

The effects of different processing parameters (cold soak and percent alcohol (v/v) at dejuicing) on the concentrations of grape glycosides and glycoside fractions and glycosidase activities in selected yeast and lactic acid bacteria.

by

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ABSTRACT

Grape-derived aroma and flavor precursors exist partially as non-volatile, sugar-bound glycosides. Hydrolysis of these compounds may modify sensory attributes and potentially enhance wine quality. Cold soak (prefermentation skin contact) at two temperatures and alcohol content (% v/v) at dejuicing were monitored to determine effects on Cabernet Sauvignon glycoside concentration. Total, phenolic-free, and red-free glycoside concentrations were estimated by the quantification of glycosyl-glucose. Cold soak (5 days at 10°C) increased total glycosides by 77%, red-free glycosides by 80%, and phenolic-free glycosides by 96%. Ambient soak (3 days at 20°C) enhanced color extraction, and increased total glycosides by 177%, red-free glycosides by 144%, and phenolic-free glycosides by 106%. Wines produced by early pressing (10% sugar) had 25% more total and red-free glycosides than late press (0.25% sugar). After post-fermentation malolactic fermentation, total glycosides were 14% lower and phenolic-free glycosides were 35% lower.

In a second study, the activities of α -L-arabinofuranosidase, β -glucosidase, and α -L-rhamnoyranosidase were determined in model systems for thirty-two strains of yeasts belonging to the following genera: *Aureobasidium*, *Candida*, *Cryptococcus*, *Hanseniaspora*, *Hansenula*, *Kloeckera*, *Metschnikowia*, *Pichia*, *Saccharomyces*, *Torulaspora*, and *Brettanomyces* (10 strains); and seven bacteria (*Leuconostoc oenos* strains). Only one *Saccharomyces* strain exhibited β -glucosidase activity, but several non-*Saccharomyces* yeast species had substantial production.

Aureobasidium pullulans hydrolyzed α -L-arabinofuranoside, β -glucoside, and α -L-rhamnopyranoside. Eight *Brettanomyces* strains had β -glucosidase activity. Location of enzyme activity was determined for those species with enzymatic activity. The majority of β -glucosidase was located in the whole cell fraction (66%), followed by the permeabilized fraction (35%), and extracellular production (2%). *Aureobasidium pullulans* was also capable of hydrolyzing grape glycosides.

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INTRODUCTION

Wine quality is influenced by grape aroma and flavor compounds which are, in part, bound to sugars, known as glycosides. Glycosidic precursors are non-volatile compounds which may be hydrolyzed during fruit maturation, vinification, and aging. Hydrolysis products (aglycones) are a complex group of chemicals with varied quantitative and qualitative effects on aroma, flavor, color, and structure. Those glycosides which contain aroma and flavor aglycones affect wine quality only after hydrolysis. Consequently, research concerning techniques to increase extraction and hydrolysis of glycosides may lead to enhanced product quality.

Complete identification and quantification of the pool of potential volatiles would be a tremendous commitment of time and resources. Hydrolysis of glycosidically-bound secondary plant metabolites produces equimolar ratios of D-glucose (known as glycosyl-glucose) and aglycones. Therefore, quantification of glycosyl-glucose (G-G) estimates the concentration of bound flavor components. By monitoring levels of G-G in juice and throughout vinification, hydrolysis and the liberation of grape-derived aroma and flavor compounds may be inferred.

Limited glycoside hydrolysis occurs during vinification by microbiological enzymes as well as acid catalyzed hydrolysis. The objective of this research was to investigate the effects of processing techniques on the concentration of Cabernet Sauvignon grape glycosides and glycoside fractions and to determine the ability of selected yeasts and bacteria to hydrolyze glycosides.

CHAPTER I: REVIEW OF THE LITERATURE

A. Glycosides

Wine quality is dependent on aroma and flavor compounds which may exist either as free volatiles or bound glycoconjugates (Abbott *et al.*, 1993, Williams *et al.*, 1995). Abbott *et al.* (1991) found a higher glycoside concentration in Shiraz wines produced from vineyards reporting high quality grapes while a low concentration coincided with low quality fruit. Glycosides are primarily located in juice, rather than the skin or pulp fractions as indicated by research on glycosylated monoterpenes (Wilson *et al.*, 1986). Formation of glycosides occurs during grape maturation and is theorized to be the result of glycosyltransferases which catalyze the relocation of carbohydrates from sugar-carrying nucleotides to aglycones (Williams *et al.*, 1982). When bound to an intermediary of glycopyranose, glycosides form disaccharide complexes such as α -L-rhamnopyranosyl- β -D-glycopyranosides or α -L-arabinofuranosyl- β -D-glycopyranosides (Cordonnier *et al.*, 1986). Complete enzyme catalysis of these compounds occurs in two steps: 1. Glucose is separated from the terminal sugar by hydrolase (α -L-arabinofuranosidase, α -L-rhamnosidase, β -apiosidase); 2. The bond between the aglycone and glucose (the monoterpenyl β -D-glucoside) is split by β -glucosidase (Gunata *et al.*, 1988). Glycosides are not volatile, and those which contain aroma and flavor aglycones only potentially affect wine quality after hydrolysis. Liberation of aglycones may occur either enzymatically through yeast β -glucosidase or via acid hydrolysis (Francis *et al.*, 1992, 1996; Gunata *et al.*, 1985; Williams *et al.*, 1982). Hydrolysis of the glycosides creates equimolar concentrations of aglycones and D-glucose (also known as glycosyl-glucose or G-G) (Williams *et al.*, 1995). As an alternative to laborious quantification of the various aglycones,

determination of G-G concentration allows an inference of the amount of glycosylated secondary metabolites (Williams *et al.*, 1995). By comparing levels of G-G in the fruit to that in the wine, the decline in glycoside concentration can be monitored and possible hydrolysis and release of grape-derived volatiles can be estimated.

Aglycones may be aliphatic residues, monoterpenes, sesquiterpenes, norisoprenoids, or shikimic acid metabolites such as phenols (Abbott, 1993; Sefton *et al.*, 1993, 1994, 1996; Winterhalter *et al.*, 1990). Sefton *et al.* (1993) found that hydrolytically released Chardonnay aglycones were comprised of 70% norisoprenoids, 10-20% benzene derivatives, 5% monoterpenes, and aliphatic compounds comprised the remaining fraction.

B. Effects of Processing and Vinification on Glycosides

Few publications explore on the effects of processing parameters on total glycosides, although numerous publications document the changes in phenolic compounds as a result of vinification techniques. Phenols affect red wine color, astringency, bitterness, and other fundamental characteristics. Because of their structural and color contributions, phenolics have been correlated to quality (Singleton and Noble, 1976). Since many phenols are glycosides, some of the observed phenol results may be applicable to glycosides.

Anthocyanins and tannins are two major types of phenolic compounds (Ribereau-Gayon and Glories, 1987). Anthocyanins provide color while tannins impart astringency and bitterness. Anthocyanins are usually extracted within the first 4 to 5 days of fermentation, although influenced by variety, fruit maturity, and temperature (Feuillat, 1987; Powers *et al.*, 1980). During wine aging free anthocyanins decline with a concomitant formation of anthocyanin and tannin complexes

(Nagel and Wulf, 1979; Somers and Verette, 1988). Polymerization of anthocyanins and other phenolics enhances long term color stability (Scudamore-Smith *et al.*, 1990; Sims and Morris, 1985). Two hypotheses exist for the mechanism of polymerization: oxidative and non-oxidative. Oxidative results in tannins and nonoxidative yields condensed tannins (Ribereau-Gayon and Glories, 1987). The oxidative mechanism involves acetaldehyde and the formation of $-\text{CH}(\text{CH}_3)$ bridges. Somers and Evans (1986) proposed that the major polymerization mechanism involves direct condensation reactions between phenolic sub-units (non-oxidative). Augmented polymerization may result in precipitation and subsequent loss of color (Somers and Evans, 1986).

Young red wine color intensity ($A_{520} + A_{420\text{nm}}$) and hue ($A_{520}/A_{420\text{nm}}$) are influenced not only by the type of anthocyanin, but the degree of equilibrium among the color and colorless forms (Berg and Akiyoski, 1956 and Liao *et al.*, 1992). Maceration releases grape phenols, giving wine color and tannic structure (Ribereau-Gayon *et al.*, 1976). Auw *et al.* (1996) found that longer skin fermentation time led to increased hue ($A_{520}/A_{420\text{nm}}$) and decreased intensity ($A_{520\text{nm}} + A_{420\text{nm}}$) in Cabernet Sauvignon. These results are indicative of increased anthocyanin-tannin polymerization.

Cold Soak

During cold soak, crushed grapes are stored at a low temperature prior to fermentation. This prefermentation skin contact increases phenolic extraction and enhances color (Heatherbell *et al.*, 1997). The absence of alcohol allows the formation of anthocyanin/phenol complexes which stabilize wine color (Zoecklein *et al.*, 1996). Lower temperatures (4°C) have been shown to give darker, less bitter wines than higher temperatures (10°C) (Heatherbell *et al.*, 1997).

A linear relationship exists between fermentation temperature (15-33°C) and the extraction of anthocyanins. Low fermentation temperature (25°C) increases fermentation aromas, and results in a fresh, fruity, aromatic wine (Ribereau-Gayon and Glories, 1987). A wine with higher tannin levels occurs after fermentation at an elevated temperature (30°C) (Ribereau-Gayon and Glories, 1987). Heatherbell *et al.* (1997) compared cold maceration at temperatures of 10 and 4 °C. The higher temperature decreased visible color and imparted a woody-tobacco aroma and flavor and increased bitterness. Heatherbell *et al.* (1997) found that cold maceration in Pinot Noir increased anthocyanins, phenols and intensity. However, these differences occurred only in the presence of sulfur dioxide. Sulfur dioxide limits polymerization and copigmentation by binding flavonoid phenols at the carbon 4 in the phenol ring.

Alcohol at Dejuicing

Although the majority of anthocyanins are extracted within the first 10 ° reduction of Brix, tannin extraction occurs throughout the skin-contact period (Berg and Akiyoski, 1956). In general, delayed pressing results in an increase in phenolic extraction, while dejuicing prior to dryness enhances fruity characteristics, gives good initial color, low astringency, low total phenols and produces a light, floral wine (Somers and Evans, 1977). Wines pressed early (at a lower alcohol level) may display color instability (Bissell, 1981). Extended skin contact time gives complexity and better color stability due to higher tannin levels. Tannins stabilize anthocyanins by forming polymeric complexes (Scudamore-Smith *et al.*, 1990; Sims and Morris, 1985; Somers and Verette, 1988). However, these complexes may be broken in the presence of ethanol (Ribereau-Gayon and

Glories, 1987). Conversely, solubilization of phenols by ethanol may lead to color enhancement (Ribereau-Gayon and Glories, 1987).

C. β -Glucosidase

Limited aroma and flavor potential is naturally revealed during fruit maturation by endogenous β -glucosidases (Cordonnier *et al.*, 1986). Grapes possess limited endogenous β -glucosidase activity (Cordonnier and Bayonove, 1974). Grape β -glucosidase activity increases throughout berry ripening, with 85% of the activity occurring in the last 10° Brix (14 to 24 °Brix in Muscat of Alexandria) (Aryan *et al.*, 1987). Additional hydrolysis may occur during vinification by microbiological enzymes. *Botrytis cinerea* has α -arabinosidase and α -rhamnosidase activities which are transferred to infected berries (Gunata *et al.*, 1989).

Acidic hydrolysis of glycosides may also occur but can be undesirable due to modification of the aromatic character of the aglycones (Gunata, 1984; Williams *et al.*, 1982). In sensory trials on Merlot and Cabernet Sauvignon grape glycosides, acid catalyzed products contributed “intense berry and plum-like aromas”, while products from enzyme hydrolysis were virtually undetectable (Sefton, 1988). Also, Chardonnay acid hydrosylates contributed important varietal characteristics such as tea, lime and honey (Francis *et al.*, 1992). In contrast, Abbott *et al.*, (1991) found that Shiraz enzymatic hydrosylates enhanced quality related aroma characteristics, particularly “non-berry” attributes. Some acid-catalysis products may cause undesirable alteration of aroma (Gunata, 1984; Winterhalter *et al.*, 1990), making enzymatic hydrolysis more favorable. These differences may be the result of contrasting mechanisms between enzymatic and acid hydrolysis (Sefton, 1998). Enzymatic hydrolysis cleaves the glycosidic linkage without altering the aglycone, while acid

hydrolysis may split alcohol aglycones and produce a reactive carbocation (Sefton, 1998). Also, the pool of glycoconjugates which can be hydrolyzed enzymatically is larger than that which can be acid hydrolyzed (Sefton *et al.*, 1996).

Hydrolytic Enzyme Activity Inhibition

Although enological yeasts may have the ability to produce hydrolases, acidic wine conditions may cause denaturation and inhibition of activity (Delcroix *et al.* 1994). Rosi *et al.* (1997) illustrated the optimum pH for hydrolytic enzyme ability for *Debaryomyces hansenii* was 3.2. The optimum pH of *Candida wickerhamii* was found to be 4.5 (Leclerc *et al.*, 1987). Delcroix *et al.* (1994) monitored three strains of *Saccharomyces cerevisiae* and illustrated a 95% loss in enzymatic activity at wine pH.

In typical wine conditions production of glycosidases and/or activity would be strongly inhibited by low pH, as well as high alcohol concentration, lack of oxygen and presence of glucose (Gunata *et al.*, 1984). Delcroix *et al.* (1994) monitored three strains of *Saccharomyces cerevisiae* for β -glucosidase in Muscat juice and found activities dropped quickly after reaching their maximum during exponential growth. Many fungal and yeast β -glucosidases are not inhibited by the concentrations of ethanol in table wine (Aryan *et al.*, 1987; Delcroix *et al.*, 1994; Leclerc *et al.*, 1987). Among the documented species are: *Hanseniaspora vineae* (Vasserot *et al.*, 1989), *Dekkera intermedia* (Blondin *et al.*, 1983) and *Candida molischiana* (Gonde *et al.*, 1985). Conversely, grape and almond β -glucosidases can exhibit a loss of activity of 60% at the same ethanol concentration (Aryan *et al.*, 1987; Gunata *et al.*, 1984). Guegen *et al.* (1994) demonstrated the β -glucosidase from *Candida entomophila* was stimulated by alcohol up to a concentration of

3.5%, and then was inhibited at higher concentrations, most likely due to protein denaturation. Permberton *et al.* (1980) hypothesized that denaturation could be due to a glycosyl transferase activity of the enzyme. Ethanol acts as an acceptor for the intermediary glycosyl cation and has better nucleophilic character than water.

The presence of glucose (>0.5% (w/v)) can inhibit β -glucosidase production as was demonstrated in *Hanseniaspora vineae* (Vasserot *et al.*, 1989). Rosi *et al.* (1997) showed the optimum glucose concentration for β -glucosidase production in *Debaryomyces hansenii* was 2-8%. Some grape berry and enological yeast enzymes seem more resistant to glucose inhibition (Aryan *et al.*, 1987; Delcroix *et al.*, 1993; Dubourdieu *et al.*, 1988). *Candida wickerhamii* has been shown to retain 44% of its activity at normal levels of glucose in grape juice (500 mM glucose medium) (Gunata *et al.*, 1994).

Plant and microbial β -glucosidases can be inhibited by gluconolactone (Lecas *et al.*, 1991; Leclerc *et al.*, 1987). Inhibition is competitive and caused by its structural analogy with an intermediate product in the enzymatic cleavage of β -D-glucoopyranosides (Beer and Vasella, 1986). Grape fungal infection may enhance inhibition due to elevated concentrations of gluconolactone (5-10 mM) (Gunata *et al.*, 1989).

The glycosidase of *Hanseniaspora vineae* displays a lack of specificity, hydrolysing almost all alkyl and arly glucosides and other sugars with β (1-4) or β (1-2) configuration (Vasserot *et al.*, 1989). Rosi *et al.* (1997) demonstrated that *Debaryomyces hansenii* hydrolyzed glycosides of primary terpenic alcohols and the extent of hydrolysis was dependent on the type of cultivar. Enzymatic hydrolysis commonly results in a faster hydrolysis rate of primary alcohols than tertiary alcohols, while acid hydrolysis has an opposite tendency (Gunata *et al.*, 1994).

D. Malolactic fermentation and lactic acid bacteria

Malolactic fermentation (MLF) by lactic acid bacteria (LAB) can alter the acidity and sensory characteristics of wine (Henick-Kling *et al.*, 1993, 1994; Kunkee, 1976). The most common lactic acid bacteria which perform an uninoculated fermentation are *Leuconostoc oenos*, *Lactobacillus*, and *Pediococcus* (Davis *et al.*, 1986). [*Leuconostoc oenos* has been recently reclassified as *Oenococcus oeni* (Dicks *et al.*, 1995)]. Hydrogen ion concentration often determines which species will conduct the fermentation, as well as the time required for completion (Fugelsang, 1997). *Pediococcus* and *Lactobacillus* occur regularly in wines with a pH higher than 3.5 and are often inhibitory towards the generally preferred species, *Leuconostoc oenos*, which is the common dominant species in wines with pH below 3.5 (Davis *et al.*, 1986). Combinations among genera, species, and strain yield numerous possibilities (including either successive or concurrent fermentations). Fleet *et al.* (1984) documented three strains of *L. oenos* performing MLF in a single red wine. Malolactic fermentation is especially prevalent in wines from cooler climates, and is used to deacidify highly acidic wines which often come from these environments (Rodriguez *et al.*, 1990). However, some non-malate degrading mutants have been isolated, and may prove useful in imparting positive flavor characteristics without changing the acidity (Cox and Henick-Kling, 1990; Renault and Heslot, 1987).

Inoculation

Lactic acid bacteria may be inoculated at three different stages during the fermentation: 1. with the yeast, 2. during various stages of alcoholic fermentation, or 3. after completion of

alcoholic fermentation (Costello, 1993). Inoculation at stages 1 or 2 minimizes the need for SO₂ and has been shown to reduce MLF time span (Beelman and Kunkee, 1985). Inoculation times prior to or during alcoholic fermentation provide lower levels of alcohol and therefore offer a more hospitable environment, but competition with yeasts for available nutrients can result in a negative situation for both types of organisms. Inoculation of heterofermentative LAB prior to completion of alcoholic fermentation can result in stuck fermentations (Huang *et al.* 1996). LAB can utilize sugar, when present, at these stages and result in the unwanted accumulation of acetic and lactic acid (Costello, 1993).

Commercial preparations of LAB provide successful inoculations and have become increasingly easier to use. Freeze-dried cultures enable winemakers to store bacteria for longer periods of time with fewer (to zero) transfers. Some of these preparations do not require reactivation and can be directly inoculated. Nielsen *et al.* (1996) demonstrated 100% survival after direct inoculation with no lag phase. These conditions lead to faster completion of MLF, allowing the winemaker to begin bottling and, therefore, provide a lower chance of spoilage. A major drawback of this type of direct inoculation is the cost.

Starter cultures of LAB are often used in industry because they allow stricter control of MLF onset, increase rate of substrate conversion with reduced risk of contamination from unwanted organisms, and provide more predictable sensory effects. However, even with the use of commercially prepared LAB, reinoculation may be necessary. The fastidious nature of these organisms is generally associated with their strict nutritional demands, but Martineau and Henick-Kling (1995a) correlated initial growth problems with different wine varieties, illustrating other factors are involved. Native malolactic fermentations are still used widely in industry (Fugelsang

and Zoecklein, 1983), but are often characterized by extended lag phase (reflecting low initial numbers), rapid die-off, and protracted conversion (Fugelsang, 1997). The unpredictability of these fermentations may have increased in recent years because of heightened awareness of winery sanitation (Nielsen *et al.*, 1996).

Strains

Most successful native MLF s occur with multiple strains, partly because this decreases the effects of phage infection and the subsequent destruction of the LAB (Edwards and Beelman, 1989). Early research led to widespread availability and use of strains ML-34 and PSU-1 (Beelman *et al.*, 1977; Ingraham *et al.*, 1960). However, new isolates appear to be heartier and more efficient; access to these strains will lead to more reliable fermentations.

Yeast Interactions

LAB have high nutritional requirements, which is one of the reasons that their growth is sometimes difficult to induce (Fugelsang, 1997). Generally, extended lees contact is advantageous for growth of LAB due to the nutritional contribution of yeast autolysates, which release vitamins, amino acids, peptides, and nucleo-bases (Edwards and Beelman, 1989; Kunkee and Amerine, 1970; Renault and Heslot, 1987). However, some yeasts, particularly certain native strains, may be inhibitory to malolactic bacteria because of production of aldehydes, sulfites, and lipophilic medium-chain fatty acids (Edwards and Beelman, 1987; Fornachon, 1968; Lonvaud-Funel *et al.*, 1988). The main fatty acid responsible for LAB inhibition is decanoic acid, present naturally in wine in concentrations ranging from 0.64 to 14.0 mg/L (Edwards and Beelman, 1987). Artificially

added, decanoic acid was shown to depress the rate of MLF at concentrations greater than 5 mg/L, but this effect could be reversed upon the addition of yeast ghosts (Edwards and Beelman, 1987). Therefore, although live yeast may be inhibitory, yeast ghosts can not only aid MLF by increasing nutrient availability, they can also serve to remove toxic compounds and further enhance growth conditions for LAB.

Metabolites

The basic mechanism of malolactic fermentation is the conversion of L-malic acid to L-lactic acid with the production of carbon dioxide. The enzyme which catalyzes the reaction is malate carboxylase which requires NAD^+ and Mn^{2+} . However, in addition to utilizing malic acid, many other metabolites may be formed as the result of MLF. Lactic acid bacteria are divided into two categories: homo- and heterofermenters. Homofermentive bacteria, i.e. *Pediococcus*, and some species of *Lactobacillus* use the Embden-Meyerhof Parnas (EMP) pathway to convert glucose to lactic acid with 2 moles of ATP used per mole of glucose. Because they lack fructose-diphosphate aldolase, heterolactic bacteria, i.e. *Leuconostoc oenos*, must use the 6-phosphogluconate pathway to hydrolyze glucose and form lactic acid, acetic acid, and CO_2 (Fugelsang, 1997). Determining secondary metabolites of MLF has been difficult, as these compounds may be caused by reactions actually catalyzed by residual grape and yeast enzymes (Davis *et al.*, 1986). *Pediococcus parvulus* has been shown to use glucose and fructose, while *L. oenos* utilizes *myo*-inositol, ribose, and arabinose (Davis *et al.*, 1986). Propionic and tartaric acids are utilized by LAB while acetic acid is produced (Avedovech *et al.*, 1992). Volatile acidity increases by 0.1-0.2 g/L after MLF completion. Tartaric acid degradation occurs more often in

wines with pH greater than 3.5; therefore, the lactobacilli and pediococci have greater ability to utilize the compound than *Leuconostoc* strains (Fugelsang, 1997). Davis *et al.* (1986) showed that metabolism of citric acid occurs with subsequent production of diacetyl, acetoin, lactic, and acetic acids as a major secondary reaction. This conversion of a dicarboxylic to a monocarboxylic acid leads to a decrease in titratable acidity. Laurent *et al.* (1993) identified post-MLF compounds which influenced aroma as diacetyl (buttery), isoamyl acetate (yeasty), and dimethoxytoluene (humus/floral), as well as other unidentified compounds. Diacetyl (2,3-butanedione) imparts a buttery flavor, and is usually one of the more prominent differences in MLF wines. Rankine *et al.* (1969) reported 2-3 times the level of diacetyl in MLF wines as compared to non-MLF wines. Also, while lees contact is stimulatory to LAB, it may decrease residual levels of diacetyl (Martineau and Henick-Kling, 1995b). Crowell and Guymon (1975) identified the "geranium tone" (2-ethoxyhexa-3,5-diene) which is sometimes undesirably formed by MLF as a derivative of sorbic acid. Lactic acid bacteria not only influence the production of aroma compounds, but can influence the degradation of others. Laurent *et al.* (1994) monitored the disappearance of ethyl decanoate (geranium), 1-octanol (urine), and 1-octene-3 ol (cooked garlic) as a result of MLF.

Aroma/Sensory

A rise in pH and a decline in titratable acidity results in a wine with a softer palate and enhanced mouthfeel (Amerine and Kunkee, 1968). Common positive sensory descriptors associated with malolactic fermentation include buttery, yeasty, fruity, and long aftertaste; for negative characteristics, the common descriptors are geranium, sweaty, bitter, and ropy (Henick-Kling *et al.*, 1994). Avedovech *et al.* (1992) evaluated Chardonnay wines that had been

fermented with the same yeast and found that there were significant variations in the aroma between wines which underwent MLF and those that did not. However, Rodriguez *et al.* (1990) discovered aroma characteristics in Chardonnay wine similar to those caused by MLF that could be induced by extended lees contact and fermentation in wood at warm temperatures. The effect of MLF on aroma is influenced by grape variety (Martineau and Henick-Kling 1995a). For example, MLF in Chardonnay can result in dramatic differences in aroma, mouthfeel, and aftertaste, but in more aromatic wines such as Riesling, MLF results in more subtle differences, such a rounder middle palate (Henick-Kling *et al.*, 1994). Sensory evaluations indicate that in Chardonnay, panelists noted significant increases in burnt-sweet and citrus characteristics, while in Riesling the burnt-sweet characteristics decreased but a maple syrup quality increased (Laurent *et al.*, 1993). Malolactic fermentation is most desirable in red wines from cool climates (Rodriguez *et al.*, 1990). These wines often have vegetative aromas, but after MLF usually possess more fruity characteristics (Henick-Kling *et al.*, 1994). Rankine *et al.* (1969) attribute the buttery aroma associated with wines which have undergone malolactic fermentation to diacetyl. Concentrations of 1-3 mg/L add desirable aroma complexity, but additional amounts are found only in spoiled wines (Rodriguez *et al.*, 1990). The minor differences in concentration of diacetyl which can cause differentiation between desirable and undesirable aroma contributions stress the need for careful strain selection. Although still lower in production compared to other LAB, different strains of *Leuconostoc oenos* can differ ten-fold in their ability to form diacetyl (Henick-Kling *et al.*, 1993). Timing of MLF can also affect the concentration of diacetyl. If MLF occurs soon after the primary fermentation, acceptable levels are formed, but protracted conversion can lead to excessive, and therefore defective, diacetyl levels (Fugelsang, 1997). Although diacetyl can reach

clearly unacceptable levels, the effect of concentration varies with wine type. Martineau *et al.* (1995) determined that threshold level in Chardonnay is 0.2 mg/L, in Pinot Noir it is 0.9 mg/L and in Cabernet Sauvignon it is 2.8 mg/L. Further complicating assessment of diacetyl production is evidence that *L. oenos* can utilize the compound as well (Martineau and Henick-Kling, 1995). The bitter compound, acrolein, which contributes to aroma complexity, is a result of glycerol hydrolysis and subsequent reactions with condensed phenols and anthocyanins (Lafon-Lafourcade, 1983). Another negative aroma characteristic associated with MLF is a mousiness or "damp urine-soaked rodent cage litter" smell (Fugelsang, 1997). Three compounds associated with this odor have been identified as ethyl lysine derivatives, 2-acetyl-1,4,5,6,-tetrahydropyridine, 2-acetyl-3,4,5,6,-tetrahydropyridine (Craig & Hereszytn, 1984).

Thus, lactic acid bacteria can be an invaluable tool for a winemaker to increase complexity. However, complexity, particularly the decrease of vegetative aroma and increase of fruity qualities, is often the result of aging. Therefore, a comparison should be made between aged wines and MLF wines, as well as the effect of aging on MLF wines. Little data is available, but non-MLF, aged wines seem to have similar qualities to MLF young wines. Therefore, MLF is most valuable to wines consumed in the early maturity stages (Henick-Kling *et al.*, 1993).

Prevention of MLF

Sometimes this secondary fermentation is undesirable, particularly when it occurs spontaneously in wine of high pH and causes ropiness and excess acetic acid (Daeschel *et al.*, 1991). Wines of elevated pH often come from warmer climates; the extended growing season leads to respiration of malic acid in the grapes which lowers acidity (Rodriguez *et al.*, 1990).

These conditions may cause MLF to be undesirable, but with more recent research highlighting the beneficial sensory changes, even warm climate wineries may choose to induce MLF. Results of Daeschel *et al.* (1991) show that unwanted MLF can be prevented with nisin (100 U/mL) without altering the sensory characteristics of the wine. Nisin, produced by *Lactococcus lactis*, subsp. *lactis*, is a bactericidal polypeptide which acts against Gram positive bacteria (Hurst, 1981). Other methods to control malolactic fermentation include the addition of sulfur dioxide or lysozyme, temperature control, and filtration (Rodriguez *et al.*, 1990). Generally, a concentration of greater than 50 mg/L of total SO₂ is sufficient as an inhibitory agent (Davis *et al.*, 1986). However, sometimes the use of SO₂ is unsatisfactory due to the allergic reactions caused in some individuals. Also, the effectiveness of SO₂ decreases as pH increases, because a low pH leads to a higher concentration of undissociated SO₂ (Beelman, 1984). Therefore, stabilization in wines of low acidity is difficult. Gerbaux *et al.* (1997) found that 500 mg/L lysozyme could inhibit MLF and 250 mg/L could stabilize post-MLF wines. Lysozyme caused no observed inhibition of alcohol fermentation or decrease in color intensity. However, the sensory effects of lysozyme have yet to be evaluated, and unlike SO₂, lysozyme has no antioxidant properties (Gerbaux *et al.*, 1997).

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Chapter II: The effects of cold soak and percent alcohol (v/v) at dejuicing on the concentration of Cabernet Sauvignon grape glycosides and glycoside fractions.

ABSTRACT

Grape-derived aroma and flavor precursors exist partially as non-volatile, sugar-bound glycosides. Hydrolysis of these compounds may modify sensory attributes and potentially enhance wine quality. Cold soak (prefermentation skin contact) at two temperatures and alcohol content at dejuicing were monitored to determine effects on Cabernet Sauvignon glycosides. Total, phenolic-free, and red-free glycoside concentrations were estimated by the quantification of glycosyl-glucose. Cold soak (5 days at 10°C) increased total glycosides by 77%, red-free glycosides by 80%, and phenolic-free glycosides by 96%. Ambient soak (3 days at 20°C) enhanced color extraction, and increased total glycosides by 177%, red-free glycosides by 144%, and phenolic-free glycosides by 106%. Wines produced by early pressing (10% sugar) had 25% more total and red-free glycosides than late press (0.25% sugar). After post-fermentation malolactic fermentation, total glycosides were 14% lower and phenolic-free glycosides were 35% lower.

INTRODUCTION

Grape-derived aroma and flavor compounds are present as free volatiles and, in part, as sugar-bound precursors including glycosides (Abbott *et al.*, 1993; Williams *et al.*, 1995). Aglycones may be aliphatic residues, monoterpenes, sesquiterpenes, norisoprenoids, or shikimic

acid metabolites such as phenols (Abbott, 1993; Sefton *et al.*, 1993, 1994, 1996; Winterhalter *et al.*, 1990). Research to increase extraction and hydrolysis of glycosides may lead to enhanced product quality (Abbott *et al.*, 1993; Francis *et al.*, 1992; Williams *et al.*, 1995).

Glycosides may exist as disaccharide complexes such as α -L-rhamnopyranosyl- β -D-glycopyranosides or α -L-arabinofuranosyl- β -D-glycopyranosides (Cordonnier *et al.*, 1986). Glycoside hydrolysis may occur either enzymatically or via acid hydrolysis, potentially releasing volatile compounds (Francis *et al.*, 1992, 1996; Gunata *et al.*, 1985; Williams *et al.*, 1982). Complete enzyme catalysis of these compounds occurs in two steps: 1. Glucose is separated from the terminal sugar by a hydrolase (α -L-arabinofuranosidase, α -L-rhamnosidase, or β -apiosidase); 2. The bond between aglycone and glucose is broken by β -glucosidase (Gunata *et al.*, 1988). Glycosides which contain aroma and flavor aglycones may potentially affect wine quality only after hydrolysis. Abbott *et al.* (1991) found a higher glycoside concentration in wines produced from vineyards reporting high quality Shiraz grapes while a low concentration coincided with low quality fruit. However, while large quality differences may be correlated with glycoside concentrations, smaller yet practical differences may not be reflected (Francis *et al.*, 1998).

Limited glycoside hydrolysis occurs during berry maturation by endogenous fruit β -glucosidases (Cordonnier and Bayonove, 1974; Cordonnier *et al.*, 1986) and during vinification by microbiological enzymes as well as by acid catalyzed hydrolysis (Gunata *et al.*, 1985; Zoecklein *et al.*, 1997). Sefton *et al.* (1998) illustrated in sensory trials that glycoside hydrolysis products contributed "intense berry and plum-like aromas". Abbott *et al.* (1991) found that enzymatic hydrosylates from Shiraz enhanced quality related aroma and flavor characteristics, particularly "non-berry" attributes.

Cold soak (pre-fermentation skin contact) can increase the extraction of phenolics, including phenolic glycosides, and enhance color intensity (Heatherbell *et al.*, 1997). The absence of alcohol allows the formation of anthocyanin/phenol complexes which help to stabilize color (Zoecklein *et al.*, 1995). Heatherbell *et al.* (1997) found that cold soak of Pinot noir increased color intensity and the concentrations of anthocyanins and phenols. Low temperature cold soak of Pinot noir (4 °C) has been shown to give darker, less bitter wines than higher temperatures (10 °C) (Heatherbell *et al.*, 1997). The higher temperature decreased visible color, imparted woody-tobacco aroma and flavor, and increased bitterness. The changes in aroma and flavor in cold soak produced wines may, in part, be the result of glycoside hydrolysis.

Although the majority of anthocyanins are extracted within the first 10° Brix reduction, tannin extraction occurs throughout the skin-contact period (Berg and Akayoshi, 1956). In general, delayed pressing increases phenolic extraction, while dejuicing prior to dryness enhances fruity characteristics, initial color, low astringency, and low total phenols (Somers and Evans, 1977).

The objective of this research was to investigate the effects of cold soak and percent alcohol at dejuicing on Cabernet Sauvignon grape glycoconjugate and conjugate fractions.

MATERIALS AND METHODS

Cabernet Sauvignon grapes (229 kg) grown in northwestern Virginia were harvested at 22 °Brix, crushed, destemmed, and equally divided into 6 replications per treatment. Treatments were assigned as follows: control musts (immediately inoculated) and cold soak (musts held prior to inoculation at 10°C for 5 days). Alcohol at dejuicing treatments consisted of early press (10 g/L

reducing sugar), and late press (the juice separated from the skins at dryness, 0.25 g/L reducing sugar).

In a second study, 181 kg of grapes (22 °Brix) from the same vineyard were used to compare two time and temperatures of pre-fermentation soak: cold soak (5 days at 10 °C) and ambient (3 days at 20 °C). Each treatment was comprised of 6 replications.

Musts were immediately treated with 600mg/L dimethyl dicarbonate (Velcorin™, Bayer Corp., Pittsburgh, PA). Control must was inoculated immediately using a 3% (v/v) actively growing culture of *Saccharomyces cerevisiae* (Fermirouge™, Gist-brocades, Cedex-France). Caps were punched manually three times daily. Fermentation occurred at ambient temperature (22 °C). Pressing was performed using a standard 10L basket press at a consistent press pressure. Juice samples were collected daily and stored at -10 °C for subsequent analysis.

Analyses: Soluble solids were determined using °Brix hydrometers and an American Optical (Warner-Lambert Technologies, Keene, NH) model 10419 temperature-compensating refractometer, pH by a Fisher (Pittsburgh, PA) Accumet® model 20 pH meter, and titratable acidity by titration with NaOH to an endpoint of pH 8.2. Reducing sugar was determined by the Rebelein method (as described by Zoecklein *et al.*, 1996). Tartaric, malic, and lactic acids were determined by HPLC, using a Hewlett-Packard (Palo Alto, CA) isocratic system model 1100 at 230 nm and a Bio-Rad (Hercules, CA) Fast acid (100 mm x 7.8 mm) column. Total phenols ($A_{280\text{ nm}} - 4$), hydroxycinnamates ($A_{320\text{ nm}} - 1.4$), and anthocyanin concentrations ($20 \times A_{520\text{ nm}}$) were estimated spectrophotometrically (Genesys5™, Spectronic Instruments Inc., Rochester, NY) as described by Somers and Evans (1977). Hue and intensity were determined spectrophotometrically as described by Zoecklein *et al.* (1990). [hue = $A_{520\text{ nm}}/A_{420\text{ nm}}$ and intensity = $A_{520\text{ nm}} + A_{420\text{ nm}}$].

Hydrolysis of grape-derived glycosides produces an equimolar ratio of D-glucose (known as glycosyl-glucose) and the aglycone. Therefore, quantification of glycosyl-glucose (GG) estimates the concentration of bound secondary plant metabolites. Glycosides were estimated in triplicate using the procedure of Williams *et al.* (1995), as modified by Iland *et al.* (1996).

Phenolic-free glycoside concentration was estimated by the procedure of Williams *et al.* (1995) and involved adjustment of sample to pH 10.0 with 20% sodium carbonate. Only glycosides lacking a phenol or functional group ionizable at pH 10 are retained on the column and subsequently estimated.

Color GG was determined by quantification of anthocyanins, measured spectrophotometrically as described by Iland (1988). Since the molar relationship between anthocyanin content and glucose is 1:1, subtraction of the color GG from total GG provides an estimation of the concentration of the color free or "red-free" GG expressed as μ mole glucose (Iland *et al.*, 1996; McCarthy *et al.*, 1996).

All data were statistically analyzed using SAS (SAS Institute, Cary, NC) and Minitab™ (Minitab, Inc., State College, PA). Statistical methods employed were ANOVA, polynomial linear regression analysis, and Student's *t*-test.

RESULTS AND DISCUSSION

Cold Soak: At the completion of the 5 day cold soak period at 10°C, total glycosides (total GG) increased by 77%, red-free glycosides (red-free GG) by 80%, and phenolic-free glycosides (phenolic-free GG) by 96% (Fig. 1). The absence of yeast or bacterial fermentation during cold soak was confirmed by lack of changes in reducing sugar, and tartaric, malic, and lactic acids. At

10°C, endogenous fruit enzyme activity would be limited. In addition, Aryan *et al.* (1987) found that native β -glucosidases had little to no activity at typical wine pH. The increase in glycoside concentration during cold soak may be due to increased aqueous solubilization (Zoecklein *et al.*, 1996). However, it may also be an indication of acid hydrolysis of complex precursors and subsequent liberation of glycosides detectable by the GG assay (Williams *et al.*, 1996). From crush throughout the pre-fermentation period the ratios of total glycosides to glycoside fractions were constant. Red-free GG comprised 75% of the total, and phenolic-free GG comprised 50% of the total.

Ambient soak (20°C) increased the rate of extraction of total GG, phenolic-free GG, and red-free GG, compared to the cold soak (10°C) (Fig. 2). At the end of the soak period, the ambient temperature soak increased total glycosides by 177% versus 103% for the cold soak. During the same period, red-free glycosides increased by 144% vs. 81%, and phenolic-free glycosides by 106% vs. 83% (Fig. 2). The ambient soak had slightly greater decline, after three days, in the ratio of phenolic-free to total glycosides (50% to 40%) compared to cold soak (50% to 45%). These differences were due to a greater increase in the total glycoside concentration with the ambient treatment. Therefore, the same trend was evident for the decline in the ratio of red-free glycosides to total (Ambient: 70% to 53%, as compared to cold soak: 70% to 63%).

Total glycosides and glycoside fractions exhibited similar behavior throughout fermentation; increasing from inoculation until a maximum concentration (approximately at 10 g/L residual sugar) (Fig. 3). A slow decline followed maximum glycoside concentration, and has been documented to continue to decrease during aging (Zoecklein *et al.*, 1997, 1998). The relative concentrations of glycoside fractions changed throughout fermentation. A reduction in the ratio

of red-free GG to the total and the ratio of phenolic free glycosides to the total occurred during fermentation. At fermentation completion, red-free GG comprised 30% of the total, and phenolic-free GG was 23% of the total glycosides.

Comparing maximum glycoside concentrations to those at completion of fermentation may provide insight into the effect of processing on these important secondary metabolites. The decline in glycoside concentration in the latter stage of fermentation may be the result of a combination of factors including precipitation, absorption, and hydrolysis. Absorption of compounds by the lees has been reported (Lebert, 1984), with variation among yeast strain due to cell wall hydrophobicity (Lubbers *et al.*, 1994). Like other compounds such as potassium bitartrate, glycosides may also precipitate from the wine. However, if the decline in glycoside concentration was due to hydrolysis and liberation of aglycones, aroma and flavor could be affected.

Total GG and red-free GG concentrations between cold soak and control had no significant differences (Table 1). Likewise, the two soak temperatures had no differences in glycoside or glycoside fraction concentrations at the end of fermentation (Tables 1 & 2). However, when the decline from maximum concentration was considered, the cold soak had a greater decline in phenolic-free glycoside concentration. Although phenolic compounds are structurally important to wine, they have limited aroma/flavor impact (Singleton and Noble, 1976). Sefton (1998) found limited sensory contribution by enzymatic hydrolyses which contained a majority of phenolic aglycones. Therefore, a significant decline in the phenolic-free glycosides might indicate hydrolysis and liberation of aglycones which have greater impact on aroma and flavor.

Cold soak wines had a higher color intensity and titratable acidity than the control wines (Table 3). The increased titratable acidity may have been caused by a lower precipitation of

potassium bitartrate. In contrast, there were no differences in hue, total anthocyanin, total phenols, or total hydroxycinnamates between the treatments at dryness (Table 3). Heatherbell *et al.* (1997) noted significant differences in anthocyanin and color intensity as a result of cold soak only with the addition of 50 and 100 mg/L SO₂. Sulfur dioxide limits polymerization and copigmentation by binding flavanoid phenols at the carbon 4 of the phenol ring.

The three day ambient soak (20°C) produced higher phenols, hydroxycinnamates, and higher absorbance at 520 nm (higher intensity, lower hue, higher anthocyanins)(Table 4). However, following the soak period differences diminished. At dryness, ambient soak had lower estimated total phenols and TA than the cold soak. These results are contrary to those of Heatherbell *et al.* (1997), who compared cold maceration at temperatures of 10 and 4 °C and noted the higher temperature decreased visible color.

Alcohol at dejuicing: At the completion of fermentation, the early press wines had a higher concentration of total and red-free glycosides than the late press; 13% higher total GG and 6% higher red-free GG (Fig. 4). These differences may be the result of a greater decline in glycoside concentration in the late press wines. In both early and late press wines, the concentration of glycosides at dryness was compared with the maximum concentration (Table 5). The total glycoside concentration of the late press decreased 47% more than the early press. Aryan *et al.*(1987) found the majority of β-glucosidase was located in the pulp. Therefore, the increased contact the late press had with the pulp may have enhanced hydrolysis and possibly the liberation of aroma and flavor compounds. However, precipitation or absorption by yeast lees may have also cause a greater decline in glycoside concentration (Lebert, 1984).

Pressing at dryness diminished color intensity, anthocyanins, total hydroxycinnamates, and elevated hue (Table 6). Young red wine color intensity ($A_{420\text{ nm}} + A_{520\text{ nm}}$) and hue ($A_{520\text{ nm}}/A_{420\text{ nm}}$) are influenced not only by the type of anthocyanin, but the degree of equilibrium among the color and colorless forms (Berg and Akiyoski, 1956; Liao *et al.*, 1992). The decreased absorbance at 520 nm and increased absorbance at 420 nm may have been a result of enhanced polymerization with non-colored phenols (Nagel and Wulf, 1979; Somers and Verette, 1988). During conventional aging, free anthocyanins decline with a concurrent increase in stable, pigmented complexes which help provide enduring color (Scudamore-Smith *et al.*, 1990; Sims and Morris, 1985; Somers and Verette, 1988). This complexation creates a shift in absorbance maxima from 520 to 420, and causes the formation of yellow-orange shades in aged red wine (Somers and Evans, 1979). Although comparing various durations of fermentation (all pressed at dryness), Auw *et al.* (1996) found that longer skin fermentation time led to increased hue ($A_{520\text{ nm}}/A_{420\text{ nm}}$) and decreased intensity ($A_{520\text{ nm}} + A_{420\text{ nm}}$) in Cabernet Sauvignon. Therefore, increased skin contact appears to lead to enhanced polymerization.

Malolactic Fermentation: In this study, MLF had mixed effects on the concentrations of glycosides and glycoside fractions. The phenolic free glycosides in both the cold soak and control wines had a 35% decrease in concentration following MLF (Table 1). Also, total glycosides declined 14% in the cold and ambient soaks following MLF (Table 2). There were no declines in the total glycoside concentrations in either the cold soak or control wines following MLF. An increase in glucose concentration coinciding with MLF has been documented (Costello *et al.*, 1985; Davis *et al.*, 1986). This increase could be caused by hydrolysis of glycosides (Lafon-Lafourcade, 1983). However, linking glycoside hydrolysis and bacterial enzymatic activity has

proven difficult; the increase in glucose concentration could also be caused by reactions catalyzed by residual grape and yeast enzymes (Davis et al., 1986). However, De Cort *et al.*, (1994) successfully isolated and purified an α -glucosidase from *Lactobacillus brevis* found during a secondary fermentation in Belgian lambic beer. Some winemakers claim native fermentations (*Leuconostoc*, *Pediococcus*, or *Lactobacillus*) exhibit enhanced sensory characteristics compared with commercial preparations (Fugelsang, 1997).

Changes in red-free glycosides were not evaluated over this time period due to the effects caused by anthocyanin concentration differences. The absorbance at 520 nm decreased, which caused an artificial increase in the red-free GG level not related to glycoside changes.

CONCLUSIONS

Cold soak caused an increase in Cabernet Sauvignon glycoside concentration and color, and ambient soak augmented the same results. However, neither glycoside nor color differences caused by soak temperatures were evident at the end of fermentation. Cold soak caused a greater decline in phenolic-free glycoside concentration, possibly signifying glycoside hydrolysis. The phenolic-free fraction comprised 10% of the total glycosides while red-free comprised 33% of the total in the fermented wines. Early press resulted in more intense color (higher spectral color at 520 nm). The changes in color in the late press seem to indicate enhanced polymerization. Late press resulted in a 47% greater decline in glycoside concentration than the early press. Malolactic fermentation resulted a 14% decline in total glycoside concentration in the cold and ambient soak fermentations and a 35% decline in phenolic free glycosides in the cold soak and control wines.

This investigation provides documentation of the effects of various processing techniques on the extraction of grape glycosides, in part, important aroma and flavor precursors. This information may lead to a greater understanding of the effect of processing on these secondary plant metabolites. Further research might focus on the corroboration of glycoside concentration decline and modification of sensory characteristics.

Table 1: Glycoside and glycoside fraction concentrations of control (immediate inoculation) and cold soak (5 days at 10°C) Cabernet Sauvignon wines post-fermentation and post-malolactic fermentation, expressed as μmol glycosyl-glucose.

Variable	Post-Fermentation		Post- Malolactic Fermentation	
	Control	Cold Soak	Control	Cold Soak
Total glycosides	1232 a	1275 a	1225 x	1255 x
Phenolic-free glycosides	283 a	271 a	211 x	235 x
Red-free glycosides	483 a	486 a	845 x	879 x

Different letters within rows of each column indicate significance of t-test of treatment means at $P \leq 0.05$. N = 6.

Table 2: Effect of two temperatures (Cold: 10°C, 5 days vs. Ambient: 20°C, 3 days) of cold soak on Cabernet Sauvignon glycoside and glycoside fraction concentrations (expressed as μmol glycosyl-glucose).

Variable	Decline		Post-fermentation		Post-MLF	
	Ambient	Cold	Ambient	Cold	Ambient	Cold
Total glycosides	254 a	134 a	1443 m	1469 m	1284 x	1239 x
Phenolic-free glycosides	370 a	257 b	228 m	255 m	298 x	288 x
Red-free glycosides	198 a	94 a	652 m	719 m	927 x	916 x

Decline signifies difference from maximum concentration to fermentation completion. Different letters within rows of each column indicate significance of t-test of treatment means at $P \leq 0.05$. N = 6.

Table 3: Effect of cold soak (5 days at 10°C) or immediate inoculation (control) on Cabernet Sauvignon wine chemistry and spectral analysis (AU), post-fermentation and post- malolactic fermentation.

Variable	Post- fermentation		Post- MLF	
	Control	Cold Soak	Control	Cold Soak
pH	3.69 a	3.68 a	3.81 x	3.76 x
TA (g/L)	7.17 a	7.72 b	5.77 x	6.05 x
Intensity $A_{420nm} + A_{520nm}$	7.05 a	8.41 b	7.29 x	7.11 x
Hue A_{420nm} / A_{520nm}	0.55 a	0.53 a	0.88 x	0.85 x
Total Phenols $A_{280nm} - 4$	28.86 a	26.86 a	26.60 x	26.24 x
Total Anthocyanin $20 * A_{520nm}$	408 a	419 a	201 x	199 x
Total Hydroxycinnamates $A_{320nm} - 1.4$	11.93 a	13.17 a	12.77 x	12.96 x

Different letters within rows of each column indicate significance of t-test of treatment means at $P \leq 0.05$. N = 6.

Table 4: Effect two temperatures (Cold: 10°C, 5 days, or Ambient: 20°C, 3 days) on Cabernet Sauvignon wine chemistry and spectral analysis (AU) at end of cold soak period and post-fermentation.

	Cold soak		Post-fermentation	
	Ambient	Cold	Ambient	Cold
TA(g/L)	5.71 a	4.90 b	6.74 x	7.77 y
Intensity $A_{420nm} + A_{520nm}$	5.92 a	3.15 b	6.45 x	7.20 x
Hue A_{420nm} / A_{520nm}	0.47 a	0.57 b	0.60 x	0.60 x
Total Phenols $A_{280nm} - 4$	18.28 a	10.93 b	23.43 x	29.71 y
Total Anthocyanin $20 * A_{520nm}$	321 a	177 b	419 x	397 x
Total Hydroxycinnamates $A_{320nm} - 1.4$	9.96 a	6.57 b	8.89 x	9.35 x

Different letters within rows of each column indicate significance of t-test of treatment means at $P \leq 0.05$.

N = 6.

Table 5: Effect of alcohol at dejuicing (Early: 10g/L reducing sugar or Late: dryness) on Cabernet Sauvignon glycoside and glycoside fraction concentrations (expressed as μmol glycosyl-glucose)

Variable	Decline		Post- Fermentation		Post- malolactic fermentation	
	Early	Late	Early	Late	Early	Late
Total glycosides	-116 a	47 b	1329 m	1177 n	1334 x	1182 x
Phenolic-free glycosides	8 a	24 a	289 m	266 m	242 x	214 y
Red-free glycosides	511 a	406 a	500 m	469 n	928 x	823 x

Decline signifies difference from maximum concentration to fermentation completion. Different letters within rows of each column indicate significance of t-test of treatment means at $P \leq 0.05$. $N = 6$.

Table 6: Effect of alcohol at dejuicing (Early: 10g/L reducing sugar or Late: dryness) on Cabernet Sauvignon wine chemistry and spectral analysis (AU) at post-fermentation and post-malolactic fermentation.

Variable	Post-fermentation		Post- MLF	
	Early	Late	Early	Late
pH	3.63 a	3.73 b	3.74 x	3.81 y
TA	7.50 a	7.36 a	6.20 x	5.77 x
Intensity $A_{420nm} + A_{520nm}$	8.05 a	7.41 a	7.73 x	6.82 y
Hue A_{420nm} / A_{520nm}	0.5220a	0.5634 b	0.8711x	0.8675x
Total Phenols $A_{280nm} - 4$	28.69 a	26.84 a	26.44 x	26.35 x
Total Anthocyanin $20 * A_{520nm}$	452 a	375 b	215 x	190 y
Total Hydroxycinnamates $A_{320nm} - 1.4$	13.87 a	11.24 b	14.87 x	11.55 y

Different letters within rows of each column indicate significance of t-test of treatment means at $P \leq 0.05$. N = 6.

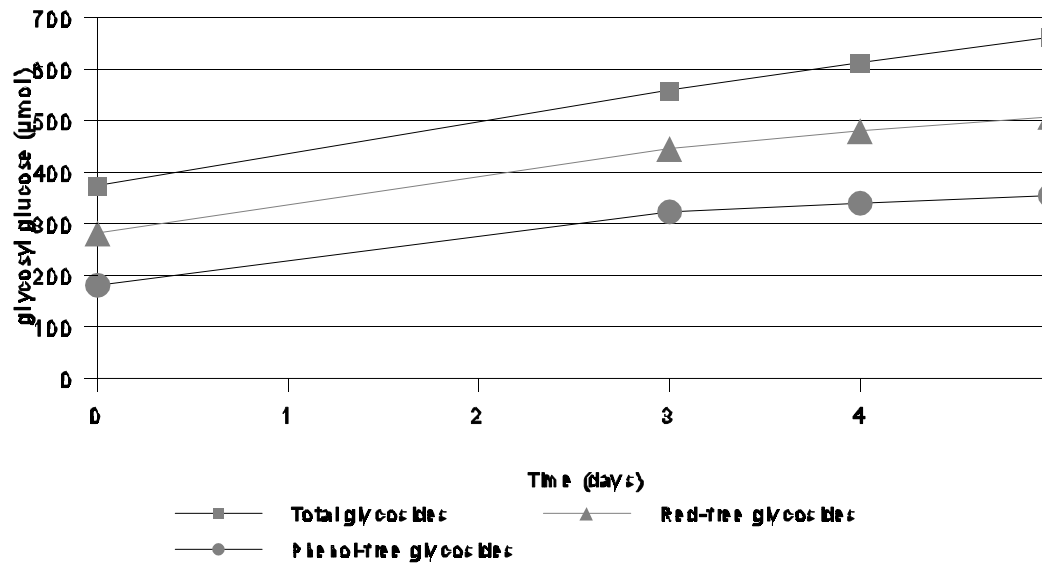


Figure 1: Effect of cold soak (prefermentation skin contact: 10°C, 5 days) on the concentration of Cabernet Sauvignon glycoside and glycoside fractions, expressed as µmole glycosyl-glucose.

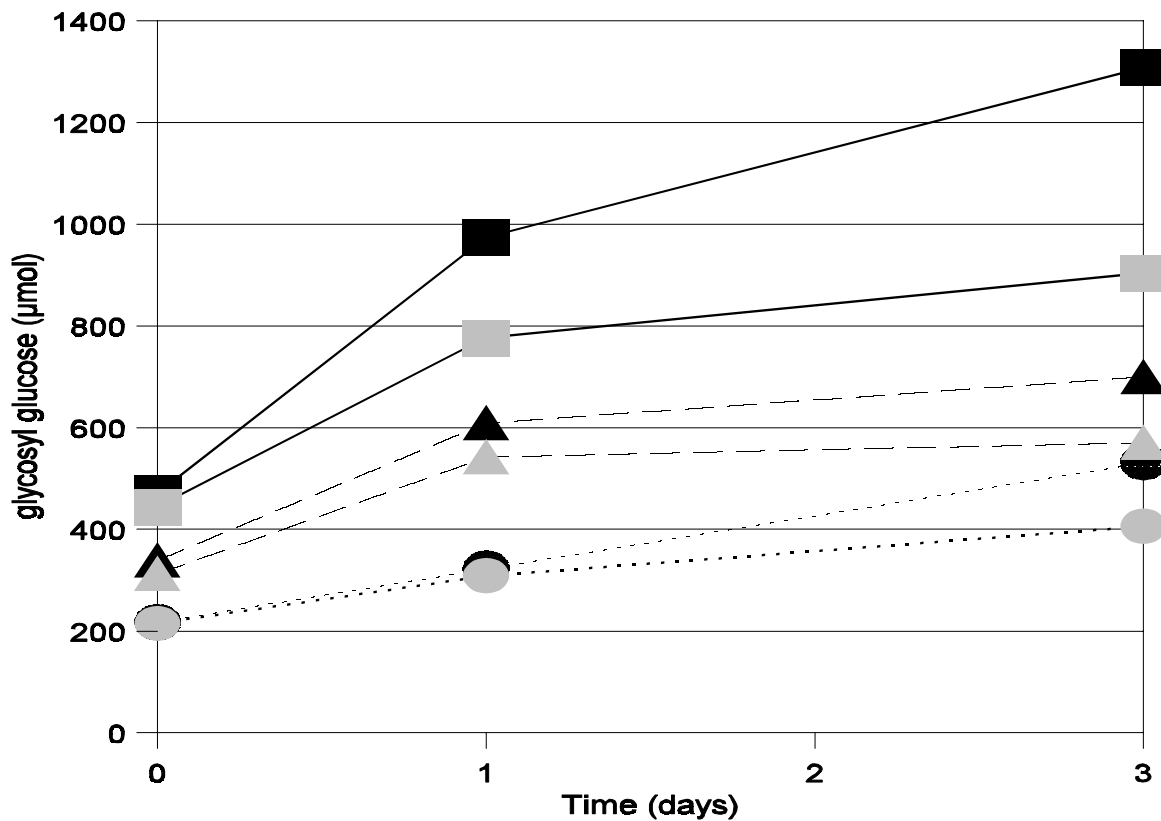
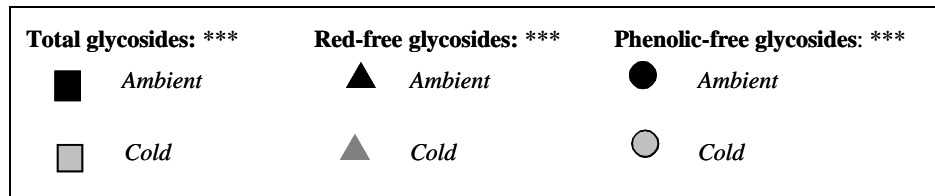


Figure 2: Effect of two temperatures (Cold: 10°C or Ambient: 20°C) of 3 day prefermentation soak on the concentration of Cabernet Sauvignon glycosides and glycoside fractions, expressed as $\mu\text{mole glycosyl-glucose}$. *** indicates differences significant between treatments at $P \leq 0.01$ level.

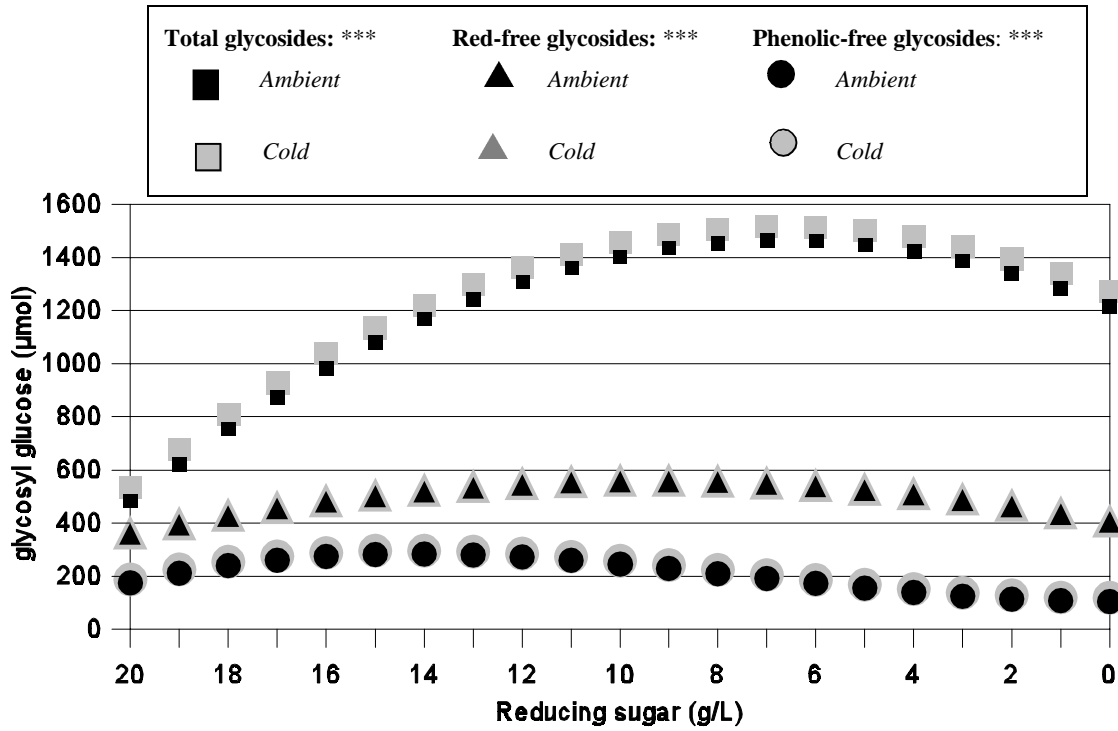


Figure 3: Effect of cold soak (prefermentation skin contact, 10°C, 5 days) or control (immediate inoculation) on the concentration of Cabernet Sauvignon glycoside and glycoside fraction concentrations during fermentation. Predicted values based on multiple linear polynomial regression of reducing sugar and cold soak or control. Differences not significant.

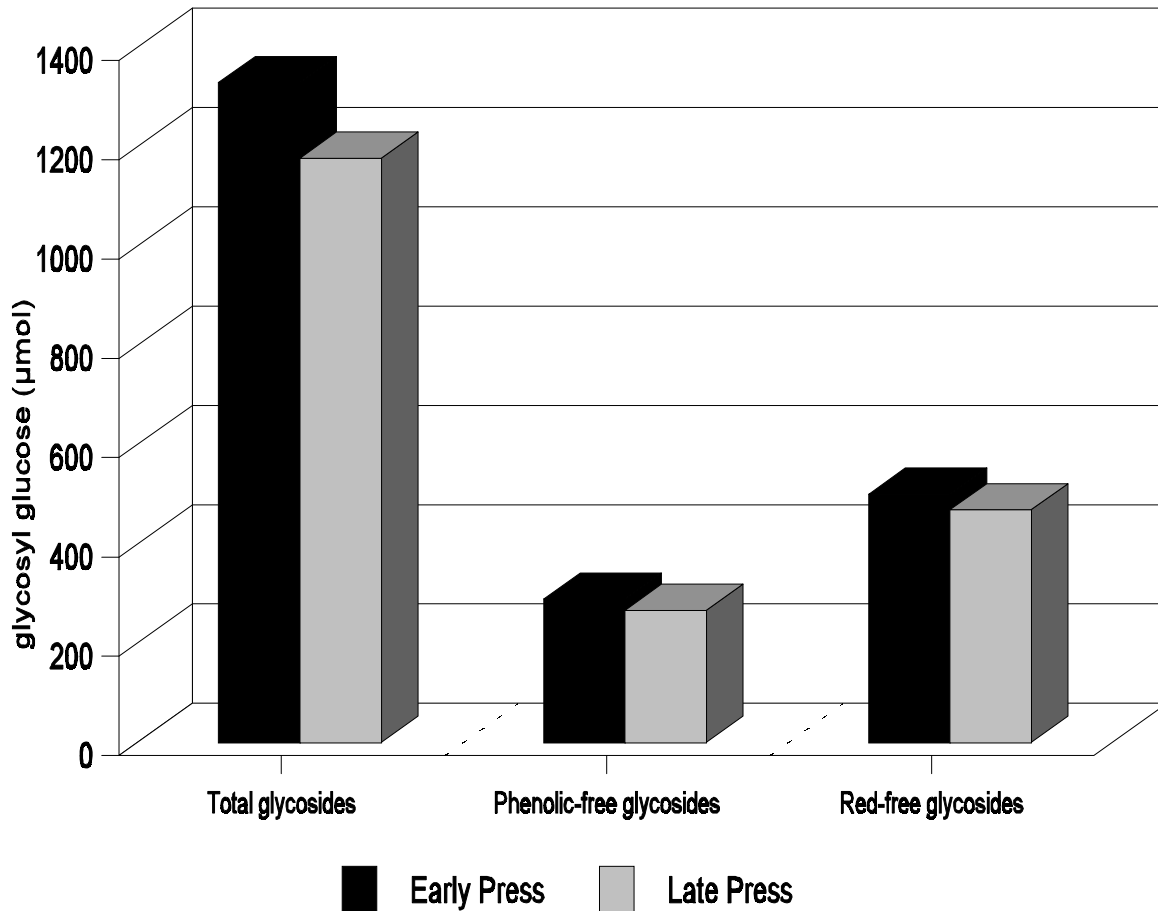


Figure 4: Effect of percent alcohol (v/v) at dejuicing on the concentration of Cabernet Sauvignon glycoside and glycoside fraction concentrations at completion of fermentation. Early press: 10 g/L reducing sugar; late press: dryness. Differences in treatments in total and phenolic free glycosides are significant at $P \leq 0.01$ level. Differences in treatments in red-free glycosides are non-significant.

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Chapter III: Quantification of glycosidase activities in selected yeasts and lactic acid bacteria.

ABSTRACT

The activities of α -L-arabinofuranosidase, β -glucosidase, and α -L-rhamnoyranosidase were determined in model systems for thirty-two strains of yeasts belonging to the following genera: *Aureobasidium*, *Candida*, *Cryptococcus*, *Hanseniaspora*, *Hansenula*, *Kloeckera*, *Metschnikowia*, *Pichia*, *Saccharomyces*, *Torulasporea*, and *Brettanomyces* (10 strains); and seven strains of bacteria (*Leuconostoc oenos*). Only one *Saccharomyces* strain exhibited β -glucosidase activity, but several non-*Saccharomyces* yeast species had substantial production. *Aureobasidium pullulans* hydrolyzed α -L-arabinofuranoside, β -glucoside, and α -L-rhamnoyranoside. Eight *Brettanomyces* strains had β -glucosidase activity. Location of enzyme activity was determined for those species with enzymatic activity. The majority of β -glucosidase was located in the whole cell fraction, followed by the permeabilized fraction, and extracellular production. *Aureobasidium pullulans* was also capable of hydrolyzing grape glycosides.

INTRODUCTION

Grape-derived aroma and flavor compounds are present as free volatiles and, in part, as sugar-bound precursors including glycosides (Abbott *et al.*, 1993; Williams *et al.*, 1995). Glycosides which contain aroma and flavor aglycones may potentially affect wine quality after hydrolysis. Aglycones may be aliphatic residues, monoterpenes, sesquiterpenes, norisoprenoids, or shikimic acid metabolites such as phenols (Abbott *et al.*, 1993; Sefton *et al.*, 1993, 1994, 1996; Winterhalter *et al.*, 1990). Research to increase glycoside production and hydrolysis may lead to enhanced product quality (Abbott *et al.*, 1993; Francis *et al.*, 1992; Williams *et al.*, 1995).

Glycosides may exist as disaccharide complexes such as α -L-rhamnopyranosyl- β -D-glycopyranosides or α -L-arabinofuranosyl- β -D-glycopyranosides (Cordonnier *et al.*, 1986). Hydrolysis may occur either enzymatically or via acid, potentially releasing volatile compounds (Gunata *et al.*, 1985; Francis *et al.*, 1992, 1996; Williams *et al.*, 1982). Complete enzyme catalysis of these compounds occurs in two steps: 1. Glucose is separated from the terminal sugar by a hydrolase (α -L-arabinofuranosidase, α -L-rhamnosidase, or β -apiosidase); 2. The bond between aglycone and glucose is broken by β -glucosidase (Gunata *et al.*, 1988). The hydrolase needed to break the disaccharide bond may have specific or broad activity.

Limited hydrolysis of glycosides occurs during berry maturation by endogenous fruit β -glucosidases (Cordonnier and Bayonove, 1974; Cordonnier *et al.*, 1986) and during vinification by microbiological enzymes and acid catalyzed hydrolysis (Gunata *et al.*, 1985; Zoecklein *et al.*, 1997).

Sefton (1998) illustrated that acid catalyzed products of Merlot and Cabernet Sauvignon contributed “intense berry and plum-like aromas” while products from enzyme hydrolysis were undetectable. In contrast, Abbott *et al.* (1991) found that enzymatic hydrolyses from Shiraz enhanced varietal aroma, particularly “non-berry” attributes. Some acid-catalysis products may cause undesirable alteration of aroma, making enzymatic hydrolysis more favorable (Gunata, 1984; Winterhalter *et al.*, 1990). These differences may be the result of contrasting mechanisms between enzymatic and acid hydrolysis (Williams *et al.*, 1982). Enzymatic hydrolysis cleaves the glycosidic linkage without altering the aglycone, while acid hydrolysis may split alcohol aglycones and produce a reactive carbonation (Sefton, 1998).

Glycosidase activities have been documented in enological yeasts (endogenous to wine or the vineyard) (Darriet *et al.*, 1988; Delcroix *et al.*, 1994; Dubourdieu *et al.*, 1988). A few *Saccharomyces* strains exhibit hydrolytic enzyme activity, but greater production has been found

in non-*Saccharomyces* yeast active in native fermentations (Gunata *et al.*, 1994). Non-*Saccharomyces* yeasts tend to be prevalent in uninoculated fermentation initiation, but decline as alcohol content increases. Strains of *Saccharomyces* generally complete fermentation (Mortimer, 1995; Ribereau-Gayon, 1985). Species isolated from native fermentations include *Hanseniaspora uvarum*, *Kloeckera apiculata*, *Metschnikowia pulcherrima*, and *Hansenula anomala*, among others (Reed and Nagodawithana, 1991). Each of these species have been shown to produce β -glucosidase *in vitro* (Rosi *et al.*, 1994). The belief that native fermentations enhance aroma (Fugelsang, 1997) may be supported, in part, by higher hydrolytic enzyme production in these genera.

Co-culture of *Brettanomyces* with *Saccharomyces cerevisiae* can cause aromas and flavors similar to malolactic fermentation, such as enhanced complexity, augmented fruitiness, and diminished vegetative odors (Fugelsang *et al.*, 1993). Although commonly viewed as a spoilage organism, *Brettanomyces* may enhance aroma (Fugelsang *et al.*, 1993). Shantha Kumara *et al.* (1993) successfully isolated and purified an α -glucosidase from *Brettanomyces lambicus* found in a secondary lambic beer fermentation.

Although enological yeasts may have the ability to produce hydrolase, acidic wine conditions may cause denaturation and inhibition of activity (Delcroix *et al.*, 1994). In typical wine conditions β -glucosidase production and activity would be strongly inhibited by low pH, as well as high alcohol concentration, lack of oxygen and presence of glucose (Gunata *et al.*, 1984). The degree to which these factors inhibit β -glucosidase production and activity depends on the species and strain of the organism involved (Aryan *et al.*, 1987; Delcroix *et al.*, 1994; Leclerc *et al.*, 1984; Rosi *et al.*, 1994).

Malolactic fermentation (MLF) by lactic acid bacteria (LAB) can alter the acidity and sensory characteristics of wine (Henick-Kling *et al.*, 1993, 1994; Kunkee and Amerine, 1970). An

increase in glucose concentration coinciding with MLF fermentation has been documented (Costello *et al.*, 1985; Davis *et al.*, 1986). This increase could be caused by hydrolysis of glycosides (Lafon-Lafourcade, 1983). Linking glycoside hydrolysis and bacterial enzymatic activity has proven difficult; the increase in glucose concentration could also result from residual grape or yeast hydrolytic enzymes (Davis *et al.*, 1986). However, De Cort *et al.* (1994) successfully isolated and purified an α -glucosidase from *Lactobacillus brevis* isolated in Belgian lambic beer during a secondary fermentation.

The objective of this study was to determine the ability of enological organisms to hydrolyze glycosides. Selected strains of enological yeasts and lactic acid bacteria strains were assayed for α -L-arabinofuranosidase, β -D-glucosidase, and α -L-rhamnopyranosidase activities and site of production. The cultures used included ten vineyard isolated yeasts, ten strains of *Brettanomyces intermedius*, five commercial *Saccharomyces cerevisiae*, and seven commercial lactic acid bacteria.

MATERIALS AND METHODS

A. Cultures: The yeast genera and species used in this study are listed in Tables 1 and 2. The vineyard isolates were obtained and identified by Jeff Cohen (1994) and provided by Dr. Kenneth Fugelsang, California State University- Fresno. The strains of *Brettanomyces intermedius* were provided by Lallemand, Inc.; Montreal, Canada. Commercial strains of *Saccharomyces cerevisiae* were obtained as follows: M1, VL1, and CV-D47 (Lallemand Inc.; Montreal, Canada), Fermiblanco (Gist-brocades, Inc.; Seclin cedex, France), and Prise de Mousse (PDM; UCD 796, Universal Foods Corp., Milwaukee, WI). All strains were isolated to obtain pure culture, and maintained on Yeast Mold Agar (Difco; Detroit, MI) slants, pH 5.0. *Leuconostoc oenos* strains were obtained as follows:

OSU, 3X, MBR, and MT-01(Lallemand Inc; Montreal Canada), *Viniflora oenos* (Chris Hansen's Laboratory, Inc., Milwaukee, WI), MCW and Bitec D (Vinqury, Healdsburg, CA). Bacterial cultures were isolated and maintained on Apple Rogosa slants as described by Fugelsang (1997).

B. Analyses: The procedure of Blondin *et al.* (1983) with modifications by Charoenchai *et al.* (1997) was used to determine hydrolytic enzyme activities on three substrates: α -L-arabinofuranoside, β -D-glucoside, and α -L-rhamnopyranoside. Cells were grown in 10 mL liquid culture consisting of 6.7 g/L Yeast Nitrogen Base (YNB; Difco, Detroit, MI) and 5g/L arbutin (Sigma, St. Louis, MO). The medium was buffered to pH 5.0 by the addition of 0.6 g/L K_2HPO_4 and 0.2 g/L tartaric acid. After 48 hours incubation at 30°C, cultures were centrifuged (5000 X g, 10 minutes, 4° C), washed with cold sterile saline (0.7%) and re-centrifuged. The pellets were each transferred to 10 mL filter-sterilized growth medium containing 6.7 g/L YNB and 1 mM substrate: p-nitrophenyl- $[\alpha$ -L-arabinofuranoside], $[\beta$ -glucopyranoside], or $[\alpha$ -L-rhamnopyranoside] (Sigma; St. Louis, MO). The medium was buffered to pH 3.5 with tartaric acid and K_2HPO_4 (approximately 0.9 and 1.0 g/L, respectively). The reaction tubes were incubated for 48 hours at 30°C. The supernatant was assayed for liberated p-nitrophenyl: 1.0 mL was mixed with 2.0 mL sodium carbonate buffer (0.2 M, pH 10.2) and measured spectrophotometrically (Genesys5™, Spectronic Instruments Inc., Rochester, NY) at 400 nm. A series of standards was prepared that contained from 0 to 200 nM pNP. A substrate blank (buffer and substrate) and sample blanks (cell preparation and buffer) were prepared and subtracted from experimental absorbance readings. All assays were performed in duplicate.

C. Enzyme activity location: Strains which demonstrated substantial enzymatic activity (>1000 nmole mL⁻¹ g dry cell mass⁻¹ for *Brettanomyces* cultures, >300 nmole mL⁻¹ g dry cell mass⁻¹ for

vineyard isolates) were further analyzed to determine generalized location of enzyme activity (whole cells, permeabilized cells, and supernatant) as described by Rosi *et al.* (1994). A loopful of culture was transferred from stock slants to 10 mL of liquid medium (YNB: 6.7 g/L, arbutin: 5 g/L, and pH to 5.0). After 48 hours, 0.2 mL of the inoculum was added to 125 mL screw-capped bottles filled to 80% of their volume and incubated at 30°C for 3 days.

1. Cell preparation:

Whole cells: Cells were harvested from 1 mL of culture (centrifuged at 5000 X g, 10 minutes, 4°C) and washed twice with cold distilled water. The pellet was resuspended in 0.2 mL of citrate-phosphate buffer (100 mM, pH 5.0).

Permeabilized cells: The procedure of Salmon (1984), modified by Rosi *et al.*, (1994) was used. 5 mL of culture was centrifuged (5000 X g, 10 minutes, 4°C) and the pellet was washed with 5 mL cold distilled water. The pellet was resuspended in 1 mL of imidazole buffer (75 x 10⁻³ mmole/L, pH 7.5) and added to 50 µL of 0.3M glutathion, 10 µL of 10 % Triton X-100, 50 µL of toluene/ethanol (1:4 v/v). The suspension was placed on a mechanical shaker for 5 minutes and then centrifuged. The pellet was suspended in 5 mL of cold distilled water, 1 mL of this suspension was centrifuged and the pellet was washed with cold distilled water. The final pellet was resuspended in 0.2 mL citrate-phosphate buffer (100 mM, pH 5.0).

2. Enzyme assay:

Unconcentrated culture supernatant fluid, whole, or permeabilized cells (0.2 mL each) were mixed with 0.2 mL of 5 mM solution of substrate in 100 mM citrate-phosphate buffer (pH 5.0). The reaction mixture was incubated at 30°C for 1 hour. After the addition of 1.2 mL of carbonate buffer (0.2 M, pH 10.2), the reaction mixture was centrifuged (Fisher 235C model, 10,000 X g) for 2.5 minutes. Liberated p-NP was measured spectrophotometrically as listed previously. A series of

standards was prepared from 0-200 nM p-NP. All assays were performed in duplicate.

D. Activity on grape glycosides:

1. *Isolation of glycosides:* Viognier grapes grown in northwestern Virginia were pressed and the juice partially fermented (4.5% alcohol, 16°Brix). Viognier glycosides were isolated using Waters (Milford, MA) C-18 reverse phase Sep-Pak columns activated by passing 10 mL of methanol followed by 10 mL of deionized water. After loading 10 mL of undiluted juice, the columns were washed three times with 15 mL of deionized water. Glycosides were eluted with 5 mL methanol. Ten elutions were combined per aliquot, concentrated to dryness, and stored at -20 °C. Glycoside concentration was estimated by the analysis of glycosyl-glucose (Iland *et al.*, 1996; Williams *et al.*, 1995).

2. *Hydrolysis of glycosides:* Culture (50 mL) grown in YNB arbutin was centrifuged and the pellet was resuspended in 50 mL of 100 mM citrate-phosphate buffer (pH 5.0) and added to an aliquot of Viognier glycosides (111 µM glycosyl-glucose). Pectinolytic enzyme (0.03 g/L) (AR2000™ - Gist-brocades, Inc.; Seclin cedex, France) in buffer was used to verify the potential for glycoside hydrolysis. After a 48 hour incubation at 30°C, liberated glycosyl-glucose was measured spectrophotometrically at 340 nm using an enzymatic glucose assay (Boehringer Mannheim, Indianapolis, IN).

E. Dry weight: Dry cell weight was determined by filtering 50 mL of culture on pre-weighed filters (0.45- µm; Pall Gelman Sciences, Ann Arbor, MI). Filters were placed in tared aluminum pans, dried overnight at 100°C, and then reweighed.

F. Statistical Analysis: All data were statistically analyzed using SAS (SAS Institute, Cary, NC) and Minitab™ (Minitab, Inc., State College, PA). Statistical method employed was Duncan's Multiple Range Test. Determination of enzyme activities required regression analysis of the series

of standards.

RESULTS AND DISCUSSION

Aureobasidium pullulans displayed arabinofuranosidase activity, and two cultures produced rhamnopyranosidase (*Aureobasidium pullulans* and *Candida guilliermondii*) (Table 1). Each of these organisms also had β -glucosidase activity. The hydrolase needed to break the disaccharide bond (α -L-arabinofuranoside, α -L-rhamnoside, or β -apioside) may have specific or broad activity. For example, *Hanseniaspora vineae* displays a lack of specificity, hydrolyzing sugars with β (1-4) or β (1-2) bonds (Vasserot *et al.*, 1989). Similar characteristics have also been reported for *Candida molischiana* (Gonde *et al.*, 1985). Genera such as *Candida* and *Kloeckera/Hanseniaspora* can grow during early fermentation stages (Heard and Fleet, 1986; Reed and Nagodawithana, 1991). The high β -glucosidase activity in *Aureobasidium pullulans*, *Candida parapsilosis*, and *Kloeckera apiculata* may support winemakers' claims that uninoculated fermentations result in a more aromatic, flavorful wine (Fugelsang, 1997). Several commercial *Saccharomyces* strains assayed were chosen based upon manufacturer's claims of "enhanced varietal expression". However, these strains conventionally used for alcoholic fermentation displayed little or no detectable enzymatic activity, similar to other *Saccharomyces cerevisiae* (Charoenchai *et al.*, 1997; Rosi *et al.*, 1994). The exception was limited β -glucosidase production by VL-1, 110 nmole mL⁻¹ g dry cell mass⁻¹ (Table 1). Zoecklein *et al.* (1997) noted fermentation by VL-1 resulted in higher total free monoterpenes and aromatic alcohols than CV-D47 or Prise de Mousse, suggesting limited hydrolysis.

Cultures with high β -glucosidase activity were further analyzed for localized site of enzyme activity, by the procedure of Rosi *et al.* (1994). On average, the whole cell fraction (parietal)

produced the majority of the enzyme activity, followed by the permeabilized fraction (intracellular), while there was limited exogenous production (supernatant) (Tables 3 and 4). Variances in the location of hydrolytic enzyme activity from the activities obtained by the screening procedure may have occurred due to the cell wall differences. The Rosi *et al.* (1994) assay is influenced by the ability of the cell culture to form a stable pellet. In cultures where cell pellets were less cohesive, some cells might have been lost in the assay, causing an artificially low concentration. The extent of this reduction would be dependent on the loss of cell mass. An extreme example was VL-1, for which the lack of parietal enzyme formation seems suspect (Table 3). The activity in the permeabilized cell fraction may have been lower than the whole cell fraction due to release of digestive enzymes. The release of β -glucosidase may have led to a higher concentration, but the enzymes may have been subsequently denatured or removed during the pellet wash. Exogenous enzyme activity averaged two percent of the total activity for all cultures, and the quantities found might have been due to cell lysis. Darriet *et al.* (1988) found that β -glucosidase is located in the periplasmic space of the yeast cell, and would be released during cell death. A small portion of the enzyme activities in the supernatant fraction may have been due to release of enzyme during cell autolysis. The extracellular enzyme activities found were slightly higher than those found by Rosi *et al.* (1994) for the species *Kloeckera apiculata* and *Hansenula anomala*.

Seven of ten *Brettanomyces intermedius* strains displayed high β -glucosidase activities, ranging from 670 to 2650 nmole mL⁻¹ g dry cell⁻¹ (Table 2). On average, the strains examined had higher permeabilized enzymatic β -glucosidase activity than the vineyard isolated yeasts. The *Brettanomyces* assayed for location of activity exhibited detectable levels of exogenous activity (Table 4). Extra and intracellularly produced (α) glucosidase activity has been previously demonstrated from *Brettanomyces lambicus* isolated in a lambic beer (Shantha Kumara *et al.*,

1993).

Hydrolytic enzyme activity was not detected in the *Leuconostoc oenos* strains (not shown). The fastidious nature of the organisms and the lack of a complex growth media may have contributed to the lack of enzymatic production. During preliminary trials, the lactic acid bacteria dried preparations were added directly to the YNB arbutin without prior isolation. Using this methodology, *Vinaflora oenos* had limited α -L-arabinofuranosidase (10 nmole mL⁻¹ g⁻¹, less than the Limit of detection) and α -L-rhamnopyranosidase (90 nmole mL⁻¹ g⁻¹) production and OSU had minor β -glucosidase activity (111 nmole mL⁻¹ g⁻¹). However when isolated colonies were used, no production was observed. The additional culture transfers might have caused cell shock and limited hydrolytic enzyme production.

Viognier glycosides (111 μ M glycosyl-glucose) were used to determine enzyme hydrolysis of a natural substrate in *Aureobasidium pullulans*, *Kloeckera apiculata*, and *Candida parapsilosis*. *Aureobasidium pullulans* was able to hydrolyze 68% of the glycosides (final concentration: 35 μ M glycosyl-glucose). However, no detectable activity was found in *Kloeckera apiculata* or *Candida parapsilosis*. Rosi *et al.* (1997) found that the extent of the glycoside hydrolytic ability of *Debaryomyces hansenii* was grape cultivar dependent. The grape glycosides may have been further substituted to form a disaccharide. In this case, the β -glucosidase would not be able to liberate the aglycones until the terminal sugars were removed. Therefore, the ability of *A. pullulans* to hydrolyze the Viognier glycosides may have been due to its arabinosidase and rhamnosidase activities. The use of a natural substrate illustrated the potential of *Aureobasidium pullulans* to hydrolyze wine glycosides; however the constraints of a fermenting juice environment may inhibit this activity (Gunata *et al.*, 1994; Rosi *et al.*, 1994).

Further investigation regarding culture conditions in both the yeast and bacteria are

warranted. Aerobic conditions, lack of ethanol, elevated pH and temperature, and removal of end product (glucose inhibition) could enhance enzyme activity (Rosi *et al.*, 1994), but these are not common fermentation parameters. Feedback inhibition through glucose concentrations less than 0.5% (w/v) is a common constraint with some hydrolytic enzyme production (Gunata *et al.*, 1994; Rosi *et al.*, 1994). Some grape enological yeast enzymes are more resistant to glucose inhibition than others (Aryan *et al.*, 1987; Delcroix *et al.*, 1994; Dubourdieu *et al.*, 1988). Rosi *et al.* (1997) showed the optimum glucose concentration for *Debaryomyces hansenii* was 2-8%. *Candida wickerhamii* has been shown to retain 44% of its activity in grape juice (500 mM glucose) (Gunata *et al.*, 1994). In this study, glucose inhibition was avoided by the use of arbutin as the carbon source in the growth media. However, the β -D bond of arbutin may have failed to induce production of α -L-arabinofuranosidase or α -L-rhamnopyranosidase and subsequently these activities could not be adequately assayed.

Grape β -glucosidases can exhibit a 60% loss of activity at ethanol concentrations of 3.5% (Aryan *et al.*, 1987; Gunata *et al.*, 1984). However, many fungal and yeast β -glucosidases are not inhibited by the concentrations of ethanol in table wine (Aryan *et al.*, 1987; Delcroix *et al.*, 1994; Leclerc *et al.*, 1984). Among the documented species are *Hanseniaspora vineae* (Vasserot *et al.*, 1985), *Dekkera intermedia* (Blondin *et al.*, 1983), and *Candida molischiana* (Gonde *et al.*, 1985). Guegen *et al.* (1994) demonstrated β -glucosidase from *Candida entomophila* was stimulated by alcohol up to a concentration of 3.5%, but was inhibited at higher concentrations, likely due to protein denaturation. Permberton *et al.* (1980) hypothesized this could be due to a glycosyl transferase activity of the enzyme. Ethanol acts as an acceptor for the intermediary glycosyl cation and has better nucleophilic character than water. Further research should focus on the influence of a model wine environment on hydrolytic activities.

CONCLUSION

Aroma and flavor changes and possibly enhanced wine quality may result from enzymatic hydrolysis of glycosides, and subsequent liberation of aglycones. Of the cultures examined, hydrolytic enzyme activities were more commonly found in non-*Saccharomyces* strains of yeast. VL-1 was the only *Saccharomyces cerevisiae* strain which had β -glucosidase activity. *Aureobasidium pullulans* hydrolyzed α -L-arabinofuranoside, β -glucoside, and α -L-rhamnopyranoside. Seven *Brettanomyces* strains had β -glucosidase activity. The lactic acid bacteria studied were unable to hydrolyze glycosides in the model system media. *Aureobasidium pullulans* was also capable of hydrolyzing Vigonier grape glycosides. Further research should focus on the optimization of hydrolytic activities in a typical fermentation environment.

Table 1: Enzyme activities for vineyard isolates and commercial yeasts (expressed as nmole of hydrolyzed β -glucoside or analogue per mL assay medium per gram dry cell mass).

Organism	Arabino- furanoside	Gluco- pyranoside	Rhamno- pyranoside
<i>Aureobasidium pullulans</i>	37 a	1774 a	653 a
<i>Candida guilliermondii</i>	n.d.	824 c	26 b
<i>Candida parapsilosis</i>	n.d.	1744 a	n.d.
<i>Cryptococcus albidus</i>	n.d.	n.d.	n.d.
<i>Hanseniaspora uvarum</i>	n.d.	n.d.	n.d.
<i>Hansenula anomala</i>	n.d.	719 d	n.d.
<i>Kloeckera apiculata</i>	n.d.	1322 b	n.d.
<i>Metschnikowia pulcherrima</i>	n.d.	633 e	n.d.
<i>Pichia guilliermondii</i>	< LOD	349 f	n.d.
<i>Saccharomyces (italicus) cerevisiae</i>	n.d.	n.d.	n.d.
<i>Torulasporea delbrueckii</i>	n.d.	n.d.	n.d.
<i>Saccharomyces cerevisiae</i> (ICV-D47)	n.d.	n.d.	n.d.
<i>Saccharomyces cerevisiae</i> (M1)	n.d.	< LOD	n.d.
<i>Saccharomyces cerevisiae</i> (VL-1)	n.d.	110 g	n.d.
<i>Saccharomyces cerevisiae</i> (Prise de Mousse)	< LOD	n.d.	n.d.
<i>Saccharomyces cerevisiae</i> (Fermiblanc)	n.d.	n.d.	n.d.

n.d. = none detectable. Values are the averages of duplicate replications. Different letters within columns indicate significance at $P \leq 0.05$. Limit of detection: 19 nmole/mL.

Table 2: Enzyme activities for *Brettanomyces intermedius* (expressed as nmole of hydrolyzed β -glucoside or analogue per mL assay medium per gram dry cell mass).

<i>Brettanomyces intermedius</i> strain	Arabinofuranoside	Glucopyranoside	Rhamnopyranoside
211	n.d	575 e	n.d
212	n.d	1694 c	n.d
213	n.d	n.d.	n.d
214	n.d	2656 a	n.d
215	n.d	1765 bc	n.d
216	n.d	2018 b	n.d
<i>brux</i>	n.d	n.d.	n.d
<i>'ave'</i>	n.d	931 d	n.d
<i>souche 'o'</i>	n.d	n.d.	n.d
<i>souche 'm'</i>	n.d	819 d	n.d

n.d. = none detectable. Values are the averages of duplicate replications. Different letters indicate significance at $P \leq 0.05$. Limit of detection: 19 nmole/mL.

Table 3: β -glucosidase activities by location of enzyme activity (whole, permeabilized, or supernatant) for vineyard isolates and a commercial yeast strain.

Organism	Whole ¹	Permeabilized ¹	Supernatant ²
<i>Aureobasidium pullulans</i>	2279 a	268 c	n.d.
<i>Candida guillermondii</i>	230 d	n.d.	23 c
<i>Candida parapsilosis</i>	1313 b	151 d	20 c
<i>Hansenula anomala</i>	587 c	84 f	6 d
<i>Kloeckera apiculata</i>	621 c	393 b	260 a
<i>Metchnikowia pulcherrima</i>	1359 b	112 e	73 b
<i>Pichia guillermondii</i>	105 e	57 g	n.d.
<i>Saccharomyces cerevisiae</i> (VL-1)	n.d.	726 a	4 d

Values are the averages of duplicate replications. Different letters within columns indicate significance at $P \leq 0.05$. ¹ Activity is expressed as nmole pNP per mg cells (dry weight).

² Activity is expressed as nmole pNP per mL. Limit of detection: 3 nmole/mL.

Table 4: β -glucosidase activities by location of enzyme activity (whole, permeabilized, or supernatant) for *Brettanomyces intermedius* strains.

<i>Brettanomyces intermedius</i> strain	Whole ¹	Permeabilized ¹	Supernatant ²
211	129 e	70 ab	LOD
212	1609 a	1568 bc	6 b
214	747 b	427 a	15 a
215	161 d	156 d	LOD
216	844 c	206 cd	< LOD

Values are the averages of duplicate replications. Different letters within columns indicate significance at $P \leq 0.05$. Means with the same letter are not significantly different. ¹ Activity is expressed as nmole pNP per mg cells (dry weight). ² Activity is expressed as nmole pNP per mL. Limit of detection: 3 nmole/mL.

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