Washington State Grape and Wine Research Program
FINAL REPORT
2021-24 Funding Cycles

Project Title: Enological Application of non-*Saccharomyces* yeasts

Principal Investigator: C.G. Edwards, Emeritus professor, School of Food Science, Washington State University, Pullman, WA 99164-6376 (email edwardsc@wsu.edu).

Summary: Over the past decade, research has focused on the possibility of using non-*Saccharomyces* yeasts present in regional vineyards as means to enhance wine quality. Past and current research indicates that one species, *Mt. pulcherrima* inoculated a few days prior to *S. cerevisiae*, may be useful as a means to reduce final ethanol concentrations of the resultant wines. Any commercial application of *Mt. pulcherrima* will require careful addition of YAN after addition of the primary fermentation yeast, *S. cerevisiae*, because of YAN consumption by *Mt. pulcherrima*.

Project Duration: Three years, July 1, 2021 – June 30, 2024

Objective(s) and Experiments Conducted to Meet Stated Objective(s): This research project represented high priorities as per the *Washington State Viticulture and Enology Research Priorities* under Fermentation Management including (but not limited to) yeast and bacteria impacts on fermentation and sensory properties. Specific objectives were to:

Objective 1. Evaluate the role of temperature and SO_2 on survivability and metabolism of selected non-*Saccharomyces* yeasts.

a. Using synthetic media, establish optimal temperatures (10° to 25°C) and inhibitory concentrations of molecular SO_2 (0 to 0.8 mg/L) for strains of *Mt. pulcherrima* and *My. guilliermondii,* potentially including other yeasts such as *My. caribbica*, *W. anomalus,* and/or commercially available strains.

Objective 2. Determine impacts of sugar concentration (*e.g.*, 24°, 26°, and 28° Brix) on sequential inoculations of non-*Saccharomyces* and *Saccharomyces* yeasts into grape juice concentrate (Chardonnay).

a. Conduct 300 L fermentations of grape musts within identified optimal conditions, focusing on the reduction in ethanol content of wines and on sensory characterization.

b. Depending on time, perform industrial-scale fermentations at regional wineries with these yeasts.

Objective 3. As time and resources permit, additional research will continue to investigate (a) specific mechanisms responsible for ethanol reduction and (b) 'mouthfeel' effects by non-*Saccharomyces* yeasts, focusing on synthesis of polysaccharides (*e.g.*, glucans) and/or biomass.

Summary of Major Research Accomplishments and Results by Objective:

While Objective 1 was completed, Objective 3 was not fully investigated and requires additional focus. Various portions of Objective 2 of this research were conducted by two graduate students, Ipek Aktuna (Ph.D. candidate, Middle Eastern Technical University, Ankara, Turkey) and Jonathan Brumley (M.S. candidate, Washington State University, Pullman, WA). To keep this report limited in size, selected aspects of the total research performed by these two students will be presented in Objective 2.

<u>*Objective 1.*</u> The synthetic grape juice medium (SGJM) developed based on the amino acid composition of Cabernet Sauvignon grapes grown in Washington was utilized (Wang et al., 2003). Here, the impacts of fermentation temperature (10°, 15°, 20°, or 25°C) and molecular SO_2 (0, 0.2, 0.4, 0.6, or 0.8 mg/L) on growth and sugar utilization of *Mt. pulcherrima*, *My. guilliermondii*, and *S. cerevisiae* were evaluated. Due to time constraints, other yeast species were not studied.

Figures 1 to 4 illustrate the growth of the yeasts at the various temperatures with different amounts of SO_2, information then summarized by Table 1. In general, *S. cerevisiae* had higher tolerances to SO_2 than either *Mt. pulcherrima* or *My. guilliermondii* as evidenced by growth in the presence of ≥0.6 molecular SO_2. Both non-*Saccharomyces* yeasts tolerated 0.2 mg/L mSO_2 but only *Mt. pulcherrima* grew with 0.4 mg/L. Interactions between mSO_2 and incubation temperature also affect the yeast as shown by *Mt. pulcherrima* which exhibited with good growth under conditions of 0.4 mg/L mSO_2 and at 20°C but which diminished with decreases (10° or 15°C) or increases (25°C) in temperature.

These data suggest two important aspects; (a) growth of *Mt. pulcherrima* or *My. guilliermondii* was limited by ≥0.6 mg/L SO_2 and (b) with 0.2 mg/L SO_2 present, both yeasts grew well 15° to 25°C. In media containing 0.4 mg/L mSO_2, *Mt. pulcherrima* or *My. guilliermondii* grew better at 20°C compared to 15°C. These data will be used next fall to set parameters for pilot-scale grape fermentations using sequential inoculations of non-*Saccharomyces* prior to *S. cerevisiae* (initial must temperature of 20°C with ≤0.2 mg/L mSO_2 added).

Table 1. Relative growth of *Mt. pulcherrima*, *My. guilliermondii*, or *S. cerevisiae* in SGJM media containing 0, 0.2, 0.4, 0.6, or 0.8 mg/L molecular SO_2 and incubated at 10°, 15°, 20°, or 25°C.

mSO_2 (mg/L)	*Mt. pulcherrima* 10°C	15°C	20°C	25°C	*My. guilliermondii* 10°C	15°C	20°C	25°C	*S. cerevisiae* 10°C	15°C	20°C	25°C
0.0	(++)	(++)	(++)	(++)	(++)	(++)	(++)	(++)	(++)	(++)	(++)	(++)
0.2	(+)	(++)	(++)	(++)	(±)	(++)	(++)	(++)	(++)	(++)	(++)	(++)
0.4	(+[d])	(+)	(++)	(-)	(±)	(-)	(±)[d]	(±)[d]	(++)	(++)	(++)	(++)
0.6	(-)	(-)	(-)	(-)	(±)	(-)	(-)	(-)	(+)	(++)	(++)	(++)
0.8	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(+)	(++)	(++)	(++)

(++) Maximum populations $>10^7$ CFU/mL.

(+) Maximum populations 10^4 to 10^6 CFU/mL.

(±) Recoverable populations prior to decline to undetectable levels.

(-) Rapid decline of populations to undetectable levels.

d After initial decline to undetectable levels, strain exhibited delayed growth.

Table 2. Percent sugar reduction (glucose and fructose) by *Mt. pulcherrima* after 3- and 10-days post-inoculation in SGJM incubated at 10°, 15°, 20°, or 25°C and in the presence of 0.0, 0.2, or 0.4 mg/L molecular SO_2.

Temperature (°C)	Molecular SO_2 (mg/L)	Day 3 Sugar Reduction (%)	Day 10 Sugar Reduction (%)
10	0.0	19.9 ± 0.3^{bc}	30.4 ± 0.5^{de}
	0.2	17.0 ± 0.3^{cd}	24.4 ± 2.1^{fgh}
	0.4	12.3 ± 1.6^{e}	26.3 ± 1.2^{efg}
15	0.0	20.0 ± 2.2^{bc}	29.2 ± 2.3^{e}
	0.2	18.9 ± 1.5^{bc}	28.1 ± 1.1^{ef}
	0.4	19.2 ± 0.5^{bc}	21.8 ± 0.7^{hi}
20	0.0	23.0 ± 1.7^{ab}	42.4 ± 0.5^{b}
	0.2	22.9 ± 1.4^{ab}	35.7 ± 1.5^{c}
	0.4	19.4 ± 0.5^{bc}	33.7 ± 2.2^{cd}
25	0.0	27.4 ± 1.5^{a}	47.5 ± 0.4^{a}
	0.2	18.1 ± 2.1^{bc}	35.7 ± 1.8^{c}
	0.4	17.4 ± 1.7^{cd}	33.9 ± 0.7^{cd}

$^{a\text{-}j}$ Mean values within columns with different superscripts are significantly different ($p \leq 0.05$).

Table 3. Percent sugar reduction (glucose and fructose) by *My. guilliermondii* after 3- and 10-days post-inoculation in SGJM at 10°, 15°, 20°, or 25°C and in the presence of 0.0, 0.2, or 0.4 mg/L molecular SO_2.

Temperature (°C)	Molecular SO_2 (mg/L)	Day 3 Sugar Reduction (%)	Day 10 Sugar Reduction (%)
10	0.0	22.3 ± 0.6^{a}	27.4 ± 1.3^{def}
	0.2	17.6 ± 1.1^{bcd}	25.2 ± 0.4^{ef}
	0.4	12.5 ± 1.2^{f}	24.2 ± 1.1^{f}
15	0.0	19.2 ± 2.1^{abc}	29.2 ± 2.2^{bcde}
	0.2	19.4 ± 0.6^{abc}	29.1 ± 0.4^{bcde}
	0.4	14.1 ± 0.9^{def}	26.2 ± 0.4^{ef}
20	0.0	17.5 ± 1.0^{bcde}	33.3 ± 1.4^{ab}
	0.2	13.2 ± 0.4^{ef}	31.9 ± 1.3^{abc}
	0.4	13.4 ± 1.2^{def}	31.5 ± 2.1^{bcd}
25	0.0	20.2 ± 0.8^{ab}	36.2 ± 1.1^{a}
	0.2	15.6 ± 2.2^{cdef}	33.2 ± 1.9^{ab}
	0.4	18.7 ± 0.8^{abc}	31.6 ± 1.4^{bcd}

$^{a\text{-}g}$ Mean values within columns with different superscripts are significantly different ($p \le 0.05$)

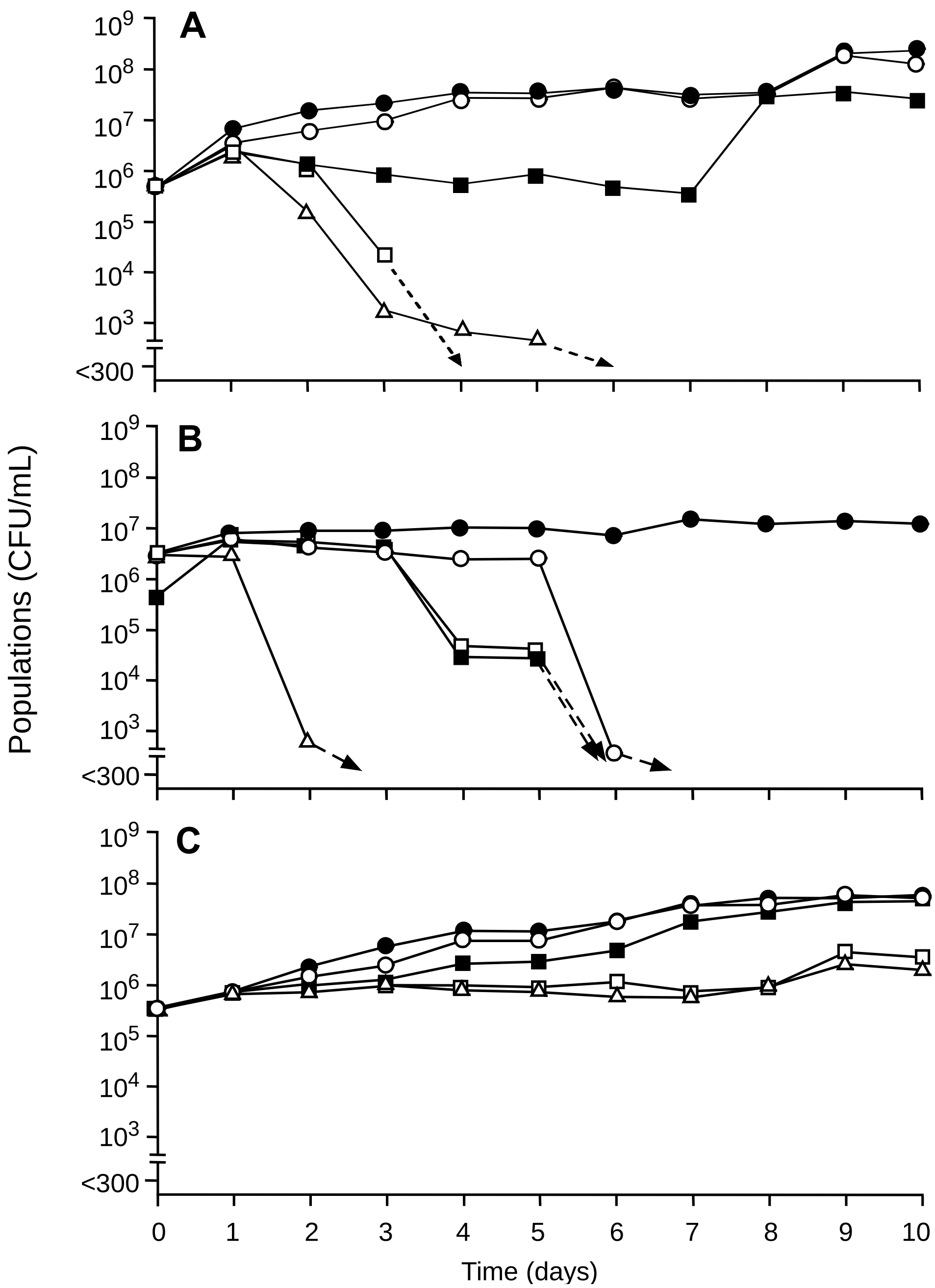

Figure 1. Culturability of *Mt. pulcherrima* (**A**), *My. guilliermondii* (**B**), or *S. cerevisiae* (**C**) in SGJM containing 0 (●), 0.2 (○), 0.4 (■), 0.6 (□), or 0.8 (△) mg/L molecular SO_2 incubated at 10°C.

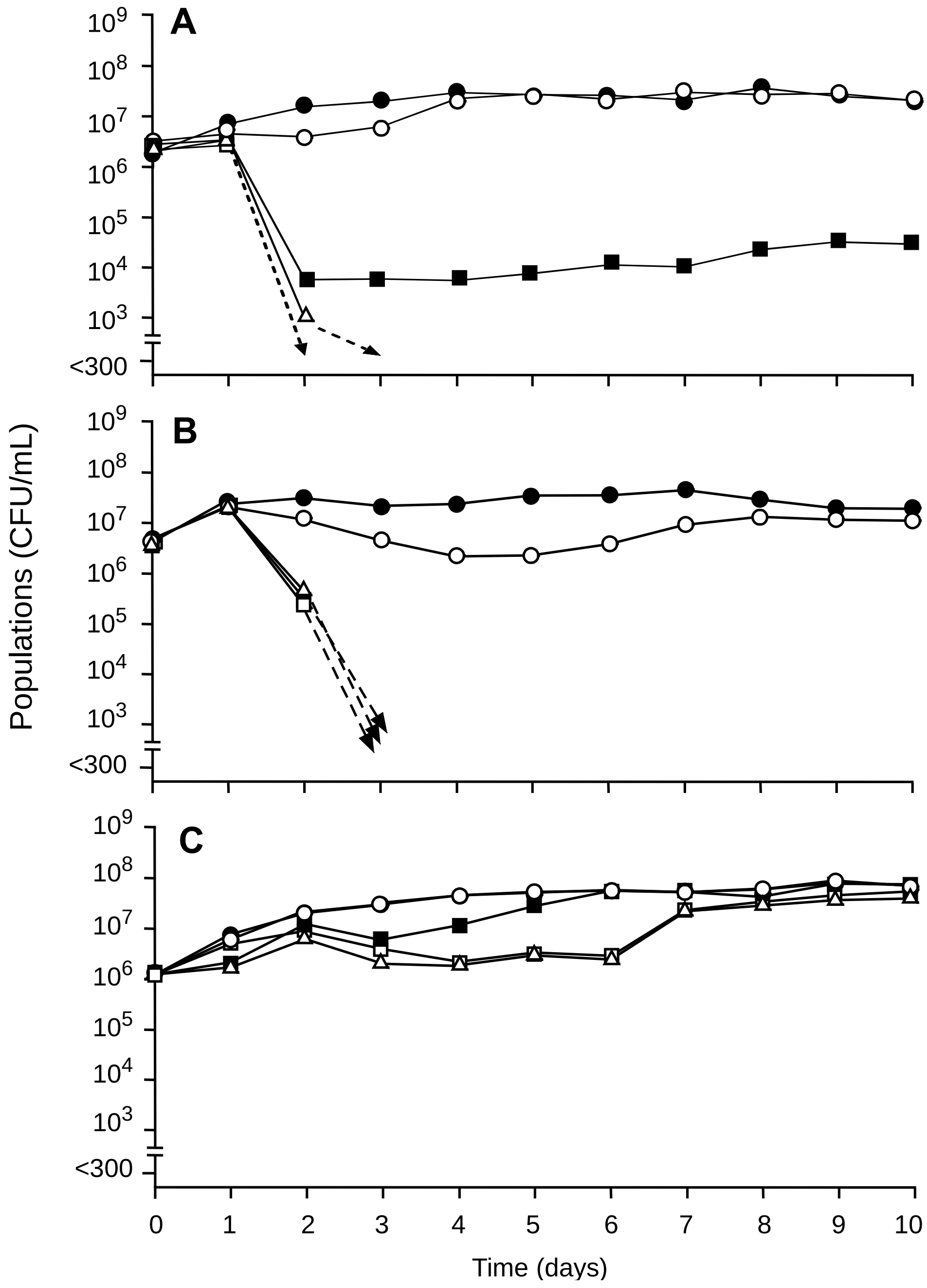

Figure 2. Culturability of *Mt. pulcherrima* (**A**), *My. guilliermondii* (**B**), or *S. cerevisiae* (**C**) in SGJM containing 0 (●), 0.2 (○), 0.4 (■), 0.6 (□), or 0.8 (△) mg/L molecular SO_2 incubated at 15°C.

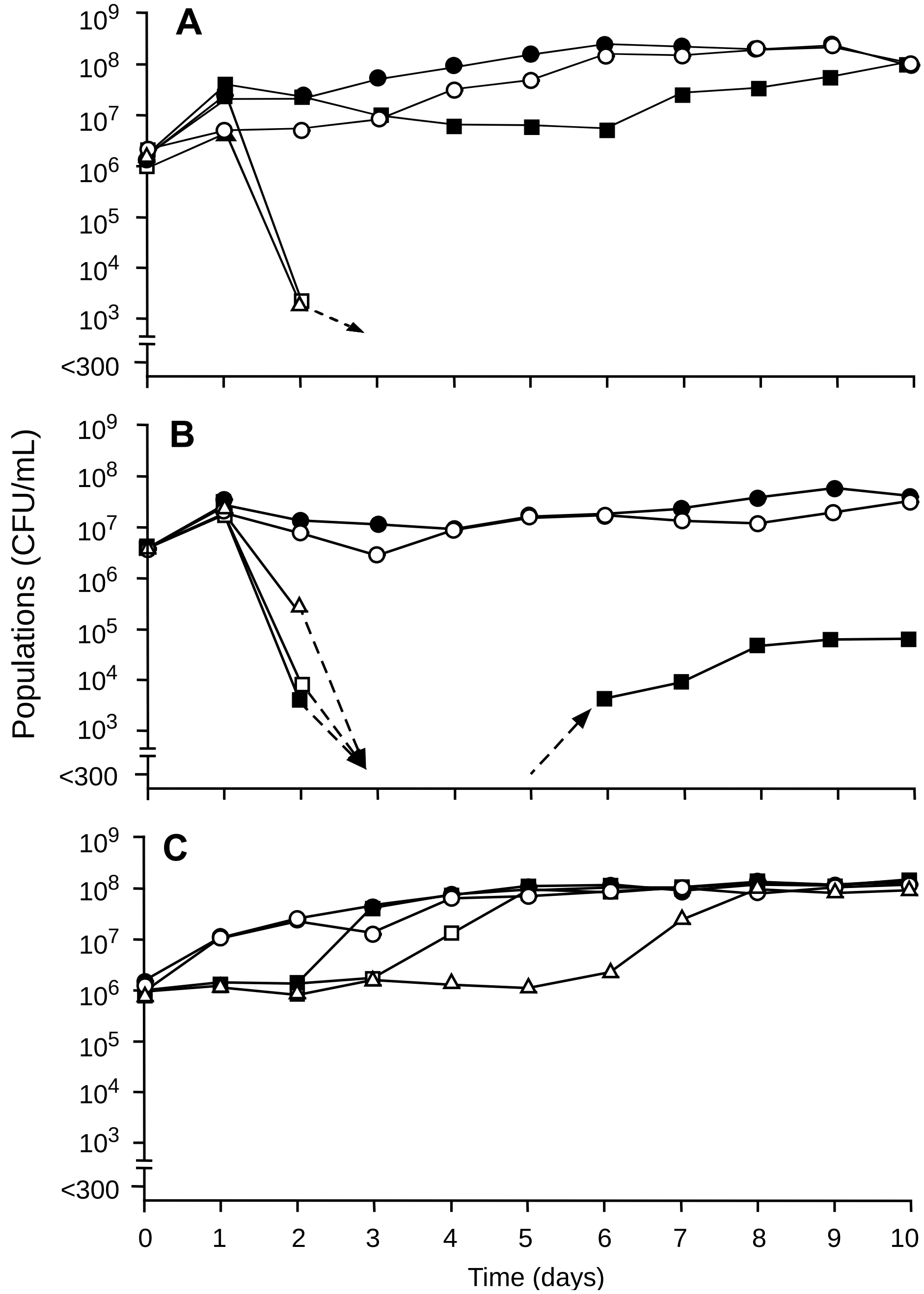

Figure 3. Culturability of *Mt. pulcherrima* (**A**), *My. guilliermondii* (**B**), or *S. cerevisiae* (**C**) in SGJM containing 0 (●), 0.2 (○), 0.4 (■), 0.6 (□), or 0.8 (△) mg/L molecular SO_2 incubated at 20°C.

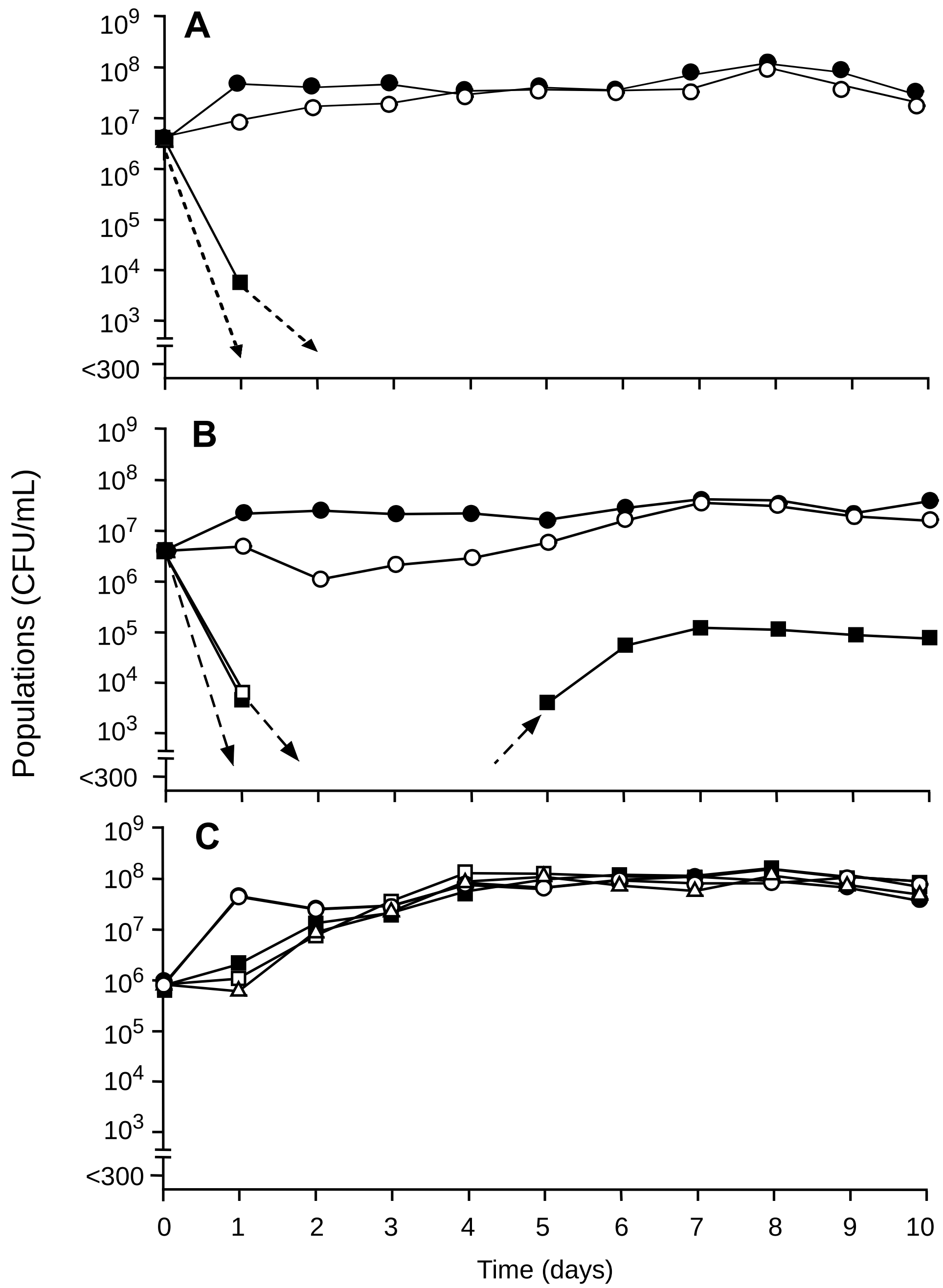

Figure 4. Culturability of *Mt. pulcherrima* (**A**), *My. guilliermondii* (**B**), or *S. cerevisiae* (**C**) in SGJM containing 0 (●), 0.2 (○), 0.4 (■), 0.6 (□), or 0.8 (△) mg/L molecular SO_2 incubated at 25°C.

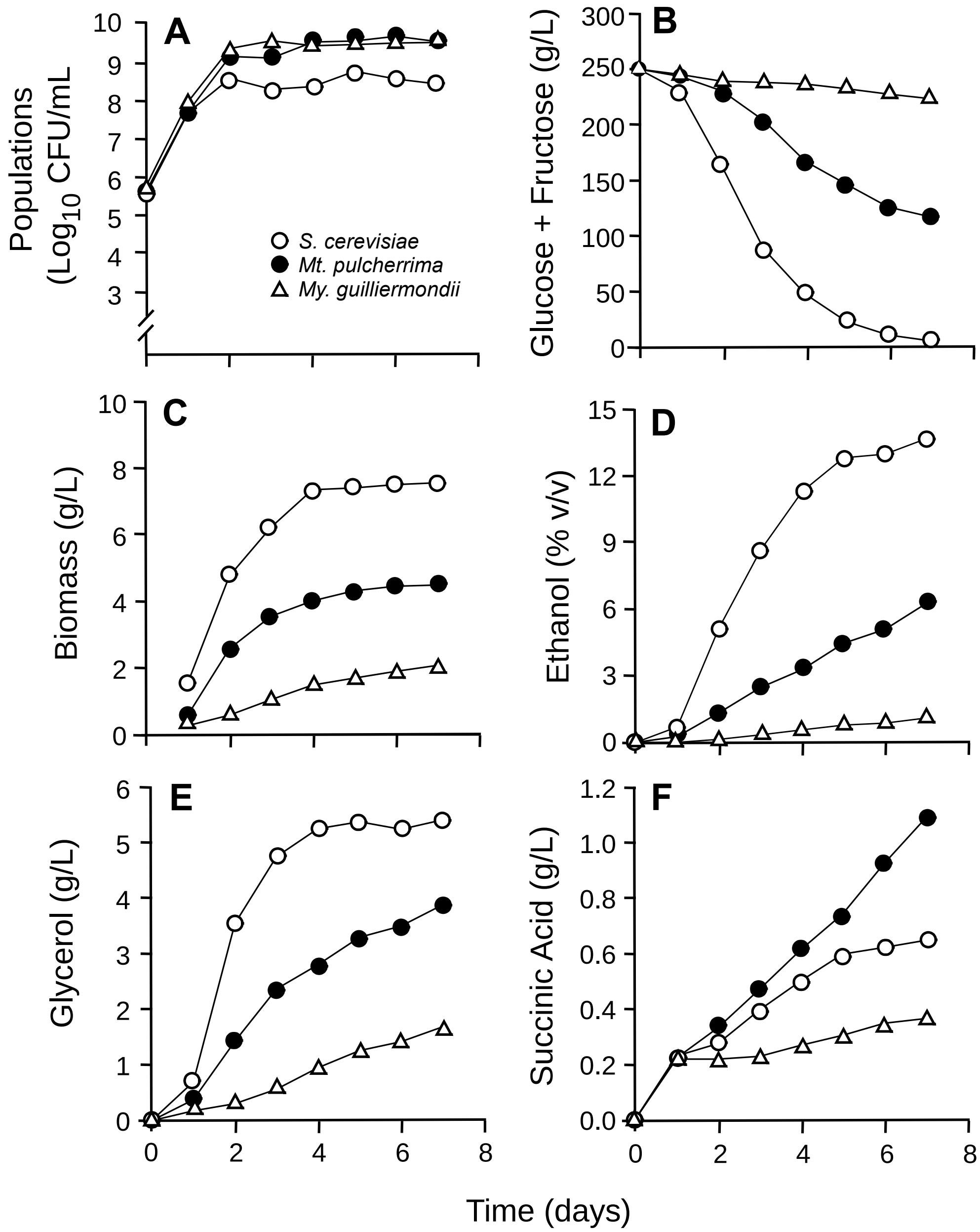

Figure 5. Yeast populations (**A**), glucose + fructose (**B**), biomass (**C**), ethanol (**D**), glycerol (**E**), and succinic acid (**F**) after single inoculations of *Mt. pulcherrima* (●), *My. guilliermondii* (△), or *S. cerevisiae* (○) in SGJM.

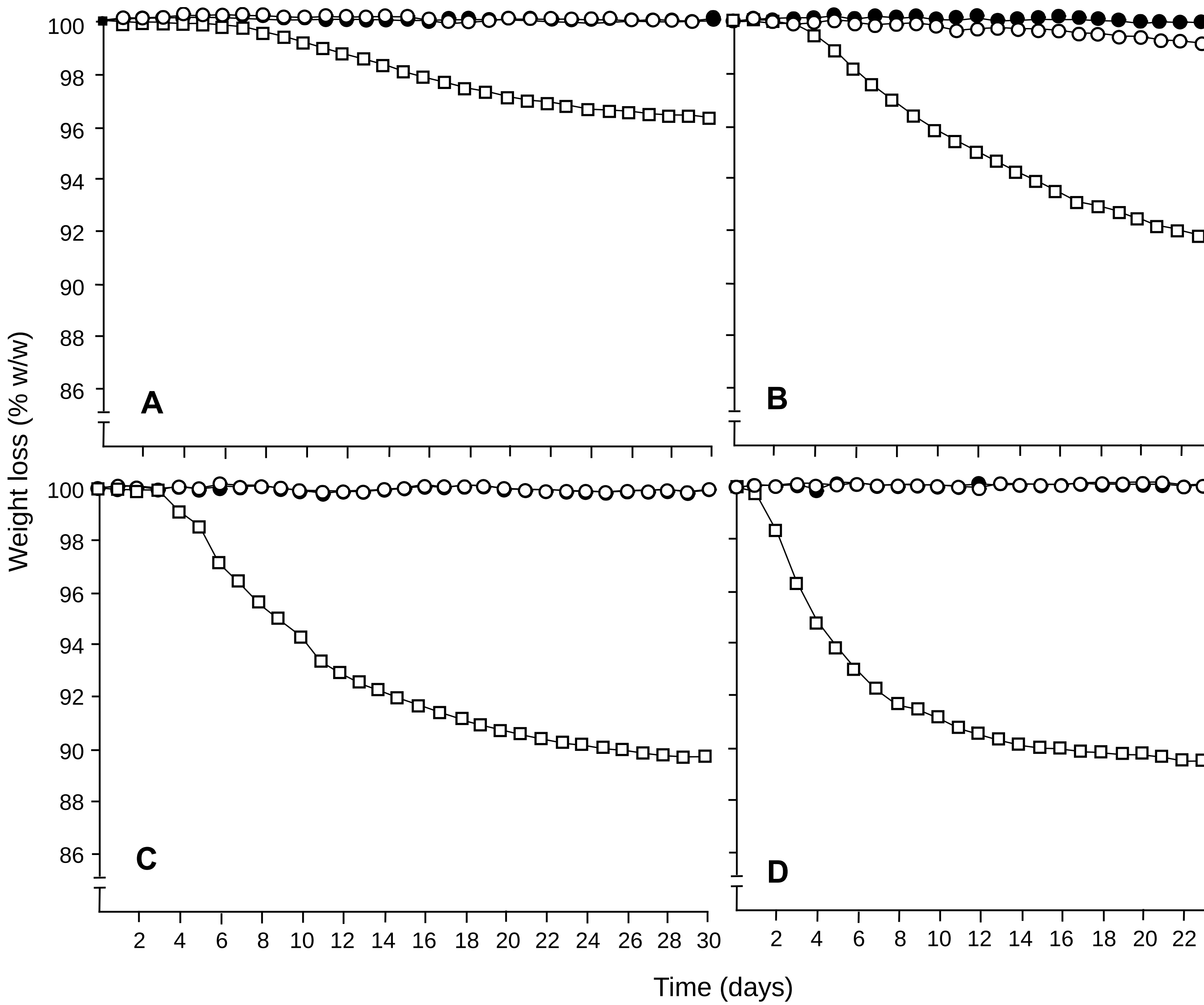

Figure 6. Weight loss of synthetic media inoculated with *Mt. pulcherrima* (○), *My. guilliermondii* (●), or *S. cerevisiae* (□) and incubated at 10°C (**A**), 15°C (**B**), 20°C

Objective 2:

Part 1. Aktuna Research
The impacts of nitrogen addition time with sequential inoculation of *M. pulcherrima* and *S. cerevisiae* during Chardonnay Fermentation were studied.

Chardonnay fermentation inoculated only *S. cerevisiae* as a starter culture was tracked with yeast population (A), total YAN (B), and soluble solids (C) illustated in Figure 1. When *Saccharomyces* and non-*Saccharomyces* populations reached 10^8 and 10^5 CFU/mL, respectively, it was observed that the YAN present on the grapes and added one day after inoculation was nearly exhausted within two days, leaving only 1.12 mg N/L remaining. Before YAN was added 4 days later after *S. cerevisiae* inoculation, the *Saccharomyces* population maintained 10^8 CFU/mL and the non-*Saccharomyces* population reached 10^6 CFU/mL. During this time, there was nearly no YAN present while it required 19 days to reach dryness.

In the fermentation where YAN was added one day after *M. pulcherrima* inoculation (treatment 1), the non-*Saccharomyces* population rose above 10^7 CFU/mL until *S. cerevisiae* inoculation on day 4. Both the preexisting and added YAN in the grapes were consumed during this period (Figures 2A–B). Not all was consumed in this treatment right away following the addition of YAN; instead, the concentration reached 1.15 mg N/L three days later. From day 4 to day 8, the amount of YAN was between 1 to 2 mg N/L, and the population of *Saccharomyces* was the same as the fermentation-only *S. cerevisiae* inoculation (10^8 CFU/mL). During this time, the amount of non-*Saccharomyces* yeast decreased from 10^7 CFU/mL to 10^6 CFU/mL. On the 31st day, fermentations reached dryness (Figure 2C).

In the fermentation to which YAN was added 3 days after *M. pulcherrima* inoculation (treatment 2), non-*Saccharomyces* and *Saccharomyces* populations were similar to those in the treatment added 1 day after *M. pulcherrima* inoculation (treatment 1) (Figure 3A). In terms of the total quantity of YAN, nearly all of it was consumed on day 4, leaving only 2.38 mg N/L of YAN, even though YAN was added on day 3 (Figure 3B). Up until day 8, the YAN concentration was 1.23-2.28 mg N/L. Similar to the prior treatment, dryness was reached in 31 days (Figure 3C).

Saccharomyces populations in the fermentation with added YAN one day after *S. cerevisiae* inoculation (day 5) were similar to the other treatments (Figure 4A). In addition, the non-*Saccharomyces* yeast population reached 10^7 CFU/mL on day 4 and decreased to 10^6 CFU/mL on day 10. When looking at total YAN, there was a similar graph compared to treatment 2, in which YAN was added 3 days after *M. pulcherrima* inoculation, and the amount of YAN on day 4 was 1.34 mg N/L (Figure 4B). The YAN addition increased from 21.56 mg N/L on day 6 and then decreased to 1.32 mg N/L again on day 7. The sample reached dryness on day 25, six days earlier than in the first and second treatments (Figure 4C).

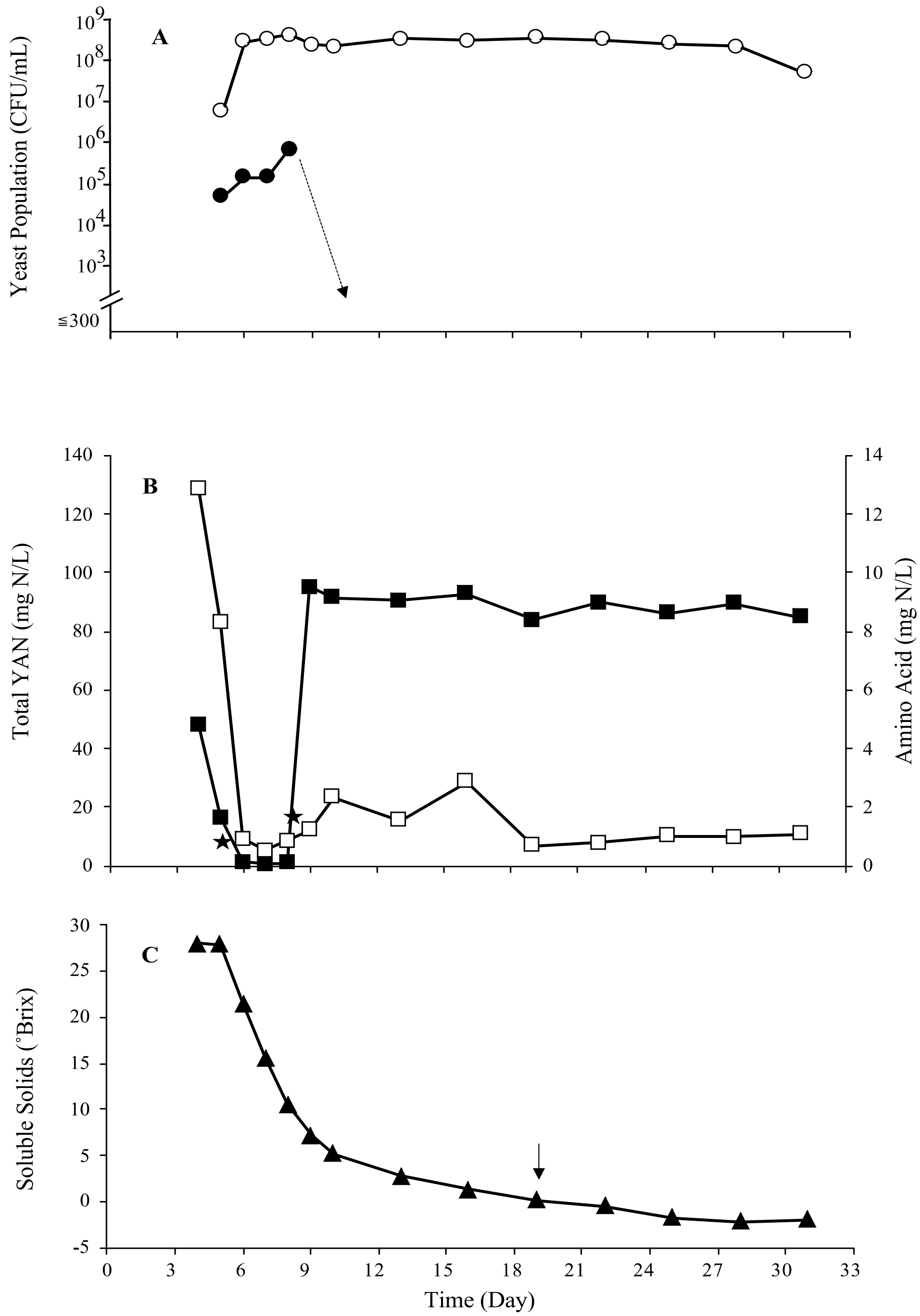

Figure 1. Changes in non-*Saccharomyces* (●) and *Saccharomyces* (○) yeast populations, total YAN (■), amino acid (□), and soluble solids (▲) during Chardonnay fermentation inoculated with *S. cerevisiae* (day 4) with YAN addition. Stars represent YAN addition time. The arrow in Figure C represents the dryness point.

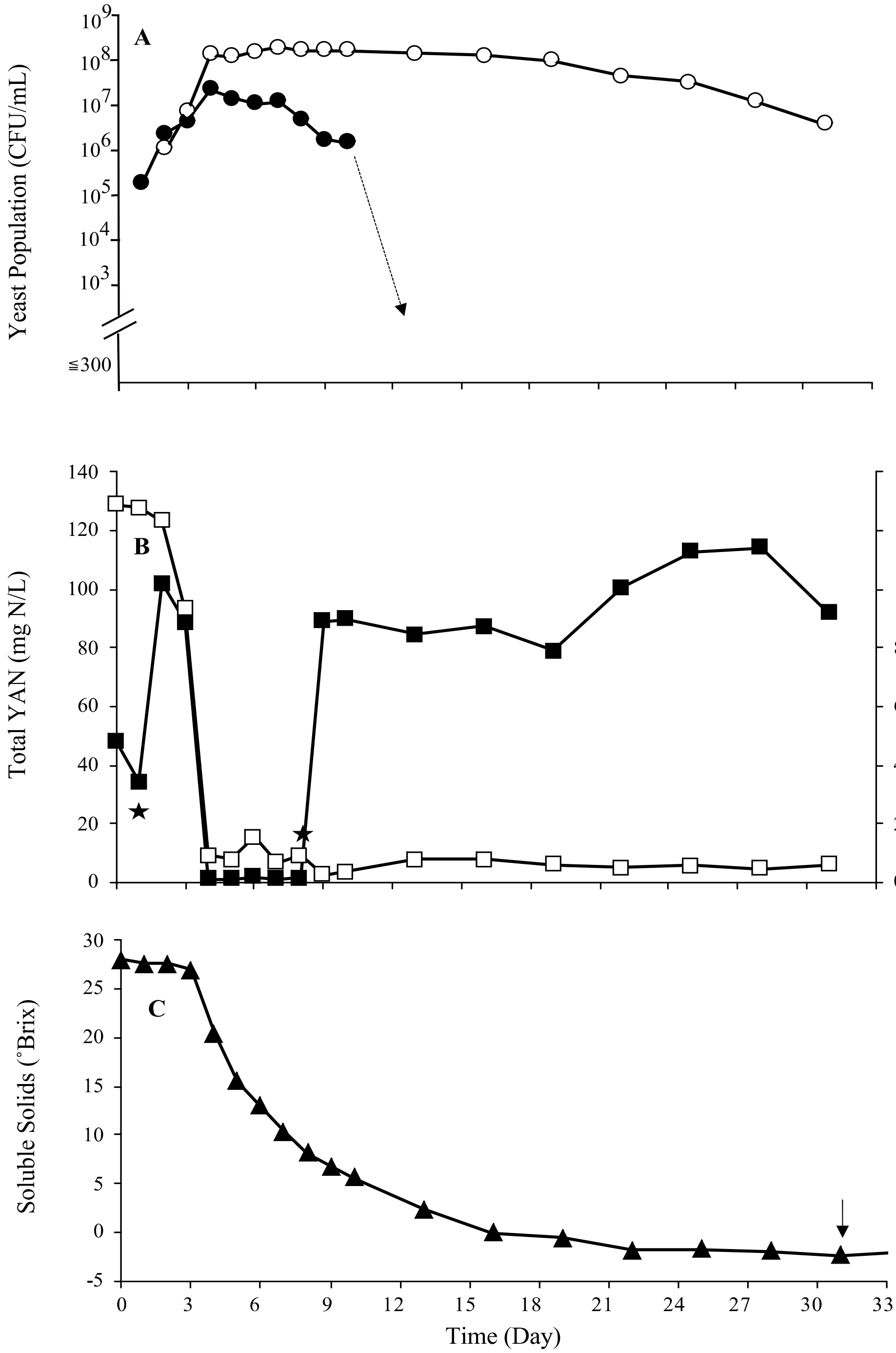

Figure 2. Changes in non-*Saccharomyces* (●) and *Saccharomyces* (○) yeast populations, total YAN (■), amino acid (□), and soluble solids (▲) during Chardonnay fermentation sequentially inoculated with *M. pulcherrima* (day 0) and *S. cerevisiae* (day 4) with day 1 YAN addition. Stars represent YAN addition time. The arrow in Figure C represents the dryness point.

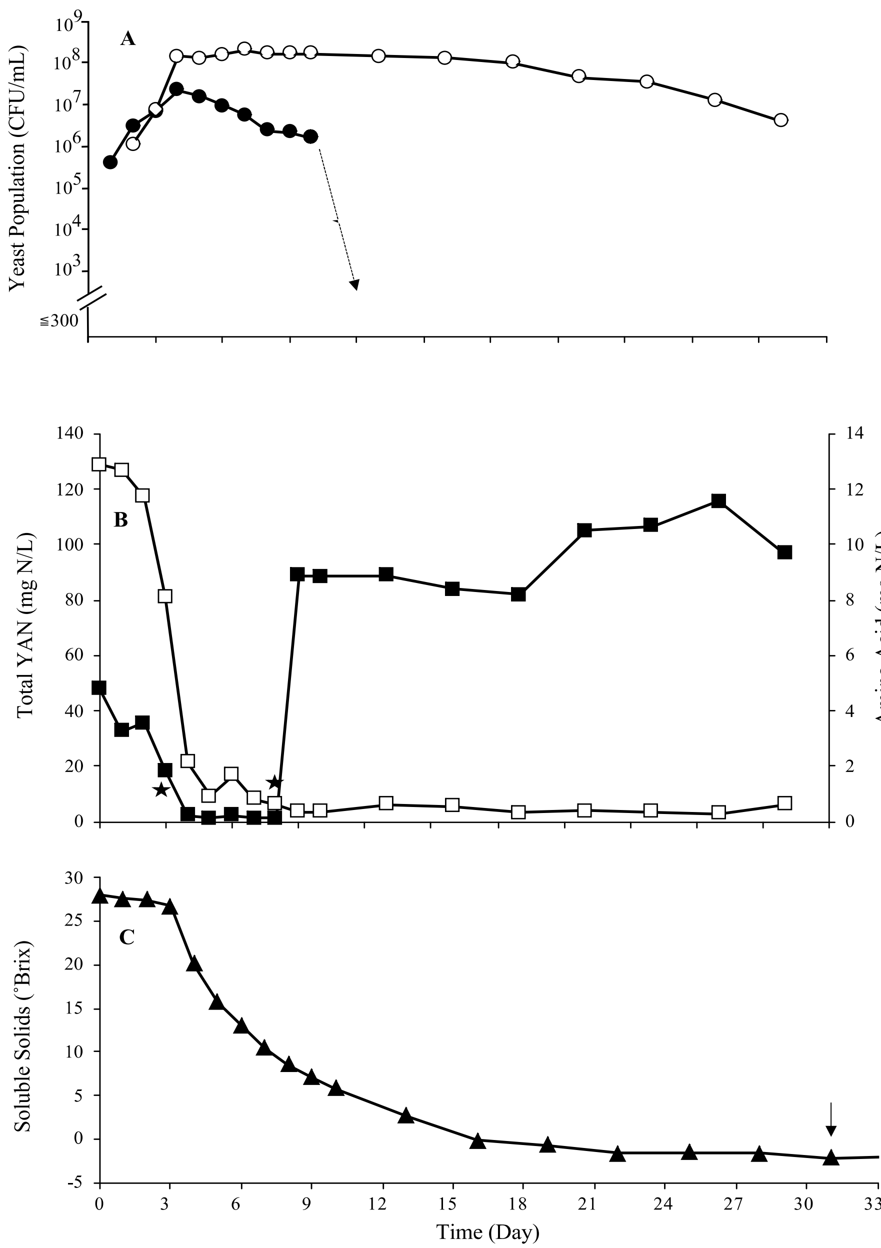

Figure 3. Changes in non-*Saccharomyces* (●) and *Saccharomyces* (○) yeast populations, total YAN (■), amino acid (□), and soluble solids (▲) during Chardonnay fermentation sequentially inoculated with *M. pulcherrima* (day 0) and *S. cerevisiae* (day 4) with day 3 YAN addition. Stars represent YAN addition time. The arrow in Figure C represents the dryness point.

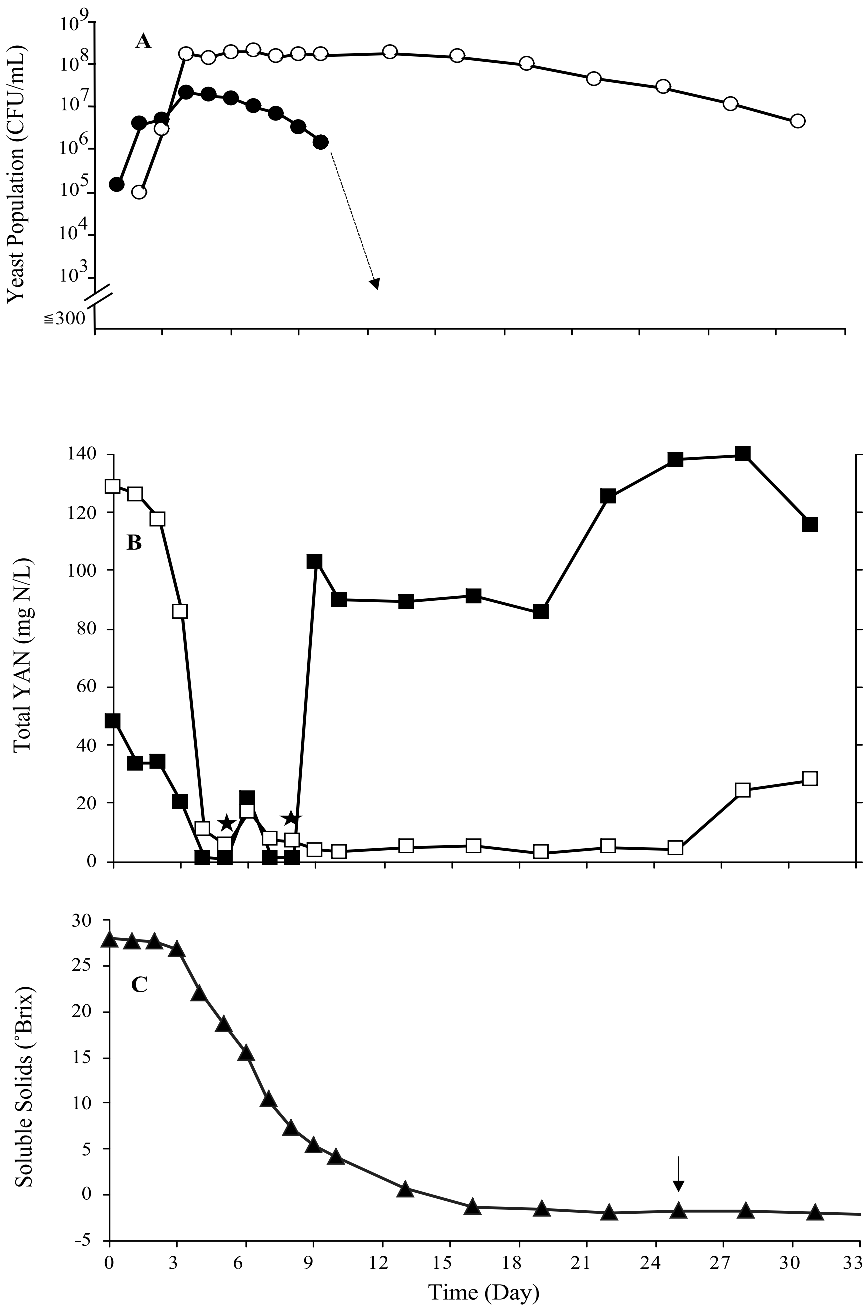

Figure 4. Changes in non-*Saccharomyces* (●) and *Saccharomyces* (○) yeast populations, total YAN (■), amino acid (□), and soluble solids (▲) during Chardonnay fermentation sequentially inoculated with *M. pulcherrima* (day 0) and *S. cerevisiae* (day 4) with day 5 YAN addition. Stars represent YAN addition time. The arrow in Figure C represents the dryness point.

Besides evaluation of the timing of nitrogen addition, Aktuna conducted several additional experimental trials including studying (a) impacts sequential inoculations of different non-*Saccharomyces* yeasts on Chardonnay fermentations, (b) optimization of adaptation conditions for starter culture preparations of *Mt. pulcherrima*, (c) determining the influence of low nitrogen addition on Chardonnay fermentations sequentially inoculated *Mt. pulcherrima* and *S. cerevisiae*. It is anticipated that all data will be published in suitable journals once Aktuna defends her dissertation.

Part 2. Brumley Research

<u>Sugar × YAN using Synthetic Grape Juice Medium</u>

The growth of *M. pulcherrima* was impacted by soluble solids and the nitrogen concentration of SGJM (Figure 3). After inoculating *M. pulcherrima* (10^5 CFU/mL), the high ˚Brix/low YAN treatment did not exceed the inoculation population until day 4 (Figure 3), as opposed to the other treatments where populations of 10^6 CFU/mL were attained one or two days ahead (Figure 3). Similarly, *M. pulcherrima* reached maximum populations of 10^7 CFU/mL in all treatments, except the high ˚Brix/low YAN treatment where the maximum population was a log lower. In the low YAN experiment, *M. pulcherrima* was detectable in the low and medium ˚Brix SGJM until day 10, while the yeast was not found in other treatments after day 7.

The amount of time required for fermentation was dependent on the YAN concentration. Fermentation progressed faster in treatments with medium YAN levels than in treatments with low YAN for all ˚Brix levels (Figure 4). In addition, YAN consumption patterns were dependent on the initial YAN concentrations (Figure 5 and 6). Notably, the amount of metabolized YAN decreased as the sugar concentration increased in low YAN treatments (Figure 5). This trend was seen during the first four days of fermentation in the low YAN where *M. pulcherrima* consumed 24.4 mg N/L compared to 17.8 or 11.8 mg N/L utilized from the medium and high ˚Brix treatments, respectively. This trend, however; was not observed in the high YAN treatment (Figure 6). In the medium YAN SGJM, 23.6, 17.0, or 29.3 mg N/L were metabolized by *M. pulcherrima* in the low, medium, and high ˚Brix treatments, respectively. Moreover, significantly more YAN was consumed by *S. cerevisiae* in the first four days of fermentation than by *M. pulcherrima* when inoculated as single cultures (Figures 5 and 6). In the low YAN experiment, *S. cerevisiae* depleted nearly all YAN in the first four days, leaving only 0.856, 1.02, or 0.729 mg N/L. Likewise, *S. cerevisiae* consumed over half of the available YAN in the medium YAN treatment, leaving only 120, 153, or 138 mg N/L residual YAN for the low, medium, and high ˚Brix treatments respectively.

Similar to microbial consumption of sugar or YAN, yields of alcohol depended on the presence/absence of *M. pulcherrima* (Tables 3 and 4). The low ˚Brix/low YAN was the only treatment where sequential inoculation reached dryness and had significantly less alcohol (0.7% v/v) compared to the control treatment (Table 3). For the medium ˚Brix/low YAN treatment, sequentially inoculated SGJM contained 1.1% v/v less alcohol than singly inoculated SGJM but contained 26.4 g/L (single) or 44.59 g/L (sequential) residual sugar. The high ˚Brix/low YAN treatment expressed no significant differences (p≤0.05) towards alcohol concentrations between the single and sequential inoculation while contained large amounts of residual sugar, > 78 g/L. For treatments with medium or high YAN, fewer differences in alcohol production were noted. As an example, single or sequential inoculations of the low ˚Brix/medium YAN treatment resulted in a dry synthetic wine (Table 4). Similarly, no significant differences were observed in the alcohol for the medium ˚Brix/medium YAN ferments yet the sequentially inoculated reached dryness while the single inoculation contained 14.8 g/L residual sugar. Unlike the low YAN treatment, the sequentially inoculated high ˚Brix/medium YAN SGJM had significantly higher alcohol concentration (1.1% v/v) than the same SGJM singly inoculated. Both the single and sequential inoculation had considerable residual sugar, 70.59 g/L and 49.6 g/L respectively.

Yields of other constituents during fermentation varied based on treatment (Table 3 and 4). In all ˚Brix treatments, no differences in acetic acid concentration can be attributed to the inoculation applied in media containing low YAN (Table 3). However, higher concentrations of YAN did affect acetic acid production. As evidence, media with high YAN contained up to 0.24 g/L more acetic acid than the low YAN (Table 3 and 4). Sequentially inoculated media (medium YAN) had less acetic acid than in the singly inoculated at either medium and high ˚Brix (Table 4). In fact, the greatest concentrations of acetic acid or another constituent, glycerol, were found in high ˚Brix treatments regardless of initial YAN (Table 3 and Table 4). Finally, no differences in glycerol concentrations were found between single and sequential inoculation in either YAN treatment (p≤0.05). However, ˚Brix did impact the glycerol concentration whereas higher ˚Brix treatments contained more glycerol (Table 3 and 4).

<u>High YAN Media</u>

In another experiment, SGJM containing 450 mg N/L was fermented by singly inoculated with *S. cerevisiae* (day 0) or sequential inoculation with *M. pulcherrima* (day 0) and *S. cerevisiae* (day 4) with the final composition presented in Table 5. Regardless of initial ˚Brix or the inoculation, none of the fermentations completed. At the end of fermentation, the low °Brix treatment had 3.22 g/L (single) or 3.38 g/L (sequential inoculation) residual sugar. Furthermore, medium or high ˚Brix SGJM had significantly less (p<0.05) residual sugar when inoculated sequentially with *M. pulcherrima* and *S. cerevisiae* (9.03 g/L or 36.47 g/L) than when singly inoculated with *S. cerevisiae* (27.7 g/L and 36.47 g/L).

Besides sugar consumption, production of other constituents was influenced by high YAN conditions. Under high YAN, glycerol concentrations increased as the initial ˚Brix increased and sequentially inoculated media contained more glycerol than singly inoculated SGJM. The highest glycerol levels were noted in the high ˚Brix treatment sequentially inoculated with *M. pulcherrima* and *S. cerevisiae* (Table 5). Unlike glycerol, the inoculation procedure had no impact on acetic acid concentrations. Finally, significant differences were observed in alcohol concentrations present in the medium and high ˚Brix treatments, where SGJM inoculated sequentially with *M. pulcherrima* and *S. cerevisiae* were 1.2% and 2.2% v/v greater than SGJM singly inoculated with *S. cerevisiae*.

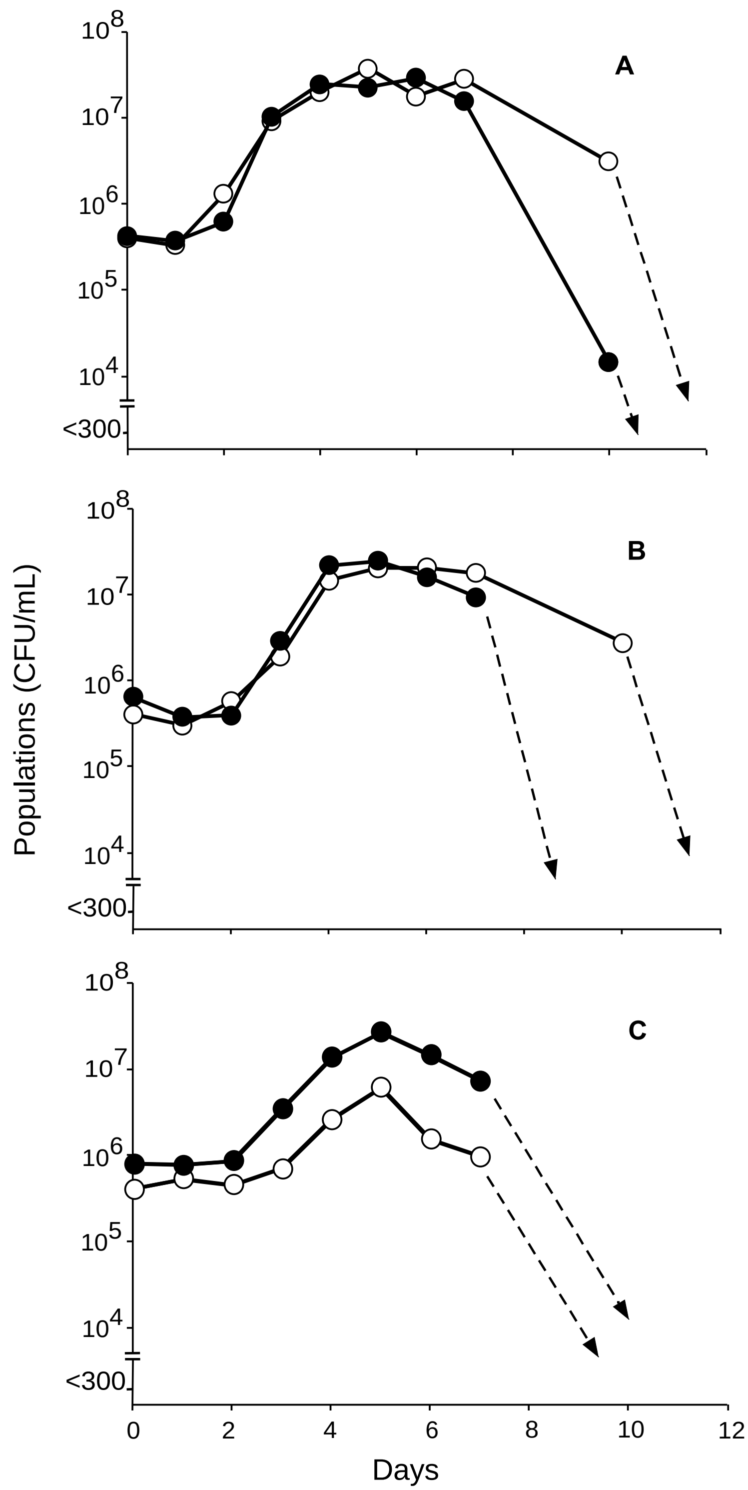

Figure 3 Populations of *M. pulcherrima* during fermentation of Synthetic Grape Juice Medium (SGJM). Prior to inoculation, SGJM was sterile filtered and contained initial soluble solids of 24˚ (A), 27˚ (B), or 30˚ (C) Brix and an initial YAN concentration of approximately 40 (○) or 280 mg N/L (●). SGJM was inoculated sequentially with *M. pulcherrima* (day 0) and *S. cerevisiae* (day 4).

Objective 3a. Analyzing media prepared from ferments as part of Objective 1, *Mt. pulcherrima* was able to metabolize upwards of 15 to 20% of available sugar (Table 2) after three days of growth. Within the same time, *My. guilliermondii* generally utilized less sugar (Table 3) but for both yeasts, the extent of sugar utilization depended temperature and added mSO_2.

Working with cooperator P. Gibney (Cornell University, Ithaca, NY), the fate of the carbon from sugar metabolized by the non-*Saccharomyces* yeasts was investigated. Though *Mt. pulcherrima, My. guilliermondii*, and *S. cerevisiae* grew well in SGJM (Figure 5A), each yeast utilized different amounts of glucose and fructose (Figure 5B). While *S. cerevisiae* consumed the most sugar and achieved dryness (<2 g/L), *Mt. pulcherrima* and *My. guilliermondii* utilized far less, approximately 50% or 10% of the original concentration (250 g/L), respectively. On the one hand, *S. cerevisiae* generated the highest amount of biomass (Figure 5C), ethanol (Figure 5D), or glycerol (Figure 5E). On the other hand, *Mt. pulcherrima* not only produced the highest amount of succinic acid (Figure 5F) but also some ethanol. Non-*Saccharomyces* yeasts are generally recognized as being less efficient than *Saccharomyces* spp. in converting sugar into ethanol thus reducing final ethanol yields in wines when sequentially inoculated with *Saccharomyces*. Though some ethanol was made by *My. guilliermondii* (<1 % v/v), *Mt. pulcherrima* synthesized amounts approaching 6% v/v after seven days of incubation. Interestingly, additional experiments indicated that the carbon was not directly respired to CO_2 likely due to the microaerophilic environment of the bottles during the experiment as determined by measuring fermentation weight loss over time (Figure 6).

These data indicate that some of the carbon being metabolized by *Mt. pulcherrima* and *My. guilliermondii* yielded biomass, glycerol, succinic acid, and, in the case of *Mt. pulcherrima*, ethanol. However, concentrations of these metabolites were still quite low in relation to the decrease in ethanol yield suggesting the synthesis of additional unidentified by-products.

Objective 3b. The ability of non-*Saccharomyces* yeasts to influence mouthfeel and sensory characteristics was evaluated in two studies by C.F. Ross.

The initial study evaluated the influence of pectinase producing non-*Saccharomyces* yeasts (*Cryptococcus adeliensis, Issatchenkia orientalis*, and *Pichia kluyveri*) had on the chemical and sensory properties of red and white wines when varying amounts of pectin were added. For both red and white wines, significant interactions were noted between the presence of non-*Saccharomyces* yeasts and pectin addition which affected pH, titratable acidity, and concentrations of D-galacturonic acid. However, no significant differences in alcohol content or mannoprotein concentration were observed when the tested non-*Saccharomyces* strains were used. Limited sensory were observed in both red and white wines. The only significant sensory attribute in white wine was ethanol burn which was decreased with the addition of pectin, regardless of non-*Saccharomyces* yeasts. Despite the lack of sensory changes, a few a strong positive correlation was found between the D-galacturonic acid and buttery aroma for Chardonnay and with flavor for Merlot. Increasing D-galacturonic acid concentrations, through utilization of non-*Saccharomyces* yeasts, may improve the wine quality as buttery aroma is often associated with high quality Chardonnay.

The second study examined the influence of storage. In this study, changes in sensory attributes of red and white wine were followed during storage when non-*Saccharomyces* yeasts (*Cryptococcus adeliensis*, *Issatchenkia orientalis*, and *Pichia kluyveri)* were utilized with one concentration of added pectin (0.05 g/L). The wines were assessed for sensory properties at 6- and 18-months of storage, and for chemical properties at 6-months of storage. For both white and red wines, the addition of non-*Saccharomyces* yeasts and added pectin increased the D-galacturonic acid, an indication of pectin breakdown, and glycerol concentration, but no changes in alcohol content or mannoprotein concentrations were observed. For sensory analysis in red wine, the interaction among the yeast utilization, pectin addition and storage time influenced the solvent flavor ($p<0.05$). Additionally, the addition of non-*Saccharomyces* yeasts increased the cherry flavor over storage. In white wines, no sensory differences were perceived with the addition of non-*Saccharomyces* yeasts; however, storage increased the intensity of most of the attributes including mouthfeel attributes.

Based on these studies, the synthesis of polysaccharides and pectinase had a limited effect on the sensory characteristics, but the addition of pectin alone reduced ethanol burn in white wine. It should be noted that no differences in mannoprotein concentrations were observed in either study, and may explain the limited differences in mouthfeel across the treatments. For both studies, the utilization of these particular non-*Saccharomyces* yeasts significantly influenced chemical properties but yielded minor sensory changes. Further research is needed to understand if other non-*Saccharomyces* yeast strains would have an increased effect on ethanol reduction and mouthfeel.

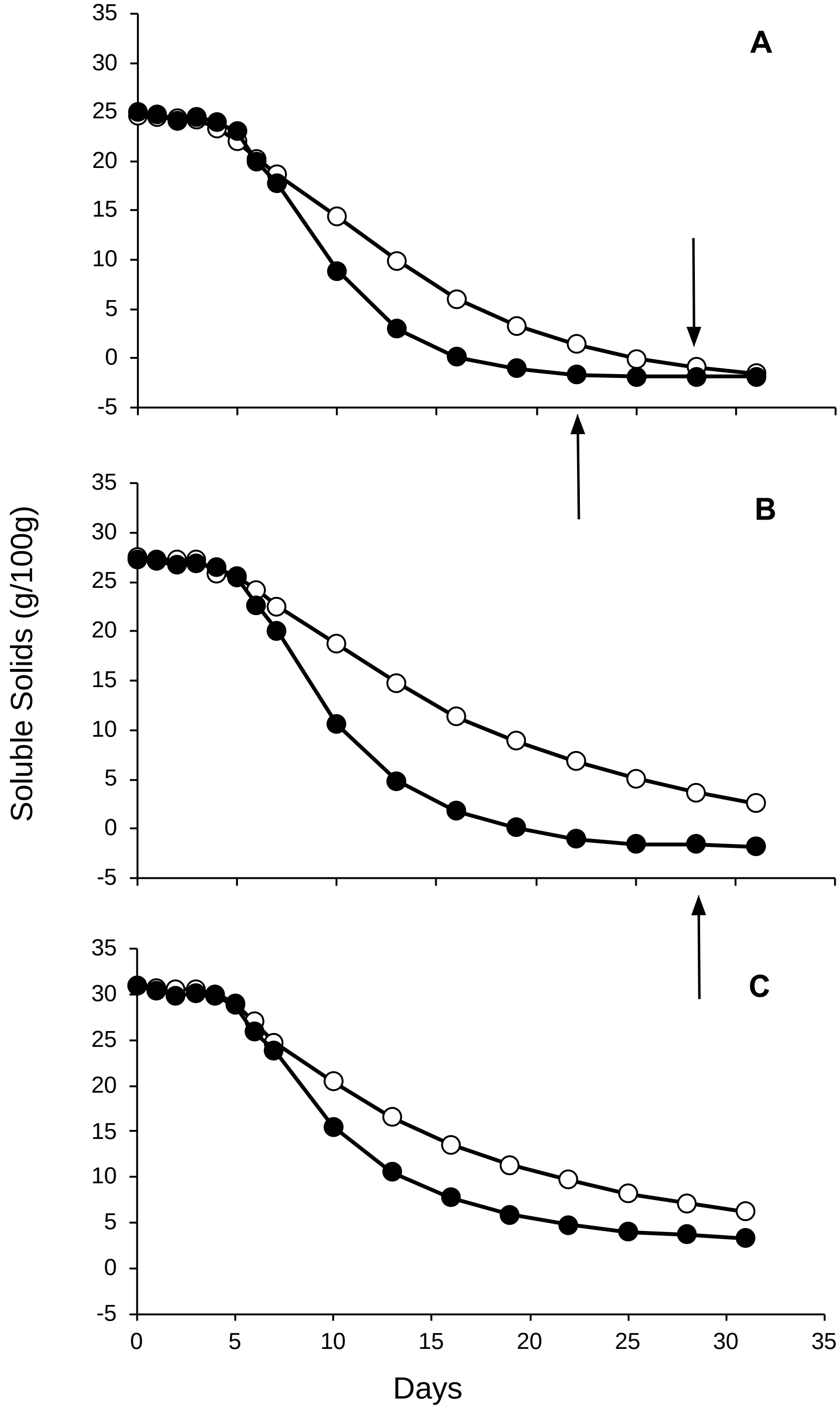

Figure 4 Change in ˚Brix during fermentation of Synthetic Grape Juice Media (SGJM), inoculated sequentially with *M. pulcherrima* (day 0) and *S. cerevisiae* (day 4). Initial soluble solids measured 24˚ (A), 27˚ (B), or 30˚ (C) Brix. SGJM was supplemented with either 40 (○) or 280 (●) mg N/L YAN. Fermentations that reached dryness are indicated by an arrow.

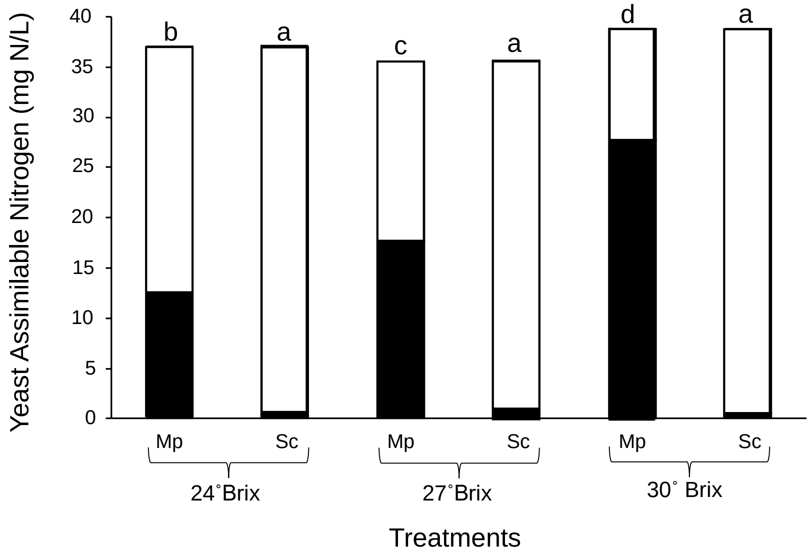

Figure 5 Metabolized (□) and residual (■) yeast assimilable nitrogen (YAN) 4 days after inoculation of either *M. pulcherrima* (Mp), or *S. cerevisiae* (Sc) into synthetic grape juice media (SGJM). SGJM was made with initial soluble solids of 24˚, 27˚, or 30˚ Brix and initial YAN (column height) of approximately 35-40 mg N/L. Lowercase letters above the column indicate significant differences between mean values of metabolized YAN ($p<0.05$).

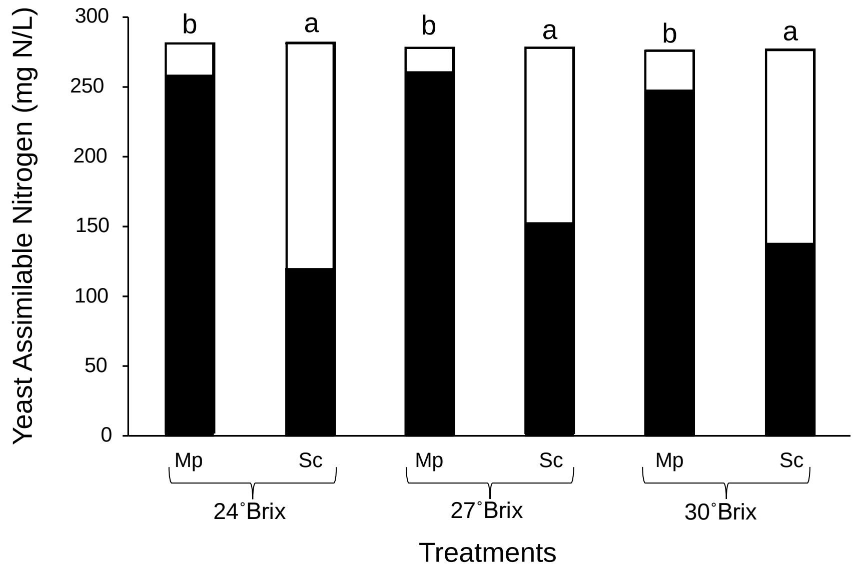

Figure 6 Metabolized (□) and residual (■) yeast assimilable nitrogen (YAN) 4 days after inoculation of either *M. pulcherrima* (Mp), or *S. cerevisiae* (Sc) into synthetic grape juice media (SGJM). SGJM was made with initial soluble solids of 24˚, 27˚, or 30˚ Brix and initial YAN (column height) of approximately 280 mg N/L. Lowercase letters above the column indicate significant differences between mean values of metabolized YAN ($p<0.05$).

Table 3 Composition of synthetic grape juice media (SGJM) after fermentation. SGJM was composed of varying initial soluble solids concentrations (˚Brix) and approximately 40 mg N/L. SGJM was inoculated singly with *S.* cerevisiae (day 0), or inoculated sequentially with *M. pulcherrima* (day 0) and *S. cerevisiae* (day 4).

	24˚Brix		27˚Brix		30˚Brix	
Compound	Sc[a]	Mp+Sc[b]	Sc	Mp+Sc	Sc	Mp+Sc
Glucose (g/L)	1.32 c[c]	1.45 c	1.79 c	5.89 b	9.26 ab	11.2 a
Fructose (g/L)	nd[d]	nd	24.6 c	38.7 b	69.3 a	69.5 a
Glycerol (g/L)	9.45 b	10.1 ab	10.5 ab	9.50 b	11.6 a	10.6 ab
Succinic Acid (g/L)	0.542 ab	0.753 a	0.493 ab	0.474 ab	0.388 b	0.346 b
Acetic Acid (g/L)	1.03 c	1.03 c	1.21 bc	1.06 c	1.52 a	1.42 ab
Alcohol (%)	14.8 a	14.1 b	14.8 a	13.7 b	14.5 ab	14.0 b

[a]Monoculture treatment with *S. cerevisiae*
[b]Sequential inoculation treatment with *M. pulcherrima* and *S. cerevisiae*
[c]Lower case letters indicate mean significant differences within a row ($p<0.05$)
[d]Not detected

Table 4 Composition of synthetic grape juice media (SGJM) after fermentation. SGJM was composed of varying initial soluble solids concentrations (˚Brix) and approximately 280 mg N/L. SGJM was inoculated singly with *S.* cerevisiae (day 0), or inoculated sequentially with *M. pulcherrima* (day 0) and *S. cerevisiae* (day 4).

	24˚Brix		27˚Brix		30˚Brix	
Compound	Sc[a]	Mp+Sc[b]	Sc	Mp+Sc	Sc	Mp+Sc
Glucose (g/L)	0.593 cd	0.608 c	0.518 d	0.538 cd	1.89 a	0.999 b
Fructose (g/L)	nd[d]	nd	14.3 c	nd	68.7 a	48.6 b
Glycerol (g/L)	8.84 c	9.09 c	9.67 b	9.09 c	10.8 a	10.7 a
Succinic Acid (g/L)	0.302 b	0.363 a	0.272 cd	0.293 bc	0.267 d	0.308 b
Acetic Acid (g/L)	1.20 d	1.11 d	1.44 c	1.18 d	1.76 a	1.60 b
Alcohol (%)	14.7 bc	14.7 bc	15.2 a	15.0 ab	14.3 c	15.4 a

[a]Monoculture treatment with *S. cerevisiae*
[b]Sequential inoculation treatment with *M. pulcherrima* and *S. cerevisiae*
[c]Lower case letters indicate mean significant differences within a row ($p<0.05$)
[d]Not detected

Table 5 Composition of synthetic grape juice media (SGJM) after fermentation. SGJM was composed of varying initial soluble solids concentrations (˚Brix) and approximately 450 mg N/L. SGJM was inoculated singly with *S.* cerevisiae (day 0), or inoculated sequentially with *M. pulcherrima* (day 0) and *S. cerevisiae* (day 4).

	24˚Brix		27˚Brix		30˚Brix	
Compound	Sc[a]	Mp+Sc[b]	Sc	Mp+Sc	Sc	Mp+Sc
Glucose (g/L)	3.22 b[c]	3.38 b	5.17 b	3.46 b	16.3 a	6.67 b
Fructose (g/L)	nd[d]	nd	22.5 b	5.57 c	49.8 a	29.8 b
Glycerol (g/L)	7.85 e	8.25 de	8.77 cd	9.31 bc	9.38 b	10.6 a
Succinic Acid (g/L)	0.407 c	0.224 d	0.479 a	0.415 c	0.473 ab	0.432 bc
Acetic Acid (g/L)	1.19 c	1.20 c	1.37 b	1.40 b	1.67 a	1.71 a
Alcohol (%)	14.5 c	14.6 bc	14.5 c	15.7 a	13.4 d	15.6 ab

[a]Monoculture treatment with *S. cerevisiae*

[b]Sequential inoculation treatment with *M. pulcherrima* and *S. cerevisiae*

[c]Lower case letters indicate mean significant differences within a row ($p<0.05$)

[d]Not detected

Outreach and Education Efforts - Presentations of Research:

Information from this research has been and will be disseminated through (a) peer reviewed publications, (b) technical meetings (*i.e.*, American Society for Enology and Viticulture annual meeting and others), and (c) workshops/shortcourses held in Washington and other locations.

Graduate students indicated by "" and presenter indicated by "†"*

Professional Articles (refereed)

Paup*, V.D., J.J. Aplin*, R.I. Potter, C.G. Edwards, J. Lee, and C.F. Ross. Sensory properties of 6- and 18-month stored wines made with pectinase producing non-*Saccharomyces* yeasts. *J. Food Sci.* 88: 462-476. DOI 10.1111/1750=3841.16418 (2023).

Carbon*, H.N., J.J. Aplin*, G.Z. Jiang*, P.A. Gibney, and C.G. Edwards. Fate of carbon in synthetic media fermentations containing *Metschnikowia pulcherrima* or *Meyerozyma guilliermondii* in the presence and absence of *Saccharomyces cerevisiae*. *Food Microbiol.* 114: 104308. DOI: 10.1016/j.fm.2023.104308 (2023).

Lee, W.-J., B. Nielsen, F. Younce, C.G. Edwards, and G. Unlu. Polyphasic identification of a *Zygosaccharomyces rouxii* isolate from grape juice concentrate and its control using thermal processing. AIMS Agric. Food 8(20: 662-673. DOI: 10.3934/agrfood.2023036 (2023).

Abstracts and Papers Presented at Technical Meetings

Aktuna, I.*, J.D. Brumley, and C.G. Edwards. Timing of nitrogen addition on Chardonnay fermentation with sequential inoculation of *M. pulcherrima* and *S. cerevisiae*. Presented at the American Society for Enology and Viticulture annual meeting, San Diego, CA. June 26-29 (2023).

Brumley, Jonathan, Ipek Aktuna, and Charles Edwards. Impact of yeast assimilable nitrogen and sugar on sequential fermentation with *Metschnikowia pulcherrima* and *Saccharomyces cerevisiae*. To be presented at the American Society for Enology and Viticulture annual meeting, Portland, OR. June 17-20 (2024).

Research Success Statements:

This research will provide winemakers options for (a) reducing ethanol concentrations in final wines and/or (b) modification of sensory characteristics using non-*Saccharomyces* yeasts. The use of this and other non-*Saccharomyces* yeasts have the great potential to improve wine quality while naturally limiting the production of alcohol as part of fermentation. However, addition of nitrogen (YAN) to fermentations with non-*Saccharomyces* yeasts will be more critical given their nutritional requirements.

Funds Status:

Funds were used to support the two graduate students (Ipek Aktuna and Jonathan Brumley). Although technically a visiting scholar, Ipek worked at WSU as part of her Ph.D. to be obtained from the Middle East Technical University (Ankara, Turkey), one of the top-ranked science universities in her country. Jonathan has now completed his research and is writing his thesis. Both Ipek and Jonathan will be defending their dissertation/thesis sometime fall semester 2024.

Acknowledgements:

Having been with WSU since 1989, it has been a true honor for the principal investigator to work with our industry and is most thankful for the years of support. While mistakes were earlier made which caused difficulties, the principal investigator hopefully learned from the past to become a better researcher and teacher. Working with such amazing folks as David Forsyth, Kay Simon, Joy Andersen, Scott Williams, Melissa Hansen, and so many others have made this truly an amazing journey.

Special thanks must also be expressed to our representative to the committee, Brian Carter. Brian kept the research program focused and moving forward by asking tough (yet very appropriate and fair) questions. Given the industry had 79 wineries at the time of hire, it is hoped that the research, extension, and teaching efforts helped (in some small way) towards the growth of the industry. Thank you, Brian, for the wonderful guidance over the years.

To all those who serve or continue to serve on this industry research review, thank you you're your assistance, guidance, and support to help the research efforts. Best wishes towards the future……cge