AIP – Activity 8 2018

Population dynamics of yeast fermenting Pinot Noir and Chardonnay Wine

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As reported in 2016, we reported that an indigenous strain of *S. uvarum* dominated all fermentations at all stages except for cold soak. We followed up this result, where we reported that *S. uvarum* had an ability to compete with *S. cerevisiae* under controlled conditions while fermenting Chardonnay juice. We found that when *S. uvarum* was inoculated at the same concentration as *S. cerevisiae*, *S. uvarum* had a greater competitive ability at 15°C than at 24°C to ferment Chardonnay juice, supporting a previous report that *S. uvarum* is cryotolerant. In the present reporting period 2017/2018, we continued our studies on *S. uvarum* in which we repeated the experiment conducted in 2015. We found *S. uvarum* again dominated all stages of the spontaneous fermentations. We also found that there were over 40 *S. uvarum* strains present in the fermentations. These results demonstrate that *S. uvarum* has the ability to dominate spontaneously conducted winery fermentations in multiple years, that the species is genetically diverse, and that it represents a yeast, which is indigenous to the Okanagan, that can potentially finish fermentations in the presence of *S. cerevisiae*.

In addition, we conducted a study at Tin Horn Creek Winery on Chardonnay wine; we found that that the spontaneous fermentations were dominated by *S. cerevisiae* strains, but unlike in previous years, they were dominated by potentially indigenous *S. cerevisiae* strains as opposed to commercial strains. There have been indications in our previous studies that South Okanagan may favour indigenous strains over commercial strains whereas we have found the opposite result in the Central and North Okanagan. Future studies that are directed at comparing the South and North Okanagan will need to be conducted to test this initial observation.

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As stated above, one of the most interesting results during this reporting was that *S. uvarum* dominated all fermentations at all stages for a second year. Along with last year's results, showing it had a greater competitive ability at 15°C than at 24°C compared with *S. cerevisiae*, we propose that these strains have the potential tofinish fermentations in the presence of *S. cerevisiae* and they have high genetic diversity to persist in a changing environment.

In addition, there have been indications in our previous studies that South Okanagan wineries may favour indigenous strains over commercial strains, whereas we have found the opposite result in the Central and North Okanagan. Future studies that are directed at comparing the South and North Okanagan will need to be conducted to test this initial observation.

Objective 1: The first objective this year was to determine whether *S. uvarum* dominates Chardonnay fermentations as it did in 2015 and whether the vineyard has an impact on the yeast conducting the fermentation.

Methods: Mission Hill Estate Winery is located on the western side of Okanagan Lake in Kelowna, British Columbia, Canada. There were three spontaneous fermentation treatments with each treatment having a different vineyard origin of grapes (this aspect was different from the 2015 study). Two levels of SO_2 were added directly to the barrels (n=3 stainless steel barrels per treatment): 0 and 40 mg/l SO_2. SO_2 will be added as potassium metabisulfite (KMS) ($K_2S_2O_5$): 0 mg/l, 40mg/l KMS will be added to the barrels of the three treatments, respectively. No inoculum will be added to the barrels, and alcoholic fermentation will be allowed to progress spontaneously. Samples for microbial analysis will be taken at four stages, as defined by °Brix levels: cold-settling, early, mid, and late. The cold-settling sample will be taken immediately after sulfite addition, and subsequent samples will be taken at the following stages: 14-19 °Brix for early, 7-12 °Brix for mid, and <2 °Brix for late. Three sub-samples will be taken from each barrel at each stage. Samples for free and total SO_2 determination will be taken at the same four stages of fermentation, and will be taken at the same time as the samples for microbial analysis. Additional SO_2 samples will be taken at two times following fermentation: (1) when the wines are transferred from the barrels into 2L glass containers, and (2) at the time of bottling. The samples taken for microbial analysis will be diluted, plated, and incubated: they will be used to determine total yeast CFU/mL as well as *S. cerevisiae* strain diversity and composition, outlined below in Section 4.2. Next generation sequencing will also be conducted on all these samples, but will be funded by and BC Investment Agriculture grant, thus detailed methods will not be discussed here.

4.2 Culture-dependent identification of S. cerevisiae *strains*

4.2.1 Yeast isolation: At the early, mid, and late stages of fermentation, each sub-sample (3) from each barrel will be serially diluted, and 100µL from various dilutions will be plated on solid YEPD media (10 g/L yeast extract, 10 g/L bacterial peptone, 20 g/L dextrose, 20 g/L granulated agar) and incubated at 28°C for two days. Samples from the cold-soak stage will not be plated, as historically *S. cerevisiae* is not found at this early stage (Zott et al., 2008). Post-incubation, plates containing 30-300 colonies will be counted, and CFU/ml will be calculated. If no plates contain 30-300 colonies for a specific sub-sample, the plate with the closest number of colonies to this range will be counted instead. From these counted plates, 24 colonies will be chosen at random and isolated onto solid YEPD media in 24-welled slant plates, and incubated at 28°C for two days. All post-incubation plates will be stored at 4°C to prevent further colony growth.

4.2.2 DNA extraction: DNA from each yeast isolate will be extracted in preparation for identification by molecular analysis using water extraction. Fifty µl molecular-grade water will be pipetted into the wells of a non-skirted, 96-welled PCR plate. Cells from each yeast isolate will be transferred into individual wells using sterile pipette tips. These plates will be gently vortexed, and then heated in an Applied Biosystems® Veriti® 96-Well Fast Thermal Cycler (Foster City, USA) at 95°C for 15 minutes. They will then be sealed and stored at -20°C. In addition, we conducted in-lab fermentations from grapes sampled from different vineyards. Isolation and identification were performed as described above.

4.5 Data analysis

Yeast species and strain diversity will be calculated both by stage and by pooling the isolates from all stages in each treatment. A repeated measures analysis of variance (ANOVA) will be performed to determine the relationship between fermentation stage and diversity of both total yeast species and *S.*

cerevisiae strains. A two-factor ANOVA will be performed to determine the relationship between sulfur dioxide treatment and diversity of total yeast species. These procedures will be repeated to determine the relationship between total yeast CFU/mL, fermentation stage, and sulfur dioxide treatment. A Bray-Curtis dissimilarity index will be used to compare the differences between the compositions of yeast species/strains present during fermentation in the different treatments. A non-metric multidimensional scaling (NMS) ordination will be performed to analyze the distances in composition of the yeast species and strains between the different sulfur dioxide treatments using PC-ORD 6.0 (MjM Software Ltd.). A multiple response permutation procedure (MRPP) will be performed as a post-hoc test to assess whether the distances between compositions are significantly different from each other. A principal component analysis (PCA) and three-factor ANOVA will be performed on the mean sensory scores to assess the relationship among the attributes of the wines and the different treatments, as well as to determine the tasting consistency of each judge between the two replicate tastings. Statistical analyses will be performed using JMP® 11 Statistical Software (SAS® Institute Inc.) as well as RStudio® software.

Results: *S. uvarum* dominated all fermentations at all stages for a second year. Along with last year's results, showing it had a greater competitive ability at 15°C than at 24°C compared with *S. cerevisiae*, we propose that these strains have the potential to finish fermentations in the presence of *S. cerevisiae* and they have high genetic diversity to persist in a changing environment.

Student Responsible: This work was conducted by Garrett McCarthy, my MSc student.

Objective 2: We determined at Tinhorn Creek Winery the yeast composition present in the vineyard and determined whether the vineyard had an impact on the yeast conducting the fermentation.

Methods: Two Chardonnay vineyards located in Oliver, BC, Tinhorn Creek (Vineyard 1) and Covert Farms (Vineyard 2), were randomly sampled by dividing each vineyard into separate blocks then numbering the rows and splitting the numbered rows into equal groups based on the number of posts supporting the vines. A random number generator was used to determine which row from each group was sampled. Then, a section on either end of each row was removed, equal to 3 posts worth of vines or about 15 vines, to ensure that the grapes chosen were not too close to a road or other factors that may influence the microbiota present. The number of sections in each row was split into equal groups and a random number generator was used to determine which section of each sampled row was to be sampled. Vineyard 1 and Vineyard 2 were sampled in the same way (Figure A1 & Figure A2). Approximately 24 hours prior to the harvest date for each of the vineyards, six samples were taken aseptically using autoclaved sampling bags. Samples were taken September 19, 2017 for Vineyard 1 and October 13, 2017 for Vineyard 2. Each sample consisted of a bag containing approximately 30 berry clusters, weighing 3-4 kg.

The samples of grapes were transported back to UBCO where they were crushed by hand for ten minutes, then transferred to sterile 500mL fermentation flasks with airlocks containing 6 mL of sterile water to maintain the sterility of the fermentation while allowing gas produced during fermentation to escape. The laboratory fermentations from Vineyard 1 were initially placed into an incubator held at 12 °C.

Fermentation progression was determined by weighing samples every day and measuring the °Brix using a refractometer. The weight of the fermentation is used to monitor the progress of

fermentation by approximating that for every 2.18 g of sugar consumed by yeast, there is 1 g of CO_2 produced for *Saccharomyces* species (Delfini and Formica, 2001). Samples for microbial analysis were taken from the laboratory fermentations at two different stages: initially after crushing (juice), and a final sample when the fermentation was completed, with no weight change over three consecutive days.

Fermentation Sampling

The grapes were harvested and processed by Tinhorn Creek Winery in Oliver, BC according to standard viticultural practices in British Columbia. Three separate stainless-steel barrels for each vineyard fermentation were set aside and served as replicates, for a total of six barrels. Two 50 mL samples were collected from each barrel by winery staff and placed on ice and shipped to the University of British Columbia Okanagan. Samples were taken immediately after crushing, at one third sugar depletion, two thirds sugar depletion, and at the end of fermentation, determined by °Brix levels. Samples generally reached the University within 24 hours of sampling.

Samples taken at the stages outlined above were serially diluted and spread-plated onto solid YEPD agar (dextrose $20gL^{-1}$, agar $20gL^{-1}$, bacterial peptone $10gL^{-1}$, yeast extract $10gL^{-1}$), and incubated for 48 hours at 28 °C. Samples taken at the juice stage, the 1/3 sugar depletion, and the 2/3 sugar depletion stage for winery samples were plated on solid YEPD agar with 10 mg/mL chloramphenicol and 15 mg/mL biphenyl. When possible, 47 yeast colonies were transferred from plates containing 30-300 colonies to Wallerstein Nutrient Agar (WLN) and incubated for 48 h at 28 °C for preliminary species identification. There was a positive control for each species present on each WLN plate, one colony of *S. cerevisiae* (Lalvin BA11®) and one colony of *S. uvarum* (CBS 7001) to serve as comparison to unknown colonies. The other half of each colony from the original 30-300 colony YEPD plate was then isolated by streaking into a single well containing YEPD in a 24-well slant plate.

DNA Extraction and Yeast Identification

DNA from each of the isolated colonies was extracted using a water-extraction protocol. Each colony was suspended in 50 µL DNA-grade water and heated at 95°C for 15 minutes in an Applied Biosystems® Veriti® 96-Well Fast Thermal Cycler (Foster City, USA). In addition to extracting DNA, each colony isolated was also transferred to a 20% glycerol solution and stored at -80°C to keep the cells intact and viable for a long period of time.

Non-*Saccharomyces* yeasts were identified to the species level to identify which yeast species were present in the vineyard, other than *S. cerevisiae*. The *S. cerevisiae* isolates, identified on WLN media, were strain-typed using microsatellite analysis of 10 unique loci. These loci have been chosen because of their variability in allele lengths and their frequencies in the population (Legras et al., 2005; Richards et al., 2009). Variable allele lengths allow for the determination of distinct strains of the same species of yeast.

Statistical Analysis

The populations present in the laboratory fermentations were analyzed and compared to determine if there is any significant difference between the populations present between vineyards and to determine which strains are sufficiently different to be classified as unique strains. This data was analyzed using the software, R. To properly categorize the isolates from the vineyards, Bruvo genetic distance measures are used. Bruvo genetic distances are a way to measure microsatellite genotype distances that takes mutations into account and is not dependent on ploidy level (Bruvo et al., 2004). To calculate genotypes using a Bruvo distance threshold of 0.3 the package “poppr” in R was used. The different genotypes present were compared to a commercial yeast database to determine which yeast isolates present are from a commercial source. To quantify the difference in community and population composition between the vineyards sampled and winery fermentations a Bray-Curtis dissimilarity index was calculated. A Principle Coordinate Analysis (PCoA) ordination was

generated to visually examine the yeast species and strains present in the vineyards. A permutational analysis of variance (PERMANOVA) was performed to determine if the yeast communities and populations present in each vineyard are significantly different from each other.

Results: We found that that the spontaneous fermentations were dominated by *S. cerevisiae* strains, but unlike in previous years, they were dominated by potentially indigenous *S. cerevisiae* strains as opposed to commercial strains. There have been indications in our previous studies that South Okanagan may favour indigenous strains over commercial strains whereas we have found the opposite result in the Central and North Okanagan. Future studies that are directed at comparing the South and North Okanagan will need to be conducted to test this initial observation.

Student Responsible: Brianne Newman

We found that *S. uvarum* could dominate fermentations in multiple years at a specific winery, but at other wineries it was found to be completely lacking in spontaneous fermentations. Based on the Tinhorn Creek study, commercial yeast are not always the dominant *S. cerevisiae* strains present in wine fermentations.

There have been indications in our previous studies that South Okanagan wineries may favour indigenous strains over commercial strains, whereas we have found the opposite result in the Central and North Okanagan. Future studies that are directed at comparing the South and North Okanagan fermentations will need to be conducted to test this initial observation.