Year Two Final Report

I. Project Title:
Impact of non-*Saccharomyces* yeast on wine quality. Part I: Isolation and identification of non-*Saccharomyces* yeast with glycosidase activity.

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Cooperator:
A. Bakalinsky (Oregon State University) will provide assistance in the identification and quantification of yeast species.

Industry Collaborators:
Archery Summit Winery and Kate Payne-Brown (Assistant Winemaker).

III. Summary:

Wine aroma is one of the most important aspects of wine quality and yeast contribute a significant amount of the volatile aroma compounds in wine. However, our understanding of the contribution of specific yeast to Pinot noir aroma is still limited. In particular, the contribution of non-*Saccharomyces* yeast species present during pre-fermentation maceration and early alcoholic fermentation is relatively unknown. This research investigated yeast population and species diversity present on Pinot noir grapes during pre-fermentation cold maceration and alcoholic fermentation and the impact these yeast had on Pinot noir wine aroma. As in 2010, yeast populations were followed during pre-fermentation cold maceration and alcoholic fermentation of Pinot noir grapes from two different Archery Summit vineyards. Fermentations were conducted at the Oregon State University research winery in 100 L tanks and samples were taken daily and plated on WL and lysine plates in order to determine *Saccharomyces* and non-*Saccharomyces* populations and identify yeast species.

The identity of isolates from 2010 fermentations that demonstrated β-glucosidase activity on 4-MUG plates (see below) was determined by DNA sequencing. DNA sequencing confirmed the identity of *Kluveromyces thermotolerans, Metschnikowia pulcherrima*, and *Hanseniaspora uvarum*. However, the isolate identified as *Hansenula* based on appearance on WL media was determined to be a *Saccharomyces cerevisiae* isolate while unknown isolate #10 was also identified as a *S. cerevisiae* isolate. These yeast isolates were then tested for β-glucosidase activity using a liquid assay (pH 3.50) at different sugar concentrations (5 g/L glucose, 20 g/L glucose, or 100 g/L glucose and 100 g/L fructose) and two temperatures (25°C and 8°C). Isolates that demonstrated β-glucosidase activity on MUG plates also had large levels of activity in media containing 5 g/L glucose. However, when the sugar content of the media was increased to better

match the concentrations in a grape juice (100 g/L glucose + 100 g/L fructose) there was a decrease in β-glucosidase activity for all isolates. While β-glucosidase activity of *Hanseniaspora* decreased dramatically (-99%), β-glucosidase activity still remained relatively high for *Metschnikowia, S. cerevisiae* isolate 1 and *S. cerevisiae* isolate 2. At 8°C β-glucosidase activity was reduced for *Metschnikowia* but activity increased for all other yeast isolates. β-glucosidase activity did not correspond with growth at 8°C. For example, while *Hanseniospora* grew best at cold temperatures it had very low β-glucosidase activity while the weaker growing *S. cerevisiae* isolate 1 had the highest β-glucosidase activity.

Yeast isolates demonstrating β-glucosidase activity were used to perform fermentations in Pinot noir grapes treated with high hydrostatic pressure (HHP). All non-*Saccharomyces* isolates grew well during a seven day cold maceration with populations increasing 3 to 4 logs. After inoculation of *S. cerevisiae* RC212 at the end of cold maceration *Metschnikowia* and *Hanseniaspora* populations remained high for 3 days prior to a rapid decline while *Kluveromyces thermotolerans* populations remained high throughout the alcoholic fermentation. In contrast, viable cell counts for *S. cerevisiae* isolate 1 and *S. cerevisiae* isolate 2 declined below detectable limits after inoculation of RC212. Alcoholic fermentations were finished in 12-15 days with fermentations containing *Kluveromyces* taken the longest to complete. Wines produced from these fermentations were analyzed for volatile aromas (GC-MS) by Dr Michael Qian. Ethyl ester concentrations were highest in the wines that did not undergo a pre-fermentation cold maceration, while concentrations of branch-chained esters were higher in the treatments with yeast present during pre-fermentation cold maceration. Pre-fermentation cold maceration with the various yeast species did not affect the concentration of β-damascenone or β-ionone. However, wines that had undergone pre-fermentation cold maceration with *S. cerevisiae* isolate 1, *S. cerevisiae* isolate 2, and a combination of all isolates resulted in over twice the concentration of β-citronellol compared to wines that did not undergo a pre-fermentation cold maceration.

IV. Objectives and Experiments Conducted to Meet Stated Objectives:

Objective 1.
Isolate non-*Saccharomyces* yeast species that have high glycosidase activities including α-L-rhamnopyranosidase, α-L-arabinopyranosidase and α-L-arabinofuranosidase and β-D-glucosidase activities from Oregon wineries.

As in 2010, samples were taken from grapes undergoing pre-fermentation cold maceration and alcoholic fermentation in order to compare yeast populations and diversity from the same vineyards in two different years. Grapes from the same vineyard lots sampled in 2010 were sampled in 2011. Grapes from both vineyards were harvested October 17th and transported to Archery Summit winery. Before the grapes were processed, 16 totes from each vineyard were immediately transported to the OSU winery and stored overnight at 4ºC before being processed the following day. Grapes were sorted (bunches with visible rot were discarded), destemmed, and randomly allocated to 100 L stainless steel tanks with cooling jackets. 50 mg/L SO_2 was added at this point. Argon gas was blanketed on top of the grapes and bladder-equipped tank lids were placed on top of the grapes and sealed. Two fermentors per vineyard were prepared.

During the pre-fermentation cold maceration tanks were maintained between 8-10ºC and sampled aseptically daily after mixing. Grapes were blanketed with argon gas after sampling and tank lids placed back on top of the grapes. After 7 days cold maceration the fermentations were warmed to approximately 25 ºC and alcoholic fermentations proceeded without inoculation.

All samples were plated onto WL media and lysine media after appropriate dilutions and incubated @ 25 ºC for 2-3 days. Plates were then counted and colonies examined on WL media in order to identify unique colony types based on color, shape, consistency, and size (Pallmann et al. 2001). Detailed descriptions of all different colony types were made. Unique colony types were restreaked and colonies purified on WL medium. These isolates were then stored on agar slants (potato dextrose agar) at 4ºC.

The identity of isolates from 2010 fermentations that demonstrated β-glucosidase activity on 4-MUG plates (see below) was determined by DNA sequencing. Select colonies were streaked from glycerol cultures on to YPD plates and incubated at 25 °C for 48 hours. Single colonies were suspended in 50 µL nuclease-free purified water. The D1/D2 domain of the 5' end of the large subunit 26S rDNA gene was amplified by direct colony PCR using the NL1 (5'-GCA TAT CAA TAA GCG GAG GAA AAG-3') and NL4 (5'-GGT CCG TGT TTC AAG ACG G-3') primers as described by Swangkeaw et al. (2011). A Thermo Hybaid PCR Express thermocycler was used. The PCR reaction was performed with an initial denaturation at 98°C for 30 seconds, followed by 30 cycles of denaturation (98 °C for 10 seconds), annealing (66 °C for 30 seconds), and extension (72 °C at 15 seconds). A final extension was performed at 72 °C for 10 minutes. The PCR products were purified using the QIAGEN QIAquick PCR Purification Kit and Sanger sequencing was performed by the Oregon State University Center for Genome Research & Biocomputing Core Laboratory (Corvallis, OR). Sequences were analyzed using the NCBI BLASTN 2.2.26+ (Zhang et al. 2000).

Yeast isolated from grapes undergoing cold maceration in 2010 were used to perform fermentations in Pinot noir grapes treated with high hydrostatic pressure (HHP). Yeast species were selected based on their β-glucosidase activities as assessed in objective 2. Pinot noir grapes (Woodhall Vineyard, Alpine, OR) were harvested at maturity and stored at 4°C overnight. Grapes were destemmed and 3 kg aliquots were placed in food saver bags and an addition of 30 mg/L SO_2 was made. The grape aliquots were then treated by HHP (5 min @ 80,000 psi) and transferred aseptically to sterile 3L micro-fermentors and allowed to warm to room temperature before being inoculated with a non-*Saccharomyces* yeast at approximately 1 x 10^4 CFU/mL. The yeast species inoculated were *Metschnikowia pulcherrim*, *Hanseniaspora uvarum*, *Kluveromyces thermotolerans, S. cerevisiae* isolate 1, and *S. cerevisiae* isolate 2. One set of fermenters was not inoculated with any yeast (control) while another set was inoculated with all five yeast isolates. All treatments were performed in triplicate. Fermenters were placed in a cold room at 10°C for seven days and sampled daily for yeast viable counts, temperature, and °Brix. At the completion of the cold maceration the fermenters were placed in a temperature controlled room at 27°C and inoculated with *S.cerevisiae* RC212 at approximately 1 x 10^5 CFU/mL. All treatments were supplemented with a sterile solution of Fermaid K at 0.25 g/L. Samples were taken daily for yeast viable cell counts, temperature, and °Brix. At the completion of the alcoholic fermentation the wines were pressed, a 30 mg/L SO_2 was made, and wines were settled at 4 °C for 120 hrs. The wines were then sterile filtered through a 0.45 µm cartridge filter, bottled, and stored at

13°C. Prior to bottling samples were taken for volatile aroma analysis (results detailed in progress report for the project "Impact of non-*Saccharomyces* yeast on wine quality-part 2, aroma and flavor development"). Bottled wine will be analyzed by a trained sensory panel at a later date.

Objective 2.
Investigate the behavior and ability of selected yeast species of hydrolyzing grape-derived volatile glycosides on isolated grape glycoside substrate.

Isolates from 2010 fermentations that demonstrated β-glucosidase activity on 4-MUG plates (Charoenchai et al. 1997) were tested for β-glucosidase activity using a liquid assay. This assay allowed quantification of β-glucosidase in conditions more representative of a grape must. Three different sugar concentrations were tested as well as two different temperatures. Media was adjusted to either 5 g/L glucose, 20 g/L glucose, or 100 g/L glucose and 100 g/L fructose (mimicking a grape juice containing 20 °Brix). The two temperatures tested were 25°C and 8°C. The conditions of 20 °Brix and 8°C were tested so as to model conditions present during a pre-fermentation cold maceration. Yeast species assessed were *Metschnikowia pulcherrim*, *Hanseniaspora uvarum*, *Kluveromyces thermotolerans, S. cerevisiae* isolate 1, and *S. cerevisiae* isolate 2

V. Summary of Major Research Accomplishments and Results:

The identity of isolates from 2010 fermentations that demonstrated β-glucosidase activity on 4-MUG plates (see below) was determined by DNA sequencing. DNA sequencing confirmed the identity of *Kluveromyces thermotolerans, Metschnikowia pulcherrima*, and *Hanseniaspora uvarum* (Table 1). However, the isolate identified as *Hansenula* based on appearance on WL media was determined to be a *Saccharomyces cerevisiae* isolate while unknown isolate #10 was also identified as a *S. cerevisiae* isolate (Table 1). β-glucosidase activity of non-*Saccharomyces* isolates from 2010 was further characterized utilizing a liquid media assay. Initial experiments demonstrated that isolates that demonstrated β-glucosidase activity on MUG plates also had large levels of activity in media at wine pH containing 5 g/L glucose (Table 2). In particular, *Hanseniaspora uvarum* had high β-glucosidase activity compared to the other species. However, when the sugar content of the media was increased to better match the concentrations in a grape juice (100 g/L glucose + 100 g/L fructose) there was decrease in β-glucosidase activity for all isolates. While β-glucosidase activity of *Hanseniaspora* decreased dramatically (-99%), β-glucosidase activity still remained high for other species such as *Metschnikowia*, and *S. cerevisiae* isolate 1 and 2 (Table 2).

Table 1. Yeast isolate identification

Tentative Identity[a]	Species designation[b,*]	GenBank accession no.	Similarity (%)[c]
Metschnikowia pulcherrima	*Metschnikowia pulcherrima*	GU080051	99%
Hanseniaspora uvarum	*Hanseniaspora uvarum*	EU386753	99%
Kluveromyces thermotolerans	*Kluveromyces thermotolerans*	U69581	99%
Hansenula anomala	*Saccharomyces cerevisiae*	JN225410	100%
Unknown isolate 10	*Saccharomyces cerevisiae*	JN225410	99%

[a]Identity based on appearance on WL media
[b]Identity based on BLASTn of D1/D2 domain of 26S rDNA sequence
[c]Percentage of similar nucleotides in domain D1/D2 between isolate and GenBank accession strain

Because a major focus of this study was the impact of non-*Saccharomyces* yeast during pre-fermentation cold maceration, experiments were conducted at 8°C as well as at 25°C. Enzyme activity is typically reduced at lower temperatures such as those present during pre-fermentation cold maceration. After 48 hrs incubation *Metschnikowia* β-glucosidase activity was reduced at 8°C compared to 25°C (-74%) but activity increased for the other yeast isolates (Table 2). The increased β-glucosidase activity was quite dramatic with an increase of 70% or more for *Kluveromyces,* and *S. cerevisiae* isolate 1 and isolate 2. These results were unexpected given the cold temperature and reasons for the elevated enzyme activity are currently being investigated. Large differences between the β-glucosidase activities of the yeast isolates were noted and so yeast growth at 8°C was investigated to determine if varying yeast growth was responsible for the varying β-glucosidase activities. Yeast were grown for seven days at 8°C to simulate a seven day cold maceration and growth and β-glucosidase activity were monitored. *Hanseniospora* grew well at 8°C increasing from an initial population of approximately 1 x 10^6 CFU/mL to almost 1 x 10^9 CFU/mL after 7 days (Figure 1) while the other yeast isolates only increased from approximately 1 x 10^6 CFU/mL to approximately 1 x 10^7 CFU/mL. However, the high growth of *Hanseniospora* did not correspond to high β-glucosidase activity (Figure 1). Instead, the highest β-glucosidase activity was observed for *Hansenula* after 5 days growth at 8°C. These results demonstrate that the high β-glucosidase activity observed for some yeast isolates is not due to elevated growth at cold temperatures but rather is likely due to increased production of β-glucosidase.

Table 2 β-glucosidase activity of various yeast species in assay media (pH 3.50, 48 hours)

	β-glucosidase activity (nmoles *p*-NP released/ g cells/ mL supernatant x 100)		
	25 °C	**25 °C**	**8 °C**
Yeast isolate	**5 g/L Glucose**	**100 g/L Glucose 100 g/L Fructose**	**100 g/L Glucose 100 g/L Fructose**
M. pulcherrima	[1]826 ± 176a	[*]183 ± 38.6a	149 ± 31a
H. uvarum	2730 ± 1130a	5.71 ± 1.14b	8.42 ± 1.72b
K. thermotolerans	208 ± 162 a	60.3 ± 12.3c	383 ± 38c
S. cerevisiae isolate 1	306 ± 123a	153 ± 18a	897 ± 113d
S. cerevisiae isolate 2	207 ± 64b	184 ± 17a	921 ± 36d

[1]Values within columns followed by the same letter do not differ significantly, $p < 0.05$, n=3

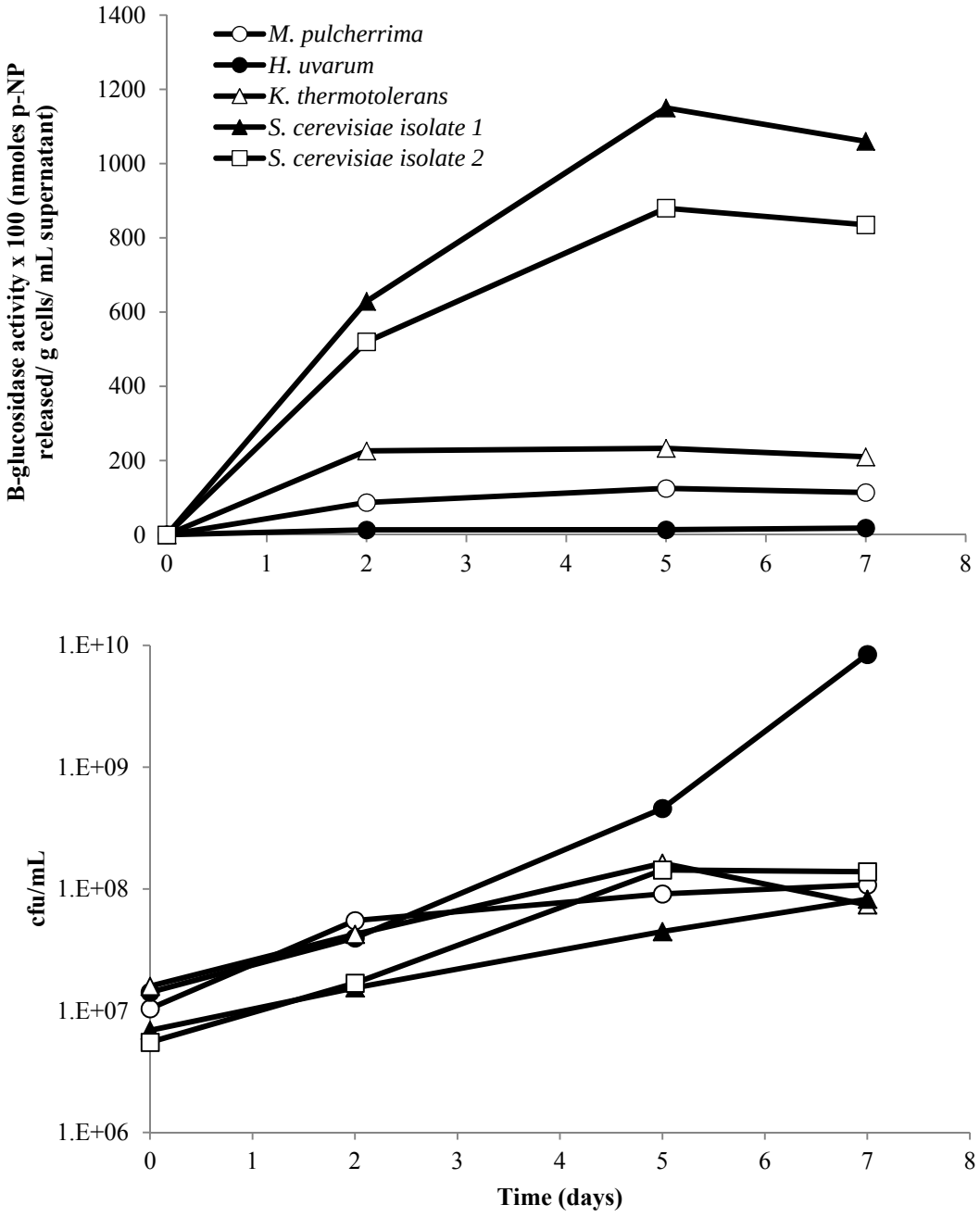

Figure 1 β-glucosidase activity (nmoles *p*-NP released/g cells/mL supernatant) and growth (cfu/mL) in *p*-NPG medium assay, 100 g/L glucose, 100 g/L fructose, pH 3.5, 8 °C, 7 days (n=3)

Yeast isolates demonstrating β-glucosidase activity were used to perform fermentations in Pinot noir grapes treated with high hydrostatic pressure. The control treatment underwent a seven day cold maceration with no inoculation of non-*Saccharomyces* yeast. After seven days grapes were inoculated with *S.cerevisiae* RC212 and alcoholic fermentation was completed after 13 days (Figure 3). The other treatments were inoculated with either a single yeast isolate or a combination of all the yeast isolates. The yeast isolates all grew well during the cold maceration. For example, *Metschnikowia pulcherrim* grew from approximately 1 x 10^4 to 1 x 10^7 CFU/mL (Figure 5) while *Hanseniaspora uvarum* grew from approximately 1 x 10^3 to 1 x 10^7 CFU/mL (Figure 6). When all the yeast isolates were inoculated together the yeast grew well and reached comparable populations as observed when inoculated individually (Figure 4).

After inoculation of *S. cerevisiae* RC212 at the end of cold maceration the growth of the non-*Saccharomyces* isolates differed. *Metschnikowia* and *Hanseniaspora* remained at high viable cell counts for 3 days prior to a rapid decline (Figure 5 & 6) while *Kluveromyces thermotolerans* viable cell counts remained high throughout the alcoholic fermentation (Figure 7). In contrast, viable cell counts for *S. cerevisiae* isolates 1 and 2 declined below detectable limits after the inoculation of *S. cerevisiae* RC212 (Figure 8 & 9). The length of alcoholic fermentation varied depending on the yeast isolate present (Figure 10). Fermentations containing *S. cerevisiae* isolates 1 and 2, and the combination of all isolates, were completed in 12 days. Fermentations containing *Metschnikowia* and *Hanseniaspora* were completed in 13 days while the fermentations containing *Kluveromyces* took 15 days to complete (Figure 10).

Wines produced from these fermentations were analyzed for volatile aromas (GC-MS) by Dr Michael Qian. Ethyl ester concentrations were highest in the wines that did not undergo a pre-fermentation cold maceration, while concentrations of branch-chained esters were higher in the treatments with yeast present during pre-fermentation cold maceration (Figure 11 & Table 4). Esters differed in both total concentrations and individual concentrations for all treatments (Figure 11). In contrast, pre-fermentation cold maceration with the various yeast species did not affect the concentration of β-damascenone or β-ionone (Table 6). However, wines that had undergone pre-fermentation cold maceration with *S. cerevisiae* isolate 1, *S. cerevisiae* isolate 2, and a combination of all isolates resulted in over twice the concentration of β-citronellol compared to wines that did not undergo a pre-fermentation cold maceration (Table 6). For more detailed volatile aroma results please results refer to the progress report for the project "Impact of non-*Saccharomyces* yeast on wine quality-part 2, aroma and flavor development" conducted by Dr Michael Qian.

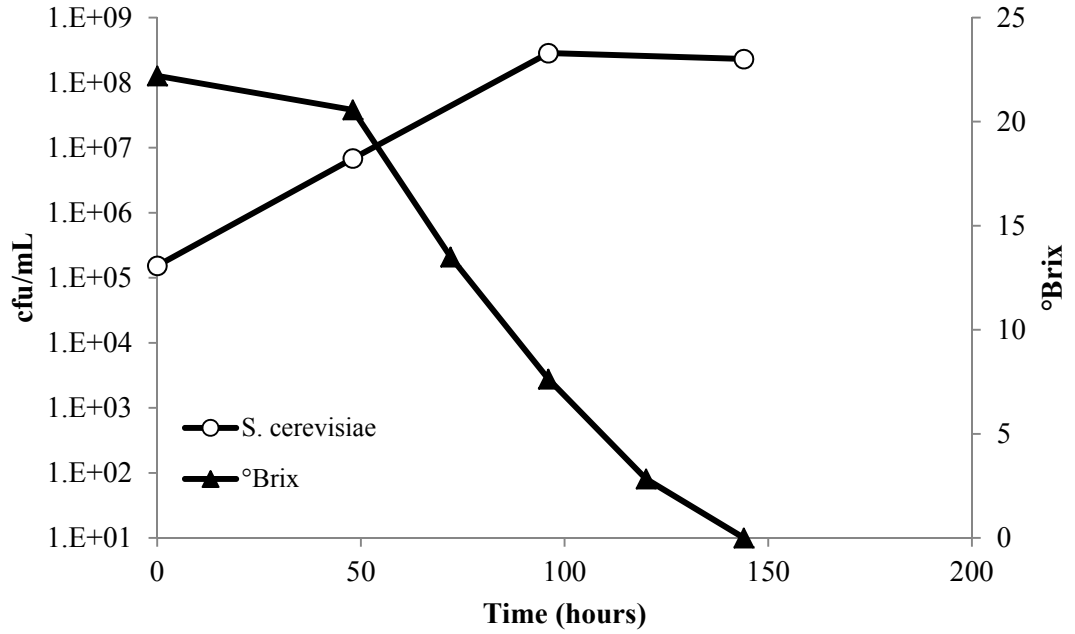

Figure 2 Growth of *Saccharomyces cerevisiae* RC212 and °Brix during alcoholic fermentation of Pinot noir grapes. No pre-fermentation cold maceration was performed. Inoculation of *S. cerevisiae* RC212 occurred at day 0.

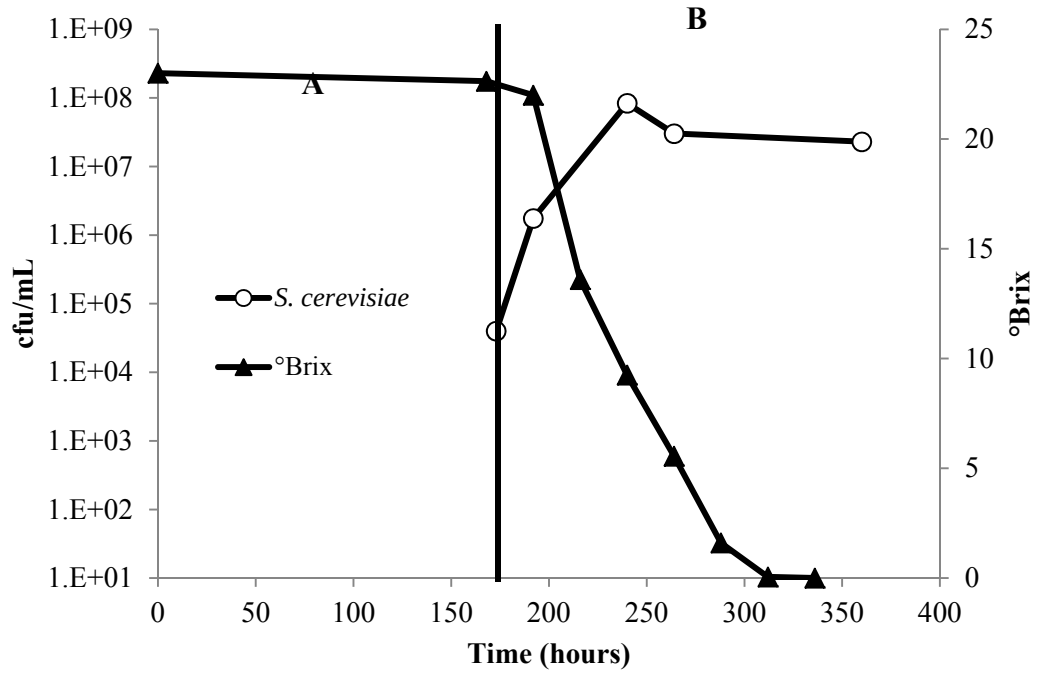

Figure 3 Growth of *Saccharomyces cerevisiae* RC212 and °Brix during pre-fermentation cold maceration (A) and alcoholic fermentation (B) of Pinot noir grapes. Inoculation of *S. cerevisiae* RC212 occurred at end of cold maceration.

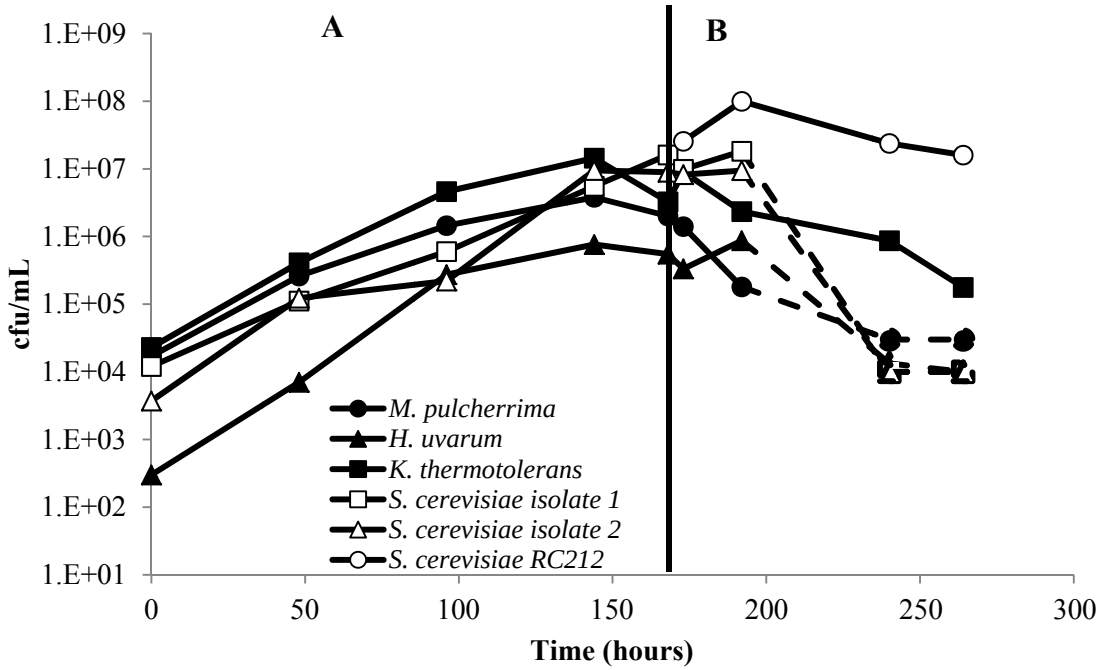

Figure 4 Growth of *Saccharomyces cerevisiae* RC212, *Metschnikowia pulcherrima*, *Hanseniaspora uvarum*, *Kluveromyces thermotolerans*, *Saccharomyces cerevisiae* isolate 1, and *Saccharomyces cerevisiae* isolate 2 during pre-fermentation cold maceration (A) and alcoholic fermentation (B) of Pinot noir grapes. Inoculation of *S. cerevisiae* RC212 occurred at end of cold maceration.

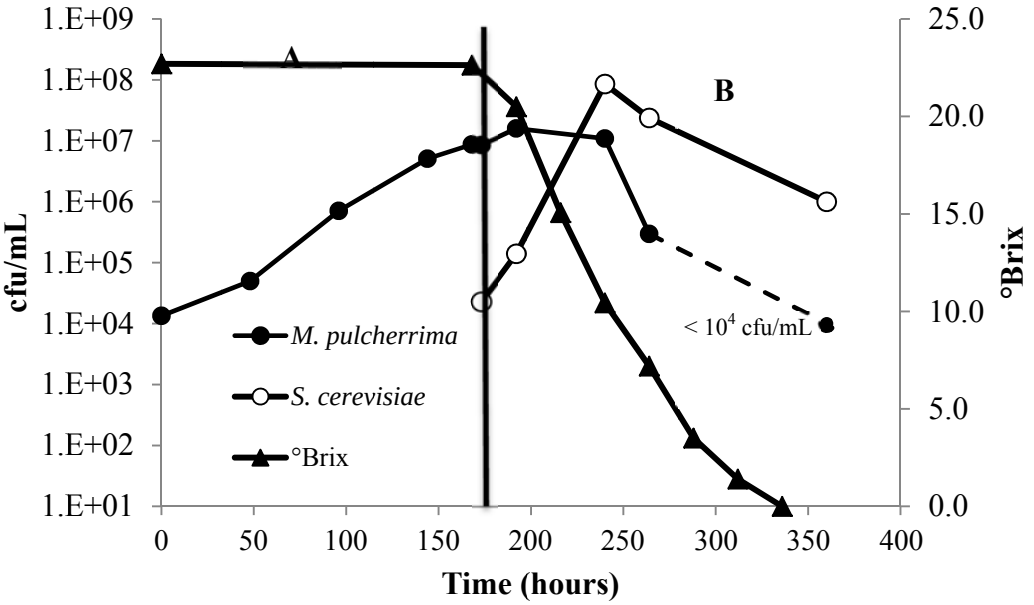

Figure 5 Growth of *Saccharomyces cerevisiae* RC212, *Metschnikowia pulcherrima*, and °Brix during pre-fermentation cold maceration (A) and alcoholic fermentation (B) of Pinot noir grapes. Inoculation of *S. cerevisiae* RC212 occurred at end of cold maceration.

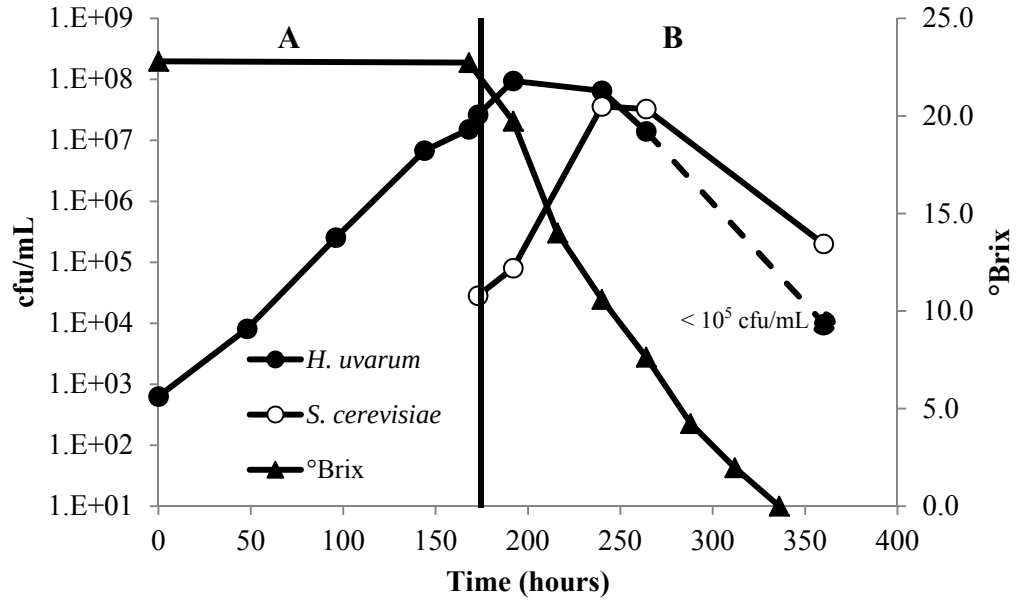

Figure 6 Growth of *Saccharomyces cerevisiae* RC212, *Hanseniaspora uvarum*, and °Brix during pre-fermentation cold maceration (A) and alcoholic fermentation (B) of Pinot noir grapes. Inoculation of *S. cerevisiae* RC212 occurred at end of cold maceration.

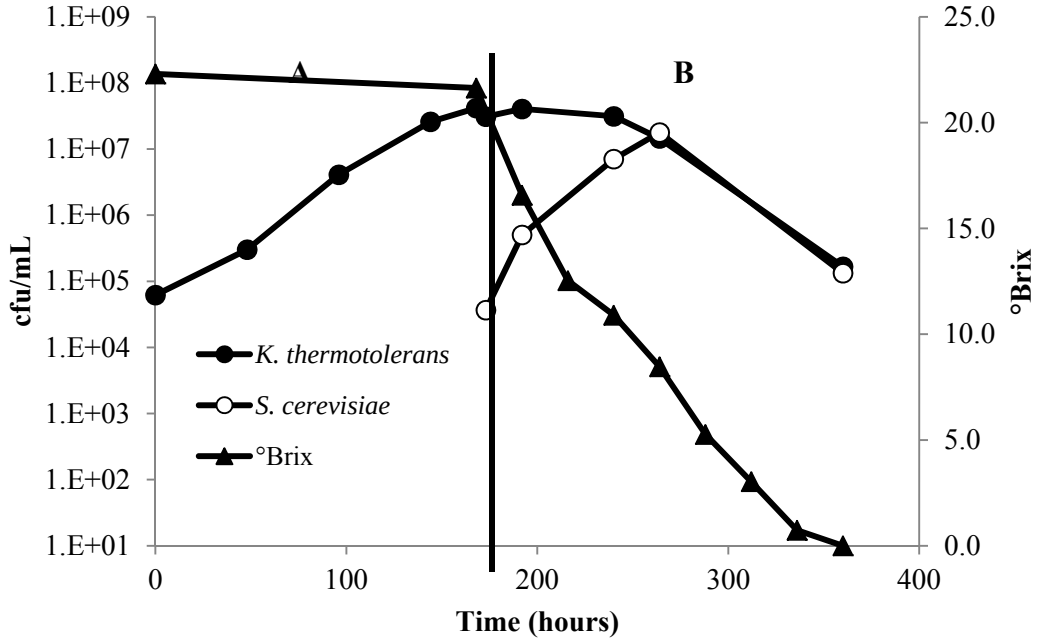

Figure 7 Growth of *Saccharomyces cerevisiae* RC212, *Kluveromyces thermotolerans*, and °Brix during pre-fermentation cold maceration (A) and alcoholic fermentation (B) of Pinot noir grapes. Inoculation of *S. cerevisiae* RC212 occurred at end of cold maceration.

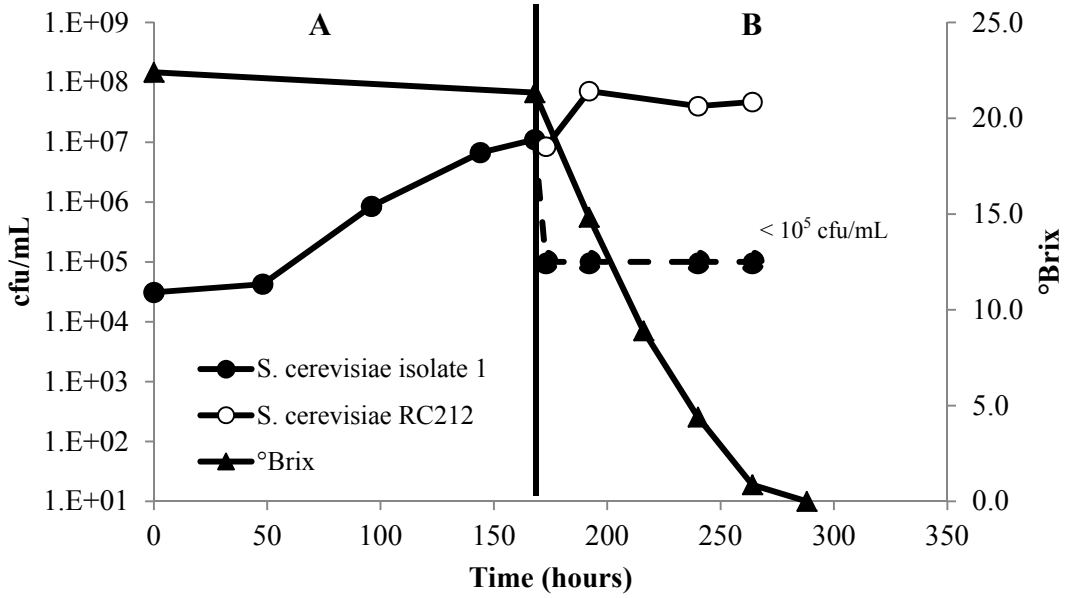

Figure 8 Growth of *Saccharomyces cerevisiae* RC212, *Saccharomyces cerevisiae* isolate 1, and °Brix during pre-fermentation cold maceration (A) and alcoholic fermentation (B) of Pinot noir grapes. Inoculation of *S. cerevisiae* RC212 occurred at end of cold maceration.

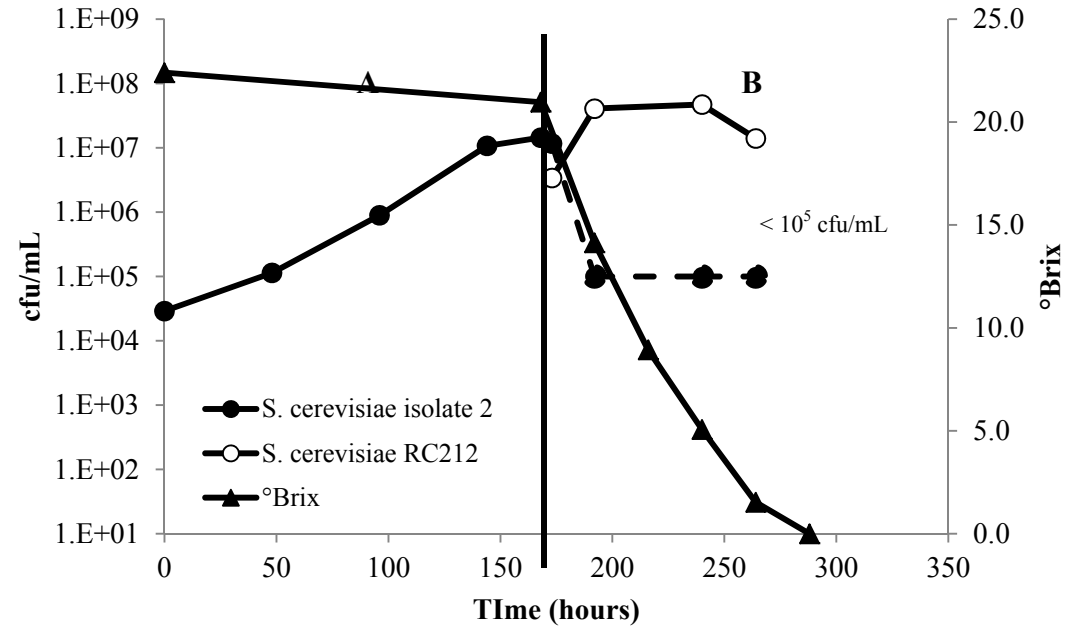

Figure 9 Growth of *Saccharomyces cerevisiae* RC212, *Saccharomyces cerevisiae* isolate 2, and °Brix during pre-fermentation cold maceration (A) and alcoholic fermentation (B) of Pinot noir grapes. Inoculation of *S. cerevisiae* RC212 occurred at end of cold maceration.

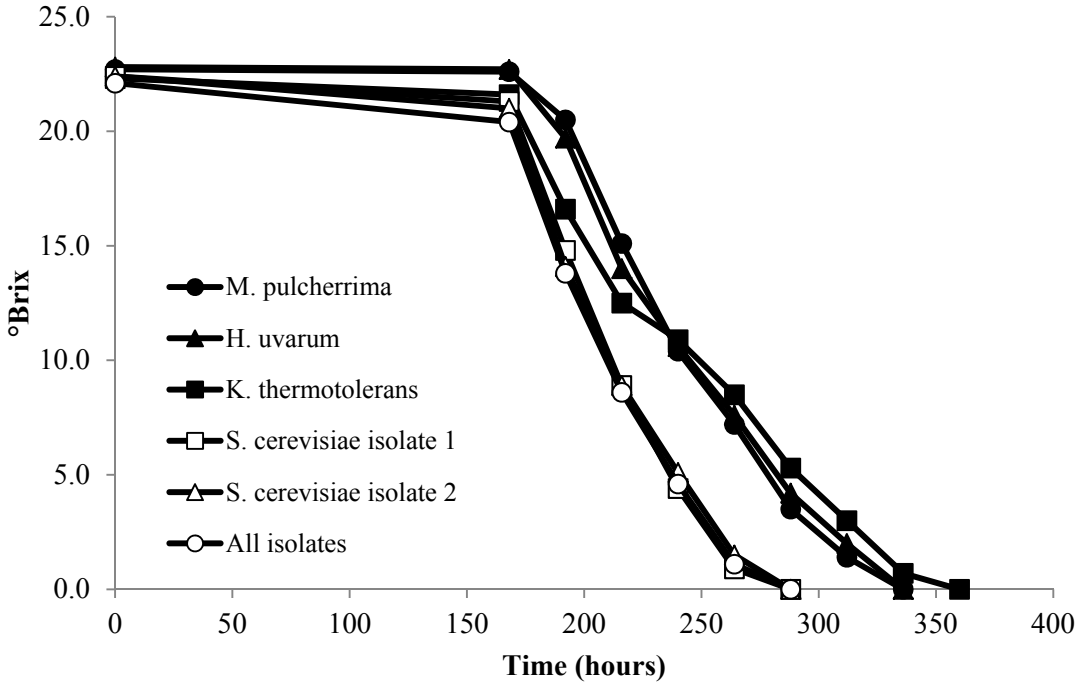

Figure 10 Change in °Brix during cold maceration (A) and alcoholic fermentation (B) of Pinot noir grapes inoculated with a single yeast isolates or combination of all isolates followed by inoculation of *S. cerevisiae* RC212 at completion of cold maceration.

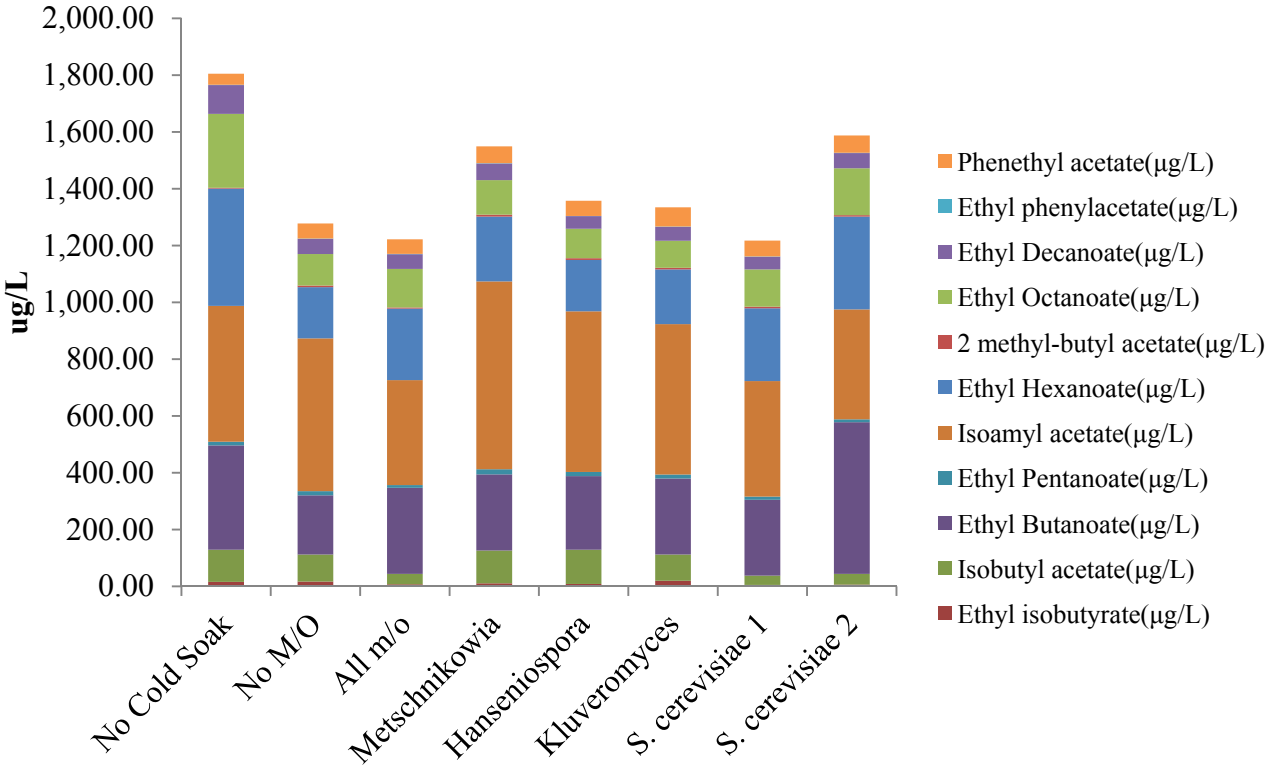

Figure 11 Concentration of esters in Pinot noir wines inoculated with various yeast isolates + *S. cerevisiae* RC212. M/O = microorganisms.

Table 4. Concentration of Esters in Pinot Noir Wines from Microscale Fermentations (n=3)

	No CS[1]	CS	CS + micoorganisms	CS + *M. pulcherrima*	CS + *H. uvarum*	CS + *K. thermotolerans*	CS + *S. cerevisiae* 1	CS + *S. cerevisiae* 2
Esters								
Ethyl acetate* (mg/L)	49.18 ± 0.88b[a]	44.34 ± 2.52b	43.97 ± 1.36b	44.27 ± 1.04b	64.12 ± 4.30a	43.60 ± 0.57b	44.36 ± 2.22b	41.99 ± 5.90b
Ethyl butanoate (µg/L)	367.58 ± 19.62b	208.68 ± 31.52c	303.32 ± 16.98bc	268.44 ± 16.33bc	258.61 ± 25.73c	267.73 ± 17.95bc	267.50 ± 24.72bc	533.87 ± 85.41a
Ethyl hexanoate (µg/L)	413.04 ± 12.35a	180.13 ± 21.43e	252.07 ± 18.04c	228.02 ± 10.72cd	180.63 ± 16.63e	192.91 ± 5.96de	256.03 ± 2.77c	326.67 ± 23.62b
Ethyl octanoate (µg/L)	260 ± 58.72a	111.07 ± 16.59cde	135.67 ± 15.79bc	121.68 ± 6.12cde	103.43 ± 8.37de	94.70 ± 4.33e	131.37 ± 6.25cd	164.95 ± 8.16b
Ethyl decanoate (µg/L)	101.26 ± 42.73a	53.78 ± 3.11bc	51.80 ± 6.71bc	58.48 ± 2.57b	44.53 ± 2.41c	49.00 ± 4.49bc	44.62 ± 8.22c	53.37 ± 1.87bc
Ethyl isobutyrate (µg/L)	16.05 ± 1.13a	16.84 ± 2.44a	7.46 ± 0.72bc	10.72 ± 1.70b	8.53 ± 0.68bc	20.06 ± 3.42a	5.06 ± 1.07c	6.43 ± 0.29bc
Isobutyl acetate (µg/L)	112.91 ± 5.41a	95.46 ± 11.78b	36.38 ± 2.47c	115.55 ± 6.99a	120.90 ± 3.36a	92.11 ± 0.97b	32.64 ± 2.70c	37.95 ± 6.92c
Isoamyl acetate (µg/L)	478.17 ± 28.42bc	538.11 ± 60.17b	369.31 ± 15.26d	661.60 ± 1.12a	565.70 ± 20.24ab	529.38 ± 9.85b	407.04 ± 39.06cd	386.64 ± 52.31cd
2-methyl-butyl acetate (µg/L)	3.20 ± 0.73c	5.53 ± 0.16ab	3.69 ± 0.57bc	6.55 ± 1.12a	6.34 ± 0.53a	5.67 ± 0.50ab	5.59 ± 0.51ab	5.53 ± 1.10ab
Ethyl phenylacetate (µg/L)	1.06 ± 0.02cd	1.24 ± 0.03ab	1.28 ± 0.05a	1.10 ± 0.11bcd	0.95 ± 0.07d	1.33 ± 0.02a	1.30 ± 0.07a	1.17 ± 0.06abc
Phenethyl acetate (µg/L)	39.22 ± 1.12d	52.92 ± 3.33bc	51.33 ± 1.37c	59.59 ± 6.38abc	53.60 ± 0.58bc	67.47 ± 2.88a	55.72 ± 1.29bc	60.77 ± 3.28ab

[1](CS) = pre-fermentation cold maceration

[a]Values within the same row followed by the same letter do not differ significantly ($p<0.05$)

*Aroma descriptions can be found in Appendix B

Table 5. Concentration of Alcohols and Volatile Acids in Pinot Noir Wines from Microscale Fermentations (n=3)

	No CS[1]	CS	CS + micororganisms	CS + *M. pulcherrima*	CS + *H. uvarum*	CS + *K. thermotolerans*	CS + *S. cerevisiae* 1	CS + *S. cerevisiae* 2
Alcohols								
Isobutyl alcohol (mg/L)	110.05 ± 6.96a[a]	109.35 ± 8.52a	50.78 ± 1.41de	93.80 ± 3.77b	89.12 ± 4.62b	75.05 ± 2.98c	40.12 ± 1.33e	60.05 ± 2.28d
Isoamy alcohol (mg/L)	178.01 ± 3.74a	162.61 ± 17.82ab	156.00 ± 4.47bcd	150.32 ± 2.99bcd	139.15 ± 4.33cd	136.62 ± 5.07d	147.33 ± 0.59bcd	158.94 ± 0.30abc
1-Hexanol (mg/L)	2.42 ± 0.17a	2.74 ± 0.44a	2.71 ± 0.27a	2.51 ± 0.12a	2.67 ± 0.09a	2.72 ± 0.09a	2.82 ± 0.28a	2.97 ± 0.33a
1-Octanol (µg/L)	19.64 ± 0.79a	17.25 ± 1.75bc	17.7 ± 0.58ab	17.23 ± 0.65bc	14.96 ± 0.30cd	13.38 ± 0.31d	17.96 ± 0.35ab	18.54 ± 0.74ab
1-Nonanol (µg/L)	9.44 ± 1.38a	6.15 ± 0.37bcd	6.87 ± 0.09bcd	5.52 ± 0.32d	5.80 ± 0.45cd	5.42 ± 0.13d	7.33 ± 0.09bc	7.55 ± 0.35b
Benzyl alcohol (µg/L)	711.23 ± 45.18b	905.7 ± 122.75a	769.76 ± 10.74ab	919.40 ± 46.16a	855.46 ± 12.75ab	783.02 ± 23.31ab	775.18 ± 101.40ab	871.30 ± 67.36ab
2-phenyl ethanol (mg/L)	34.59 ± 0.78ab	29.28 ± 1.65cd	37.42 ± 2.28a	28.21 ± 0.30de	25.16 ± 0.78e	32.12 ± 0.19bc	34.69 ± 1.41ab	37.05 ± 0.87a
Acids								
Hexanoic acid (µg/L)	299.83 ± 8.70a	155.49 ± 19.76de	206.65 ± 11.72b	161.17 ± 6.29cd	124.93 ± 6.21e	126.36 ± 7.52de	192.12 ± 17.39bc	265.65 ± 16.42a
Octanoic acid (µg/L)	5722.85 ± 201.51a	3079.55 ± 294.91de	3805.60 ± 43.35c	3164.51 ± 99.13de	2617.38 ± 123.90ef	2376.85 ± 119.38f	3278.95 ± 280.92cd	4641.35 ± 252.47b
Decanoic acid (µg/L)	466.47 ± 103.93a	347.57 ± 13.08ab	377.05 ± 16.32ab	352.59 ± 8.08ab	278.39 ± 9.66b	275.06 ± 16.66b	304.32 ± 95.62b	387.62 ± 10.69ab
Dodecanoic acid (µg/L)	66.54 ± 19.91a	67.05 ± 4.74a	68.58 ± 4.06a	76.28 ± 2.45a	66.37 ± 1.71a	60.99 ± 5.64a	56.97 ± 12.20a	75.96 ± 7.35a

[1](CS) = pre-fermentation cold maceration

[a]Values within the same row followed by the same letter do not differ significantly ($p<0.05$)

[*]Aroma descriptions can be found in Appendix B

Table 6. Concentration of Terpene Alcohols and C_{13}-Norisoprenoids in Pinot Noir Wines from Microscale Fermentations (n=3)

	No CS[1]	CS	CS + microorganisms	CS + *M. pulcherrima*	CS + *H. uvarum*	CS + *K. thermotolerans*	CS + *S. cerevisiae* 1	CS + *S. cerevisiae* 2
C_{13}-Norisoprenoids								
[*]β-Damascenone (µg/L)	3.51 ± 0.10a[a]	3.55 ± 0.17a	3.27 ± 0.26b	3.47 ± 0.07a	3.19 ± 0.19b	2.90 ± 0.09b	3.49 ± 0.22a	3.55 ± 0.16a
β-Ionone (µg/L)	1.36 ± 0.01a	1.34 ± 0.01a	1.34 ± 0.01a	1.35 ± 0.01a	1.34 ± 0.01a	1.35 ± 0.02a	1.35 ± 0.01a	1.35 ± 0.01a
Terpene Alcohols								
Linalool (µg/L)	11.16 ± 0.16ab	11.78 ± 0.82a	8.30 ± 0.36d	11.33 ± 0.41a	10.09 ± 00.04bc	11.49 ± 0.19a	8.22 ± 0.27d	9.46 ± 0.33c
α-Terpineol (µg/L)	9.16 ± 0.14cd	10.62 ± 0.46a	8.37 ± 0.32d	9.44 ± 0.33bc	8.90 ± 0.39cd	10.27 ± 0.36ab	7.27 ± 0.17e	8.85 ± 0.31cd
β-Citronellol (µg/L)	8.72 ± 0.07b	11.16 ± 0.45b	18.87 ± 3.42a	9.86 ± 1.28b	9.30 ± 0.56b	7.26 ± 0.43b	21.33 ± 1.87a	19.35 ± 0.61a
Nerol (µg/L)	2.39 ± 0.23ab	2.53 ± 0.28a	1.79 ± 0.28b	2.40 ± 0.25ab	2.32 ± 0.26ab	1.91 ± 0.20ab	2.05 ± 0.28ab	2.25 ± 0.20ab
Trans-Geraniol (µg/L)	20.20 ± 1.39a	20.43 ± 2.88a	11.34 ± 1.17e	19.54 ± 0.47ab	18.12 ± 0.88abc	16.31 ± 0.66bcd	12.85 ± 1.28de	15.26 ± 0.56cd

[1](CS) = pre-fermentation cold maceration

[a]Values within the same row followed by the same letter do not differ significantly ($p<0.05$)

[*]Aroma descriptions can be found in Appendix B

VI. Outside Presentations of Research:

Results from this research were presented at the 2011 OWRI Research Colloquium (August 25th) and the 2012 ASEV annual meeting in Portland (June 20th – 24th). Results have also been presented at Willamette Valley enology technical group meetings.
Presentations:

Harper, H., Qian, M., and Osborne, J.P. 2011. Impact of non-*Saccharomyces* yeast on wine quality: isolation of yeast with β-glycosidase activity. 2011 OWRI Research Colloquium (August 25th).

Zhou, Q., Osborne, J.P., and Qian, M.C. 2011. Non-*Saccharomyces* yeast on Pinot noir wine volatile composition. 2011 OWRI Research Colloquium (August 25th)

Harper, H., Zhou, Q., Qian, M., and Osborne, J.P. 2012. Impact of non-*Saccharomyces* yeast on wine quality: isolation of yeast with β-glycosidase activity. 2012 ASEV national meeting, Portland, OR, June 20th – 21st.

Zhou, Q., Osborne, J.P., and Qian, M.C. 2012. The effect of Non-*Saccharomyces* yeasts on Pinot noir wine volatile composition. 2012 ASEV national meeting, Portland, OR, June 20th – 21st.

VII. Research Success Statements:

The first year of this project identified non-*Saccharomyces* species present during cold maceration and alcoholic fermentation of Pinot noir grapes from two different vineyards. These isolates were then screened for β-glucosidase activity. The β-glucosidase activity of these isolates has been further characterized revealing that high sugar content repressed β-glucosidase activity for some non-*Saccharomyces* species but not others. Furthermore, at cold maceration temperatures (8-10°C) the β-glucosidase activity of many of the yeat isolates increased dramatically. β-glucosidase activity was not related to yeast growth and rather may be due to elevated production of the enzyme at cold temperatures. These results demonstrated that many of the yeast isolates maintain high β-glucosidase activity under the high sugar and low temperature conditions present during a cold maceration. Fermentations of Pinot noir with these yeast isolates allowed the specific contribution of yeast species to Pinot noir aroma to be determined. Results from the volatile aroma analysis demonstrated that the presence of *M. pulcherrima*, *K. thermotolerans*, *H. uvarum*, and cold tolerant *S. cerevisiae* isolates during pre-fermentation cold maceration impacted the volatile chemical composition of Pinot Noir wine. In particular, the composition of esters, higher alcohols, and volatile acids were affected. In addition, the composition of certain terpene alcohols was highly impacted by the presence of the two *S. cerevisiae* isolates during pre-fermentation cold maceration indicating the potential of this organism to have an impact in the release of bound volatile compounds important for wine aroma. In addition, because fermentations were performed with HHP treated grapes the fermentation of grapes where cold maceration occurred with no microorganisms present was able to be compared to fermentations where yeast have been inoculated during cold maceration. This allowed us to determine whether wine aroma changes due to cold maceration were primarily due to the physical and chemical processes occurring during the cold maceration or to the action of yeast. Results from this study demonstrate that cold maceration does changes in the volatile aroma composition of Pinot noir wine. Differences in volatile aroma composition were noted for wines that underwent cold maceration whether yeast were present or not. In addition, differences occurred between wine that had undergone cold maceration with or without yeast. In summary,

changes in the volatile composition of wines that undergo cold maceration are likely due to a combination yeast action and chemical changes that occur during the process.

Future work will include the analysis of the grape must after pre-fermentation cold maceration. Determination of the volatile profiles as well as the amino content of the will allow for a better understanding the specific impact of these yeast isolates before inoculation of alcoholic fermentation. In addition, sensory analysis of the wines produced will be performed to determine how the unique chemical profiles of the wines may impact the wine aroma. The ability of some of these isolates to impact the concentration of free terpenes will be investigated using a grape variety with higher concentrations of terpene alcohols such as Riesling. In addition, further studies should be performed inoculating these organisms in different combinations to determine what impact interactions between these species during pre-fermentation might have.

VIII. Fund Status:

A graduate student, Harper Hall, continues to work on his project. Funds have been spent for media and other consumables required for determining β-glucosidase activity as well as for conducting fermentations, running the high pressure unit, and microbiological and chemical analysis. The majority of remaining funds for year two are allocated for salary and supplies and will be expended by end of summer 2012.

VIV. References

Charoenchai, Fleet, G.H. Henschke, P.A. Todd, B.E.N. 1997. Screening of non-*Saccharomyces* wine yeasts for the presence of extracellular hydrolytic enzymes. *Aust. J. Grape Wine Res.* 3: 2-8.

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