PROGRESS REPORT 2011

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.

Yeast isolates from 2010 fermentations that demonstrated β-glucosidase activity on 4-MUG plates were 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, Hansenula,* and unknown isolate #10. 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 *Hansenula* had the highest β-glucosidase activity.

Non-*Saccharomyces* yeast 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 *Hansenula anomala* and unknown isolate #10 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 are currently be analyzed for volatile aromas (GC-MS) and will be assessed by a trained sensory panel.

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 4°C 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 8-10°C 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 °C and alcoholic fermentations proceeded without inoculation.

All samples were plated onto WL media and lysine media after appropriate dilutions and incubated @ 25 °C 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 4°C.

Non-*Saccharomyces* 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, Hansenula anomala,* and an unknown non-*Saccharomyces* isolate (suspected to be *Toralospora*). One set of fermenters was not inoculated with any yeast (control) while another set was inoculated with all five non-*Saccharomyces* yeast species. 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 non-*Saccharomyces* 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. Non-*Saccharomyces* species assessed were *Metschnikowia pulcherrim, Hanseniaspora uvarum, Kluveromyces thermotolerans, Hansenula anomala,* unknown #10 (suspected to be *Toralospora*), and a commercially available *Toralospora delbrueckii* (PRELUDE, Chr. Hansen).

V. Summary of Major Research Accomplishments and Results:

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 1). 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, Hansenula,* and unknown isolate #10 (Table 1).

Table 1. β-glucosidase activity after 48 hrs of various non-*Saccharomyces* yeast species in media (pH 3.50) containing different sugar concentrations at 25°C.

	β-glucosidase activity (nmole p-np/ mL cells/g cells)		
Yeast isolate	5 g/L (glucose)	200g/L (glucose/fructose)*	Change
Metschnikowia pulcherrima	82650	33200	-60%
Hanseniaspora uvarum	273000	571	-99%
Kluveromyces thermotolerans	20770	6030	-70%
Hansenula anomala	30794	15300	-50%
Unknown isolate #10	32305	18400	-43%
Torulospora delbrueckii	37446	9820	-74%

*media contained 100 g/L glucose and 100 g/L fructose

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, Hansenula,* and *Torulospora*. 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 2). 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 after 48 hrs of various non-*Saccharomyces* yeast species in media (pH 3.50, 100 g/L glucose and 100 g/L fructose) at 25°C and 8°C.

	β-glucosidase activity (nmole p-np/ mL cells/g cells)		
Yeast isolate	25°C	8°C	Change
Metschnikowia pulcherrima	33200	8710	-74%
Hanseniaspora uvarum	571	1320	+56%
Kluveromyces thermotolerans	6030	22600	+73%
Hansenula anomala	15300	62900	+76%
Unknown isolate #10	18400	52000	+65%
Torulospora delbrueckii	9820	47500	+79%

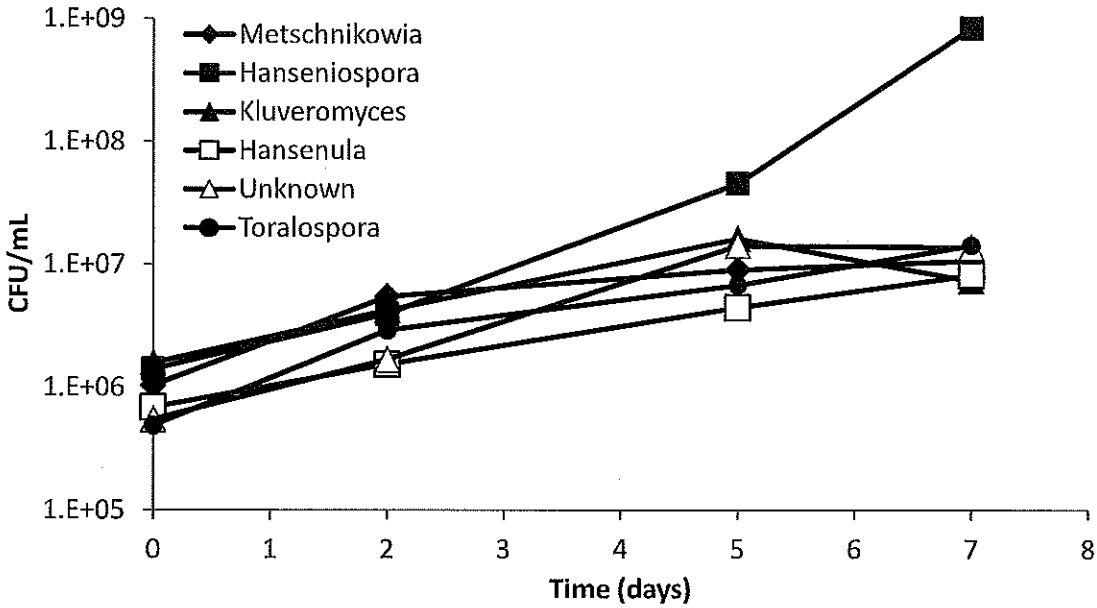

Figure 1. Growth of various non-*Saccharomyces* yeast species in media (pH 3.50, 20 °Brix) at 8°C.

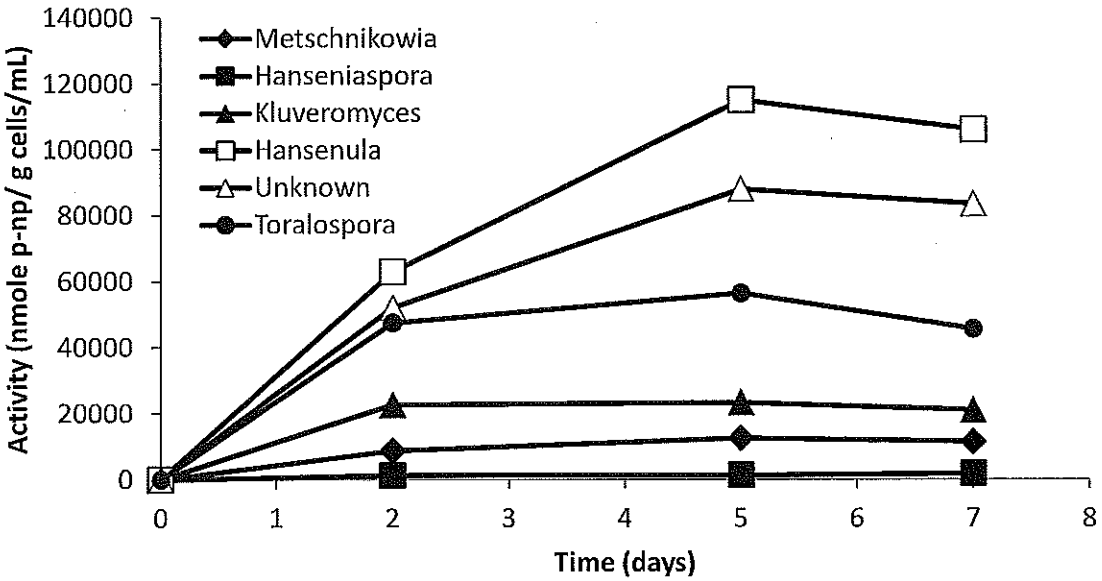

Figure 2. β-glucosidase activity of various non-*Saccharomyces* yeast species during growth in media (pH 3.50, 20 °Brix) at 8°C.

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 non-*Saccharomyces* yeast or a combination of all the non-*Saccharomyces* yeast. The non-*Saccharomyces* yeast 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 4) while *Hanseniaspora uvarum* grew from approximately 1 x 10^3 to 1 x 10^7 CFU/mL (Figure 5). When all the non-*Saccharomyces* isolates were inoculated together (Figure 7) the yeast grew well and reached comparable populations as observed when inoculated individually.

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 4 & 5) while *Kluveromyces thermotolerans* viable cell counts remained high throughout the alcoholic fermentation (Figure 4). In contrast, viable cell counts for *Hansenula anomala* and unknown isolate #10 declined below detectable limits after the inoculation of *S. cerevisiae* RC212 (Figure 5 & 6). The length of alcoholic fermentation varied depending on the non-*Saccharomyces* isolate present (Figure 8). Fermentations containing *Hanesnula,* unknown isolate #10, 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 8).

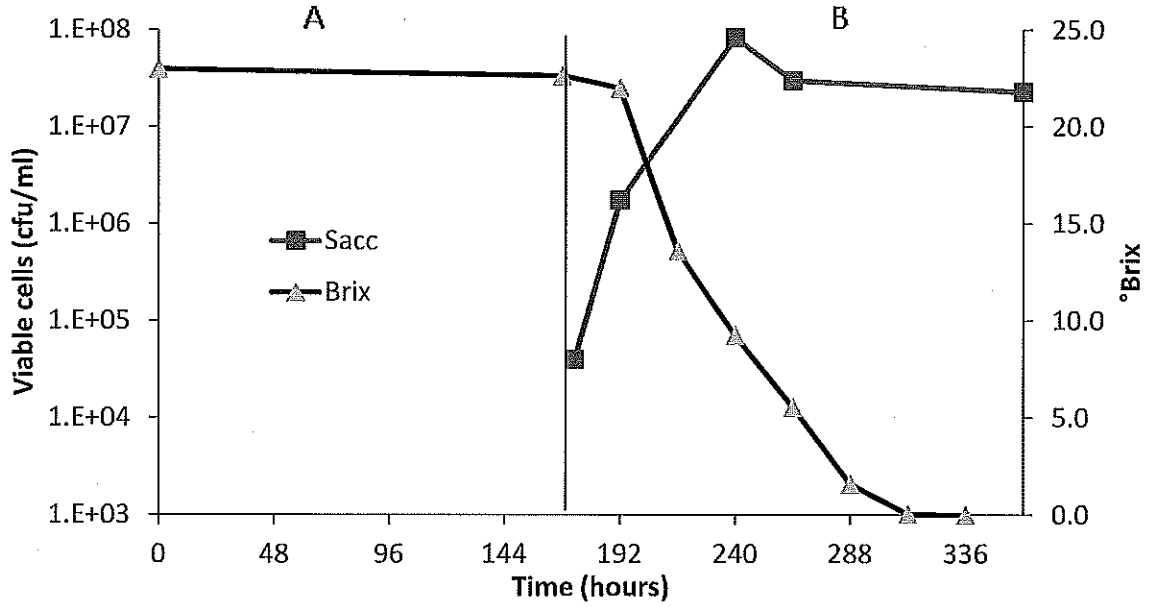

Figure 3. Growth of *Saccharomyces cerevisiae* RC212 and Brix during cold maceration (A) and alcoholic fermentation (B) of Pinot noir grapes. Inoculation of *S. cerevisiae* RC212 occurred at end of cold maceration.

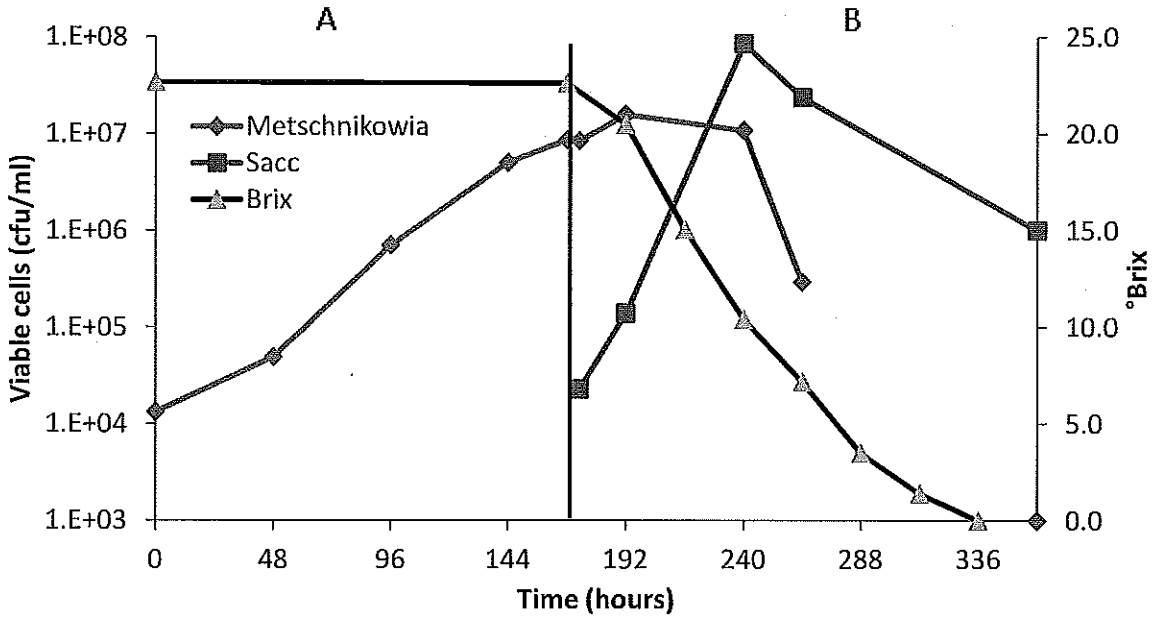

Figure 2. Growth of *Saccharomyces cerevisiae* RC212, *Metschnikowia pulcherrim*, and Brix during cold maceration (A) and alcoholic fermentation (B) of Pinot noir grapes. Inoculation of *S. cerevisiae* RC212 occurred at end of cold maceration.

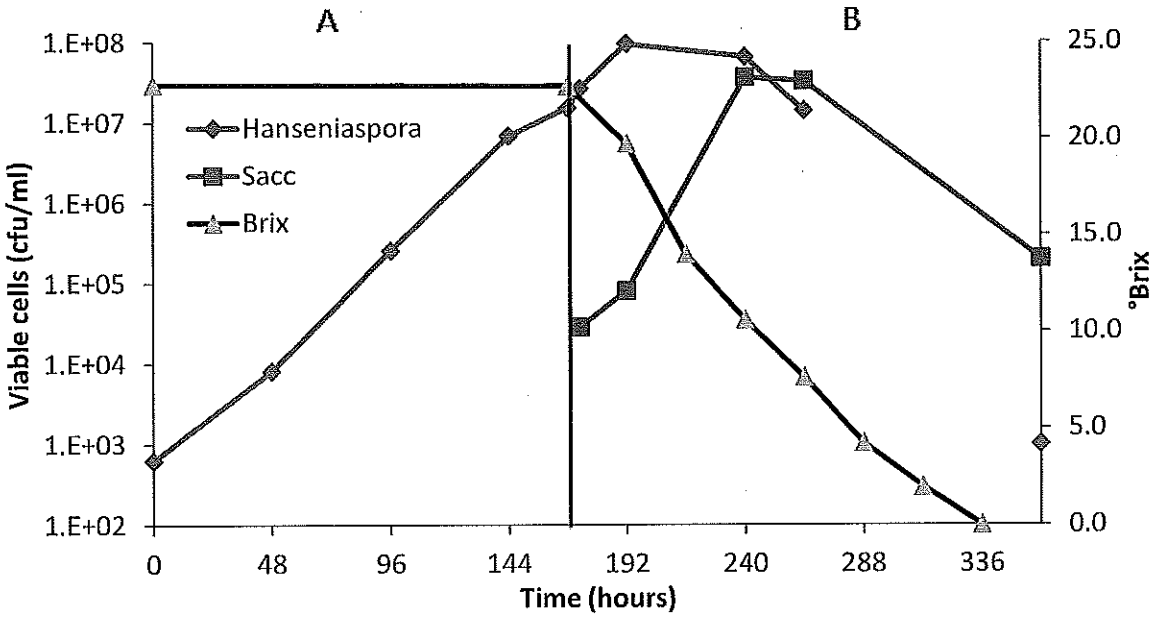

Figure 3. Growth of *Saccharomyces cerevisiae* RC212, *Hanseniaspora uvarum*, and Brix during cold maceration (A) and alcoholic fermentation (B) of Pinot noir grapes. Inoculation of *S. cerevisiae* RC212 occurred at end of cold maceration.

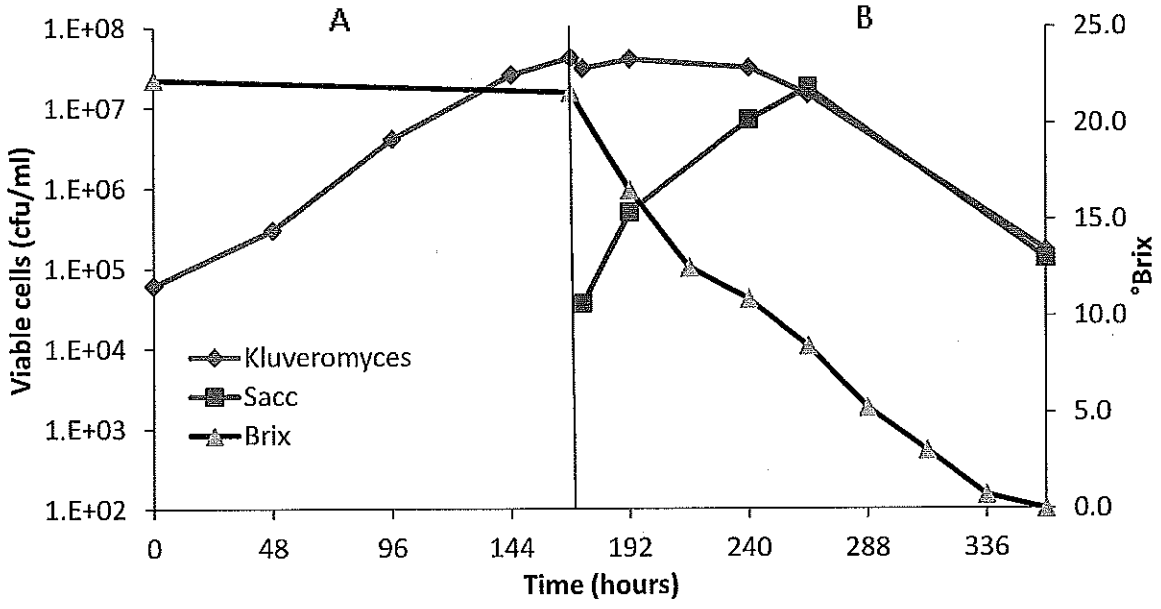

Figure 4. Growth of *Saccharomyces cerevisiae* RC212, *Kluveromyces thermotolerans*, and Brix during cold maceration (A) and alcoholic fermentation (B) of Pinot noir grapes. Inoculation of *S. cerevisiae* RC212 occurred at end of cold maceration.

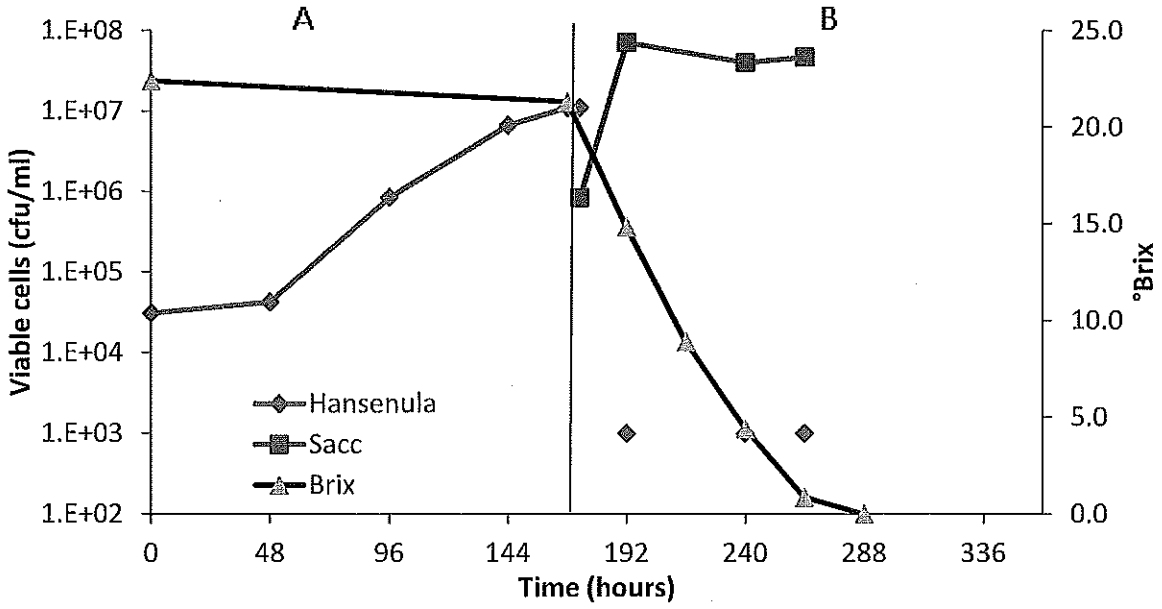

Figure 5. Growth of *Saccharomyces cerevisiae* RC212, *Hansenula anomala*, and Brix during cold maceration (A) and alcoholic fermentation (B) of Pinot noir grapes. Inoculation of *S. cerevisiae* RC212 occurred at end of cold maceration.

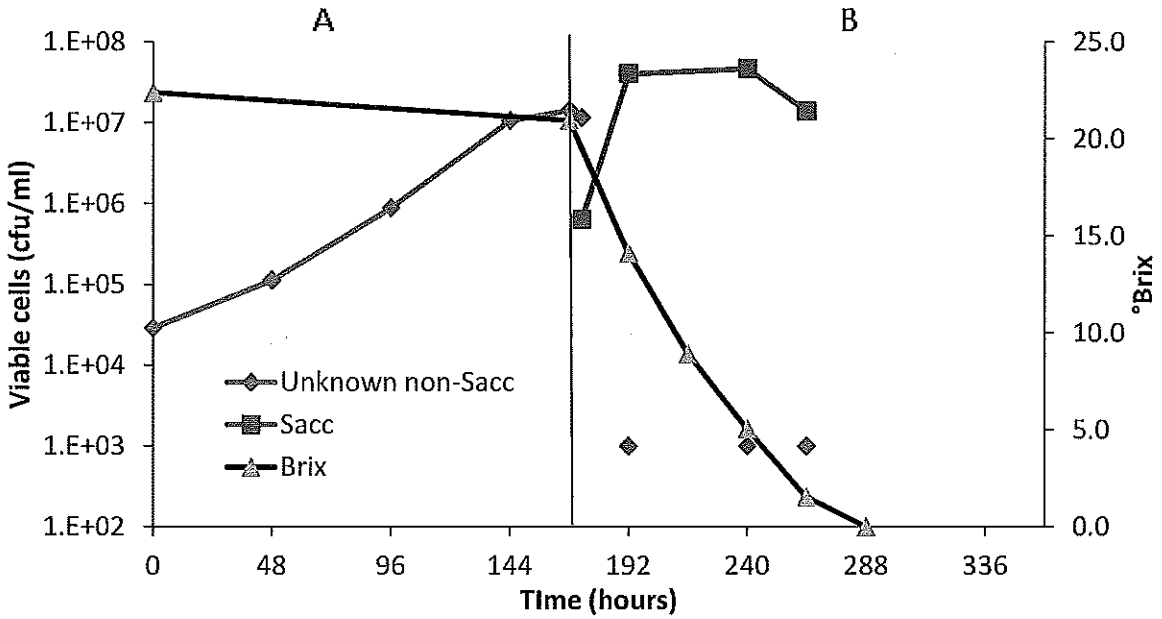

Figure 6. Growth of *Saccharomyces cerevisiae* RC212, unknown non-*Saccharomyces* #10, and Brix during cold maceration (A) and alcoholic fermentation (B) of Pinot noir grapes. Inoculation of *S. cerevisiae* RC212 occurred at end of cold maceration.

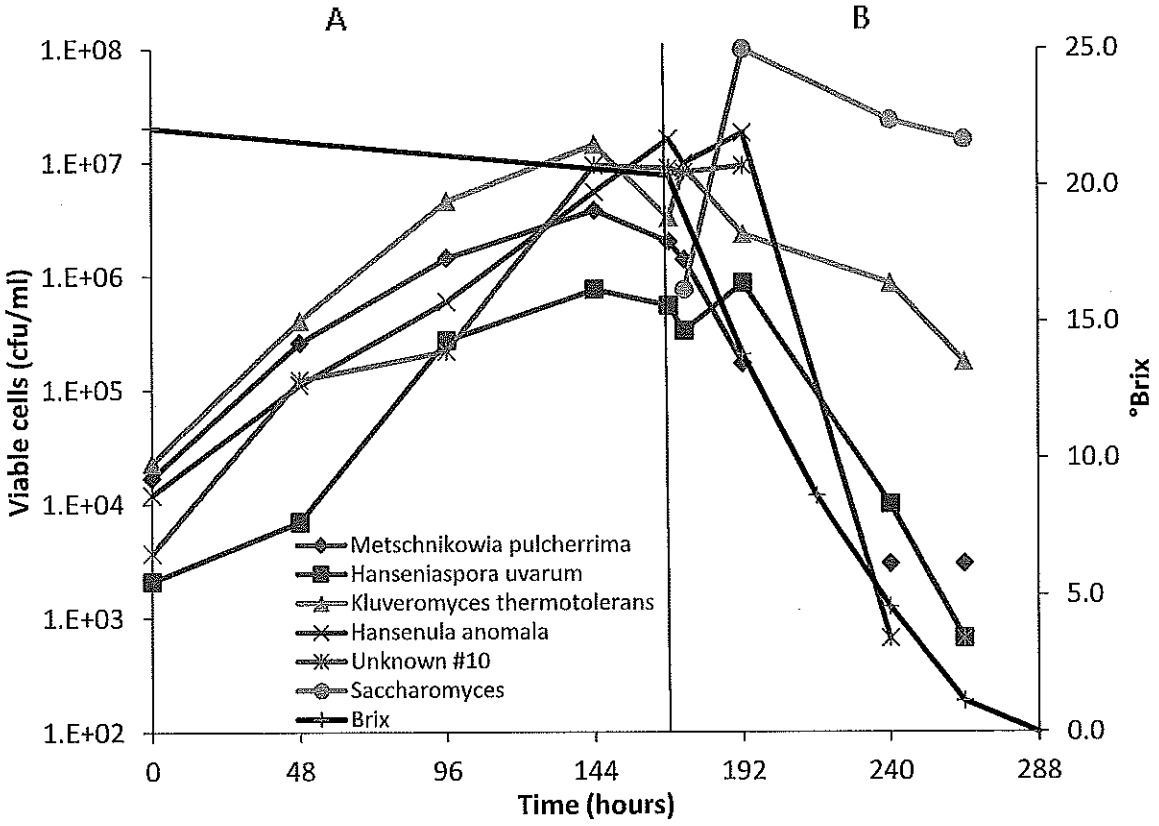

Figure 7. Growth of *Saccharomyces cerevisiae* RC212, *Metschnikowia pulcherrim*, *Hanseniaspora uvarum*, *Kluveromyces thermotolerans, Hansenula anomala,* unknown non-*Saccharomyces* #10, and Brix during cold maceration (A) and alcoholic fermentation (B) of Pinot noir grapes. Inoculation of *S. cerevisiae* RC212 occurred at end of cold maceration.

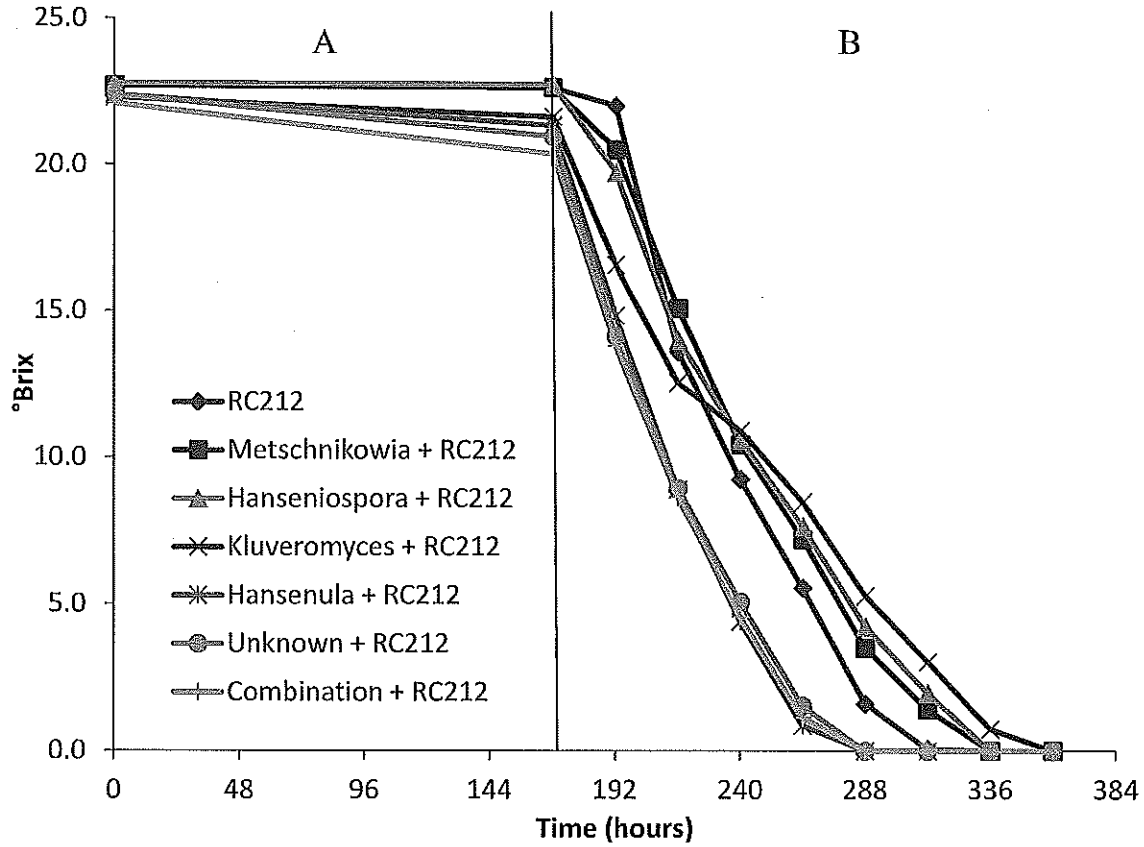

Figure 8. Change in Brix during cold maceration (A) and alcoholic fermentation (B) of Pinot noir grapes inoculated with a single non-*Saccharomyces* yeast species or combination of species followed by inoculation of *S. cerevisiae* RC212 at completion of cold maceration.

VI. Outside Presentations of Research:

Results from this research were presented at the 2011 OWRI Research Colloquium (August 25th) and will be presented at 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, (to be submitted).

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 (to be submitted).

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 non-*Saccharomyces* 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 non-*Saccharomyces* yeast maintain high β-glucosidase activity under the high sugar and low temperature conditions present during a cold maceration. Current experiments will help determine whether these yeast have β-glucosidase activity during cold maceration of Pinot noir grapes and the impact this has on Pinot noir wine aromas. Because fermentations have been undertaken in HHP treated grapes the specific contribution of the individual non-*Saccharomyces* yeast can be determined. In addition, fermentation of grapes where cold maceration occurred with no microorganisms present will be compared to fermentations where non-*Saccharomyces* yeast have been inoculated during cold maceration. This will determine whether wine aroma changes due to cold maceration are primarily due to the physical and chemical processes occurring during the cold maceration or are due to the action of non-*Saccharomyces* yeast.

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.

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.

Pallmann, C.L. Brown, J.A. Olineka, T.L. Cocolin, L. Mills, D.A. Bisson, L.F. 2001. Use of WL medium to profile native flora fermentations. *Am. J. Enol. Vitic.* 52: 198-203.