EVALUATION OF COMMON AND NOVEL SANITIZERS AGAINST SPOILAGE YEASTS FOUND IN WINE ENVIRONMENTS

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

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January 2014

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Cornell University 2014

Wine is subjected to many sources of microbial contamination throughout the wine making process, including but not limited to fermentation, barrel maturation, bottling, etc. In wineries, sanitation protocols should consider not only the type of microorganisms that need to be challenged, but also the type of surface that is going to be sanitized, since contact surfaces need to be treated differently according to physical and chemical properties. In the past, chlorinated compounds were used as sanitizers in wine industry, however we now know that they can be involved in the formation of trichloroanisoles (TCA), resulting in wine defects. Chlorine dioxide unlike other chlorinated compounds does not form TCA, or at least at very low levels. However, this research concluded its poor efficacy to sanitize wine barrels, likely due to the organic nature of the barrels.

Alternative sanitizers in wine industry also include: sulfur dioxide, peroxyacetic acid, hot water, steam, ozone, etc. On the other hand, Velcorin ® (Dimethyl dicarbonate or DMDC) is currently used as a wine sterilant, however, due to its high disinfection effectiveness against yeast, we investigated its potential as a sanitizer for wine contact surfaces. None of the sanitizers mentioned here have been evaluated under strict controlled and/or standardized conditions (concentration, time, kind of surface, etc.) against common wine spoilage microorganisms. This is of utmost importance for wine industry, since instituted changes should be implemented for the improvement of winery sanitation practices. We evaluated the majority of these sanitizers under both *in vitro* and *in vivo* conditions in order to validate their effective parameters. Other sanitizers

were only evaluated for their *in vitro* or *in vivo* effectiveness, due to regulatory restrictions for the currently unapproved applications. Of all the sanitizers evaluated, steam treatment for 10 minutes and peroxyacetic acid at 200 mg/L for one week exposure were the most effective. During this research, an important observation was made, that *Brettanomyces bruxellensis* displays unique colony morphology when isolated from different sources. This wine spoilage yeast is known to cause off aromas in wines and the varying colony morphology may suggest varying susceptibilities to sanitizers, depending on morphology type.

BIOGRAPHICAL SKETCH

Alejandra, or Ale, was born in Mexico City, most of her life she lived with her grandparents and her mother. She has a younger brother, Emmanuel, who is studying to become a chef, with a specialization in bakery. Ale became interested in Biological and Chemical Sciences when she was 11 years old, but still she did not have any specific idea of what career she would choose in the future. Alejandra's mother always assured that her daughter studied the English language because she knew it was going to be useful someday. With that, Ale started her academic pursuit when she was 4 years old and she never stopped since then. At the age of 12, she was attending secondary school and at the same time a technical degree for being a computer technician, it was a very busy time for her but rewarding in the end. At the age of 15, she was accepted in a technical high school to study a specialization in Biotechnology. She was in her second year when she started to take a course called "Fermentation Processes", and that was when she realized that she wanted to study "Wine Science". Since in Mexico there are no any degrees related to enology, she decided to study in a field that eventually would help her to continue with the study of wine in the future. In 2007, she had received a degree in Chemistry, Bacteriology and Parasitology from ENCB-IPN (National School of Biological Sciences-Polytechnic National Institute), and her next step was to secure a scholarship to study Enology and Viticulture abroad. She was awarded a scholarship from the National Council of Science and Technology in Mexico, and came to Cornell in 2009. Since then, she has met many different personalities, worked in different work environments, became in a very independent person, and discovered herself. She has been fortunate to meet some of her best friends here. She also met Sharon (her beloved counselor), her advisors Randy, David and Alan, who have been an extraordinary wealth of wisdom and guidance.

This dissertation is dedicated to my uncle Alejandro, my grandparents (Anselmo and Guillermina), my mother (Bertha Araceli), Luis, Paula (beloved uncle and aunt), Sharon, Ramiro, Margarita, Emmanuel and to that Superior Power who is always guiding me...

To Randy:

For the freedom to create and beyond

ACKNOWLEDGMENTS

I would like to thank Dr. Randy W. Worobo for becoming my major advisor and for his guidance during my time in Cornell.

I would like to thank Dr. David M. Gadoury for his invaluable help with my research, for all the laughs when I visit his lab, the cups of coffee, and most importantly, for all his advice.

I would like to thank Dr. Alan Lakso for being part of this wonderful committee and for his ideas on the use of temperature probes for the steam experiments in wine cooperage.

I would also like to thank to all my lab members and all the people in the Food Science Department, who for one reason or other gave me a hand.

Finally, I would also like to thank to Constellation Brands for the kind donation of the barrels and access to the wine facilities for me to conduct my *in vivo* sanitation studies.

My PhD studies in Cornell University were financially supported by the National Council of Science and Technology (CONACYT-Mexico) and Cornell University.

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CHAPTER 1

JUSTIFICATION AND INTRODUCTION

Microbial spoilage is not easily defined, particularly in fermented foods and beverages, where the metabolites produced contribute to the flavor, aroma, and taste of the final products. In fact, for cultural or ethnic reasons, there is sometimes little difference between what is perceived as spoilage or organoleptic attribute (Loureiro and Malfeito Ferreira, 2003). There are three well known yeasts able to cause spoilage in wine, namely *Brettanomyces/Dekkera bruxellensis*, *Zygosaccharomyces bailii* and *Saccharomyces cerevisiae.* The concept of wine spoilage yeasts *sensu stricto* includes only those species able to affect wines that have been processed and packaged according to GMP (Good Manufacturing Practices). *S. cerevisiae* strains isolated from dry white wines for instance, seem to be a more potential spoilage yeast than *Z. bailii* due to its sorbic acid and sulfite tolerance at high ethanol levels. Furthermore, strains of *S. cerevisiae* have frequently been associated with re-fermentation of bottled ‘‘dry’’ red wines due to the presence of residual sugars in high ethanol (>13% v/v) wines (Loureiro and Malfeito Ferreira, 2003). On the other hand, *Dekkera bruxellensis* is probably the major cause of microbial wine spoilage worldwide, and results in substantial economic losses within the wine industry. Wines infected by *D. bruxellensis* are said to have “Brett” character: they may smell mousy or medicinal, wet wool, burnt plastic, or horse sweat. The mousy taint is the result of pyridines synthesized by *D. bruxellensis* from lysine and ethanol, while medicinal or barnyard odors are caused by the volatile phenols 4-ethylguaiacol and 4-ethylphenol, secondary metabolites produced by *D. bruxellensis*

from phenolic acids naturally present in the grape must (Woolfit et al, 2007). Physiological traits of *B. bruxellensis* vary depending on strain, growth phase, and environmental conditions (Zuehlke et al, 2013).

Alternatively, *Zygosaccharomyces bailii* is an effective spoilage microorganism of many foods and beverages (Thomas and Davenport, 1985). *Z. bailii* is exceptionally tolerant to low pH, being able to grow at pH as low as 2.2, in the presence of >600 μg/ml benzoic or sorbic acids and in laboratory media containing 2% of acetic acid. Tolerance has been reported to be due to its ability to metabolize sorbic and benzoic acids and to control the rate of acid uptake and internal pH (Makdesi and Beuchat, 1996). Typically, products spoiled by *Z. bailii* have low pH, low water activity, contain sufficient amounts of fermentable sugars and/or other assimilable carbon compounds (e.g., alcohol, glycerol and acetic acid), a nitrogen source, and a source of essential B group vitamins, and contain preservatives such as acetic, sorbic and benzoic acids or sulfur dioxide (Thomas and Davenport, 1985). *Zygosaccharomyces bailii* is also an important causative spoilage agent of sweet and dry wines. In addition to causing undesirable properties (off-flavors, hazing), the vigorous alcoholic fermentation that occurs in spoiling foods may lead to explosion of beverages (Rodrigues et al, 2001).

Due to problems of wine spoilage, wineries should implement sanitation protocols capable of keeping the whole winemaking process safe, from grape to bottle. These protocols in fact, should be supported with the pertinent scientific validation using different sanitizers and common wine spoilage microorganisms (surrogate microorganisms). Adequate sanitizers should be used

accordingly, that is: at the proper concentration according to the regulatory affairs), type of surface to be sanitized, the type of microorganisms to be challenged, and for a sufficient contact time, just to name a few important consideration factors. Thus, when sanitation is properly achieved, it is because the sanitizer is able to cause a significant log reduction of the microbial population. Indeed some sanitizers become more useful than others, not only because of the log reduction they yield, but also because no residual effects occur after their use.

Ozone for instance is a sanitizer commonly used in the wine industry. It leaves no hazardous residues on food or food-contact surfaces. The sanitizer is effective against a wide spectrum of microorganisms, and it can be used in an environment-friendly manner. Ozone treatment requires no heat and hence saves energy. It must be produced on-site; this leads to considerable savings in the costs of transporting and storing sanitizers. Stability and efficacy of ozone at chilling temperatures constitute attractive savings to the industry which is already burdened by rising energy costs (Khadre et al, 2001).

On the other hand, chlorine dioxide (ClO_2) is a strong disinfectant that is effective over a wide pH range. In addition to disinfection, ClO_2 can eliminate bad odors that may be present in food and beverage facilities. It is very effective in killing bacteria and particularly efficient in deactivating viruses. A smaller dosage and less reaction time are required for ClO_2 to produce the same disinfection effects as hypochlorites (Chang et al, 2000). Chlorine dioxide has a different reaction pathway than other chlorine-based sanitizers, and forms different disinfection by-products (DBPs). For example, it has been shown that while it can react with naturally occurring organics to form

organohalogens, the concentrations are typically much lower than when using free chlorine, with little formation of trihalomethanes and haloacetic acids (Volk et al, 2002). However, its efficacy against yeasts has not been studied more deeply due to fact that yeasts may occur in clustered cells, as pseudohyphae, which confer protection against undesirable environments, in a process called filamentation (Perissatto Meneghin et al, 2008). Indeed, *B. bruxellensis* is an example of a typical yeast species that can present pseudohyphae.

Alternatively, peroxyacetic acid (PAA) is known as a strong oxidant and it is another sanitizer commonly used in many segments of the food industry (Fan et al, 2009; Vandekinderen et al, 2009). It is commercially available as a quaternary equilibrium mixture containing acetic acid, hydrogen peroxide, PAA and water. It is produced from the reaction of acetic acid or acetic anhydride with hydrogen peroxide in the presence of sulfuric acid, which functions as a catalyzing agent. PAA is not susceptible to peroxidases and it retains its activity better in the presence of organic loads or food residue when compared with chlorine and in a broad temperature range. It can be used over a broad spectrum of pH (3.0–7.5) (Vandekinderen et al, 2009).

DMDC (Dimethyl dicarbonate) is currently used as a wine sterilant but not a sanitizer, however, this research is aiming its future use as a sanitizer for wine industry due to the effective results it achieved under *in vitro* conditions against target wine spoilage yeasts. DMDC is a microbial control agent with legal limits of 200 mg/L in wine. The US Food and Drug Administration regulations (21 CFR Part 172.133) require reduction of viable microbial loads to ≤ 500/mL by good manufacturing practices (heat treatment and/or sterile filtration) prior to DMDC

addition (FDA 2001). DMDC is hydrolyzed to carbon dioxide and methanol in aqueous solutions. The low concentration of residual byproducts produced is not considered harmful (Threlfall and Morris, 2002).

Similar to DMDC, sulfur dioxide (SO_2) has been used as a preservative in wines and other food for centuries. It works as an antioxidant and has antimicrobial activity against wild yeasts and bacteria (Ough and Crowell, 1987). Its effectiveness is strongly influenced by pH, its use can result in off-odors and flavors, and may cause allergic responses (Threlfall and Morris, 2002).

Equally important are heat treatments, which are the most frequently used methods to assure the safety and stability of foods. The use of heat treatments as a food preservation method requires knowledge of the kinetics of microbial inactivation, since it would allow us to calculate the intensity of the heat treatment necessary to reach a desirable level of microbial inactivation without altering the sensorial and nutritional properties of foods (Hassani et al, 2005). In the wine industry, studies of microbial inactivation kinetics for wine spoilage microorganisms using hot water or steam could also be highly useful. The use of heat in the form of steam, is widely used to sanitize different surfaces in wineries. Indeed, wine cooperage sanitation has become in one of the many applications that steam has been used in the wine industry. The lethal effect for a specific spoilage microorganism at a given temperature is estimated by calculating the decimal reduction time (*DT* value) that represents the time to reduce the microbial population one Log_{10} cycle. The *DT* value allows us to compare the heat resistance of different microorganisms and to calculate the treatment time to achieve a desirable level of inactivation at a given temperature (Hassani et al,

2005).

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CHAPTER 2

AN *IN VITRO* AND *IN VIVO* EVALUATION OF PEROXYACETIC ACID AS AN ALTERNATIVE SANITIZER FOR WINE BARRELS

Short version of title: Peroxyacetic Acid: Sanitizing Efficacy on Wine Barrels

ABSTRACT

Peroxyacetic acid is a common sanitizer used in the food and wine industry, but its use as a sanitizer for wine barrels has not been reported. We are reporting the findings for *in vitro* studies using three different concentrations of peroxyacetic acid (0, 60, and 120 mg/L) as sanitization challenges against seven strains of wine spoilage yeast representing three different genera: *Brettanomyces bruxellensis* (three strains), *Saccharomyces cerevisiae* (three strains) and *Zygosaccharomyces bailii* (one strain). Sensitivity to peroxyacetic acid concentration varied *in vitro*. A *post hoc* study (*in vivo*) using the highest concentration from the *in vitro* studies (120 mg/L) was used to validate a sanitization method for wine barrels. Exposure of barrels to an elevated concentration of 200 mg/L of peroxyacetic acid for one week resulted in no detectable levels of wine spoilage microorganisms after treatment. These findings provide a basis for establishing protocols to assure the maximum reduction of microbial contaminants with potential to degrade wine quality within wine cooperage.

Key words: peroxyacetic acid, barrel sanitation, validation, *Brettanomyces*

INTRODUCTION

The food and beverage industry has used a variety of different sanitizers that have been scientifically shown to be effective against targeted microorganisms. However, comparatively little research has focused upon scientific evaluation of sanitizers specifically relevant to the wine industry. Wine spoilage microorganisms interfere with the winemaking process, causing stuck fermentations or degraded wine quality causing off flavors that develop during the aging process as a consequence of poor sanitation practices.

Peroxyacetic acid (PAA) is a sanitizer approved for fresh produce by the United States (US) Food and Drug Administration (Neo et al. 2013). PAA has been used for food contact surface sanitizing and aseptic packaging. Its efficacy is a function of concentration, exposure time, and treatment surface (Gonzalez Aguilar et al. 2012). The potential application of PAA to reduce populations of wine spoilage microorganisms has not been comprehensively evaluated on the variety of different surfaces used in the vinification process. PAA is a strong oxidant due to its chemical composition. In its commercially-available form, it is a quaternary equilibrium mixture containing acetic acid, hydrogen peroxide, PAA and water. PAA solutions are produced from the reaction of acetic acid or acetic anhydride with hydrogen peroxide in the presence of sulfuric acid, which functions as a catalyzing agent (Vandekinderen et al. 2009). In spontaneous decomposition, peracetic acid is decomposed to form acetic acid and oxygen, thus representing loss of the oxidation power (Yuan et al. 1997). The PAA efficacy against yeasts is reduced at lower temperatures. This is not surprising, as disinfection, like other chemical processes, almost invariably takes place at a slower rate as the temperature falls (Baldry, 1983). PAA acts primarily on lipoproteins in the cell

membrane, and it may be equally effective against outer membrane lipoproteins (Silveira et al. 2008).

Microbial spoilage of wine can occur at any stage of the vinification process due to non-*Saccharomyces* yeasts and lactic and acetic acid bacteria. Of these potential contaminants, wild species of non-*Saccharomyces* yeasts in the genera *Brettanomyces*, *Candida*, *Hanseniaspora*, *Pichia*, *Metschnikowia*, *Saccharomycodes*, *Schizosaccharomyces* and *Zygosaccharomyces* have been involved in wine spoilage (Enrique et al. 2007). Moreover, if the aging conditions are not perfectly controlled, barrel-aged wines may be more easily exposed to several types of microbiological contamination likely to have a negative impact on their composition (Chatonnet et al. 2010). Indeed, the maintenance of sanitary practices during the various steps of wine production is essential to prevent the contamination of wine.

The selection of detergents and disinfectants in the food and beverage industry is dependent upon several factors, such as the efficacy in removing a wide range of microorganisms, handling safety, the rinsability of the agent, its corrosiveness on contact surfaces, and its impact on the sensory quality of the products manufactured (Tristezza et al. 2010). Wood is one of the surfaces to be sanitized in wineries and has played a historically significant role in the history of wine, and continues to be important in production today. Wooden barrels are used as containers in the wine making process, and wineries often reuse barrels for several cycles of wine production due to the initial high investment costs. The microporous structure of wood allows the penetration of microorganisms into the internal structure of the wood, increasing the difficulty of cleaning and sanitization, and increasing the risk of wine spoilage due to contamination during the fermentation

and aging of wines (Gonzalez Arenzana et al. 2013). Wineries around the world, in an attempt to improve their product and process, have been requesting that the scientific community develop effective, safe, and reliable methods to eliminate the microorganisms responsible for wine spoilage. There are very few reports of the efficacy of PAA against yeasts and more specifically towards wine spoilage yeasts. Moreover, PAA has not been evaluated under controlled conditions to sanitize wine barrels or any other winery surface, where wine spoilage microorganisms could be harbored. In this study we assessed three different concentrations of PAA *in vitro* by challenging seven strains yeast commonly found in wine environments and known for causing spoilage of wines. Those results were used to achieve a *post hoc* study in naturally contaminated barrels (*in vivo*) with *Brettanomyces* and general yeast populations. This study was designed to explore an alternative method for sanitizing wine barrels using a moderate concentration of PAA.

MATERIALS AND METHODS

Strain selection. *Brettanomyces/Dekkera bruxellensis* isolates (CE261, 2080, CE149). Three isolates were obtained by donation or from the Department of Food Science collection at Cornell University.

Saccharomyces cerevisiae isolates (CE81, CE9 and CE78). Three isolates were obtained from the Department of Food Science collection at Cornell University.

Zygosaccharomyces bailii isolate (4A1). One Isolate was obtained from the Department of Food Science collection at Cornell University.

Preparation of starter culture and inoculation. The yeast cultures were stored at -80°C

in glycerol 15% (w/v), and revitalized and maintained on YPD agar (yeast extract 10 g/L, peptone 20g/L, dextrose 20g/L, agar 15 g/L; Difco^{TM}; Sparks, MD, USA). All the strains were grown until stationary phase (200 rpm, 30°C). The growth time varied according to the strain (growth curve data not shown). All yeast strains were grown in YPD broth (yeast extract 10 g/L, peptone 20g/L, dextrose 20g/L) (Difco^{TM}; Sparks, MD, USA). Once the cultures reached between 10^6-10^8 CFU/mL, the target concentration was verified via a viable count. To prepare the target yeast for treatment, culture volumes of 1 mL were centrifuged (4500 rpm, 5 min and ambient temperature), the supernatant discarded, and the cells re-suspended in 1 mL of sterile deionized water. This washing step was repeated. Concurrently, a flask of the test solution: sterile deionized water and PAA (peroxyacetic acid formulation: 15.2%, hydrogen peroxide, 11.2%, inert ingredients 73.6%) (Tsunami 100 ECOLAB USA Inc.; St. Paul, MN, USA) at the desired concentration (0 mg/L, 60 mg/L and 120 mg/L) was prepared. Subsequently, 1 mL of the microbial suspension was added to the flask to yield 100 mL (total volume). Then, samples were taken from this flask at different times (0, 1, 5, and 15 min).

Microbiological enumeration. Yeast were enumerated by serially diluting samples in BPW (0.1%) (Hardy Diagnostics; Santa Maria CA, USA) and 0.1 % sodium thiosulfate (Fisher Scientific; Fair Lawn, NJ, USA) and immediately plated in duplicate on YPD agar (yeast extract 10 g/L, peptone 20g/L, dextrose 20g/L, agar 15 g/L; Difco^{TM}; Sparks, MD, USA). When necessary, direct plating of the sample (10^0 dilution) was performed to enumerate low concentrations of cells after treatment. The plates were incubated at 30°C for 48 to 72 h for *S. cerevisiae* and *Z. bailii*. Genera such as *Brettanomyces/Dekkera bruxellensis* grew slowly, and required 3-4 weeks of incubation. For the control experiments, the same experimental procedures were performed but

deionized water was added in place of PAA. The counts were averaged and expressed on a log10 scale. The reduction due to treatment was likewise calculated and expressed on a log10 scale for each strain. Every experiment was performed in triplicate with duplicate plating.

***In vivo* decontamination experiments using naturally contaminated barrels.** Twenty barrels naturally contaminated with both *Brettanomyces bruxellensis* and general yeast populations, were split in two groups of ten barrels each and treated with two different concentrations of peroxyacetic acid (PAA formulation: 5.1%, hydrogen peroxide 21.7%, inert ingredients 73.2%) (VigorOx®LS&D FMC Corporation; Philadelphia PA, USA): 120 mg/L and 200 mg/L. The 120 mg/L concentration was applied for 15 min and the 200 mg/L concentration was applied for 1 week. Each barrel had a code number to be identifiable in cellar and those numbers were also used to present our results in this article. The PAA solutions were prepared immediately prior to usage in distilled water and added quickly to the barrels that were fully filled with the working solution and the barrels were stored bung side up for their respective treatment time. Samples were taken before and after treatment and transferred to sterile bottles. The first portion of the water was discarded in order to "rinse" the bung hole which was additionally sprayed with 70% ethanol. Samples were stored at 4°C until analysis. The samples were analyzed for microbial population by filtration (EZ-Fit™ Manifold for universal laboratory filtration; Concord Road Billerica, MA USA). Some samples required pertinent dilutions. If samples needed to be diluted, 0.1% (wt/vol) buffered peptone water (Hardy Diagnostics; Santa Maria CA, USA) was used.

For the filtration, 0.22 μm nitrocellulose membrane filters (GE* Nitrocellulose-Mixed Esters of

Cellulose Membrane Filters; Pittsburg, PA, USA) were used and the samples were filtered twice, and the results were averaged. The maximum volume filtered was 100 mL and the results were calculated as CFU/100mL and then transformed to percentage to normalize the results since not all barrels had the same initial microbial load. After filtration, the membrane filters were transferred with sterile forceps to both WL and YPD agar. WL agar (yeast extract 4 g/L, tryptone 5g/L, glucose 50 g/L, potassium dihydrogen phosphate 0.55 g/L, potassium chloride 0.425 g/L, calcium chloride 0.125 g/L, magnesium sulphate 0.125 g/L, ferric chloride 0.0025, manganese sulphate 0.0025 mg/L, bromocresol green 0.022 g/L, agar 15 g/L) (Oxoid, LTD; Basingstoke Hampshire, England) was used to detect *Brettanomyces/Dekkera bruxellensis* and was incubated at 30°C for up to 3-4 weeks. WL agar contained 10 mg/L of cycloheximide (Sigma Aldrich; St. Louis, MO, USA) to make it selective for *B./D. bruxellensis* (dissolved in 50% ethanol and filter sterilized), 150 mg/L of biphenyl (Acros Organics; Fair Lawn, New Jersey, USA) (dissolved in ethanol and filter sterilized) to avoid the growth of mold, 30 mg/L of chloramphenicol (MP Biomedicals LLC; Solon, OH, USA) (dissolved in 100% ethanol) to prevent the growth of lactic acid bacteria and 25 mg/L of kanamycin sulfate (AMRESCO; Solon, OH, USA) (dissolved in sterile distilled H_2O) to prevent the growth of acetic acid bacteria. YPD agar (yeast extract 10 g/L, peptone 20g/L, dextrose 20g/L, agar 15 g/L) (Difco™; Sparks, MD, USA) was used to enumerate the general yeast population and was incubated at 30°C for 48-72 h. YPD agar was supplemented with 150 mg/L of biphenyl (Acros Organics; Fair Lawn, New Jersey, USA; dissolved in ethanol and filter sterilized) to prevent the growth of mold, 30 mg/L of chloramphenicol (MP Biomedicals LLC; Solon, OH, USA; dissolved in 100% ethanol) to prevent the growth of lactic acid bacteria, and 25 mg/L of kanamycin sulfate (AMRESCO; Solon, OH, USA; dissolved in sterile distilled

H_2O) to prevent the growth of acetic acid bacteria.

Extraction of wood cores and isolation of *Brettanomyces* spp.. Wood cores were extracted from each barrel before and after sanitation treatment using a borer attached to a drill. This borer penetrated up to 17 mm from the outside surface of the barrel, reaching 8 mm from the inside of the barrel (total standard thickness of a stave is 25 mm). These cores were cut at a depth of 8 mm, quickly flame-disinfected using 70% ethanol and transferred to liquid YPD broth (Yeast Extract 10 g/L, Peptone 20g/L, Dextrose 20g/L) (Difco™; Sparks, MD, USA) and incubated at 200 rpm and 30ºC until visible growth was observed. Cores were taken from the bilge and head (either upper or lower part of the head). After growth was observed in liquid culture, the culture was streaked on WL agar and colonies with the hallmark morphology of *Brettanomyces* were selected. The colonies were re-streaked up to 7 times for purification purposes and due to *Brettanomyces* isolates from barrels were observed to present a wrinkled and dusty morphology that eventually changed over time to a smooth morphology.

Statistical analysis. For the *in vitro* experiments, all CFU/mL data were transformed to $\log_{10}$ CFU/mL. The reductions in yeast were calculated from the initial concentration of yeast cells (target inoculum) at time zero minus the last concentration of yeast after fifteen min of treatment time. The analysis currently used was a two-way ANOVA where all pairwise multiple comparison procedures were done with a Holm-Sidak method at an alpha level of 0.05. For the reduction of *Brettanomyces* and general yeast populations in naturally contaminated barrels using PAA

solutions (*in vivo* experiments), a Fisher's exact test was performed in order to see if the two study groups (120 mg/L or 200 mg/L) differ in the proportions of presence or absence of microorganisms. Another Fisher's exact test was performed for the wood cores experiment in order to see if the two study groups (120 mg/L or 200 mg/L) differ in the proportions of presence or absence of *Brettanomyces*. Statistical analyses were conducted using SigmaPlot 12.0; Systat Software Inc., San Jose CA.

RESULTS AND DISCUSSION

***In vitro* reduction of yeast.** We first evaluated the efficacy of PAA under *in vitro* conditions where three concentrations (120 mg/L, 60 mg/L and 0 mg/L) were used to challenge seven strains of yeast commonly found in wine environments and that are known for causing spoilage of wine. Multiple comparisons were performed at the strain level for the different concentrations used in order to determine if there were statistical differences. The results showed that at a concentration of 120 mg/L, the strain *Zygosaccharomyces bailii* 4A1 was the most resistant. In fact, *Z. bailii* 4A1 showed significant differences at a concentration of 120 and 60 mg/L of the sanitizer (Table 2.1 and 2.2) in comparison with all the strains.

Table 2.1 Comparison between strains using 120 mg/L of PAA

Comparison	**P**	**P<0.05**
CE149 vs. 4A1	<0.001	Yes
CE261 vs. 4A1	<0.001	Yes
CE78 vs. 4A1	<0.001	Yes
CE81 vs. 4A1	<0.001	Yes
CE9 vs. 4A1	<0.001	Yes
2080.000 vs. 4A1	<0.001	Yes
CE78 vs. CE149	1	No
CE149 vs. 2080.000	1	No
CE81 vs. 2080.000	1	No
CE261 vs. CE149	1	No
CE261 vs. 2080.000	1	No
CE81 vs. CE149	1	No
CE78 vs. CE261	1	No
CE9 vs. CE78	1	No
CE81 vs. CE9	1	No
CE78 vs. 2080.000	1	No

Table 2.1 (Continued)

CE81 vs. CE78	1	No
CE81 vs. CE261	1	No
CE9 vs. CE261	1	No
CE9 vs. CE149	1	No
CE9 vs. 2080.000	1	No

This table represents all the possible comparisons among strains with regards the effects of PAA at a concentration of 120 mg/L.
Brettanomyces/Dekkera bruxellensis isolates (CE261, 2080, CE149).
Saccharomyces cerevisiae isolates (CE81, CE9 and CE78).
Zygosaccharomyces bailii isolate (4A1).

Table 2.2 Comparison between strains using 60 mg/L of PAA

Comparison	**P**	**P<0.05**
2080.000 vs. 4A1	<0.001	Yes
CE149 vs. 4A1	<0.001	Yes
CE261 vs. 4A1	<0.001	Yes
CE9 vs. 4A1	<0.001	Yes
CE81 vs. 4A1	<0.001	Yes
CE149 vs. CE78	<0.001	Yes
2080.000 vs. CE78	<0.001	Yes
CE261 vs. CE78	<0.001	Yes
CE9 vs. CE78	<0.001	Yes
CE81 vs. CE78	<0.001	Yes
CE78 vs. 4A1	0.008	Yes
CE261 vs. CE81	0.054	No
CE149 vs. CE81	0.048	Yes

Table 2.2 (Continued)

CE9 vs. CE81	0.043	Yes
2080.000 vs. CE81	0.038	Yes
CE9 vs. CE261	1	No
CE9 vs. 2080.000	1	No
CE261 vs. CE149	1	No
CE9 vs. CE149	1	No
CE261 vs. 2080.000	1	No
CE149 vs. 2080.000	1	No

This table represents all the possible comparisons among strains with regards the effects of PAA at a concentration of 60 mg/L.
Brettanomyces/*Dekkera bruxellensis* isolates (CE261, 2080, CE149).
Saccharomyces cerevisiae isolates (CE81, CE9 and CE78).
Zygosaccharomyces bailii isolate (4A1).

This is, in fact, expected since our results showed that *Z. bailii* 4A1 was highly resistant to the 60 and 120 mg/L PAA concentrations, whereas, the other strains showed more susceptibility to the sanitizer (Figure 2.1).

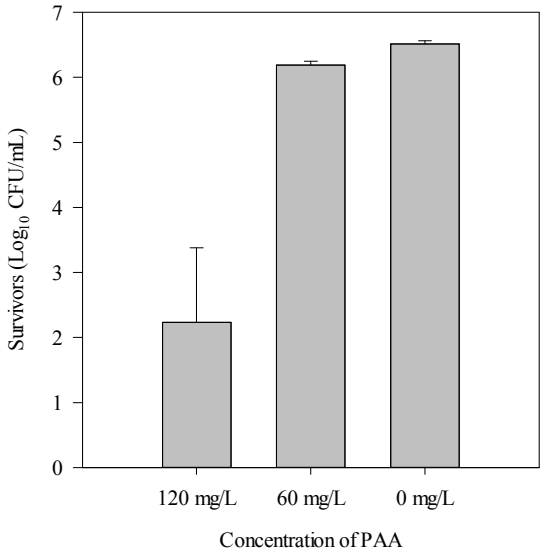

Figure 2.1. The efficacy of PAA on the reduction of suspended cells of *Z. bailii* (4A1) at three different concentrations. Error bars represent the standard error of the mean from triplicate experiments at the longest exposure time (15 min).

Moreover, *Z. bailii* 4A1 showed significant differences at all comparison concentrations (120 mg/L *vs* 0 mg/L and 120 *vs* 60 mg/L) except for 60 mg/l *vs* 0 mg/L. Hilgren and Salverda (2000) performed a study using *Z. bailii* and peroxyacetic acid at a concentration of 80 mg/L using different times of exposure. They used 30 s, 2 min, and 5 min, and an initial concentration of yeast cells of 5.98 Log_{10} CFU/mL, whereas our initial concentration was 6.59 Log_{10} CFU/mL (log mean of three replicates). Additionally, we used longer exposure times (0,1, 5, and 15 min) and none of our exposure times decreased the population of *Z. bailii* to below detectable levels even though a higher concentration of peroxyacetic acid was used. Hilgren and Salverda (2000) claimed to have

log reductions that ranged between 0.16 and 0.94 Log_{10} CFU/mL, whereas our log reduction at our longest exposure time (15 min exposure), with a higher concentration of peroxyacetic acid (120 mg/L) and with higher initial number of yeast cells (6.59 Log_{10} CFU/mL) was 4.36 Log_{10} CFU/mL, thus leaving 2.23 Log_{10} of live populations (Figure 2.1). This is interesting because the initial concentration of cells that Hilgren and Salverda (2000) used is similar to ours, yet they obtained a considerable higher reduction. However, other factors such as strain variability and associated resistance could be taken into account for these differing results. Similarly, the log reduction was immediate for *S. cerevisiae* CE9 and CE81 with initial number of yeast cells of 6.08 and 6.15 Log_{10} CFU/mL respectively (Figures 2.2-2.3) and after 1 minute of exposure, no detectable levels of these strains were found. However, *S. cerevisiae* CE78 with initial number of yeast cells of 6.63 Log_{10} CFU/mL did not show any reduction until 5 min of exposure (Figure 2.4). *S. cerevisiae* CE81 and CE78 showed significant differences at all concentration comparisons (120 mg/L *vs* 0 mg/L, 120 mg/L *vs* 60 mg/L and 60 mg/l *vs* 0 mg/L). *S. cerevisiae* CE9 also showed significant differences when concentration comparisons were done, except for the comparison 120 mg/L *vs* 60 mg/L. Baldry (1983) exposed *S. cerevisiae* to different concentrations of PAA, however different temperatures and pH were used (5.0, 6.5 and 8.0) versus our experiment where pH was not a factor to control, since only deionized water was used to prepare the working solutions that were added to the flasks (*in vitro* experiments) and where temperature was stable during the whole experiment. Baldry (1983) found that the efficacy of PAA against two strains of *S. cerevisiae* decreases with increasing pH, since when alkalinity of the solution increases, peracetic acid is

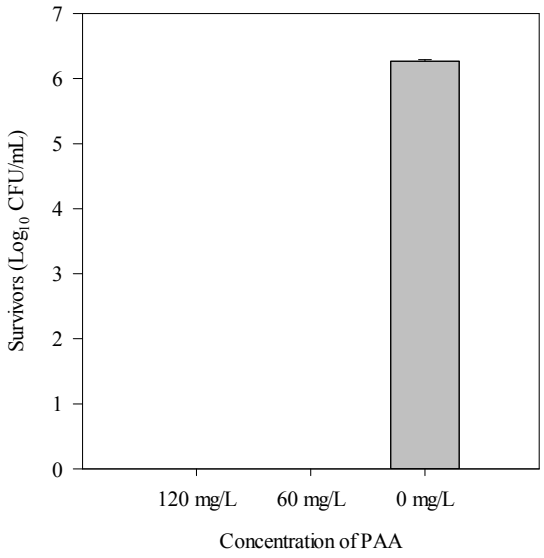

Figure 2.2 The efficacy of PAA on the reduction of suspended cells of *S. cerevisiae* (CE9) at three different concentrations. Error bars represent the standard error of the mean from triplicate experiments at the longest exposure time (15 min).

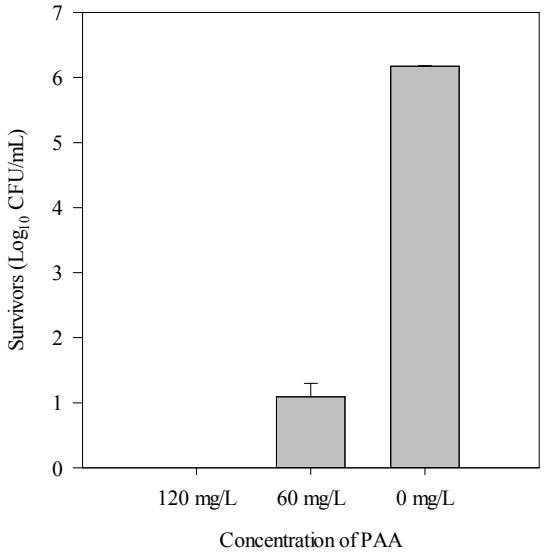

Figure 2.3. The efficacy of PAA on the reduction of suspended cells of *S. cerevisiae* (CE81) at three different concentrations. Error bars represent the standard error of the mean from triplicate experiments at the longest exposure time (15 min).

hydrolyzed to form acetic acid and hydrogen peroxide (Yuan et al. 1997). Accordingly, Baldry (1983) found that resistance among genera (particularly between *Z. bailii* and *S. cerevisiae*) is variable, this latter aspect also

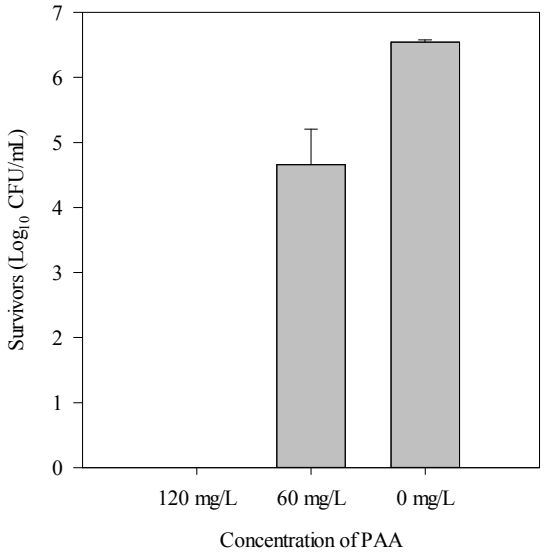

Figure 2.4. The efficacy of PAA on the reduction of suspended cells of *S. cerevisiae* (CE78) at three different concentrations. Error bars represent the standard error of the mean from triplicate experiments at the longest exposure time (15 min).

found in our experiments. With regards *B./D. bruxellensis* strains CE149, 2080 and CE261 had 6.73, 4.19 and 5.66 Log_{10} CFU/mL initial number of yeast cells respectively, and the reduction was immediate when 120 mg/L was used. After 1 min exposure, no detectable levels of any of the three strains were found (Figures 2.5-2.7). The statistical analysis for all *S. cerevisiae* and *B./D. bruxellensis* strains did not show any significant differences when comparisons where performed between them at a concentration of 120 mg/L (Table 2.1). However, when concentration comparisons where done in each of the three *B./D. bruxellensis* strains significant differences were found between 120 mg/L *vs* 0 mg/L and 60 mg/l *vs* 0 mg/L except for the comparison 120 mg/L *vs* 60 mg/L.

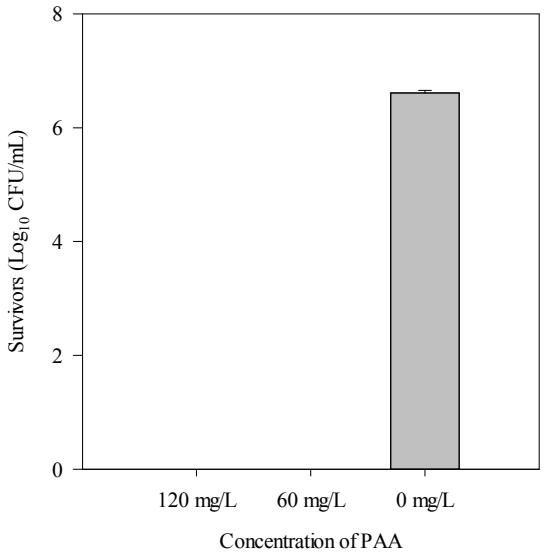

Figure 2.5. The efficacy of PAA on the reduction of suspended cells of *B./D. bruxellensis* (CE149) at three different concentrations. Error bars represent the standard error of the mean from triplicate experiments at the longest exposure time (15 min).

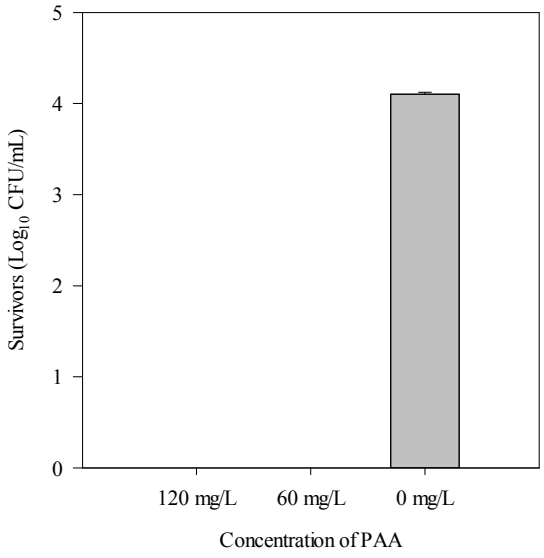

Figure 2.6. The efficacy of PAA on the reduction of suspended cells of *B./D. bruxellensis* (2080) at three different concentrations. Error bars represent the standard error of the mean from triplicate experiments at the longest exposure time (15 min).

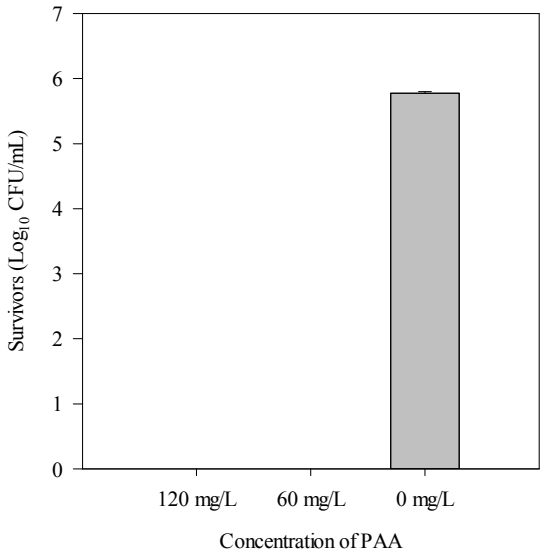

Figure 2.7. The efficacy of PAA on the reduction of suspended cells of *B./D. bruxellensis* (CE261) at three different concentrations. Error bars represent the standard error of the mean from triplicate experiments at the longest exposure time (15 min).

Duarte et al. (2011) also studied the efficacy of PAA to sanitize stainless tanks that were in contact with inoculated wine with populations of yeasts (including *Dekkera/Brettanomyces* yeasts), lactic acid and acetic acid bacteria (> 3000 CFU/mL). However, the PAA concentration used was 1000 mg/L, three times higher than the lowest PAA concentration recommended by the manufacturer in Portugal where this study was performed. PAA used at 1000 mg/L was highly effective for achieving non-detectable levels of yeasts. It is worth to mention that Portugal belongs to the EU, where at present, no such substances containing PAA are authorized for use, but permission may be given under EU legislation when preceded through scientific evaluations (Gonzalez Aguilar et al. 2012). In contrast, in the US, up to 500 mg/L of PAA can be used in wineries (www.epa.gov/fedrgstr/). However, there are many different surfaces that need to be evaluated separately, and wood should be one of these.

When 60 mg/L of peroxyacetic acid was used, the comparisons among strains showed that only 7

comparisons out of 21 did not show statistical significance ($p < 0.05$) (Table 2.2). With regards to the three strains of *S. cerevisiae*, the initial number of yeast cells for CE9, CE81 and CE78 were 6.64, 6.17 6.54 Log_{10} CFU/mL respectively and only CE9 (Figure 2.2) showed a log reduction that was below detectable levels right after the addition of PAA. However, CE81 and CE78 (Figures 2.3-2.4) showed a maximum log reduction of 5.08 and 1.88 log10 CFU/mL, respectively (average of three replicates), after 15 min of exposure. These findings suggest that the log reduction for *S. cerevisiae* strains at 60 mg/L is strain and time dependent. In contrast, *Z. bailii* 4A1 (Figure 2.1) had an initial concentration of cells of 6.64 Log_{10} CFU/mL and it had a 0.45 log reduction (average of three replicates). Finally, for the *B./D. bruxellensis* strains, the initial number of yeast cells for CE149, 2080 and CE261 were 6.55, 5.20, 5.68 Log_{10} CFU/mL respectively. The reduction was almost immediate, with both CE149 and 2080 being reduced to non-detectable levels after 1 min exposure (Figures 2.5-2.6). However, *B./D. bruxellensis* CE261 (Figure 2.7) was reduced to non-detectable levels only after 5 min of PAA exposure. This suggests that at this concentration for *B./D. bruxellensis* strains, the reduction is strain and time dependent, since the log reduction varied with time and the strain used. Finally, with regards the 0 mg/L (control) of PAA, no statistical differences were observed among all the possible comparisons of strains, since no variability is expected when the sanitizer is not present in the working solution.

In vivo **Reduction of *Brettanomyces* and general yeast populations in naturally contaminated barrels.** PAA at 200 mg/L and a contact time of one week decreased levels of *Brettanomyces* and general yeast populations below detectable levels (Tables 2.3 and 2.4). However, when concentration of PAA was reduced to 120 mg/L and contact time of 15 minutes,

detectable levels of both yeast populations were present (Tables 2.3 and 2.4). The statistical analysis was performed using a Fisher's exact test where the response in terms of reduction is recorded as either yes or no. Statistical differences were found between the 15 min and one week treatments for both *Brettanomyces* and general yeast populations, with $P = 0.011$ and $P = <0.001$ respectively. This means that the proportion of sanitation efficacy is not the same in the 15 min treatment and the one week treatment. The one week treatment (200 mg/L) is more effective than the 15 min treatment (120 mg/L).

Microbiological examination of wood cores for the presence of *Brettanomyces*. The efficacy of peroxyacetic acid treatments in barrels was studied at both the surface level (0 mm) with the liquid samples taken before and after PAA treatment, and a depth of 8 mm also before and after PAA treatment. The depth of 8 mm was selected as the maximum reported wood penetration depth for wine in barrel staves (Malfeito Ferreira et al. 2004). *Brettanomyces* is most commonly isolated at the bottom of a barrel (bilge) and also at the top of the barrels due to higher levels of oxygen that stimulate its growth (Van de Water, 2010), so the cores were purposely taken from the bilge and the heads (upper head and lower head) positions. Our results showed that at a depth of 8 mm, the barrels with the numbers 4A1M124747 (bilge position, lower head position and 120 mg/L of peroxyacetic acid), 4AD9120919 (bilge position and 200 mg/L of peroxyacetic acid), 4AD9120720 (bilge position and 200 mg/L of peroxyacetic acid), 4AD9120721 (bilge position and 200 mg/L of peroxyacetic acid) were positive for the growth of *Brettanomyces* before treatment.

Table 2.3 *Brettanomyces* populations pre-treatment and post-treatment

Treatment	Barrel	CFU/100ML INITIAL	CFU/100ML INITIAL Scientific Notation	CFU/100ML FINAL	CFU/100ML FINAL Scientific Notation	REDUCTION %	TIME (MIN OR WEEKS)	CONCEN-TRATION mg/L
PAA	4A1F125001	4000	4.00E+03	134	1.34E+02	96.65	15 MIN	120
PAA	4A1F125002	390000	3.90E+05	220	2.20E+02	99.943	15 MIN	120
PAA	4AD9120915	800	8.00E+02	63	6.30E+01	92.187	15 MIN	120
PAA	4AD9120916	ND[a]	ND[a]	ND[a]	ND[a]	-	15 MIN	120
PAA	4ALL119189	2325000	2.33E+06	23	2.30E+01	99.999	15 MIN	120
PAA	4ALL119188	9950	9.95E+03	80	8.00E+01	99.195	15 MIN	120
PAA	4A1M124748	10950	1.10E+04	80	8.00E+01	99.269	15 MIN	120
PAA	4A1M124747	4000	4.00E+03	80	8.00E+01	98	15 MIN	120
PAA	4ALL119242	11450	1.15E+04	4	4.00E+00	99.965	15 MIN	120

Table 2.3 (Continued)

PAA	4ALL119243	8000	8.00E+03	1	1.00E+00	99	15 MIN	120
PAA	4A1M125066	3100000	3.10E+06	0	0.00E+00	100	1 WEEK	200
PAA	4A1M125069	11400	1.14E+04	0	0.00E+00	100	1 WEEK	200
PAA	4AD9120925	15000	1.50E+04	0	0.00E+00	100	1 WEEK	200
PAA	4AD9120926	8000	8.00E+03	0	0.00E+00	100	1 WEEK	200
PAA	4AD9120922	27600	2.76E+04	0	0.00E+00	100	1 WEEK	200
PAA	4AD9120921	18900	1.89E+04	0	0.00E+00	100	1 WEEK	200
PAA	4AD9120919	1600	1.60E+03	0	0.00E+00	100	1 WEEK	200
PAA	4AD9120920	4000	4.00E+03	0	0.00E+00	100	1 WEEK	200
PAA	4AD9120720	80	8.00E+01	0	0.00E+00	100	1 WEEK	200
PAA	4AD9120721	11300	1.13E+04	0	0.00E+00	100	1 WEEK	200

[a] ND No detected.

This table represents the *in vivo* reduction of *Brettanomyces* in natural contaminated barrels.

Table 2.4 General yeast populations pre-treatment and post-treatment

Treatment	Barrel	CFU/100ML INITIAL	CFU/100ML INITIAL Scientific Notation	CFU/100ML FINAL	CFU/100ML FINAL Scientific Notation	REDUCTION %	TIME (MIN OR WEEKS)	CONCEN-TRATION mg/L
PAA	4A1F125001	1600	1.60E+03	4	4.00E+00	99.765	15 MIN	120
PAA	4A1F125002	37500	3.75E+04	3	3.00E+00	99.991	15 MIN	120
PAA	4AD9120915	800	8.00E+02	30	3.00E+01	96.25	15 MIN	120
PAA	4AD9120916	40	4.00E+01	5	5.00E+00	87.5	15 MIN	120
PAA	4ALL119189	37000	3.70E+04	0	0.00E+00	100	15 MIN	120
PAA	4ALL119188	13200	1.32E+04	15	1.50E+01	99.88	15 MIN	120
PAA	4A1M124748	19800000	1.98E+07	57	5.70E+01	99.999	15 MIN	120
PAA	4A1M124747	3150	3.15E+03	0	0.00E+00	100	15 MIN	120
PAA	4ALL119242	23100000	2.31E+07	80	8.00E+01	99.999	15 MIN	120

Table 2.4 (Continued)

PAA	4ALL119243	3500	3.50E+03	80	8.00E+01	97.714	15 MIN	120
PAA	4A1M125066	200000000	2.00E+08	0	0.00E+00	100	1 week	200
PAA	4A1M125069	5400	5.40E+03	0	0.00E+00	100	1 week	200
PAA	4AD9120925	1600	1.60E+03	0	0.00E+00	100	1 week	200
PAA	4AD9120926	7000	7.00E+03	0	0.00E+00	100	1 week	200
PAA	4AD9120922	24200	2.42E+04	0	0.00E+00	100	1 week	200
PAA	4AD9120921	181500	1.82E+05	0	0.00E+00	100	1 week	200
PAA	4AD9120919	533	5.33E+02	0	0.00E+00	100	1 week	200
PAA	4AD9120920	4000	4.00E+03	0	0.00E+00	100	1 week	200
PAA	4AD9120720	260	2.60E+02	0	0.00E+00	100	1 week	200
PAA	4AD9120721	1085	1.09E+03	0	0.00E+00	100	1 week	200

This table represents the i*n vivo* reduction of general yeast populations in natural contaminated barrels.

However, after treatment, none of the wood cores extracted from these barrels showed positive growth for *Brettanomyces*. Interestingly, one barrel (4AD9120915) that had no positive growth before treatment, but was positive after treatment (120 mg/L PAA for 15 min treatment time) in the bilge position core sample. Because only one barrel was positive for the presence of *Brettanomyces* after treatment, a Fisher's exact test was performed to determine if this was just due to random variability, or in fact that there was a difference between the two treatments at a depth of 8 mm. The statistical analysis showed that at that depth, there were no significant differences between treatments (P = 1.000).

CONCLUSIONS

Effective methods to sanitize wine barrels and other common surfaces in wineries are required due to the high replacement cost of barrels for the wine industry. Our study has demonstrated that PAA is effective to decontaminate wine cooperage when used at 200 mg/L and one week exposure, however 120 mg/L and 15 minutes proved to be ineffective under *in vivo* conditions, regardless of the fact that this concentration was highly effective under *in vitro* conditions. The use of the appropriate concentration of sanitizers must adhere to food regulations, even though higher concentrations could provide improved efficacy. Different surfaces in wineries must be assessed for the best sanitation protocols, since not all surfaces will be sanitized with the same concentrations and contact time. Moreover, autochthonous microbiota should also be taken into account when protocols of sanitation are validated, since microorganisms may present different levels of sensitivity to the common sanitizers used in wine industry.

ACKNOWLEDGMENTS

This research project was funded by CONACYT-Mexico, New York State Agricultural Experiment Station Federal Formula Funds, Cornell University Research Travel Awards. The authors thank the industry donors at Constellation Brands California, USA, Sean McKenzie and Zoran Ljepović (Constellation Brands, California, USA).

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CHAPTER 3

AN *IN VITRO* AND *IN VIVO* EVALUATION OF BARREL SANITATION METHODS: EFFICACY AND COLLATERAL EFFECTS ON ENVIRONMENTAL MORPHOLOGIES OF *BRETTANOMYCES* SPP.

Short version of title: Sanitation methods for wine barrels

ABSTRACT

Chlorine dioxide and ozone are commonly used as sanitizers in the food and wine industry. However, few studies of the *in vivo* efficacy of ozone for winery sanitation purposes and more specifically for barrel sanitation have been reported. Moreover, chlorine dioxide has not been evaluated against barrels that are naturally contaminated with *Brettanomyces* or other contaminant yeasts. DMDC (dimethyl dicarbonate) has been widely used in the wine industry as a wine sterilant, but not as a sanitizer. We evaluated the efficacy of chlorine dioxide and ozone to sanitize wine cooperage. Additionally, DMDC was evaluated for its *in vitro* efficacy as a sanitizer against seven spoilage yeasts strains within the species *Zygosaccharomyces bailii*, *Brettanomyces bruxellensis* and *Saccharomyces cerevisiae*. At 250 mg/L of DMDC, all strains were reduced below detectable levels. At 150 mg/L, one strain of *S. cerevisiae* was reduced by 2.99 log10, but other strains were again reduced below detectable levels. Ozone at 1 mg/L for 5 and 10 minutes effectively reduced *Brettanomyces* spp. and other contaminant yeasts in naturally contaminated barrels to undetectable levels in the majority of the barrels. Chlorine dioxide at 5 or 10 mg/L yielded no or slight reductions (1 log) for all yeast populations, regardless of concentration. *Brettanomyces* spp. presented different morphologies when isolated from naturally contaminated barrels than those from *Brettanomyces* spp. from the collection samples, suggesting that *Brettanomyces* spp. grow different according to the environment where it is isolated.

Key words: dimethyl dicarbonate, ozone, chlorine dioxide, barrels, morphology, *Brettanomyces*.

INTRODUCTION

Ozone use may have many advantages in the food industry. It is an effective alternative to chlorine, and a sanitizer with superior antimicrobial properties due to its potential oxidizing capacity. It is capable of inactivating bacteria, bacterial spores, molds, yeasts, protozoan cysts and viruses at relatively low concentrations and in short exposure times when applied to pure cell suspensions (Guzel Seydim et al. 2004 a; Kim et al. 2003). Ozone exhibits high reactivity and spontaneous decomposition to nontoxic products. It decomposes rapidly in water (Trindade et al. 2012) and is easily manufactured by addition of an oxygen atom to molecular diatomic oxygen (O_2) (Guzel Seydim et al. 2004 a). O_3 is generated commercially by passing oxygen molecules (O_2) through an electrical charge or ultraviolet light radiation. It is highly unstable and rapidly degrades back to molecular oxygen (O_2) with the released free oxygen atom ($O^{\bullet}$) combining with another free oxygen atom ($O^{\bullet}$) to form molecular oxygen (O_2) or combining with other chemical moieties to cause oxidation. Upon release of the third oxygen atom, O_3 acts as a strong oxidizing agent (Guzel Seydim et al. 2004 b).

Currently, many wineries use ozone as a means to sanitize wine barrels and other surfaces. Some studies have been performed to determine the efficacy of ozone and these studies have been published as brief technical reports (Hampson, 2000; as found on the Wines and Vines website (www.winesandvines.com). A more quantitative analysis of the effective reduction of common wine spoilage microorganisms has been reported only by Guzzon et al (2013), where ozone dissolved in water was evaluated *in vitro*. The foregoing conditions do not reproduce the inner barrel environment, where physical characteristics of the surface previous sanitation practices, and prior uses of the barrel could substantially impact the sanitizing efficacy of ozone. Guzzon et al.

(2011) did report the *in vivo* use of ozone in barrels, but to date no other *in vivo* trials involving ozone have been reported. Cantacuzene et al (2003), evaluated the efficacy of ozone in oak cubes, but again these experimental conditions did not reproduce the internal conditions of naturally contaminated barrels. Within barrels, contaminant yeasts and/or bacteria may penetrate into the wood at different depths over the years of usage, and where previous sanitation practices will have an influence on the initial microbial load. Other researchers have used ozone together with high power ultrasound (HPU) (Yap and Bagnall 2009), to increase the efficacy of ozone (O_3) as sanitizer of wine cooperage.

Another sanitizer known for its broad-spectrum biocidal activity and selective oxidant capacity is chlorine dioxide (ClO_2) (Gordon and Rosenblatt 2005). According to Sharma and Sohn (2012), the main advantage of using ClO_2 over other chlorine-containing disinfectants such as chlorine gas or hypochlorites is in controlling the formation of harmful trihalomethanes (THMs) when reacting with natural organic matter. It is used in aqueous form in over 400 drinking water treatment plants in the United States and is widely used in Europe (Lee et al. 2004). Experimental evidence of the efficacy of chlorine dioxide to sanitize wine barrels has not been previously reported. Its efficacy should be evaluated under controlled conditions that include concentrations permitted by current legislation, time, type of surface and type of microorganisms present. ClO_2 disinfection efficiency is independent of pH (Kunzmann and Schütz 2009) and it can be used to sanitize food contact surfaces and food surfaces as a gas or in an aqueous form (Han et al. 1999). It has about 3.5 times the oxidation capacity of chlorine gas (Lee et al. 2004). In contrast, dimethyl dicarbonate (DMDC) (Velcorin™) is used as a yeast inhibitor in wine and ready-to-drink tea beverages, and it was first FDA approved in 1998 (Basaran Akgul et al. 2009; Delfini et al. 2002). It is approved for use in the United States for table as well as low-alcohol and dealcoholized wines at a maximum concentration of 200 mg/L over the life of the wine. DMDC has never been used as a sanitizer, however we investigated its use as such because it does not possess any residual activity due to

hydrolysis to carbon dioxide and methanol (Fugelsang and Edwards 2007). DMDC acts by inhibiting select glycolytic enzymes that include alcohol-dehydrogenase and the glyceraldehyde-3-phosphate-dehydrogenase, by methoxycarbonylation of the nucleophilic residues (imidazoles, amines, thiols). DMDC yields no residual odors or flavors. In addition, its effect is not directly pH-dependent (Renouf et al. 2008). Its antimicrobial activity is more effective against yeasts than against bacteria (Delfini et al. 2002). The purpose of this study was to test different concentrations of DMDC (Velcorin; 99.8%; Lanxess Corp., Pittsburgh, PA, USA) under *in vitro* conditions, to assess its ability to sanitize wine barrels effectively. Since the purpose of this research was not the sterilization of wine but investigating the possible use with wine barrels, the experimental design for DMDC used a matrix free of wine. In addition, the sanitizers O_3 and ClO_2 were assessed under *in vivo* conditions, using naturally contaminated barrels. Many of the current sanitation practices used by the wine industry have not been evaluated under controlled conditions, and the assessment of these two sanitizers will offer the wine industry a means to evaluate alternative sanitizers for the treatment of wine cooperage.

MATERIALS AND METHODS

Strain selection. *Brettanomyces/Dekkera bruxellensis*. Three Isolates (2080, CE149 and CE261) were obtained from the Department of Food Science collection at Cornell University.

Saccharomyces cerevisiae. Three Isolates (CE78, CE9 and CE81) were obtained from the Department of Food Science collection at Cornell University.

Zygosaccharomyces bailii. One Isolate (4A1) was obtained from the Department of Food Science collection at Cornell University.

Preparation of starter culture and inoculation for DMDC experiment. The yeasts were stored at -80°C in glycerol 15% (w/v), and were revitalized and maintained on YPD agar (Yeast Extract

10 g/L, Peptone 20 g/L, Dextrose 20 g/L, Agar 15 g/L; Difco™; Sparks, MD, USA). All the strains were grown until stationary phase (10^8 -10^7 CFU/mL) or at least 10^6 CFU/mL (200 rpm, 30°C). The growth time varied according to the strain (previous data of growth characteristics for all strains used). The strains were grown in YPD broth without any pH adjustment. Once the cultures reached 10^8-10^7 CFU/mL or 10^6 CFU/mL, the target inoculum was verified by viable count. Volumes of 1 mL of culture were placed in sterile Eppendorf tubes, centrifuged (4500 rpm, room temperature), the supernatant was discarded and resuspended with 1 mL of 0.1% (wt/vol) buffered peptone water (Hardy Diagnostics; Santa Maria CA, USA). A 1 mL of culture was inoculated into 99 mL of BPW (0.1%) to achieve final cell concentrations of 10^6-10^4 organisms per mL. Analyses of all treatments, including controls, were performed in triplicate.

Preparation of DMDC stock solution. DMDC (Velcorin; 99.8%; Lanxess Corp., Pittsburgh, PA, USA) solutions were prepared before each experiment by initially performing a 1:4 dilution in 100% ethyl alcohol to achieve a 390 X 10^3 mg/L stock solution. DMDC stock solutions were immediately added to spiked buffered peptone water (0.1%) with 1 ml of cells to obtain a final volume of 100 mL. The final concentration of DMDC was 0, 150, and 250 mg/L.

Sampling and Culturability for DMDC experiment. Each flask was aseptically sampled at different times. The flasks were left at room temperature with no agitation and sampled at 0 min, 15 min, 30 min and 60 min. At each sampling time, 1 ml from the flask was taken and ten-fold dilutions in 0.1% (wt/vol) buffered peptone water (Hardy Diagnostics; Santa Maria CA, USA) were performed and immediately plated in duplicate onto YPD agar (Yeast Extract 10 g/L, Peptone 20 g/L, Dextrose 20g /L, Agar 15 g/L) (Difco™; Sparks, MD, USA). The plates were incubated at 30°C for 48-72 h for *Z. bailii* and *S. cerevisiae*, and up to 3-4 weeks for *B./D. bruxellensis.* When necessary, direct plating of the sample (10^0 dilution) was performed to enumerate low

concentrations of cells after treatment.

Microbiological enumeration for DMDC experiment. Plates were enumerated for total microbial count. The counts were averaged and expressed as log numbers. The log reduction was then calculated for each strain and expressed as log numbers. Each inactivation experiment and respective controls were performed in triplicate for each strain.

Barrel treatments using ozone and chlorine dioxide. Forty barrels naturally contaminated with both *B. bruxellensis* and general yeast populations, were split into groups of ten barrels and treated whether with O_3 or ClO_2. For the O_3 experiments, two groups of ten barrels were treated for 5 and 10 minutes respectively (DEL Ozone; San Luis Obispo, CA, USA). With both groups of barrels, a concentration of 1 mg/L of O_3 and a water temperature of 21.3˚C were used. For the ClO_2 (Selectrocide 12G; Columbus, OH, USA) experiment, two groups of ten barrels were treated whether with 5 mg/L or 10 mg/L and the treatment time was 15 minutes for both groups of barrels. Concentrations used are the FDA permitted concentrations of up to 10 mg/L (www.fda.gov). Briefly, 7 liters of distilled water was added to the 40 barrels before and after the use of O_3 or ClO_2. The barrels were rolled several times to enhance the contact of water with the inner surface of the barrel and then stored bung side up for 24 hrs. The water was then removed and placed in sterile bottles at 4°C for subsequent microbiological enumeration. Samples were taken before and after the disinfection treatments. The samples were analyzed to determine the initial and final populations by filtration (EZ-Fit™ Manifold for universal laboratory filtration; Concord Road Billerica, MA USA) using 0.22 μm nitrocellulose filtration membranes and/or pertinent dilutions of the samples, since the microbial loads differed for each barrel. If samples needed to be diluted, 0.1% (wt/vol) buffered peptone water (Hardy Diagnostics; Santa Maria CA, USA) was used to perform 10 fold dilutions.

For the filtration method, 0.22 µm nitrocellulose filters (GE* Nitrocellulose-Mixed Esters of Cellulose Membrane Filters; Pittsburg, PA, USA) were used and the samples were filtered twice, and the results were averaged. The maximum volume filtered was 100 mL and the results were calculated as CFU/100 mL and then transformed in percentage to normalize results since not all the barrels had initially the same microbial load. Expressing the results in percentage allowed for easier comparison across biological samples. The nitrocellulose filters were placed on both WL and YPD agar using sterile forceps. WL agar (Yeast extract 4 g/L, tryptone 5 g/L, glucose 50 g/L, potassium dihydrogen phosphate 0.55 g/L, potassium chloride 0.425 g/L, calcium chloride 0.125 g/L, magnesium sulphate 0.125 g/L, ferric chloride 0.0025, manganese sulphate 0.0025 mg/L, bromocresol green 0.022 g/L, Agar 15 g/L) (Oxoid, LTD; Basingstoke Hampshire, England) was used to detect *B./D. bruxellensis* and was incubated at 30°C for up to 3-4 weeks. WL agar containing 10 mg/L cycloheximide (Sigma Aldrich; St. Louis, MO, USA; dissolved in 50% ethanol and filter sterilized) was used to allow for selection of *B./D. bruxellensis*, 150 mg/L of biphenyl (Acros Organics; Fair Lawn, New Jersey, USA; dissolved in ethanol and filter sterilized) to prevent the growth of mold, 30 mg/L of chloramphenicol (MP Biomedicals LLC; Solon, OH, USA; dissolved in 100% ethanol) to prevent the growth of lactic acid bacteria and 25 mg/L of kanamycin sulfate (AMRESCO; Solon, OH, USA; dissolved in sterile distilled water) to prevent the growth of acetic acid bacteria. YPD agar (Yeast Extract 10 g/L, Peptone 20 g/L, Dextrose 20 g/L, Agar 15 g/L) (Difco™; Sparks, MD, USA) was used to detect general yeasts populations and was incubated at 30°C for 48-72 hr. YPD agar was supplemented with 150 mg/L of biphenyl (Acros Organics; Fair Lawn, New Jersey, USA; dissolved in ethanol and filter sterilized) to prevent the growth of mold, 30 mg/L of chloramphenicol (MP Biomedicals LLC; Solon, OH, USA; dissolved in 100% ethanol) to prevent the growth of lactic acid bacteria, and 25 mg/L of kanamycin sulfate (AMRESCO; Solon, OH, USA; dissolved in sterile distilled H_2O) to prevent the growth of

acetic acid bacteria.

Isolation of *Brettanomyces* spp. from wood cores. Since the treatment using ClO_2 had minimal microbial reduction at a surface level (liquid samples), no wood cores were analyzed from these barrels. However, since O_3 treatment showed higher levels of microbial reductions in the liquid samples, wood cores were analyzed from each barrel, before and after treatment, in order to determine if from the barrels that presented *Brettanomyces* spp. initially, were still present and viable post-treatment. Since *Brettanomyces* spp. are more typically found at the bottom of a barrel (bilge) and also at the top of the barrels due to higher levels of oxygen that stimulate its growth (Van de Water 2010), the cores were strategically taken from bilge and heads (upper head and lower head) using a borer attached to a drill. This borer penetrated up to 17 mm from the outside surface of the barrel, reaching 8 mm from the inside of the barrel (total standard thickness of a stave is 25 mm). These cores were then excised at a depth of 8 mm, quickly flamed using 70% ethanol and placed in liquid media YPD media (Yeast Extract 10 g/L, Peptone 20 g/L, Dextrose 20 g/L; Difco™; Sparks, MD, USA) at 30ºC with 200 rpm agitation until visible growth was observed. After growth was observed in the liquid culture, a loop of the culture was streaked on WL (Yeast extract 4 g/L, tryptone 5 g/L, glucose 50 g/L, Potassium dihydrogen phosphate 0.55 g/L, potassium chloride 0.425 g/L, calcium chloride 0.125 g/L, magnesium sulphate 0.125 g/L, ferric chloride 0.0025, manganese sulphate 0.0025 mg/L, bromocresol green 0.022 g/L, Agar 15 g/L; Oxoid, LTD; Basingstoke Hampshire, England) and colonies with the particular morphology of *Brettanomyces* were selected.

Statistical analysis. For the dimethyl dicarbonate experiments, all CFU/mL data were transformed to $\log_{10}$ CFU/mL. The reductions in yeasts were calculated from the initial concentration of yeast cells (target inoculum) at time zero minus the final concentration of yeast at 60 min. All experiments and controls were performed in triplicate. For dimethyl dicarbonate and chlorine

dioxide experiments, no statistical analyses were performed because there was not enough variability in the response (in the case of dimethyl dicarbonate) or no reduction was observed (ClO_2) regardless of the concentration. For the O_3 experiment, a Fisher's exact test was performed where the response in terms of reduction was observed or not. Statistical analyses were conducted using SigmaPlot 12.0; Systat Software Inc., San Jose CA.

RESULTS AND DISCUSSION

DMDC effect on yeasts *in vitro.* Several studies have demonstrated the effectiveness of DMDC in terms of toxicity and germicidal activity in dry, semi-sweet, and sweet wines ready for bottling (Delfini et al. 2002). In this study, we assessed the efficacy of DMDC in a matrix different from wine, as we intend for it to be used as a wine barrel sanitizer and not as a wine or grape must sterilant. For that purpose, each strain was re-suspended in BPW (0.1%) and 0, 150 and 250 mg/L concentrations of DMDC were added to the flask (room temperature). At a concentration of 250 mg/L of DMDC, *Z. bailii* (4A1) was reduced to non-detectable levels. Divol et al. (2005) used concentrations above 200 mg/L and the cells were unable to resuscitate, however, they do not mention at which temperature the study was performed. According to Steels et al. (1999) *Z. bailii* can grow in YPD at 250 mg/L. The foregoing illustrates why experiments should be standardized, as different experimental conditions can lead to different results. It is worth mentioning that the authors also used ethanol to prepare the DMDC solution, although these results might be also due to strain variability. According to Porter and Ough (1982) DMDC is more effective as a fungicide at high temperatures (20°C and 30°C) and high alcohol levels, since both temperature and alcohol create a synergistic effect. Moreover, alcohol increases the water solubility of DMDC, which is 3.65 g•(100 g^{-1} of water) (Delfini et al. 2002). The same authors found that *Z. bailii* is inhibited in grape must using 400 mg/L of DMDC, 20ºC and 10^6 cells/mL. It is worth to mention that we also

used 10^6 cells/mL for the three concentrations tested. However, Delfini et al. (2002) weighed out the pure commercial DMDC and added it directly to a fermentation flask containing only 10% of the uninoculated medium (grape must or synthetic nutrient media) to favor dissolution. Then, the remaining 90% of the inoculated medium was added to restore the desired DMDC and yeast concentrations. In contrast, we previously prepared a stock solution of DMDC with 100% ethyl alcohol to favor dissolution, and we performed the experiments at ambient temperature (20-25°C). These findings suggest that the conditions we used in our experiment were even more effective at a lower concentration. We also found growth inhibition of *Z. bailii* (4A1) using 150 mg/L of DMDC, however, Divol et al. (2005) treated *Z. bailii* with lower concentrations of DMDC (less than 200 mg/l) in fermenting must, and the yeast were able to resuscitate, even in the case of the combined addition of SO_2 and DMDC. The authors did not mention if DMDC was previously diluted in ethanol to enhance its effectiveness. With regards to *S. cerevisiae*, Delfini et al. (2002) found that a DMDC concentration of 250 mg/L at 20ºC was sufficient to inhibit it in grape must. However, the same study showed that *S. cerevisiae* was resistant to 200 mg/L of DMDC in grape must using 10^6 cells/mL. According to Steels et al. (1999), *S. cerevisiae* can grow in YPD at 200 mg/L of DMDC. In our case, using 250 mg/L of DMDC caused non-detectable levels for all three of our experimental strains (CE9, CE78 and CE81). The initial concentration of cells were 10^6 cells/mL for CE78 and CE81 and 10^5 cells/mL for CE9; however, at 150 mg/L, strain CE78 was reduced only 2.99 log units, while strains CE9 and CE81 were reduced to undetectable levels. These findings suggest two things: a) that the response to DMDC is both strain- and dose-dependent, and maybe also that b) DMDC efficacy is subject to variation according to the menstrum where it is applied. According to Divol et al. (2005), DMDC does not have any interaction with sugars, however other components in the media where DMDC is applied could exert an influence in its efficacy. *B./D. bruxellensis* strains (CE149, 2080 and CE261) had undetectable levels after using both 250 mg/L or 150 mg/L of DMDC at ambient temperature (20-

25°C). Renouf et al. (2008) found that *B. bruxellensis* is inhibited at a concentration of 150 mg/L (whether at the beginning of the alcoholic fermentation or in grape juice) and using 25 ºC. However, they only used 10^3 cells/mL as their initial concentration of cells, whereas our experiment used 10^4 for CE261 and 2080, and for CE149 10^6 cells/mL (the number of cells is dependent of the maximum growth shown per each strain).

Efficacy of chlorine dioxide and ozone in decreasing general yeast populations and *Brettanomyces* spp. populations. We performed a study using two different concentrations of ClO_2 in order to see its efficacy to sanitize naturally contaminated barrels. The study showed that no consistent reduction was achieved in either the general yeast populations or *Brettanomyces* spp. yeast populations, regardless of the concentration used (5 or 10 mg/L), (Table 3.1 and Table 3.2 respectively). In some barrels we observed minimal reductions in yeast populations, but with other barrels we observed an increase in the microbial load after treatment. The higher levels of yeast counts after treatment could be attributed to the microorganisms being drawn from the interior portions of the cooperage due to the known degradation that ClO_2 can cause to lignin (Lemeune et al. 2004), causing an increase of the microbial load instead of a reduction. This information reveals that the use of chlorine dioxide might not be an appropriate sanitizer, at least not for wood porous surfaces. Additional *in vitro* studies of the efficacy of this sanitizer should be performed targeting both different surfaces and wine spoilage microorganisms, since there is very little information in the literature of its use against wine spoilage yeasts.

Ozone is used in a gaseous form or solubilized in water, as a means to sanitize processing equipment and surfaces. In the wine industry, O_3 and ozonated water are used for sanitation of stainless steel tanks, surfaces, oak barrels, and clean-in-place (CIP) systems (Zuehlke et al. 2013). We used ozone to sanitize two groups of wine barrels and the treatment was shown to be effective in decreasing both general yeast populations and *Brettanomyces* spp. populations, however, the

statistical analysis showed no significant difference between 5 and 10 minutes use of ozone, for Brettanomyces and general yeast populations (Table 3.3 and Table 3.4 respectively).

Table 3.1. General yeast populations with chlorine dioxide treatment

Treatment	Barrel	CFU/100ML INITIAL Scientific Notation	INITIAL Log10	CFU/100ML FINAL Scientific Notation	FINAL Log10	TIME (MIN)	CONCENTRATION mg/L	% Reduction[b]
ClO_2	4ALL119193	8.10E+08	8.91E+00	9.70E+07	7.99E+00	15	5	90
ClO_2	4ALL119186	1.82E+04	4.26E+00	9.60E+07	7.98E+00	15	5	N/A[a]
ClO_2	4AD9120725	3.40E+04	4.53E+00	5.10E+08	8.71E+00	15	5	N/A[a]
ClO_2	4ALL119187	8.15E+07	7.91E+00	6.25E+07	7.80E+00	15	5	23.31
ClO_2	4AD9120724	2.19E+08	8.34E+00	1.73E+08	8.24E+00	15	5	21
ClO_2	4ALL119192	1.50E+08	8.18E+00	1.53E+09	9.18E+00	15	5	N/A[a]
ClO_2	4A1F124972	1.44E+09	9.16E+00	9.45E+08	8.98E+00	15	5	90
ClO_2	4A1F124971	3.60E+08	8.56E+00	8.65E+07	7.94E+00	15	5	90
ClO_2	4ALL119245	2.17E+09	9.34E+00	4.40E+08	8.64E+00	15	5	90
ClO_2	4ALL119244	6.15E+07	7.79E+00	4.50E+08	8.65E+00	15	5	N/A[a]
ClO_2	4A1M124745	4.20E+05	5.62E+00	8.45E+08	8.93E+00	15	10	N/A[a]
ClO_2	4A1F124974	3.80E+07	7.58E+00	5.05E+07	7.70E+00	15	10	N/A[a]

Table 3.1 (Continued)

ClO_2	4A1M124746	1.26E+08	8.10E+00	1.37E+08	8.14E+00	15	10	N/A[a]
ClO_2	4ALL119185	1.15E+07	7.06E+00	2.96E+08	8.47E+00	15	10	N/A[a]
ClO_2	4A1F124973	4.10E+06	6.61E+00	2.94E+07	7.47E+00	15	10	N/A[a]
ClO_2	*4A1M124717	7.85E+08	8.89E+00	5.70E+08	8.76E+00	15	10	27.38
ClO_2	4A1M125068	1.36E+07	7.13E+00	3.00E+08	8.48E+00	15	10	N/A[a]
ClO_2	4ALL119184	1.70E+08	8.23E+00	1.62E+08	8.21E+00	15	10	4.7
ClO_2	4A1M125067	1.24E+08	8.09E+00	6.25E+08	8.80E+00	15	10	N/A[a]
ClO_2	4A1M124718	1.92E+05	5.28E+00	9.30E+08	8.97E+00	15	10	N/A[a]

[a]N/A not applicable due to increase in microbial load instead of reduction
[b]Percent reductions were determined by comparing the initial microbial load with the final microbial load after being exposed to the sanitizer.
This table represents the *in vivo* reduction of general yeast populations in natural contaminated barrels.

Table 3.2. *Brettanomyces* spp. populations with chlorine dioxide treatment

Treatment	Barrel	CFU/100ML INITIAL Scientific Notation	INITIAL Log10	CFU/100ML FINAL Scientific Notation	FINAL Log10	TIME (MIN)	CONCENTRATION mg/L	% Reduction[b]
ClO_2	4ALL119193	8.35E+05	5.92E+00	3.50E+07	7.54E+00	15	5	N/A[a]
ClO_2	4ALL119186	2.19E+04	4.34E+00	1.05E+05	5.02E+00	15	5	N/A[a]
ClO_2	4AD9120725	1.58E+05	5.20E+00	7.70E+07	7.89E+00	15	5	N/A[a]
ClO_2	4ALL119187	2.41E+06	6.38E+00	2.25E+06	6.35E+00	15	5	0
ClO_2	4AD9120724	1.28E+07	7.11E+00	3.00E+05	5.48E+00	15	5	97.656
ClO_2	4ALL119192	6.90E+05	5.84E+00	1.62E+08	8.21E+00	15	5	N/A[a]
ClO_2	4A1F124972	1.32E+06	6.12E+00	1.16E+07	7.06E+00	15	5	N/A[a]
ClO_2	4A1F124971	ND[c]	ND[c]	ND[c]	ND[c]	15	5	-
ClO_2	4ALL119245	2.17E+07	7.34E+00	1.60E+02	2.20E+00	15	5	99.999
ClO_2	4ALL119244	1.82E+07	7.26E+00	7.10E+05	5.85E+00	15	5	96.077
ClO_2	4A1M124745	3.75E+05	5.57E+00	8.05E+07	7.91E+00	15	10	N/A[a]
ClO_2	4A1F124974	ND[c]	ND[c]	ND[c]	ND[c]	15	10	-

Table 3.2 (Continued)

ClO_2	4A1M124746	9.10E+05	5.96E+00	1.16E+07	7.06E+00	15	10	N/A[a]
ClO_2	4ALL119185	ND[c]	ND[c]	ND[c]	ND[c]	15	10	-
ClO_2	4A1F124973	ND[c]	ND[c]	ND[c]	ND[c]	15	10	-
ClO_2	4A1M124717	1.46E+07	7.16E+00	1.21E+07	7.08E+00	15	10	17.12
ClO_2	4A1M125068	3.70E+03	3.57E+00	5.15E+03	3.71E+00	15	10	N/A[a]
ClO_2	4ALL119184	8.00E+03	3.90E+00	1.85E+07	7.27E+00	15	10	N/A[a]
ClO_2	4A1M125067	9.20E+05	5.96E+00	1.58E+07	7.20E+00	15	10	N/A[a]
ClO_2	4A1M124718	7.50E+06	6.88E+00	3.80E+06	6.58E+00	15	10	49.33

[a]N/A not applicable due to increase in microbial load instead of reduction
[b]Percent reductions were determined by comparing the initial microbial load with the final microbial load after being exposed to the sanitizer.
[c]ND Not detected
This table represents the i*n vivo* reduction of *Brettanomyces* populations in natural contaminated barrel.

Table 3.3 *Brettanomyces* spp. populations with ozone treatment

Treatment	Barrel	CFU/100ML INITIAL Scientific Notation	INITIAL Log10	CFU/100ML FINAL Scientific Notation	FINAL Log10	TIME (MIN)	CONCENTRATION mg/L	% Reduction[b]
O_3	327427	2.60E+02	2.41E+00	1.00E+00	0.00E+00	5 MIN	1	99.615
O_3	327438	8.00E+01	1.90E+00	0.00E+00	0.00E+00	5 MIN	1	100
O_3	327548	ND[a]	ND[a]	ND[a]	ND[a]	5 MIN	1	-
O_3	325920	3.70E+01	1.57E+00	0.00E+00	0.00E+00	5 MIN	1	100
O_3	325919	8.00E+01	1.90E+00	0.00E+00	0.00E+00	5 MIN	1	100
O_3	327547	3.00E+01	1.48E+00	0.00E+00	0.00E+00	5 MIN	1	100
O_3	335938	7.10E+01	1.85E+00	0.00E+00	0.00E+00	5 MIN	1	100
O_3	339051	3.80E+02	2.58E+00	0.00E+00	0.00E+00	5 MIN	1	100
O_3	335621	5.73E+02	2.76E+00	0.00E+00	0.00E+00	5 MIN	1	100
O_3	335620	6.60E+01	1.82E+00	0.00E+00	0.00E+00	5 MIN	1	100

Table 3.3 (Continued)

O_3	335812	8.00E+03	3.90E+00	0.00E+00	0.00E+00	10 MIN	1	100
O_3	335813	9.10E+07	7.96E+00	8.00E+00	9.03E-01	10 MIN	1	99.999
O_3	323593	8.00E+01	1.90E+00	2.00E+00	3.01E-01	10 MIN	1	97.5
O_3	323596	3.50E+01	1.54E+00	1.00E+00	0.00E+00	10 MIN	1	97.142
O_3	323934	ND[a]	ND[a]	ND[a]	ND[a]	10 MIN	1	-
O_3	323937	ND[a]	ND[a]	ND[a]	ND[a]	10 MIN	1	-
O_3	323335	6.00E+02	2.78E+00	0.00E+00	0.00E+00	10 MIN	1	100
O_3	329009	ND[a]	ND[a]	ND[a]	ND[a]	10 MIN	1	-
O_3	335631	1.60E+02	2.20E+00	0.00E+00	0.00E+00	10 MIN	1	100
O_3	335795	2.50E+01	1.40E+00	0.00E+00	0.00E+00	10 MIN	1	100

[a]ND Not detected

[b]Percent reductions were determined by comparing the initial microbial load with the final microbial load after being exposed to the sanitizer.

This table represents the *in vivo* reduction of *Brettanomyces* populations in natural contaminated barrels.

Table 3.4 General yeast populations with ozone treatment

Treatment	Barrel	CFU/100ML INITIAL Scientific Notation	INITIAL Log10	CFU/100ML FINAL Scientific Notation	FINAL Log10	TIME (MIN)	CONCENTRATION mg/L	% Reduction[b]
O_3	327427	5.33E+02	2.73E+00	1.60E+01	1.20E+00	5 MIN	1	96.998
O_3	327438	7.00E+00	8.45E-01	0.00E+00	0.00E+00	5 MIN	1	100
O_3	327548	1.00E+00	0.00E+00	0.00E+00	0.00E+00	5 MIN	1	100
O_3	325920	1.00E+00	0.00E+00	0.00E+00	0.00E+00	5 MIN	1	100
O_3	325919	2.64E+02	2.42E+00	1.00E+00	0.00E+00	5 MIN	1	99.621
O_3	327547	1.60E+02	2.20E+00	0.00E+00	0.00E+00	5 MIN	1	100
O_3	335938	8.00E+01	1.90E+00	0.00E+00	0.00E+00	5 MIN	1	100
O_3	339051	8.00E+02	2.90E+00	0.00E+00	0.00E+00	5 MIN	1	100
O_3	335621	9.00E+02	2.95E+00	0.00E+00	0.00E+00	5 MIN	1	100
O_3	335620	ND[a]	ND[a]	ND[a]	ND[a]	5 MIN	1	-
O_3	335812	1.13E+08	8.05E+00	8.00E+02	2.90E+00	10 MIN	1	99.999

Table 3.4 (Continued)

O_3	335813	3.00E+06	6.48E+00	8.00E+02	2.90E+00	10 MIN	1	99.999
O_3	323593	ND[a]	ND[a]	ND[a]	ND[a]	10 MIN	1	-
O_3	323596	ND[a]	ND[a]	ND[a]	ND[a]	10 MIN	1	-
O_3	323934	1.52E+02	2.18E+00	2.00E+00	3.01E-01	10 MIN	1	98.684
O_3	323937	ND[a]	ND[a]	ND[a]	ND[a]	10 MIN	1	-
O_3	323335	1.60E+02	2.20E+00	0	0.00E+00	10 MIN	1	100
O_3	329009	ND[a]	ND[a]	ND[a]	ND[a]	10 MIN	1	-
O_3	335631	3.20E+02	2.51E+00	0.00E+00	0.00E+00	10 MIN	1	100
O_3	335795	ND[a]	ND[a]	ND[a]	ND[a]	10 MIN	1	-

[a]ND Not detected
[b]Percent reductions were determined by comparing the initial microbial load with the final microbial load after being exposed to the sanitizer.
This table represents the *in vivo* reduction of general yeast populations in natural contaminated barrels.

Interestingly, when ozone was used to decrease the *Brettanomyces* spp. populations, one barrel (no. 335813) (Table 3.3) had a high initial microbial load (9.10E+07) and 99.999% reduction was found after treatment. In contrast, two other barrels (nos. 323593 and 323596) had lower initial microbial loads (8.00E+01 and 3.50E+01 respectively), and the percentages of reduction were 97.5% and 97.142%, respectively. We attribute this due to experimental variation only, likely due to poor contact of the inner surface of these two barrels with the ozonated water. As for the general yeast populations, similar results were observed in barrels no. 327427 and no. 323934 (Table 3.4) that did not show 100% reduction when compared to other barrels, such as 335812 that had higher initial microbial loads but showed a 99.999% reduction after ozone treatment.

Extraction of wood cores and isolation of *Brettanomyces*. The efficacies of ClO_2 and ozone treatments were studied at the surface level (0 mm) with liquid samples and in wood cores (a depth of 8mm) taken before and after sanitation treatment from different sites of the barrels, specifically bilge and heads (upper head and lower head) (Figure 3.1). However, the analysis of wood cores was only performed with the ozone treated barrels due to our observation that high microbial reductions of *Brettanomyces* spp. populations was achieved in the liquid samples, versus observations of the ClO_2 treated barrels that was shown to be ineffective. A depth of 8 mm was chosen for the examination of wood cores, because 8 mm is the level of wine penetration, and because *Brettanomyces* spp. has been found at that depth (Malfeito Ferreira et al. 2004). At a depth of 8 mm, only two barrels out of twenty were positive for *Brettanomyces* spp. before treatment (Table 3.5) and these same barrels after treatment did not have positive isolation of *Brettanomyces* spp.

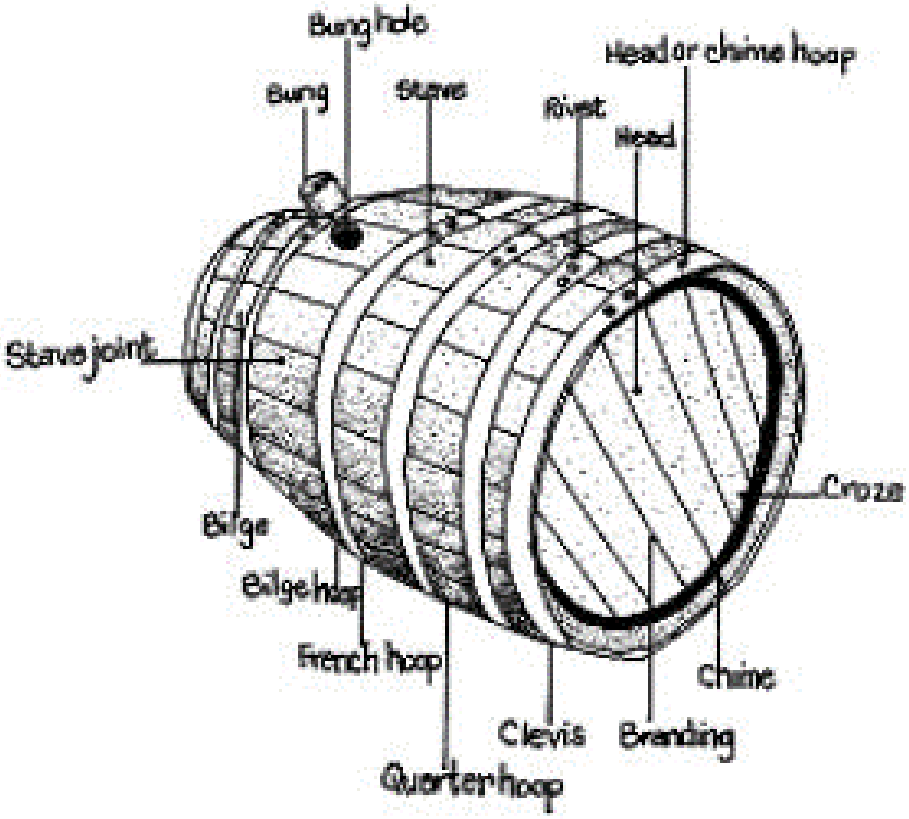

Figure 3.1. Barrel sites

However, there was a third barrel that pre-treatment did not show positive growth of *Brettanomyces* spp., but after treatment *Brettanomyces* spp. was isolated. This suggests that ozone, although effective at a surface level (liquid samples), might not be effective at the holding times and concentration used at a depth of 8 mm.

Other interesting findings were observed during the isolation of *Brettanomyces* spp. from the wood cores of naturally contaminated barrels. We know from prior experience that isolates from naturally contaminated barrels, often the colony morphology may be wrinkled, dusty and irregular shaped (Figure 3.2), or may exhibit

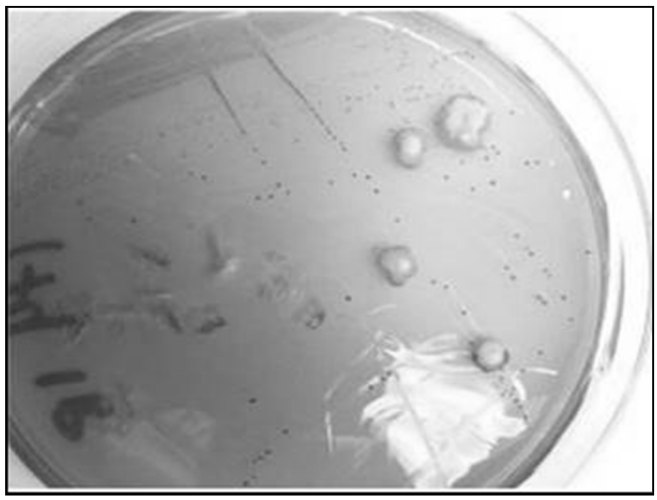

Figure 3.2. *Brettanomyces* spp. wrinkled yeast morphology isolated from naturally contaminated barrels.

Table 3.5 Presence of *Brettanomyces* spp. pre- and post-treatment wood cores

Treatment	Barrel	Pre-treatment	Post-treatment	Location	Time
Ozone	327438	positive	negative	lower head	5 minutes
Ozone	325920	negative	positive	lower head	5 minutes
Ozone	323934	positive	negative	upper head	10 minutes

This table represents the presence of *Brettanomyces* spp. in wood cores.

a smooth morphology as observed with laboratory strains of *Brettanomyces* spp.. Fugelsang and Edwards (2007), reported that *Brettanomyces* spp. grown on solid agar substrate may appear considerably different from *Brettanomyce*s spp isolated from barrel aged wine. When grown on solid media, colonies appear white to yellowish and may be glistening, moist and smooth, or dull and wrinkled. Perissatto Meneghin et al. (2008) isolated yeasts from an alcoholic operating units, and found similar results to what was observed in our study (smooth, bright yeast colonies and wrinkled, opaque colonies). They collected the samples from fermented must collected from an alcohol-operating unit in the 2006/2007 sugar cane harvest, and observed two different morphologies, as reported by Fugelsang and Edwards (2007). Perissatto Meneghin et al. (2008) also evaluated the effectiveness of ClO_2 as a means to decrease yeast populations, and found that wrinkled yeast colonies required 150 mg/L ClO_2 to decrease their growth, while smooth yeast colonies only required 100 mg/L to result in the same effect. In our case, smooth morphologies were observed with the liquid samples. Conversely, the yeasts isolated from the 8 mm wood cores for O_3 treatment and other sanitizer treatments, showed irregular morphologies. In additional ozone and chlorine dioxide studies, *Brettanomyces* spp. were isolated from naturally contaminated barrels in NY and yielded wrinkled morphologies associated with the inner space of staves (wood cores). PCR analysis was performed on the isolates to confirm their identification as *Brettanomyces* (unpublished). Perissatto Meneghin et al. (2008) suggested that additional research is required to investigate if traits associated with this wrinkled morphology confer certain resistance to chlorine dioxide, and possibly other sanitizers. Despite the concentrations used in these studies, use of concentrations greater than 10 mg/L are not presently permitted in the US.

CONCLUSIONS

Our results showed that DMDC could be an effective barrel sanitizer since the highest concentration used (250 ppm) showed high efficacy against the yeasts used in this study. However, a *post hoc* validation method should be performed in order to confirm its effectiveness in wine barrels. The wine industry is searching for alternative methods to sanitize wine barrels without compromising the integrity of the wood and the quality of wines during aging. New sanitizing methods for wine barrels should be implemented due to the high cost they represent for wine industry. The results obtained with DMDC in this study will be useful to standardize the conditions in our next series of experiments for wine barrel sanitation. Furthermore, ozone proved to be very effective at decreasing both yeast populations studied, however its efficacy was not optimal at 8 mm depth. In contrast, chlorine dioxide showed no reductions in either yeast populations examined. Further research is required to investigate the different morphologies that *Brettanomyces* spp. present, since this will increase the knowledge of this important microorganism for the wine industry and its improved identification at not only a microscopic level, but also at a macroscopic level.

ACKNOWLEDGMENTS

This research project was funded by CONACYT-Mexico, New York State Agricultural Experiment Station Federal Formula Funds, and Cornell University Research Travel Awards. The authors thank the industry donors at Constellation Brands California, USA for the barrels used in this study.

The authors would like to thank Erik Arthur Smith (Cornell University) for his valuable editorial contributions to this article and Ms. Amy Andersen (Cornell University) for the barrel drawing.

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CHAPTER 4

EFFICACY OF SULFUR DIOXIDE AND SULFUR DISCS AGAINST YEASTS COMMONLY FOUND IN WINE ENVIRONMENTS: *IN VIVO* AND *IN VITRO* TRIALS

Short version of title: Efficacy of sulfur dioxide and sulfur in sanitation

ABSTRACT

Our objective was to evaluate the efficacy of sulfur dioxide using 150 mg/L at three pH levels (3.0, 3.2 and 3.4) against various yeasts commonly found in wine environments. A six log reduction in *Zygosaccharomyces bailii* was achieved using 150 mg/L of potassium metabisulfite at pH 3.0 and 3.2, but no reduction was noted at pH 3.4 (4A1). Among three strains of *Saccharomyces cerevisiae*, none of the pH levels evaluated (3.0, 3.2 and 3.4) significantly affected contaminant survival. Three strains of *Brettanomyces/Dekkera bruxellensis* (2080, CE261, CE149) were also included in the study. Strain 2080 was reduced by 1.9 $\log_{10}$ units at a pH 3.0, and by 0.5 and 0.3 $\log_{10}$ units at pH 3.2 and 3.4, respectively. *B./D. bruxellensis* (CE 261) was similarly reduced by a factor of 1.78 $\log_{10}$ at a pH 3.0, and 1.2 and 1.0 $\log_{10}$ at pH 3.2 and pH 3.4. Strain CE149 of *B. bruxellensis* was reduced by a factor of 3.0 $\log_{10}$ at a pH of 3.0, and 2.5 and 2.4 $\log_{10}$ at pH 3.2 and 3.4, respectively. Efficacy of sulfur dioxide gas (discs) was also evaluated for decontamination of naturally contaminated barrels, with treatment times of three or six weeks using 5 g discs of sulfur per barrel. The level of decontamination was determined at the internal wood surface of the barrel (0 mm depth) by taking liquid samples for both general yeast populations and *Brettanomyces*, and at a depth of 8 mm by aseptically extracting wood cores from the interior of the barrel. Liquid

samples permitted quantification of both yeast populations, and wood cores for the presence or absence of *Brettanomyces* spp., before and after treatment.

Key words: Sulfur dioxide, *Brettanomyces/Dekkera bruxellensis*, *Zygosaccharomyces bailii, Saccharomyces cerevisiae*, barrels, sanitizer.

INTRODUCTION

Winemaking involves the multiplication and metabolism of numerous yeasts and bacteria in grape juice. After the fermentation process, the fermented juice is stabilized by the addition of sulfites (sulfur dioxide) to protect against re-fermentation or the growth of deleterious microorganisms that could spoil the wine through production of various undesirable flavors and aromas (Lustrato et al. 2010). Sulfur dioxide (SO_2) is an antioxidant and antimicrobial (Usseglio Tomasset, 1992), and it is also used to sanitize empty barrels prior to reuse or storage. Sulfur dioxide has not been systematically evaluated to sanitize wine cooperage and the current knowledge of SO_2 application is largely based upon studies performed in wine, where carbonyl compounds, sugars, etc. and other uncontrolled conditions may confound the observed antimicrobial effects of SO_2. Glucose present in wine is one of the main SO_2-binding compounds. Moreover, SO_2 forms additional compounds with aldehydes and, to a lesser extent, with ketones (King et al. 1981). Sulfur dioxide exists in equilibrium in aqueous solutions between molecular SO_2 (SO_2•H2O), bisulfite (HSO_3^-), and sulfite (SO^{2-}_3) species, but this equilibrium is strictly dependent on pH (Fugelsang and Edwards 2007; Usseglio Tomasset, 1992). It is generally believed that the molecular sulfur species is the antimicrobial form of sulfur dioxide which is predominant at low pH. Because SO_2•H_2O does not

have a charge, the molecule enters the cell and undergoes rapid pH-driven dissociation at cytoplasmic pH (generally near 6.5) to yield bisulfite and sulfite. As the intracellular concentration of molecular SO_2 decreases due the internal equilibrium, more molecular SO_2 enters the cell, further increasing intracellular concentrations (Fugelsang and Edwards 2007).

Oak barrels have been commonly used for aging wines and spirits due to their positive effects on the finished product that include increased color stability, spontaneous clarification, and desirable complex aromas (Rodriguez Rodriguez and Gomez Plaza 2011). However, spoilage sometimes occurs during ageing, or even after bottling, due to the continued growth or residual secondary metabolites of contaminant microorganisms such as the yeast *Dekkera/Brettanomyces* that causes widespread losses in the wine industry due to degraded wine quality (Lustrato et al. 2010). The growth of yeasts belonging to *Dekkera / Brettanomyces* during the production of red wine, especially during aging, can seriously affect the organoleptic quality of the finished product. These yeasts grow slowly during wine ageing in wooden barrels, particularly when the SO_2 concentration is low (molecular SO_2 <0.5 mg /L), the pH is high (>3.8) and the temperature is above 15°C (Benito et al. 2009). Although barrel disinfection methods are becoming more sophisticated (water vapor, ozonization, etc.), the shape and microstructure of wooden barrels offer undesirable microorganisms niches that provide a great degree of protection from direct contact by introduced sanitizers (Suarez et al. 2007). It is now generally accepted that control of *Dekkera/Brettanomyces* spp. cannot be achieved by mere cleaning or incomplete sanitation of all cellar equipment, but demands much more stringent microbiological control and judicious utilization of sulfite or dimethyl dicarbonate (Loureiro and Malfeito Ferreira 2003)**.** The ability of yeast strains to survive high alcohol concentrations (both wild and those introduced purposely in the winemaking process)

allows them to carry out re-fermentation in barrels or bottles, thereby modifying the alcohol/sugar balance and the aroma of the wine, thus reducing its quality. This process is mainly carried out by *Z. bailii* and *S. cerevisiae*, due to their tolerance to high concentrations of alcohol and SO_2 (Salinas et al. 2009), and their survival of primary alcoholic fermentation (Divol et al. 2006). The presence of yeasts of the genus *Zygosaccharomyces*, in particular *Z. bailii*, is well known in wineries producing sweet or sparkling wines using juice concentrate or sulfited grape juice. Their high level of resistance to preservatives, particularly in *Z. bailii*, means that addition of high, but sub-lethal doses, decreases the incidence of competing microorganisms, making the survival of the resistant strains even more problematic (Loureiro and Malfeito-Ferreira 2003). To determine the antimicrobial efficacy of SO_2 alone, we conducted *in vitro* experiments where potassium metabisulfite was the sole antimicrobial compound. Additionally, SO_2 was monitored throughout the experiment using the Ripper analysis (direct iodometric titration). Because the Ripper method involves the use of a solution with poor stability, with the consequent possibility of parallel reactions and difficulties associated with the end-point observation, particularly in red wines (Moreira Gonçalves et al. 2010), we used a free wine sample wherein the end point could easily be identified. Sulfur dioxide was also evaluated using 5 g sulfur discs in wine barrels (*in vivo* experiment). The purpose of the *in vitro* study was to test sulfur dioxide at three different pH levels, in order to assess its efficacy against wine spoilage yeasts, while measuring free and total sulfur dioxide, and then contrast these results with the use of sulfur discs (≈5g) (*in vivo* study) in naturally contaminated barrels held for three and six weeks. The generated results with these experiments will serve as a guide to more efficient sanitation practices using sulfur dioxide as a sanitizer. Preliminary accounts of our findings have been published elsewhere (Aguilar Solis et al. 2012 a);

Aguilar Solis et al. 2013 b)).

MATERIALS AND METHODS

Strain selection. *Brettanomyces/Dekkera bruxellensis*. Three Isolates (2080, CE149 and CE261) were obtained from the Department of Food Science collection at Cornell University.

Saccharomyces cerevisiae. Three Isolates (CE78, CE9 and CE81) were obtained from the Department of Food Science collection at Cornell University.

Zygosaccharomyces bailii. One isolate (4A1) was obtained from the Department of Food Science collection at Cornell University.

Preparation of starter culture and inoculation. The yeasts, stored at -80°C in glycerol 15% (w/v), were revitalized and maintained on YPD agar (Yeast Extract 10 g/L, Peptone 20 g/L, Dextrose 20 g/L, Agar 15 g/L; Difco™; Sparks, MD, USA). All the strains were grown until stationary phase (10^8 CFU/mL) or at least 10^6 CFU/mL (200 rpm, 30°C). The growth time varied according to the strain (previous data not shown of growth curve for each strain). Each strain was grown in YPD broth (Yeast Extract 10 g/L, Peptone 20g/L, Dextrose 20g/L) (Difco™; Sparks, MD, USA) adjusted to different pH levels (3.0, 3.2 and 3.4) with HCl 1M and/or NaOH 1M. Once the cultures reached 10^8 CFU/mL or 10^6 CFU/mL, the target inoculum was verified via a viability assessment. Volumes of 1 mL of culture were placed in sterile eppendorf tubes, centrifuged (4500 rpm, ambient temperature), supernatant discarded and re-supended with 1 mL of McIlvaine buffer (Dawson et al. 1986) at the different pH levels stated above. A 1 mL of culture was inoculated into 99 mL of McIlvaine buffer at the different pH levels mentioned above, reaching a concentration of 10^4-10^6 CFU/mL. Analyses were performed in triplicate.

Preparation of sulfur dioxide solutions. The solutions of sulfur dioxide were prepared before each experiment by dissolving the required amount of potassium metabisulfite ($K_2S_2O_5$) (Fisher Scientific; Fair Lawn, NJ, USA) in McIlvaine buffer (Dawson et al. 1986) at the different pH values used for this study (3.0, 3.2 and 3.4). The final concentrations of sulfur dioxide were 0 mg/L and 150 mg/L. Each sulfur dioxide solution was spiked with 1 mL of cells to achieve a final volume of 100 mL.

Sampling and microbiological isolation. Each flask was aseptically sampled at different times. The flasks were incubated in a water bath (Polytherm Benchscale Equipment; Dayton Ohio, USA) at 30 °C with no agitation and sampled at 0, 15, 30, 60 and 90 min. At each sampling time, 1 mL from the flask was taken and properly diluted in 0.1% (wt/vol) buffered peptone water (Hardy Diagnostics; Santa Maria CA, USA), immediately plated in duplicate onto YPD agar (Yeast Extract 10 g/L, Peptone 20g/L, Dextrose 20g/L, Agar 15 g/L) (Difco™; Sparks, MD, USA), and incubated at 30°C for 48-72 hr for *Zygosaccharomyces bailii* and *Saccharomyces cerevisiae*, and up to 3-4 weeks for *Brettanomyces/Dekkera bruxellensis.* When necessary, direct plating from the flask was also performed, in order to increase the level of detection threshold.

Microbiological enumeration. Plates were enumerated for total microbial count. The counts were averaged and expressed as log values. The log reduction was then calculated for each strain and expressed as log values. Each inactivation experiment and controls were performed in triplicate

for each strain.

Determination of free and total sulfur dioxide. Throughout the sulfur dioxide experiment, samples were taken at different times (0, 30 and 90 min). The samples were analyzed in triplicate for the free and total sulfur dioxide level. Briefly, for free SO_2 analysis, 25 mL of sample were volumetrically transferred to a clean 250 mL Erlenmeyer flask, then 5 mL of starch indicator (Fisher Scientific; Fair Lawn, NJ, USA), a pinch of sodium bicarbonate (Mallinckrodt Chemical Works; St. Louis, NY, USA) and 5 mL of 3:1 water:sulfuric acid were added (JT Baker; NJ, USA). Rapidly the sample was titrated with freshly prepared 0.02 N iodine from 0.1 N iodine stock solution (JT Baker; NJ, USA) until to a blue end point that was stable, approximately 20 sec. The formula used to calculate the free sulfur dioxide was: free sulfur dioxide (mg/L) = (mL iodine)(N iodine)(32)(1000)/mL wine sample (Zoecklein et al. 1995). For total sulfur dioxide analysis, 25 mL of sample were volumetrically transferred into a clean 250 mL Erlenmeyer flask, then 25 mL of 1N sodium hydroxide was added. The flask was swirled and stoppered for 10 min for the hydrolysis reaction to occur. Five milliliters of starch indicator (Fisher Scientific; Fair Lawn, NJ, USA), a pinch of sodium bicarbonate (Mallinckrodt Chemical Works; St. Louis, NY, USA) and 10 mL of 3:1 water:sulfuric acid were added (JT Baker; NJ, USA). Rapidly the sample was titrated with freshly prepared 0.02 N iodine from 0.1 N iodine stock solution (JT Baker; NJ, USA) until to a blue end point that persisted for approximately 20 sec. The formula used to calculate the total sulfur dioxide is the same as above (Zoecklein et al. 1995).

Sulfur disc treatment of barrels. Twenty barrels naturally contaminated with both

Brettanomyces bruxellensis and general yeast populations, were split in two groups of ten barrels each and treated with sulfur discs of ≈5g (La Littorale; Béziers Cedex, France). Each barrel had an identifier number. The treatments were held for 3 and 6 weeks. Briefly, 7 liters of distilled water was added to each of the 20 barrels, before and after burning the sulfur discs inside the barrels. The barrels were rolled several times to enhance the contact of water with the inner surface of the barrel and then stored bung side up for 24 hrs. The barrels were sampled before and after burning the sulfur discs. Water samples from the barrel rinse were collected into sterile bottles. The first portion of the water was discarded, and served as a "rinse" for the bung hole (outer portion of the bung), and then 70% ethanol was sprayed around the bung hole of the barrel. The samples were placed at 4°C until their analysis. The samples were analyzed to determine the initial and final populations, either by filtration (EZ-Fit™ Manifold for universal laboratory filtration; Concord Road Billerica, MA USA) using discs of 0.22 µm and/or pertinent dilutions of the samples, since the microbial loads varied for each barrel. If samples needed to be diluted, 0.1% (wt/vol) buffered peptone water (Hardy Diagnostics; Santa Maria CA, USA) was used.

For the filtration method, 0.22 µm cellulose filters (GE* Nitrocellulose-Mixed Esters of Cellulose Membrane Filters; Pittsburg, PA, USA) were used, and the samples were divided into two samples and filtered. The results of the two filters were then averaged. The maximum volume filtered was 100 mL and the results were calculated as CFU/100mL and then transformed in percentage to homogenize results since not all the barrels had initially the same microbial load. Expressing the results in percentage allowed for easier interpretation. The cellulose filters were aseptically placed onto WL and YPD agar. WL agar (yeast extract 4g/L, tryptone 5g/L, glucose 50 g/L, potassium dihydrogen phosphate 0.55 g/L, potassium chloride 0.425 g/L, calcium chloride 0.125 g/L,

magnesium sulphate 0.125 g/L, ferric chloride 0.0025, manganese sulphate 0.0025 mg/L, bromocresol green 0.022 g/L, agar 15 g/L) (Oxoid, LTD; Basingstoke Hampshire, England) was used to detect *Brettanomyces/Dekkera bruxellensis* and was incubated at 30°C for up to 3-4 weeks. WL agar contained 10 mg/L of cycloheximide (Sigma Aldrich; St. Louis, MO, USA) to allow for selection of *B./D. bruxellensis* (dissolved in 50% ethanol and filter sterilized), 150 mg/L of biphenyl (Acros Organics; Fair Lawn, New Jersey, USA; dissolved in ethanol and filter sterilized) to prevent the growth of mold, 30 mg/L of chloramphenicol (MP Biomedicals LLC; Solon, OH, USA; dissolved in 100% ethanol) to prevent the growth of lactic acid bacteria and 25 mg/L of kanamycin sulfate (AMRESCO; Solon, OH, USA; dissolved in sterile distilled H_2O) to inhibit the growth of acetic acid bacteria. YPD agar (Yeast Extract 10 g/L, Peptone 20g/L, Dextrose 20g/L, Agar 15 g/L) (Difco™; Sparks, MD, USA) was used to detect general yeasts populations and was incubated at 30°C for 48-72 hr. YPD agar contained all of the above selective agents except cycloheximide.

Isolation of *Brettanomyces* spp. from wood cores. Wood cores were extracted from each barrel before and after sanitation treatment using a borer attached to a drill. This borer penetrated up to 17 mm from the outside surface of the barrel, reaching 8 mm from the inside of the barrel (total standard thickness of a stave is 25 mm). These cores were excised at a depth of 8 mm, quickly flamed and transferred to YPD broth (Yeast Extract 10 g/L, Peptone 20g/L, Dextrose 20g/L) (Difco™; Sparks, MD, USA) at 200 rpm and 30ºC until growth was observed. The zones from where the cores were extracted were the bilge and head (whether upper or lower part of the head). After growth was observed in liquid culture, a loop of the broth was streaked on WL and colonies

with the typical morphology of *Brettanomyces* were selected. The colonies were re-streaked up to 7 times for purification purposes and due to *Brettanomyces* isolates from barrels were observed to present a wrinkled and dusty morphology that eventually changed over time to a smooth morphology.

Statistical analysis. All CFU/mL data were transformed to $\log_{10}$ CFU/mL. The reductions in yeasts were calculated from the initial concentration of yeast cells (target inoculum) at time zero minus the last concentration of yeast at time ninety (min) for the *in vitro* experiments. All experiments, including the controls, were performed in triplicate. The software used for the statistical analysis was SigmaPlot Version 12.0; Systat Software Inc., San Jose CA. The analysis used was a three-way ANOVA and the simple comparisons were performed using the Tukey test with a p value < 0.05. For the reduction of *Brettanomyces* and general yeast populations using sulfur discs experiment, a Fisher's exact test was performed, where the response in terms of reduction was reduced “yes” or “no”.

RESULTS AND DISCUSSION

Effect of Sulfur Dioxide on strains. Sulfite acts as a powerful antimicrobial agent and is highly toxic to most non-*Saccharomyces* yeasts (Mendoza et al. 2009). In this work, we studied the effect of pH at a high concentration of potassium metabisulfite (KMB) (150 mg/L) using a matrix with no SO_2-binding compounds (i.e. carbonyl compounds) commonly found in wines. Indeed, the majority of studies that have been performed to date, have been in wine menstruum, wherein uncontrolled variables related to differences in grape cultivars, winemaking techniques, and dose of sulfur dioxide, could potentially confound observed treatment effects. In this study, we used a non-interfering matrix with the antimicrobial effect of sulfur dioxide and seven strains: one *Zygosaccharomyces bailii,* three *Saccharomyces cerevisiae* and three *Brettanomyces/Dekkera bruxellensis*. The difference in the mean values among the different concentrations and pH used, were evaluated for all the strains. At a pH of 3.0 and 3.2 and 150 mg/L of sulfur dioxide, total log reductions were observed for 4A1 (*Z. bailii)*. The initial number of cells were 6.32, 6.39 and 6.42 log_{10} for pH 3.0, 3.2 and 3.4 respectively. Moreover, statistically significant difference was found in comparison with their respective control at pH 3.0 and 3.2 ($P=<0.001$). However, at pH 3.4 and 150 mg/L, the log reduction was null, and thus, no significant statistical difference was found when compared to its control ($P=0.992$). The null reduction at pH 3.4 is due to the decreased availability of the molecular form (SO_2), which is the germicidal form of the sanitizer (Figure 4.1). In contrast, Divol et al. (2005) found that with 200 mg/L of sulfur dioxide, *Z. bailii* cells did not lose their capacity to grow on a solid medium. However, the cited study was achieved in wine and the pH was not mentioned as a factor, which has an important influence on the antimicrobial effect of sulfur dioxide. Our study was conducted using a non-interfering matrix where SO_2-bound

compounds are not expected to be formed, and where the pH factor was controlled. Moreover, statistical significance (P = <0.001) was found when different pH values were compared and 150 mg/L were used. In fact, the comparison between pH 3.0 and 3.4 and between pH 3.2 and 3.4 showed statistically significant differences, this was expected since at pH 3.0 and pH 3.2 there were significant log reductions. However, the comparison between pH 3.0 and

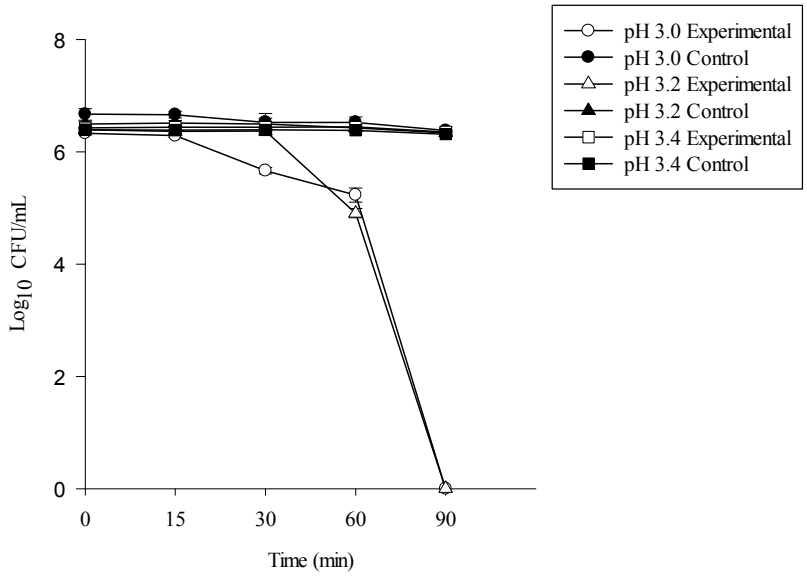

Figure 4.1 Strain 4A1 (*Z. bailii*). Effect of sulfur dioxide at three different pH levels. Each data represents a mean of three replicates and error bars represent standard error. Log Reduction equal to zero indicates the absence of colonies in a volume of 0.1 mL.

3.2 showed no statistically significant differences (P=1.000), since both pH conditions behaved the same way, achieving a total log reduction or not detectable levels of yeasts. As expected for the control (0 mg/L), when the different pH levels were compared no statistically significant differences were found among the three of them (P=0.844).

For strain 2080 (*B./D. bruxellensis)* (Figure 4.2), 1.9 log reduction was achieved at a

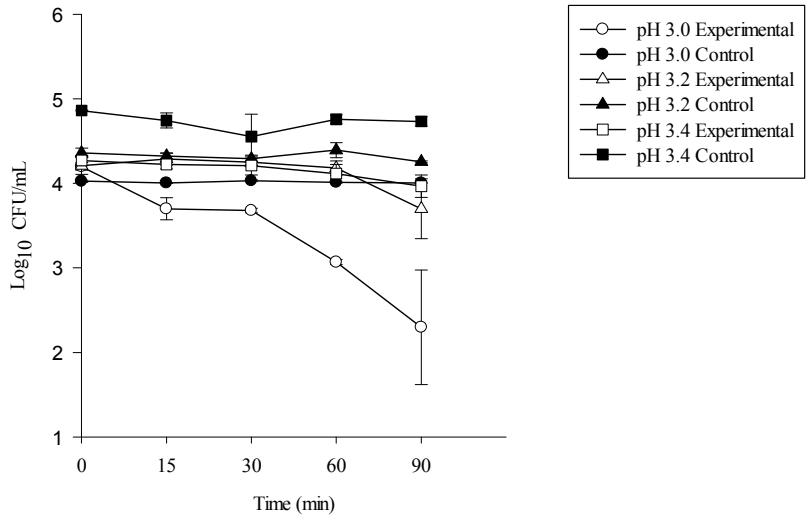

Figure 4.2 Strain 2080 (*B./D. bruxellensis*). Effect of sulfur dioxide at three different pH levels. . Each data represents a mean of three replicates and error bars represent standard error.

pH of 3.0. At pH 3.2 and 3.4, there was only a log reduction of 0.5 and 0.3, respectively. The initial number of yeasts cells were 4.19, 4.20 and 4.27 $\log_{10}$ at a pH of 3.0, 3.2 and 3.4 respectively. At a pH of 3.0 and 150 mg/L there were significant differences (P=0.001) when compared to its control (0 mg/L). However, at pH of 3.2 and 3.4, no statistical differences were found with their respective controls (P=0.069 and P=0.401, respectively). Statistically significant differences were found when comparing the pH values between 3.0 and 3.4 and 3.0 and 3.2 and 150 mg/L were used (P=<0.001 for both pH). However, this was not true for the comparison between pH 3.2 and 3.4, where pH 3.2 and 3.4 had similar $\log_{10}$ reductions (almost null for both pH values) (P=0.611). When the different pH levels were compared and 0 mg/L (control) were used, no significant differences were found among the three of them (P=0.912). With regards the strain CE149 (*D./B. bruxellensis*) (Figure 4.3), there was a 3 $\log_{10}$ reduction at a pH of 3.0, and 2.5 and 2.4 log

reductions at a pH 3.2 and 3.4, respectively. The initial number of yeast cells were

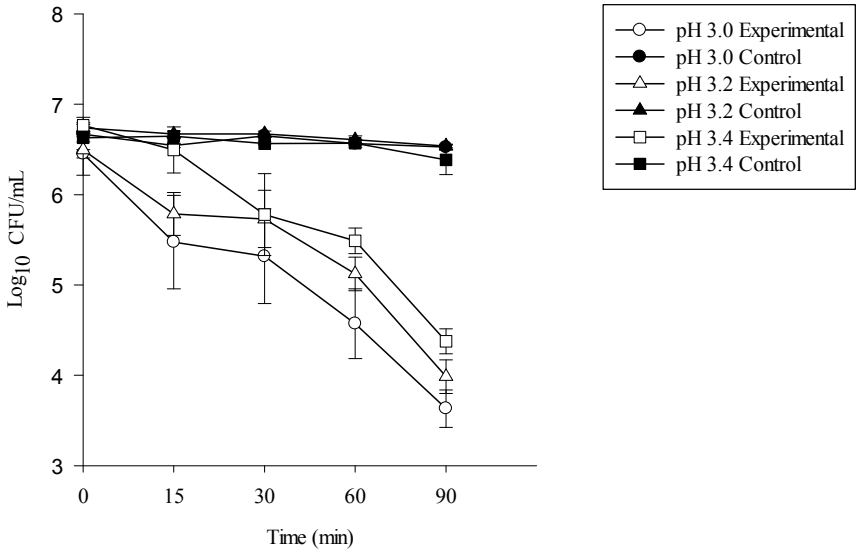

Figure 4.3 Strain CE149 (*B./D. bruxellensis*). Effect of sulfur dioxide at three different pH levels. Each data represents a mean of three replicates and error bars represent standard error.

6.45, 6.49 and 6.76$\log_{10}$ at a pH of 3.0, 3.2 and 3.4 respectively. CE 261 (*B./D.bruxellensis)* (Figure 4.4) was reduced 1.78 log units at a pH 3.0, and 1.2 and 1.0 at a pH of 3.2 and 3.4, respectively. The initial number of yeast cells were 6.01, 6.61 and 6.69 $\log_{10}$ at a pH of 3.0, 3.2 and 3.4, respectively. The statistical analysis revealed that for both strains there were significant differences (P=<0.001) in comparison to their respective controls (0 mg/L) at all levels of pH and using 150 mg/L of SO_2. In contrast, no significant differences were found when comparisons of the three different pH levels and 150 mg/L or 0 mg/L were used for both strains (CE149 and CE261) (P=0.654 and P=0.175, respectively). Interestingly, reports of the effect of sulfur dioxide on *D./B. bruxellensis* inactivation are often contradictory. Some authors refer its sensitivity to values higher than 30 mg/L SO_2, while others state that it should be regarded as resistant and growth has been reported when greater than 30 mg/l of free sulfur dioxide. This controversy probably arises from

differences

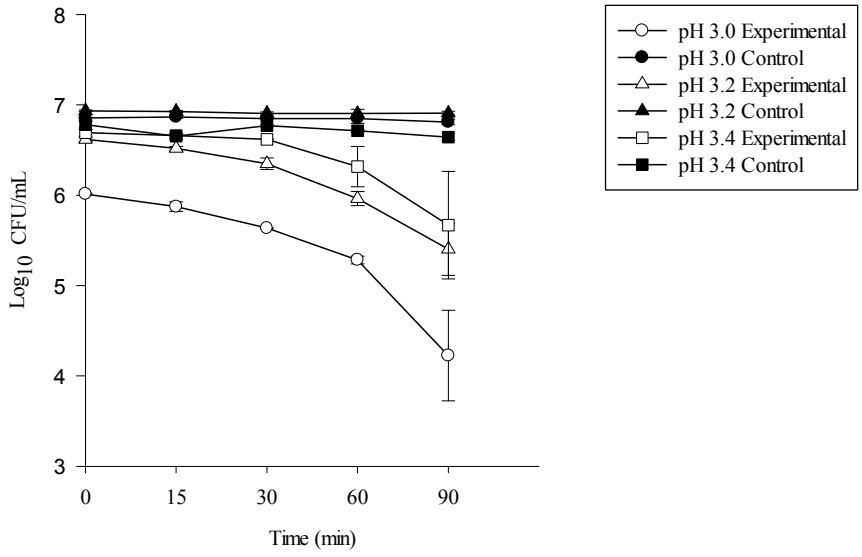

Figure 4.4 Strain CE261 (*B./D. bruxellensis*). Effect of sulfur dioxide at three different pH levels. Each data represents a mean of three replicates and error bars represent standard error.

in experimental conditions and strain behavior variability (Barata et al. 2008). Our results showed that 150 mg/L was not sufficient to reduce the yeasts tested to non-detectable levels, despite the fact that the concentration of sulfur dioxide was always much higher than 30 mg/L for all the experiments (Table 4.1).

With regards to the three *S. cerevisiae* strains, the log reduction was very low. At pH 3.0, only strain CE78 was reduced in 1.0 $\log_{10}$ unit, and at pH 3.2 and 3.4 there was only a reduction of 0.7 and 0.5 $\log_{10}$ units, respectively (Figure 4.5). The initial number of yeast cells were 6.53, 6.62 and 6.67 $\log_{10}$ at a pH of 3.0, 3.2 and 3.4 respectively. Statistically significant differences were found when 150 mg/L were used for all pH levels (3.0, 3.2 and 3.4), when compared to their respective controls (0 mg/L) ($P=<0.001$). However, no significant different were found

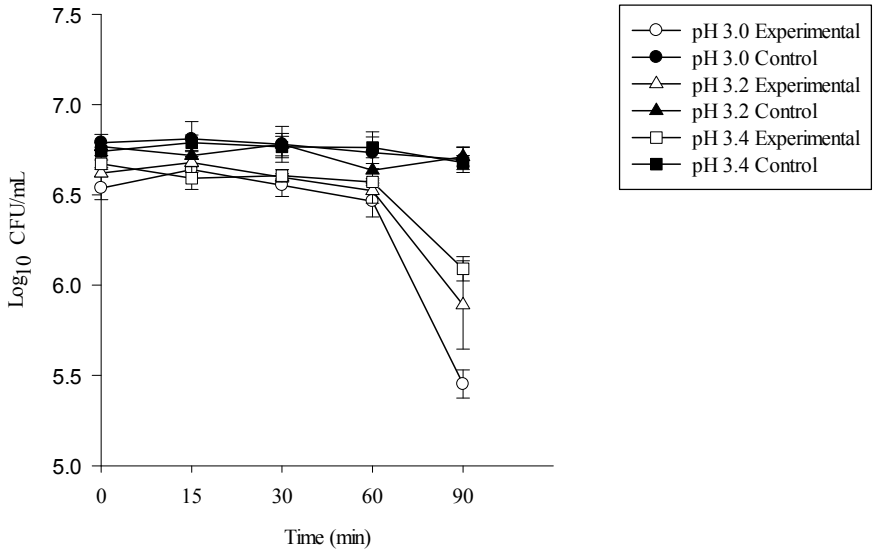

Figure 4.5 Strain CE78 (*S.cerevisiae*). Effect of sulfur dioxide at three different pH levels. Each data represents a mean of three replicates and error bars represent standard error.

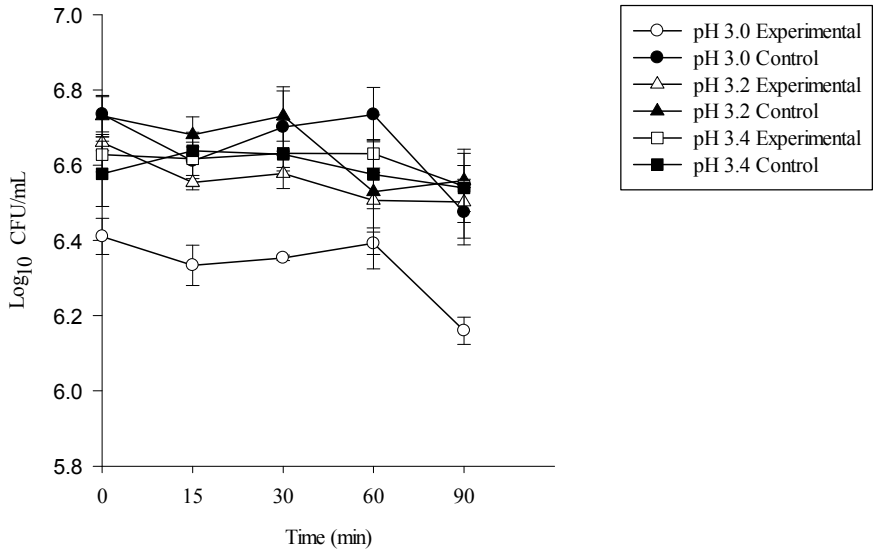

Figure 4.6 Strain CE81 (*S.cerevisiae*). Effect of sulfur dioxide at three different pH levels. Each data represents a mean of three replicates and error bars represent standard error.

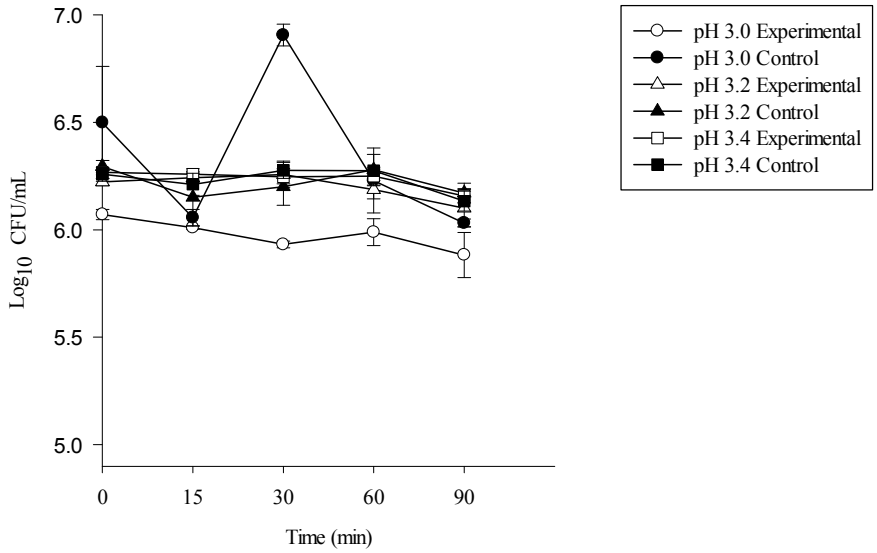

Figure 4.7 Strain CE9 (*S.cerevisiae*). Effect of sulfur dioxide at three different pH levels. Each data represents a mean of three replicates and error bars represent standard error.

regardless of the concentrations used (0 or 150 mg/L) when compared among the three different pH levels (P=0.522). On the other hand, the other two *S. cerevisiae* strains, CE81 and CE9, were only reduced 0.2 and 0.1 $\log_{10}$ units at a pH of 3.0, respectively (Figures 4.6 and 4.7). The initial number of yeast cells for CE81 were 6.41, 6.66 and 6.62 $\log_{10}$ at a pH of 3.0, 3.2 and 3.4 respectively. The initial number of yeast cells for CE9 were 6.07, 6.22 and 6.26 $\log_{10}$ at a pH of 3.0, 3.2 and 3.4, respectively. The remaining pH 3.2 and 3.4 treatments had very small reductions ranging from 0.1-0.03 for both strains. In fact, the statistical analysis for CE81 and CE9 using 150 mg/L of SO_2 showed no significant difference (P=0.951 and P=0.664, respectively) when compared to their respective controls in any of the pH levels tested. Likewise, no significant differences were found for both strains (CE81 and CE9) when three different pH were compared using either 150 or 0 mg/L of SO_2 (P=0.738 and P=0.646, respectively). In fact, the effect of the sanitizer was expected to be negligible. In reality, *S. cerevisiae* strains could be considered as

controls since they are generally recognized as being sulfite-resistant yeasts. Among the strains of *S. cerevisiae*, differences in resistance have been attributed to the production of certain compounds, particularly acetaldehyde, that bind sulfite to form α-hydroxysul-phonates (Pilkington and Rose 1988). Indeed, *S. cerevisiae* strains produce relatively high levels of acetaldehyde, from 50 to 120 mg/l (Romano et al. 1994). However, the study of the production of sulfite-binding compounds is beyond the purpose of this study.

***In vitro* availability of free sulfur dioxide.** Free and total sulfur dioxide was measured throughout the experiment for all the strains at different times (0, 30 and 90 min) with the aim to monitor how free sulfur dioxide changed over the course of the experiment. The values reflected a wide variability of free sulfur dioxide in solution. As we can see from Table 4.1, at the lowest pH (3.0) the amounts of free sulfur dioxide tend to diminish as the time goes by (for the majority of the strains) which could suggest intracellular accumulation of SO_2 (Duckitt, 2012) or the production of binding compounds to it by the yeasts. Although, we could not suggest any specific mechanism as a response to SO_2 exposure, we know that certain yeast species have cellular and molecular mechanisms that include sulfite reduction, sulfite oxidation, acetaldehyde production, sulfite efflux, etc. that help them to cope with the antimicrobial effect of the sanitizer (Divol et al. 2012). Our results showed fluctuations in the free SO_2 values throughout the different pH used. However, as suggested by Duckitt (2012), this could be related to how the yeast is coping with sulfur dioxide when it is present. Furthermore, we observed few values higher than the initial amount of SO_2 added (150 mg/L), and we think this is mostly due to inaccuracies of the Ripper method and the availability of free SO_2 as the pH changes.

Table 4.1 Free and Total SO_2 levels (Ripper method)

		Experimental			Control		
STRAIN		Time (min)	Free SO_2 (mg/L)	Total SO_2 (mg/L)	Time (min)	Free SO_2 (mg/L)	Total SO_2 (mg/L)
		0	143.36	181.76	0	12.80	12.80
CE 149	pH 3.0	30	134.83	180.91	30	13.65	14.51
B./D. bruxellensis		90	187.73	150.19	90	13.65	14.51
		0	204.80	151.89	0	9.39	11.95
	pH 3.2	30	183.47	190.29	30	9.39	12.80
		90	175.79	190.29	90	11.09	12.80
		0	185.17	192.00	0	12.80	12.80
	pH 3.4	30	197.12	199.68	30	12.80	12.80
		90	182.61	180.05	90	10.24	12.80
CE 261	pH 3.0	0	96.43	134.83	0	12.80	12.80
B./D. bruxellensis		30	153.60	136.53	30	12.80	12.80

Table 4.1 (Continued)

		90	125.44	107.52	90	12.80	12.80
	pH 3.2	0	132.27	132.27	0	10.24	12.80
		30	121.17	145.07	30	12.80	12.80
		90	113.49	137.39	90	12.80	12.80
	pH 3.4	0	136.53	157.01	0	11.09	12.80
		30	153.60	89.60	30	12.80	12.80
		90	150.19	307.20	90	11.95	12.80
2080	pH 3.0	0	138.24	139.09	0	6.83	7.68
B./D. bruxellensis		30	131.41	156.16	30	7.68	7.68
		90	112.64	182.61	90	12.80	12.80
	pH 3.2	0	118.61	164.69	0	12.80	14.51
		30	151.89	163.84	30	12.80	12.80
		90	145.92	148.48	90	12.80	13.65

Table 4.1 (Continued)

	pH 3.4	0	160.43	160.43	0	17.92	13.65
		30	132.27	152.75	30	14.51	14.51
		90	136.53	170.67	90	17.07	15.36
CE 78	pH 3.0	0	133.97	154.45	0	7.68	10.24
S. cerevisiae		30	86.19	131.41	30	7.68	6.83
		90	122.03	135.68	90	8.53	7.68
	pH 3.2	0	97.28	118.61	0	8.53	8.53
		30	250.03	99.84	30	7.68	7.68
		90	131.41	170.67	90	7.68	7.68
	pH 3.4	0	162.99	164.69	0	7.68	7.68
		30	162.13	174.08	30	6.83	7.68
		90	177.49	144.21	90	7.68	7.68
CE 81	pH 3.0	0	119.47	133.97	0	8.53	7.68
S. cerevisiae		30	208.21	151.04	30	7.68	10.24

Table 4.1 (Continued)

		90	136.53	130.56	90	7.68	8.53
	pH 3.2	0	128.00	117.76	0	6.83	6.83
		30	162.99	126.29	30	7.68	7.68
		90	225.28	159.57	90	9.39	10.24
	pH 3.4	0	127.15	238.93	0	7.68	8.53
		30	155.31	221.01	30	7.68	7.68
		90	175.79	136.53	90	7.68	7.68
CE 9	pH 3.0	0	107.52	114.35	0	7.68	8.53
S. cerevisiae		30	118.61	169.81	30	8.53	12.80
		90	99.84	204.80	90	8.53	7.68
	pH 3.2	0	156.16	127.15	0	7.68	7.68
		30	156.16	147.63	30	7.68	7.68
		90	111.79	198.83	90	6.83	7.68
	pH 3.4	0	135.68	171.52	0	7.68	7.68

Table 4.1 (Continued)

		30	114.35	174.93	30	7.68	7.68
		90	111.79	250.03	90	7.68	7.68
4A1	pH 3.0	0	141.65	162.99	0	7.68	7.68
Z. bailii		30	132.27	153.60	30	7.68	7.68
		90	121.17	163.84	90	7.68	7.68
	pH 3.2	0	219.31	147.63	0	7.68	7.68
		30	262.83	149.33	30	6.83	7.68
		90	140.80	128.00	90	7.68	8.53
	pH 3.4	0	165.55	174.93	0	7.68	7.68
		30	160.43	156.16	30	7.68	7.68
		90	153.60	181.76	90	7.68	7.68

This table shows the values of free and total SO_2 found throughout the *in vitro* experiment (Ripper method).

Our results showed that the lowest value of free sulfur dioxide for *S. cerevisiae* strains was 86.19 mg/L at a pH of 3.0 for strain CE78, and the highest value was 250.03 mg/L at a pH of 3.2 for strain CE78 (Table 4.1). For *Z bailii* (4A1), the lowest value found was 121.17 mg/L at a pH of 3.0, and the highest value was 262.83 mg/L at a pH of 3.2 (Table 4.1). For *B./D. bruxellensis* strains, the lowest sulfur dioxide value was 96.43 mg/L at a pH of 3.0 for CE261, and 204.80 mg/L at a pH 3.2 of CE149.

Reduction of *Brettanomyces* and general yeast populations using sulfur discs. The six-week treatments were shown to be highly effective in decreasing both *Brettanomyces* and general yeast populations, since no colonies where found after filtration of post-treatment liquid samples (see Table 4.2 and 4.3 respectively). The exceptions were only two barrels, where the percentage of elimination was 94.73% for the analysis of *Brettanomyces* populations, and 98.71% for general yeast populations. When the three-week treatment was used, non-detectable levels of *Brettanomyces* populations was found. However, in the case of general yeast populations, there were two barrels where the percentage of elimination was 37.31% and 87.87%, respectively, versus 100% for the other barrels in that same group. This can be attributable to an incomplete burning of the sulfur discs, since the data showed that for other barrels with higher microbial loads (up to 10^3 CFU/100mL was the highest number found), a 100 % elimination or non-detectable levels of microorganisms was achieved. This suggests that sulfur discs of 5 g were sufficient to sanitize the barrels. A statistical analysis was performed using a Fisher's exact test where the response in terms of reduction was reduced or not; no statistical differences were found between the three and six week

Table 4.2 *Brettanomyces* yeast populations

Treatment	Barrel	CFU/ 100ML INITIAL	CFU/100ML INITIAL Scientific Notation	CFU/ 100ML FINAL	CFU/100ML FINAL Scientific Notation	REDUCTION %	TIME
SO_2	4A1F124975	104500000	1.05E+08	8	8.00E+00	99.999	3 WEEKS
SO_2	4A1F124976	ND[a]	ND[a]	ND[a]	ND[a]	-	3 WEEKS
SO_2	4A1M124749	13200	1.32E+04	0	0.00E+00	100	3 WEEKS
SO_2	4A1M124750	1600	1.60E+03	0	0.00E+00	100	3 WEEKS
SO_2	4A1F124996	80	8.00E+01	0	0.00E+00	100	3 WEEKS
SO_2	4A1F124995	ND[a]	ND[a]	ND[a]	ND[a]	-	3 WEEKS
SO_2	4ALL119280	4000	4.00E+03	0	0.00E+00	100	3 WEEKS
SO_2	4ALL119281	3	3.00E+00	0	0.00E+00	100	3 WEEKS
SO_2	4AD9120823	6	6.00E+00	0	0.00E+00	100	3 WEEKS
SO_2	4AD9120824	ND[a]	ND[a]	ND[a]	ND[a]	-	3 WEEKS
SO_2	327439	ND[a]	ND[a]	ND[a]	ND[a]	-	6 WEEKS
SO_2	327428	320	3.20E+02	0	0.00E+00	100	6 WEEKS

Table 4.2 (Continued)

SO_2	335861	ND[a]	ND[a]	ND[a]	ND[a]	-	6 WEEKS
SO_2	335810	11	1.10E+01	0	0.00E+00	100	6 WEEKS
SO_2	328480	80	8.00E+01	0	0.00E+00	100	6 WEEKS
SO_2	328481	8	8.00E+00	0	0.00E+00	100	6 WEEKS
SO_2	327545	ND[a]	ND[a]	ND[a]	ND[a]	-	6 WEEKS
SO_2	S080227T	81	8.10E+01	0	0.00E+00	100	6 WEEKS
SO_2	327555	38	3.80E+01	2	2.00E+00	94.736*	6 WEEKS
SO_2	327556	160	1.60E+02	0	0.00E+00	100	6 WEEKS

[a]ND No detected

Table 4.3 General yeast populations

Treatment	Barrel	CFU/ 100ML INITIAL	CFU/100ML INITIAL Scientific Notation	CFU/ 100ML FINAL	CFU/100ML FINAL Scientific Notation	REDUCTION %	TIME
SO_2	4A1F124975	1600	1.60E+03	0	0.00E+00	100	3 WEEKS
SO_2	4A1F124976	80	8.00E+01	0	0.00E+00	100	3 WEEKS
SO_2	4A1M124749	80	8.00E+01	0	0.00E+00	100	3 WEEKS
SO_2	4A1M124750	1600	1.60E+03	4	4.00E+00	99.75	3 WEEKS
SO_2	4A1F124996	160	1.60E+02	0	0.00E+00	100	3 WEEKS
SO_2	4A1F124995	268	2.68E+02	33	3.30E+01	87.87*	3 WEEKS
SO_2	4ALL119280	2667	2.67E+03	0	0.00E+00	100	3 WEEKS
SO_2	4ALL119281	67	6.70E+01	42	4.20E+01	37.31*	3 WEEKS
SO_2	4AD9120823	77	7.70E+01	0	0.00E+00	100	3 WEEKS
SO_2	4AD9120824	ND[a]	ND[a]	ND[a]	ND[a]	-	3 WEEKS
SO_2	327439	445	4.45E+02	0	0.00E+00	100	6 WEEKS
SO_2	327428	160	1.60E+02	0	0.00E+00	100	6 WEEKS

Table 4.3 (Continued)

SO_2	335861	ND[a]	ND[a]	ND[a]	ND[a]	-	6 WEEKS
SO_2	335810	146	1.46E+02	0	0.00E+00	100	6 WEEKS
SO_2	328480	264	2.64E+02	0	0.00E+00	100	6 WEEKS
SO_2	328481	78	7.80E+01	1	1.00E+00	98.71	6 WEEKS
SO_2	327545	ND[a]	ND[a]	ND[a]	ND[a]	-	6 WEEKS
SO_2	S080227T	ND[a]	ND[a]	ND[a]	ND[a]	-	6 WEEKS
SO_2	327555	320	3.20E+02	0	0.00E+00	100	6 WEEKS
SO_2	327556	4000	4.00E+03	0	0.00E+00	100	6 WEEKS

[a]ND No detected

treatment for both *Brettanomyces* and general yeast populations. This suggests that the levels of disinfection are the same in three or six week treatment times. Since there was not enough variability in the response, further statistical analysis was not attempted. In any case, if superficial elimination needs to be pursued, sulfur dioxide rings used and held for 3 and 6 weeks are sufficient to decrease the microbial loads at non-detectable levels. However, some issues still need to be taken into consideration, such as the environment inside of the barrel that cannot be controlled because once the disc is burned, the bung hole must be immediately closed and no more intervention can be applied. Several variables can influence the effectiveness when the microbial elimination is taken into consideration i.e.: the amount of oxygen that needs to be consumed inside of the barrel, the initial microbial load, presence of debris or residues of organic matter, and the quantity of the sulfur disc burned. As an example, the barrel that showed the lowest microbial reduction in the three-week treatment (37.31%) (Table 4.3), the weight of the sulfur disc after the treatment was 2.327 g, and the initial weight was 5.789 g. It is important to note that the lowest weight found after burning discs was 0.123 g (data not shown) versus 2.327 g for the barrel that had the lowest microbial elimination. Unfortunately, a total recovery of ashes from all our barrels was not possible. However, this finding corroborates the importance of the sulfur disc combustion, theoretically burning 1 g of sulfur, 2 g of sulfur dioxide are formed, but only a portion of the sulfur dioxide is absorbed, while the rest escapes (Farkas, 1988). The combustion reaction is influenced by many factors, e.g., the relative humidity and/or temperature in the cellar may provoke physical alterations of the barrels causing leaking of the gas, etc. Moreover, non-stoichiometric reactions might have occurred and variable production of SO_2, or losses of SO_2 from the barrel could have occurred. Furthermore, the sulfur discs by the manufacturer had a stated weight of 5 g, but the

weights were highly variable, and all of them weighed more than 5g (Table 4.4). In this regard, Bursen (2010) highlighted that there is nothing in the literature suggesting the negatives of slightly higher use. However, there is a certain heterogeneity in the quantity of sulfur dioxide produced by the combustion of the same weight wick or ring (discs) according to their preparation conditions or storage (fixation of humidity). However, rings are more sensitive to their external environment i.e. (moisture) (Ribereau Gayon et al. 2006).

Wood core isolation of *Brettanomyces*: post-treatment. The efficacy of the treatment of sulfur dioxide in barrels was studied at the surface level (0 mm) with the liquid samples taken after sanitation treatment (Table 4.2), and at a depth of 8 mm using core extraction, since this depth has been found to be the level of wine penetration (Malfeito Ferreira et al. 2004). Since *Brettanomyces* is more prone to be found at the bottom of a barrel (bilge) and also at the top of the barrels due to higher levels of oxygen that stimulate its growth (Van de Water, 2010), the cores were strategically taken from bilge and heads (upper head and lower head). Our results showed that at a depth of 8 mm, the barrels with the numbers 4A1M124750 and 4ALL119281 were positive for *Brettanomyces* growth in the locations of head down for 4A1M124750, and bilge for the barrel 4ALL119281. This was for post-treatment since the cores extracted before treatment were negative for *Brettanomyces*. Both barrels corresponded to the three-week treatment, where all the barrels had a 100% elimination of *Brettanomyces*

Table 4.4 Initial weight of sulfur discs (rings)

BARREL	WEIGHT (g)	TREATMENT
4AIF124976	6.138	SO_2
4AIF124975	5.403	SO_2
4AD9120823	5.944	SO_2
4ALL119280	5.594	SO_2
4AIM124750	5.605	SO_2
4ALL119281	5.789	SO_2
4AIF124996	5.347	SO_2
4AD9120824	5.862	SO_2
4AIF124995	5.626	SO_2
4AIM124749	5.704	SO_2
327439	6.05	SO_2
327428	5.523	SO_2

Table 4.4 (Continued)

335861	6.262	SO_2
335810	5.404	SO_2
328480	5.611	SO_2
328481	5.692	SO_2
327545	5.66	SO_2
S080227T	5.588	SO_2
327555	5.902	SO_2
327556	5.483	SO_2

This table shows the weight of the sulfur discs before being burned.

at a surface level (0 mm) based on the liquid sample microbial isolation. On the other hand, none of the cores (pre- or post-treatment) for the 6 week treatment barrels were positive for the growth of *Brettanomyces* at the 8 mm depth.

CONCLUSIONS

In this study, we considered the use of a non-interfering matrix an important variable to prove the maximum efficacy of sulfur dioxide, whether in solution and/or the headspace of a 225 L barrel. The highly variable results found in the literature made it difficult to precisely define an optimal protocol for the use of sulfur dioxide as a sanitizer of surfaces in wineries. Its use as a wine preservative in many different studies involving an array of experimental conditions has led to generalization of results that may not be applicable to specific sanitation purposes. When used in solution, sulfur dioxide provided relative efficacy using the strains and initial microbial concentration levels for this study. The fact of substantial importance for winemakers, is that when sulfur dioxide is to be used in the liquid form, the pH must be sufficiently low to favor the production of the germicidal form of SO_2. However, under laboratory conditions, sulfur dioxide had no extraneous conditions or variables to influence the germicidal action, versus the ones encountered in the barrel trials. This should be considered when sanitation protocols are going to be developed. The lack of a systematic approach to evaluating sulfur dioxide in common protocols of winery sanitation has resulted in the generalized use of certain protocols, that might not be applicable to all the surfaces in wineries i.e. barrels, equipment, etc. High microbial loads were used under laboratory conditions, representing the worst case scenario of barrel contamination. However, when we extrapolated this same approach under winery conditions, we realized that the

use of sulfur dioxide as a gas, although effective, it was only challenged with up to 10^3 CFU/ml for general yeast populations and up to 10^8 CFU/ml for *Brettanomyces* populations. This fact should be taken into consideration by winemakers since microbial loads in naturally contaminated barrels may vary according to their previous sanitation practices. The barrels used here, although always sanitized by the wineries that donated them, presented a residual contamination, factors necessary to evaluate current sanitation practices.

Winemakers in general must keep in mind that prevention is more effective than correction. Pre-sanitation practices (rinsing at the right temperatures to avoid thermally adhering debris on the surface) and the correct concentration of sulfur dioxide that is effective for the surface to be sanitized, is the best approach to adopt when optimal sanitation is being attempted. The cost of water that pre-rinses consume, should be taken into consideration by the wineries as additional expense. However, pre-rinses and thorough cleaning are crucial to achieve optimal disinfection. In general, winemakers and the wine industry should consider the re-assessment of their practices, in terms of the use of scientifically validated information and utilize it to protect their wine from future contamination.

ACKNOWLEDGMENTS

This research project was funded by CONACYT-Mexico, New York State Agricultural Experiment Station Federal Formula Funds, and Cornell University Research Travel Awards. The authors thank Constellation Brands, California, USA, for the donation of barrels and resources to conduct the *in vivo* trials.

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CHAPTER 5

THERMAL INACTIVATION OF WINE SPOILAGE YEASTS TO VALIDATE STEAM SANITATION PROTOCOLS IN WINERIES

Short version of title Thermal inactivation of wine spoilage yeasts

ABSTRACT

The time (D) required to kill 90% of a target microorganism and the temperature (z) required to reduce D by one log cycle varied according to the microorganism and medium inhabited by the microbial contaminant. Three genera of wine spoilage yeasts -*Dekkera*/*Brettanomyces bruxellensis*, *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii*- were used for this study. Values for D and z were determined to guide thermal treatments of wine cooperage. A maximum log reduction of 3.2 at 50°C in *Dekkera/Brettanomyces bruxellensis* populations was observed. The highest temperature after which *Brettanomyces* was detected was 55°C. Temperatures of 45ºC, 50ºC, 52.5ºC and 55ºC were used to determine values of D and z for these strains. Strains of *Saccharomyces cerevisiae* were investigated at 45ºC, 50ºC, 52.5ºC, 55ºC, 57.5ºC and 60ºC, and were reduced by a maximum of 4 log units at 50ºC. Finally, survival of *Zygosaccharomyces bailii* was studied at 50ºC, 55ºC and 57.5ºC, and was reduced by a maximum of 3.4 log units at 50ºC. Changes in wood temperature at depths of 8 and 14 mm were monitored during steam treatments of barrels naturally contaminated with *Brettanomyces* spp. Duration of steam treatments was

either 5 min or 10 min and the temperatures of 57.5ºC or 42.5ºC were reached after 10 min. at depths of 8 mm or 14 mm, respectively. Wood cores extracted to a depth of 8 mm from barrels exposed to steam treatment for 10 minutes were free of *Brettanomyces* indicating that steam treatments lasting 10 min produced temperatures that were lethal to *Brettanomyces*.

Key words: steam, D and z values, spoilage yeasts, wine, barrels.

INTRODUCTION

Several microbial contaminants appear to survive on walls and other interior surfaces of wineries; including interior surfaces of presses and fermentation tanks, and within wooden barrels (Woolfit et al. 2007). Bulk wines and bottled wines are often spoiled by fermentative species of *Zygosaccharomyces*, *Dekkera* (anamorph *Brettanomyces*), *Saccharomyces* and *Saccharomycodes*. *Dekkera*/*Brettanomyces* is associated with the production of unpleasant mousy and medicinal taints, due to their production of tetrahydropyridines and volatile phenolic substances such as 4-ethylguaiacol and 4-ethyl phenol (Fleet 2003). *D. bruxellensis*, *Z. bailii*, and *S. cerevisiae* are spoilage yeasts *sensu stricto*. However, *S. cerevisiae* appears to be more problematic than indicated by the above mentioned authors, as some strains isolated from dry white wines seem to be more of a potential spoilage yeast than *Z. bailii*, due to its sorbic acid and sulfite tolerance at high ethanol levels (Loureiro and Malfeito Ferreira 2003). Management of these types of spoilage is generally done by following good manufacturing practices and hygiene in the winery (Fleet 2003). Wine cooperage, although useful for wine ageing has some disadvantages when sanitation needs to be performed, since the micro-porous structure of wood purportedly allows penetration of

microorganisms into the wood to depths that makes their subsequent eradication a challenge, thereby increasing the risk wine spoilage when cooperage is reused. Development of undesirable microbiota within cooperage may significantly degrade wine, rendering both the wine and barrels, unusable (González Arenzana et al. 2013). Certain spoilage microorganisms are able to survive within barrels even under "starvation" conditions. *Brettanomyces* has been reported to survive within the porous wood structures of barrels that have not been filled with wine in weeks or months (M. de L. A. Aguilar Solis, unpublished data, 2012). While methods of sanitation used in wineries are effective on surfaces such as stainless steel, plastic, and glass; sterilization of wood surfaces has proven more difficult. Steam sanitation of wood is a standard method used for wine cooperage. However, the temperatures required to inactivate spoilage microorganisms, and the times required to reach these temperatures within the wood are poorly understood. Heat and mass transfer in capillary porous materials such as wood, has been discussed by Younsi et al. (2006), particularly in regard to thermal conductivity of wood materials and development of thermal inactivation regimes. Wood is a natural polymer of complex chemical composition and microstructure (Qing-Xian 2001). Its hygroscopic and porous medium result in heat transfer by conduction and convection, as well as radiation (Khattabi and Steinhagen 1993). Various methods of heat sterilization of wood are currently under investigation as a means of killing exotic insects or pathogens within imported goods. An important factor in thermal inactivation, is the amount of time (D) required to heat wood of various cross-sectional sizes and configurations to a temperature that will kill 90% of the insects or pathogens (Simpson 2001, Narang 2004). The value of D is then used as a predictor for responses beyond the data to estimate the time required for disinfection (10^{-3} CFU/mL) or sterilization (10^{-6} CFU/mL). The underlying assumption when utilizing this measure

is that the relationship between the $\log_{10}$ number of survivors and time is linear (Sutton et al. 1991). If the logarithms of the D values obtained at various temperatures are plotted against temperature, and the best straight line is drawn through the points, the reciprocal of the slope of this line is the value of z: the number of degrees by which the temperature has to be raised or lowered to bring about 90% reduction or tenfold increase in D (Narang 2004). In our research, steam treatment in barrels was used as a validation method after having obtained the D and z values for different species of spoilage yeasts. The validation method was achieved in naturally contaminated barrels where we measured the temperatures reached at different times and depths of the staves. The understanding of temperature changes and times needed to achieve the sanitation as depth is increased, together with D and z values, will be useful to understand what are the more necessary parameters to be used when steam is the preferred sanitation method for wine cooperage.

MATERIALS AND METHODS

Strain selection. *Brettanomyces/Dekkera bruxellensis* isolates (CE261, CE149). The isolates were obtained one by donation and the other one from the Department of Food Science collection at Cornell University.

Saccharomyces cerevisiae isolates (CE81, CE9, and CE78). Three isolates were obtained from the Department of Food Science collection at Cornell University.

Zygosaccharomyces bailii isolates (4A1). One isolate was obtained from the Department of Food Science collection at Cornell University.

Preparation of starter culture and inoculation. The yeasts were stored at -80°C in glycerol 15% (w/v) and revitalized and maintained on YPD agar (Yeast Extract 10 g/L, Peptone 20g/L, Dextrose

20g/L, Agar 15 g/L; Difco™; Sparks, MD, USA). All strains were grown until stationary phase (200 rpm, 30°C). The growth time varied according to the strain (previous data not shown of growth curve of each one of them). Each strain was grown in YPD broth (Yeast Extract 10 g/L, Peptone 20g/L, Dextrose 20g/L) (Difco™; Sparks, MD, USA). Once the cultures reached the stationary phase, the target inocula were injected in sterile glass capillary tubes and groups of five tubes were sealed with a direct flame and put into tubes with water already tempered in a water bath at the temperatures used for this study. The capillary tubes were removed at different times and put into tubes with ethanol (70%) to decontaminate the exterior surface and then left in ice until proper dilutions to detect the residual microbial activity were performed. The dilutions were plated in YPD agar (Yeast Extract 10 g/L, Peptone 20g/L, Dextrose 20g/L, Agar 15 g/L) (Difco™; Sparks, MD, USA) and incubated at 30°C for 48 to 72 hr for *Zygosaccharomyces bailii* and *Saccharomyces cerevisiae* and up to 3 to 4 weeks for *Brettanomyces/Dekkera bruxellensis.*

Microbiological enumeration. Plates were enumerated for total microbial count. The counts were averaged and expressed as log numbers. The log reduction was then calculated for each strain and expressed as log numbers. Each experiment was performed until the best linear correlation coefficients were obtained (r^2= 0.9).

Steam treatment of barrels. Twenty barrels naturally contaminated with both *Brettanomyces bruxellensis* and general yeast populations, were split in two groups of ten barrels each and treated with steam 5 and 10 min, respectively. Briefly, the 20 barrels were added with 7 L of distilled

water before the steam treatment. The barrels were rolled several times to enhance the contact of water with the inner surface of the barrel, and then stored bung side up for 24 hr and then sampled. Afterwards, the steam treatment was achieved in a 4 cabinet barrel washer (TomBeard Santa Rosa California, USA) using a steam generator (ARS Enterprises Santa Fe Springs California, USA) and a pressure of 70 psi. The treatment was as follows: Pre-rinsing for 30 sec (cold rinsing) at a temperature of 15.5 ºC, then 5 or 10 min of steam treatment, bung hole steaming for 5 min, cold rinsing for 30 sec at 15.5 ºC. The temperature that was reached inside of the staves of these barrels was monitored using four probes (type "T") at two different depths (17 mm and 11 mm from the outside/ being the equivalent 8 and 14 mm from the inside) and a USB data logger thermometer (Omega; Stamford, Connecticut, USA) that measured the temperature at 1 second intervals, until five min or ten min steam treatments were completed. After the steam treatment, the remaining water that was generated from the steam treatment was collected and placed in sterile bottles for microbiological enumeration. The bung hole was sprayed with 70% ethanol (before and after treatment) and the samples were taken and placed at 4°C until analysis was performed. The samples were analyzed to determine the initial and final *Brettanomyces* and general yeast populations, either by filtration (EZ-Fit™ Manifold for universal laboratory filtration; Concord Road Billerica, MA USA) using discs of 0.22 µm and/or pertinent dilutions of the samples, since the microbial loads differed for each barrel. If samples required dilution, 0.1% (w/v) buffered peptone water (Hardy Diagnostics; Santa Maria CA, USA) was used.

For the filtration method, 0.22 µm nitrocellulose membranes (GE* Nitrocellulose-Mixed Esters of Cellulose Membrane Filters; Pittsburg, PA, USA) were used and the samples were filtered twice and the results were averaged. The maximum volume filtered was 100 mL and the results were

calculated as CFU/100 mL and then transformed in percentage to homogenize the results since not all the barrels had initially the same microbial load. Expressing the results in percentage made the interpretation easier. The membranes were placed onto WL and YPD agar using sterile forceps. WL agar (yeast extract 4g/L, tryptone 5g/L, glucose 50 g/L, potassium dihydrogen phosphate 0.55 g/L, potassium chloride 0.425 g/L, calcium chloride 0.125 g/L, magnesium sulphate 0.125 g/L, ferric chloride 0.0025 g/L, manganese sulphate 0.0025 g/L, bromocresol green 0.022 g/L, agar 15 g/L) (Oxoid, LTD; Basingstoke Hampshire, England) was used to detect *B./D. bruxellensis* and was incubated at 30°C for up to 3 to 4 weeks. WL agar containing 10 mg/L cycloheximide (Sigma Aldrich; St. Louis, MO, USA) for the selection of *B./D. bruxellensis* (dissolved in 50% ethanol and filter sterilized), 150 mg/L of biphenyl (Acros Organics; Fair Lawn, New Jersey, USA) (dissolved in ethanol and filter sterilized) to prevent the growth of mold, 30 mg/L of chloramphenicol (MP Biomedicals LLC; Solon, OH, USA) (dissolved in 100% ethanol) to prevent the growth of lactic acid bacteria, and 25 mg/L of kanamycin sulfate (AMRESCO; Solon, OH, USA) (dissolved in sterile distilled H_2O) to prevent the growth of acetic acid bacteria. YPD agar (yeast extract 10 g/L, peptone 20g/L, dextrose 20g/L, agar 15 g/L) (Difco™; Sparks, MD, USA) was used to detect general yeast populations and was incubated at 30°C for 48 to 72 hr. YPD agar was supplemented with 150 mg/L of biphenyl (Acros Organics; Fair Lawn, New Jersey, USA; dissolved in ethanol and filter sterilized) to prevent the growth of mold, 30 mg/L of chloramphenicol (MP Biomedicals LLC; Solon, OH, USA; dissolved in 100% ethanol) to prevent the growth of lactic acid bacteria, and 25 mg/L of kanamycin sulfate (AMRESCO; Solon, OH, USA; dissolved in sterile distilled H_2O) to prevent the growth of acetic acid bacteria.

Extraction of wood cores and isolation of *Brettanomyces*. Wood cores were extracted from each barrel before and after sanitation treatment using a borer attached to a drill. This borer penetrated up to 17 mm from the outside surface of the barrel, reaching 8 mm from the inside of the barrel (total standard thickness of a stave is 25 mm). These cores were cut at a depth of 8 mm, quickly flamed using 70% ethanol and placed in liquid YPD media (yeast extract 10 g/L, peptone 20g/L, dextrose 20g/L) (Difco™; Sparks, MD, USA) at 200 rpm and 30ºC until visual growth was observed. The zones where the cores were extracted from the barrels were the bilge and the head (whether upper or lower part of the head). After growth was observed in liquid culture, a loop of the sample was streaked on WL agar and isolated colonies with the typical morphology of *Brettanomyces* were selected. The colonies were re-streaked up to 7 times for purification purposes and due to *Brettanomyces* isolates from barrels were observed to present a wrinkled and dusty morphology that eventually changed over time to a smooth morphology.

Statistical analysis. For the *in vitro* experiments, D values were calculated as the negative reciprocal slope of the linear regression of survivor curves obtained by plotting logarithms of the survival counts versus time (min). Z values were calculated using the negative reciprocal slope of the linear regression from the plots of the D values versus temperatures. Only linear correlation coefficients of ≥ 0.9 were used ($r^2=0.9$).

For the reduction of *Brettanomyces* and general yeast populations in naturally contaminated barrels using steam, a Fisher's exact test was performed in order to see if the two study groups (5 or 10 min) differ in the proportions of presence or absence of microorganisms. Statistical analyses were conducted using SigmaPlot 12.0; Systat Software Inc., San Jose CA.

RESULTS AND DISCUSSION

The vegetative cells of yeasts possess low heat resistance. The medium or food in which the vegetative cells are heated has a marked effect on their heat resistance. Sugars provide protection, as do sodium chloride and citric acid (Splittstoesser 1986). Three different genera of common wine spoilage yeasts were used for this study, where thermal inactivation was achieved in hot water at different temperatures. Two strains of *D./B. bruxellensis* were challenged at different inactivation temperatures. The highest temperature that permitted the survival of these yeasts was 55ºC (131ºF) for CE261 (Figure 5.1) and 52.5ºC (126.5ºF) for CE149 (Figure 5.2) (Table 5.1). The lowest temperature that was capable of inactivating both yeast strains was 45ºC (113ºF). With regards the *S. cerevisiae* strains used in this study, the highest temperature that permitted survival was 60ºC (140ºF) by strain CE78 (Figure 5.3).

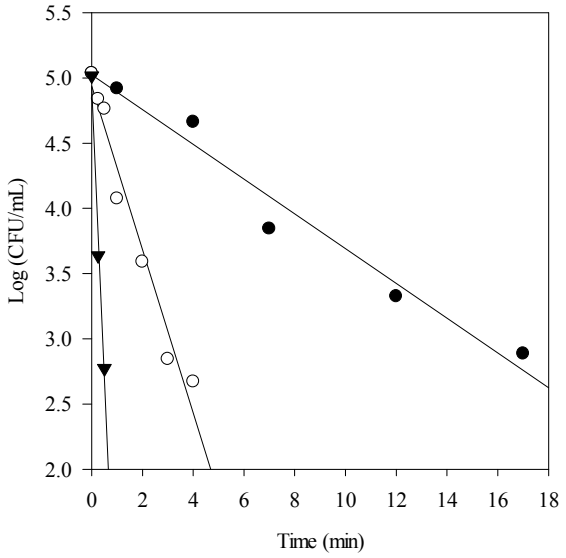

Figure 5.1 Thermal Inactivation of *B./D. bruxellensis* (CE261) at 45 ●, 50 ○ and 55 ▼ºC.

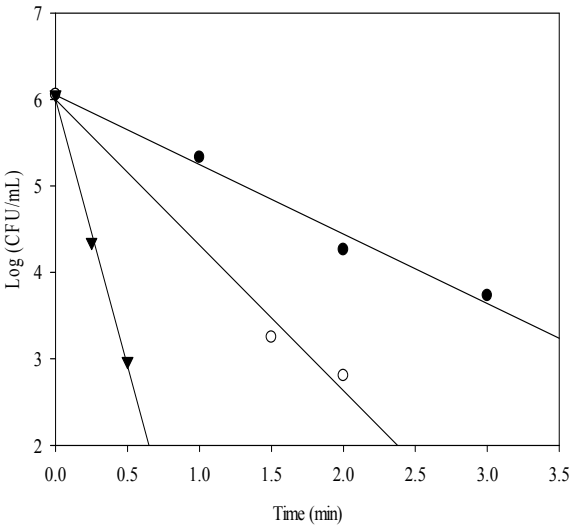

Figure 5.2 Thermal Inactivation of *B./D. bruxellensis* (CE149) at 45 ●, 50 ○ and 52.5 ▼ºC.

Table 5.1 D and z values determined in hot water at different temperatures

Strain		T(°C)	T(°F)	D (min)	r^2	Log Reduction	z (°C)	z (°F)
Z. bailii	4A1	50	122	14.26	0.99	3.40	4.79	8.62
		55	131	3.52	0.95	2.41		
		57.5	135.5	0.30	0.95	3.22		
S. cerevisiae	CE78	55	131	3.22	0.97	2.42	4.97	8.94
		57.5	135.5	1.24	0.99	2.38		
		60	140	0.32	0.95	3.91		
S. cerevisiae	CE81	50	122	15.82	0.96	1.51	4.21	7.58
		55	131	1.30	0.97	3.56		
		57.5	135.5	0.25	0.95	3.62		
S. cerevisiae	CE9	45	113	3.27	1.00	0.30	54.20	97.56
		50	122	14.00	0.98	4.00		
		52.5	126.5	1.57	0.98	2.68		
B./D. bruxellensis	CE261	45	113	7.51	0.97	2.15	6.55	11.79

Table 5.1 (Continued)								
		50	122	1.59	0.96	2.37		
		55	131	0.22	0.98	2.24		
B./D. bruxellensis	CE149	45	113	1.24	0.99	2.32	9.07	16.32
		50	122	0.59	0.99	3.25		
		52.5	126.5	0.16	1.00	3.08		

This table represents the D and z values for all the strains at different temperatures.

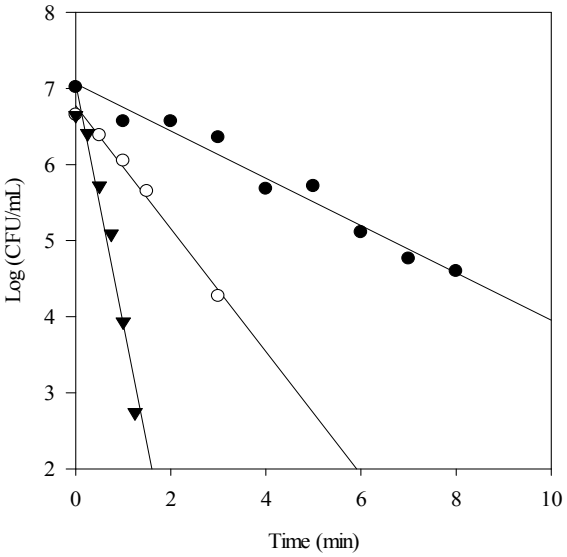

Figure 5.3 Thermal Inactivation of *S. cerevisiae* (CE78) at 55 ●, 57.5 ○ and 60 ▼ºC.

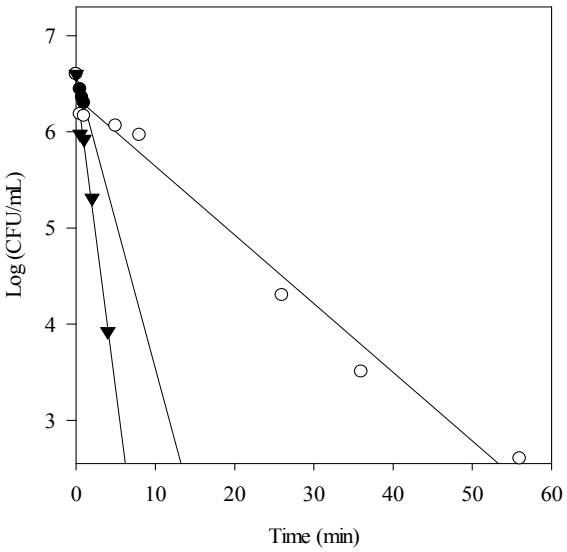

Figure 5.4 Thermal Inactivation of *S. cerevisiae* (CE9) at 45 ●, 50 ○ and 52.5 ▼ºC.

The lowest temperature resulting in a reduction was 45ºC (113ºF) for strain CE9 (Figure 5.4) (Table 5.1). For strain CE81 (Figure 5.5), the highest temperature permitting survival was

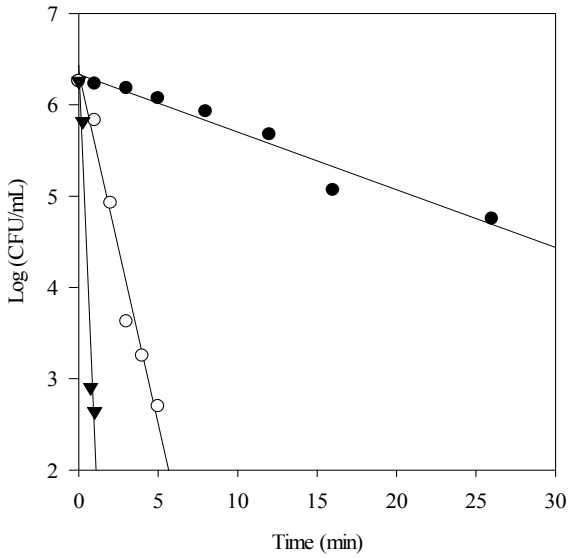

Figure 5.5 Thermal Inactivation of *S. cerevisiae* (CE81) at 50 ●, 55 ○ and 57.5 ▼ºC.

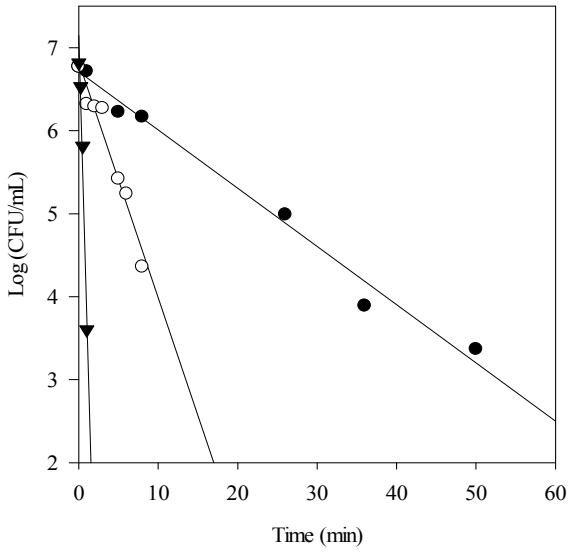

Figure 5.6 Thermal Inactivation of *Z. bailii* (4A1) at 50 ●, 55 ○ and 57.5 ▼ºC.

57.5ºC (135.5ºF). Finally, for *Z. bailli* (4A1) (Figure 5.6), the highest survival temperature was 57.5ºC (135.5ºF) and the lowest was 50ºC (122ºF) (Table 5.1). The lowest and highest temperatures that permitted survival of the strains were useful to validate the steam sanitation

protocols that we achieved in wine cooperage, where steam was applied for 5 and 10 min to two groups of ten barrels each, where the analysis of the presence of *Brettanomyces* and general yeast populations were studied. The statistical analysis showed that for the general yeast populations, the 5 and 10 min treatments were not significantly different than is expected from random occurrence ($P = 1.000$). This means that the proportions of presence or absence of microorganisms are the same in five or ten minute treatments (Table 5.2). Whereas for *Brettanomyces* yeast populations, none of the post-treatment samples were positive, so no statistical analysis was performed, since both 5 and 10 min treatments had no detectable levels of microorganisms (Table 5.3). In other words, there is not sufficient variability in the response to conduct statistical analysis. Alternatively, the internal temperature inside of the staves of these barrels was monitored at two different depths (17 mm and 11 mm from the outside/ being the equivalent 8 and 14 mm from the inside) and a USB data logger thermometer registered the temperature on 1 sec intervals, until five min or ten min were reached. Once the steam treatment was completed, a bung was immediately placed in the barrel opening to generate a vacuum inside the barrel, and thus the more extraction of debris from the barrel. The analysis of the internal temperature at different depths in the barrel staves revealed that the steam application is non-uniform, and the temperature profiles showed that probes located deeper within the staves reached much lower temperatures than that from the most distal point to the steam source

Table 5.2 General yeast populations

Treatment	Barrel	CFU/ 100ML INITIAL	CFU/100ML INITIAL Scientific Notation	CFU/ 100ML FINAL	CFU/100ML FINAL Scientific Notation	REDUCTION %	TIME (MIN)
Steam	4A1M124743	12750000	1.28E+07	0	0.00E+00	100	10 MIN
Steam	4A1M124744	735	7.35E+02	0	0.00E+00	100	10 MIN
Steam	4ALL 119288	300000	3.00E+05	50	5.00E+01	99.983	10 MIN
Steam	4ALL119289	41500	4.15E+04	0	0.00E+00	100	10 MIN
Steam	4AD9120722	8750	8.75E+03	420	4.20E+02	95.2	10 MIN
Steam	4AD9120723	10950000	1.10E+07	0	0.00E+00	100	10 MIN
Steam	4A1M124753	700000000	7.00E+08	20	2.00E+01	99.999	10 MIN
Steam	4ALL119196	59000000	5.90E+07	0	0.00E+00	100	10 MIN
Steam	4AD9120937	264000	2.64E+05	0	0.00E+00	100	10 MIN

Table 5.2 (Continued)

Steam	4AD9120938	1295000	1.30E+06	0	0.00E+00	100	10 MIN
Steam	4ALL119197	105250	1.05E+05	0	0.00E+00	100	5 MIN
Steam	4A1F124977	38000	3.80E+04	0	0.00E+00	100	5 MIN
Steam	4A1F124978	590000000	5.90E+08	60	6.00E+01	99.999	5 MIN
Steam	4A1M124754	21100	2.11E+04	1600	1.60E+03	92.417	5 MIN
Steam	4A1F124980	17000	1.70E+04	275	2.75E+02	98.38	5 MIN
Steam	4A1F124979	14300	1.43E+04	0	0.00E+00	100	5 MIN
Steam	4ALL119195	80500	8.05E+04	0	0.00E+00	100	5 MIN
Steam	4ALL119194	16000	1.60E+04	150	1.50E+02	99.062	5 MIN
Steam	4A1M124722	190000000	1.90E+08	0	0.00E+00	100	5 MIN
Steam	4A1M124721	101250	1.01E+05	0	0.00E+00	100	5 MIN

This table represents the *in vivo* reduction of general yeast populations in natural contaminated barrels.

Table 5.3 *Brettanomyces* yeast populations

Treatment	Barrel	CFU/ 100ML INITIAL	CFU/100ML INITIAL Scientific Notation	CFU/ 100ML FINAL	CFU/100ML FINAL Scientific Notation	REDUCTION %	TIME (MIN)
Steam	4A1M124743	5100	5.10E+03	0	0.00E+00	100	10 MIN
Steam	4A1M124744	20000	2.00E+04	0	0.00E+00	100	10 MIN
Steam	4ALL 119288	17050	1.71E+04	0	0.00E+00	100	10 MIN
Steam	4ALL119289	1600	1.60E+03	0	0.00E+00	100	10 MIN
Steam	4AD9120722	160	1.60E+02	0	0.00E+00	100	10 MIN
Steam	4AD9120723	18600	1.86E+04	0	0.00E+00	100	10 MIN
Steam	4A1M124753	80	8.00E+01	0	0.00E+00	100	10 MIN
Steam	4AD9120937	180000	1.80E+05	0	0.00E+00	100	10 MIN
Steam	4AD9120938	160	1.60E+02	0	0.00E+00	100	10 MIN
Steam	4ALL119196	6200	6.20E+03	0	0.00E+00	100	10 MIN

Table 5.3 (Continued)

Steam	4A1F124978	8000	8.00E+03	0	0.00E+00	100	5 MIN
Steam	4A1F124977	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]	5 MIN
Steam	4A1M124754	17400	1.74E+04	0	0.00E+00	100	5 MIN
Steam	4A1F124980	12600	1.26E+04	0	0.00E+00	100	5 MIN
Steam	4A1F124979	14200	1.42E+04	0	0.00E+00	100	5 MIN
Steam	4ALL119197	57900	5.79E+04	0	0.00E+00	100	5 MIN
Steam	4ALL119195	19100	1.91E+04	0	0.00E+00	100	5 MIN
Steam	4ALL119194	8300	8.30E+03	0	0.00E+00	100	5 MIN
Steam	4A1M124722	6200000	6.20E+04	0	0.00E+00	100	5 MIN
Steam	4A1M124721	99750	9.98E+04	0	0.00E+00	100	5 MIN

[a] ND Not detected

This table represents the *in vivo* reduction of *Brettanomyces* populations in natural contaminated barrels.

(82˚C) (Fugelsang 2010). In fact, much lower temperatures than the previously one mentioned were registered when less time (5 min treatment) and deeper depths (14 mm) were used. Moreover, even when 10 min of steam treatment was used, none of the temperatures were more than 57.5°C (135.5ºF). However, *in vitro* thermal inactivation studies showed that the highest temperature that permitted the survival of *Brettanomyces/Dekkera* strains was 55ºC (131ºF), with a corresponding D value of 0.22 min and z value of 6.55 ºC (11.79ºF) (Table 5.1). Our results showed that ten minutes of steam treatment was more consistent in reaching lethal temperatures in the interior of the staves, than with 5 minutes of steam treatment. In fact, the highest temperature reached at 8 mm stave depth after 5 min of steam treatment was 47.4ºC (117.32 ºF); however, using 14 mm depth and 5 min of steam treatment resulted in a maximum temperature of 42.4ºC (108.32ºF). Conversely, using 10 minute steam treatment and a depth of 8 mm the highest temperature consistently reached was 57.5°C (135.5ºF). Using 10 min and 14 mm depth, 42.5ºC (108.5ºF) was the highest temperature reached. These findings suggest that a minimum of 10 minute steam treatment is necessary to reach temperatures capable of killing harbored wine spoilage microorganisms at a depth of 8 mm. If this data was extrapolated to that from the *in vitro* studies, the highest temperatures that permitted the survival of the genera studied ranged between 55ºC (131º F) and 60ºC (140ºF) (Table 5.1). Consequently, if we consistently steam treat for 10 min or more, it is possible to consistently reach a temperature of 57.5 ºC (135.5ºF), and this is a sufficient temperature to kill wine spoilage yeasts at a depth of 8 mm, where *Brettanomyces* can harbor (Malfeito Ferreira et al. 2004). This finding is supported by the use of the D and z values obtained from the slope equations obtained with the *in vitro* experiments, that in turn will help us to predict information that might not be plotted in the scatter plot and with the cumulative

lethalities data obtained using reference temperatures of each genera studied in the *in vitro* experiments. The cumulative lethality is the summation of the lethality accumulated during the heating and cooling process (Kabir and Shoukat Choudhury, 2012) that occurred inside of the barrel. Once cooling was started (after 5 or 10 minutes treatment in this case), the lethalities cannot be controlled (Kabir and Shoukat Choudhury, 2012). Therefore, total cumulative lethality is controlled by the heating process and a predicted value of lethality required for the cooling process (Kabir and Shoukat Choudhury, 2012). This was actually calculated using a reference temperature from the D and z values obtained from in the *in vitro* experiments, and the temperatures registered over time using the data from barrels treated 10 minutes with steam. The total cumulative lethalities for the three genera used in the *in vitro* experiments are shown in table 5.4. These values show a range (minimum value-maximum value) of the time in minutes after 10 minutes of steaming the barrels, and these range of values show the necessary minutes to cause lethality after finishing the steam treatment for ten minutes. Thus, even if not all graphs (Figures 5.7-5.16) show more than 10 minutes of steam, the values shown in Table 5.4 can help to predict, the necessary minutes after 10 minutes to cause lethality in the studied wine spoilage yeasts in barrels.

Table 5.4 Total cumulative lethalities (minutes)

Strain	Temperature Probes (8mm depth)	min	max
Z. bailii	Probe 1	0.0031	2.3252
	Probe 2	0.0030	3.4691
S. cerevisiae	Probe 1	0.0013	0.7498
	Probe 2	0.0012	1.1055
B./D. bruxellensis	Probe 1	0.0552	6.9829
	Probe 2	0.0541	9.5244

Table 5.4 shows the calculation results to cause lethality for each genera of yeast studied in the *in vitro* experiments using 10 minutes steam treatment, at 8mm depth and using the highest temperatures (°F) that caused heat inactivation (Table 5.1). This process of lethality determination model was done to provide wine industry with a science-based validation tool that can be used to demonstrate the effectiveness of a specific heat process to destroy a microorganism of concern, in this case wine spoilage yeasts (http://www.meatami.com/). The table also shows 2 ranges because the temperatures were measured by duplicate using two temperatures probes at 8 mm depth.

The temperature at deeper depths, such as 14 mm, were also monitored, however the cumulative lethalities were only studied at 8 mm depth, since this is the maximum isolation depth for *Brettanomyces* in barrels. Another reason to study 14 mm depth was because it is not known if microorganisms could reach that depth, despite of the wine penetration is only 8 mm (Malfeito Ferreira et al. 2004), since other sources of carbon, such as cellobiose that is produced during the toasting of barrels, could be a nutrient source for *Brettanomyces*, and thus survive in the wood regardless of the level of wine penetration (Blomqvist et al. 2010). It is important to recognize that *Brettanomyces* can utilize cellobiose as a carbon source for growth, as well as residual nutrients in dry fermented wines. In addition to the percentage of microbial reduction presented on Table 5.2 and 5.3, graphs showing the temperature change over time at 8 mm depths for 10

minutes steam treatments are shown. Since 10 min steam treatments and 8 mm depths proved to be the most effective parameters to reach temperatures that are able to cause heat inactivation of common spoilage yeasts (Figure 5.7 to Figure 5.16), graphs for 5 minutes steam treatment of the change of temperature over time are not shown due to very low temperatures being achieved.

Moreover, the understanding of these findings should also be referred to a more theoretical aspect, since the thermal conductivity of wood is affected by a number of basic factors that include wood density, moisture content, extractive content, grain direction, structural irregularities (checks and knots), fibril angle, and temperature (Simpson and TenWolde 2007). In fact, thermal conductivity increases as density, moisture content, temperature, or extractive content of the wood increases. Furthermore, conductivity along the grain has been reported as 1.5 to 2.8 times greater than conductivity across the grain (Simpson and TenWolde 2007). Thus, when we interpret the data found with these experiments we must understand that many different factors may affect the thermal conductivity in wood, however, we observed that sufficiently lethal temperatures were consistently achieved if steam treatments of more than 10 min are applied.

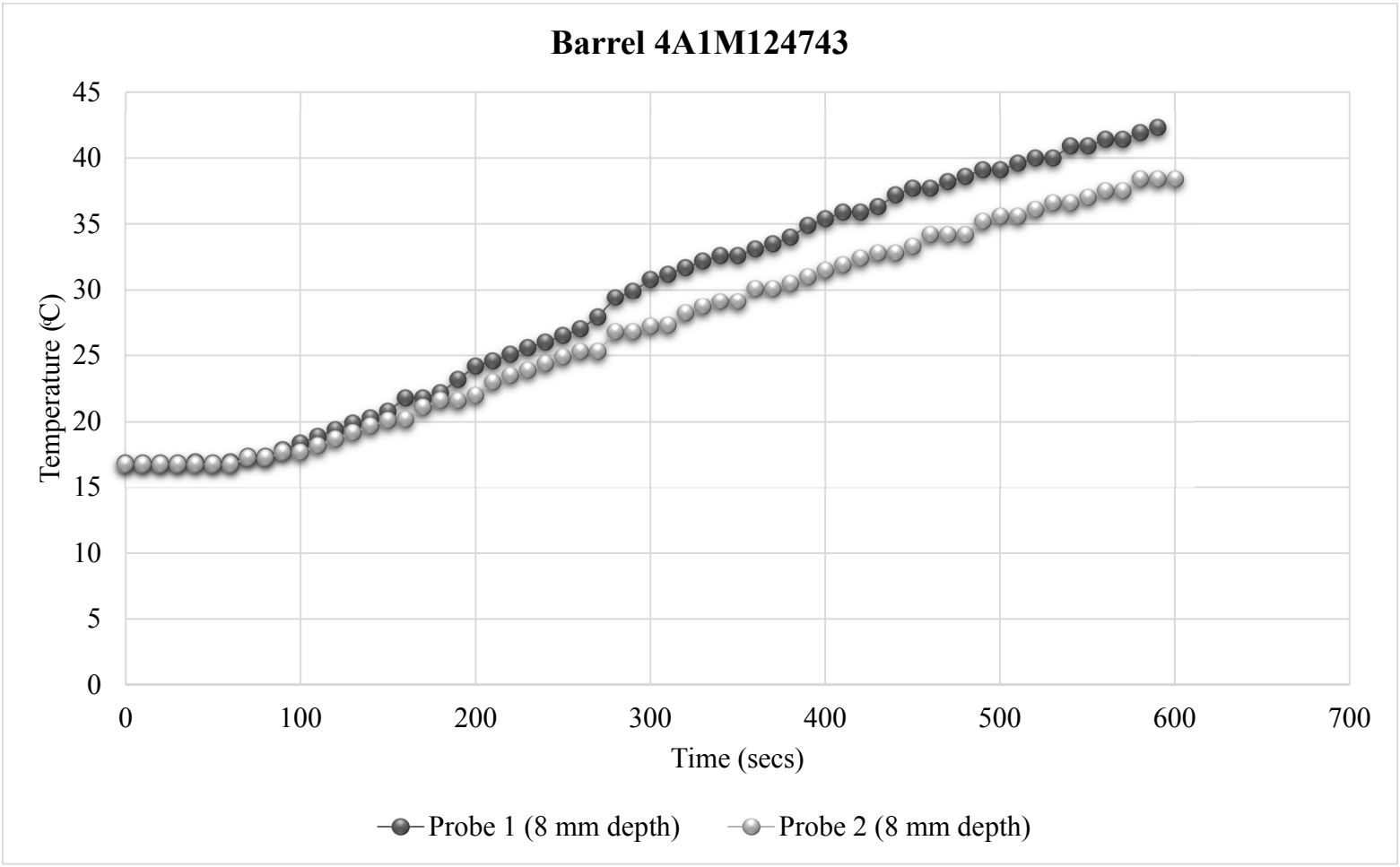

Figure 5.7 Internal barrel temperature over time at 8 mm depth with steam treatment.

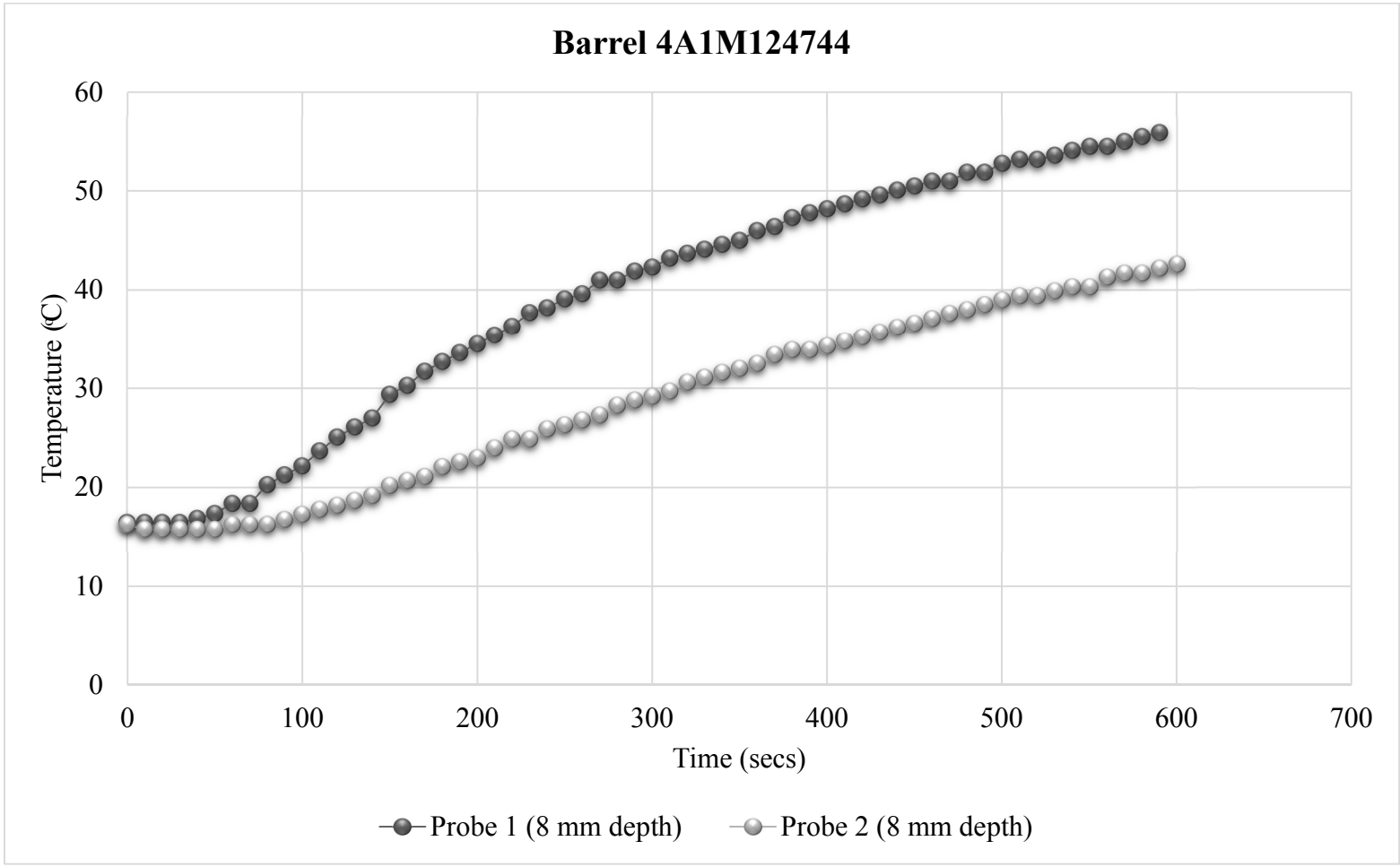

Figure 5.8 Internal barrel temperature over time at 8 mm depth with steam treatment.

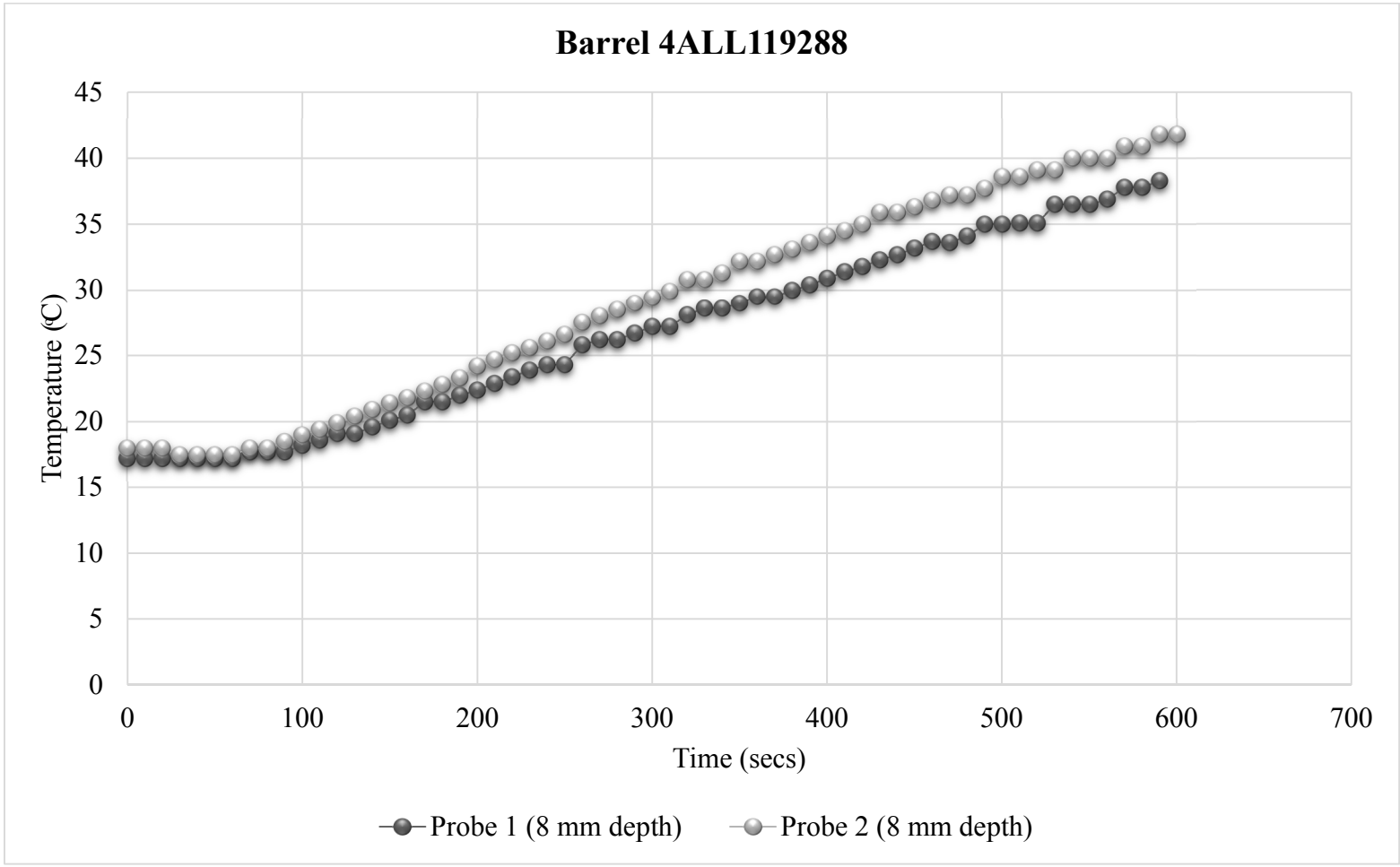

Figure 5.9 Internal barrel temperature over time at 8 mm depth with steam treatment.

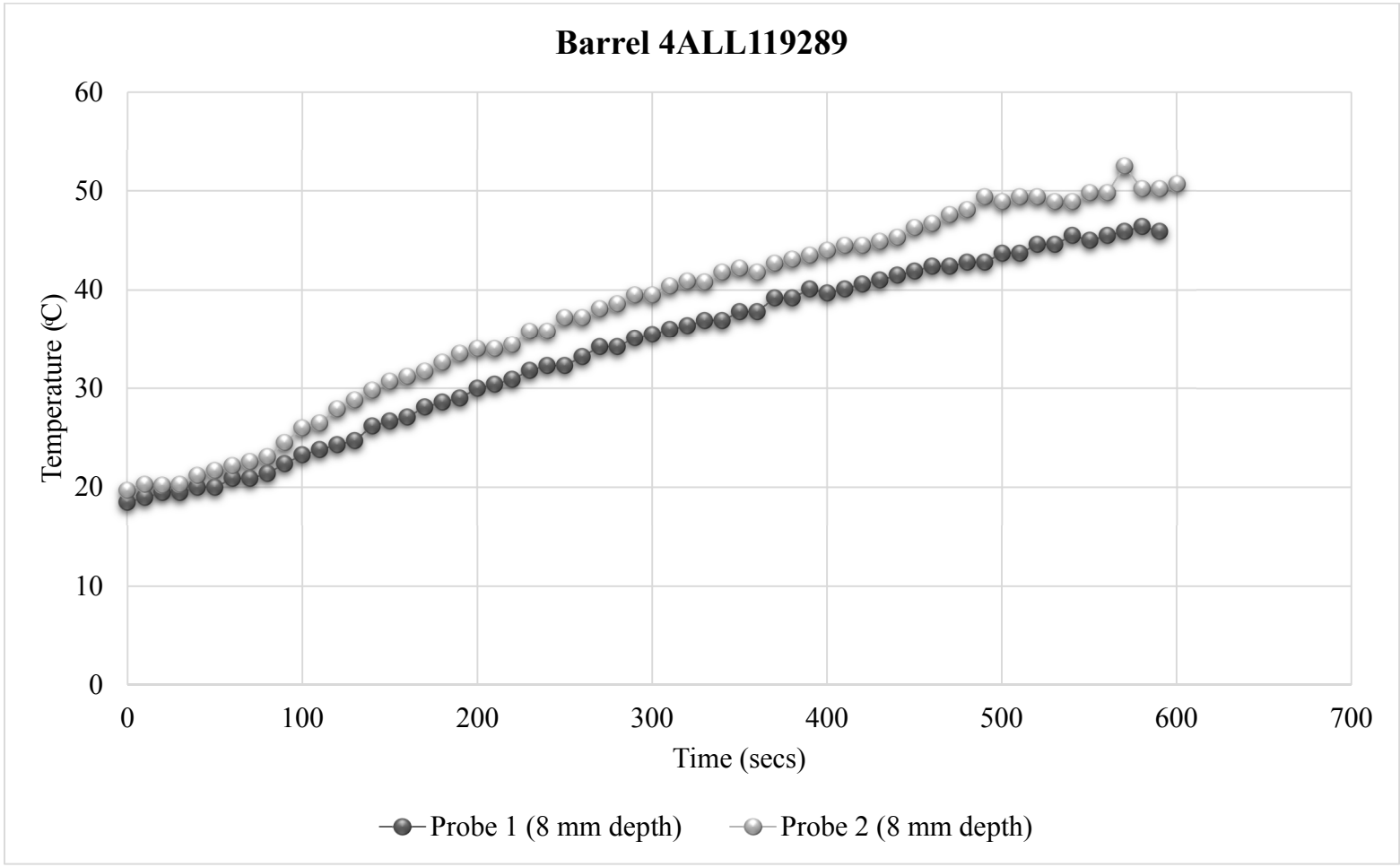

Figure 5.10 Internal barrel temperature over time at 8 mm depth with steam treatment.

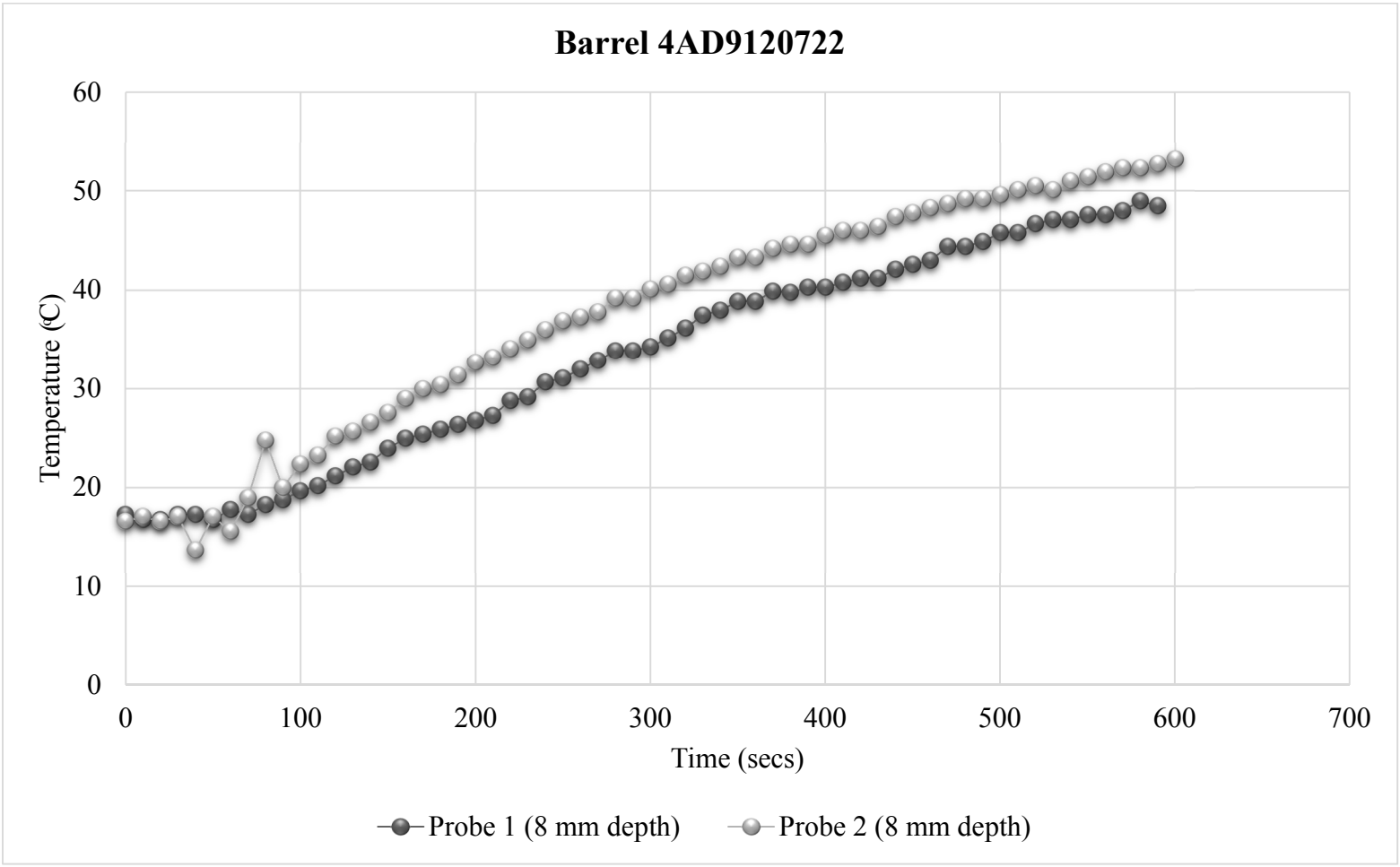

Figure 5.11 Internal barrel temperature over time at 8 mm depth with steam treatment.

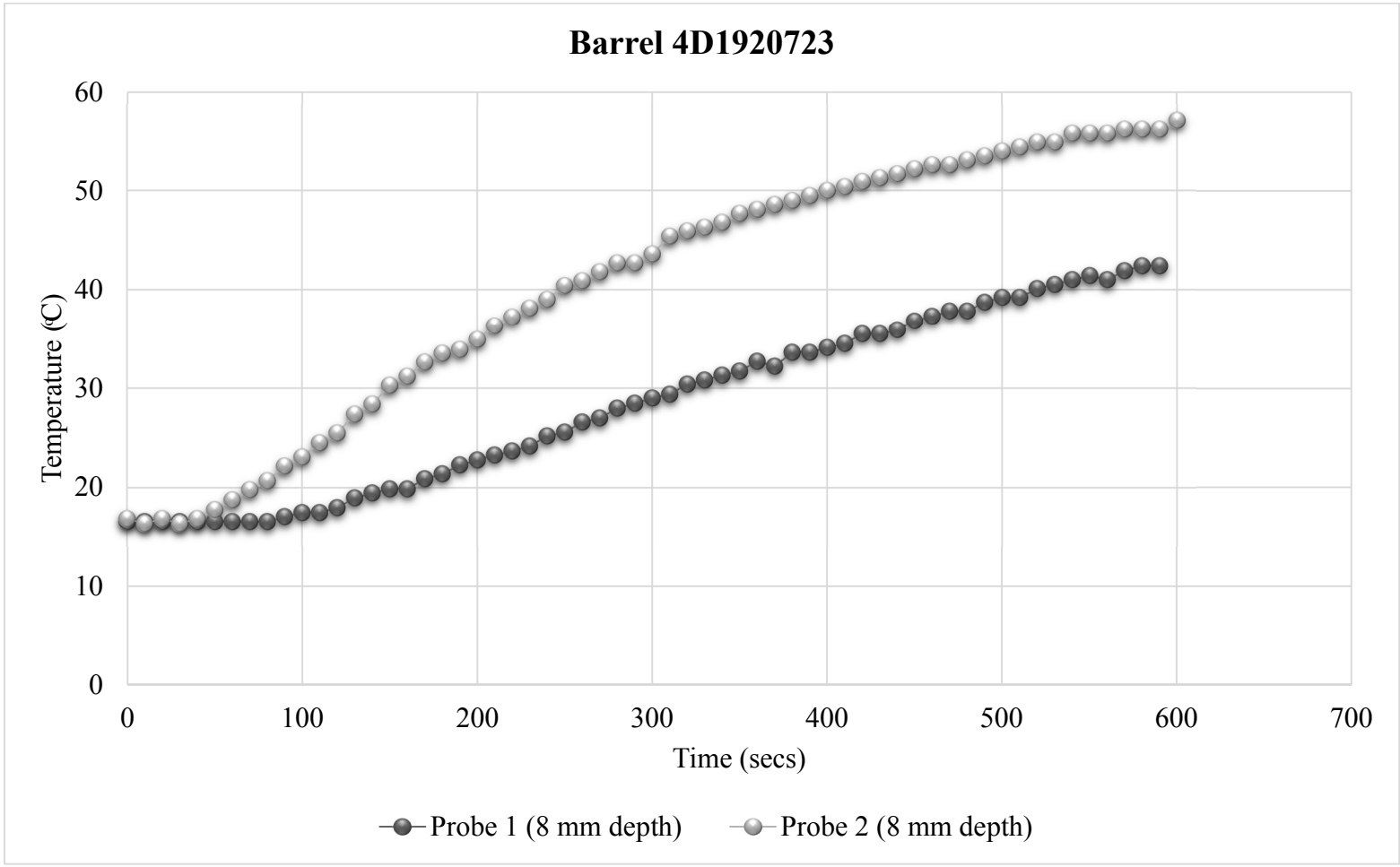

Figure 5.12 Internal barrel temperature over time at 8 mm depth with steam treatment.

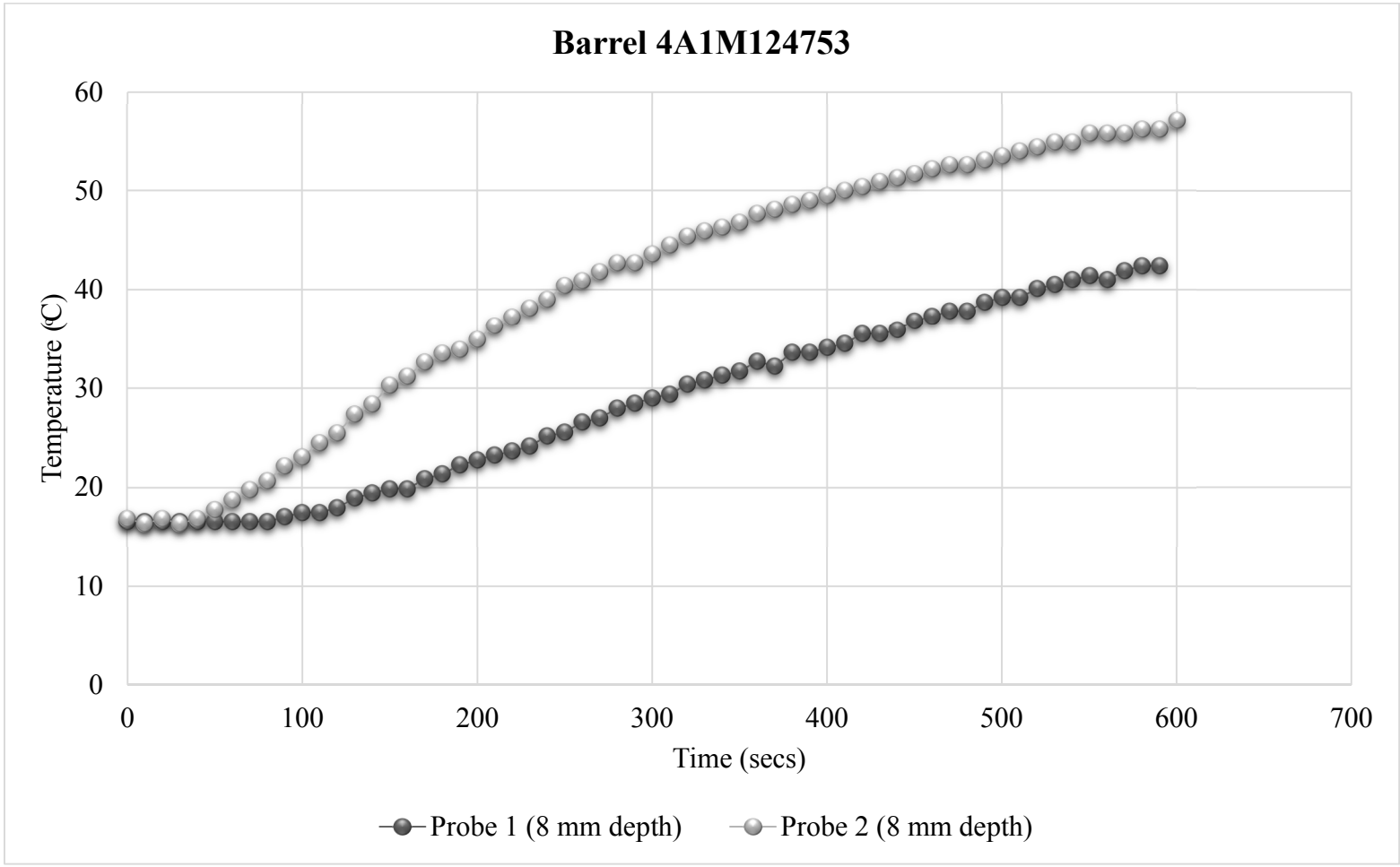

Figure 5.13 Internal barrel temperature over time at 8 mm depth with steam treatment.

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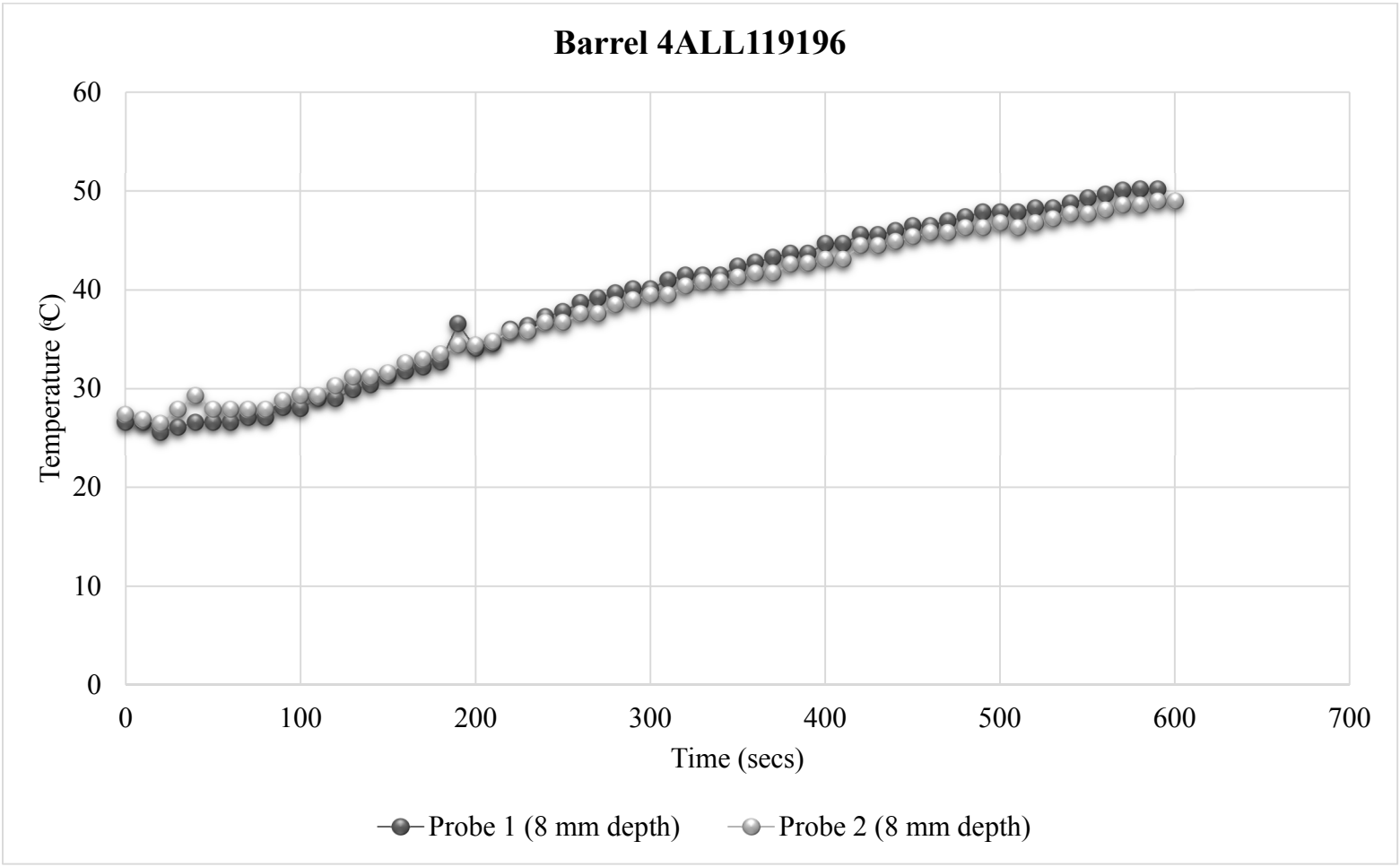

Figure 5.14 Internal barrel temperature over time at 8 mm depth with steam treatment.

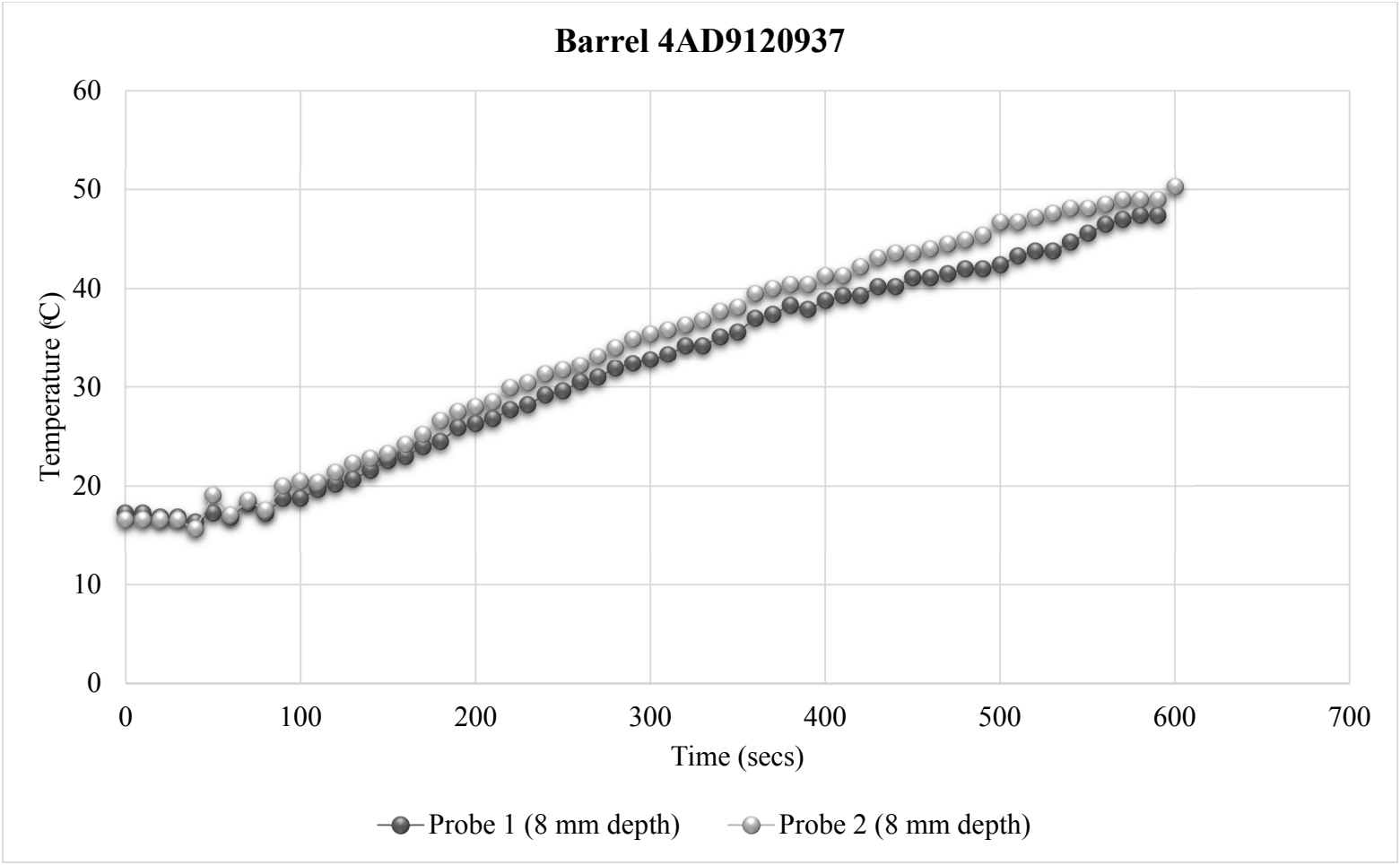

Figure 5.15 Internal barrel temperature over time at 8 mm depth with steam treatment.

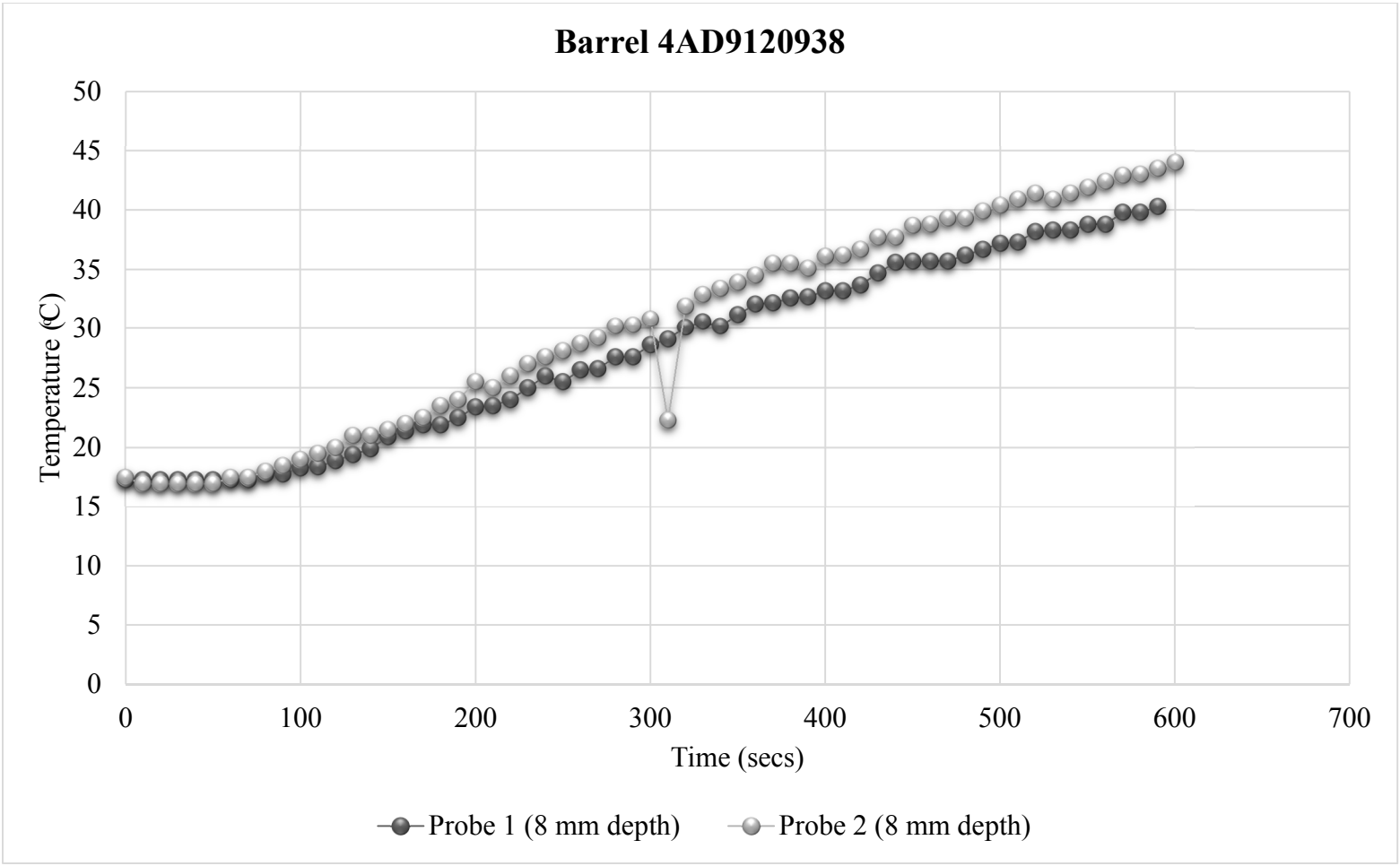

Figure 5.16 Internal barrel temperature over time at 8 mm depth with steam treatment.

The concept of thermal conductivity should be understood and taken into account, when steam treatment is used for wood surfaces such as wine cooperage, and it should be understood as a measure of the rate of heat flow through one unit thickness of a material subjected to a temperature gradient (Simpson and TenWolde 2007).

Wood core examination for the presence of *Brettanomyces*: post-treatment. The efficacy of the steam treatment in barrels was studied at a surface level of the barrel interior (0 mm) with the liquid samples taken after sanitation treatment (Table 5.3) and at a depth of 8 mm, since this depth has been found to be the level of wine penetration (Malfeito Ferreira et al. 2004). Since *Brettanomyces* is more prone to be found at the bottom of a barrel (bilge) and also at the top of the barrel due to higher levels of oxygen that stimulate its growth (Van de Water 2010), the cores were strategically taken from bilge and heads (upper head and lower head). Our results showed that at a surface level (0 mm) none of the barrels were positive for the growth of *Brettanomyces* spp. after steam treatment for both treatment times (5 and 10 min). When wood cores were analyzed at a depth of 8 mm (pre-treatment), only two barrels were positive for the growth of *Brettanomyces*, however, after treatment none of these cores from these two barrels showed *Brettanomyces* growth.

CONCLUSIONS

Steam sanitation should ensure that sufficient temperatures be reached at the deeper depths of the staves to kill any wine spoilage microorganisms that may be harbored in the pores of wood. To our knowledge, wineries in the USA and other parts of the world have evaluated various sanitation practices but a lack of a scientific studies exist that validate the use of steam as a means to sanitize

wine cooperage. In this study, we employed the use of steam to reach lethal temperatures, and the *in vitro* thermal inactivation studies showed that temperatures between the ranges 45 ºC (113ºF) to 60ºC (140ºF) were sufficient to kill common spoilage yeasts found in wine environments. However, there are several factors that must be taken into consideration to ensure that steam treatments of barrels are effective. These include treatment times, treatment temperatures, target microorganisms, and barrel penetration depth. The design of this experiment was performed taking into account several factors to be controlled; one of them was penetration depth. We know that 8 mm is the depth that has been found to be the level of wine penetration and assumed also the depth at which *Brettanomyces* and other microorganisms can be found. Based on these results, steam treatment for 10 min should be an effective decontamination method to ensure that a depth of 8 mm, and perhaps even deeper, the temperature is sufficient to kill the wine spoilage microorganisms found there. Consequently, depth, time, target microorganisms, and temperature, should be four important factors to be considered when using steam as a sanitation method for wine cooperage to consistently reach temperatures that are sufficient to kill target wine spoilage microorganisms. The findings in this article indicate that the correct use of steam as a sanitation method in wineries can control spoilage yeast including *Brettanomyces* spp. in cooperage, and therefore should be re-assessed as one of the preferred sanitation methods.

ACKNOWLEDGMENTS

This research project was funded by CONACYT-Mexico, New York State Agricultural Experiment Station Federal Formula Funds, and Cornell University Research Travel Awards. The authors thank the industry donors at Constellation Brands California, USA.

The authors would like to thank Mark Nisbet (Cornell University) for his valuable editorial contributions to this article.

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CHAPTER 6

CONCLUSIONS AND PROSPECTUS

CONCLUSIONS

Numerous different sanitizers are currently used in wine industry. However, none has been validated for their specific effectiveness either *in vitro* or *in vivo*. Furthermore, some sanitizers are used at concentrations that are not adequate. The objective of this research was the evaluation of common and novel sanitizers *in vitro* and *in vivo* conditions challenging common wine spoilage yeasts. The major conclusions of this study were:

- Sulfur dioxide in aqueous solution was effective at the lowest pH levels used (3.0 and 3.2) for *Zygosaccharomyces bailii* and *Brettanomyces/Dekkera bruxellensis*. However, for *Saccharomyces cerevisiae* strains the log reduction was almost null. When sulfur dioxide was used in the gaseous form (burning sulfur discs), it was effective when the contact time was six weeks for both general yeast and *Brettanomyces* spp. yeast populations. However, when contact times were three weeks, the treatments were only effective in lowering *Brettanomyces* spp. populations to undetectable levels *versus* general yeast populations that still presented reminiscent populations.
- The use of sulfur discs to sanitize wine cooperage is highly dependent on the complete burning, or at least the majority of the disc which is dependent on the oxygen that is present in the headspace of the barrel that needs to be consumed, the initial microbial loads present at the moment of the sanitation, and humidity inside the barrel.

- Sulfur discs although proven to be effective when used for 3 weeks to reduce *Brettanomyces* spp. yeast populations to undetectable levels at the surface, it was shown not to be effective at a level of 8 mm depth (wine level penetration), since *Brettanomyces* spp. were isolated from wood cores.
- Peroxyacetic acid (PAA) used *in vitro* conditions at a concentration of 120 mg/L is highly effective against *Brettanomyces/Dekkera bruxellensis* and *Saccharomyces cerevisiae,* since within few minutes it resulted in undetectable levels of the strains; however, detectable levels of *Zygosaccharomyces bailii* were found even after the longest exposure time.
- Of all the three genera used *in vitro* conditions, *Zygosaccharomyces bailii* proved to be the most resistant to PAA.
- PAA when used in barrels (*in vivo* conditions) proved to be ineffective at 120 mg/L, however when 200 mg/L and one week exposure were used, undetectable levels of both general yeast and *Brettanomyces* spp yeast populations were found.
- PAA wood cores analyzed for the presence of *Brettanomyces* spp at a depth 8 mm (wine penetration level) showed negative isolation of the microorganism only when 200 mg/L and 1 week exposure were used.
- Chlorine dioxide proved to be ineffective to sanitize wine cooperage regardless of the concentration used (5 or 10 mg/L) or the treatment times.
- DMDC used at a concentration of 250 mg/L proved to be highly effective in reducing to undetectable levels the three genera studied (*Saccharomyces cerevisiae, Zygosaccharomyces bailii* and *B./D. bruxellensis*) after 15 minutes of treatment time.

- DMDC when used at a concentration of 150 mg/L is effective in reducing *Zygosaccharomyces bailii* and *B./D. bruxellensis* strains to undetectable levels, however, *S. cerevisiae* showed strain differences, suggesting that the effect of DMDC is strain and dose dependent.
- Ozone although effective in the majority of the barrels treated at a concentration of 1 mg/L for 5 or 10 minutes for both general yeast and *Brettanomyces* spp yeast populations, some barrels still had reminiscent yeast populations.
- Ozone although effective at the surface level, was not effective at a level of 8 mm depth, since isolation of *Brettanomyces* spp was positive from barrel cores after treatment.
- The heat inactivation from the *in vitro* experiments revealed that the highest temperatures that permitted the survival of the three genera studied (*Saccharomyces cerevisiae, Zygosaccharomyces bailii* and *B./D. bruxellensis*) ranged between 55ºC (131º F) and 60ºC (140ºF).
- At a depth of 8 mm with 10 minutes treatment of steam, the highest temperature that can be reached in a barrel is 57.5°C (135.5ºF) taking 10 minutes and 4 seconds to reach it consistently. However, at a depth of 14 mm and using 10 minutes, 42.5°C (108.5ºF) was the highest temperature reached.
- Steam treatment for 5 minutes was shown insufficient to reach inactivation temperatures capable of killing the more common wine spoilage microorganisms found in wine environments. However, 10 minutes of steam treatment resulted in more consistent temperatures, with the results from the *in vitro* experiments, since this time was sufficient to reach a temperature capable of causing heat inactivation for the three genera studied. This temperature was 57.5ºC, which is more than enough to cause heat inactivation of the three genera studied, in just few minutes depending on the strain.

PROSPECTUS

Based on the results and conclusions of this work, the following suggestions are recommended for future work:

- DMDC proved to be highly effective under *in vitro* conditions at the highest concentration used (250 mg/L), and testing DMDC at these concentrations under *in vivo* conditions could be useful to evaluate its potential for application as a sanitizer for wine cooperage.
- All the sanitizers used in this research should also be tested against lactic acid bacteria and acetic acid bacteria under *in vitro* and *in vivo* conditions, since these two groups are also common contaminants of wine.
- The observation that other colony morphologies exist with *Brettanomyces* spp isolated from barrels, suggests better identification strategies are needed when isolated from different sources in wineries.
- It has been suggested that the wrinkled morphology found in yeasts is related to the presence of pseudomycelia (a state related to a protective effect against adverse conditions), it is likely that this state could attribute certain resistance to sanitizers. This increased resistance was observed with this particular morphology in *Brettanomyces* spp isolated from empty barrels, where conditions of lack of nutrients and other stressors could exert and influence for the presence of this kind of morphology. Additional research is needed to conclusively establish the link between this atypical morphology and its response to different sanitation methods.