Fall 2015

Efficacy of Ultraviolet Radiation to Control Lactic Acid Bacteria in Wine Must

Brian D. Williams
Central Washington University, desertlifter@gmail.com

EFFICACY OF ULTRAVIOLET STERILIZATION TO CONTROL LACTIC ACID BACTERIA IN WINE MUST

A Thesis

Presented To

The Graduate Faculty

Central Washington University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

Biology

---

by

Brian David Williams

November 2015

CENTRAL WASHINGTON UNIVERSITY

Graduate Studies

We hereby approve the thesis of

Brian David Williams

Candidate for the degree Master of Science

APPROVED FOR THE GRADUATE FACULTY

_____	_____
	Dr. Holly Pinkart, Committee Chair
_____	_____
	Dr. Jennifer DeChaine
_____	_____
	Dr. Jim Johnson
_____	_____
	Dean of Graduate Studies

ABSTRACT

EFFICACY OF ULTRAVIOLET STERILIZATION

TO CONTROL LACTIC ACID BACTERIA

IN WINE MUST

by

Brian David Williams

November 2015

The use of shortwave ultraviolet (UV_c) radiation to control lactic acid bacteria (LAB) in wine production was studied. A simulated wine sterilizer was built using a commercially sourced ultraviolet (UV) sterilizer commonly used in aquariums and ponds. After growing cultures in test tubes, samples of five different species of LAB were introduced into white grape juice adjusted for brix and pH to match that of wine must commonly found in the Yakima Valley American Viticultural Area. The mixture was then agitated and allowed time to evenly distribute the bacteria throughout the juice. The juice was sent through the sterilizer in a single pass using an aquarium pump. LAB were quantified pre- and post-treatment using a dilution series on MRS agar. The UV_c treatment resulted in a significant reduction of LAB by an average of 52.7% with a 95% confidence

interval for three replicates of three trials. These data are compared to industry standards and applications of UV_c sterilization in the wine industry with suggested areas for further study are discussed.

ACKNOWLEDGMENTS

Huge thanks are owed to all and sundry who have supported me in this journey. I would like to thank Dr. Jennifer Dechaine for the opportunity to undertake my program of study – without your work in putting the program together; I would never have had the opportunity to study at Central Washington University. I also owe huge thanks to everybody who provided logistical support throughout the research process, including Jonathan Betz and Mary Bottcher, who always have gone out of their way to help me find what I need. Eric Foss and everybody in lab prep were all instrumental in having materials ready and in locating them when I needed things in the lab.

Without Dr. Holly Pinkart's patience and guidance I never would have gotten off the ground. Coming from a physics background and mindset, I was lost without your help and guidance. As a science teacher, I hope that I will someday be as inspirational as both you and Dr. Dechaine. I also owe a huge debt to the winemakers at Bonair Winery in Zillah and Gooseridge Estate Vineyard in Kennewick for their input.

Finally, I owe everything to my wife and family who have been my rock through this entire journey. Working multiple jobs while completing this work would have been impossible without your love and support.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

The Winemaking Industry

Evidence of winemaking exists from as early as 7000 $_{B.C.}$ in China, and wine has been made since 3000 $_{B.C.}$in Egypt (Jackson 2008). While differences exist as to the source of sugar – be it rice, honey, or fruit - the winemaking process is basically the same today. Sugar is exposed to yeast and given time to ferment to an alcoholic end-product. Perhaps the greatest change from 7000 $_{B.C.}$ in China to "modern" winemaking in ancient Egypt was when wine was produced using cultured yeasts rather than wild ones, which began around 3150 $_{B.C.}$ Wines made from that period in Egypt onward may be considered to be using modern winemaking techniques because they were made using *Saccharomyces cerevisiae*, a yeast used in modern winemaking that was not native to grapevines or areas in which they grew (Jackson 2008).

Today grapes are grown in greater quantity worldwide than oranges, bananas, or apples, with well over half of the grape crop being used in wine production (Jackson 2008). Locally, wine is a growth industry in the U.S. and Washington State (WA). A report prepared for the Washington State Wine Commission (Stonebridge Research 2012) showed that the Washington winemaking industry is worth over $8.6 billion, directly and indirectly providing full time employment for nearly 30,000 workers in the state. Since 2009, the total acreage of vineyards in Washington increased by 7,000 acres, with nearly 100

new wineries opening between 2009 and 2011 (Stonebridge Research 2012). The number of jobs and impact on the economy of Washington State alone is an indicator of the importance of quality control of Washington wines. The necessity to minimize product loss and to ensure a positive public image of the product in order to ensure the continual viability of this industry cannot be over-emphasized.

Spoilage in winemaking

Spoilage in winemaking may occur at different points in the winemaking process and take different forms. It may range in degree from minor flavor and/or odor issues to rendering wine unfit for consumption. In either case, loss of product and lowered commodity prices impact winemakers by increasing costs and lowering profits. Wine spoilage may occur due to issues ranging from storage and aging caused by the bottle or cork to those caused by bacteria or yeasts (Jackson 2008). The focus of this study is on the latter.

The Timing of Spoilage

Microbial wine spoilage may occur at three points in the winemaking process: with the raw material and equipment that handles it prior to fermentation, during fermentation, and after fermentation (du Toit and Pretorius 2000).

Wine grapes are brought to the winery having been exposed to handling by workers, contamination introduced by birds and insects, and from the equipment that delivers it and in which it is stored. Grapes may be even become infected on the vine, and that infection may be then be spread to more of the fruit

when it is in contact with batches of grapes during pre-production and transportation. When grapes arrive at the winery, they come into contact with an assortment of equipment from storage tanks to pumps and the equipment used to crush the fruit. If any of the equipment is not properly sterilized, it may pass on undesirable wild yeasts or bacteria (du Toit and Pretorius 2000).

After the grapes are crushed and fermentation begins, the juice may be exposed to contamination by natural flora on the fruit or in the air. The pH of the must (the juice from the crushed grapes), its sugar content, and the addition of sulfites will all impact the survival and growth of these spoilage organisms at this phase. Selective pressures caused by the viability of the species and their population will also determine whether organisms present in the must cause spoilage (du Toit and Pretorius 2000).

The final step in which spoilage may occur takes place after fermentation. Spoilage may occur in the bottle or in oak barrels used for aging, or even by the corks used to stopper the bottles. While ensuring the wine is not exposed to oxygen helps at this phase, many organisms that can cause wine spoilage are anaerobes or selective anaerobes. Quality control of the final product is often ensured by adding antimicrobial agents to the wine at this stage (du Toit and Pretorius 2000).

The Process of Winemaking

Regardless of the fruit used, winemaking is a simple process. Fruit of some kind if harvested and its juice is extracted, yeast is added to the juice, and

then once fermentation is complete to some degree, it is either placed into casks or bottles for aging. These steps are outlined in greater detail in Figure 1 below.

Harvesting and Crushing the Grapes

The major steps for winemaking are the same for red and white wines. The timing of harvesting grapes for winemaking typically revolves around measurements of their sugar content and acidity, the optimal levels for which change according to cultivar and region. After the determination is made that a crop is ready, grapes are harvested mechanically or by hand. The grapes are then sorted to remove foreign materials such as insects as well as grapes that are sub-standard. They are then de-stemmed and crushed to extract their juice, which is then referred to as must. After crushing the grapes, sulfites are added to prevent spontaneous fermentation by wild yeasts and bacteria (Jackson 2008).

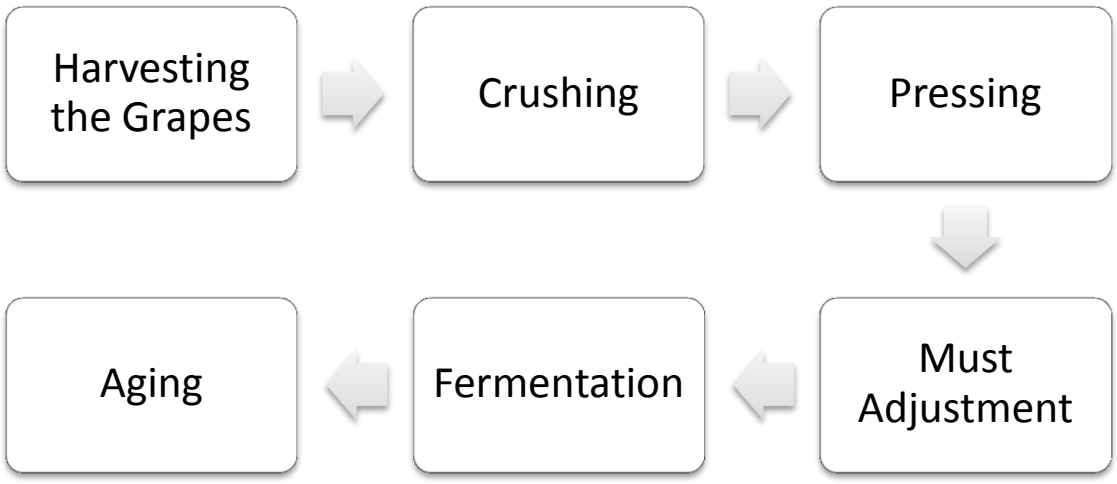

Figure 1 Major steps in the winemaking process

Maceration

The methods for producing white and red wines then diverge. While the majority of white wine musts move on to the next stage, red wine musts and a few whites are first macerated, meaning that they are left in contact with their skins and stems. Maceration of red wines takes as few as 3-5 days to as long as 15 days or in some cases up to 3 weeks. Maceration is much briefer in white wines than red, taking no more than 30 hours. In either case, maceration extracts nutrients and other chemicals from the physical constituents of the fruit. These nutrients – in particular sugars, nitrogen, lipids and phenols – are critical to make a final product that is well fermented and results in higher tannin levels, which improve the quality of wines as they age. Lipids provide essential nutrients needed by yeasts, while phenols improve the final product by enhancing flavors and mouth-feel. Maceration at cooler temperatures produces fruity wines, while at warmer temperatures it results in darker, more complex wines. The shorter maceration time for white wine must results in a much lower phenolic content. The must of both red and white wines is then pressed to complete extraction (Jackson 2008).

Must Adjustment

After removing the must from its constituent fruit, white wine is clarified by either centrifuging it or allowing solids to settle and pouring the clarified must off of the solids – a process called “racking.” Sugar may be added to the must if its

levels are too low, and pH may be adjusted if the pH of the must is outside of 3.1 to 3.4 for white wines and 3.3 to 3.6 for red wines. Note that alkalinity or acidity here is relative and refers to reference levels for wine, which is acidic and does not imply that the must is basic – it may be just too alkaline for effective fermentation. During must adjustment, the sulfites added to the must in the previous step abate through enzymatic action. Once free sulfite levels drop to a level where it will remain viable, yeast is added to the must to begin fermentation (Jackson 2008).

Fermentation

Fermentation of wine serves to not only produce alcohol but improve flavor profile and mouthfeel. While largely similar, there are differences in how fermentation is carried out between red and white wines. The focus here is on the latter. There are two types of fermentation in wines – alcoholic and malolactic fermentation. Malolactic fermentation (MLF) is carried out by LAB and is in general more beneficial to red wines than white, as most white wines have a more delicate bouquet and flavor profile that can be adversely affected by MLF. MLF for white wines is limited to certain varietals as well as to cooler climates for this reason (Jackson 2008). The generally adverse impact of MLF on white wines is one reason that LAB were chosen as the model organisms for this study.

Alcoholic Fermentation

Alcoholic fermentation was first described by Henry Pasteur in 1857 when he studied winemaking and the “diseases of wines” (Willey and others 2008).

Alcoholic fermentation is a microbial anaerobic fermentation where pyruvate loses one CO_2, forming acetaldehyde. This process results in the alcohol in wines and enhances the fragrance of wines emphasizing characteristics unique to different varietals (Jackson 2008).

In alcoholic fermentation's early phase, sugars are broken down into pyruvate via an anaerobic metabolic pathway called glycolysis. Fermentation then takes place in two basic steps. During the first step, pyruvate decarboxylase removes CO_2, which produces acetaldehyde [Figure 2 (Denniston and others 2007)].

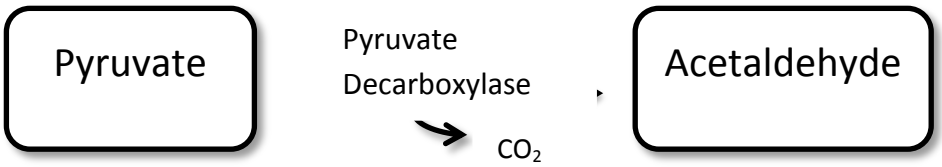

Figure 2: Step 1 of alcoholic fermentation

During the second step of alcoholic fermentation, alcohol dehydrogenase reduces the acetaldehyde to ethyl alcohol [Figure 3 (Denniston and others 2007)]. Two of the products of alcoholic fermentation are then CO_2 and ethanol (Denniston and others 2007).

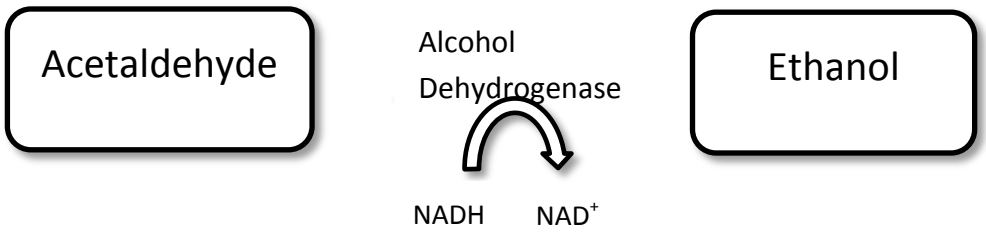

Figure 3: Step 2 of alcoholic fermentation

Malolactic Fermentation

Like alcoholic fermentation, MLF can be described as a two-step process: first, malic acid is decarboxylated to pyruvic acid, and then the pyruvic acid is reduced to lactic acid (Jackson 2008). Malolactic fermentation is performed by LAB with musts that are relatively high in pH (for wine) or sugars (Edwards 1992). Typical LAB populations on the vine average around 10^2 CFU/mL and rise to 10^4 during alcoholic fermentation. Malolactic fermentation begins once yeast activity lowers enough to taper alcoholic fermentation so the bacteria are not competing with the yeast for the remaining sugars, and MLF populations rise to 10^6 CFU/mL (Lonvaud-Funel 1999).

The wines that MLF benefits the most are those in which it has a comparably difficult time completing since the conditions in those wines are not optimal for LAB growth; those with lower pH and brix. In contrast, MLF takes place more readily in the opposite conditions of higher sugar/pH (relative to wines) where it is more detrimental (Jackson 2008). In wines where it is beneficial, MLF raises pH and lowers their perceived acidity. Malolactic fermentation with beneficial strains of LAB also controls wild yeasts such as *Brettanomyces,* a strain of yeast that is of particular concern to winemakers because it can cause spoilage that introduces unwanted esters to the wine, and is commonly found in wood barrels (du Toit and Pretorius 2000). Malolactic fermentation may also serve to increase microbial stability during cellaring and aging, although there is some question as to whether MLF itself is the engine for this effect (Jackson 2008).

Lactic Acid Bacteria

Lactic acid bacteria (LAB) are Gram-positive, non-motile, and non-spore forming bacteria. They are aero-tolerant anaerobes and can be both rod and coccus shaped. LAB are well adapted to living in wine. They are tolerant of low pH and have even adapted to survive in environments with ethanol and SO_2 (du Toit and Pretorius 2000). LAB can be either beneficial (as in MLF as discussed previously) or detrimental to wines, depending on the variety and desired fermentation (Lonvaud-Funel 1999).

Wine spoilage caused by LAB is of greater concern to winemakers in Washington State because of the high overall pH of Washington wines, which commonly exceeds 3.5 (Edwards 1992). LAB were chosen for this study due to their prevalence in the local region as well as to the higher potential for negative outcomes due to the typical pH of wine grapes in the area, which is nominal for MLF. Five species of LAB were used in the study, each having its own potential impacts on wine quality: *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *Oenococcus oeni*, *Pediococcus* damnosus, and *Pediococcus pentosaceus.*

Lactobacillus plantarum can cause tartaric acid reduction in wines, which completely spoils the wine (du Toit and Pretorius 2000). It can also increase diacetyl levels in wine, which give the wine buttery, nutty, or caramel notes (Bartkowsky 2009). *Lactobacillus rhamnosus* is more commonly used in food production, such as in yogurt, but may cause issues similar to those of other

Lactobacillus species, from bitterness and geranium odors to pungent or vinegar flavors.

Oenococcus oeni is the most common species of LAB present in wine grapes and the most beneficial due to its activity in MLF (Jackson 2008). Even so, it can cause stuck fermentations and increase diacetyl levels so much that the wine gains a buttery flavor (du Toit and Pretorius 2000). It can compete with yeast for sugars that are used in alcoholic fermentation, so *O. oeni* has to be controlled during that phase of winemaking (Jackson 2008).

Of the model LAB chosen for the experiment, *Pediococcus damnosus and Pediococcus pentosaceus are* the most detrimental to wines. P*. damnosus* and *P*. *pentosaceus* can both produce polysaccharides that cause a viscous condition in wine called “ropiness” in lay terms (du Toit and Pretorius 2000). Ropy wines have strands (ropes) of cloudy mucous-like structures that render wines unfit for consumption. Du Toit and Pretorius noted that lowering pH below 3.5 controls ropiness (2000). Since Washington wines tend to be more alkaline (Edwards 1992), *P. damnosus* and *P*. *pentosaceus* present a particularly onerous problem for winemakers.

This study was undertaken to determine whether UV_c could be used to control spoilage bacteria in wine. There is little literature addressing this question currently, although many alternatives including other chemical methods (Blättel and others 2009; Defini and others 2002) have been examined. Questions about the practicality of using UV_c were addressed by using a readily available UV_c

sterilizer, making the research of value for both the hobbyist as well as commercial operations. Data were created to examine how close this UV_c system could maintain LAB populations below the threshold where acid reduction due to MLF occurs, 10^8 cells per mL (Jackson 2008).

CHAPTER II

LITERATURE REVIEW

Bacterial Infection of Wine

There are many points during the winemaking process at which infection can occur. This includes native flora and fauna on the vine, handling the grapes in the field, equipment sterilization in the winery, and sterilization of the must itself (Mendes-Ferreira and others 2010). The sterilization of must prior to pitching yeast and beginning fermentation is an important step in which to assess the effectiveness UV sterilization due to its place at the beginning of fermentation, where the action of wild yeasts and bacteria are the most likely. One of those types of bacteria – lactic acid bacteria – is common to winemaking.

LAB are not always detrimental to wine production. They are sometimes used in later fermentation stages (in particular malolactic fermentation) to improve wine quality. LAB prefer alkaline environments. American wines are fairly acidic – except in Washington State, where alkaline soils produce wines of unusually high pH (Edwards 1992). This makes the control of LAB particularly poignant in Washington as Washington wines present a more beneficial environment for LAB to grow. The typical method for sterilization in this case is to add more sulfite. This has the potential to lower wine quality and initiate a stuck fermentation (when fermentation stops even though there are adequate sugars to produce more alcohol and CO_2). If ultraviolet radiation can effectively control

LAB, the use of sulfites can be minimized in winemaking, allowing for better quality control as well as opening the market to those who are allergic to sulfites.

Current Sterilization Methods

Bacterial (Lonvaud-Funel 1999) and wild yeast infections (Loureiro and Malfeito-Ferreira 2003) in wine affect both quality and efficiency of wine production. Unwanted yeast and bacteria compete for resources with desirable organisms and release chemicals that impart off flavors, unwanted consistencies (such as ropiness), and/or off-odors to the wine. Sanitation is perhaps the most important factor in producing high and consistent quality wines, but the most prevalent methods slow production as the winemaker must wait until their effects are abated or compensate for their presence by pitching more yeast to compensate for chemical controls that are still controlling microbial populations (Delfini and others 2002).

There are several problems with current sterilization methods of equipment and must used in the wine industry. Chemical sterilization using sulfites can directly affect wine quality (Blättel and others 2009) and can change the rate of release for H_2S during fermentation, which also negatively affects wine quality (Mendes-Ferreira and others 2010). Another problem with chemical controls is that they affect both unwanted and wanted organisms. Chemical measures must abate before fermentation can begin; otherwise the winemaker will induce stuck fermentation or worse (Delfini and others 2002), delaying the start of fermentation.

Issues with Sulfites

Sulfites are among the oldest compounds used in winemaking, dating back to the Egyptian and Roman empires (du Toit and Pretorius 2000). The addition of sulfites to wine must is the source of some controversy. Its efficacy is in question (Jackson 2008) and some people have adverse reactions to sulfites when they consume them (Simon 2003). Meanwhile, its utility in controlling microbes that are implicated in spoilage is supported in the same literature that speaks to its drawbacks (Garde-Cerdán and Ancín-Azpilicueta 2007).

Sulfiting has “clearly been shown . . . to be the cause of serious and potentially life-threatening asthmatic reactions” (Simon 2003). Prior reports of adverse reaction to consumption of sulfited foods led to the FDA to ban them in fresh foods in 1986 (Simon 2003).

Sulfites have an additional image problem in winemaking. On one hand they are a necessary part of the winemaking process. They act as a preservative for the wine – increasing its shelf life – and are thus instrumental in the aging process, which is critical in the production of finer wines. On the other hand, they can also impart off-odors and flavors to the wine if they are over-used, usually described as mousy, ropy, or smelling of rotten eggs (Mendes-Ferreira and others 2010).

Sulfites also are perceived as a source of headaches and other problems by consumers. Individuals with sulfite allergies react to them in different ways, and the degree of reaction is more pronounced with those who are allergic to

skin contact of sulfites (Simon 2003). For those who are allergic to them, sulfites can cause symptoms ranging from headaches and nausea to stomach upset and breathing difficulties in asthma sufferers (Santos and others 2012). Even though sulfites are not actually causing headaches in the vast majority of wine consumers, they are still saddled with that image. Coupled with the requirement lowering the allowable threshold for sulfites allowed for use in winemaking, this indicates a need for developing alternative methods of microbial control that maintain the level of quality and advantages provided by sulfites while avoiding their disadvantages (Santos and others 2012).

Physical Sterilization Methods in the Wine Industry

Physical sterilization methods are used in the wine industry largely for preparing the equipment and the operating environment (air, tabletops, work surfaces, etc.). High pressure steam is used to sterilize wine barrels and other equipment, and UV is used to kill airborne wild yeast and bacteria. Heat can be used to sterilize equipment, but repeated heat cycles can damage it and shorten its lifespan, ultimately increasing production cost. Wine and other food production has seen testing of ultra-high pressure treatment, ultrasound, and pulsed electrical fields (Bartowsky, 2009). Both ultrasound and pulsed electrical fields have been shown to be effective in preliminary trials, however they both can accelerate the aging process for wines, and high pressure treatment can lead to decreases in both antioxidant activity and anthocyanin content (Santos and others 2011). While ultrasound appears to be promising, it is of note that all of the physical controls for bacteria are effective only while they are being applied.

Bacteria that form spores are a problem for these methods, as spores may be viable after treatment. The exception to this is high pressure treatments, which disrupt cell membranes. Ultrasound treatment was also less effective in treating LAB than other microbes (Santos and others 2011).

Although it is currently used only for equipment, UV is a potentially effective method for sanitizing the wine itself. UV is already used as a sterilization method in water treatment and other food services (Koutchma 2009), thus adaptable equipment is already in production. While theoretical application is important, the feasibility for actual implementation in the field is still largely unknown, and further research is required to evaluate the efficacy of ultraviolet sterilization of wine (Guerrero-Beltrán and Barbosa-Cánovas 2004).

UV in the Control of Microbes in Liquid Media

UV radiation is effective in controlling viruses, bacteria, and other microorganisms when it is applied in the frequency range of 250-260nm. It controls microorganisms by damaging their DNA, preventing cellular division (Bintsis and others 2000). The process does not produce by-products that might lower product quality, and it is cheaper than other methods (Guerrero-Beltrán and Barbosa-Cánovas 2004; Santos and others 2012). In addition to liquid food products, UV has been used effectively in industrial applications to treat filtered effluent and solid foods such as fruits and vegetables (Bintsis and others 2000).

However, UV_c has limitations in its application. While effective to treat clear water, factors such as turbidity, color, and high microbial load (Fredericks

and others 2011) may all negatively impact its utility to control unwanted microbes (Bintsis and others 2000). Even humidity negatively impacts the action of UV on bacteria in air, and in a liquid medium the problem is even greater. The depth of penetration for UVc radiation in juices is only about 1mm for 90% absorption (Guerrero-Beltrán and Barbosa-Cánovas 2004). To counter this shortcoming, systems using laminar flow and turbulent flow are used to improve results. Both systems seek to increase the probability of exposure to microbes to UV with as little depth of penetration as possible, however laminar flow systems have been shown to be less effective in controlling bacterial populations in grape juice and wine (du Toit and Krügel 2011). Even clumps of bacteria will block UV's potency, so the distance it must penetrate to act on microbiota is critical in ensuring that it has a germicidal effect in liquids (Bintsis and others 2000). Its effectiveness also may vary according to species and life stage of microbes, particularly in spore-forming microbes whose dormant state may increase survival after treatment (Guerrero-Beltrán and Barbosa-Cánovas 2004). Even with these considerations, UV_c has been used to successfully reduce total colony counts in goat milk between 50% and 60%, and it even reduced coliform bacteria in the milk at up to 90%. The FDA was even able to successfully treat fruit juices using turbulent flow systems (Bintsis and others 2000). The potential for success in white wine was at least positive considering these results – both milk and fruit juices are more turbid and opaque than white wine and the white grape juice used to produce it.

Ultraviolet radiation was thus chosen for study as a potential method to control LAB in white wine production. While UVc presents challenges to use in liquid media, its effectiveness in similar applications – in particular as tested in fruit juices as discussed above – and low cost make it a viable candidate to minimize or even end the use of sulfites in some cases. This is important because it would allow for access to a consumer group that currently is unable or reluctant to consume sulfited wines. It was further undertaken in order to seek cost effective methods to control LAB in white wines. While LAB are beneficial to many red wines, they are not typically so in white wines.

CHAPTER III

MATERIALS AND METHODS

Grape Juice

Safeway Kitchens brand 100% white grape juice (Safeway, inc., Pleasanton, CA) was used in the study. Initial tests were undertaken using locally sourced must, however its use was problematic because it was difficult to ensure that the juice was only infected by the model organisms mentioned below. Chemical controls could have skewed the results, and pasteurizing or autoclaving the must resulted in browning. Grape juice was chosen because it was similar in opacity to juices used in white wines and similar work undertaken by du Toit and Krügel (2011). The juice's brix (sugar content) was adjusted to ~250 g/L using 92 g/L of sugars, including 45 g dextrose, 45 g sucrose, and 2 g yeast nitrogen base/liter according to measures published by Margalit 2004. Samples taken from local wineries averaged pH of 3.9, so the juice was adjusted to a pH of 3.9 pH ±.05 to match that of must samples taken from Gooseridge Estates in Kennewick, Washington. This range is indicative of typical higher pH in Washington State as opposed to other American wine growing regions (Edwards 1992).

Sterilization Apparatus

The sterilization apparatus (Figure 4) was assembled on a lab cart with an Aqua Medic "Helix Max" 55 watt Ultraviolet Sterilizer (Bissendorf, Germany) using an Aquamedic Electronic Ballast (Model UV-55) to power the unit. The

Helix Max sterilizer uses a 55 watt Weipro dual bulb (Zhongshan, China). The sterilizer unit was mounted to 2 pieces of plywood that were first glued and screwed together. All lines were secured with zip ties, and the ballast was secured to the board with the sterilizer with a conduit strap.

Two sections of 12.8 Durometer (Shore A) 85 PVC bubble tubing were attached to the inlet and outlet ports of the sterilizer unit, and then standard 1.3 cm thick flexible plastic aquarium tubing was attached to the bubble tubing as a reduction fitting to attach the inlet line to a Marineland Mini-Jet model 606 submersible adjustable flow pump (Blacksburg, VA). The same 1.3 cm tubing was attached to the outlet tube to maintain steady fluid flow. The plastic connections were silicon sealed at the reduction fittings. A hose clamp was installed on the outlet line's plastic hose to control flow rate. Two 4000 mL plastic beakers were used to handle the juice at the inlet and outlet ends.

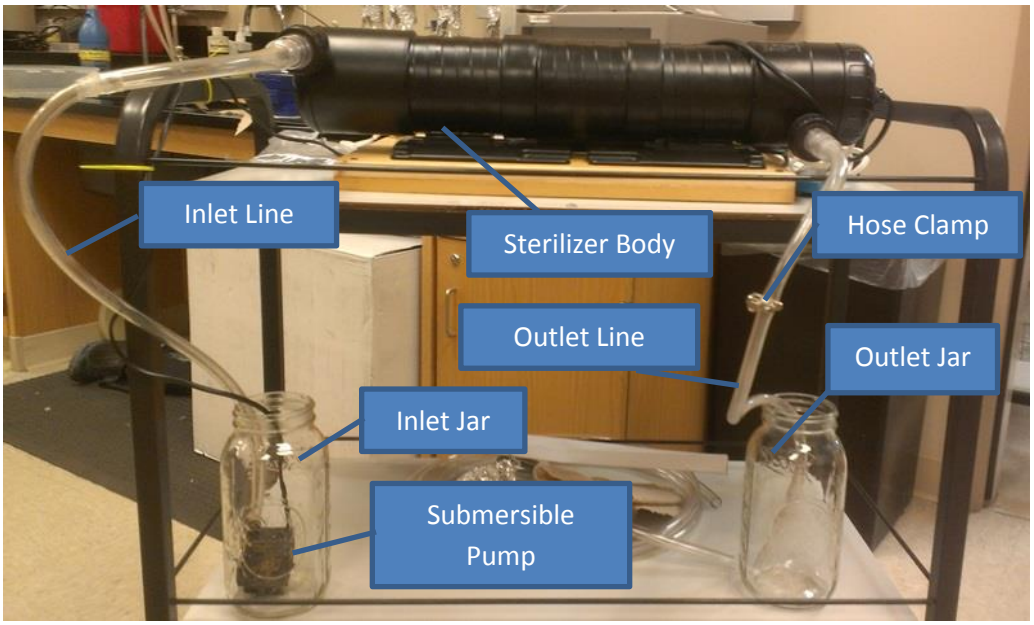

Figure 4 UVc apparatus installed on lab cart

The pump is rated for 579 liters per hour and the apparatus has a total volume of 2.12 L, including the hoses. Both the inlet and outlet containers were changed during the course of the experiment as needed.

Bacterial Cultures

Sample cultures of LAB were grown on MRS agar plates. The plates were inoculated and then placed in Ziploc bags containing Becton Dickinson (Franklin Lakes, NJ) CO_2 gas generators (130mg Sodium Bicarbonate) and incubated at room temperature (25˚C-28˚C) for 48 hours. Species of LAB used in this study are shown in Table 1.

Table 1. Bacterial Strains Used in This Study

Strain	Source*
Lactobacillus plantarum	ATCC 8014
Lactobacillus rhamnosus	ATCC 53103
Oenococcus oeni	Viniflora, CHR Hansen (Hørsholm, Denmark)
Pediococcus damnosus	ATCC 29358
Pediococcus pentosaceus	ATCC 33316

*ATCC: American type Culture Collection, Manassas, VA

Media Preparation

Test tubes (16x150 mm) were sterilized in an autoclave at 18psi / 121º C. MRS broth was then prepared using Himedia *Lactobacillus* MRS medium (Mumbai, India), and pH was adjusted to 6.46 using HCl. Thirty-six test tubes were then filled with 8 mL of the broth and capped.

MRS agar was prepared according to formula using 1 L distilled water and 5 g peptone, 5 g beef extract, 2.5 g yeast extract, 10 g dextrose/glucose, 0.5 mL Tween (Polysorbate 80), 2.5 g ammonium citrate, 2.5 g sodium acetate, 0.05 g magnesium sulfate, 0.025 g manganese sulfate, 1 g dipotassium phosphate, and 7.5 g agar. The pH was adjusted to 6.46, the MRS medium autoclaved, and then poured into sterile Petri dishes (approximately 20 mL each plate).

Procedure

Two test tubes with liquid agar were inoculated with a colony of each LAB species listed above and allowed to grow for 48 hours at 29º C. A cocktail of 0.5 mL of each of the five LAB species was added to the grape juice that was adjusted for brix and pH above. This was then mixed and allotted 10 minutes for the cultures to distribute evenly through the juice.

A dilution set was then made by pipetting 0.5 mL of the infected juice into a large test tube, then pipetting 1 mL of the next sample in line for reductions of 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} mL of the solution. Each of the pre-treatment dilutions was then plated on the MRS agar.

The sterilizer was prepped by first being purged with a diluted bleach/water solution and then rinsed with 2 L of distilled water. The must was then run through the sterilizer in one pass. The above dilution procedure was then repeated on the treated juice, and both the inoculated and sterilized plates were then sealed with parafilm, placed into a 2 gallon Ziploc bag with 4 Benton Dickinson CO_2 generators and placed in a dark cabinet at 26º C.

A count of CFU was taken and CFU/mL determined after two days. Gross organism removal rates were then determined by a simple removal algorithm ((N_i – N_p / N_i)100 where N_i = initial population, and N_p = post-treatment population, adapted from Vlachos and others (2006) and ISO 10718.)

Data were analyzed with paired t-tests using GraphPad Prism and InStat software (GraphPad Software Inc, San Diego, CA).

CHAPTER IV

RESULTS

Three replicates were run in each trial. Each replicate was run through the apparatus, and then diluted prior to enumeration on MRS media. Data are shown in Table 2 below. Trials one and three did not show growth at dilutions of 10^{-4}, 10^{-5}, or 10^{-6} and trial two did not at dilutions of 10^{-5} and 10^{-6}. Dilutions that did not result in growth are removed for brevity. A baseline was also run through the apparatus, with three replicates run through without the apparatus being turned on. These results are shown in Table 2 below.

Table 2 Dilution Sets for UV_c Trials

		Pre-treatment			**Post Treatment**		
Trial I		**Dil**	**colonies**	**CFU mL^{-1}**	**Dil**	**colonies**	**CFU mL^{-1}**
	Sample 1	$1.00x10^{2}$	175	$1.75x10^{5}$	$1.00x10^{2}$	60	$6.00x10^{4}$
		$1.00x10^{3}$	1	$1.00x10^{4}$	$1.00x10^{3}$	8	$8.00x10^{4}$
	Sample 2	$1.00x10^{2}$	50	$5.00x10^{4}$	$1.00x10^{2}$	44	$4.40x10^{4}$
		$1.00x10^{3}$	3	$3.00x10^{4}$	$1.00x10^{3}$	1	$1.00x10^{4}$
		$1.00x10^{4}$	1	$1.00x10^{5}$	$1.00x10^{4}$		
	Sample 3	$1.00x10^{2}$	141	$1.41x10^{5}$	$1.00x10^{2}$	101	$1.01x10^{5}$
		$1.00x10^{3}$	9	$9.00x10^{4}$	$1.00x10^{3}$		
		$1.00x10^{5}$			$1.00x10^{5}$		
	Ave Trial 1			**$8.51x10^{4}$**			**$5.90x10^{4}$**
	Std dev			**59666.93**			**31086.97**
Trial II		**Dil**	**colonies**		**Dil**	**colonies**	
	Sample 1	$1.00x10^{2}$	41	$4.10x10^{4}$	$1.00x10^{2}$	110	$1.10x10^{5}$
		$1.00x10^{3}$	17	$1.70x10^{5}$	$1.00x10^{3}$	2	$2.00x10^{4}$
	Sample 2	$1.00x10^{2}$	114	$1.14x10^{5}$	$1.00x10^{2}$	160	$1.60x10^{5}$

Table 2 (Continued)

Trial II		**Dil**	**Colonies**		**Dil**	**colonies**	
		$1.00x10^{3}$	13	$1.30x10^{5}$	$1.00x10^{3}$	70	$7.00x10^{5}$
		$1.00x10^{4}$	21	$2.10x10^{6}$	$1.00x10^{4}$		
	Sample 3	$1.00x10^{2}$	81	$8.10x10^{4}$	$1.00x10^{2}$	46	$4.60x10^{4}$
		$1.00x10^{3}$	13	$1.30x10^{5}$	$1.00x10^{3}$	1	$1.00x10^{4}$
	Ave Trial 2			**$3.95x10^{5}$**			**$1.74x10^{5}$**
	Std dev			**752880.3**			**263804.2**
Trial III		**Dil**	**colonies**		**Dil**	**colonies**	
	Sample 1	$1.00x10^{2}$	52	$5.20x10^{4}$	$1.00x10^{2}$	2	$2.00x10^{3}$
		$1.00x10^{3}$	2	$2.00x10^{4}$	$1.00x10^{3}$		
		$1.00x10^{4}$	1	$1.00x10^{5}$	$1.00x10^{4}$		
	Sample 2	$1.00x10^{2}$	78	$7.80x10^{4}$	$1.00x10^{2}$	86	$8.60x10^{4}$
		$1.00x10^{3}$	64	$6.40x10^{5}$	$1.00x10^{3}$	6	$6.00x10^{4}$
	Sample 3	$1.00x10^{2}$	121	$1.21x10^{5}$	$1.00x10^{2}$	22	$2.20x10^{4}$
		$1.00x10^{3}$	3	$3.00x10^{4}$	$1.00x10^{3}$		
	Ave Trial 3			**$1.49x10^{5}$**			**$4.25x10^{4}$**
	Std dev			**219665.4**			**37678.46**

The average reduction in LAB colonies for the three trial series is then summarized in Table 3.

Table 3 Difference Between Average Pre- and Post-Treatment LAB Populations in Dilution Series.

Trial	**Pre-Treatment Average (CFU/mL)**	**Post-Treatment Average (CFU/mL)**	**Removal**
1	**8.51×10^{4}**	**5.90×10^{4}**	**30.7%**
2	**3.95×10^{5}**	**1.74×10^{5}**	**55.9%**
3	**1.49×10^{5}**	**4.25×10^{4}**	**71.5%**

Three replicates of three single-pass trials with the apparatus resulted in decreases in post-treatment CFU/mL from 2.61 x 10^4 in trial one to 1.06 x 10^5 in the third, with an average reduction of 52.7%. Each trial resulted in final CFU/mL concentrations below 10^8 cells per mL, the threshold to begin MLF (Jackson 2008). Note that while all beginning concentrations were below that threshold to begin with, they were all well above those typically found both initially when the grapes are brought in from the vineyard as well as those found during typical growth occurring alcoholic fermentation as discussed earlier.

To test for statistical significance, data were log-transformed, then analyzed using a paired t-test with Bonferroni post-test. Results are shown in Figures 5 and 6. In the pretrial control (Figure 5), simply running the inoculated must through the apparatus in the absence of UVc had no effect on the LAB populations (P=0.2074). However, a significant drop in populations was observed in each trial following exposure of the must to UVc radiation.

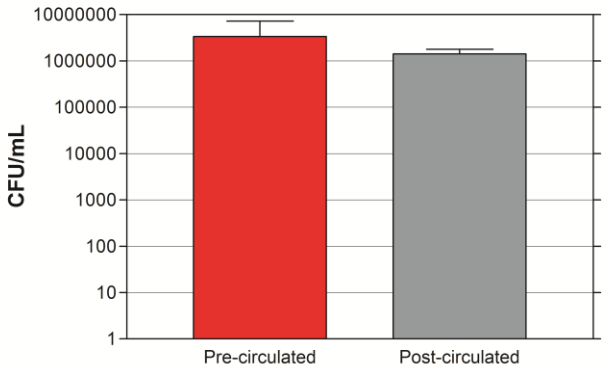

Figure 5. LAB counts before and after circulation through the apparatus in the absence of UVc. No significant difference in populations was observed by passing inoculated must through the apparatus. N=3, P=0.2074 (paired t-test).

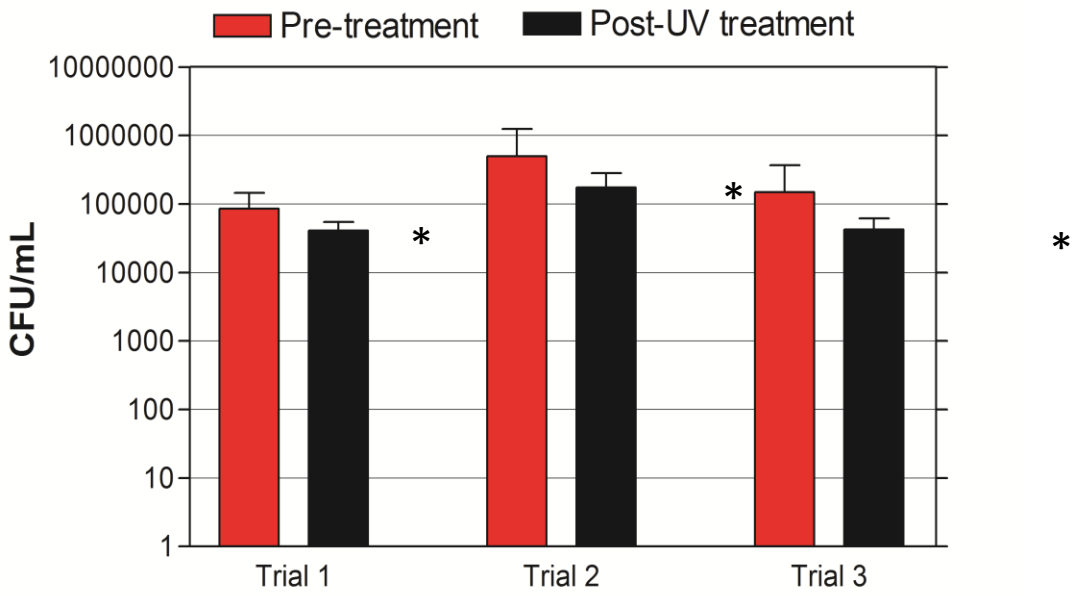

Figure 6. LAB counts before and after UV treatment. Significant reductions were seen in each trial. N=3 for each trial, *P<0.05, **P<0.01.

CHAPTER V

DISCUSSION

Ultraviolet radiation was shown to be an effective control mechanism for LAB in wine production. At least in the case of white wine, UV_c is effective in controlling LAB and should work similarly with other microorganisms. That said, there are additional questions left for inquiry from the trials.

Testing with Other Microbes

The LAB species used in the trials were common in winemaking, with both pathogenic and beneficial characteristics depending on the grape varietal and species of LAB. No wild yeasts were tested, nor were other microorganisms that might create quality control issues in winemaking. Although the literature does not indicate this as a problem, spore forming microorganisms and even non-spore forming bacteria and yeast might be controlled at different levels of efficacy. This dictates a need for testing UV_c with yeasts and spore-forming bacteria to confirm this assertion.

Equipment Modification

There are changes to the system that would likely increase the lethality of the system in treating both white and red wine must. Since exposure to radiation is what leads to germicidal effect with UV_c, changes in the system that increase the probability of microbes coming into close enough proximity to the ultraviolet source would be beneficial. This could be done by using either multiple passes

with multiple systems, or longer units with more lengthy bulbs that would increase the amount of time that the wine is exposed to UV_c radiation. The goal would not be to increase the time of exposure to radiation, since UV_c is instantaneously lethal. The increase in time of exposure would provide more opportunity for the bacteria in the fluid column to come within the ~1 mm germicidal range of the bulb since the juice would be flowing for a longer period within the sterilizer. This would increase the probability of exposure and thus control of bacteria by the system.

Adapting the equipment to a commercial scale would be relatively simple since as flow rate approaches the maximum for UV_c sterilizers, they become more effective. UVc sterilization is already in use in the commercial brewing as well as with other applications in food the industry (Bintsis and others 2000).

The Aquamedic unit used in this test fits one of two designs described by Koutchma as being effective for sterilization of liquid foods – laminar flow and those creating a turbulent Taylor-Couette flow (2009). While laminar flow units work by varying fluid velocity within strata layers, turbulent channel reactors ensure that the entire liquid column comes into close enough proximity for a germicidal effect from the UV_c lamp. Turbulent channel reactors are also more efficient when using secondary flow causing a Dean effect, where a secondary, perpendicular flow is caused by differences in centrifugal forces caused by the channel reactors (Koutchma 2009). Current research has indicated laminar flow systems are less effective than turbulent flow units (du Toit and Krügel 2011), so the answer to better efficiency is likely to be found with turbulent flow units.

As discussed above, the need is for exposure of the microorganisms to UV_c rather than a particular duration for exposure. The sterilizers would not be in continual use, so maintenance would be easily carried out between batches when needed. It would be very simple to install UV_c sterilizers as a modular system that can be moved between multiple batches, so wineries would not necessarily need separate units for individual batches – they would then be easily moved within the winery and make the system even easier to use. At any time when the product is being moved from one vessel to another, the system could be placed in line between them since the sterilizers work as quickly as the material is passed through them.

Red Wine

While red wine was not tested, if it is similar in turbidity the results may be similar, although further testing is needed to confirm this. The main limiting factor to using UVc for red wine is the coefficient of absorption, a measure of penetrance of UV_c through liquids of varying turbidity and opacity. While white wine is well within the effective range for its application, red wine's coefficient of absorption approaches maximal ranges, sitting beyond that of both beer and white wine (Guerrero-Beltrán and Barbosa-Cánovas 2004). Adjustment for the lack of absorption of UV radiation in colored media would need to be accounted for by decreasing the depth of the liquid column, increasing the number of passes, or another method to ensure microbial exposure to radiation at germicidal levels. Similar challenges exist with must treated with this method prior to being filtered or otherwise clarified, as physical barriers presented by

turbidity lead to the same issues as opacity (Guerrero-Beltrán and Barbosa-Cánovas 2004).

Conclusions

This study has shown that ultraviolet radiation is an effective germicidal control for LAB in white wine production. With the emergence of research contraindicating the use of sulfites to treat wine must for myriad reasons, the development of effective and cost-effective alternatives to sulfites must be vetted by research and made available to winemakers to ensure continued profitability for an industry that has a significant impact in Washington State both monetarily and in the number of jobs that it generates.

Since UV_c systems do not incur significant cost and are easy to maintain (with only bulb changes to be completed to keep the equipment operational), they are a viable method to sanitize wine, lessening or removing the reliance on sulfites. Ultraviolet treatment uses less electricity, is more effective in controlling bacteria, and does not introduce off-flavors or odors into the wine. That is not the case with other mechanical sterilization techniques. Some of them are ineffective in some applications (such as LAB), most are not cost effective and have a larger footprint, and they may also impact the quality of the final product.

For this and the other reasons above, UV_c is a valid candidate for use in winemaking to either supplant or abate the use of sulfites. Further testing is warranted before broad-scale implementation of the system, however compared to the others it has far more advantages than disadvantages at this juncture. If

the wine industry is going to continue to expand, alternatives such as UV_c must be fully studied and put into use in wine production. While sulfites do have their uses, their drawbacks necessitate this work in order to ensure the continued viability of the industry.

REFERENCES

Bartowsky EJ. 2009. Bacterial spoilage of wine and approaches to minimize it. Lett. Appl. Microbiol 48:149–156.

Bintsis, T, Litopoulou-Tzanetaki, E, Robinson, R. 2000. Existing and potential applications of ultraviolet light in the food industry – a critical review. J Sci. Food Agric. 80: 637-645.

Blättel V, Wirth K, Claus H, Schlott B, Pfeiffer P, König H. 2009. A lytic enzyme cocktail from Streptomyces sp. B578 for the control of lactic and acetic acid bacteria in wine. Appl Microbiol Biotechnol 83:839-848.

Delfini C, Gaia P, Schellino R, Strano M, Pagliara A, Ambrò S. 2002. Fermentability of Grape Must after Inhibition with Dimethyl Dicarbonate (DMDC). J. Agric. Food Chem. 50:5605-5611.

Denniston K, Topping J, Caret R. 2007. General, Organic, and Biochemistry, 5th ed. New York (NY): McGraw Hill. 801p.

du Toit M., Krügel M. 2011. Efficacy of ultraviolet radiation as an alternative technology to inactivate microorganisms in grape juices and wines. Food Microbiology. 28:510-517.

du Toit M., Pretorius, I.S. 2000. Microbial Spoilage and Preservation of Wine: Using Weapons from Nature's Own Arsenal – A Review. S. Afr. J. Enol. Vitic., 21: Special Issue.

Edwards, C. Lactic Acid Bacteria Native to Washington State Wines. Research Bulletin XB1026E for Agricultural Research Center, Washington State University, Pullman (WA), 1992.

Fredericks, I., du Toit, M., Krügel, M. 2011. Efficacy of Ultraviolet Radiation as an Alternative Technology to Inactivate Microorganisms in Grape Juices and Wines. Food Microbiology. 28 (2011) 510-517.

Garde-Cerdán, T, Ancín-Azpilicueta, C. 2007. Effect of Sulfites on the formation and evolution of volatile compounds in wines. Food Control 18(12):1501-1506.

Guerrero-Beltrán, JA, Barbosa-Cánovas, GV. 2004. Advantages and Limitations on Processing Foods by UV Light. Food Science and Technology International 10:137–147.

Jackson, R. 2008. Wine Science: Principles and Applications. Burlington (MA): Academic Press: 751 p.

Koutchma T. 2009. Advances in Ultraviolet Light Technology for Non-thermal Processing of Liquid Foods. Food and Bioprocess Technology 2:138–155.

Lonvaud-Funel, A. 1999. Lactic acid bacteria in the quality improvement and depreciation of wine. Antonie von Leeuwenhoek. 76: 317-331.

Loureiro, V., Malfeito-Ferreira, M. 2003. Spoilage yeasts in the wine industry. Intl. Journal of Food Microbiology. 86: 23-50.

Margalit, Y. 2004. Concepts in Wine Chemistry, 2nd Ed. San Francisco (CA): Wine Appreciation Guild, Ltd. 486 p.

Mendes-Ferreira A, Barbosa C, Inês A, Mendes-Faia A. 2010. The timing of diammonium phosphate supplementation of wine must affects subsequent H2S release during fermentation. Journal of Applied Microbiology 108:540-549.

Santos M, Nunes C, Saraiva J, Coimbra M. 2012. Chemical and Physical Methodologies for the Replacement/Reduction of Sulfur Dioxide use During Winemaking: Review of their Potentialities and Limitations. European Food Research and Technology 231:1-12.

Simon RA. 2003. Adverse reactions to food additives. Curr Allergy Asthma Rep 3:62-66.

Vlachos P, Kampioti A, Kornaros M, Lyberatos G. 2006. Development and evaluation of alternative processes for sterilization and deodorization of cork barks and natural cork stoppers. Eur Food Res Technol 225:653-663.

Stonebridge Research [Internet]. The Economic Impact of Washington State Wine and Grapes; 2012 [cited 2013 July 6]. Available from: http://www.wawgg.org/files/documents/2012_Economic_Impact_WA_Wine-Grapes.pdf.

Willey J, Sherwood L, Woolverton, C. 2008. Microbiology. 7th ed. New York (NY): McGraw-Hill. 1088 p.