Washington State Grape and Wine Research Program

ANNUAL PROGRESS/FINAL REPORT FORMAT
2021-22 FUNDING CYCLE

Title: Microbiology and Chemistry of Washington Wines (3 year project 2018-2021)

Principal Investigator: C.G. Edwards, School of Food Science, Washington State University, Pullman, WA 99164-6376 (ph. 509-335-6612; email edwardsc@wsu.edu).

Section 1. Summary:

One approach to limit spoilage associated with *Brettanomyces bruxellensis* is application of so-called "hurdle technology" where synergies between two or more antagonistic factors are used to inhibit the target microorganism to a greater extent than relying upon only one factor. To this end, the current project has studied how interactions between molecular SO_2, ethanol, and storage temperature affect this spoilage yeast under vinification conditions. While yeast growth was influenced by ethanol and storage temperature, mSO_2 greatly impacted the culturability of the yeast. Statistical analyses may indicate significant interactions between these three factors which affect the yeast including formation of off-odors associated with 4-ethylphenyl and 4-ethylguaiacol.

Additional research has illustrated the importance of wine composition on growth of *B. bruxellensis.* Here, a single strain of the yeast was inoculated into six different red wines (primarily Merlot) which had similar concentrations of mSO_2 (none) and ethanol (13.5% v/v) and were all stored at 18°C. While the yeast grew well in some wines, rapid declines in culturabilities were noted for others. Differences in growth do not appear to be due to a lack of nutrients but suggested the presence of unidentified inhibitory factors.

Work involving commercial application of non-*Saccharomyces* yeasts as a means to reduce ethanol production has continued. Merlot grapes were obtained from a commercial vineyard and fermented under industrial conditions in 2018 (120 kg must per fermenter). Two different non-*Saccharomyces* yeasts, originally obtained from a regional vineyard, were inoculated and fermentations proceeded under either temperature regime A (maximum 15°C for 72 hr. followed by 25°C) or B (maximum 17.5°C for 72 hr. followed by 25°C). Those musts inoculated with *Mt. pulcherrima* and vinified under temperature regime B contained the lowest amounts of ethanol, compared to those with only *S. cerevisiae* present (14.3% vs. 15.0% v/v). Sensory analysis confirmed that wines produced with *Mt. pulcherrima* (temperature regime B) were deemed to have less 'dried fruit' and 'herbaceous' (aroma), 'bitter' (taste), and, most importantly, less 'ethanol' (flavor) and 'hot/ethanol' (mouthfeel) in comparison to control fermentations with only *S. cerevisae*. However, research conducted in 2019 was inconclusive due to hindrance of non-*Saccharomyces* yeasts through reduced initial must temperatures as a result of harvest conditions. It therefore appears that must temperature is a critical factor affecting ethanol reductions use of *Mt. pulcherrima* and *My. guilliermondii*. With optimization of conditions, reducing final ethanol concentrations is achievable without quality loss and possible improvement.

Section 2. Annual or Final Report:

Final report.

Section 3. Project Title:

Microbiology and Chemistry of Washington Wines.

Section 4. Principal Investigator/Cooperator(s):

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Section 5. Objective(s) and Experiments Conducted to Meet Stated Objective(s):

Objective 1: *Brettanomyces* spoilage of wines.

(a) Compare single and continuous additions of molecular SO_2 to wines inoculated with *B. bruxellensis*.

(b) Investigate three-way antagonistic effects of molecular SO_2, alcohol concentration, and storage temperature on growth and metabolism (*i.e.*, production of volatile phenols, volatile acidity, etc.) of *B. bruxellensis*.

Eight, commercially produced red wines representing five different brands were obtained from California (Wineries A and B) and from Washington (Wineries C, D, and E). Three wines were obtained from Winery A, two non-vintage, "burgundy-style" (bulk) wines which were purchased in 2018 (wine 1) and 2019 (wine 2) as well a 2018 Merlot (wine 3). An additional 2018 Merlot was received from Winery B (wine 4). Three wineries from Washington provided four Merlot wines of different vintages; 2014 from Winery C (wine 5), 2014 (wine 6) and 2015 (wine 7) from Winery D, and a 2016 from Winery E (wine 8).

A 4 x 2 x 2 factorial design with concentrations of molecular SO_2 (0 to 0.8 mg/L), ethanol (13.5% or 14.5% v/v), and storage temperature (12° or 18°C) as variables was conducted using wine 7 (pH 3.85, 13.5% v/v ethanol). Sulfites originally present in the wine were initially removed to <0.02 mg/L by adding enough 0.9% v/v H_2O_2 as confirmed by the aeration-oxidation method (Buechsenstein and Ough 1978). Various amounts of potassium metabisulfite were then added to yield "low" (0.25 to 0.28 mg/L), "medium" (0.36 to 0.42 mg/L), or "high" (0.86 to 0.92 mg/L) concentrations of molecular SO_2. Fixed volumes containing different proportions of ethanol:water were added to produce wines of varying ethanol concentrations. Ethanol concentrations were confirmed by an Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA). After SO_2 removal and ethanol adjustment, wines were supplemented with 0.5 g/L glucose, 0.5 g/L fructose, 0.1 g/L yeast extract while pH was adjusted to 3.70 using 5 M NaOH or 50% v/v H_3PO_4. The wines were then sterile-filtered through 0.2 µm PES Express Plus Bottle-top filters (MilliporeSigma) into 100 mL sterilized milk dilution bottles.

After incubating the bottles at 12° or 18°C for 24 hr., the wines were inoculated with 10^5 CFU/mL *Brettanomyces bruxellensis* I1a (Jensen et al. 2009) previously maintained on

Wallenstein Laboratory Differential (WLD) medium agar (Becton Dickinson and Co., Sparks, MD). Starter cultures were prepared from single colonies inoculated into 10 mL of yeast mold (YM) broth (Becton Dickinson and Co.) adjusted to pH 3.85 using 50% v/v H_3PO_4. Ten mL cultures were incubated at 27°C for 96 hr. before 100 µL was transferred into 50 mL YM broth containing 5% v/v ethanol. After 60 hr., cells were harvested by centrifugation at 2000 x *g* for 20 min, washed twice with 0.1% w/v peptone, and suspended in wine or inoculation (10^5 cfu/mL). SO_2 was added to bottles on day 13 in the form of a 40% w/v potassium metabisulfite solution (Sigma Aldrich, St. Louis, MO). During incubation, three bottles of each treatment were randomly selected and removed for analysis.

Yeast culturability was evaluated by plating on WLD using an Autoplate 4000 spiral plater (Spiral Biotech, Bethesda, MD) and incubated at 27°C. After incubation of 90 days, wines were analyzed for organic acids, glucose/fructose, and ethanol using an Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA) outfitted with refractive index detectors and a UV-VIS diode array (Eyéghé-Bickong et al. 2012). Concentrations of 4-ethylphenol and 4-ethylguaiacol were measured by gas chromatography-mass spectroscopy (Oswald and Edwards 2017). Analysis of variance (ANOVA) and Tukey's HSD were carried out for mean separation at probability $p \leq 0.05$ using Minitab statistical software (Minitab LLC, 2019).

Objective 2: Impact of non-*Saccharomyces* yeasts on wine quality.

(a) Complete sensory and chemical analyses of Merlot wines produced in 2017 and 2018 inoculated with non-*Saccharomyces* yeasts.

(b) Evaluate the role of temperature and timing of inoculation on survivability of selected non-*Saccharomyces* yeast strains and wine composition (*i.e.*, reduction in ethanol content).

The two species of non-*Saccharomyces* used were *Metschnikowia pulcherrima* P01A016 and *Meyerozyma guillermondii* P40D002, previously isolated from vineyards located in Washington state (Bourret et al. (2013). *Saccharomyces cerevisiae* D254 was acquired from Lallemand Inc. (Montréal, Quebec, Canada). Strains were maintained on yeast peptone dextrose (YPD) agar plates incubated at 28°C.

Starter cultures were prepared in YPD broth from single colonies grown on YPD agar. Upon reaching late exponential phase, cells were harvested by centrifugation at 3000 x *g* for 15 min, washed twice with 0.2 M phosphate buffer (pH 7.0), and resuspended in grape must diluted 1:1 with sterile water prior to inoculation.

In 2018, Merlot grapes were obtained from a commercial vineyard located in south central Washington state. After crushing/destemming, grape must (145 g/L glucose, 142 g/L fructose, and 61 mg N/L yeast assimilable nitrogen) was distributed into 300 L stainless steel, jacketed fermenting tanks with 120 kg must per fermenter. Potassium metabisulfite was added to each fermenter to achieve 25 mg/L total SO_2.

Control tanks were inoculated, in duplicate, with rehydrated active dry yeast (*S. cerevisiae*) per manufacturer's instructions, temperature controls were set to a maximum of 25°C and fermentation proceeded with lids attached. Grape musts were inoculated with either *Mt.*

pulcherrima or *My. guilliermondii* at 10^6 CFU/mL, in duplicate. Temperature controls were set to a maximum at 15°C (temperature regime A) or 17.5°C (temperature regime B) for fermentation without lids for 3 days. After this time, these tanks were inoculated with *S. cerevisiae*, lids were attached, and maximum temperatures were set to 25°C. For all fermentations, cap management consisted of twice daily punch-downs and two additions of 0.25 g/L Fermaid-K (Lallemand), one at 12 hr. after inoculation of *S. cerevisiae* and another 48 hours later.

When soluble solids reached approximately 0°Brix, fermentations were pressed into 100 L stainless steel tanks and stored at ambient temperature (21±2°C). All wines underwent spontaneous malolactic fermentation prior to addition of potassium metabisulphite (30 mg/L total SO_2) and storage at 9°C. Wines were then adjusted to 0.4 mg/L molecular SO_2 and sterile-filtered through 0.45 µm polyvinylidene fluoride cartridges (MilliporeSigma, Bellerica, MA) housed in stainless-steel filter housings (Pall, Port Washington, NY) into sterile 750 mL screw-capped bottles which had been flushed with N_2 gas. After bottling, the wines were stored at 7°C prior to chemical and sensory analyses.

Wines produced in 2018 were evaluated by a trained sensory panel. Panelists (n=10) were trained to evaluate a range of attributes associated with aroma, taste, mouthfeel and flavor. Following training, panelists evaluated the five treatments in triplicate utilizing a 15 cm, unstructured line scale. Data were analyzed with ANOVA and Fisher's least significant difference.

In 2019, additional Merlot grapes were obtained from the same vineyard block as in 2018. After crushing/destemming, 85 kg grape must (135 g/L glucose, 140 g/L fructose) was distributed into the 300 L fermentation tanks. Potassium metabisulfite was added to each fermenter to achieve 25 mg/L total SO_2. Musts were inoculated, in triplicate, with *Mt. pulcherrima* on day 0 followed by *S. cerevisiae* on days 0, 1, or 3 with additional musts only inoculated with *S. cerevisiae* on day 0. All tanks were set to a maximum of 17.5°C and fermented without lids for 3 days. Following this time, temperature controls were adjusted to 25°C and fermentation proceeded with lids attached. For all fermentations, cap management consisted of twice daily punch-downs. Two additions of 0.25 g/L Fermaid-K (Lallemand) were made, one 12 hours after inoculation of *S. cerevisiae* and another 48 hours later. When soluble solids reached approximately 0°Brix, free run was collected in five-gallon glass carboys and stored at ambient temperature until dry (<2 g/L glucose and fructose).

Section 6. Summary of Major Research Accomplishments and Results by Objective:

Objective 1. As illustrated by Figures 1 to 4, growth of *B. bruxellensis* declined upon addition of any concentration of molecular SO_2 (mSO_2). The exceptions were wines adjusted to 13.5% ethanol and stored at 18°C where scattered culturable populations were detected in some replicates upon addition of 0.2 mg/L mSO_2. Declines in the concentrations of mSO_2 depended upon initial concentration as well as temperature of storage (higher reduction with higher temperature) but not ethanol content. Concentrations of 4-ethylphenyl and 4-ethylguaiacol are being statistically analyzed at the time of writing.

Even taking into account SO_2, ethanol, and storage temperature, growth of *B. bruxellensis* was highly dependent on the specific wine (Figure 5). As evidence, better growth was illustrated in wines 2, 4, 5, and 6 with the yeast exhibiting a longer lag phase when inoculated into wine 3. Upon inoculation in wine 1, capturability of *B. bruxellensis* reached undetectable levels within only 20 days. Explanation for differences in growth remain unknown but does not appear to be due to a lack of any nutrients (data not presented).

Objective 2. Grape musts (120 kg must per fermenter) were either inoculated with *S. cerevisiae* alone or with *Mt. pulcherrima* or *My. guilliermondii*, the latter two under either temperature regime A or B. Without inoculation of non-*Saccharomyces* yeasts, *S. cerevisiae* quickly reached populations of almost 10^9 cfu/mL while those non-*Saccharomyces* yeasts originally present in the musts declined from 10^5 cfu/mL to undetectable levels (Figure 6). Inoculation of *Mt. pulcherrima* resulted populations of non-*Saccharomyces* yeasts to peak at 10^7 cfu/mL under temperature regime B before declining (Figure 7). Populations of non-*Saccharomyces* yeasts including *My. guilliermondii* reached a lower peak ($>10^6$ cfu/mL) prior to die-off by day 13 (Figure 8).

Those wines not inoculated with non-*Saccharomyces* yeasts contained 15.0% v/v ethanol while residual glucose or fructose were not detected (Table 1). Although musts with *Mt. pulcherrima* or *My. guilliermondii* inoculated contained lower amounts of ethanol, most values were not statistically different from wines only inoculated with *S. cerevisiae*. The exceptions were those grape musts inoculated with *Mt. pulcherrima* and fermented under temperature regime B with only 14.3% v/v present. These experiments illustrate the importance of temperature towards encouragement of non-*Saccharomyces* yeasts. Few other compositional differences were noted regarding yeast production glycerol, succinic acid, or acetic acid (Table 1).

Sensory analysis of the 2018 Merlot wines by a trained panel focused on a total of 44 attributes associated with aroma, taste, flavor, and mouthfeel (Table 2). Comparing *Mt. pulcherrima* (temperature regime B) to those with only *S. cerevisiae* present, wines were deemed to have less ‘dried fruit’ and ‘herbaceous’ (aroma), ‘bitter’ (taste), less ‘ethanol’ (flavor), and less ‘hot/ethanol’ (mouthfeel) but higher for ‘round’ (‘balanced’). It should be noted that the replicate fermentations containing *My. guilliermondii* (temperature regime A) were thought to be too different sensorily so wines were not pooled for sensory analysis. Many of the sensory differences between these two replicates were related to mouthfeel, notably ‘roughness’, ‘drying’, ‘puckering’, and ‘sharp.’

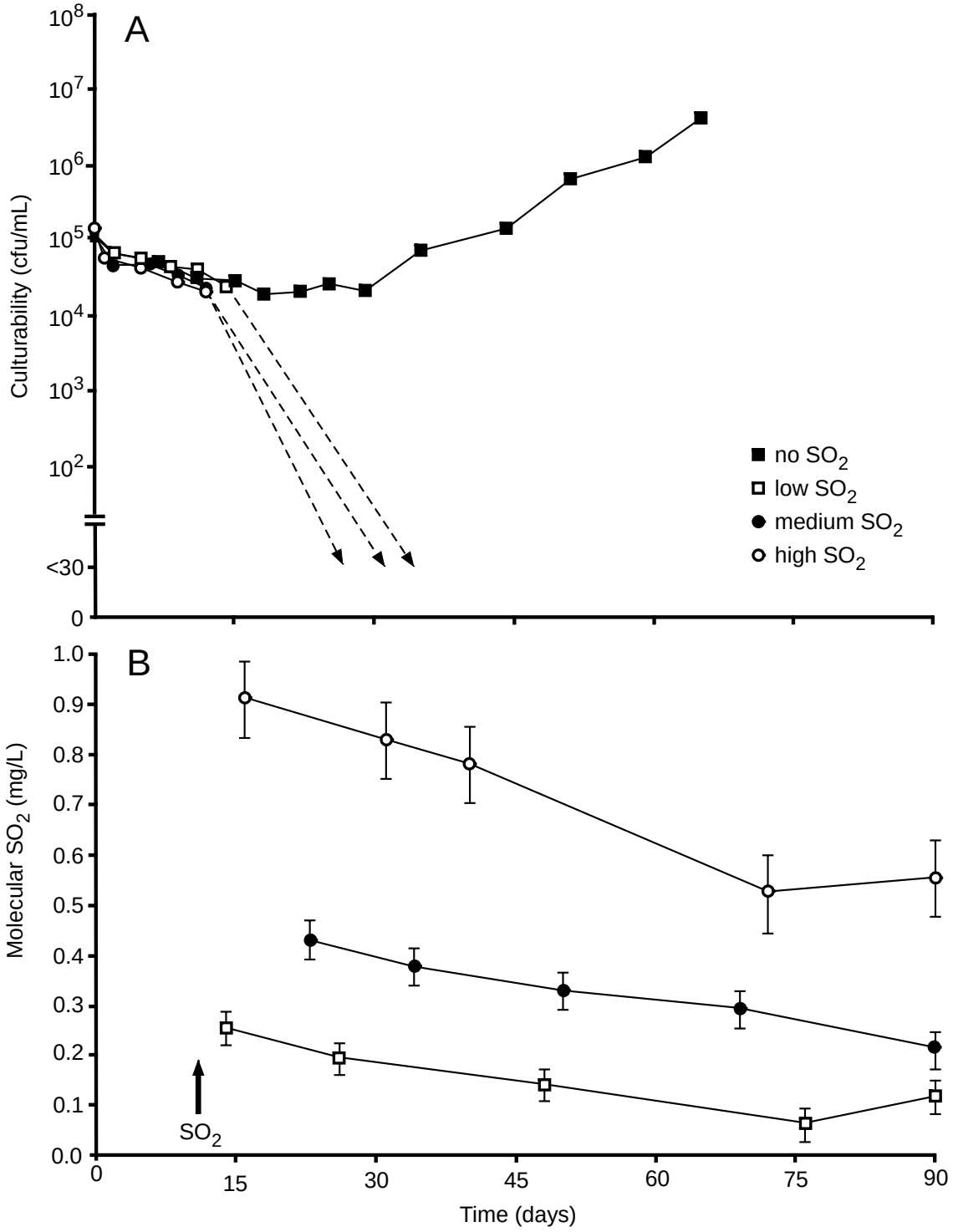

Figure 1. Culturability of *B. bruxellensis* I1a (A) and decline in concentrations of molecular SO_2 (B) in Merlot wine at 12°C and adjusted to 13.5% v/v ethanol. Various concentrations of SO_2 were added on day 13 as indicated by an arrow.

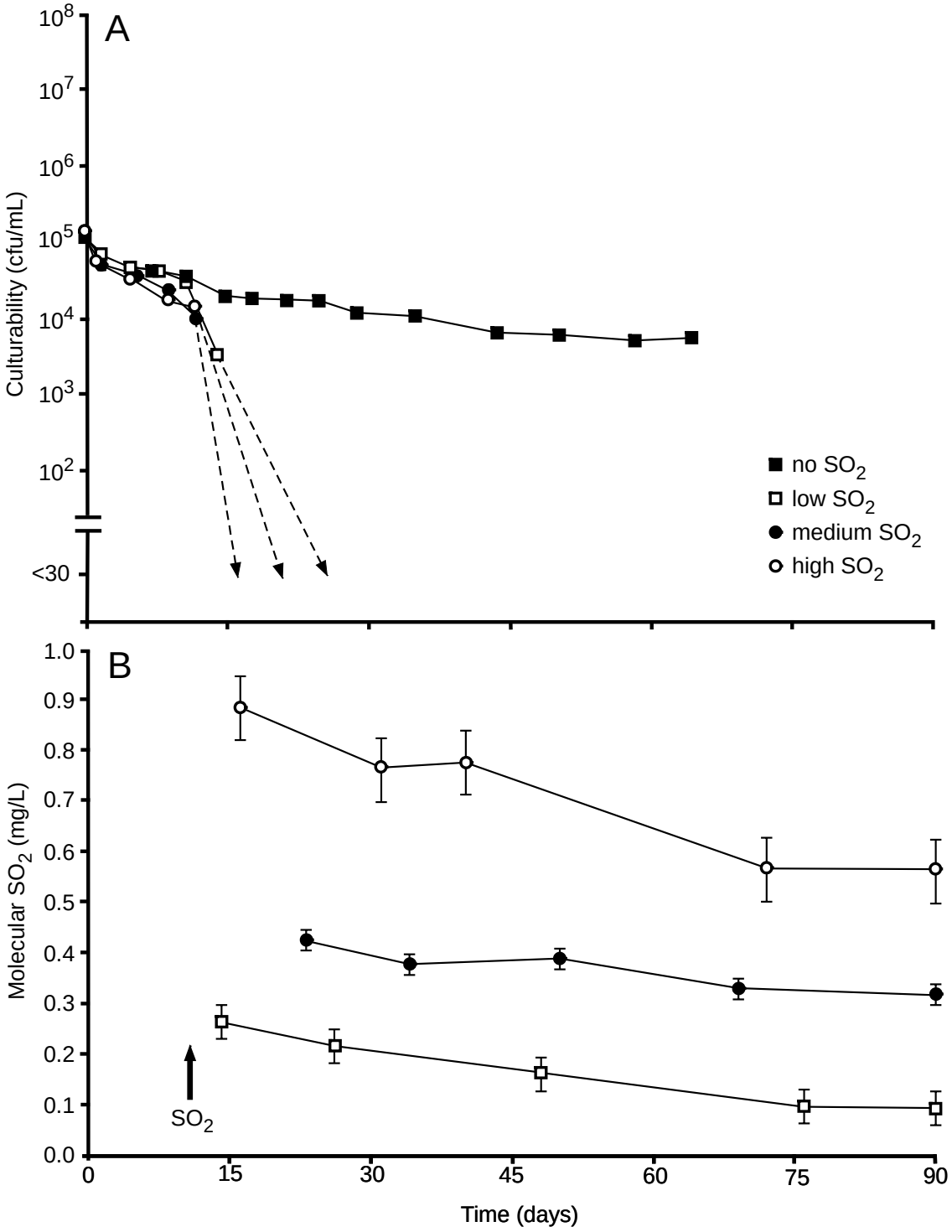

Figure 2. Culturability of *B. bruxellensis* I1a (A) and decline in concentrations of molecular SO_2 (B) in Merlot wine at 12°C and adjusted to 14.5% v/v ethanol. Various concentrations of SO_2 were added on day 13 as indicated by an arrow.

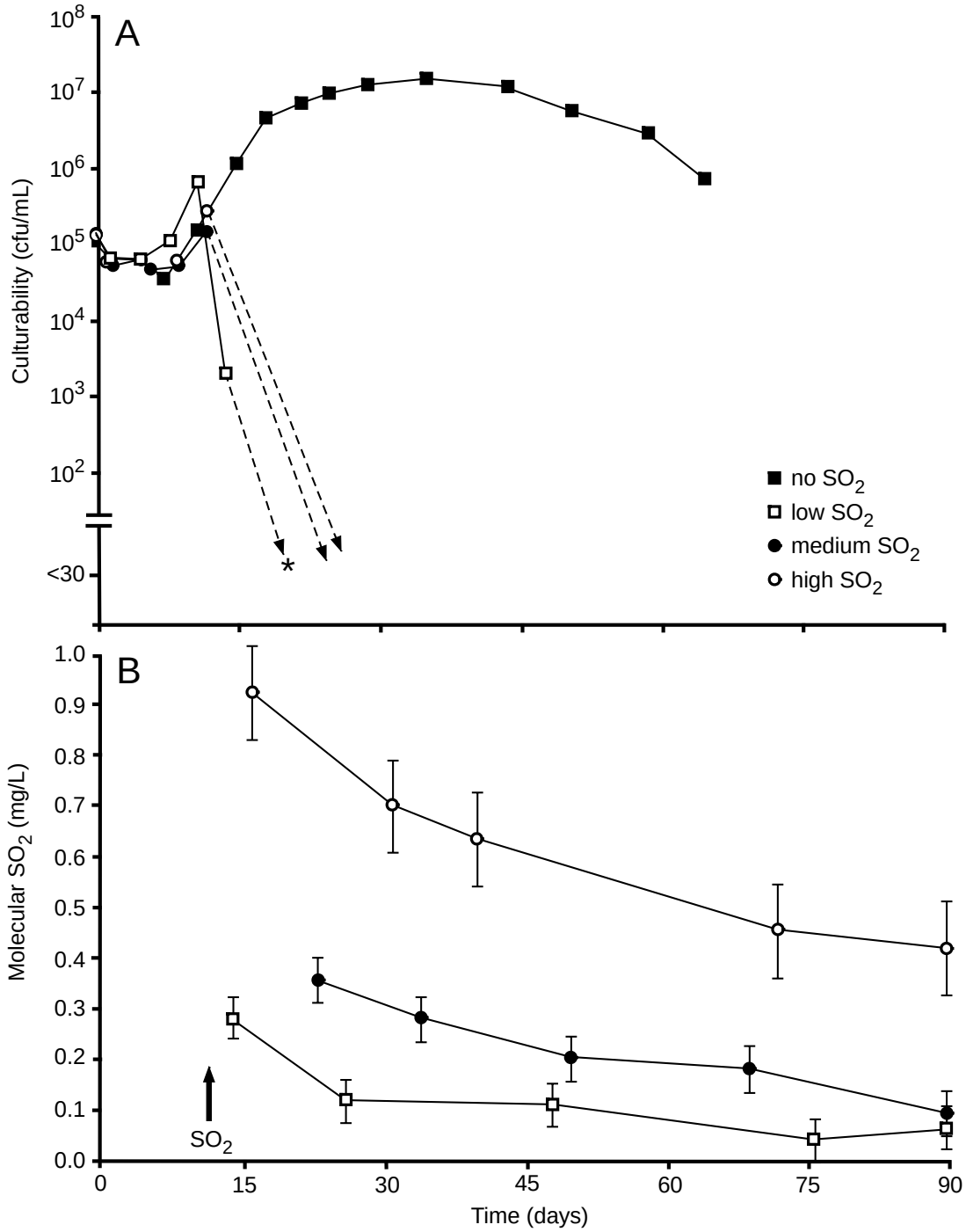

Figure 3. Culturability of *B. bruxellensis* I1a (A) and decline in concentrations of molecular SO_2 (B) in Merlot wine at 18°C and adjusted to 13.5% v/v ethanol. Various concentrations of SO_2 were added on day 13 as indicated by an arrow. After day 15 indicated by "*", culturability was expressed in some, but not all, of replicates containing 0.2 mg/L molecular SO_2.

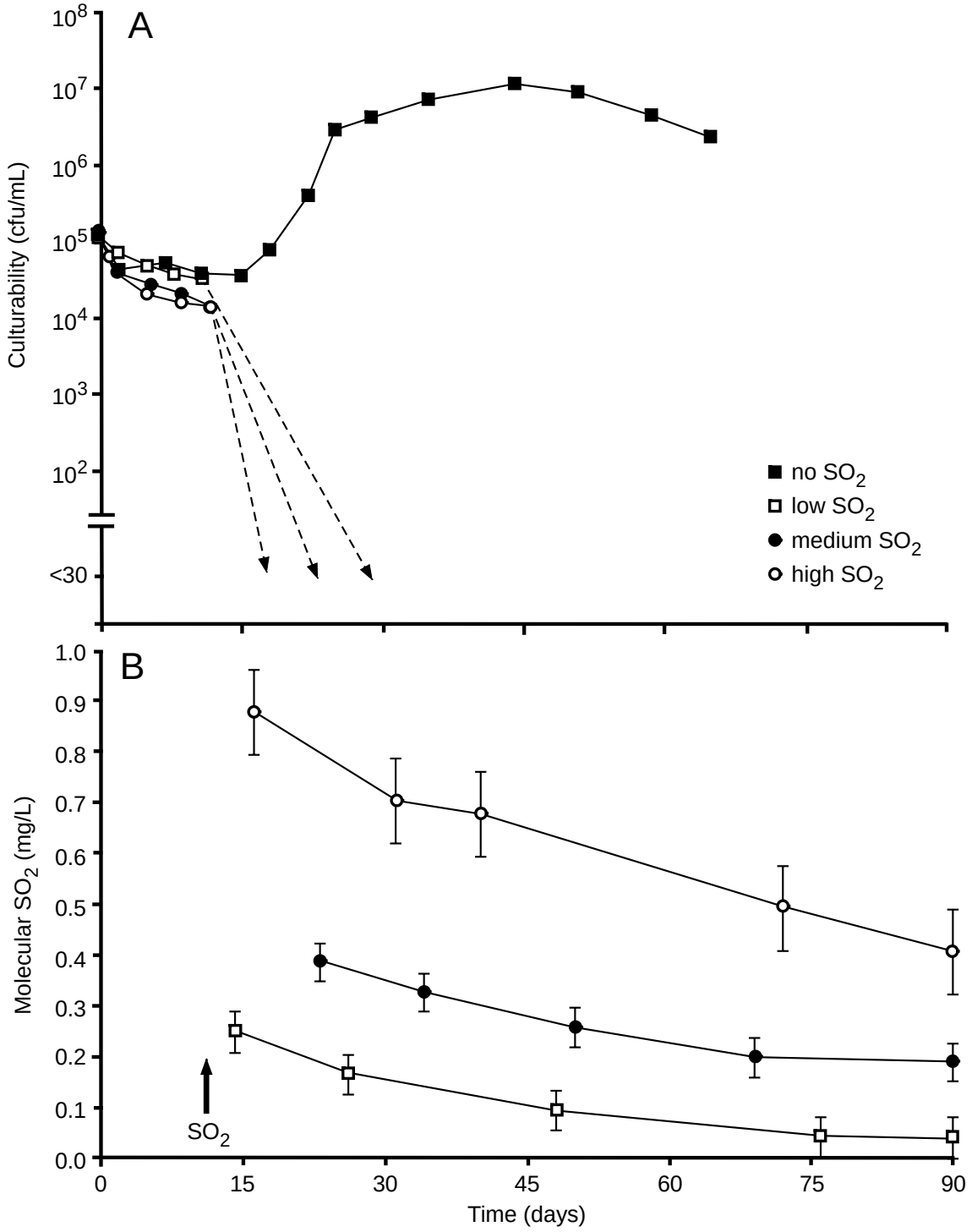

Figure 4. Culturability of *B. bruxellensis* I1a (A) and decline in concentrations of molecular SO_2 (B) in Merlot wine at 18°C and adjusted to 14.5% v/v ethanol. Various concentrations of SO_2 were added on day 13 as indicated by an arrow.

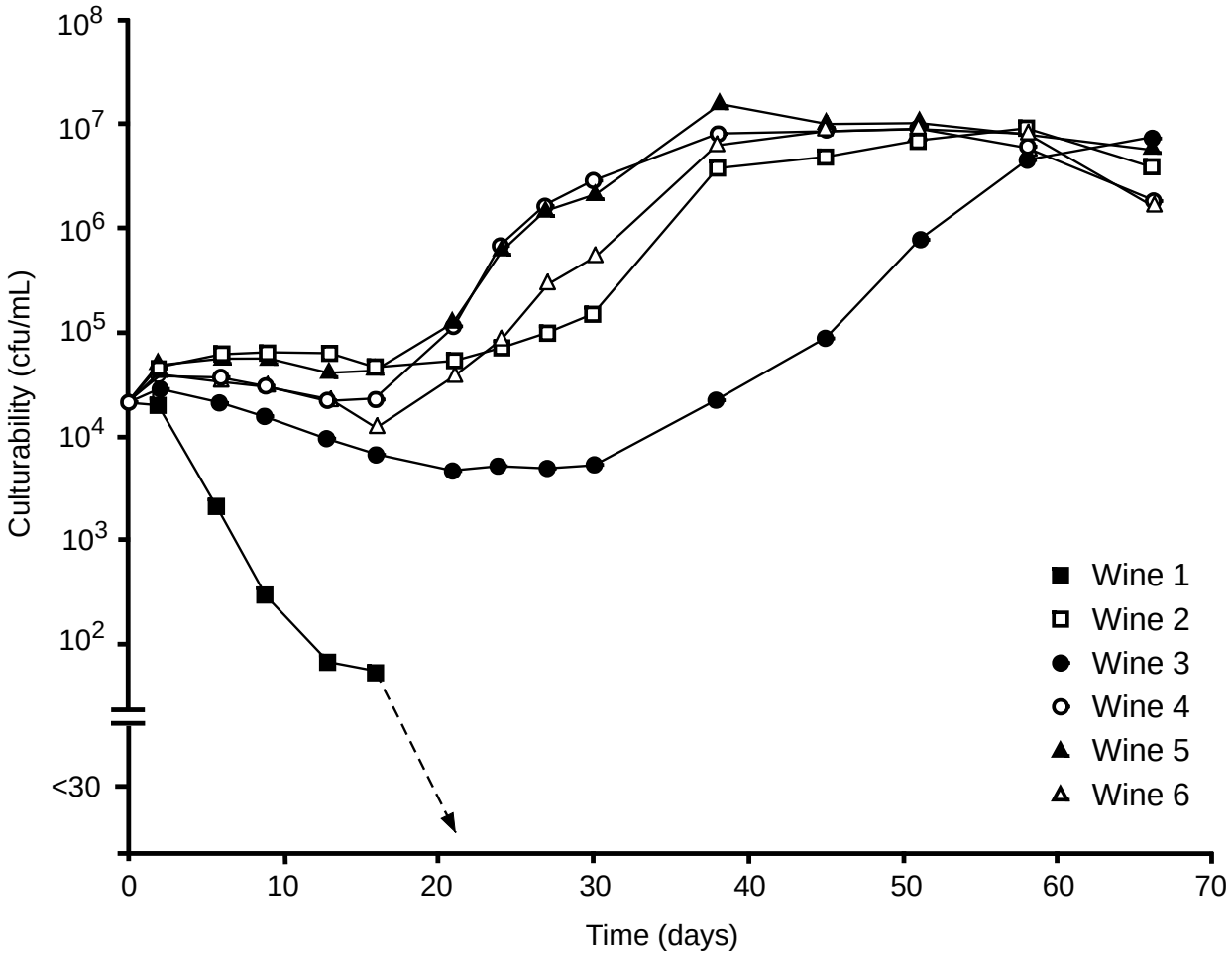

Figure 5. Growth of *B. bruxellensis* I1a in six different commercial red wines incubated at 18°C and adjusted to 13.5% v/v ethanol and without any SO_2 present.

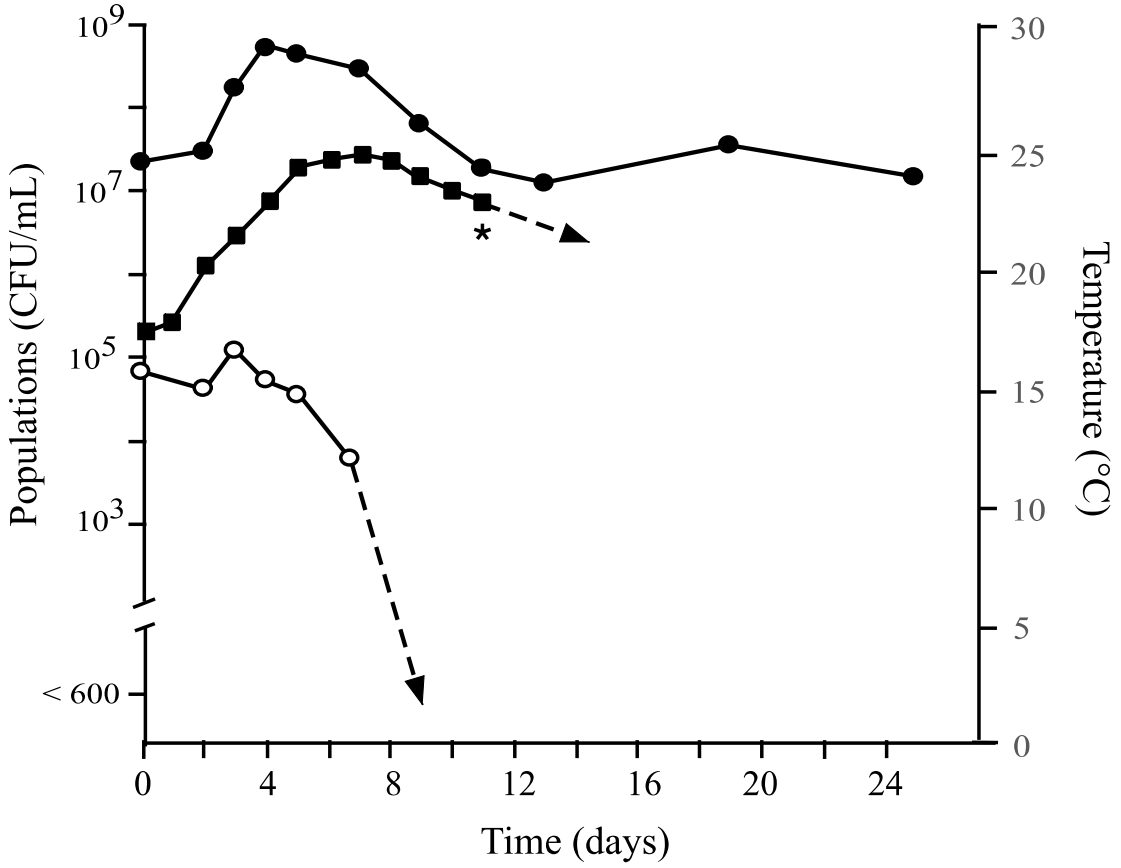

Figure 6. Culturable populations of *S. cerevisiae* (●), total non-*Saccharomyces* yeasts (○), and temperatures (■) of 2018 Merlot fermentations inoculated on day 0 *S. cerevisiae*. *Asterisk denotes wine pressed at 0 °Brix and stored at 21°C.

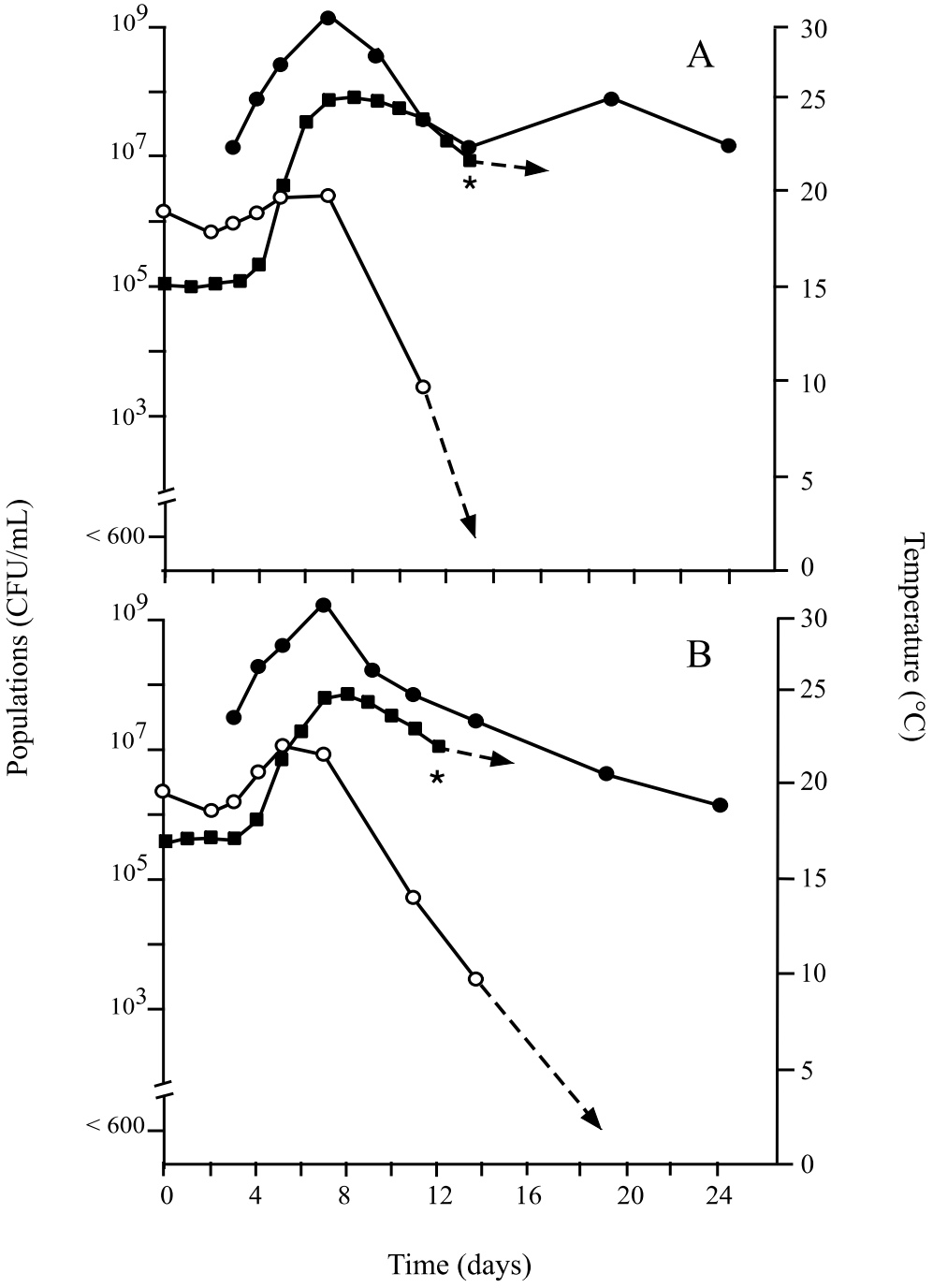

Figure 7. Culturable populations of *S. cerevisiae* (●), total non-*Saccharomyces* yeasts (O), and temperatures (■) of 2018 Merlot fermentations inoculated on day 0 with *Mt. pulcherrima* following temperature regime A or B, until sequentially inoculated with *S. cerevisiae* on day 3. *Asterisk denotes wines pressed at 0 °Brix and kept at 21°C.

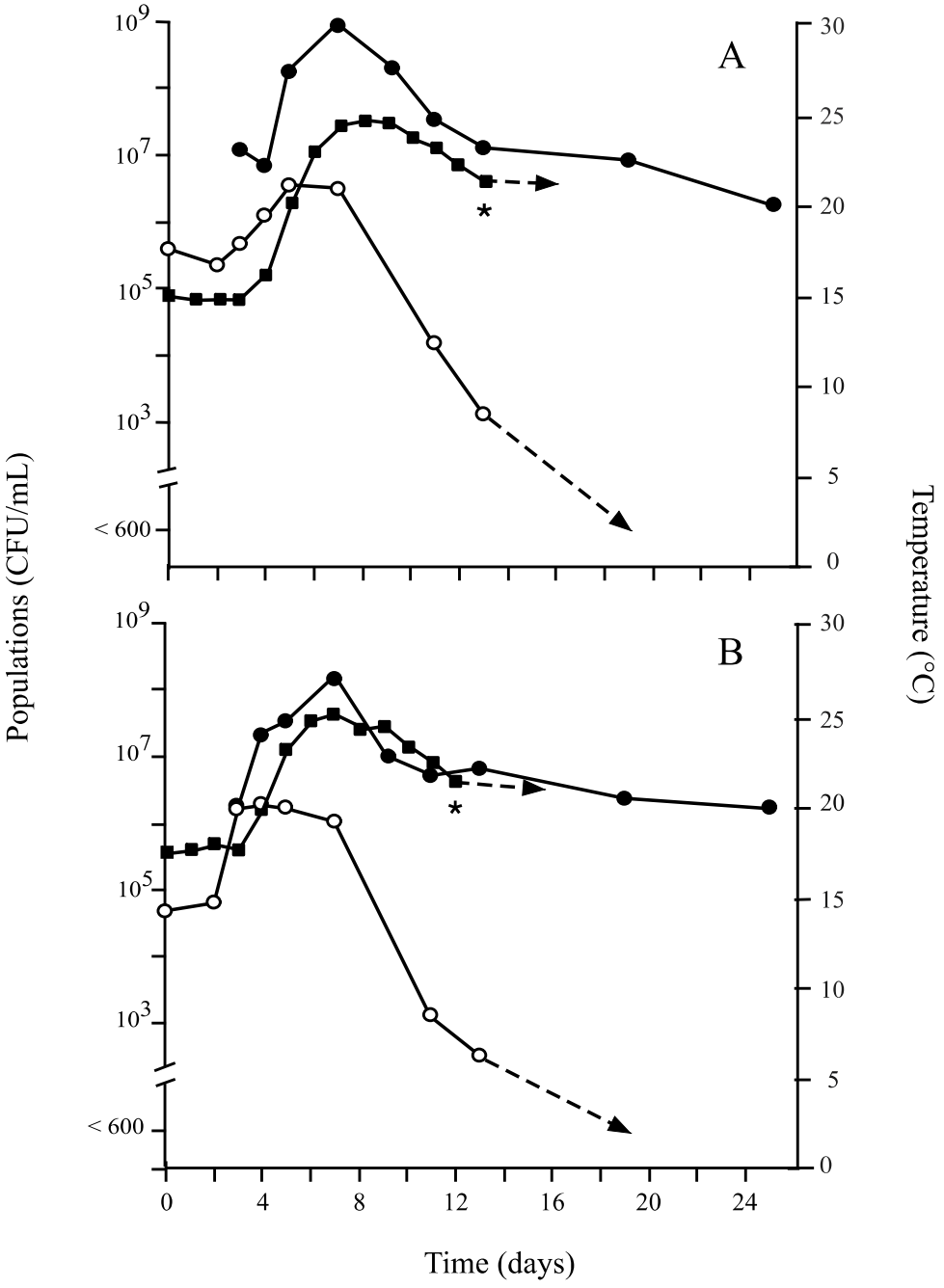

Figure 8. Culturable populations of *S. cerevisiae* (●), total non-*Saccharomyces* yeasts (ʘ), and temperatures (■) of 2018 Merlot fermentations inoculated on day 0 with *My. guilliermondii* following temperature regime A or B, until sequentially inoculated with *S. cerevisiae* on day 3. *Asterisk denotes wines pressed at 0 °Brix and kept at 21°C.

Table 1. Composition of 2018 Merlot wines inoculated without/with non-*Saccharomyces* yeasts following temperature regime A (maximum 15°C for 72 hours) or B (maximum 7.5°C for 72 hours) prior to inoculation of *S. cerevisiae* on day 3 when maximum increased to 25°C.

Yeast strain & temperature regime	Glu + Fru (g/L)	Ethanol (% v/v)	Glycerol (g/L)	Succinic Acid (g/L)	Acetic Acid (g/L)
S. cerevisiae	nd	15.0[a]	9.96[a]	2.19[a]	0.704[a]
Mt. pulcherrima A	nd	14.8[ab]	9.41[bc]	1.88[c]	0.569[ab]
Mt. pulcherrima B	nd	14.2[c]	9.27[c]	1.99[b]	0.526[b]
My. guilliermondii A	nd	14.7[b]	9.34[bc]	2.01[b]	0.486[b]
My. guilliermondii B	nd	14.6[b]	9.52[b]	2.01[b]	0.483[b]

nd: not detected (<1.5 g/L)
[a-b]Mean values within columns with different superscripts are significantly different (p<0.05)

Table 2. Mean scores for sensory attributes of 2018 Merlot wines inoculated without/with non-*Saccharomyces* yeasts following temperature regime A or B.

	Treatment					
Attribute	*S. cerevisiae*	*My. guilliermondii* (A)*	*My. guilliermondii* (A)*	*My. guilliermondii* (B)	*Mt. pulcherrima* (A)	*Mt. pulcherrima* (B)
Aroma						
'Fruity'	5.6^{a}	5.7^{a}	5.7^{a}	5.7^{a}	5.8^{a}	5.9^{a}
'Floral'	5.2^{a}	5.5^{a}	5.5^{a}	5.6^{a}	5.4^{a}	5.6^{a}
'Berry'	5.4^{a}	5.4^{a}	5.5^{a}	5.5^{a}	5.3^{a}	5.6^{a}
'Chocolate'	3.8^{a}	4.0^{a}	3.9^{a}	3.9^{a}	3.8^{a}	3.9^{a}
'Sweaty'	4.5^{a}	4.4^{a}	4.4^{a}	4.2^{a}	4.4^{a}	4.1^{a}
'Dried fruit'	5.1^{a}	5.1^{ab}	4.9^{ab}	5.1^{a}	5.1^{a}	4.7^{b}
'Ethanol'	6.9^{a}	6.9^{a}	6.7^{a}	6.9^{a}	6.7^{a}	6.6^{a}
'Sulfur'	5.6^{ab}	5.4^{ab}	5.8^{a}	5.5^{ab}	5.6^{ab}	5.3^{b}
'Solvent'	5.7^{a}	5.8^{a}	5.7^{a}	5.7^{a}	5.6^{a}	5.5^{a}
'Buttery'	4.1^{a}	4.2^{a}	4.0^{a}	4.1^{a}	4.2^{a}	4.0^{a}
'Woody'	4.7^{ab}	4.5^{ab}	4.4^{ab}	4.5^{ab}	4.7^{a}	4.3^{b}
'Animal'	4.4^{a}	4.5^{a}	4.4^{a}	4.3^{a}	4.3^{a}	4.2^{a}
'Herbaceous'	4.6^{a}	4.3^{ab}	4.4^{ab}	4.4^{ab}	4.5^{ab}	4.2^{b}
'Spicy'	3.9^{b}	4.2^{a}	3.7^{b}	3.8^{b}	3.8^{b}	3.7^{b}
'Yeasty'	4.6^{ab}	4.5^{ab}	4.5^{ab}	4.5^{ab}	4.8^{a}	4.3^{b}
Taste						
'Sweet'	6.2^{ab}	5.9^{bc}	6.0^{abc}	6.0^{abc}	5.8^{c}	6.3^{a}
'Bitter'	6.6^{a}	6.5^{a}	6.5^{a}	6.5^{a}	6.2^{ab}	5.9^{b}
'Sour'	6.8^{abc}	7.1^{a}	6.9^{ab}	7.0^{ab}	6.7^{bc}	6.4^{c}
Flavor						
'Fruity'	5.5^{a}	5.3^{a}	5.5^{a}	5.5^{a}	5.3^{a}	5.3^{a}
'Floral'	5.4^{ab}	5.2^{ab}	5.5^{a}	5.1^{b}	5.2^{ab}	5.1^{b}
'Berry'	3.8^{a}	3.7^{a}	3.8^{a}	3.6^{a}	3.7^{a}	3.7^{a}
'Chocolate'	4.3^{ab}	4.5^{a}	4.3^{ab}	4.4^{ab}	4.2^{ab}	4.1^{b}
'Sweaty'	5.0^{abc}	5.3^{a}	5.1^{ab}	4.8^{bc}	5.0^{abc}	4.7^{c}
'Dried fruit'	7.2^{a}	7.2^{a}	7.0^{a}	7.1^{a}	7.2^{a}	6.5^{b}
'Ethanol'	5.7^{a}	5.8^{a}	5.7^{a}	5.8^{a}	5.6^{ab}	5.2^{b}
'Sulfur'	6.1^{a}	6.0^{ab}	6.1^{a}	6.1^{a}	5.9^{ab}	5.6^{b}
'Solvent'	4.1^{a}	4.1^{a}	4.2^{a}	4.0^{a}	4.2^{a}	4.0^{a}
'Buttery'	4.5^{a}	4.5^{a}	4.6^{a}	4.5^{a}	4.4^{a}	4.5^{a}
'Woody'	4.5^{ab}	4.5^{ab}	4.6^{ab}	4.7^{a}	4.4^{ab}	4.2^{b}
'Animal'	4.8^{a}	4.8^{a}	4.7^{ab}	4.6^{ab}	4.5^{ab}	4.3^{b}
'Herbaceous'	4.1^{a}	3.9^{a}	3.9^{a}	4.0^{a}	3.8^{a}	3.8^{a}
'Spicy'	4.7^{a}	4.7^{a}	4.5^{a}	4.6^{a}	4.6^{a}	4.4^{a}
'Yeasty'	5.5^{a}	5.3^{a}	5.5^{a}	5.5^{a}	5.3^{a}	5.3^{a}
Mouthfeel						
'Tingle'	5.7^{a}	5.8^{a}	5.5^{ab}	5.6^{ab}	5.5^{ab}	5.2^{b}
'Viscosity'	6.7^{a}	6.7^{a}	6.8^{a}	6.6^{a}	6.8^{a}	6.7^{a}
'Weight'	6.1^{a}	6.2^{a}	6.3^{a}	6.1^{a}	6.3^{a}	6.2^{a}
'Hot'/'ethanol'	7.3^{ab}	7.5^{a}	7.3^{ab}	7.3^{ab}	7.0^{bc}	6.7^{c}
Roughness'	6.0^{ab}	6.3^{a}	5.9^{bc}	5.8^{bc}	6.0^{ab}	5.6^{c}
''Astringent'	6.8^{ab}	7.1^{a}	6.8^{ab}	6.8^{ab}	6.6^{b}	6.5^{b}
'Drying'	6.3^{ab}	6.6^{a}	6.2^{b}	6.1^{b}	6.2^{b}	6.0^{b}
'Puckering'	6.0^{bc}	6.5^{a}	6.0^{bc}	6.2^{b}	6.1^{bc}	5.8^{c}
'Sharp'	6.1^{ab}	6.4^{a}	6.0^{b}	6.0^{b}	6.0^{bc}	5.5^{c}
'Mouth coat'	6.0^{b}	6.5^{a}	6.1^{ab}	6.2^{ab}	6.3^{ab}	6.0^{b}
'Round'	6.6^{b}	6.7^{b}	6.9^{ab}	6.8^{ab}	7.0^{ab}	7.3^{a}

$^{a\text{-}c}$Means within a row with different superscripts are significant at $p<0.05$.

*Replicates of temperature treatment A could not be pooled.

Section 7. Outreach and Education Efforts - Presentations of Research:

Information from this research conducted from 2017 to 2020 was disseminated through a number of outreach opportunities. Graduate students funded by this project (*) as well as presenters (†) are indicated below.

Professional Articles (refereed)

Oswald*, T.A. and C.G. Edwards. Interactions between storage temperature and ethanol that affect growth of *Brettanomyces bruxellensis* in Merlot wine. *Am. J. Enol. Vitic.* 68: 188-194 (2017).

Edwards, C.G., and T.A. Oswald*. Interactive effects between total SO_2, ethanol, and storage temperature against *Brettanomyces bruxellensis*. *Lett. Appl. Microbiol.* 66: 71-76 (2018).

Cartwright*, Z.M., D.A. Glawe, and C.G. Edwards. Reduction of *Brettanomyces bruxellensis* populations from oak barrel staves using steam. *Am. J. Enol. Vitic.* 69: 400-409 (2018).

Cartwright*, Z.M., B.R. Bondada, and C.G. Edwards. Survival of *Brettanomyces bruxellensis* in grape pomace and reduction of populations by application of heat and sulphites. *Aust. J. Grape Wine Res.* 25: 109-115 (2018).

Edwards, C.G. and Z.M. Cartwright*. Application of heated water to reduce populations of *Brettanomyces bruxellensis* present in oak barrel staves. *S. Afr. J. Enol. Vitic.* 40: DOI: http://dx.doi.org/10.21548/40-1-3008 (2019).

Aplin*, J.A., K.P. White*, and C.G. Edwards. Growth and metabolism of non-*Saccharomyces* yeasts isolated from Washington state vineyards in media and high sugar grape musts. *Food Microbiol.* 77: 158-165 (2019).

Edwards, C.G. and Z.M. Cartwright*. Application of heated water to reduce populations of *Brettanomyces bruxellensis* present in oak barrel staves. *S. Afr. J. Enol. Vitic.* 40: DOI: http://dx.doi.org/10.21548/40-1-3008 (2019).

Cartwright*, Z.M. and C.G. Edwards. Efficacy of warmed wine against *Brettanomyces bruxellensis* present in oak barrel staves. *Am. J. Enol. Vitic.* 71:249-255 (2020).

Aplin*, J.A., and C.G. Edwards. Impacts of non-*Saccharomyces* species and aeration on sequential inoculation with *Saccharomyces cerevisiae* to produce lower alcohol Merlot wines. *J. Sci. Food Agric.* 101: 1715-1719 (2021).

Aplin*, J.A., V.D. Paup*, C.F. Ross, and C.G. Edwards. Chemical and sensory profiles of Merlot wines produced by sequential inoculation of *Metschnikowia pulcherrima* or

Meyerozyma guilliermondii followed by *Saccharomyces cerevisiae*. *Food Microbiol.* (submitted, 2021).

Carbon*, H.N., J.J. Aplin*, T.L. Barton (Cook)*, P. Gibney, G. Jiang*, J.D. Brumley, and C.G. Edwards. Metabolism of *Metschnikowia pulcherrima* and *Meyerozyma guilliermondii* in synthetic media in the absence and presence of *Saccharomyces cerevisiae*. *Fermentation* (in preparation, 2021).

Barton (Cook)*, T.L. and C.G. Edwards. Optimization of fermentation temperature and inoculation timing for use of non-*Saccharomyces* yeasts to reduce ethanol production in red wines. *Am. J. Enol. Vitic.* (in preparation, 2021).

Abstracts and Papers Presented at Technical Meetings

Cartwright†*, Z.M., D.A. Glawe, and C.G. Edwards. Eradication of the spoilage yeast, *Brettanomyces bruxellensis*, from oak barrels using various heat treatments. Institute of Food Technologists annual meeting, Las Vegas, NV, June 25-28 (2017).

Cartwright†*, Z.M. and C.G. Edwards. Survival of *Brettanomyces bruxellensis* in grape pomace. American Society for Enology and Viticulture annual meeting, Bellevue, WA, June 26-29 (2017).

Cartwright†*, Z.M. and C.G. Edwards. Reduction of *Brettanomyces bruxellensis* populations from oak barrel staves using steam. American Society for Enology and Viticulture annual meeting, Bellevue, WA, June 26-29 (2017).

Aplin†*, J.J. and C.G. Edwards. Sequential inoculation of different yeasts to reduce alcohol contents of red wines. American Society for Enology and Viticulture annual meeting, Bellevue, WA, June 26-29 (2017).

Oswald*, T.A. and C.G. Edwards†. Impact of storage temperature and ethanol on *Brettanomyces bruxellensis* inoculated into Merlot wine. American Society for Enology and Viticulture annual meeting, Bellevue, WA, June 26-29 (2017).

Cartwright†*, Z.M. and C.G. Edwards. Reduction of *Brettanomyces bruxellensis* populations from oak barrel staves using heat. Washington Winegrowers Association annual meeting, Kennewick, WA. February 6-8 (2018).

Aplin†*, J.J. and C.G. Edwards. Use of non-*Saccharomyces* yeasts to reduce alcohol content of red wines. Washington Winegrowers Association annual meeting, Kennewick, WA. February 6-8 (2018).

Edwards[†], C.G., Z.M. Cartwright*, and T.A. Oswald*. Survivability of *Brettanomyces bruxellensis* in wine and barrels under vinification conditions. American Society for Enology and Viticulture annual meeting, Monterey, CA, June 18-21 (2018).

Cook*[†], T.L., J.J. Aplin*, and C.G. Edwards. Changes in wine quality caused by non-*Saccharomyces* yeasts and the effects of temperature on their survivability during fermentation. Washington Winegrowers Association annual meeting, Kennewick, WA. February 12-13 (2019).

Dietsche*[†], S. and C.G. Edwards. The effects of ethanol, storage temperature, and molecular sulfur dioxide on *Brettanomyces bruxellensis* in red wine. Washington Winegrowers Association annual meeting, Kennewick, WA. February 12-13 (2019).

Aplin IV*[†], J.J., V.D. Paup*, C.F. Ross, and C.G. Edwards. Impacts of non-*Saccharomyces* yeasts on chemical and sensory attributes of Merlot wine with reduced amounts of alcohol. American Society for Enology and Viticulture annual meeting, Napa, CA. June 17-20 (2019).

Cook*, T., V. Paup*, C.F. Ross, and C.G. Edwards. Optimization of non-*Saccharomyces* yeasts for production of lower ethanol red wines. Accepted for presentation at the Washington Winegrowers Association annual meeting, Kennewick, WA. March 2-5 (2020).

Dietsche*, S., C.G. Edwards, and K. Bowlby. Factors influencing the growth and spoilage of *B. bruxellensis* in red wine. Accepted for presentation at the Washington Winegrowers Association annual meeting, Kennewick, WA. March 2-5 (2020).

Cook*, T., C. Merrick*, and C.G. Edwards. Optimization for use of non-*Saccharomyces* in red wines. Accepted for presentation at the International Cool Climate Wine symposium, St. Catharines, Ontario, Canada. July 12-17 (2020). (meeting delayed until 2022).

Professional Articles (refereed)

Cartwright*, Z.M. and C.G. Edwards. What can a winemaker do about *Brettanomyces bruxellensis* present in oak barrels? *Wine and Viticulture Journal* 34: 17-21 (2019).

Press Releases/Newspaper Articles/Radio Interviews (research)

Schrand. B. Turning up the heat on 'Brett.' WSU Insider. September 16 (2019).

Paterson, L. Fighting invasive yeast in red winemaking. WSU Insider. December 14 (2020).

Paterson, L. Fighting invasive yeast in red winemaking. Morning Ag Clips. December 16 (2020).

Invited Presentations

Edwards†, C.G. What we've learned about wine spoilage and *Brettanomyces*. Oregon Wine Symposium, Portland, OR February 22 (2017).

Edwards†, C.G. What we've learned about wine spoilage and *Brettanomyces*. E&J Gallo Technical Seminar, Modesto, CA May 12 (2017).

Edwards†, C.G. What we've learned about wine spoilage and *Brettanomyces*. Presented at the Ste. Michelle Wine Estates Winemaker Council meeting, Clore Wine Center, Prosser, WA, June 6 (2017).

Edwards†, C.G. Managing Brett in the winery. Presented at the Washington Advancements in Viticulture and Enology annual meeting, Clore Wine Center, Prosser, WA, April 4 (2018).

Edwards†, C.G. Managing *Brettanomyces* in the winery. Presented at the *Brettanomyces* Symposium, American Society for Enology and Viticulture annual meeting, Monterey, CA, June 18 (2018).

Edwards†, C.G. Controlling *Brettanomyces*. Presented at The Pennsylvania State University, Halifax, PA, July 17 and at New York State Agricultural Experiment Station, Cornell University, Geneva, NY, July 18 (2018).

Edwards†, C.G. Controlling *Brettanomyces*. Presented at the Fermentation Problems: Causes, Prevention, Detection, and Solutions workshop, Santiago, Chile, October 23 (2018).

Edwards†, C.G. Controlling *Brettanomyces*. Presented at the Washington Advancements in Viticulture and Enology annual meeting, Woodinville, WA, July 23 (2019).

Edwards†, C.G. Wine microbiology and climate change…thoughts from an 'el anciano.' Presented at the Effectos del Cambio Climático en la Microbiología del Vino virtual conference, Santiago, Chile, November 24 (2020).

Section 8. Research Success Statements:

This research has provided winemakers to; (a) limit risk of the spoilage by *Brettanomyces bruxellensis* using hurdle technology involving combinations of molecular SO_2, ethanol, and storage temperature, (b) provide specifications regarding methods to sanitize oak barrels infected by the yeast, and (c) evaluate an alternative processing method to lower ultimate ethanol concentrations by encouraging certain species/strains of non-*Saccharomyces* yeasts prior to alcoholic fermentation.

Section 9. Funds Status:

Funds had been used in to support three graduate students conducting research in support of *Objective 1* (Dietsche) and *Objective 2* (Aplin and Cook). Dietsche is continuing to write her thesis (2021) while Cartwright graduated in 2018, Aplin in 2019, and Cook in 2020.