once all the factors having a major impact on fermentation kinetics are defined, it should be possible to use the trained neural network as a warning of an impending fermentation problems prior to inoculation, thus allowing corrective action to be taken from the beginning of the fermentation.

Objective 3: Analysis of the Microbial Flora in Grape Must Fermentations: Profiling of Fermentations by A) Temperature Gradient Gel Electrophoresis and B) Cellular Fatty Acid Analysis

Objective 3A: The first three years of this grant were devoted to development and implementation of molecular methods for direct analysis of yeast and bacterial populations in wine fermentations. Initial work focused on yeast populations using PCR primers specific to the

18S and 26S rDNA segments as well as an evaluation of different yeast DNA purification methods. In addition both temperature gradient gel electrophoresis (TGGE) and denaturing gradient gel electrophoresis (DGGE) methods were evaluated for separation of yeast amplicons. DNA purification using a bead mill method was found to be superior for isolation of both yeast and bacterial DNA. It was found that DGGE is far superior to TGGE in the differentiation of yeast strains and that the 26S region provides more discrimination than the 18S region. The 26S region was favored since this region has been used in the phylogenetic analysis of yeast diversity. Sequences are available for most of the known ascomycete [17] and basidiomycete yeasts [9].

Once purification and PCR-DGGE methods were developed, we examined mixed populations of yeasts in synthetic juice (triple M media; [13]). These studies clearly demonstrated that the PCR-DGGE methodology could identify individual yeast populations within a dynamic mixed culture. More importantly, the presence or absence of DGGE bands correlated with the presence or absence of different yeast populations as determined by standard plating. Our experiments also indicated that yeast populations as low as 10^4 cells per mL could be identified by PCR-DGGE. The PCR-DGGE methodology and synthetic juice studies were presented in our recent publication [4]. Another aspect to this study was the use of WL plating media for discrimination of the different yeast populations, a strategy that was fully developed by LFB to differentiate wild yeast populations in commercial fermentations at a local winery [19].

While we could readily monitor co-inoculated yeast dynamics in synthetic juice, we found that it is challenging to recreate the same mixed culture fermentation profile between different runs. This is likely due to differences in the metabolic state of the mixed culture inocula. Since a goal of this objective was to examine parameter effects on yeast ecology, we decided to examine the samples already generated by DEB as part of Objective 2. Currently these samples are being evaluated (Erica Orr, MS thesis).

In order to use PCR-DGGE on true wine fermentation samples additional challenges needed to be overcome. DNA preparation from wine can often be problematic, since plant tissues (polysaccharides and phenolics) are common contaminants known to disrupt PCR reactions [8,31]. In order to circumvent these issues we examined several methods and developed a procedure, using a modification of a currently available commercial plant DNA purification kit, to obtain PCR-quality DNA [5].

As an initial test of out methods, we chose to examine samples from an indigenous fermentation at Far Niente winery (the Dolce fermentation). This wine was chosen specifically because it was known to possess a large microbial diversity in the initial stages of the fermentation (A. Heisey, personal communication). Using PCR-DGGE we were able to identify, by sequence analysis, major populations of yeast present throughout the Dolce fermentation [5]. Several aspects to this study were noteworthy. Firstly we found that we could get viable PCR-DGGE “reads” by purifying DNA from very small amounts of fermentation broth (as small as 100 uL). Secondly, wine samples that were held in the freezer for up to 6 months gave clear microbial profiles. The microbial profile of the Dolce fermentation also proved interesting. Fungal DGGE bands (*Botrytis* sp.) were observed in samples taken from the settling tank. Moreover a *Candida stellata* population was identified which persisted throughout the complete fermentation (in the presence of *Saccharomyces* population).

In the second year we examined another Dolce vintage by both plating and PCR-DGGE. This analysis clearly demonstrated the presence of PCR-DGGE bands to which no corresponding population was identified on plates (Johannsen et al. 2000). This result suggests the presence of viable but non-culturable (VBNC) yeasts within the Dolce fermentation. Like the previous year, this work also identified the *Candida* population that persisted throughout the Dolce fermentation. Currently we are planning to purify RNA from the Dolce samples for RT-PCR-DGGE. The goal of this effort will be to determine if the putative VBNC DGGE bands are still revealed using an RNA template. This may help determine the viability of different yeast populations identified by PCR-DGGE.

The fact that we were able to directly freeze wine samples and still obtain good microbial profiling using PCR-DGGE prompted another study. The goal of this effort is to more rigorously evaluate the "normal" microflora present in chardonnay fermentations. To date, we have collected nearly 1000 samples from barrel-fermented chardonnays at three different wineries (~15 barrels in each winery, half inoculated, half indigenously fermented). This data set is currently being evaluated by PCR-DGGE. The goal will be to determine the variability in yeast ecology between barrels in a winery and between similar fermentations at wineries in the same region (Napa). This study will help us understand differences between indigenous and inoculated wine ecology, differences in yeast ecology that occurs at different locales, as well as aid us in the design of future mixed-culture experiments relative to stuck fermentations. This work is ongoing (Erica Orr, MS thesis).

Several "offshoot" projects based on the PCR-DGGE methods developed in this work have also been initiated. We have demonstrated that PCR-DGGE can be used to differentiate the microflora present on the grape surface. Methods have been developed to sterilely wash and collect the microbes that reside on the grape surface. Moreover, Dr. Jean VanderGheynst (a collaborator) has developed methods for isolation of PCR-quality microbial DNA from grape pomace. Dr. VanderGheynst and I have recently submitted a proposal to the Viticulture Consortium to evaluate further these methods and characterize the effects of certain viticultural treatments on the fungal and bacterial populations present on the grape surface. Once fully developed, these methods will be idea for rapidly characterizing the microflora on the grapes prior to harvest.

Studies to discriminate only the *Saccharomyces* species/strains present within wine fermentations are also underway in collaboration with Dr. Marisa Manzano at the Department of Food Science in Udine, Italy. The goal of this work is to develop methods to specifically identify and profile various *Saccharomyces* populations within wine fermentations. *Saccharomyces* specific primers were designed to the ITS regions of *Saccharomyces* rDNA and employed to differentiate various *Saccharomyces* species. To date, this work has moved slowly and differentiation of *Saccharomyces* strains by PCR-DGGE of ITS regions has been problematic. Currently we are examining alternative loci for differentiation of *Saccharomyces* species/strains by DGGE.

Studies to evaluate the bacterial flora have also been initiated. Numerous primers specific for bacterial 16S rDNA have been used in PCR-DGGE studies for determination of bacterial communities [18]. We evaluated four commonly-used primer pairs and developed DGGE

methodology for differentiation of wine-related bacteria. Unfortunately, we recently demonstrated that all of these primer pairs amplify yeast and fungal 18S genes from wine samples. This was a surprise given the repeated use (and publication!) of these primer sets to examine bacterial communities where fungal or yeast populations were also present. The development of *specific* bacterial primers for PCR-DGGE from wine samples possessing a predominant level of yeast, remains a key goal for future work

Objective 3B: As noted in last year's report, the MIDI system for fatty acid profiling was found to be insufficiently discriminating of yeasts in mixed population. This analysis was used as described in Objective 1 to profile the fatty acids and fatty acid derivatives released by yeast upon both up and down shift of temperature. Grape juice fermentations were initially started at two initial temperatures were shifted in 5° C increments to + and - 15°C. Numerous fatty acids were released upon either an upshift or downshift in temperature. This data set is still being analyzed.

Literature Cited:

1. Blum, H., H. Beier and H. J. Gross. (1987) Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophor. 8:93-99.

2. Bonhivers, M., J. M. Carbrey, S. J. Gould and P. Agre. (1998) Aquaporins in *Saccharomyces*. J. Biol. Chem. 273:27565-27572.

3. Clauser, K. R., P. Baker and A. L. Burlingame. (1999). Role of accurate mass measurement (±10 ppm) in protein identification strategies employing MS or MS/MS and database searching. Anal. Chem. 71: 2871-2882.

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

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

6. Craig, E. (1992) The Heat-Shock Response of *Saccharomyces cerevisiae*. In: The Molecular and Cellular Biology of the Yeast *Saccharomyces*: Gene Expression. (Jones, E., Pringle, J. R. and Broach, J. R., Eds.), pp.501-538. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

7. Cramer, A. C., Vlassides, S., and D. E. Block. "A mechanistic model for nitrogen-limited wine fermentation kinetics." Submitted.

8. Demeke, T. and R. P. Adams (1992). The effects of plant polysaccharides and buffer additives on PCR. Biotechniques 12:332-4.

9. Fell, J. W., T. Boekhout, A. Fonseca, G. Scorzetti and A. Statzell-Tallman (2000). Biodiversity and systematics of basidiomycetous yeasts as determined by large-subunit rDNA D1/D2 domain sequence analysis. Int. J. Syst. Evolut. Microbiol. 50:1351-1371.

10. Francois, J. M., S. Thompson-Jaeger, J. Skroch, U. Zellenka, W. Spevak and K. Tatchell. (1992). *GAC1* may encode a regulatory subunit for protein phosphatase type 1 in *Saccharomyces cerevisiae*. EMBO J. 11:87-96.

11. Garay-Arroyo, A. and A. A. Covarrubias. (1999). Three genes whose expression is induced by stress in *Saccharomyces cerevisiae*. Yeast 15:879-892.

12. Gharahdaghi, F., C. R. Weinberg, D. A. Meagher, B. S. Imai and S. M. Mische. (1999). Mass spectrometric identification of proteins from silver-stained polyacrylamide gel: A method for the removal of silver ions to enhance sensitivity. Electrophor. 20:601-605.

13. Giudici, P. and R. E. Kunkee. (1994). The effect of nitrogen deficiency and sulfur-containing amino acids on the reduction of sulfate to hydrogen sulfide by wine yeasts. Am. J. Enol. Vitic. 45: 107-112.

14. Gygi, S. P., G. L. Corthals, Y. Zhang, Y. Rochon and R. Aebersold. (2000). Evaluation of two-dimensional gel electrophoresis-based proteome technology. Proc. Natl. Acad. Sci. 97:9390-9395.

15. Johannsen, E., L. Cocolin and D. A. Mills. (2000). Direct characterization of the yeast ecology in commercial wine fermentations. 51th Annual Meeting, American Society for Enology and Viticulture, Seattle, WA, June

16. Klein, C. and K. Struhl. (1994). Protein kinase A mediates growth-regulated expression of yeast ribosomal protein genes by modulating *RAP1* transcriptional activity. Molec. Cell. Biol. 14:1920-1928.

17. Kurtzman, C. P. and C. J. Robnett. (1998). Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. Antonie Van Leeuwenhoek 73:331-71.

18. Muyzer, G. and K. Smalla (1998). Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie Van Leeuwenhoek 73:127-41.

19. Pallman, C., B. J. B., T. L. Olineka, C. L., D. A. Mills and L. F. Bisson (submitted). Use of WL medium to profile native flora fermentations. Am. J. Enol. Vitic.

20. Puig, S. and J. E. Perez-Ortin. (2000). Stress response and expression patterns in wine fermentations of yeast genes induced at the diauxic shift. Yeast 16:139-148.

21. Rabilloud, T. (1992). A comparison between low background silver diammine and silver nitrate protein stains. Electrophor. 13:429-439.

22. Rosenfeld, J., J. Capdevielle, J. C.Guillemot and P. Ferrara. (1992). In gel digestion of proteins for internal sequence analysis after one- or two-dimensional electrophoresis. Anal. Chem. 230: 173-179.

23. Sagliocco, F., J. C. Guillemot, C. Montribot, J. Capdevielle, M. Perrot, E. Ferran, P. Ferrara and H. Boucherie. (1996). Identification of proteins of the yeast protein map using genetically manipulated strains and peptide-mass fingerprinting. Yeast 12:1519-1533.

24. Shevchenko, A., O. Jensen, A. V. Podtelejniko, A. Sagliocco, M. Wilm, O. Vorm, P. Mortensen, A. Shevchenko, H. Boucherie and M. Mann. (1996). Linking genome and proteome by mass spectrometry: identification of yeast proteins from two dimensional gels. Proc. Natl. Acad. Sci. USA 93:14440-14445.

25. Shevchenko, A., M. Wilm, O. Vorm, and M. Mann. (1996). Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. Anal. Chem. 68:850-858.

26. Tadi, D., R. N. Hasan, F. Bussereau, E. Boy-Marcotte and M. Jacquet. (1999). Selection of genes repressed by cAMP that are induced by nutritional limitation in *Saccharomyces cerevisiae*. Yeast 15:1733-1746.

27. Takada, Y and T. Noguchi. (1985). Characteristics of alanine:glyoxylate aminotransferase from *Saccharomyces cerevisiae*, A regulatory enzyme in the glyoxylate pathway of glycine and serine biosynthesis from tricarboxylic acid-cycle intermediates. Biochem. J. 231:157-163.

28. Vandenbol, M., P. Durand, P. A. Bolle, C. Dion, D. Portelle and F. Higler. (1994). Sequence analysis of a 40.2 kb DNA fragment located near the left telomere of yeast chromosome X. Yeast 12:1657-1662.

29. Versele, M., J. H. de Winde, and J. M. Thevelein. (1999). A novel regulator of G protein signaling in yeast, Rgs2, downregulates glucose-activation of the cAMP pathway through direct inhibition of Gpa2. EMBO J. 18:5577-5591.

30. Vlassides, S., Ferrier, J. G., and D. E. Block (2001). "Using historical data for bioprocess optimization: Modeling wine characteristics using artificial neural networks and archived process information." Biotechnology and Bioengineering (in press).

31. Wilson, I. G. (1997). Inhibition and facilitation of nucleic acid amplification. Appl. Environ. Microbiol. 63:3741-3751.

Outside Presentation of Research:

Results from Objective 1 have been presented at the annual meeting of the Lallemand Corporation, May 2000 and in a seminar at the University of British Columbia (June 2000). This work featured prominently in the Research Lecturer presentation given by LFB at the annual meeting of the American Society for Enology and Viticulture (Seattle, 2000). A paper describing the results of the microarray analysis has been submitted for publication. Two student theses on this topic are in final draft stages (Jason Henrie and Jason Burrus).

During the past year, data from Objective 2 has been presented as part of oral presentation and a poster at the American Society for Enology and Viticulture meeting (Seattle, 2000). One paper on this topic is in press, one submitted, and several are in preparation.

Data from Objective 3A has been presented in the following venues: Gallo Winery (Feb, 10,1999); Department of Food Science, UC-Davis (March, 1999); CERA meeting of the Microbiology Group, (June 25,1999); ASEV Annual Meetings (June 30,1999 & June 23, 2000); Kendall Jackson Technical Retreat (July 27, 1999); New England BioLabs (September 8, 1999); Unified Wine and Grape Symposium (January 25, 2000); the meeting of the Napa Technical Group (February 17, 2000); Issues in Red Wine Short Course (March 23, 2000); Department of Food Science, Utah State University (July 10, 2000); Department of Microbiology, Catholic University, Piacenza, Italy (September 29, 2000).

Publications:

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., Vlassides, S., and D. E. Block. "A mechanistic model for nitrogen-limited wine fermentation kinetics." Submitted.

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. (submitted).

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).

Subramanian, V. (2000). "Use of database mining and artificial intelligence in bioprocess optimization." M.S. Thesis.

Vlassides, S., Ferrier, J. G., and D. E. Block (2001). "Using historical data for bioprocess optimization: Modeling wine characteristics using artificial neural networks and archived process information." Biotechnology and Bioengineering (in press).

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 two years and to identify key yeast strain and physiological input factors needed for full optimization of the predictive potential of neural networks. In addition, we have demonstrated that artificial neural networks can be used to predict wine fermentation kinetics when all critical juice characteristics and processing are known. A means of using simple optical density measurements one to two days into a fermentation in order to predict problems has been identified. We have also adapted TGGE and DGGE technologies for the analysis of the microbial complement of wine samples. This capability now allows us to detect the presence of all common wine microbes in a juice, must or wine sample without the need for cultivation. This permits a more statistically robust sampling of a fermentation and will provide data of sufficient quality to be useful in the development of neural networks for the prediction of fermentation behavior.

Funds Status: The following personnel will be working on this project for the 2000-01 academic year. Researchers receiving full or partial support from this grant are indicated in bold. It is anticipated that all funds will be spent or encumbered (salary) by the end of this grant cycle.

Objective 1:

Jim Brown (PhD)
Kathryn Weiss (Postdoctoral Fellow)
Magda Lopez Barajas (Postdoctoral Fellow)
Jason Burrus (MS)
Chris Cooney (MS)
Jason Henrie (MS)
Kurt Niznik (MS)
Russ Robbins (MS)
Alice Sprouse (MS)
Viyada Kunathigan (PhD)
Vidhya Ramakrishnan (PhD)
Tammi Olineka (Research Technician)

Objective 2:

Kristan Buck (Postdoctoral Fellow)
Amanda Cramer (MS)
Julie Schrieber (MS)
Venkat Subramanian (MS)

Jennifer Hall (UG)

Objective 3

Luca Cocolin (Postdoctoral Fellow)
Eric Johannson (MS student)
Marisa Manzano (Visiting scientist)
Kevin Kelley (Undergraduate)
Erica Orr (MS student)