AN ABSTRACT OF THE THESIS OF

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Title: Impact of Yeast Present During Pre-Fermentation Cold Maceration on Pinot Noir Wine Aroma

Abstract approved:

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This research investigated yeast populations and diversity during pre-fermentation cold maceration and alcoholic fermentation of *Vitis vinifera* L. cv. Pinot noir grapes from a commercial vineyard (Dayton, OR). Fermentations were conducted at the Oregon State University research winery in 100 L tanks while grapes from the same vineyard lot were fermented at a commercial winery. Samples were taken daily during pre-fermentation maceration (9°C) and alcoholic fermentation (27°C) and plated on WL and lysine media to determine *Saccharomyces* and non-*Saccharomyces* populations and diversity. Total non-*Saccharomyces* populations increased from 1 x 10^3 cfu/mL to 1 x 10^5 cfu/mL during pre-fermentation cold maceration and reached a maximum of 1 x 10^7 cfu/mL during alcoholic fermentation. Thirteen distinct yeast species were tentatively identified based on appearance on WL media and were initially screened for β-glucosidase activity using 4-methyllumbelliferyl-β-D-gluconopyranoside (4-MUG) plates. The identity of the isolates screening positive for β-glucosidase activity was determined by sequence analysis of the D1/D2 domain of the 26S rDNA gene. The five isolates identified were *Metschnikowia pulcherrima*, *Hanseniaspora uvarum*, *Kluveromyces thermotolerans*, and two *Saccharomyces*

cerevisiae isolates. β-glucosidase activity was further characterized and quantified using a liquid media representing grape must conditions (pH 3.5, 20° Brix) at two temperatures (25°C and 8°C). While increasing sugar concentration suppressed the β-glucosidase activity of *H. uvarum* (-99%), β-glucosidase activity still remained relatively high for *M. pulcherrima*, *S. cerevisiae* isolate 1, and *S. cerevisiae* isolate 2. At 8°C, β-glucosidase activity was reduced for *M. pulcherrima* compared to activity at 25°C, but activity increased for *K. thermotolerans*, *S. cerevisiae* isolate 1, and *S. cerevisiae* isolate 2.

The yeast isolates possessing β-glucosidase activity were used in fermentations of *Vitis vinifera* L. cv. Pinot Noir grapes. The grapes were treated with high hydrostatic pressure (HHP) to inactivate naturally occurring yeast and bacteria. All yeast isolates grew during pre-fermentation cold maceration (7 days at 9°C) and populations increased 3 to 4 logs. Following pre-fermentation cold maceration, all ferments were warmed to 27°C and inoculated with *S. cerevisiae* RC212. Alcoholic fermentations were all complete within eight days and after pressing wines were analyzed for volatile aroma compounds by SPME-GC-MS. The presence of different yeast isolates during pre-fermentation cold maceration resulted in wines with unique aroma profiles. Ethyl ester concentrations were highest in the wine 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 yeast isolates demonstrating β-glucosidase did not affect the concentration of β-damascenone or β-ionone. 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 over wines that did not undergo a pre-fermentation cold maceration.

Impact of Yeast Present During Pre-Fermentation Cold Maceration on Pinot Noir Wine Aroma

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CONTRIBUTION OF AUTHORS

Dr. Michael Qian and Qin Zhou of OSU collaborated on the project and performed the volatile aroma analysis. Dr. Alan Bakalinsky and Jun Ding of OSU provided guidance and assistance in the DNA sequence analysis. Kate Payne-Brown of Archery Summit Winery collaborated on the project, as did Virginia Usher of OSU.

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Impact of yeast present during pre-fermentation cold maceration on Pinot noir wine aroma

LITERATURE REVIEW

Wine Aroma and Quality

Wine quality, while not easy to define, is based on wine evaluation by four senses: sight, smell, taste, and touch (Jackson and Lombard 1993, Sweigers et al. 2005). The perception of these factors will vary from taster to taster and any determinations made about wine based on these characteristics will be subjective (Jackson and Lombard 1993). However, the organoleptic quality of wine including the appearance, aroma, flavor, and mouthfeel are used by consumers to make decisions about the value of a wine and are therefore very important (Sweiger et al. 2005).

Wine aroma is one of the most important components of wine quality (Swiegers et al. 2005, Swangkeaw et al. 2001). It is extremely complex with more than 800 compounds having been identified as contributing to wine aroma (Mendes-Pinto 2009). This complex matrix consists of volatile compounds that are derived from the grape, microbial flora, and aging (Swiegers et al. 2005, Styger et al. 2011). The types and concentrations of volatile compounds can differ greatly depending on the grape variety, viticultural practices, and winemaking procedures (Reynolds and Wardle 1997, Esti and Tamborra 2006, Piñeiro et al. 2005). Winemaking procedures differ based on the type of wine being made and can be used to manipulate the final organoleptic quality of the wine (Jackson 2000). In general, all wine grapes are destemmed following harvest (Jackson 2000). White wine varieties are pressed following destemming to extract the juice and typically have little to no skin contact prior to the

initiation of alcoholic fermentation (Jackson 2000). On the other hand, red wine varieties are kept in contact with the skin during fermentation (Jackson 2000). For some red wine varieties, such as Pinot noir, additional skin contact is desired and can be performed at cold temperatures prior to alcoholic fermentation (Jackson 2000). This procedure is called pre-fermentation maceration although it is more commonly referred to as a 'cold soak'. The grape must is generally held at < 10°C for 1-14 days so as to prevent the growth of *Saccharomyces cerevisiae* and delay the beginning of alcoholic fermentation. Winemakers choose to employ this process for two major reasons: to improve the color of the wine and/or to modify the flavor and aroma of the wine (Zoecklein, 1995). Improvement in color is thought to be due to increased extraction of the water-soluble anthocyanin pigments while changes in flavor and aroma are less well understood although may be due to extraction of grape derived aroma compounds or the action of cold-tolerant yeast (Reynolds and Wardle 1997, Esti and Tamborra 2006, Piñeiro et al. 2005).

Yeast-Derived Aroma

The major contributors to wine aroma are derived from microbial fermentations, specifically the yeast present during fermentation (Fleet 2008, Varela et al. 2009, Styger et al. 2011). Yeast are responsible for synthesis of flavor active primary and secondary metabolites, the biotransformation of grape must constituents into flavor-active compounds, and the production of enzymes that can transform odorless compounds present in grapes into aroma-active compounds (Fleet 2008, Varela et al. 2009, Styger et al. 2011, Swangkeaw et al. 2011).

The aroma compounds present in the highest concentrations in wine are the products and by-products of yeast fermentation. These include ethanol, glycerol, acetic acid, and acetaldehyde (Millán and Ortega 1998, Swiegers et al. 2005). Ethanol is formed from the catabolism of

glucose and fructose and its final concentration depends on the level of these sugars present in the grape berry (Ebeler 2001). Compared to other volatile components of wine, ethanol is present in relatively high amounts and therefore can have an impact on wine aroma (Stygers et al. 2011). For example, ethanol can enhance aroma characteristics such as fruitiness or floral notes (Styger et al. 2011). However, in excess ethanol can have a negative impact, masking aroma and producing a perceived hotness in wine (Swiegers et al. 2005, Robinson et al. 2008, Jones et al. 2009).

Acetaldehyde is formed as a precursor to ethanol and can also have an important impact on wine aroma (Pronk et al. 1996, Styger et al. 2011). At low levels it can lend a pleasant fruitiness to wine, but at high levels (> 100 mg/L) it can result in aromas of bruised apples and green grass or can impart a nutty character (Schreier 1979). The production of acetaldehyde is dependent on yeast strain, fermentation conditions, and is also an indication of wine oxidation (Millán and Ortega 1998, Swiegers et al. 2005, Styger et al. 2011). As a result, acetaldehyde levels change throughout fermentation and during aging, with acetaldehyde production reaching a peak when carbon dissimilation is at a maximum and then decreasing at the end of fermentation (Millán and Ortega 1998, Swiegers et al. 2005, Styger et al. 2011).

Acetic acid can also be produced by yeast during alcoholic fermentation with concentrations produced varying by strain and fermentative conditions although they are typically low (< 500mg/L) (Millán and Ortega 1998, Swiegers et al. 2005). At higher levels (> 1000 mg/L), acetic acid can have a negative impact on aroma resulting in a vinegar character (Corison et al. 1979). However, high levels are usually the result of metabolism of ethanol by aerobic bacteria, such as *Acetobacter* (Swiegers et al. 2005).

An additional product of alcoholic fermentation that can impact wine quality is glycerol. While considered an important factor in mouthfeel, the impact of glycerol on the perceived aroma of wine is still under investigation (Styger et al. 2011). In a study involving white wine and model white wine, glycerol was found to have no impact on the volatile aroma profile (Lubbers et al. 2001). However Jones et al. (2008) reported that glycerol enhanced volatiles in the presence of 11% ethanol compared to 13% ethanol suggesting a relationship between these two compounds and the aroma of a model white wine. More investigation is necessary to determine the impact glycerol may have on wine aroma.

Aside from the major yeast products of alcoholic fermentation, yeast can produce secondary metabolites that have a significant impact on wine aroma. These include higher alcohols and their associated esters and volatile acids (Styger et al. 2011). These compounds are derived, in part, from amino acids present in the grape must and are synthesized by the yeast during fermentation (Sweigers and Pretorius 2005, Styger et al. 2011). Their production is therefore dependent not only on the yeast strain and fermentation kinetics, but also on the nitrogen composition of the grape must (Swiegers and Pretorius 2005, Styger et al. 2011).

Higher alcohols (fusel alcohols) are synthesized via the Ehrlich pathway. This involves the degradation of the branched-chain amino acids leucine, isoleucine, and valine to branched-chain higher alcohols (Sweigers et al. 2005, Styger et al. 2011). The first step in the pathway is a transamination of α-ketoglutarate to form an α-keto acid and glutamate. The α-keto acid is then decarboxylated into an aldehyde. The final step involves the NADH-dependent reduction of the aldehyde to the corresponding higher alcohol. The aldehyde can also be oxidized in a NAD^+-dependent reaction into a volatile carboxylic acid (Derrick and Large 1993, Dickinson Norte 1993). The main higher alcohols present in wine include, 2- and 3-methyl butanol, 2-methyl propanol and propanol (Ebeler 2001) with the total concentration of higher alcohols in

wines ranging from 45-490 mg/L (Styger et al. 2011). Higher alcohols have been reported to have a positive impact on wine aroma at concentrations below 300 mg/L, imparting fruity characteristics and a negative impact at concentrations exceeding 400 mg/L that result in harsh or pungent aromas (Sweigers et al. 2005).

Esters represent the secondary metabolites with the greatest impact on wine aroma and their presence contributes to the overall fruity aroma of the wine. The esters with the greatest impact on wine aroma are ethyl acetate (fruity, nail polish), 2-phenylethyl acetate (honey, fruity, flowery, rose), isoamyl acetate (pear, banana), isobutyl acetate (banana), and ethyl caproate (apple) (Sweigers and Pretorius 2005, Sweigers et al. 2005, Styger et al. 2011). Yeast form esters by an enzyme-catalyzed reaction that is linked to lipid and acetyl-CoA metabolism (Sweigers et al. 2005, Styger et al. 2011). The two-stage formation requires an alcohol, a fatty acid, coenzyme A (CoA), and an ester-synthesizing enzyme (Sweigers and Pretorius 2005). First, the acid is combined with a coenzyme donor to activate it, and then is reacted with the alcohol to form an ester (Park et al. 2009). In the formation of ethyl esters, the coenzyme donor is acyl-CoA, the alcohol is ethanol, and the acid is derived from a medium-chain fatty acid (Malcorps and Dufour 1992). In the formation of acetate or branch chained esters, the coenzyme donor is acetyl-CoA and the alcohol is derived from the degradation of amino acids, carbohydrates, and lipids (Miller et al. 2007).

The formation of esters is impacted by yeast strain, fermentation conditions, and nutrient availability (Herraiz and Ough 1993, Sweigers et al. 2005, Miller et al. 2007). For example, Miller et al. (2007) found that ester production increased for one strain of *S. cerevisiae* in fermentation of Chardonnay juice that had been supplemented with ammonium over juice that had been supplemented with amino acids. However, different nitrogen sources did not have any significant impact on ester production for the other two strains involved in the study. In

addition to fermentation conditions and nutrient availability, certain grape varieties have been shown to have unique ester profiles (Sweigers et al. 2005). For example, Pinot noir wine typically contains ethyl anthranilate (sweet-fruity, grape-like), ethyl cinnamate (cinnamon-like, sweet balsamic, sweet-fruity, plum, cherry), 2,3-dihydrocinnamate, and methyl anthranilate (Moio and Etievant 1995, Sweigers et al. 2005). These esters contribute to the characteristic plum, cherry, strawberry, raspberry, black currant and blackberry aromas characteristic of Pinot noir wines (Moio and Etievant 1995, Sweigers et al. 2005).

In addition to producing compounds with a positive impact on wine aroma, yeast also produce several compounds that are considered to negatively impact wine aroma. This includes the sulfur containing compounds hydrogen sulfide, dimethyl disulfide, and mercaptans (Sweigers and Pretorius 2005). These compounds impart a rotten egg, cabbage, sulfurous, garlic, and onion character to wine (Sweigers et al. 2005). Yeast are involved in either the production or development of these sulfur containing compounds via the degradation of sulfur-containing amino acids or the degradation of sulfur-containing pesticides (Sweigers et al. 2005).

Hydrogen sulfide production by yeast is one of the most common problems encountered in winemaking. The sensory threshold of this highly volatile compound is very low (50-80 μg/L) and it contributes the characteristic rotten egg aroma to wine (Sweigers et al. 2005). Sulfide is produced by the yeast as an intermediate in the reduction of inorganic sulfur compounds for the biosynthesis of organic sulfur compounds (Sweigers et al. 2005). Yeast need organic sulfur compounds such as cysteine, methionine, *S*-adenosyl methionine, and glutathione for growth and must biosynthesize them using inorganic forms of sulfur if they are not present (Sweigers et al. 2005). In *S. cerevisiae*, sulfide is formed as an intermediate in the Sulfate Reduction Sequence (SRS) (Sweigers et al. 2005). First, sulfate is transported into the cell and then reduced to sulfide in a series of steps catalyzed by ATP-sulfurylase and sulfite reductase.

The sulfide is then combined with either *O*-acetylserine to form cysteine or *O*-acetylhomoserine to form homocysteine (Sweigers et al. 2005). Sulfides can accumulate in the cell and are released in the form of hydrogen sulfide if there are insufficient levels of precursors (*O*-acetylserine and *O*-acetylhomoserine) present (Sweigers et al. 2005). The production of hydrogen sulfide varies with yeast strain and can be impacted by factors other than nitrogen deficiencies in the must, such as fermentation conditions (Mendes Ferreira et al. 2002, Ugliano et al. 2011).

Grape and Yeast Derived Aroma

Although yeast may produce a large portion of the aroma compounds present in a wine, compounds found in the grape also play an important role in wine aroma and in particular unique cultivar specific flavors and aromas (Reynolds and Wardle 1997, Piñeiro et al. 2005). For example, the presence of monoterpene alcohols (linalool, geraniol, nerol, and citronellol) is responsible for the cultivar specific floral, fruity, spicy, and vegetal aroma of Muscat wines (Ebeler 2001, Fleet 2008, Styger et al. 2011). The concentrations of these grape derived aroma compounds can be impacted by viticulture practices, climate conditions, soil, and maturity at harvest (Reynolds and Wardle 1997, Piñeiro et al. 2005, Mendes-Pinto 2009, Styger et al. 2011). In addition, the aroma compounds found in *Vitis vinifera* are present in either a free volatile form or a glycosidically bound non-volatile form (Williams et al. 1981,Sefton et al. 1993, Takatoshi et al. 1998). The type and concentration of the free volatile forms and the release of the bound forms during winemaking play a major role in determining the intensity of the cultivar specific aroma and the quality of the wine.

An example of the importance of grape derived aroma compounds to wine quality is the concentration of thiols in Sauvignon blanc wines. The thiol compounds 4-mercapto-4-methylpentan-2-one (4MMP), 3-mercaptohexan-1-ol (3MH), and 3-mercaptohexyl acetate

(3MHA) contribute grapefruit, citrus peel, and passion fruit aromas (Takatoshi et al. 2000, Styger 2011) and are present in the grape as non-volatile, cysteine-bound compounds (Takatoshi et al. 1998). However, during fermentation, yeast cleave the cysteine from the thiol rendering it volatile (Takatoshi et al. 1998, Swiegers et al. 2005, Styger et al. 2011).

C_{13}-norisoprenoids are also present in the grape in free and glycosidically bound forms (Sefton et al. 1993). The C_{13}-norisoprenoids are derived from the oxidation of carotenoids in grapes as they ripen (Ebeler 2001, Baumes et al. 2002, Fang and Qian 2006, Styger et al. 2011). Based on their structure, C_{13}-norisoprenoids are divided into two groups, the megastigmanes and the non-megastigmanes (Sweigers et al. 2005). The aromas of megastigmanes are complex and include the C_{13}-norisoprenoids β-damascenone and β-ionone (Sweigers et al. 2005). These compounds are important contributors to wine aroma due to their high concentration in wine and low sensory threshold (Sweigers et al. 2005, Fang and Qian 2006). They typically contribute aromas of exotic flowers and heavy fruit (β-damascenone) and berry or violet (β-ionone) (Fang and Qian 2006, Styger et al. 2011) and are thought to be important to the aroma of Pinot noir wines (Fang and Qian 2006).

An additional class of aroma compounds present in the grape in either free or glycosidically bound forms is the monoterpenes (Williams et al.1981). Linalool, geraniol, and nerol usually represent a majority of the free forms (Mateo and Jiménez 2000). The ratio of free to bound forms of monoterpenes changes as berries mature. In general, a greater proportion of bound monoterpenes is found in mature berries (Mateo and Jiménez 2000, Fang and Qian 2006, Vilanova et al. 2012). Monoterpene aroma precursors are typically present in mono or disaccharide complexes (Williams et al. 1982). For example, the aglycone can be directly bound to β-D-glucopyranoside or bound to a disaccharide such as α-L-rhamnopyranosyl- β-D-glucopyranoside, α-L-arabinofuranosyl- β-D-glucopyranoside, or β-D-apiofuranosyl- β-D-

glucopyranoside (Mateo and Jiménez 2000, Sweigers et al. 2005). Monoterpene alcohols contribute floral, fruity, spicy, and vegetal aromas to wines (Ebeler 2001, Fleet 2008, Styger et al. 2011).

The release of C_{13}-norisoprenoid and monoterpene aroma precursors can occur via acid or enzymatic hydrolysis (Williams et al. 1981, Sefton et al. 1993). Acid hydrolysis involves the cleavage of the ester linkage between the glucose and the aglycone, which results in a reactive carbocation that may undergo rearrangement (Williams et al. 1981, Sefton et al. 1993, Mateo and Jiménez 2000). For example, Williams et al. (1982) found that while the acid hydrolysis of linalyl β-D-glucoside resulted in the production of linalool, geraniol, (Z)-ocimene, α-terpinene, and myrcene were also formed. Acid hydrolysis is thought to contribute to the free fraction of aroma precursors derived from the grape, and may occur overtime with wine aging (Williams et al. 1982, Gunata et al. 1986). Under enzymatic hydrolysis, cleavage occurs at the glycosidic linkage, retaining the chemical structure of the aglycone and retaining the relative proportions aroma compounds that were present as precursor compounds (Williams et al. 1981, Williams et al. 1982, Sefton et al. 1993, Mateo and Jiménez 2000). Enzymatic hydrolysis can occur in two steps. First, an α-L-rhamnosidase and an α-L-arabinofuranosidase or a β-D-apiofuranosidase cleave the 1, 6-glycosidic linkage, followed by the cleavage of the β-D-glucoside from the monoterpene or norisoprenoid by a β-glucosidase (Gunata et al. 1988).

Several sources for β-glucosidases have been examined in the grape and wine system that are thought to impact the release of bound aroma compounds. A number of studies have reported that the source of the enzyme, as well as the structure of the aglycone, can determine the efficiency of the enzyme in hydrolyzing bound aroma precursors (McMahon et al. 1999, Sweigers et al. 2005). In addition, certain criteria have been formed to evaluate the impact and usefulness of β-glucosidases based on their activity in a grape or wine system (Sweigers et al.

2005). First, the enzyme should have a high hydrolytic activity towards grape-derived aglycones. Second, the enzyme should be active at wine pH (2.5-3.8), resistant to glucose inhibition, and stable in high ethanol environments (Sweigers et al. 2005). A discussion of the sources of β-glucosidase in the winemaking process follows.

Grapes (*Vitis vinifera*) possess an endogenous β-glucosidase (Aryan et al. 1987, Mateo and Jiménez 2000). However, in conditions similar to the grape must or wine environment (low pH, high glucose content, or high ethanol content) the enzyme has been found to have little to no activity (Aryan et al. 1987, Mateo and Jiménez 2000). In addition, β-glucosidases isolated from grapes have low hydrolytic activity on monoglucosides of tertiary terpene alcohols such as linalool (Aryan et al. 1987, Mateo and Jiménez 2000). Based on this, it seems unlikely that *V. vinifera* would be a source for enzyme activity that can significantly impact wine aroma (Aryan et al. 1987, Mateo and Jiménez 2000, Sweigers et al. 2005).

The β-glucosidase activity of *S. cerevisiae* has also been investigated as a potential source for releasing bound aroma precursors. Commercial strains of *S. cerevisiae* have been reported to possess β-glucosidase activity, however the amount of activity, as well as the conditions under which the enzyme is active can vary by yeast strain (Delcroix et al. 1994, McMahon et al. 1999, Hernández et al. 2003, Ugliano et al. 2006). For example, Delcroix et al. (1994) found that the activity of β-glucosidase isolated from a commercial *S. cerevisiae* was severely inhibited by pH levels found in wine (2.8-3.8), but only slightly inhibited by glucose (100 g/L) and ethanol (15% v/v) levels typically encountered in wine. The hydrolytic activity of these β-glucosidases towards aroma precursors in Muscat juice was found to be low (Delcroix et al. 1994). On the other hand, Ugliano et al. (2006) reported the ability of two commercial strains of *S. cerevisiae* and one strain of *S. bayanus* to release high levels of volatiles (monoterpene alcohols, terpene oxides, terpene diols, C_{13}-norisoprenoid 3-oxo-α-ionol) in the fermentation

of a synthetic medium containing white Frontignac grape glycosidic precursors. The authors also found that of the monoterpene alcohols measured (linalool, gernaiol, nerol, α-terpineol) the highest decline in precursor levels following fermentation was seen for linalool, indicating a potential pattern of substrate specificity for the *Saccharoymces* strains used in this study (Ugliano et al. 2006). In order to fully understand the impact *S. cerevisiae* can have on the release of bound aroma precursors, more research is necessary. The activity of *S. cerevisiae* appears to vary with strains as well as the glycosidic pool (Delcroix et al. 1994, McMahon et al. 1999, Hernández et al. 2003, Ugliano et al. 2006). Current research focuses on the use of white wine glycosides, and little work has been done using red grape derived precursors (Delcroix et al. 1994, McMahon et al. 1999, Hernández et al. 2003, Ugliano et al. 2006, Ugliano and Moio 2008).

Non-*Saccharomyces* species have also been screened for β-glucosidase activity. At this time yeasts belonging to the genera *Aureobasidium*, *Brettanomyces*, *Candida*, *Debaryomyces*, *Hanseniaspora*, *Hansenula*, *Metschnikowia*, *Torulaspora*, and *Pichia* have screened positive for β-glucosidase activity under a variety of assay conditions (Charoenchai et al. 1997, McMahon et al. 1999, Belancic et al. 2003, Roderíguez et al. 2004, Mateo et al. 2011, Swangkeaw et al. 2011). When the β-glucosidase activity for non-*Saccharomyces* species is quantified it is typically two to three times greater than that of *S. cerevisiae* (Charoenchai et al. 1997, McMahon et al. 1999). However, the β-glucosidase activity of non-*Saccharomyces* species can be inhibited by glucose content as low as 5 g/L (Charoenchai et al. 1997, Mateo et al. 2011). The degree of inhibition is between 10 and 100 fold and is species and in some cases strain dependent (Charoenchai et al. 1997, Belancic et al. 2003, Roderíguez et al. 2004, Mateo et al. 2011, Swangkeaw et al. 2011). For example, Mateo et al. (2011) found that while the β-glucosidase activity of *Hanseniaspora uvarum*, *Hanseniaspora vineae*, and *Torulaspora delbrueckii* retained only 25-30% activity at 20 g/L glucose, the enzyme of *Pichia anomala*

retained 40% activity at this glucose concentration. The β-glucosidase activity of some non-*Saccharomyces* has also been found to be stable at low pH values and even possess maximum activity at pH values found in grape must and wine (3.2-3.5) (Charoenchai, et al. 1997, McMahon et al. 1999, Mateo et al. 2011). However, this may be species dependent as well. Belancic et al. (2003) observed an 80% reduction of activity in *Debaryomyces vanrijiae* when the pH was lowered from 5.0 to 3.2.

The ability of β-glucosidases isolated from non-*Saccharomyces* yeast to release bound aroma compounds from grape derived glycosides has also been investigated. However, as is the case with *S. cerevisiae*, the research focuses on the use of glycosides derived from white wine varieties (McMahon et al. 1999, Mendes Ferreira et al. 2001, Belancic et al. 2003, Swangkeaw et al. 2011). The ability of β-glucosidases isolated from non-*Saccharomyces* species to release bound aroma compounds appears to depend on the yeast strain, the enzyme's ability to act on disaccharides in addition to monosaccharides, the tolerance of the enzyme to high sugar or high ethanol environments, and the composition of the glycosidic pool (McMahon et al. 1999, Mendes Ferreira et al. 2001, Belancic et al. 2003, Swangkeaw et al. 2011). For example, McMahon et al. (1999) found that of three species that possessed high β-glucosidase in assay conditions, only one species (*Aureobasidium pullulans*) was capable of hydrolyzing glycosides of Viognier grapes. The authors hypothesized that this was due to the ability of *A. pullulans* to hydrolyze terminal sugars as well as β-D-glucose (McMahon et al. 1999).

Non-*Saccharomyces* Yeast

Given the enzymatic potential of non-*Saccharomyces* yeast to impact wine aroma there is increased interest in understanding the ecology of these yeast in the winemaking process (Suárez-Lepe and Morata 2012). Until recently, the complex ecology of yeast during wine fermentation was not well understood (Fleet 2008). Inoculated *S. cerevisiae* was assumed to

be dominant and therefore the main driver of wine character (Fleet 2008). It is now known that the yeast ecology of fermentation is much more complex and as a result, the impact on wine quality is much more diverse (Fleet 2008). Therefore, research is now focused on identifying the yeast species present during wine fermentation, observing their growth kinetics throughout fermentation, and finally correlating this information with changes in wine aroma (Mercado et al. 2007, Fleet 2008, Romancino et al. 2008, Zott et al. 2008).

Grape must typically contains natively occurring yeast mostly from the genera *Candida*, *Issatchenkia*, *Kluveromyces*, *Metschinikowia*, *Pichia*, *Torulaspora*, and in low numbers, *Saccharomyces* (Hierro et al. 2006, Mercado et al. 2007, Fleet 2008, Romancino et al. 2008, Zott et al. 2008, Ocón et al. 2010). Less frequently, yeast from the genera *Dekkera*, *Schizosaccharomyces*, and *Zygosaccharomyces*, may also be found (Hierro et al. 2006, Fleet 2008, Romancino et al. 2008, Ocón et al. 2010). The yeast found in grape must originate from the microbial flora present on the grape berry as well as the microbial flora present in the winery (Mercado et al. 2007, Fleet 2008). Research has shown that both inoculated and spontaneous wine fermentations involve the growth and succession of non-*Saccharomyces* and *Saccharomyces* species as well as the successions of strains within each species (Mercado et al. 2007, Fleet 2008). *S. cerevisiae* (native or inoculated) dominates the later stages of fermentations and is largely responsible for completing the fermentation (Jolly et al. 2003). Non-*Saccharomyces* species typically have low fermentation vigor and alcohol tolerance and are not able to finish a wine fermentation (Jolly et al. 2003).

Non-*Saccharomyces* species are initially present in populations ranging from 10^3 to 10^5 cfu/mL and can reach a maximum of 10^6 to 10^7 cfu/mL during the early stages of fermentation of both inoculated and spontaneous alcoholic fermentations (Fleet and Heard 1985, Hierro et al. 2006, Zott et al. 2008). The diversity of species present typically reaches a peak in the first

24-72 hours (Fleet and Heard 1985, Hierro et al. 2006, Zott et al. 2008, Ocón et al. 2010) although under certain conditions, such as pre-fermentation cold maceration, the population of non-*Saccharomyces* species remains relatively high due to their cold tolerance (Fleet 2008, Zott et al. 2008). Zott et al. (2008) found initial populations of non-*Saccharomyces* yeast in Merlot must to be between 10^4 and 10^5 cfu/mL. Populations reached a maximum of 5 x 10^6 cfu/mL following pre-fermentation cold maceration (15°C, 6 days) and by the end of the alcoholic fermentation (initiated by inoculation of *S. cerevisiae*) the population was 5 x 10^4 cfu/mL. Hierro et al. (2006) found that while the non-*Saccharomyces* population did not increase from initial levels (10^6-10^7 cfu/mL) during pre-fermentation cryogenic maceration (4 °C), the population of non-*Saccharomyces* yeast was 10^5 cfu/mL at the end of inoculated alcoholic fermentation. The authors also noted that while the overall population levels reached by non-*Saccharomyces* yeast were not significantly impacted by the pre-fermentation cold maceration treatment, fermentations that included a pre-fermentation cryogenic maceration had a greater diversity of non-*Saccharomyces* yeast species persist during cold maceration (Hierro et al. 2006). The presence of cold tolerant yeast during the pre-fermentation maceration therefore has the potential to impact the chemical composition of the wine (Fleet 2008).

The impact of non-*Saccharomyces* yeast on wine aroma has been investigated in a number of studies although these tend to have been conducted in white wines (Lema et al. 1996, Ciani and Maccarelli 1998, Toro and Vazquez 2002, Jolly et al. 2003, Garde-Cerdán and Ancín-Azpilicueta 2006, Varela et al. 2009). For example, Garde-Cerdán and Ancín-Azpilicueta (2006) reported that Parellada juice fermented with a mixed inoculum of native yeast and commercial *S. cerevisiae* resulted in wine with higher levels of total esters over juice fermented with *S. cerevisiae* alone or juice fermented spontaneously. The authors suggested that the increased production was due to competition between the native yeast and *S.*

cerevisiae (Garde-Cerdán and Ancín-Azpilicueta 2006). Varela et al. (2009) found similar results when analyzing the volatile aroma profile of Chardonnay wine with lower levels of acetate esters being produced in spontaneously fermented juice compared with juice that contained both native yeast and commercial *S. cerevisiae*. Jolly et al. (2003) reported that sequential inoculation of sterile Chardonnay juice with native isolates *Candida stellata*, *Kloeckera apiculata*, or *Candida pulcherrima* and commercial *S. cerevisiae* (VIN 13) significantly increased the levels of total esters over the fermentation with *S. cerevisiae* alone. The same non-*Saccharomyces* species inoculation resulted in lower total ester levels in a Sauvignon blanc, and did not have any impact on total esters in Chenin blanc (Jolly et al. 2003). However, when analyzed by a sensory panel, the Chenin blanc wines produced by sequential inoculation were judged to be of better quality than those produced with *S. cerevisiae* alone suggesting the positive impact of non-*Saccharomyces* yeast on wine quality is not driven by ester production alone (Jolly et al. 2003).

Summary

The number of studies investigating the impact of yeast on red wine aroma is very limited compared to studies in white wines. The focus on white wine aroma is partly due to the importance of aroma to white wine quality, but aroma can also be important to the quality of some red wines such as Pinot noir (Fang and Qian 2006). Furthermore, the increased cold-tolerance of non-*Saccharomyces* yeast, their persistence throughout cold maceration, and their potential to alter wine aroma suggest that these yeast may play a larger role in red wine aroma than is currently believed. The second challenge when investigating the effect of a specific yeast strain on red wine aroma, is the difficultly of minimizing the impact of naturally occurring background organisms such as yeast and bacteria that are present on the grapes. Unlike in white wines studies it is not possibly to sterile filter the grape must to remove

microorganisms and studies often have to rely on the inoculation of high populations of a commercial *S. cerevisiae* strain that will hopefully dominate the fermentation. However, inoculation with a commercial strain of *S. cerevisiae* does not guarantee a monoculture of the inoculated yeast strain (Howell et al. 2004). An alternative method to eliminate yeast and bacteria on the grapes was recently developed involving high hydrostatic pressure (HHP) (Takush and Osborne 2011). The researchers were able to inactivate microorganisms from Pinot noir grape must prior to fermentation using HHP processing and while not creating any significant differences in the sensory properties of the wine. In addition, through the use of autoclavable microscale fermentors, aseptic sampling and fermentation management can be achieved. Therefore, the aim of this study was to monitor, isolate, and identify non-*Saccharomyces* yeast species present during pre-fermentation cold maceration and alcoholic fermentation of *Vitis vinifera* L. cv. Pinot noir grapes; to characterize β-glucosidase activity of isolated non-*Saccharomyces* species; and to investigate the volatile aroma generation by specific non-*Saccharomyces* species during pre-fermentation maceration and alcoholic fermentation of HHP treated Pinot noir grapes.

Isolation and identification of yeast present during pre-fermentation cold maceration of Pinot noir and characterization of their β-glucosidase activity

ABSTRACT

Yeast populations and diversity were monitored during pre-fermentation cold maceration and alcoholic fermentation of Pinot noir grapes from a commercial vineyard (Dayton, OR). Fermentations were conducted at the Oregon State University research winery in 100 L tanks while grapes from the same vineyard lot were fermented at a commercial winery and were not inoculated for alcoholic fermentation. Samples were taken daily during pre-fermentation maceration (9°C) and alcoholic fermentation (27°C) and plated on WL and lysine media to determine *Saccharomyces cerevisiae* and non-*Saccharomyces* populations and diversity. Total non-*Saccharomyces* populations increased from 1 x 10^3 cfu/mL to 1 x 10^5 cfu/mL during pre-fermentation cold maceration and reached a maximum of 1 x 10^7 cfu/mL during alcoholic fermentation. Thirteen distinct yeast species were tentatively identified based on appearance on WL media. Yeast were initially screened for β-glucosidase activity using 4-methyllumbelliferyl-β-D-gluconopyranoside (4-MUG) plates. The identity of the isolates screening positive for β-glucosidase activity was determined by sequence analysis of the D1/D2 domain of the 26S rDNA gene. The five isolates identified were *Metschnikowia pulcherrima*, *Hanseniaspora uvarum*, *Kluveromyces thermotolerans*, and two *Saccharomyces cerevisiae* isolates. β-glucosidase activity was further characterized and quantified using a liquid media representing grape must conditions (pH 3.5, 20° Brix) at two temperatures (25°C and 8°C). While increasing sugar concentration suppressed the β-glucosidase activity of *Hanseniaspora uvarum* (-99%), β-glucosidase activity still remained relatively high for *M. pulcherrima, S. cerevisiae* isolate 1, and *S. cerevisiae* isolate 2. At 8°C, β-glucosidase activity was reduced for *M. pulcherrima* compared to activity at 25°C, but activity increased for *H. uvarum*, *Kl. thermotolerans, S. cerevisiae* isolate 1, and *S. cerevisiae* isolate 2.

INTRODUCTION

Yeast species present on wine grapes and winery surfaces have been reported to grow and persist during the winemaking process (Hierro et al. 2006, Mercado et al. 2007, Fleet 2008, Romancino et al. 2008, Zott et al. 2008, Ocón et al. 2010) and potentially impact wine aroma. Inoculated and spontaneous wine fermentations involve the growth and succession of non-*Saccharomyces* and *Saccharomyces* species as well as the succession of strains within each species (Mercado et al. 2007, Fleet 2008) with a dominant *S. cerevisiae* strain completing the fermentation (Jolly et al. 2003). Furthermore, under conditions of pre-fermentation cold maceration, the population of non-*Saccharomyces* yeast has been reported to grow and persist in relatively high numbers through the end of alcoholic fermentation even in conjunction with a high population of *S. cerevisiae* due to their cold tolerance (Fleet 2008, Zott et al. 2008). The populations of certain *Saccharomyces* and non-*Saccharomyces* yeast has been reported to impact wine aroma (Fleet and Heard 1985, Ciani and Maccarelli 1998, Hierro et al. 2006, Zott et al. 2008) although often the specific contributions of the yeast are unclear. Yeast play an important role in aroma generation through the production of primary and secondary metabolites, such as esters, alcohols, and volatile acids. In addition, there is increased interest in the production of β-glucosidase enzymes by yeast that may release glycosidically bound aroma compounds derived from the grape (Fleet 2008, Zott et al. 2008). These grape-derived aroma precursors such as, monoterpenes and C_{13}-norisoprenoids, are important to wine aroma and can contribute unique cultivar specific flavors and aromas to wine (Reynolds and Wardle 1997, Piñeiro et al. 2005).

Non-*Saccharomyces* yeast have been reported to possess β-glucosidase activity (Charoenchai et al. 1997, McMahon et al. 1999, Belancic et al. 2003, Roderíguez et al. 2004, Mateo et al. 2011, Swangkeaw et al. 2011) and in general possess two to three times greater activity than

that of *S. cerevisiae* (Charoenchai et al. 1997, McMahon et al. 1999). However, many of these studies were conducted in model systems and also reported that β-glucosidase activity is often inhibited by the presence of glucose in a species dependent manner (Charoenchai et al. 1997, Belancic et al. 2003, Roderíguez et al. 2004, Mateo et al. 2011, Swangkeaw et al. 2011). Furthermore, most studies have focused on white wine production with very few reports of yeast being isolated from red wine fermentations. However, in red wine production the contribution of non-*Saccharomyces* yeast to wine aroma may be significant. In particular their increased cold tolerance (Fleet 2008, Zott et al. 2008) may be important during the red winemaking processes of pre-fermentation cold maceration and their persistence and growth could potentially impact red wine aroma. Therefore, the aim of this study was to monitor, isolate, and identify non-*Saccharomyces* yeast species present during pre-fermentation cold maceration and alcoholic fermentation of Pinot noir grapes and to characterize their β-glucosidase activity under conditions of pre-fermentation cold maceration such as high sugar and low temperature.

MATERIALS AND METHODS

Grapes

Vitis vinifera L. cv. Pinot noir grapes were harvested from two commercial vineyards on October 18, 2010, in Dundee, OR, USA. Vineyard 'A' is located in the Dundee Hills AVA (Jory soil), Oregon. The vines are clone 777 planted on Riparia rootstock (14 year old vines). Vineyard 'B' is located in the Ribbon Ridge AVA (Willakenzie soil), Oregon. The vines are also clone 777 planted on Riparia rootstock (10 year old vines). The grapes were sorted and destemmed at the commercial winery and 50 mg/L SO_2 was added at the crusher. Grapes from Vineyard A were placed in an 8-ton fermentor while grapes from Vineyard B were placed in a 4-ton fermentor. Initial samples were taken for plating.

Approximately 300 kg of grapes from each vineyard (A and B) were also transported to Oregon State University (OSU) winery and stored overnight at 4 °C. Grapes were destemmed in a Velo DPC 40 crusher/destemmer (Altivole, Italy) and randomly allocated to 100 L stainless steel tanks. 50 mg/L SO_2 was added, argon gas was blanketed on top of the grapes, and bladder-equipped tank lids were placed on top of the grapes and sealed. Two tanks per vineyard (A and B) were prepared and each contained approximately 70-80 L of grape must.

Pre-fermentation cold maceration and fermentation

Tanks at the commercial winery and OSU were maintained between 8-10 °C during pre-fermentation cold maceration. After eight days cold maceration, the tanks were warmed to approximately 25 °C and alcoholic fermentation proceeded without inoculation at both the commercial winery and OSU. °Brix and temperature were monitored using an Anton-Paar DMA 35N Density Meter (Graz, Austria)

Enumeration, Isolation, and Identification

Samples were aseptically taken daily after mixing at the commercial winery. Two samples per tank were taken at the commercial winery, transported (chilled) daily to OSU, and plated within three hours of sampling. At the OSU winery, two samples (one per duplicate tank) from each lot were taken. All samples were plated on WL (Difco™, Franklin Lakes, NJ, USA) supplemented with 0.15 g/L Biphenyl (Sigma) and lysine (Difco™, Franklin Lakes, NJ, USA) media using appropriate dilutions in 0.1% peptone and incubated at 25 °C for 48 hours. Plates were counted and colonies were examined on WL media in order to identify unique colony types. Colonies were described in detail based on color, shape, consistency, and size. Unique colony types were re-streaked on WL medium for isolation. Purified colonies were maintained

on potato dextrose agar (Difco™, Franklin Lakes, NJ, USA) slants and stored at 4 °C. Glycerol cultures (15% v/v glycerol) were prepared for long-term storage at -80 °C.

DNA Sequence Analysis

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).

β-Glucosidase Activity

Yeast isolates were streaked on 4-MUG media (40 mg/L 4-methylumbelliferyl-β-D-glycopyransoside (Sigma), 1.7 g/L YNB (Difco™, Franklin Lakes, NJ, USA), 5 g/L glucose, 20 g/L agar, pH 5.0). 4-MUG was filter sterilized and added to the media before pouring. The hydrolysis of the substrate (4-MUG) resulted in the release of the fluorescent compound, 4-methylumbelliferone. The activity of β-glucosidase enzyme resulted in a blue fluorescent zone surrounding the yeast growth that was visible under long wave ultraviolet light.

β-Glucosidase Quantification

Yeast isolates that gave positive results on the 4-MUG plates were further assayed for β-glucosidase activity according to the method described by Charoenchai et al. (1997). Isolates were streaked from glycerol cultures on YPD media and incubated at 25 °C for 48 hours. Single colonies were inoculated in 10 mL of Wickerman's MYGP medium (3 g/L malt extract, 3 g/L yeast extract, 10 g/L glucose, 5 g/L peptone, pH 5.5) and incubated 24 hours at 25 °C. Yeast cells were harvested by centrifugation at 4650 g for 10 minutes and washed with sterile saline twice. The cells were re-suspended in sterile saline and inoculated in triplicate in 10 mL filter-sterilized ρ-NPG medium. The ρ-NPG medium contained 6.7 g/L YNB, 5 g/L glucose, 0.9 g/L tartaric acid, 1.0 g/L potassium phosphate, dibasic, 1mM ρ-nitrophenyl-β-D-glucopyranoside (Sigma) and was buffered at pH 3.5. The cultures were incubated at 25 °C for 48 hours. Assays were also conducted with media containing 5 g/L glucose or 100 g/L glucose and 100 g/L fructose as well as at 8°C. An assay was also conducted with media containing 100 g/L glucose and 100 g/L fructose at 8 °C for 7 days and growth was monitored by plating on YPD media.

Following incubation, cultures were centrifuged at 4650 g for 10 minutes. The supernatant (1.0 mL) was mixed with 2.0 mL sodium carbonate (0.2 M, pH 10.2) and the absorbance of the solution was measured at 400 nm on a Thermo Scientific Genesys 10 UV Spectrophotometer (Madison, WI, USA). To determine dry cell mass, 1.0 mL of the culture was removed prior to enzyme analysis and transferred to pre-weighed, dry tubes. The cells were harvested by centrifugation (4650 g for 10 minutes) and washed twice with sterile saline. The cells were then dried 24 hours in 60 °C oven and weighed following cooling. Liberated ρ-nitrophenyl was determined using the extinction coefficient of 18,300/M cm. β-glucosidase activity was reported as nmole ρ-nitrophenyl released per g of dry cells per mL of supernatant.

Statistical Analysis

All statistical analysis was performed in Microsoft® Excel® 2008 (Version 12.3.3) using the two-tailed, Student's t-test.

RESULTS

Pre-fermentation cold maceration and alcoholic fermentation

The populations of non-*Saccharomyces* yeast and *Saccharomyces cerevisiae* were monitored during pre-fermentation cold maceration (day 0-8) and alcoholic fermentation (day 9-14) of grapes from two different vineyards at a commercial winery (Figure 1.1) and at the OSU winery (Figure 1.2). At the commercial winery total population growth was similar for both fermentations with the initial non-*Saccharomyces* populations being approximately 10^3 cfu/mL. In the fermentation of must from vineyard B, the non-*Saccharomyces* population rose to approximately 10^5 cfu/mL by the end of pre-fermentation cold maceration. In the fermentation of must from vineyard A, the population reached a peak of 10^4 cfu/mL during the first 48 hours of pre-fermentation cold maceration. However, by the end of pre-fermentation cold maceration the population was approximately 10^3 cfu/mL. The non-*Saccharomyces* populations in both musts increased during alcoholic fermentation and reached a maximum population of 10^7 cfu/mL by the fourth day of alcoholic fermentation (day 11). The population of non-*Saccharomyces* yeast was dominated by *Hanseniaspora uvarum* and *Kluveromyces thermotolerans* during pre-fermentation cold maceration and alcoholic fermentation (data not shown). *Saccharomyces cerevisiae* was not detected in the fermentation of must from vineyard B until the grape must was warmed (day 9). In fermentation of must from vineyard A, *S. cerevisiae* was detected on day 2 and day 6, and did not show up in significant numbers until the must was warmed (day 9). The maximum population of *S. cerevisiae* in both fermentations was approximately 10^9 cfu/mL. During pre-fermentation cold maceration, the must from

vineyard A had an average temperature of 11.0 °C and the average temperature of must from vineyard B must was 11.2 °C. Alcoholic fermentation commenced two days after the must was warmed (day 10) and was complete by day 14 for both fermentations.

The fermentations performed at the OSU winery were prepared in duplicate. As with the samples from the commercial winery, the populations of non-*Saccharomyces* yeast and *Saccharomyces cerevisiae* were monitored during pre-fermentation cold maceration (day 0-7) and alcoholic fermentation (day 9-13). The initial population of non-*Saccharomcyes* yeast in must from vineyard A was approximately 3 x 10^2 cfu/mL (Figure 1.2). While the initial poplulation in must from vineyard B was approximately 10^3 cfu/mL. The non-*Saccharomyces* population in both fermentations rose to approximately 10^5 cfu/mL by the end of pre-fermentation cold maceration and reached a maximum of approximately10^8 cfu/mL on the third day of alcoholic fermenation (day 10). As seen in the commercial winery fermentations the population of non-*Saccharomyces* yeast was dominated by *Hanseniaspora uvarum* and *Kluveromyces thermotolerans* during pre-fermentation cold maceration and alcoholic fermentation (data not shown). *S. cerevisiae* was not detected until the must was warmed, day 9 in vineyard A musts and day 10 in vineyard B musts. The maximum population of *S. cerevisiae* was approximately 2 x 10^8 cfu/mL for both treatments. The average temperature for all the musts during pre-fermentation cold maceration was 9.9°C. Alcoholic fermentation commenced three days after the must was warmed in vineyard A musts (day 10) and two days after the must was warmed in vineyard B musts (day 9). Alcoholic fermentation was complete in all musts on day 13.

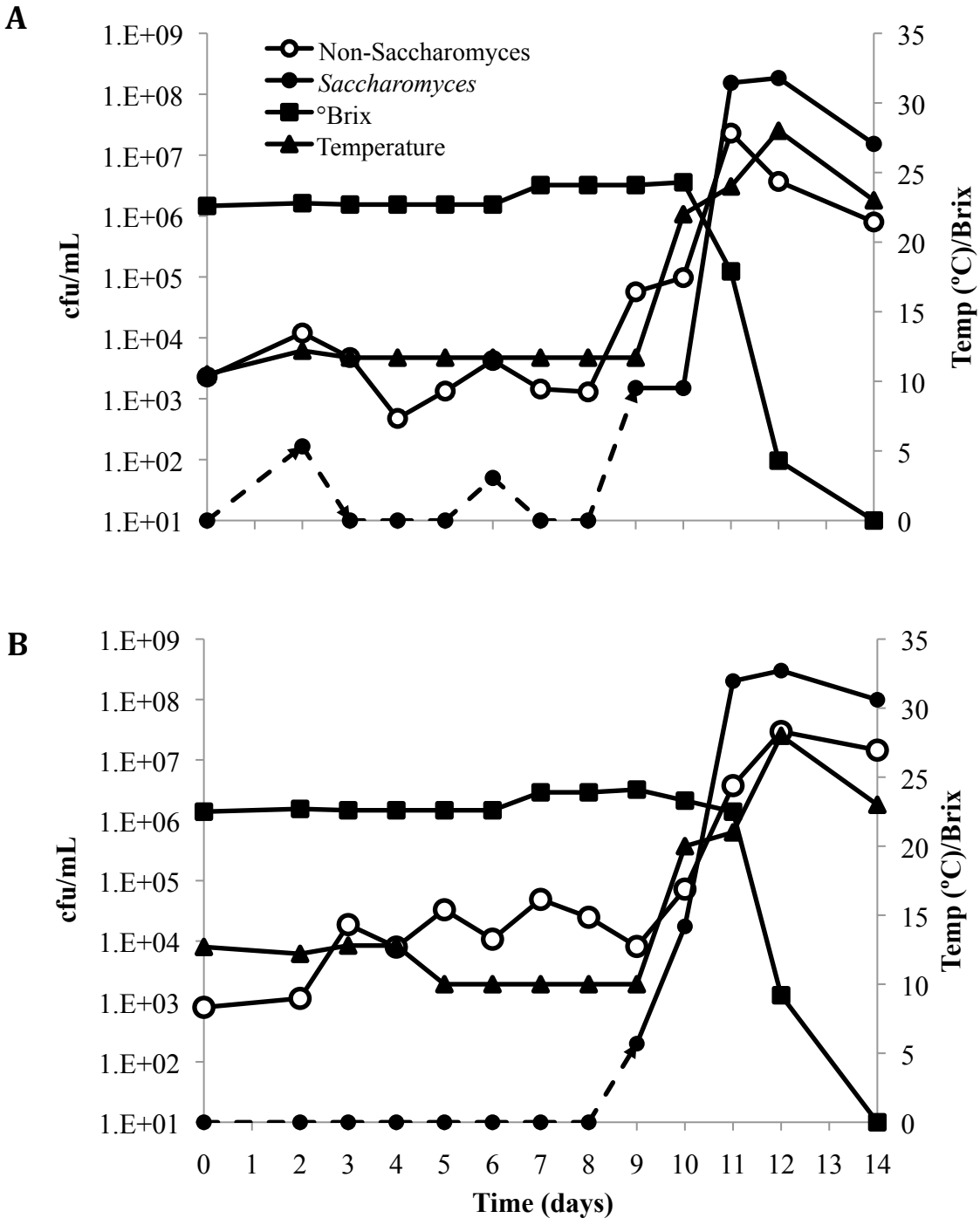

Figure 1.1 Growth of non-*Saccharomyces* yeast and *Saccharomyces cerevisiae* and change in °Brix and temperature during pre-fermentation cold maceration (day 0-8) and alcoholic fermentation (day 8-14) of Pinot noir grapes from vineyard **A** and **B**. Fermentations performed at a commercial winery.

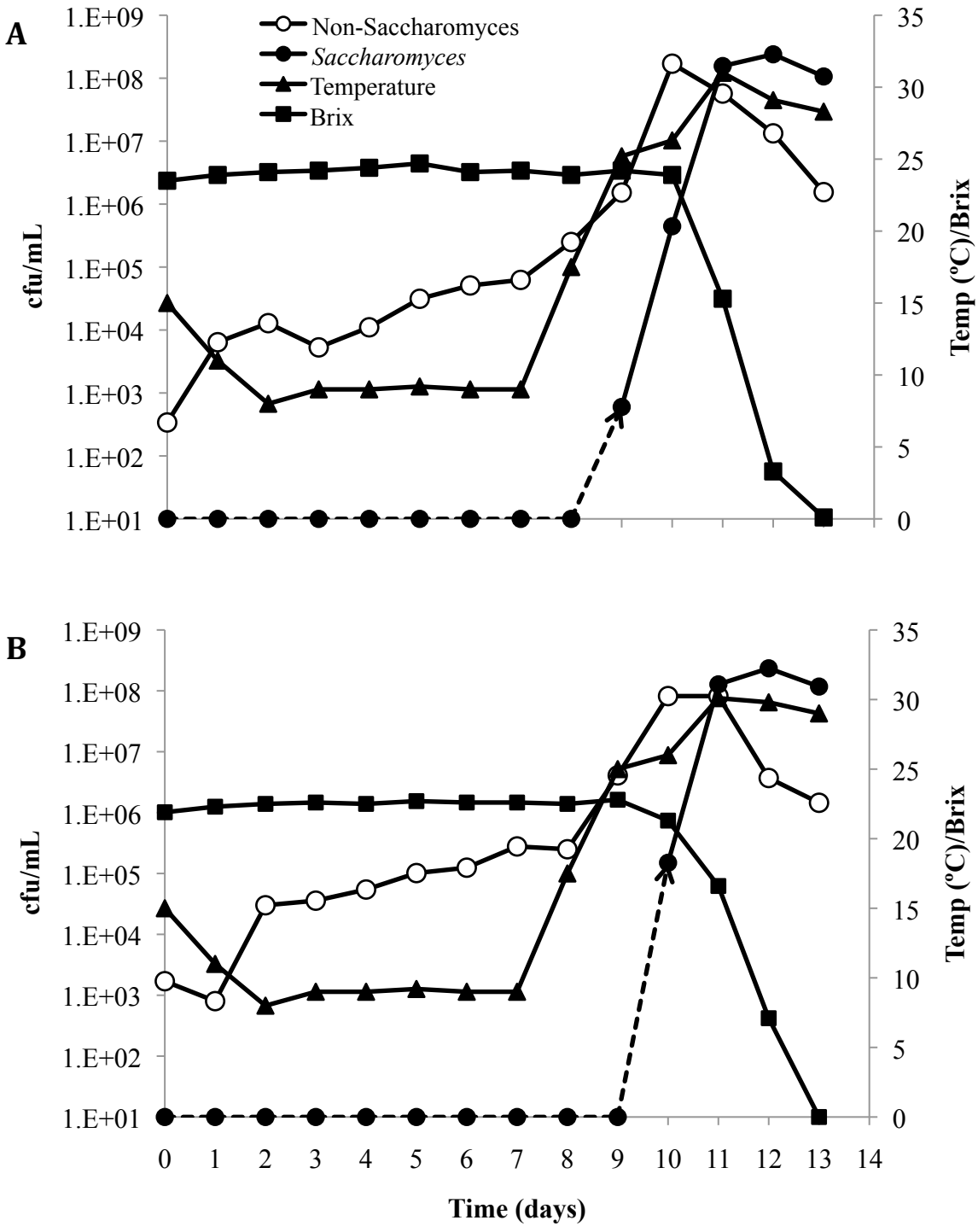

Figure 1.2 Growth of non-*Saccharomyces* yeast and *Saccharomyces cerevisiae* and change in °Brix and temperature during pre-fermentation cold maceration (day 0-7) and alcoholic fermentation (day 7-13) of Pinot noir grapes from vineyard **A** and **B**. Fermentations performed in duplicate at OSU winery.

Isolation and Identification

Colonies present on WL media were described in detail based on color, shape, consistency, and size. Table 1.1 lists the unique colony types isolated from vineyard A and B musts and the tentative species identification according to Pallman et al. (2001) or Edwards (2005). Thirteen unique colony types were isolated.

Table 1.1 Yeast tentatively identified during pre-fermentation maceration by appearance on WL media

Yeast Isolate	Appearance on WL media
Metschnikowia pulcherrima[a]	small, round, white, knoblike, with dark brick red haze and bottom
Hanseniaspora uvarum[a]	small-medium sized, round, flat, dark pea green with white edge
Issatchenkia orientalis[b]	round, white, flat, matte-looking
Candida oleophilia[a]	matte, frosty, white dark bright green/teal with white edge
Candida vini[b]	tall, white-ice blue, mountain-like
Pichia membranaefaciens[b]	round, blue center, frosty, flour like consistency
Kluveromyces thermotolerans[b]	small-medium sized, round, flat bright teal/green
Hansenula anomala[a]	round, light blue, opaque, knoblike
Unknown isolate 8	small, round, glossy, clear-looking, light blue
Unknown isolate 9	small, flat, wet-looking, bright straw
Unknown isolate 10	round, white, knoblike; sometimes with dark green center
Unknown isolate 11	white, round, flat, bright green with concentric circles
Unknown isolate 12	round, white with dark blue center, flat bright dark teal/green with darker center and lighter concentric rings to white edge

[a]Pallman et al. 2001, [b]Edwards 2005

β-Glucosidase Activity

Eleven isolates were screened for β-glucosidase activity on 4-MUG plates (Table 1.2). Five of the eleven isolates screened positive for activity. *M. pulcherrima*, *H. uvarum* isolate A, and *K. thermotolerans* isolate A had weak activity while, *H. anomala* and Unknown isolate 10 had strong activity.

Table 1.2 β-glucosidase acitivity of yeast species on 4-MUG media

Yeast isolate	**β-glucosidase actvity**[1]
M. pulcherrima	+
H. uvarum (A)	+
H. uvarum (B)	-
P. membranaefaciens	-
K. thermotolerans (A)	+
K. thermotolerans (B)	-
K. thermotolerans (C)	-
H. anomala	++
Unknown isolate 10	++
Unknown isolate 11	-
Unknown isolate 12	-

[1](-) no activity, (+) weak activity, (++) strong activity

DNA Sequence Analysis

The species identity of the yeast isolates that screened positive for β-glucosidase activity was determined by DNA sequence analysis of the D1/D2 domain of the 26S rDNA. The results of the BLASTn search can be found in Table 1.3. The *H. anomala* isolate and Unknown isolate

10 were determined to be *Saccharomyces cerevisiae* isolates. *H. anomala* will be referred to as *S. cerevisiae* isolate 1 and Unknown isolate 10 will be referred to as *S. cerevisiae* isolate 2.

Table 1.3 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
[*]Sequences are presented in Appendix A

β-Glucosidase Quanitification

The β-glucosidase activity of the isolates that screened positive for activity on 4-MUG plates was quantified using the *p*-NPG assay (Table 1.4, Figure 1.3). All of the isolates were found to have activity under the assay conditions of 5 g/L glucose and pH 3.5 at 25 °C. The greatest activity under these conditions was seen for the *H. uvarum* isolate. The lowest activity was seen for the *K. thermotolerans* and *S. cerevisiae* isolate 2. There was a reduction of activity for all isolates when the sugar content of the assay media was increased from 5 g/L glucose to 100 g/L glucose and 100 g/L fructose. The greatest reduction was seen for the *H. uvarum* isolate with a 99.8% reduction in activity, while the least reduction was seen for *S. cerevisiae* isolate 1 with an 11% reduction. When the temperature of the assay was reduced to 8 °C while maintaining the glucose and fructose concentration as well as the pH, the activity of *M.*

pulcherrima was further reduced. However, the acitivities of *K. thermotolerans*, *S. cerevisiae* isolate 1, and *S. cerevisiae* isolate 2 increased, and were even higher than the levels seen in the 5 g/L glucose, 25 °C assay medium. The activity of *H. uvarum* also increased when the temperature was reduced, however it did not recover all of the activity present in the 5 g/L glucose assay.

Table 1.4 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=2, **n=3

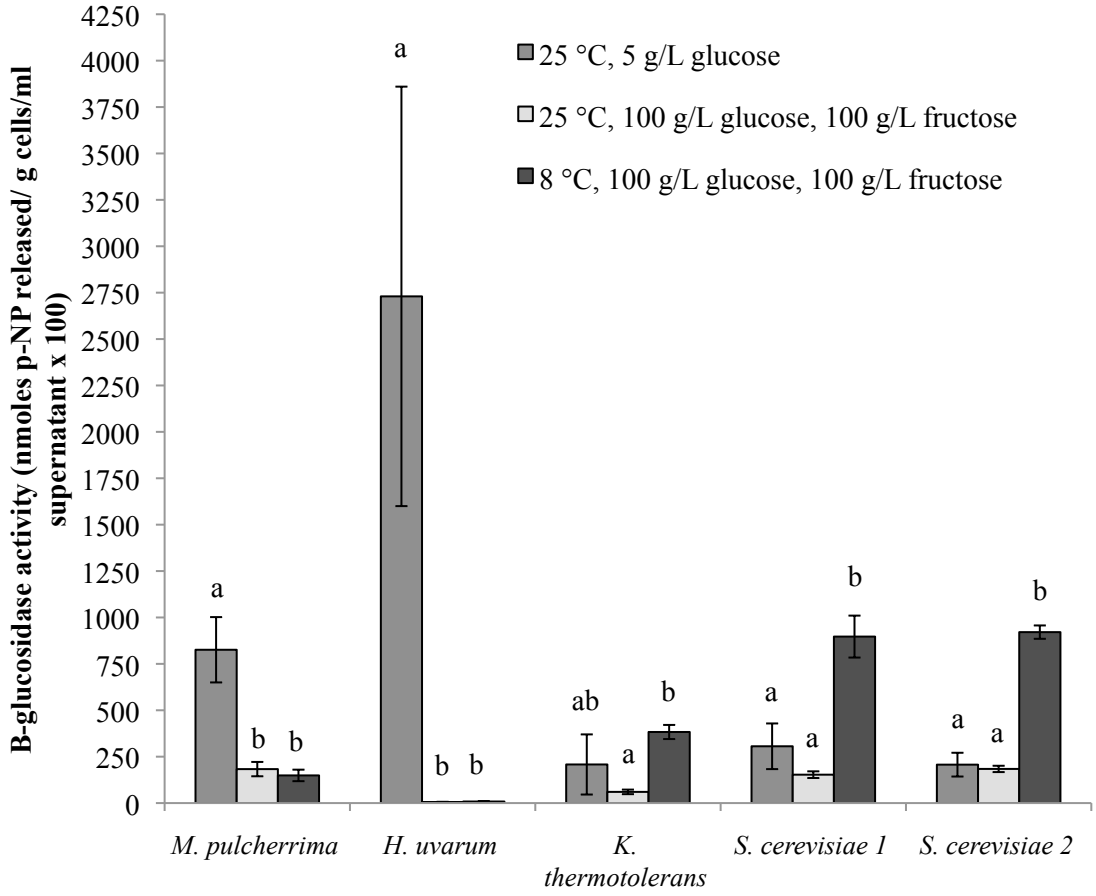

Figure 1.3 β-glucosidase activity of various yeast species in assay media (pH 3.50, 48 hours). Columns within the same category with the same letter do not differ significantly ($p < 0.05$). Standard deviation indicated with error bars, n=2 for all 25 °C, 5 g/L glucose results and 25 °C, 100 g/L glucose, 100 g/L fructose results for *M. pulcherrima*, n=3 for all 8 °C, 100 g/L glucose, 100 g/L fructose results.

The activity and growth of the yeast isolates in the p-NPG assay medium over 7 days was determined (Figure 1.4). All isolates grew in the assay medium (100 g/L glucose, 100 g/L fructose, pH 3.5, 8 °C). However, higher growth did not correspond with higher activity as shown by the low growth and high activity of the *S. cerevisiae* isolates and the high growth and low activity of the *H. uvarum* isolate

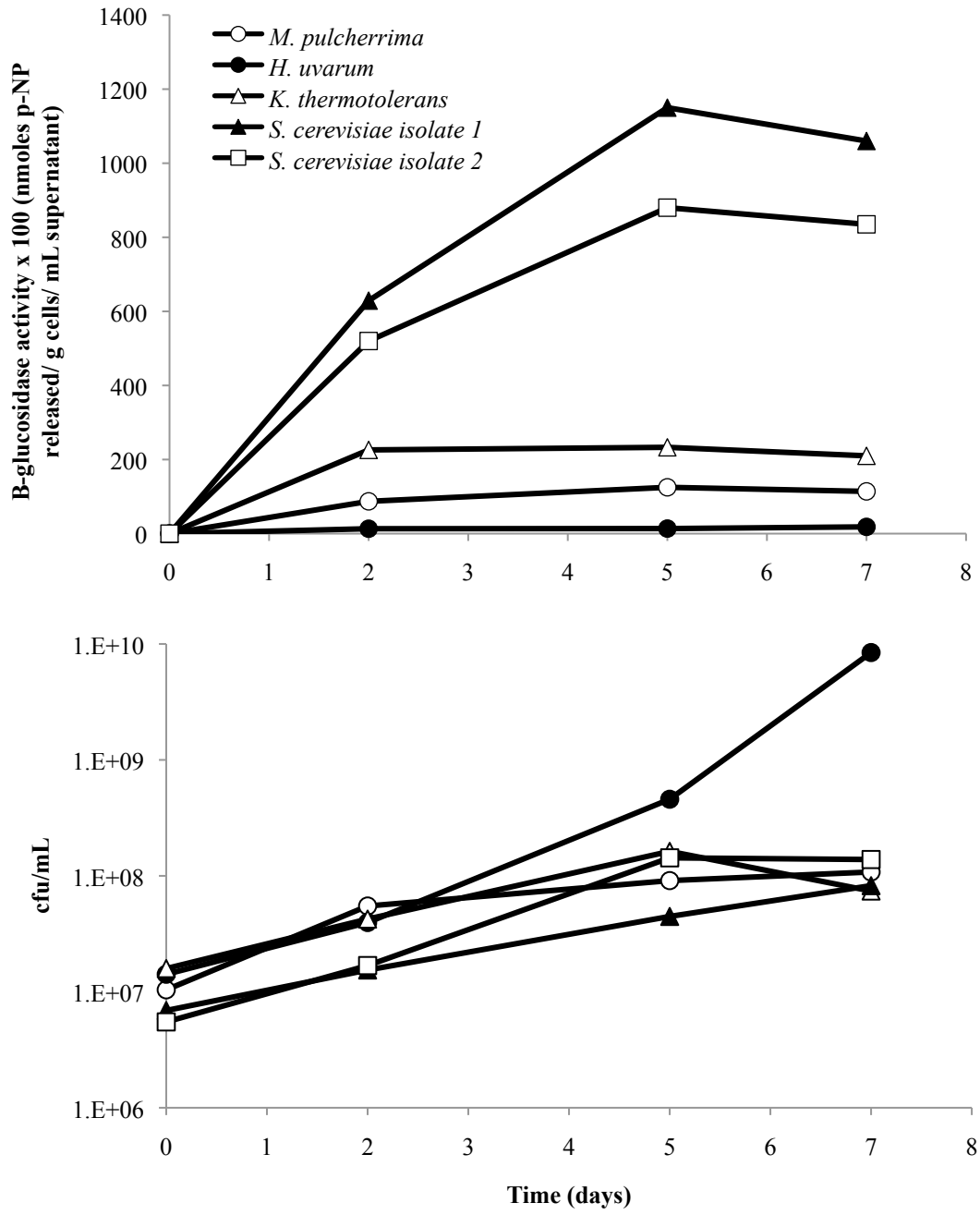

Figure 1.4 β-glucosidase activity (nmoles *p*-NP released/g cells/mL supernatant) and growth (cfu/mL) in *p*-NPG medium assay, 100 g/L glucose, 100 g/L fructose, pH 3.5, 8 °C, 7 days (n=3)

DISCUSSION

Numerous yeast species present on wine grapes and winery surfaces may persist during the winemaking process and potentially influence wine quality. In this study a total of thirteen unique colony types were identified during pre-fermentation cold maceration and alcoholic fermentation of Pinot Noir grapes. The genera identified were consistent with what others have observed in the grape must environment (Hierro et a l. 2006, Jolly et al. 2006, Mercado et al. 2007, Fleet 2008, Romancino et al. 2008, Zott et al. 2008, Ocón et al. 2010) as were the initial population levels. High population levels of non-*Saccharomyces* yeast were monitored despite the addition of 50 mg/L SO_2 at the beginning of pre-fermentation cold maceration suggesting that higher SO_2 additions may be required to suppress growth of these yeast if required. The population of non-*Saccharomyces* yeast was dominated by *Hanseniaspora uvarum* and *Kluveromyces thermotolerans* during pre-fermentation cold maceration and alcoholic fermentation. *H. uvarum* is often reported as the dominant species during the early stages of fermentation in grape must (Torija et al. 2001, Pallman et al. 2001, Ocón et al. 2010) while *K. thermotolerans* has also been observed in high populations in some studies (Mills et al. 2002, Romancino et al. 2008). However, in other studies *Kluveromyces* was not detected or found only to be a minor constituent of grape must (Torija et al. 2001, Zott et al. 2008, Ocón et al. 2010).

Although high populations of non-*Saccharomyces* yeast were found in the grape must, the *S. crevisiae* isolate that dominated the alcoholic fermentation was present at a very low population or was not detected at all until the grape must was warmed. This finding is typical of what is reported in the literature where levels of *S. cerevisiae* during the early stages of spontaneous fermentation are low or undetectable (Torija et al. 2001, Jolly et al. 2006, Romancino et al. 2008, Zott et al. 2008, Ocón et al. 2010). In contrast, two additional *S.*

cerevisiae isolates that had distinct colony morphology on WL media were present in higher populations during the pre-fermentation cold maceration. These isolates demonstrated greater cold tolerance as well as slow growth on lysine media and were initially characterized as non-*Saccharomyces* isolates until sequencing results revealed that they were in fact *Saccharomyces* yeast. Whether the *S. cerevisiae* isolate that dominated the alcoholic fermentation was originally present on the grapes is undetermined at this point as the genetic identity of the *S. cerevisiae* isolates was not determined at each point of the winemaking process. Further genetic analysis of the *Saccharomyces cerevisiae* isolates will need to be performed in order to determine any strain divergence.

Total yeast population increased during pre-fermentation maceration despite the cold temperatures (8-9°C). Growth of non-*Saccharomyces* yeast during pre-fermentation cold maceration has been reported in previous studies (Jolly et al. 2006, Fleet et al. 2008, Zott et al. 2008) although a number of the studies were performed at higher temperatures than the present study. The growth and persistence of yeast species throughout the cold maceration and during the alcoholic fermentation suggests that these yeast could cause changes in a wines composition and the specific contributions of these yeast should not be discounted.

Because of the role yeast play in the development of wine aroma it is important to understand the impact of different yeast species and strains on volatile aromas. Aside from the production of primary and secondary metabolites such as esters, alcohols, and volatile acids, a number of recent studies have focused on the production of β-glucosidases enzymes that can release glycosidically bound aroma compounds derived from the grape (Delacroix et al. 1994, McMahon et al. 1999, Ferreira et al. 2001, Belancic et al. 2003, Ugliano et al. 2006, Swangkeaw et al. 2011). In this study five isolates present during the pre-fermentation cold maceration demonstrated β-glucosidase activity. These isolates included both non-

Saccharomyces and *Saccharomyces* species, consistent with results of other investigations (Charoenchai, et al. 1997, McMahon et al. 1999, Mateo et al. 2011). As reported by others (Charoenchai et al. 1997, Belancic et al. 2003, Roderíguez et al. 2004, Mateo et al. 2011, Swangkeaw et al. 2011) the activity of β-glucosidases were inhibited by conditions present in the grape must such as high sugar content. In the present study the degree of β-glucosidase inhibition was species dependent, with some species experiencing severe inhibition, while others only slight inhibition. In particular, the β-glucosidase activity of *H. uvarum* was strongly inhibited by high sugar concentration while *M. pulcherrima, K. thermotolerans*, and the two *S.cerevisiae* isolates still demonstrated high β-glucosidase activity even in the presence of 100 g/L glucose and 100g/L of fructose. Charoenchai et al. (1997) and McMahon et al. (1999) reported that the β-glucosidase activity of *Saccharomyces* species was two to three times lower than that of non- *Saccharomyces* species. This was also observed in this study but only under assay conditions of low sugar (5 g/L glucose). When the sugar content was increased the activity of the *Saccharomyces* isolates was among the highest with only one of the three non- *Saccharomyces* species (*M. pulcherrima*) having a significantly similar level. Furthermore, most previous studies had reported inhibition in assays containing only up to 20 g/L glucose or fructose while in this study higher sugar concentrations were tested that more closely simulated conditions in a grape must.

Aside from high sugar concentration, the other condition present in during a pre-fermentation cold maceration that may impact β-glucosidase activity is low temperature. However, in this study when the β-glucosidase assay was conducted at 8°C an increase in activity was observed for both *Saccharomyces* isolates and *K. thermotolerans* compared to activity at 25°C. The increased β-glucosidase activity was significant with an increase of 70% or more. To our knowledge, this is the first report of yeast β-glucosidase activity increasing due to colder temperatures. Although the β-glucosidase assay takes into account yeast growth (activity

expressed per dry cell weight), a further experiment was conducted to determine if varying yeast growth was responsible for the increased β-glucosidase activity at cold temperatures. The results from the growth study in the assay medium indicated that higher levels of viable cells in the medium did not correspond with higher enzyme activity values. In fact, the highest activity was found for those yeast isolates with the lowest populations of viable cells indicating that the enzyme activity was not related to increased cell growth at lower temperatures. Conversely, when enzyme activity was considered regardless of dry cell mass, the same trend was observed with the *Saccharomyces* isolates and *K. thermotolerans* possessing increased activity at the high sugar concentrations and low temperature used in the assay.

CONCLUSION

The persistence and growth of certain yeast species during the pre-fermentation maceration and the high β-glucosidase activities observed for some species under conditions of high sugar and low temperature demonstrate the potential impact these yeast may have on wine aroma. Yeast such as *M. pulcherrima, K. thermotolerans, H. uvarum*, and cold tolerant *S. cerevisiae* could potentially change wine aroma through production of esters, higher alcohols, and/or fatty acids while β-glucosidase could result in release of bound volatile compounds important for wine aroma. Future work should focus on assessing the specific contributions of these yeast to wine aroma and whether encouraging or discouraging their growth would be beneficial to wine quality.

Impact of yeast species present during pre-fermentation cold maceration on the aroma of Pinot noir wine

ABSTRACT

Isolates of *Metschnikowia pulcherrima, Hanseniaspora uvarum, Kluveromyces thermotolerans,* and two different *Saccharomyces cerevisiae* strains that demonstrated β-glucosidase activity were used in fermentations of *Vitis vinifera* L. cv. Pinot noir grapes. The grapes were treated with high hydrostatic pressure (HHP) to inactivate naturally occurring yeast and bacteria before inoculation of specific yeast. All yeast grew during pre-fermentation cold maceration (7 days at 9°C) and populations increased 3 to 4 logs. Following pre-fermentation cold maceration, all ferments were warmed to 27°C and inoculated with *S. cerevisiae* RC212. Alcoholic fermentations were all complete within eight days and after pressing wines were analyzed for volatile aroma compounds by SPME-GC-MS. 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 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.

INTRODUCTION

Wine aroma is one of the most important components of wine quality and microbial flora, specifically the yeast present during fermentation, contribute significantly to the volatile aroma compounds in wine (Ebeler 2001, Sweigers et al. 2005). Because of the importance of yeast to wine aroma, many studies have investigated the impact of yeast species and strain on

wine aroma (Lema et al. 1996, Garde-Cerdán and Ancín-Azpilicueta 2006, Viana et al. 2008, Comitini et al. 2011). For example, Lema et al. (1996) reported that Albariño wine fermentations with a dominant *S. cerevisiae* strain in combination with the growth of non-*Saccharomyces* species during the first days of fermentation resulted in higher concentrations of ethyl esters and volatile fatty acids. Furthermore, Comitini et al. (2011), found that fermentations of select non-*Saccharomyces* yeast in combination with *Saccharomyces cerevisiae* produced wines with unique aromatic profiles based on the yeast strain combinations and inoculum rates.

Because of the role yeast play in the development of wine aroma it is important to understand the impact of different yeast species and strains on volatile aromas. Aside from the production of primary and secondary metabolites such as esters, alcohols, and volatile acids, a number of recent studies have focused on the production of β-glucosidases enzymes that can release glycosidically bound aroma compounds derived from the grape (Delacroix et al. 1994, McMahon et al. 1999, Ferreira et al. 2001, Belancic et al. 2003, Ugliano et al. 2006, Swangkeaw et al. 2011). In particular, a number of studies have reported on the β-glucosidase activity of non-*Saccharomyces* yeast (Charoenchai et al. 1997, Belanic et al. 2003, Roderíguez et al. 2004, Mateo et al. 2011) that may lead to greater liberation of glycosidically bound aroma compounds such as C_{13}-norisoprenoids and terpene alcohols (McMahon et al. 1999, Mendes Ferreira et al. 2001). In addition, previous research in our laboratory had identified five yeast isolates present during the pre-fermentation cold maceration that demonstrated high β-glucosidase activity even under conditions of high sugar and low temperature (see Chpt. 1). The purpose of the present research was therefore to utilize these yeast in studying the specific impact of yeast on wine aroma. Furthermore, pre-fermentation macerations would be conducted to determine if the presence and growth of these cold-tolerant yeast play a role in

impacting the aroma of wines that undergo pre-fermentation maceration compared to those that do not.

One of the challenges presented when investigating the effect of specific yeast species or strains on red wine aroma is the presence of naturally occurring yeast and bacteria on the grapes prior to fermentation. In this present study a recently developed method to inactivate microorganisms from Pinot noir grape must prior to fermentation using high hydrostatic pressure (HHP) processing (Takush and Osborne 2011) will be utilized. When coupled with the use of autoclavable microscale fermenters and aseptic sampling, the specific impact of yeast strains possessing β-glucosidase activity on Pinot noir wine aroma will be able to be determined.

MATERIALS AND METHODS

Microorganisms

Yeast isolates (*Metschnikowia pulcherrima, Hanseniaspora uvarum, Kluveromyces thermotolerans, Saccharomyces cerevisiae* isolate 1*,* and *Saccharomyces cerevisiae* isolate 2) were streaked from glycerol cultures on YPD media and incubated at 25 °C for 48 hours. Single colonies were inoculated in 250 mL acidic grape juice broth (25% v/v grape juice, 5/L yeast extract, 0.125 g/L magnesium sulfate, 0.00275 g/L manganese sulfate, 0.5% v/v Tween, pH 4.5) and incubated at 25 °C for 48 hours. The cells were harvested by centrifugation at 4650 g for 10 minutes and washed once with sterile phosphate buffer (27.8 g/L $NaH_2SO_4 \bullet H_2O$, 28.38 g/L Na_2HPO_4, pH 7.0). The cells were re-suspended in sterile phosphate buffer prior to inoculation.

Grapes

Vitis vinifera L. cv. Pinot noir grapes were harvested at Oregon State University's Woodhall Vineyard (Alpine, OR, USA) on October 27, 2011 and stored overnight at 4 °C. The following day, grapes were destemmed and crushed using a Velo DPC 40 crusher/destemmer (Altivole, Italy) before being pooled and divided into 3 kg aliquots. Each aliquot was placed in a Food Saver® bag (Jarden Corp., Boca Raton, FL, USA) and 30 mg/L SO_2 was added before the bags were sealed. The grape must was processed by high hydrostatic pressure (5 minutes at 80,000 psi) as described by Takush and Osborne (2011). The HHP unit was made by National Forge Company (Irvine, Pennsylvania) and has a 22L maximum capacity and a 689 MPa maximum pressure. The high pressure intensifier pump was made by Flow International Corporation (model 7XS-6000, Kent, Washington) and has a maximum capacity of 620 MPa.

Must analysis was performed following HHP processing. Titratable acidity (TA) was determined by titration with 0.1 NaOH and recorded as g tartaric acid/100 mL. °Brix was determined using an Anton-Paar DMA 35N Density Meter (Graz, Austria). pH was determined using a Mettler-Toledo Delta 320 pH meter (Shanghai, China). Yeast assimilable nitrogen (YAN) status was determined by measuring primary amino acids according to the procedure outlined by Dukes and Butzke (1998) and measuring ammonia content (r-Biopharm® Ammonia Assay). Must TA was 0.798 g tartaric acid/100 mL, with a pH of 3.53, 22.0 °Brix, and contained 147.6 mg/L YAN.

Pre-fermentation cold maceration

Microscale fermentors (4L) were used as described by Takush and Osborne (2011). The fermentors were autoclaved at 121 °C for 30 minutes and cooled before use. HHP treated grape must (3 kg) was aseptically transferred to fermentors in a laminar flow hood. The must

was inoculated with either *Metschnikowia pulcherrima, Hanseniaspora uvarum, Kluveromyces thermotolerans, Saccharomyces cerevisiae* isolate 1, or *Saccharomyces cerevisiae* isolate 2 at approximately 10^4 cfu/mL. A treatment containing all of the isolates was prepared with each isolate being inoculated at the same rate as the individual fermentations (10^4 cfu/mL). In addition, an uninoculated control was also prepared. Fermentations were conducted in triplicate. Fermaid K (filter sterilized) was added to all fermentations post inoculation at 0.25 g/L. The fermentors were incubated in a temperature controlled room at 9 °C for 7 days. Samples were taken before and after inoculation and then every 48 hours. The samples were plated on WL (Difco™, Franklin Lakes, NJ, USA) media supplemented with 0.15 g/L Biphenyl (Sigma) after appropriate dilutions in 0.1% peptone.

Microscale Fermentation

After 7 days at 9 °C, fermentors were transferred to a temperature controlled room at 27 °C and allowed to warm (approximately 3 hours). *Saccharomyces cerevisiae* RC212 was streaked from glycerol cultures on YPD media and incubated at 25 °C for 48 hours. A Single colony was inoculated in 250 mL acidic grape juice broth (25% v/v grape juice, 5/L yeast extract, 0.125 g/L magnesium sulfate, 0.00275 g/L manganese sulfate, 0.5% v/v Tween, pH 4.5) and incubated at 25 °C for 48 hours. The cells were harvested by centrifugation at 4650 g for 10 minutes and washed once with sterile phosphate buffer. The cells were re-suspended in sterile phosphate buffer prior to inoculation. The warm must was then inoculated with *S. cerevisiae* RC212 at approximately 10^4 cfu/mL. A fermentation that did not undergo pre-fermentation cold maceration was also prepared and inoculated with *S. cerevisiae* RC212 at the same rate. Samples were taken before and after inoculation with *S.* cerevisiae and every 48 hours through the completion of alcoholic fermentation. Viable cell populations were monitored by plating on WL-Biphenyl and lysine (Difco™, Franklin Lakes, NJ, USA) media after appropriate dilutions in 0.1% peptone. °Brix and temperature was monitored using an Anton-Paar DMA

35N Density Meter (Graz, Austria). Fermentations were pressed at dryness (< 0.5 g/L reducing sugar as measured by CliniTest®) using a modified basket press with an applied constant pressure of 0.1 MPa for 5 minutes. No malolactic fermentation was conducted. A 30 mg/L SO_2 addition was made to all wines before settling at 4 °C for five days. Samples were taken from each replicate for volatile aroma analysis. Preliminary sensory evaluation revealed no apparent differences between replicates and these were combined before being sterile filtered (0.45μm, PES, sterile cartridge filter (Pall Corp., East Hills, NY, USA)), bottled (375 mL brown bottles with crown-cap closure), and stored at 13 °C.

Volatile Aroma Analysis

Wine samples (2 mL) were diluted with 8 mL saturated salt water in a clean 20ml auto-sampler glass vial and 20 μL of internal standard solution (containing 1.5mg/mL methyl propionate, 38.4 mg/L 3-heptanone, 47.5 mg/L hexyl formate, 43.9 mg/L 4-octanol and 41.7 mg/L octyl propionate) were added. The vials were tightly capped with Teflon-faced silicone septa. A SPME fiber coated with divinyl benzene/Carboxen/polydimethylsiloxane phase (2 cm long, 50/30 μm) was used for the extraction of volatile compounds. The vials were placed in an automatic headspace sampling system and samples were pre-incubated at 50 °C for 30 min, and then extracted for 30 min at 50 °C.

The volatile compounds extracted by SPME fiber were analyzed using a HP6890 gas chromatograph equipped with a 5973 mass spectrometry detector (MSD) system (Agilent Technologies, Palo Alto, CA) and fitted with a ZB-Wax capillary column (30 m X 0.25mm X 0.5 μm). Helium was used as the carrier gas at a flow rate of 1.2 mL/min. The inlet temperature was 250 °C. The desorption was performed in the split mode with a split ratio of 2:1. The oven temperature program was 35°C for 4 min, followed by an increase of 5 °C/min to 230 °C. The final temperature was held for 10 min. The MSD in scan mode was used. The

electron impact (EI) energy was 70 eV, the MS transfer line and ion source temperature were 230 °C respectively. Electron impact mass spectrometric data from m/z 35-350 were collected.

Statistical Analysis

Statistical analysis of volatile aroma compounds was performed using SPS 15 with Tukey's HSD test for mean separation.

RESULTS

Microscale Fermentations

All microscale fermentations completed alcoholic fermentation within eight days (Figures 2.1 and 2.9). Samples were plated on WL-Biphenyl media following HHP treatment of the must and aseptic transfer to microscale fermentors. No microorganisms were detected in the must prior to inoculation (data not shown). In addition, no microorganisms were detected in the un-inoculated, pre-fermentation cold maceration treatment prior to inoculation with *S. cerevisiae* RC212 (Figure 2.2). In the pre-fermentation cold maceration treatment containing all isolates (Figure 2.3), the individual populations rose to approximately 10^5-10^7 cfu/mL during pre-fermentation cold maceration with the total population reaching 3.5 x 10^7 cfu/mL. *M. pulcherrima* and *H. uvarum* were not detected after the first day of alcoholic fermentation (192 hours). *K. thermotolerans* persisted through the end of alcoholic fermentation with a final population of 10^5 cfu/mL. Once *S. cerevisiae* RC212 was inoculated it grew well and reached a maximum population of approximately 10^8 cfu/mL in all treatments during alcoholic fermentation. In the treatments containing individual yeast isolates, populations all increased during pre-fermentation cold maceration, reaching approximately 10^7 cfu/mL (Figures 2.4-2.8). However, only *K. thermotolerans* persisted through the end of alcoholic fermentation (Figure 2.6). In general, *S. cerevisiae* reached a maximum population of 10^8 cfu/mL in these treatments.

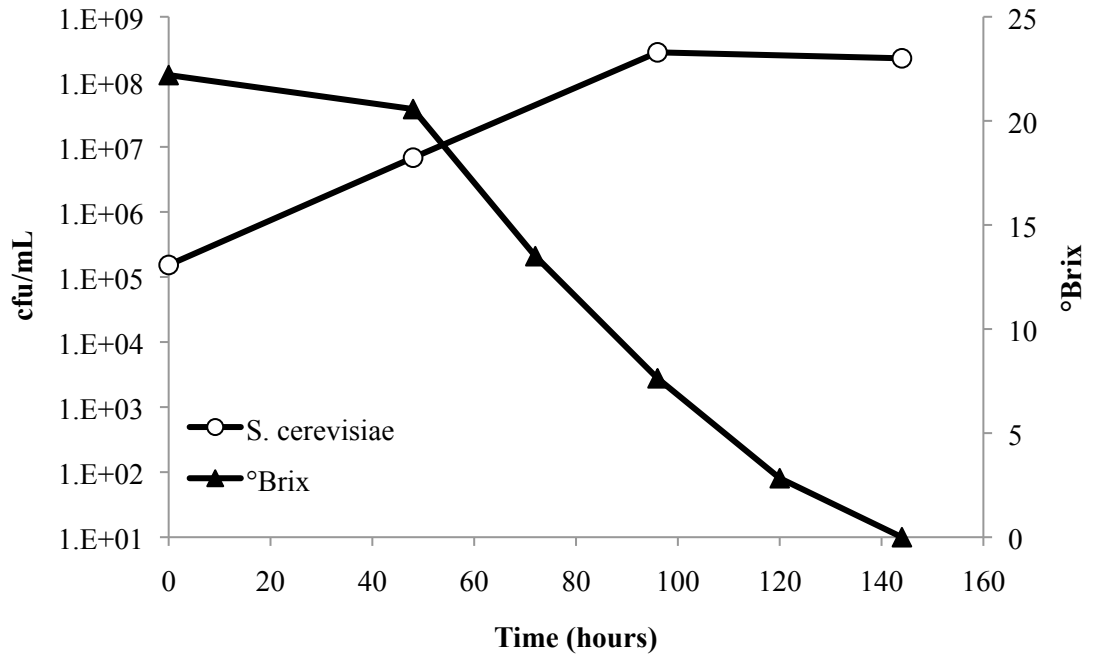

Figure 2.1 Growth of *Saccharomyces cerevisiae* RC212 and °Brix during alcoholic fermentation of Pinot noir grapes. No pre-fermentation cold maceration was performed. Inoculation of *S. cerevisiae* RC212 occurred at day 0.

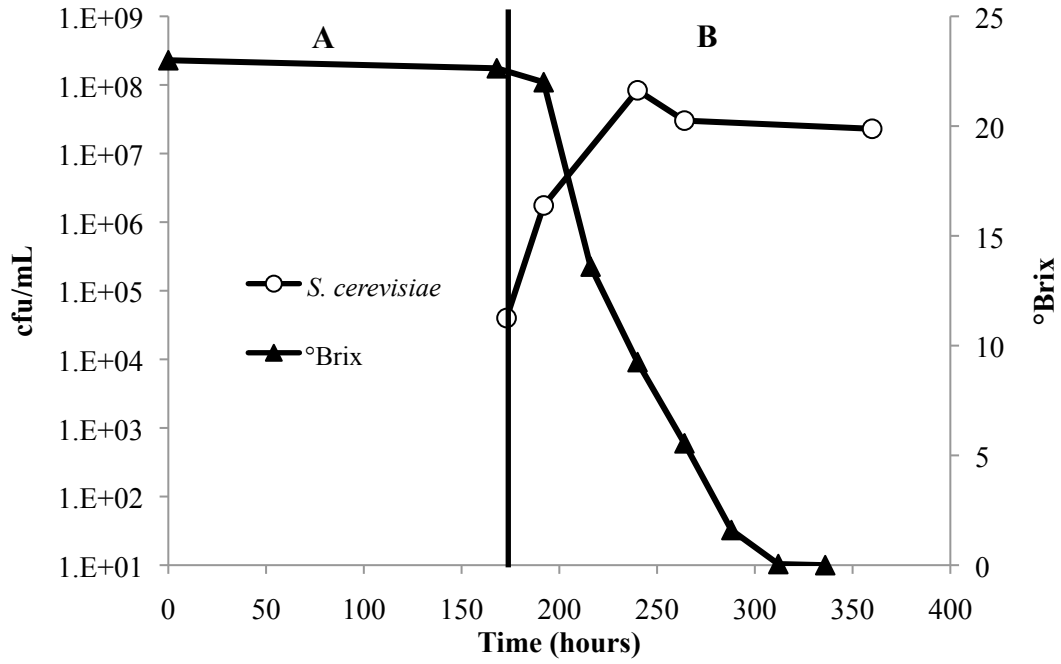

Figure 2.2 Growth of *Saccharomyces cerevisiae* RC212 and °Brix during pre-fermentation cold maceration (A) and alcoholic fermentation (B) of Pinot noir grapes. Inoculation of *S. cerevisiae* RC212 occurred at end of cold maceration.

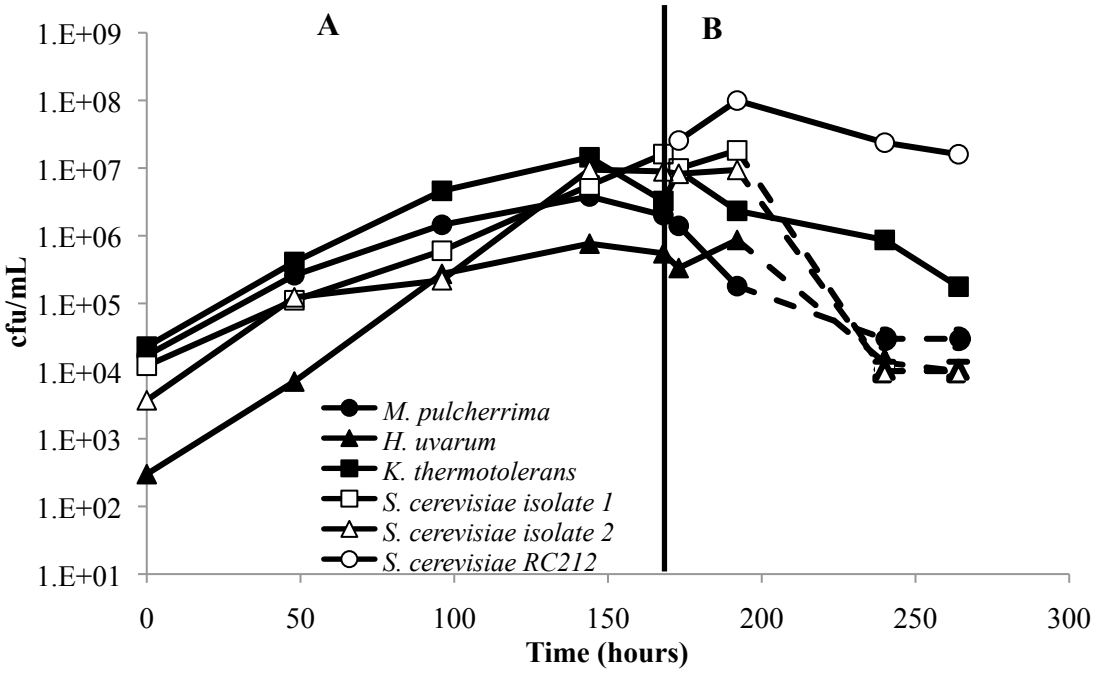

Figure 2.3 Growth of *Saccharomyces cerevisiae* RC212, *Metschnikowia pulcherrima*, *Hanseniaspora uvarum*, *Kluveromyces thermotolerans*, *Saccharomyces cerevisiae* isolate 1, and *Saccharomyces cerevisiae* isolate 2 during pre-fermentation cold maceration (A) and alcoholic fermentation (B) of Pinot noir grapes. Inoculation of *S. cerevisiae* RC212 occurred at end of cold maceration.

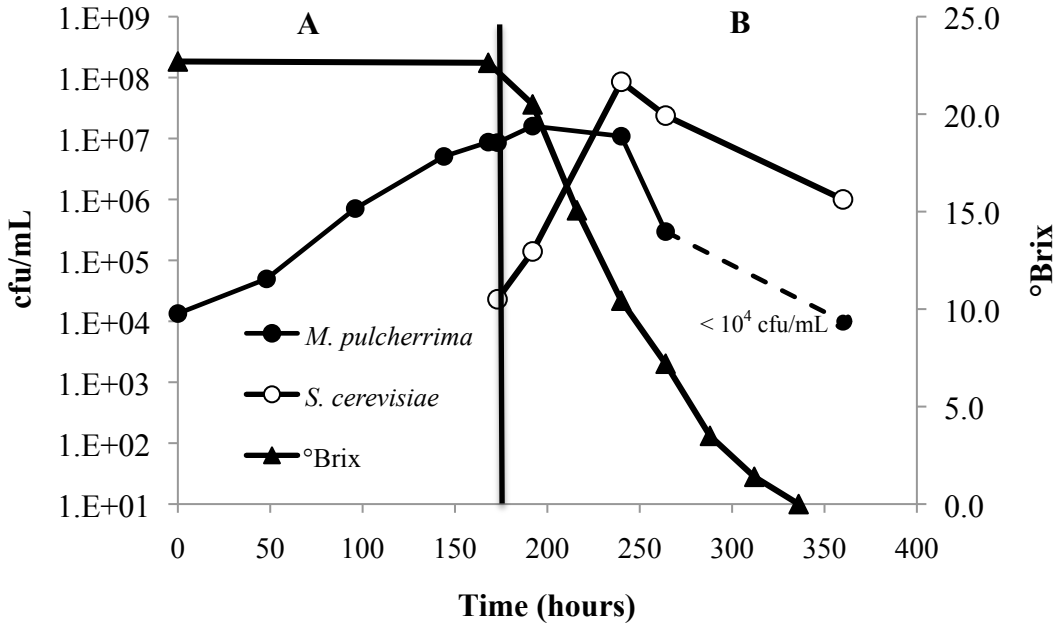

Figure 2.4 Growth of *Saccharomyces cerevisiae* RC212, *Metschnikowia pulcherrima*, and °Brix during pre-fermentation cold maceration (A) and alcoholic fermentation (B) of Pinot noir grapes. Inoculation of *S. cerevisiae* RC212 occurred at end of cold maceration.

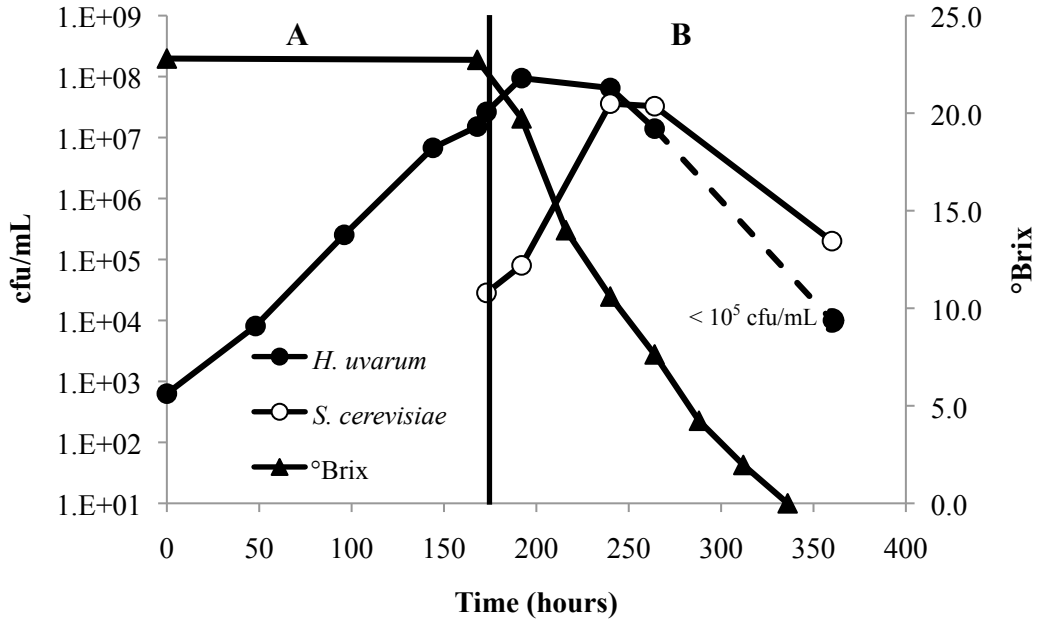

Figure 2.5 Growth of *Saccharomyces cerevisiae* RC212, *Hanseniaspora uvarum*, and °Brix during pre-fermentation cold maceration (A) and alcoholic fermentation (B) of Pinot noir grapes. Inoculation of *S. cerevisiae* RC212 occurred at end of cold maceration.

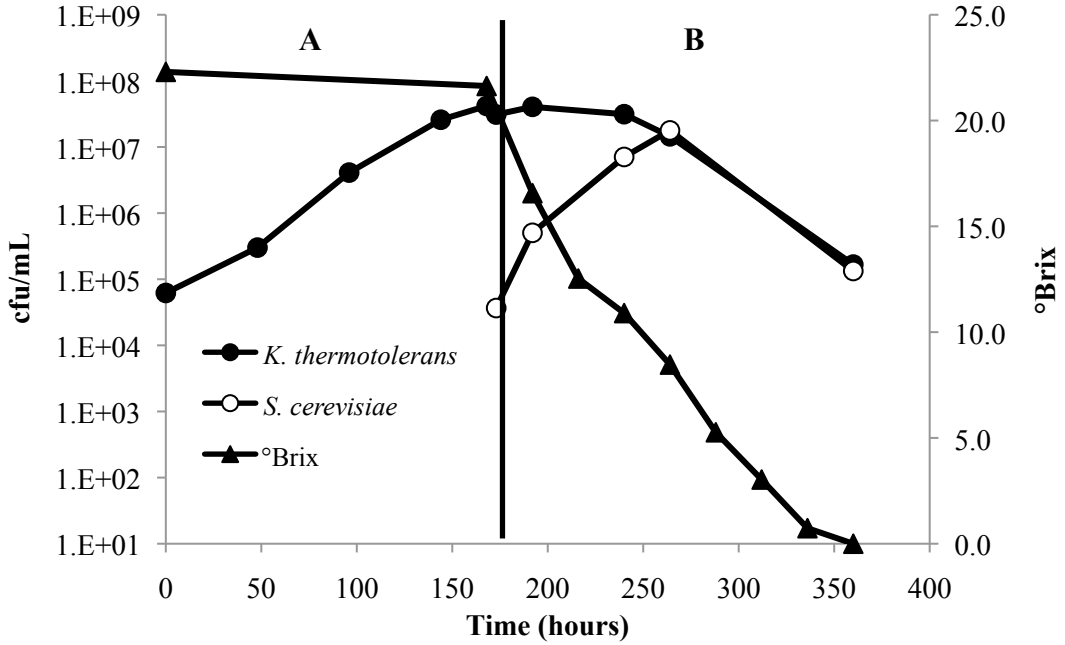

Figure 2.6 Growth of *Saccharomyces cerevisiae* RC212, *Kluveromyces thermotolerans*, and °Brix during pre-fermentation cold maceration (A) and alcoholic fermentation (B) of Pinot noir grapes. Inoculation of *S. cerevisiae* RC212 occurred at end of cold maceration.

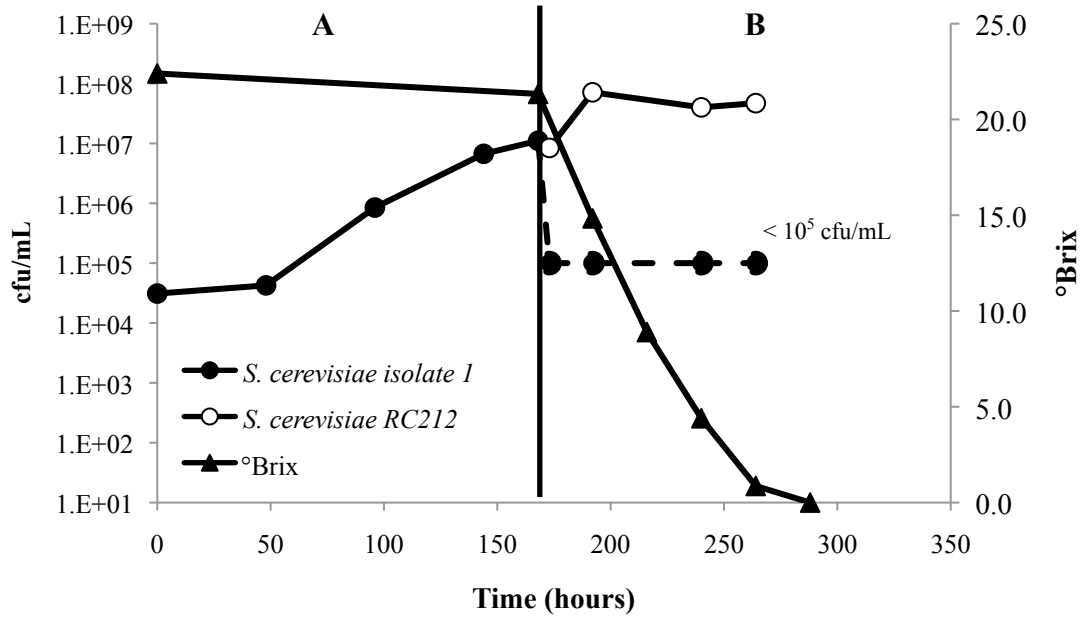

Figure 2.7 Growth of *Saccharomyces cerevisiae* RC212, *Saccharomyces cerevisiae* isolate 1, and °Brix during pre-fermentation cold maceration (A) and alcoholic fermentation (B) of Pinot noir grapes. Inoculation of *S. cerevisiae* RC212 occurred at end of cold maceration.

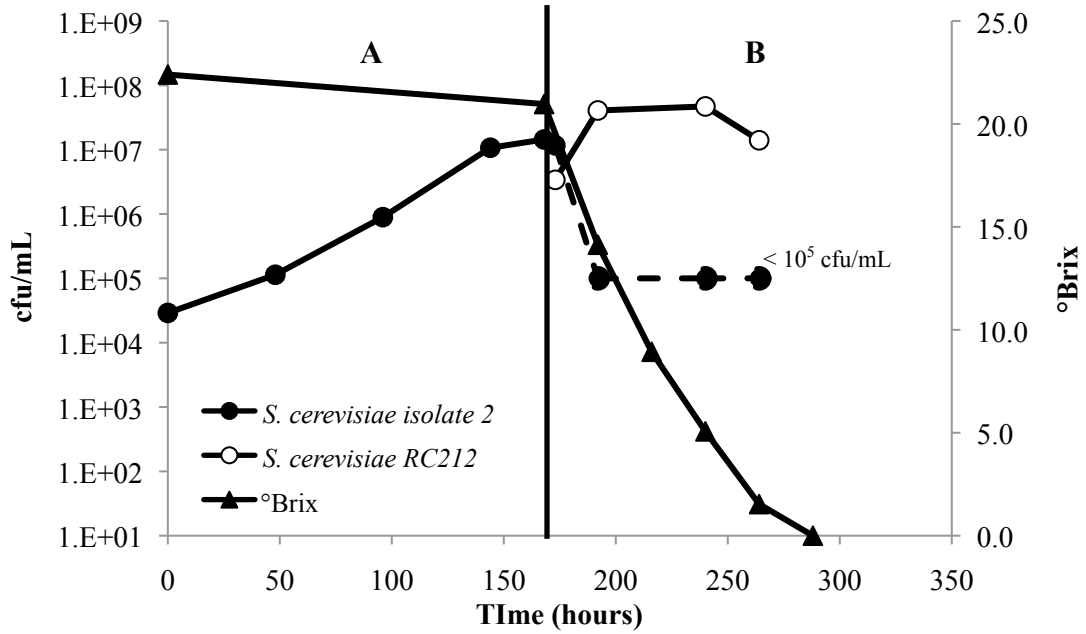

Figure 2.8 Growth of *Saccharomyces cerevisiae* RC212, *Saccharomyces cerevisiae* isolate 2, and °Brix during pre-fermentation cold maceration (A) and alcoholic fermentation (B) of Pinot noir grapes. Inoculation of *S. cerevisiae* RC212 occurred at end of cold maceration.

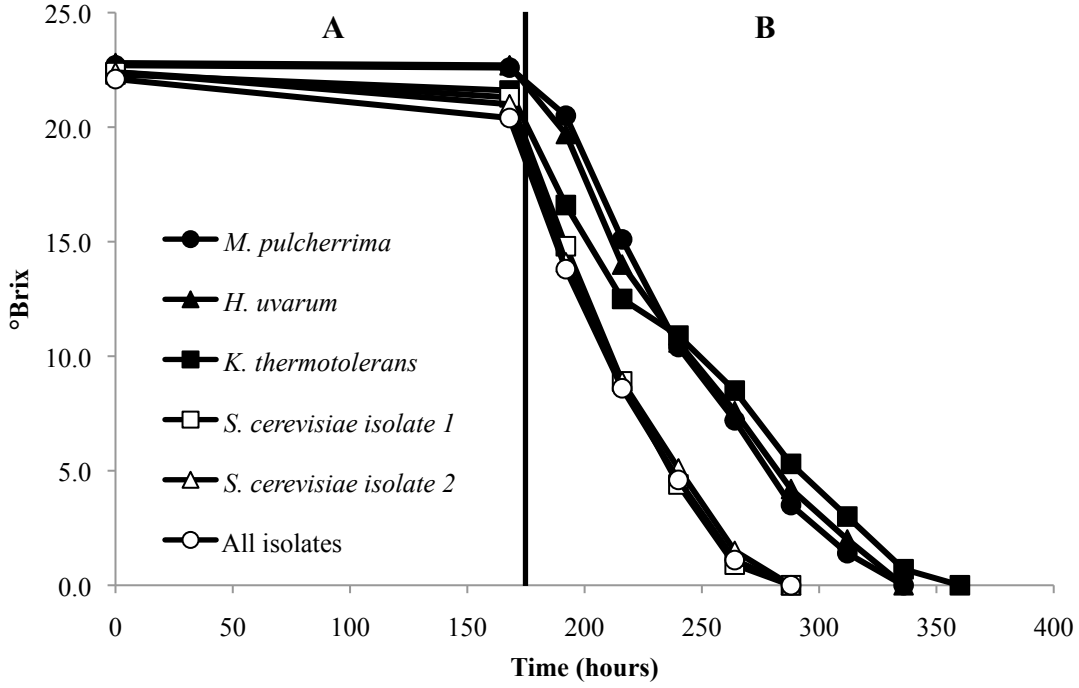

Figure 2.9 Change in °Brix during cold maceration (A) and alcoholic fermentation (B) of Pinot noir grapes inoculated with a single yeast isolates or combination of all isolates followed by inoculation of *S. cerevisiae* RC212 at completion of cold maceration.

Table 2.1 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 2.2 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 2.3 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

Volatile Aroma

A total of more than 50 compounds were identified in the wines with the SPME-GC-MS method. Of those identified, 40 compounds were quantified. A selection of key aroma-active compounds, including esters, alcohols, acids, terpene alcohols, and C_{13}-norisoprenoids, is presented. The esters listed in Table 2.1 include the ethyl esters (ethyl acetate, ethyl butanoate, ethyl hexanoate, ethyl octanoate, and ethyl decanoate), branch-chained esters (ethyl isobutyrate, isobutyl acetate, and 2-methyl-butyl acetate), and aromatic esters (ethyl phenylacetate and phenethyl acetate). In general, the treatment that did not include a pre-fermentation cold maceration (No CS) had higher levels of ethyl esters while the treatment that included a pre-fermentation without microorganisms (CS) had the lower levels compared to all the treatments (Table 2.1). The treatment inoculated with *H. uvarum* (CS + *H. uvarum*) had the highest levels of ethyl acetate. However, of the treatments with single isolate inoculation prior to pre-fermentation cold maceration, the treatment inoculated with *S. cerevisiae* isolate 2 (CS + *S. cerevisiae* 2) had the highest levels of ethyl butanoate, ethyl hexanoate, and ethyl octanoate.

The levels of branch-chained esters were generally higher in pre-fermentation treatments with individual species present. The CS + *M. pulcherrima* treatment had the highest levels of isoamyl acetate and 2-methyl-butyl acetate and second highest level of isobutyl acetate. While the CS + *K. thermotolerans* had the highest level of ethyl isobutyrate and CS + *H. uvarum* had the highest level of isobutyl acetate. In general, the levels of aromatic esters were lowest in the treatment that did not include a pre-fermentation cold maceration (No CS).

The alcohols and organic acids quantified are listed in Table 2.2. In general, the highest levels of alcohols were found in the No CS treatment. However, the pre-fermentation cold

maceration treatment containing a mixture of all isolates (CS + microorganisms) and CS + *S. cerevisiae* 2 had the highest levels of 2-phenylethanol. The levels of decanoic and dodecanoic acid were similar among all treatments. The No CS treatment had the highest levels of hexanoic and octanoic acid. While the CS + *S. cerevisiae* 2 treatment had the highest levels of hexanoic and octanoic acid among the pre-fermentation cold maceration treatments.

The C_{13}-norisoprenoids and terpene alcohols are listed in Table 2.3. The concentrations of β-damascenone were very similar for all treatments. However, the CS + microorganisms, CS + *H. uvarum*, and CS + *K. thermotolerans* treatments had slightly lower concentrations. The concentration of β-ionone did not differ significantly among the treatments. In this study five monoterpene alcohols were compared. In general, the pre-fermentation treatment without microorganisms had the highest concentration of terpene alcohols, except for β-citronellol. The CS + *S. cerevisiae* 1, CS + *S. cerevisiae* 2, and CS + microorganisms all had as much as twice the concentration of β-citronellol compared to the other treatments.

DISCUSSION

There is increased interest in the contribution of various yeast species and strains to wine aroma, in particular the contribution of non-*Saccharomyces* yeast that may produce different levels of aroma volatile compounds than *S. cerevisiae*. In this study a number of different yeast species isolated from Pinot noir wines undergoing pre-fermentation cold maceration were investigated to determine their impact on Pinot noir aroma. All yeast grew well during the cold maceration period with similar increases in population being observed. In addition, growth of high populations of non-*Saccharomyces* yeast did not hinder the growth of *S. cerevisiae* RC212 and alcoholic fermentation was completed in a similar time for all treatments. This finding is in contrast to the suggestion by Bisson (1999) that excessive growth of non-*Saccharomyces* yeast may result in stuck or sluggish fermentations. Whether

stuck or sluggish fermentations are the result of growth of non-S*accharomyces* yeast is probably more determined by the starting nutritional composition of the grape must. If the grape must originally contains low levels of nitrogen then further depletion of nitrogen by yeast during cold maceration could contribute to stuck or sluggish fermentations. This was not the case in the present study as the starting yeast available nitrogen (YAN) was approximately 150 mg/L, a level considered adequate for a healthy fermentation (Bisson and Butzke 2000).

Despite similarities in yeast growth, the concentrations of volatile compounds present in the wines produced from each treatment were different. In general, wines that underwent a pre-fermentation cold maceration contained lower concentrations of ethyl esters. Ethyl esters are derived from medium chain fatty acids formed by yeast during fatty acid biosynthesis (Malcorps and Dufour 1992) and their production is reported to vary with differing grape nitrogen composition (Herraiz and Ough 1993, Miller et al. 2007). Therefore, differences in ethyl ester concentrations may have been due to compositional changes caused by the growth of the yeast present during the cold maceration. The high population of yeast may have reduced the pool of nutrients available for the synthesis of ethyl esters by *S. cerevisiae* RC212. However, this does not fully explain the differences observed. For example, the un-inoculated pre-fermentation cold maceration treatment contained higher levels of ethyl esters than the treatment that did not undergo a pre-fermentation cold maceration despite having no yeast present during the cold maceration. Analysis of the amino acid composition of the grape must at the end of the cold maceration should be conducted to determine if the growth of yeast caused any alteration of the amino acid profile. In addition, the concentration of ethyl esters at the end of cold maceration should be assessed to determine the levels of esters produced by the yeast prior to inoculation with *S. cerevisiae* RC212.

In contrast to the ethyl esters, the concentrations of branch-chained esters were higher in wines that had undergone a pre-fermentation cold maceration. Branch-chained esters are derived from the products (alcohols) of the degradation of amino acids, carbohydrates, and lipids (Miller et al. 2007) and amino acid composition of the must can impact their production in wine (Herraiz and Ough 1993, Miller et al. 2007). It is possible that the increase in branch-chained esters in the wine that had undergone pre-fermentation cold maceration was due to greater pool of amino acids being available in these wines compared to the wine that did not undergo a pre-fermentation cold maceration. Again, analysis of the composition of the grape must post cold maceration would be required before any conclusions could be made regarding the mechanism by which yeast growth during cold maceration impacts ester production.

In addition to the overall trends observed in ester production, the growth of individual yeast isolates during pre-fermentation cold maceration resulted in wines with higher concentrations of particular esters. The treatment inoculated with *H. uvarum* had the highest concentration of ethyl acetate among all treatments. This finding was not surprising given that many other studies have reported high production of ethyl acetate by certain strains of *H. uvarum* (Ciani and Maccarelli 1998, Rojas et al. 2001, Viana et al. 2008). However, the concentration of ethyl acetate in this treatment (64 mg/L) was below the 150 mg/L level considered to produce an off odor in wine (Garde-Cerdán and Ancín-Azpilicueta 2006) and in fact at lower levels ethyl acetate may be considered a desirable wine aroma (Etievant 1991). The treatment inoculated with *H. uvarum* also had high concentrations of the branch-chained esters, isoamyl acetate and isobutyl acetate along with the treatment inoculated with *M. pulcherrima*. The impact of non-*Saccharomyces* yeast on ester production has been reported in other studies (Lema et al. 1996, Viana et al. 2008). However, many of these studies focused on ester production during white wine production. To our knowledge this is one of the few studies that has reported the impact of non-*Saccharomyes* yeast on red wine, and in particular Pinot noir.

In addition, this study reports the impact of yeast growth during pre-fermentation maceration on ester production and is unique in this respect.

There were some differences in the concentrations of higher alcohols among the treatments, but in general aromatic alcohol concentrations were higher in the treatment that did not undergo a pre-fermentation cold maceration and the un-inoculated pre-fermentation cold maceration treatment. Higher alcohols are synthesized by yeast and are derived from branch-chained amino acids (Sweigers et al. 2005). Their production can be impacted by the amino acid content of grape must as well as yeast strain (Giudici et al. 1990, Hernández-Orte et al. 2006). The increased concentrations of higher alcohols in the treatment that did not undergo a pre-fermentation cold maceration and in the un-inoculated pre-fermentation cold maceration treatment could be due to the presence of a lower total yeast population and therefore a greater pool of available amino acids. It has also been reported that non-*Saccharomyces* yeast can produce varying levels of higher alcohols, but usually lower than that of *S. cerevisiae* (Jolly et al. 2006). This is consistent with the findings of this study regarding the pre-fermentation cold maceration treatments. The treatments inoculated with non-*Saccharomyces* isolates (*M. pulcherrima, H. uvarum, K. thermotolerans*) had lower concentrations of higher alcohols than those treatments inoculated with the *S. cerevisiae* isolates. In addition, the treatments inoculated with *S. cerevisiae* 1, *S. cerevisiae* 2, and all of the isolates prior to pre-fermentation cold maceration had significantly higher concentrations of 2-phenylethanol. This compound is has been reported as an important odorant for Pinot Noir and can contribute honey or rose aromas to the wine (Fang and Qian 2006).

In addition to generating aroma active compounds during fermentation, some yeast also possess β-glucosidase activity that can result in the release of sugar bound grape derived aroma compounds (Delcroix et al. 1994, Charoenchai et al. 1997, McMahon et al. 1999,

Hernández et al. 2003, Ugliano et al. 2006, Swangkeaw et al. 2011). The yeast isolates used in this study had previously demonstrated varying levels of β-glucosidase activity under conditions of pre-fermernation cold maceration (see Chpt. 1). However, that study had assessed the activity of the yeast towards a synthetic glycoside, p-NPG, rather than grape glycosides. In this research the concentrations of some grape derived aroma compounds were impacted by yeast while others were not. For example, the concentrations of β-ionone and β-damascenone were very similar among all the treatments while the concentrations of terpene alcohols differed. β-ionone and β-damascenone are very important to the aroma of Pinot Noir, contributing aromas of berry or violet (β-ionone) and exotic flowers or heavy fruit (β-damascenone) (Fang and Qian 2006) and although the concentrations of these compounds were within the range of what has been reported for Pinot Noir (Fang and Qian 2006) their concentrations were not impacted by yeast that had previously demonstrated high β-glucosidase activity. In addition, the concentration of C_{13}-norisoprenoids was not impacted by the pre-fermentation cold maceration itself, whether yeast were present or not. This finding suggests that cold maceration does not increase the concentrations of these compounds in Pinot noir wine.

In contrast to the findings for the C13-norisoprenoids, some differences in the concentrations of terpene alchols among the treatments were observed. Linalool, α-terpineol, nerol, and geraniol were present in the highest concentrations in the un-inoculated pre-fermernation cold maceration treatment and their concentrations were consistent with what has been reported for Pinot noir (Fang and Qian 2006). However, the concentration of β-citronellol was as much as twice as high in the treatments inoculated with *S. cerevisiae* isolate 1, *S. cerevisiae* isolate 2, and a combination of all the yeast. These results support the previous finding where *S. cerevisiae* isolate 1 and *S. cerevisiae* isolate 2 demonstrated increased β-glucosidase activity under conditions of pre-fermentation cold maceration (see Chpt. 1). To our knowledge this is

the first report of yeast increasing the free terpene alcohol content of Pinot noir wine. It should be noted however, that the concentrations of β-citronellol observed, although high for Pinot noir, were still below the published sensory threshold in wine (Guth 1997). Never the less, the results of this study will be valuable in determining future research directions. For example, fermentations of grape varieties containing higher concentrations of terpene alcohols, such as Riesling, should be conducted utilizing the high β-glucosidase producing *S. cerevisiae* isolate 1 and isolate 2 yeast.

CONCLUSION

Yeast strains known to have β-glucosidase activity were inoculated into Pinot noir grape must prior to pre-fermentation cold maceration. The presence of these different yeast isolates resulted in wines with unique volatile composition demonstrating the influence these yeast can have during pre-fermentation cold maceration. Pre-fermentation cold maceration altered the volatile aroma profile of the wines compared to wines produced with no pre-fermentation cold maceration. In addition, the presence of microorganisms during the pre-fermentation cold maceration also produced wines with a distinct volatile aroma profile. This demonstrates that pre-fermentation cold maceration does impact wine aroma and that the changes are likely due to a combination of chemical extractive processes as well as microbial activity. In some cases the concentrations of certain grape derived compounds were unaffected by either the pre-fermentation cold maceration and/or microbial activity (β-ionone) while in other cases the concentrations of certain compounds were impacted (β-citronell) demonstrating yeast β-glucosidase activity. Future work should involve sensory evaluation of the wines produced with different yeast present during pre-fermentation cold maceration as well as the fermentations of grape varieties containing higher concentrations of terpene alcohols, such as Riesling.

SUMMARY

Wine aroma is one of the most important aspects of wine quality. An increased understanding of the contribution of certain yeast strains to wine aroma and how their growth might be encouraged or discouraged would be beneficial to wine quality. The persistence and growth of certain yeast species during pre-fermentation maceration as well as the high β-glucosidase activities observed for some species under conditions of high sugar and low temperature demonstrated the potential impact these yeast may have on wine aroma. The presence of *M. pulcherrima*, *K. thermotolerans*, *H. uvarum*, and cold tolerant *S. cerevisiae* during pre-fermentation cold maceration impacted the volatile chemical composition of Pinot Noir wine. Pre-fermentation cold maceration in the absence of microorganisms altered the volatile profile of the wine as well, indicating that several factors are involved in the volatile aroma profile of red wine varieties such as Pinot noir and further research is required to fully understand all the mechanisms involved.

Future work should include volatile and amino acid analysis of the grape must after pre-fermentation cold maceration in order to better understand the specific impact of these yeast isolates on aroma and nutrient status before inoculation for alcoholic fermentation. In addition, sensory analysis of the wines produced should be performed to determine how the unique chemical profiles of the wines may have impacted the perception of the wine aroma. The ability of certain yeast isolates to impact the concentration of free terpenes should be investigated using a grape variety with higher concentrations of terpene alcohols such as Riesling. In addition, further studies should be performed with these yeast isolates in different combinations to determine what impact interactions between these species during pre-fermentation cold maceration might have on the final aroma of the wine.

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APPENDICIES

APPENDIX A
Yeast Isolate DNA sequences of D1/D2 domain of 26S rDNA gene

Metschnikowia pulcherrima, 518 bp

ANNNNNNNNNGNCCNCCNTCACTGCATAGTGAGCGGCAAAAGCTCAAATTTGAA
ATCCCCCGGGAATTGTAATTTGAAGAGATTTGGGTCCGGCCGGCAGGGGTTAAGT
CCACTGGAAAGTGGCGCCACAGAGGGTGACAGCCCCGTGAACCCCTTTAACGCCC
TCATCCCAGATCTCCAAGAGTCGAGTTGTTTGGGAATGCAGCTCTAAGTGGGTGG
TAAATTCCATCTAAAGCTAAATACCGGCGAGAGACCGATAGCGAACAAGTACAGT
GATGGAAAGATGAAAAGCACTTTGAAAAGAGAGTGAAAAAGTACGTGAAATTGT
TGAAAGGGAAGGGCTTGCAAGCAGACACTTAACTGGGCCAGCATCGGGGCGGCG
GGGAGCAAAACCACCGGGGAATGTACCTTTCGAGGATTATAACCCCGGTCCTTAC
TCCCTCACCATCCCGAGGCCTGCAATCTAAGGATGCTGGCGTAATGGTTGCAAGT
CGCCCGTCTTGAANCACGGACCANNA

Hanseniaspora uvarum, 596 bp

NNNNNGNGGNNNTNCCTTAGTNNNGGCGAGTGAAGCGGTAAAAGCTCAAATTTG
AAATCTGGTACTTTCAGTGCCCGAGTTGTAATTTGTAGAATTTGTCTTTGATTAGG
TCCTTGTCTATGTTCCTTGGAACAGGACGTCATAGAGGGTGAGAATCCCGTTTGGC
GAGGATACCTTTTCTTTGTAAGACTTTTTCGAAGAGTCGAGTTGTTTGGGAATGCA
GCTCAAAGTGGGTGGTAAATTCCATCTAAAGCTAAATATTGGCGAGAGACCGATA
GCGAACAAGTACAGTGATGGAAAGATGAAAAGAACTTTGAAAAGAGAGTGAAAA
AGTACGTGAAATTGTTGAAAGGGAAGGGCATTTGATCAGACATGGTGTTTTTTGC
ATGCACTCGCCTCTCGTGGGCTTGGGCCTCTCAAAAATTTCACTGGGCCAACATCA
ATTCTGGCAGCAGGATAAATCATTAAGAATGTAGCTACTTCGGTAGTGTTATAGC
TTTTTGGAATACTGTTAGCCGGGATTGAGGACTGCGCTTCGGCAAGGATGTTGGC
ATAATGGTTAAATGCCGCCCGTCTTGAACCACGGGACCANAAAA

Kluveromyces thermotolerans, 590 bp

NNNNNNNGNNTNNNNNTNAGTACGGCGAGTGAAGCGGCAAAAGCTCAAATTTGA
AATCTGGCACCTTCGGTGTCCGAGTTGTAATTTGAAGAAGCTACTTTGGGGCTAGT
CCTTGTCTATGTTCCTTGGAACAGGACGTCATGGAGGGTGAGAATCCCGTATGGC
GAGGAGTCTAGTCCTATGTAAAGTGCTTTCGACGAGTCGAGTTGTTTGGGAATGC
AGCTCTAAGTGGGTGGTAAATTCCATCTAAAGCTAAATATTGGCGAGAGACCGAT
AGCGAACAAGTACAGTGATGGAAAGATGAAAAGAACTTTGAAAAGAGAGTGAAA
AAGTACGTGAAATTGTTGAAAGGGAAGGGCATTTGATCAGACATGGTGTTTTGCG
ACCCTCGCTCCTTGTGGGTGGGGATCTCGCAGCTCACTGGGCCAACATCAGTTTTG
GCGGTAGGATAAATCTTTGGGAACGTGGCTTGTCTTCGGAGAAGCGTTATAGCCC
AGGGGAATACTGCCAGCCGGGACTGAGGACTGCGACTTTTGTCAAGGATGTTGGC
ATAATGGTTAAATGCCGCCCGTCTTGAANCANGGGACCAN

Saccharomyces cerevisiae isolate 1, 598 bp

NNNNNNNGNNNNNTNNNTNANTNAANNGNNNGNGAAGCGGCAAAAGCTCAAAT
TTGAAATCTGGTACCTTCGGTGCCCGAGTTGTAATTTGGAGAGGGCAACTTTGGG
GCCGTTCCTTGTCTATGTTCCTTGGAACAGGACGTCATAGAGGGTGAGAATCCCGT
GTGGCGAGGAGTGCGGTTCTTTGTAAAGTGCCTTCGAAGAGTCGAGTTGTTTGGG
AATGCAGCTCTAAGTGGGTGGTAAATTCCATCTAAAGCTAAATATTGGCGAGAGA
CCGATAGCGAACAAGTACAGTGATGGAAAGATGAAAAGAACTTTGAAAAGAGAG
TGAAAAAGTACGTGAAATTGTTGAAAGGGAAGGGCATTTGATCAGACATGGTGTT
TTGTGCCCTCTGCTCCTTGTGGGTAGGGGAATCTCGCATTTCACTGGGCCAGCATC
AGTTTTGGTGGCAGGATAAATCCATAGGAATGTAGCTTGCCTCGGTAAGTATTAT
AGCCTGTGGGAATACTGCCAGCTGGGACTGAGGACTGCGACGTAAGTCAAGGAT
GCTGGCATAATGGTTATATGCCGCCCGTCTTGAAACACGGACCAAANNAA

Saccharomyces cerevisiae isolate 2, 596 bp

NNNNNGGNNNNNGCCCTTTAGTTAACGGGCGANTGAAGCGGCAAAAGCTCAAAT
TTGAAATCTGGTACCTTCGGTGCCCGAGTTGTAATTTGGAGAGGGCAACTTTGGG
GCCGTTCCTTGTCTATGTTCCTTGGAACAGGACGTCATAGAGGGTGAGAATCCCGT
GTGGCGAGGAGTGCGGTTCTTTGTAAAGTGCCTTCGAAGAGTCGAGTTGTTTGGG
AATGCAGCTCTAAGTGGGTGGTAAATTCCATCTAAAGCTAAATATTGGCGAGAGA
CCGATAGCGAACAAGTACAGTGATGGAAAGATGAAAAGAACTTTGAAAAGAGAG
TGAAAAAGTACGTGAAATTGTTGAAAGGGAAGGGCATTTGATCAGACATGGTGTT
TTGTGCCCTCTGCTCCTTGTGGGTAGGGGAATCTCGCATTTCACTGGGCCAGCATC
AGTTTTGGTGGCAGGATAAATCCATAGGAATGTAGCTTGCCTCGGTAAGTATTAT
AGCCTGTGGGAATACTGCCAGCTGGGACTGAGGACTGCGACGTAAGTCAAGGAT
GCTGGCATAATGGTTATATGCCGCCCGTCTTGAAACACGGACCANAN

APPENDIX B
Aroma descriptors of volatile analysis compounds

Compound	Descriptor
Ethyl acetate (mg/L)	Fruity, solvent
Ethyl butanoate (μg/L)	Banana
Ethyl hexanoate (μg/L)	Pineapple, fruity, apple
Ethyl octanoate (μg/L)	Waxy, apple skin, fruity
Ethyl decanoate (μg/L)	Waxy, soapy, fruity
Ethyl isobutyrate (μg/L)	Fruity
Isobutyl acetate (μg/L)	Waxy, fruity, apple, banana
Isoamyl acetate (μg/L)	Banana, fruity, sweet
2-methyl-butyl acetate (μg/L)	Fruity, fatty, pleasant
Ethyl phenylacetate (μg/L)	Flowery, rose
Phenethyl acetate (μg/L)	Pleasant, flowery
Isobutyl alcohol (mg/L)	Fusel, alcohol
Isoamy alcohol (mg/L)	Alcohol, harsh
1-Hexanol (mg/L)	Green, grass
1-Octanol (μg/L)	Orange, rose
1-Nonanol (μg/L)	Green
Benzyl alcohol (μg/L)	Sweet, fruity
2-phenylethanol (mg/L)	Roses
Hexanoic acid (μg/L)	Sweat, cheese
Octanoic acid (μg/L)	Rancid, harsh, cheese
Decanoic acid (μg/L)	Fatty, unpleasant
Dodecanoic acid (μg/L)	Dry, metallic, laurel oil
β-Damascenone (μg/L)	Apple, heavy fruit, exotic flowers
β-Ionone (μg/L)	Berry, violet
Linalool (μg/L)	Flowery, fruity, Muscat
α-Terpineol (μg/L)	Root, grass, anise
β-Citronellol (μg/L)	Green lemon
Nerol (μg/L)	Sweet rose
Trans-Geraniol (μg/L)	citric