Annual/Final Report

(Project Title) Biological Effects of Phenolic Compounds on Saccharomyces cerevisiae
(Principal Investigators)

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(Summary)

In this current grant year, it was discovered that some of the variability in response to phenolic compounds displayed by different commercial strains was possibly due to subtle differences in preparation of the synthetic juice medium, Triple M. This was confirmed using slightly different protocols and the Premier Cuvee yeast strain. Therefore, the preparation and composition of this Medium has been redesigned this year in order to avoid this problem. Analysis of the impact of phenolic compounds is continuing. To date, gallic acid appears to be uniformly stimulatory at concentrations found in wine while ferulic acid may be neutral, stimulatory or inhibitory to sugar consumption under laboratory conditions mimicking natural fermentations. The hexose transporters encoded by the HXT9/HXT11 genes that are under multidrug resistance control were shown to be expressed under enological conditions. The presence of these genes has been shown to be stimulatory for the uptake of inhi!
bitory drugs. They may therefore play a role in uptake or release of phenolic compounds inside of the cell by coupling such movements to the uptake of glucose., Objective 1: The aim of objective 1 was to define the conditions under which phenolic compounds could be demonstrated to have a stimulatory or alternately inhibitory effect on yeast metabolic rates as determined from differences in the rate of weight loss (carbon dioxide evolution). In the first year of the grant, we found that the impact of the phenolic compounds was most apparent late in the fermentation. Subsequent analysis indicated that the cells were switching from anaerobic fermentative growth to respiratory growth at this time. This suggested the simple hypothesis that carbon dioxide evolution could be

stimulated during aerobic growth in the presence of antioxidants because antioxidants could react with oxygen radicals generated during respiration and that the ability to eliminate these toxic molecules some!
how regulates flux through the electron transport chain. In other words, because oxygen radicals are not accumulating, the rate of respiration remains maximal. Another alternative hypothesis is equally likely, that the phenolic compounds stimulate gene and protein expression leading to the presence of higher concentrations of respiratory or other enzymes resulting in faster fermentation rates. These two hypotheses can be distinguished by looking at the profile of gene/protein expression of the cells, as is proposed in this study. Such an analysis is also inherently unbiased and will reveal other changes in protein expression patterns that might divulge important information regarding the physiological status of the cells grown in the presence of natural phenolic compounds.

In this current grant year, we launched the comparative analysis of the impact of phenolic compounds on different yeast strains (conducted by Laura Lange). She initially found that the effects were quite variable and there appeared to be no consensus as to whether the effects of a given compound were stimulatory or inhibitory with the exception of gallic acid. In the course of her work, we discovered that the inherent variability in the composition of the Triple M medium led to variation in the response of the yeast. The pH of this medium is adjusted using ammonium hydroxide. We found that since the pH of our water supply varied, so did the ammonium content of the medium. Further analysis revealed that this difference in nitrogen content was not impacting the results. However, the medium has been redesigned to contain a constant concentration of nitrogen. Another problem with the medium was the low concentration of potassium. Previous work in my laboratory indicated that !
potassium deficiency could lead to a sluggish fermentation and that this effect was somewhat strain dependent. We therefore decided to use potassium hydroxide to adjust the pH of the medium to assure a reasonably high concentration of potassium. Finally, the micronutrients were prepared as a stock solution and diluted when added to the medium. We noticed that a precipitate formed in the stock upon storage and that the age of the stock solution was correlated with variability in the effect of the phenolic compounds. This makes sound physiological sense since the cofactors derived from the vitamins and minerals in this mix are critical for respiratory activity of the yeast. To eliminate this problem, we now make the micronutrient cocktail fresh each time and use the Triple M medium within 48 hours of preparation. This has eliminated the variability in the response. This important optimization of the medium slightly delayed progress on the grant, but we are now conducting !
the comparative studies across strains. This will not be finished as originally hoped by the end of this grant year, so will continue into the first three months of the next grant year.

Objective 2: A rich literature exists extolling the virtues of dietary antioxidants in human health. Critics of this body of work claim that until the mechanisms by which antioxidants are shown to be beneficial are determined, the positive effect of dietary antioxidants remains unproven and speculative. Speculative though the dietary studies

may be, the correlation they suggest between antioxidants and health is significant. Many of the current diseases that are leading causes of death are the result of oxidative damage to the tissues and cells of the body. It is logical therefore that antioxidant compounds through the simple chemical reaction with radical oxygen and oxidizing agents could prevent or mitigate this damage. It is also known that the main source of damaging oxidizing agents is the process of respiration. The yeast Saccharomyces cerevisiae is a typical eukaryote and shares many physiological characters in common with mammalian cells. Our goal is to use thi!
s yeast as a model system to determine the impact of phenolic compounds on metabolism. The aim of objective 3 is to assess the impact of these compounds in preventing oxidative damage to sensitive target molecules within the cell (DNA lipids). The goal of objective 2 is to determine the impact of these compounds on gene expression and metabolic activity of yeasts.

The completion of the sequence of the yeast genome has ushered in a new era of "functional genomics" the ability to simultaneously evaluate expression of the total complement of genes of the organism in a single experiment. Two types of technologies have emerged, those that evaluate gene expression based upon analysis of the mRNA (the "transcriptome" analyses: Microarray, SAGE, GeneFilters) and those that analyze the protein complement of the cell, the "proteome" analysis. The transcriptome analyses are easier to perform but problematic. We have used the microarray analysis in collaboration with Dr. Joe DeRisi to evaluate the effectiveness of this analysis in the profiling of the physiological status of yeast cells during enological fermentations. AS described in detail in other reports to the American Vineyard Foundation, the limitations of this technique severely restrict its utility in evaluation of our and commercial samples. Principle among these limitations is the l!
ack of correlation between the microarray and proteome data, and the quality of the microarray data itself (which largely accounts for the lack of correlation). Most of the mRNA species of interest are outside of the linear range of the microarray analysis, and thus cannot be quantified using this technique. The protein content can be readily quantified on the proteome gels, especially since we are using the large format 2D gel electrophoresis. Therefore, we are evaluating the impact of the presence of phenolic compounds using the proteome analysis. We are currently in the process of collecting samples and running the 2D gels, so have no preliminary data that we wish to report at this time on the differences that seem to be present. However, some conclusions can be reached from the initial microarray analysis. First, the two hexose transporters, HXT9/HXT11 that have been shown to be under the control of the multidrug response in yeast are expressed under enological condit!
ions in nutrient sufficient, but not in nutrient limited cells. This result needs to be confirmed at the protein level. These two transporters are expressed in the presence of antifungal compounds and appear to make the cells more sensitive to the compound, meaning that more is taken into the cell. At the same time the multidrug resistance transporters are also expressed which expel these compounds. At face value, this strategy does not make any sense. We have long thought that the true substrates for this process were natural phenolic compounds that are taken up in a reduced state then excreted in an oxidized state and that the purpose of this system is not for the elimination of artificial

drugs. It appears that the transporters are expressed under normal (absence of phenolic compounds) media conditions in the absence of the full multidrug resistance response. We are currently determining whether or not the native phenolic compounds can lead to the induction of protein!
s controlled by the multidrug response. Such a finding would explain the physiological relevance of this response in yeast as well as document the effectiveness of dietary antioxidants.

Objective 3: Preliminary studies have been initiated to investigate the ability of phenols to protect against in vivo lipid peroxidation in Saccharomyces (Prise de Mousse, UCD594). Yeast were grown in the presence of either catechin (0.3 to 25 mM) or resveratrol (0.4 mM) and cell growth was monitored approximately every five hours (by absorbance at 580 nm). Samples for further analysis of antioxidant activity were taken during logarithmic growth and stationary phases. After cells had been counted, they were washed 4 times by centrifugation and resuspended in 50 mM Tris HCl (pH 7.6).

Conditions for the oxidative experiments on live cells were established. Basically, cells were resuspended in phosphate buffered saline solution to a density of 2 x 108 cells/mL. A mixture of FeSO4 (1 mM), H202 (75 mM) and ascorbic acid (2.5 mM) was then added to initiate oxidation and the cells were incubated at 30°C for two hours. The oxidation reactions was stopped by adding EDTA, the cells were lysed, and lipid peroxidation products (i.e., aldehydes) in the cells were analyzed by derivatizing with TBA (thiobarbituric acid) using standard procedures (Slater et al., 1984 Ebeler et al., 1994).

Preliminary data indicate a protective effect for resveratrol at low levels (0.4 mM). Further studies at other resveratrol concentrations are underway. Catechin also provided a protective effect against lipid peroxidation the decrease in lipid peroxidation was dependent on catechin concentration with the greatest effect seen at a concentration of 25 mM. Growth curves for Saccharomyces in the presence of both catechin and resveratrol were normal and no toxic effects were observed. We have also observed differing effects of yeast species, growth media and cell lysis conditions on the effects o oxidative status in the preliminary conditions. Therefore, optimization and standardization of the methodologies for analysis of oxidative status is crucial to the evaluation of the impact of the phenolic compounds on in vivo lipid peroxidation.

We are also carrying out experiments to evaluate the effect of phenols on the oxidation of DNA in the living yeast cell. Initial experiments consist of growing yeast cultures in the presence of specific phenolic compounds, introducing non-lethal oxidative conditions (described above), extracting DNA and analysing levels of oxidized DNA bases. The interpretation of the data obtained from these analyses by this and other labs has been hampered, however, by artifactual oxidation which occurs during sample handling and anlysis. We are addressing this problem in two ways. First, we are modifying our GC/MS method to minimize oxidation which might occur during the analysis. Second, we are evaluating the use of a recently described immunochemical technique for measuring levels of DNA oxidation. This potentially less problematic technique uses

highly specific antibodies to the DNA oxidation product malonaldehyde-deoxyguanosine (M1G) and has been successfully used to determine M1G!
levels from a number of tissue types.

We are also conducting preliminary experiments with a recently described mutant of S. cerevisiae designated mgm101 (Meeuson et al., 1999). This mutant appears to be deficient in the repair of oxidatively damaged mitochondiral DNA making it possible to study in vivo DNA oxidation without introducing artificial oxidizing conditions. For this reason and others, mgm101 may be an excellent model for studying oxidative process in vivo, especially as they effect mitochondria and respiration.

Results from this objective will begin to identify specific phenols which can act as antioxidants in vivo. 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.

(Objectives)

Plant phenolic compounds are biologically active, affecting many functions of eucaryotic cells. The goal of this work is to investigate the effect of phenolic compounds on growth and fermentation rates in Saccharomyces cerevisiae and to evaluate the role of these compounds as antioxidants in yeast. There are three specific sub-objectives of this proposal:

1. Analysis of the ability of specific phenolic compounds to stimulate yeast growth and fermentation rates.

2. Identify genes regulated by the presence of phenolic compounds using proteome analysis and HXT display.

3. Investigate the ability of phenolic compounds to protect against oxidative damage in yeast.

These analyses are being conducted in a synthetic juice medium, Triple M, which was redesigned this year due to problems in reproducibility. Both commercial and natural yeast isolates are being evaluated for the effect of phenolic compounds on fermentation and respiration rates as well as on growth. To date, no significant effects on growth have been observed in contrast to the initial report of Cantarelli. Objective 2 was originally going to be accomplished using the microarray analysis in collaboration with Dr. Joe DeRisi, now of UCSF. However, preliminary studies indicated that this technique would not suit our purposes at all, so we have switched to using the proteome analysis.

(Outside Presentations) There have been no outside presentations of the results obtained in Objectives 1,2 , and 3 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.

(Reasearch Success) This research program has already shown that dietary phenolic antioxidants have an effect on the metabolic activity of yeast cells. It will now be possible to determine the nature of that effect. One of the main criticisms of the body of work demonstrating the benefits of dietary antioxidants to human health is that the mechanism of this effect has not been demonstrated. This research proposal will determine the mechanism of the stimulation of yeast cells by phenolic compounds. Much of this information will be directly applicable to mammalian cells due to the high conservation of function among the eukaryotes.

(Funds Status) Funds from this grant were used in partial support of the salary of Laura Lange. She has subsequently left to take a position in the biotechnology industry and her part of the project has been taken over by Tammi Olineka. Funds were also used to partially support a PhD student, Mike Webb who has focused on the work described in Objective 3. In addition, two students, Mike Gray (MS) and Warren Place (PhD), have worked on aspects of this proposal during this grant year and were supported from other funds.

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