**PROGRESS REPORT**

January 30, 2015

Virginia Wine Board

# Identification and Quantification of Wine-related Microorganisms via Real-Time PCR

Principal Investigator:

**Amanda Stewart**

Assistant Professor of Enology and Fermentation

Virginia Tech

Department of Food Science and Technology

360 Duck Pond Dr., FST Bldg. MC 0418

Blacksburg, VA 24061

Phone: 540.231.0868

Fax: 540.231.9293

Email: amanda.stewart@vt.edu

Start date: July 1, 2014

Type of project: Research

Amount funded: $19,855

## ACCOMPLISHMENTS TO DATE

### A. OBJECTIVE:

**Overall Objective:** To develop in-house methods for rapid identification and quantification of yeasts and bacteria in juice and wine in support of Enology research and extension programs by leveraging Real-Time PCR equipment and molecular biology expertise in the Department of Food Science and Technology at Virginia Tech.

**Specific Objective 1:** Validate Real-Time PCR method for detection of target wine microorganisms in juice and wine down to concentrations of 1-10 cfu/mL including but not limited to *Brettanomyces bruxellensis*, *Oenococcus oeni*, and *Saccharomyces cerevisiae*.

**Specific Objective 2:** Employ this technology in preliminary research related to the impact of nitrogen concentration and composition on growth of common wine microorganisms such as *Brettanomyces bruxellensis*, *Oenococcus oeni*, and *Saccharomyces cerevisiae*.

### B. SUMMARY

During wine fermentation, microbial composition and growth are crucial factors that positively or negatively impact the characteristics of wine. Therefore, studies on nitrogen content, optimization of fermentation conditions and other strategies to control the wine microbiota have been conducted. To assess the impact of these studies, not only analyzing the chemicals of end products but also monitoring the growth of microorganisms by quantification is an essential step. The Virginia Tech Enology Service lab currently utilizes traditional methods to monitor yeast and bacteria by microscopy and plate counting in a selection media. Although this method provides reliable results, it is time consuming and doesn't take in to account viable but not culturable state (VNC) of microorganisms. Real-time PCR is a more advanced DNA-based technique which rapidly identifies and quantifies microorganisms including VNC. Our lab recently acquired a Qiagen Rotor Gene 6000 Real-Time PCR machine previously used by Food Microbiology researchers in the Department of Food Science and Technology. Using the current funds provided by the Virginia Wine Board, supplies and materials for real-time PCR method development were purchased and set up in

the new Enology and Fermentation lab to bring the real-time PCR methods online. With respect to specific objective 1, reference strains of wine yeasts and bacteria including *Brettanomyces bruxellensis*, *Saccharomyces cerevisiae*, *Oenococcus oeni* were obtained from ARS Culture Collection (NRRL), cultured and subjected to species-specific PCR of *B. bruxellensis*. This step was to confirm the specificity of real-time PCR primer of *B. bruxellensis* designed based on studies published by others (Phister and Mills, 2013). Our results showed that the primer for *B. bruxellensis* did not amplify other wine microorganisms (it was specific to *B. bruxellensis*) and therefore was chosen to use in the real-time PCR experiment.

**C. LAB SET UP**

To set up the lab for the real-time experiment utilizing the Qiagen Rotor Gene 6000 equipment, a laptop PC was purchased and connected to the real-time PCR machine (Fig 1.). Rotor-Gene Software was installed in the laptop PC and is currently ready to use. Moreover, supplies and materials such as genomic DNA extraction reagents (lyticase, 2-mercaptoethanol, Tris pH 8.0), PCR reagents, micropipettors and consumables (microcentrifuge tubes, filtered pipet tips) essential for the experiment were purchased.

**D. RESEARCH PROGRESS**

Prior to conducting real-time quantitative PCR, gDNA extraction and primer specificity were evaluated by using conventional PCR. This was achieved using equipment in other labs in the Food Science & Technology Department on Virginia Tech's Blacksburg campus. Five common wine yeasts *Brettanomyces bruxellensis* Y1413, *Saccharomyces cerevisiae* Y12632, *Candida apicola* Y6688, *Hanseniaspora osmophila* Y1613 and *Pichia membranifaciens* were obtained from ARS Culture Collection (NRRL) and genomic DNA of each strain was extracted. We selected the method for genomic DNA extraction which had the highest sensitivity and effectiveness among 6 methods tested and reported by Portugal and Ruiz-Larrea (Portugal and Ruiz-Larrea 2012). After extracting the genomic DNA, each concentration was measured by NanoDrop spectrophotometer and it was confirmed that all samples had sufficient amount of genomic DNA (around 1000 ng/ul).

In order to specifically amplify the DNA of *B. bruxellensis*, DBRUX-F, DBRUX-R primer set (PCR product size : 79 bp) developed by (Phister and Mills 2003) and DB90F, DB 394R primer set (PCR product size: 305 bp) designed by (Cocolin, Rantsiou et al. 2004) were

evaluated in this experiment. The results of conventional PCR using each primer set are shown in Fig 2. When DBRUX primer set was used (Fig 2a.), a thick band having a size of 79 bp was observed in lane 2 (*B. bruxellensis*). However, other lanes including the negative control which should contain no band, (lane 1) also showed a faint band having similar size. It is thought that primer dimers were formed or other kinds of undesirable reactions occurred. We will continue to troubleshoot this method until suitable specificity is demonstrated. When primers DB 90F and DB 394R were used (Fig 2b.), only Lane 2 *(B. bruxellensis)* showed a thick band and no other bands were observed in the other lanes, which is the desired result. Therefore, it was confirmed that primers for *B. bruxellensis* were specific and genomic DNA was properly extracted from the reference strains. Expenditures to date to complete the work reported here are summarized in Table 1.

**E. FUTURE PLANS**

To elaborate the next step which will be conducted in the near future, *B. bruxellensis* will be cultured in a liquid media to the point of $O.D_{600}$=1 corresponding to 2~4 $\times 10^7$ CFU/ml and after serially diluting the culture, each sample will be plate counted and the genomic DNA will be extracted to perform the real-time quantitative PCR. A Linear regression model will be generated by plotting the log CFU/ml and Ct value of each sample to validate the real-time quantitative PCR method. Commercial wine or wine artificially contaminated by *B. bruxellensis* will then be used to actually quantify this strain. Using the same approach, *S. cerevisiae* and *O. oeni* can be quantified as well. The supplies and materials to complete the work have been purchased and are being used. An undergraduate student is conducting an independent research project in Spring 2015 to gather preliminary data on the impact of nitrogen sources in wine on growth of *B. bruxellensis,* to begin to address Objective 2. The remaining expenditures for this project consist primarily of graduate student support, which will be used to pay for the remaining labor needed to complete the project. Travel support funds will be used to attend and present at a state meeting on the topic of PCR methods for detection and quantification of wine microorgansims.

## F. REFERENCES

Cocolin, L., et al. (2004). "Molecular Detection and Identification of Brettanomyces/Dekkera bruxellensis and Brettanomyces/Dekkera anomalus in Spoiled Wines." Applied and Environmental Microbiology 70(3): 1347-1355.

Phister, T. G. and D. A. Mills (2003). "Real-Time PCR Assay for Detection and Enumeration of Dekkera bruxellensis in Wine." Applied and Environmental Microbiology 69(12): 7430-7434.

Portugal, C. and F. Ruiz-Larrea (2012). "Comparison of Specific Real-Time PCR and Conventional Culture for Detection and Enumeration of Brettanomyces in Red Wines." American Journal of Enology and Viticulture 64(1): 139-145.

## APPENDIX

### Impact Statement:

Setting up real-time quantitative PCR methods in the Enology & Fermentation Lab at Virginia Tech expands research and analytical capabilities for the Enology program to support the continued growth and success of the Virginia Wine Industry. This project leverages existing equipment and expertise in the Department of Food Science & Technology, adding breadth to resources available for Enology research and extension.

### Figures and Tables:


**Figure 1. Qiagen Rotor Gene 6000 and laptop PC in new Enology & Fermentation Lab.**

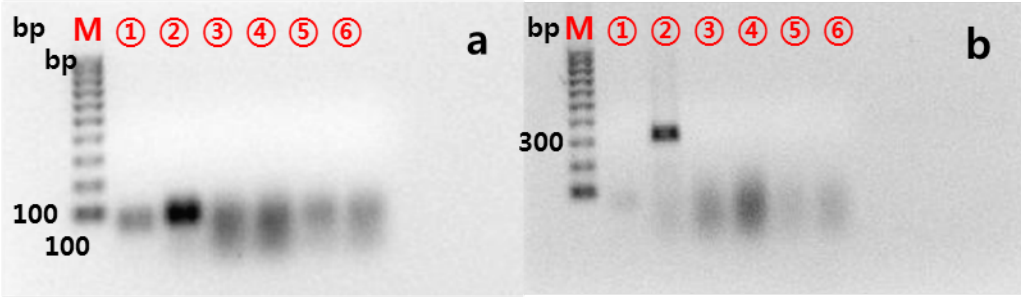


**Figure 2.** ***Brettanomyces*****-specific PCR. (a) PCR using DBRUX-F and DBRUX-R primer (b) PCR using DB90F and DB394R**

**M: Marker, ①: negative control, ②: *B. bruxellensis*, ③: *Candida apicola*, ④: *H. osmophilla*, ⑤: *P. membranifaciens*, ⑥: *S. cerevisiae***

**Table 1. Expenditures to date (as of January 15, 2015).**

| Expense | Allocation | % of category spent as of 1/2015 |
|---|---|---|
| GRA step 11 and fringe (33%) | 7,912 | 45% |
| Tuition and academic fees, 2 semesters (AY) | 3,943 | 100% |
| Materials and supplies | 7,200 | 108% |
| Travel support | 800 | 0% |
| Total | $19,855 | 77% |