AN ABSTRACT OF THE THESIS OF

Uri Hetz for the degree of Master of Science in Food Science and Technology. Presented on June 22 2001. Title: Lysozyme as an Aid in Preventing Stuck Wine Fermentations.

Abstract approved: _____.
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To provide a possible alternative for the antimicrobial action of sulfur dioxide in winemaking, and address the issue of stuck fermentations, I studied the efficacy of chicken lysozyme (EC 3.2.1.17) as an antimicrobial in grape juice. Two different forms of lysozyme were used: native lysozyme (NL), that is known to be an effective inhibitor of lactic acid bacteria in wine, and partially unfolded lysozyme (PUL), that has been reported to have antimicrobial activity against both Gram-positive and Gram-negative bacteria. *Lactobacillus kunkeei* and *Acetobacter pasteurianus*, two bacteria associated with the induction of stuck fermentations were used in the experiments.

Chardonnay and Pinot Noir juices were inoculated with *L. kunkeei* and two days later with yeast strain EC1118 and then incubated for 10 days. The addition of 250ppm of either NL or PUL reduced populations of *L. kunkeei* to less than 10 CFU/mL in 24 hours while in inoculated grape juice that did not contain any lysozyme, the bacteria grew to 10^9 CFU/mL within two days. Grape juices supporting the growth of *L. kunkeei* developed up to 14 times more volatile acidity (VA) than the control or either of the lysozyme treatments. No differences were observed in the antimicrobial action of NL and PUL or in their effects on the composition of the wine.

Native lysozyme and PUL were tested against *A. pasteurianus*, in peptone water and in broth medium. In peptone water, 200ppm of PUL caused more than 95% reduction in populations of *A. pasteurianus* within two hours of exposure. When inoculated into a mixture of broth and grape juice, no inhibition was observed and the bacteria grew to similar numbers in all treatments. The addition of 200ppm of NL did not cause any inhibition of *A. pasteurianus* either in peptone water or in broth medium.

Lysozyme as an Aid in Preventing Stuck Wine Fermentations

By
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A THESIS

Submitted to
Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Presented June 22, 2001
Commencement June 2002

Master of Science thesis of Uri Hetz presented on June 22, 2001.

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I understand that my thesis will become part of the permanent collection of Oregon State University Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Uri Hetz, Author

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INTRODUCTION

Stuck Fermentations of Wine

General

Despite being an old and familiar problem and in light of considerable research, winemakers are still waiting for an effective cure to stuck and sluggish fermentations. Though there doesn't necessarily seem to be an increase in the frequency of their occurrence, they nevertheless continue to cause considerable losses to the wine industry as they have for hundreds of years (1,2,9).

Stuck fermentations are those that stop prematurely, leaving a wine with a higher than desirable residual sugar concentration and a correspondingly low alcohol level. Sluggish fermentations may reach the desired final sugar concentration, but take much longer than average to do so (2). Both these cases are equally troublesome in the sense that not only do such wines require more time and attention from the winemaker but they also take up tank space that is often needed for other activities such as racking, cold stabilizing, and blending (9). Another concern with stuck and sluggish fermentations is that once the fermentation rate decreases and with it the rate of CO_2 production, the wine becomes more susceptible to spoilage, both by simple oxidation and by the action of bacteria that can thrive in the low alcohol high sugar environment (2). Furthermore, a wine containing residual sugar and insufficient alcohol requires special care when bottled, as it is inherently unstable and can start refermenting in the bottle by either bacteria or yeast, resulting in effervescence or sensory defects. Finally, even if all these problems are successfully overcome, a wine with a higher than

desired residual sugar concentration might be simply unfit for sale or at the very least would not command as high a price as it might otherwise have.

Why then hasn't a solution been found for this chronic problem? To begin with, the environment of fermenting grape juice is highly complex; the large number of factors involved and their intricate interplay makes the determination of the cause and the subsequent remediation of stuck fermentations extremely difficult (2,9). The main factors identified so far as possible causes are nutrient deficiency, ethanol toxicity, temperature extremes, growth of spoilage microorganisms, the presence of pesticides and fungicides, phenol composition, pH and potassium concentrations, and winemaking practices such as aeration, clarification and sulfur dioxide (SO_2) (1,2,9,24,29,35). To complicate things further, some of these factors have a cumulative or synergistic effect. For example the presence of acetic acid, even at sub-toxic levels, can hinder the uptake of thiamine by the yeast; the thiamine content can be further reduced by the growth of wild yeast (and possibly bacteria) and by the reaction with SO_2, which ironically is added to control the growth of such organisms (2,3).

A better understanding of these factors and processes is wanting, yet the gathering of information is a challenging task. Stuck fermentations occur in the winery and not in the lab, and more often than not, their occurrence is not reported and their causes left uninvestigated. Many of the factors involved, such as the chemical and microbiological makeup of the must, are highly variable (depending on site, year, variety, etc.) and are rarely monitored, others are highly transient in their nature and are hard to track retroactively. For example, a momentary temperature shock, which can bring a premature end to a fermentation, is hard to recognize by the time the problem is noticed; microorganisms that might have thrived during the first few days of the fermentation and produced some inhibitory metabolites may be dead or uncultivable once the fermentation reaches a halt. Lastly, our understanding of the fermentation process comes from lab studies, which often employ sterile synthetic media rather than grape juice. Most of these studies tend to focus on the nature of the

growth phase and not the stationary phase, the one at which most stuck fermentations occur (2).

Considering the complexity of the problem, and the limited success treating stuck fermentations usually encounters, prevention rather than remediation is most likely the more practical approach.

Microbial spoilage of fermenting grape juice and its role in stuck fermentations

One of the factors involved in the induction of stuck fermentations that has been receiving more attention lately is bacterial spoilage (2,3,5,7,8,15). When considering the problem of bacterial spoilage in wine, it is only natural that most of the attention is given to those bacteria that can survive and grow in wine and not those whose presence in the wine is limited to the first few days of the fermentation. Nevertheless, numerous types of bacteria, although not suited for growth in wine, can thrive during this short period before enough ethanol is produced to inhibit or kill them (5,7,10,15,26,36). Bacteria growing in grape juice can compete for nutrients as well as produce inhibitory substances. Their growth may often go unnoticed, as they don't always leave an impact on the quality of the wine, yet certain types of bacteria can rapidly reach large populations and in a short time produce enough metabolites that can render the wine defective (5,7,15). In California alone, about one million gallons of wine are processed annually by commercial operations for the reduction of volatile acidity (VA); about two thirds of the cases are believed to be due to bacterial spoilage during fermentation (Clark Smith, personal communication, 2001). In more severe cases, bacterial spoilage can not only cause aroma defects or illegal levels of VA but also bring the fermentation to a premature end. Even a limited proliferation, as inconspicuous as it might be, can simply add another hurdle to the long list of those challenging the yeast and thus lead to a stuck fermentation (2,5,7,15).

Lactic acid bacteria (LAB) are associated with the spoilage of wine and grape juice as well as the induction of stuck fermentations (3,7,10,12,15). These bacteria are

usually better known for their activity in wine: either desirable such as the conversion of malate into lactate, or undesirable such as the production of VA and biogenic amines (3,12,14). However, recent studies have shown that some LAB that can grow during fermentation may lead to the development of stuck and sluggish fermentations (7,14). Huang et al. (15) isolated three strains of LAB from musts (crushed grapes) that were undergoing sluggish fermentations and demonstrated their ability to inhibit the fermentative activity of several strains of yeast. Two of those bacterial strains were later identified as wild types of *Oenococcus oeni* while the third was a newly recognized species of *Lactobacillus* named *Lactobacillus kunkeei* (7,8). Edwards et al. (7) studied the growth of *L. kunkeei* in grape juice and its effect on the fermentation process. They observed after 8 days of fermentation by *Saccharomyces cerevisiae* in the presence of *L. kunkeei* that alcohol production started to level off at 6% while the control (containing only *S. cerevisiae*) reached 12%. Grape juice fermented in the presence of *L. kunkeei* displayed acetic and lactic acid levels of 4g/L and 8g/L respectively. Although these levels of acetic acid are generally regarded as inhibitory to some strains of *S. cerevisiae* (7,15,35), the addition of comparable amounts of acetic acid to fermentations free of *L. kunkeei* did not result in a stuck fermentation, suggesting that the production of acetic acid was not the sole culprit and that the bacterium may be producing other inhibitory substances (7).

Another group of bacteria associated with the production of high levels of VA are the acetic acid bacteria (AAB) (4,11). Depending on the condition of the grapes, different species of AAB can be found in must at levels as high as 10^6 CFU/mL (4,9,26). Once again, these bacteria are better known for their ability to cause spoilage in wine (by oxidizing ethanol into acetic acid) and not for their ability to grow in grape juice (6,9,26). For years it was generally believed that AAB such as *Acetobacter aceti* and *Acetobacter pasteurianus* couldn't grow in the anaerobic conditions that predominate during fermentation. However, some studies have shown that these bacteria do not only have the ability to survive for long periods of time in wines kept under anaerobic conditions but also to grow during the alcoholic fermentation (5,26,30,39). Furthermore, evidence suggests that even when denied alternative

electron acceptors, such as phenolic compounds, these bacteria can survive in wine in a viable but non-culturable state (30).

Drysdale et al. (5) observed that when *A. aceti, A. pasteurianus* and *Gluconobacter oxydans* were inoculated into grape juice together with various species of yeast, they managed to multiply in fermenting grape juice and to cause varying degrees of yeast inhibition. In one example from their study, grape juice inoculated with both *S. cerevisiae* and *A. pasteurianus* still contained 8g/L residual sugar after 12 days, while the control reached dryness. Compared to the control, the same wine also showed a pH drop of >0.5 and an increase of 2.2g/L in the acetic acid content. *Gluconobacter oxydans*, though it cannot survive in the alcoholic environment of wine, managed to grow during the first few days of fermentation while producing 1.4g/L of acetic acid and 17g/L of gluconic acid and limiting the ethanol production to 5.4% (compared with 11.2% in the control) (5).

The growth of LAB and AAB in must can cause varying degrees of spoilage ranging from off flavors to yeast inhibition. Controlling the growth of such bacteria is essential for the quality control of wine production and may be a crucial step in avoiding the occurrence of stuck and sluggish fermentations. Currently, the main tool available for this task is sulfur dioxide.

The use of sulfur dioxide

The proliferation of spoilage microorganisms in fermenting grape juice is generally avoided by the appropriate application of SO_2 (3,9,11,41). Once added to the juice, SO_2 enters an equilibrium of three species. The specie most potent as an antimicrobial is known as the molecular form; it is generally considered that levels of 0.5-0.8ppm of this form are sufficient to inhibit the growth of most spoilage microorganisms in wine. The concentration of this specie is highly pH-dependent and only starts becoming dominant at pH values lower than 1.77; in a typical juice with a pH of 3.5, less than 1% of the total SO_2 added will exist in the molecular form (41).

The antimicrobial effect of SO_2 varies depending on the specific strain of microorganism and the wine composition. Many studies have been conducted attempting to determine the amounts of SO_2 necessary for inhibition of different microorganisms yet the variability in the media and the conditions used in all those different studies makes the drawing of definite guidelines very difficult (3,9,11). Most sources agree that 10ppm free SO_2 are sufficient to inhibit the growth of LAB in wine and 15-30ppm is enough for inhibition of AAB (11). However, these are general guidelines and strains of *Acetobacter* are often isolated from wines with as much as 100ppm total SO_2 (9).

Despite SO_2's proven efficacy both as an antimicrobial and as an antioxidant, many winemakers lean towards minimizing its use (9,11,41). Reasons for avoiding its use are its general toxicity, which can make it a hazard in the cellar, its allergenicity towards some sensitive individuals, its ability to impact the character of the wine, and its toxic effect on the yeast (9,11,14,40,41). Since the portion of the molecular form of SO_2 becomes smaller with rising pH, the use of SO_2 is especially problematic in high pH juices. For example, to maintain a level of 0.8ppm of molecular SO_2 in a grape juice of pH 3.7, one has to adjust the concentration of free SO_2 to *ca* 80ppm; however, since 50-70% of the SO_2 added to the juice get bound up, a total of more than 200ppm SO_2 has to be added (9,11). Grape juice made of high percentage of damaged fruit is even more problematic as the tendency of the juice to bind SO_2 increases with increasing levels of rot. Lastly, in the case of stuck and sluggish fermentations, SO_2 can also act as a stress factor on the fermenting yeast through its toxicity and its tendency to degrade certain nutrients (2,3,9).

Despite its inherent limitations, finding a substitute for SO_2 and its application in wine is a challenging task. Nevertheless, the mandatory labeling of SO_2 in wine (when higher than 10ppm), the growing public concern of food additives and the general trend to prefer organic and "natural" substances, have all led to extensive studies. It is no surprise that the Pasteur institute in France has offered a 10,000FF award to anyone who finds such a substitute (41), an award that hasn't been claimed yet.

Lysozyme

General

Lysozyme is an enzyme that catalyzes the breakdown of peptidoglycan – the polysaccharide-peptide complex composing the cell walls of bacteria. Chicken lysozyme, extracted from egg white, is made of 129 residues, and has four disulfide bridges, a MW of 14.4kDa and a pI value of 10.5 (25,34).

Lysozyme was discovered in 1922 by Alexander Fleming, who noticed that human mucal secretions formed clearings in bacterial lawns. It was later verified, as Fleming claimed, that lysozyme was the active antimicrobial in many seemingly unrelated substances formerly described by scientists as having bactericidal properties (25). Lysozyme is a widespread enzyme in nature; different variants of it can be found in viruses, bacteria, fungi, plants, birds, and mammals. They differ in many structural aspects but common to them all is the ability to catalyze the breakdown of peptidoglycan (25). Commercially, the most readily available source of lysozyme is chicken egg white, where it can be found in a concentration of *ca* 0.5% by weight and can easily be extracted by using ion-exchange resins (34).

Peptidoglycan, the substrate for lysozyme's catalysis, is a polymer of repeating units of N-acetylglucosamine and N-acetylmuramic acid. Lysozyme breaks the β 1-4 glycosidic bond between these sugars and thus gradually degrades the bacterial cell wall. Once the bacterium is stripped of its wall, it becomes vulnerable and can easily rupture at the slightest change in osmotic pressure (25,34). However, not all bacteria are susceptible to the action of lysozyme. Some bacteria are protected by an additional coating that acts as a barrier between the peptidoglycan and the cell's surroundings. These bacteria are known as Gram-negative bacteria and are insensitive to the action of lysozyme under most conditions (11,34).

Partially unfolded lysozyme

Despite the specificity of its enzymatic action, abundant evidence exists suggesting that lysozyme's action is not limited to the breakdown of bacterial cell walls and its function not restricted to the killing of Gram-positive bacteria (20,22,25,32). For example in humans, it has been shown to participate in fighting infections by viruses and Gram-negative bacteria, to stimulate the immune system and to limit tumor growth (25). In egg, lysozyme continues to ward off infections even as the pH of the egg rises gradually from 7.6 to 9.5 – a pH at which the enzyme loses its catalytic activity! (22,27,34) Furthermore, studies have shown that during the storage of eggs, lysozyme goes through some structural changes such as deamidation and dimerization that may alter or even deny access to its active site (22,27,39).

In an effort to explore the behavior of lysozyme and possibly enhance the spectrum of its antimicrobial activity, scientists have studied the effects of structural changes on the functional properties of lysozyme (16,17,18,19,20,21,22,23,25,31,33). Ibrahim et al. used different approaches, such as attachment of fatty acids or addition of hydrophobic residues, to enhance the hydrophobic character of lysozyme, in hope of enabling it to penetrate the lipopolysaccharide and phospholipid bilayer of the outer membrane of Gram-negative bacteria and thus reach the bacterial cell wall (16,17,18,19,21). They found that these modifications and others resulted in a partial loss of catalytic activity coupled with an increased activity against Gram-negative bacteria (16,17,18,19,21).

A most remarkable finding was stumbled upon when heating the enzyme; this induced an irreversible partial unfolding of the protein and resulted in a compound bactericidal to both Gram-positive and Gram-negative bacteria (20,22). Ibrahim's group monitored the functional and structural changes in lysozyme as a function of heating parameters and found that as the temperature, time, and pH of the heat treatment increased, lysozyme gradually lost its catalytic activity until eventually, after 20 minutes of heating at 90^{0}C and pH 7, no enzymatic activity could be detected at all. However, despite the loss of catalytic activity, the various heated forms of lysozyme

continued to cause inhibition of Gram-positive bacteria (20). The most potent variant was obtained by heating the enzyme for 20 minutes at 80^{0}C and pH 6 (20/80/6). This form retained 50% of its catalytic activity and exhibited a novel antimicrobial activity against different types of Gram-negative bacteria while at the same time it lost none of its potency against Gram-positive bacteria (22). Structural analysis revealed some profound structural changes: 60% of the 20/80/6 form was found to be in dimeric and oligomeric forms, one disulfide bridge was broken, surface hydrophobicity increased 14-fold and binding affinity to both peptidoglycan and Gram-negative outer membrane lipopolysaccharide was greatly enhanced (22). The new form also displayed an ability to fuse liposomes made from *Escherichia coli* membrane; a shift in the intrinsic tryptophan fluorescence also suggested strong interaction between the new lysozyme and the phospholipid layer of bacterial membranes. Finally, scanning electron micrographs of *E. coli* exposed to this novel form clearly showed severe damage to the bacterium's membrane (within 30 minutes of exposure) with no similar effect when exposed to native lysozyme (22).

Being a potent antimicrobial of natural origin makes lysozyme a useful tool in the production of food. However, food spoilage often involves both Gram-positive and Gram-negative bacteria and therefore can't be completely avoided using lysozyme. A modified form of the enzyme, such as the 20/80/6, may prove to be an excellent preservative as it can offer protection against both groups of bacteria.

The use of lysozyme in wine

The use of enzymes as natural preservatives in food presents an alternative to chemical agents. Using lysozyme to prevent microbial spoilage of food has been explored in many countries but most extensively in Japan, where products such as tofu, fresh produce, seafood, meat and sake have all been shown to benefit from preservation by lysozyme (34). In Europe and the USA lysozyme is currently used for the control of spoilage bacteria in cheese (11,14).

The use of lysozyme as an antimicrobial in winemaking is a relatively recent development. The application of the enzyme has been studied for several years now both in Europe and in the USA; its final approval, however, is still pending. Until such ruling, the Bureau of Alcohol Tobacco and Firearms has authorized wineries to use lysozyme under the self-affirmation principle.

Gerbaux et al. (12) studied the efficacy of lysozyme in controlling the growth of LAB in wine, both as a mean of avoiding malolactic fermentation (MLF) and as a way of preventing post MLF spoilage. They found that the addition of 500ppm of lysozyme to wines inoculated with *Oenococcus oeni* delayed the MLF for 90 days, comparable to the addition of 40ppm SO_2. In wines that were not inoculated with malolactic bacteria, 500ppm lysozyme prevented MLF for at least 6 months while the control completed MLF within 100 days. The addition of lysozyme to wines inoculated with various LAB after the completion of MLF, suppressed bacterial growth and prevented the production of VA and biogenic amines. As little as 125ppm of lysozyme was enough to reduce the content of biogenic amines by 70-80% compared with control; the use of SO_2 only gave a 55-70% reduction. Gerbaux observed no deleterious effects that may be attributed to the use of the enzyme.

In further studies Gerbaux (13) has shown that lysozyme can also be applied to stabilize sluggish fermentations and thus reduce VA production and also to delay the onset of MLF in the production of Beaujolais wine. Gerbaux also found that not only can lysozyme be used instead of SO_2, but also that wines made with lysozyme show an overall higher quality than those made with SO_2. On the other hand, wines made with lysozyme exhibited protein instability when heated for 30 minutes at 80^0C; no instabilities developed under normal storage conditions.

The heat instability of lysozyme in wine was also studied by Daeschel (Mark A. Daeschel, personal communication, 2001), who found that although considerable haze developed after 30 minutes at 80^0C, exposure to 55^0C for 2 days did not result in any haze as long as lysozyme concentrations were lower than 100ppm (see appendix C). Bentonite fining can be used to remove lysozyme from the wine prior to bottling and thus avoid any heat instability issues. A lysozyme concentration of 300ppm can be

reduced to less than 100ppm by using 3lb of bentonite per 1000 gallons of wine (see appendix C).

Green (14) studied the efficacy of lysozyme in preventing and stopping MLF as well as the interaction of lysozyme with wine components. He found that despite some reduction in enzymatic activity, caused by some wine constituents, lysozyme was able to suppress the growth of both wild and commercial malolactic bacteria at levels of 250-500ppm. The addition of 300ppm lysozyme to wines undergoing MLF reduced bacterial population to undetectable levels within a week. Visual and sensory analysis of the finished wines showed that lysozyme can cause changes in the phenol composition of some wines, resulting in a color shift towards pink and a reduction in astringency.

Conclusion

The growth of certain species of LAB and AAB in must can result in unacceptable wines and may lead to the development of stuck and sluggish fermentations. The use of SO_2 to avoid such events is not always an ideal solution. Native lysozyme has been shown to be an effective substitute for SO_2 as an inhibitor of LAB yet its efficacy in grape juice and the possible effects of its use during fermentation have not been studied. Furthermore, lysozyme's activity is limited to Gram-positive bacteria and it is therefore not likely to be an effective tool in the control of AAB. The partially unfolded form of the enzyme, reported to have activity against both Gram-positive and Gram-negative bacteria, may be effective against AAB as well and thus provide an alternative to the use of SO_2 in inhibiting the growth of both LAB and AAB.

The main objective of this research was to evaluate the efficacy of NL and PUL as antimcrobials in grape juice and to investigate their effect on the fermentation. In addition I wanted to study the ability of the two forms to minimize VA production and thus reduce the risk of experiencing stuck or sluggish fermentations.

LYSOZYME INHIBITION OF ACETIC ACID BACTERIA

Abstract

To find a natural antimicrobial that can inhibit both lactic acid and acetic acid bacteria in wine, I studied the efficacy of native lysozyme (NL) and partially unfolded lysozyme (PUL) in inhibiting the growth of acetic acid bacteria. Populations of *Acetobacter pasteurianus* were exposed to 200ppm of PUL and NL in peptone water as well as in a growth medium. In peptone water, populations exposed to PUL for two hours showed more than 95% decrease in numbers compared to the control while NL caused no inhibition. When incubated in a 1:1 mixture of yeast-extract broth and grape juice, no inhibition was observed with either NL or PUL and the bacteria grew to similar numbers overnight.

Introduction

Acetic acid bacteria (AAB) are known to cause spoilage in wine primarily by the oxidation of ethanol into acetic acid (4,11). In addition, they are also able to grow in fermenting grape juice and produce high levels of metabolites such as acetic acid, gluconic acid and ethyl acetate, resulting in an unacceptable wine. In extreme cases such spoilage can result in a stuck or a sluggish fermentation (5).

Usually such problems can be avoided by the addition of SO_2. However, public concerns and market trends, as well as some inherent limitations, have led winemakers to try to minimize the use of SO_2 (9,11,41). Not only is SO_2 toxic to certain individuals but it can also become a stress factor in stuck and sluggish fermentations as it can be toxic to yeast and cause nutrient depletion (2,3,9). In these scenarios and others, such as organic wines, where the permitted level cannot exceed 100ppm, or in high pH wines where its efficacy is greatly reduced, an alternative antimicrobial is needed.

Lysozyme is a bacteriolytic enzyme commercially produced from hen egg white, which has been shown to be effective in the control of malolactic fermentation and the protection against microbial spoilage by LAB (12,13,14). Since it targets the bacterial cell wall, lysozyme has limited activity against Gram-negative bacteria (such as AAB), which are protected by an outer membrane (15,25,34). This limits the ability of lysozyme to replace the role of SO_2 as an antimicrobial in wine. However, recent studies have shown that lysozyme's activity can be altered to include Gram-negative bacteria as well (20,22). One such alteration involves the partial unfolding of lysozyme in hot water. Lysozyme heated for 20 minutes at 80°C and pH 6 goes through irreversible structural changes resulting in a 50% loss of its catalytic activity. Surprisingly, this form of lysozyme not only retains its bactericidal effect against Gram-positive bacteria but also becomes a potent killer of Gram-negative bacteria (20,22). This form of the enzyme may therefore be used in wine to inhibit both the Gram-positive LAB and the Gram-negative AAB.

The objective of this study was to evaluate the efficacy of PUL in inhibiting the growth of acetic acid bacteria in grape juice.

Materials and Methods

Microorganisms and media

Acetobacter pasteurianus ATCC9432 was obtained from the American Type Culture Collection. The bacteria were cultivated in a 2% yeast extract 2% glucose broth and enumerated on agar plates made of the same medium with the addition of 50ppm of Nystatin (Sigma, St. Louis, MO), to prevent the growth of yeasts and molds.

Lysozyme

Native chicken lysozyme was obtained from Fordras (Lugano, Switzerland) while PUL was a gift from Professor Hisham Ibrahim (Yamaguchi University, Japan). Both were rehydrated from a freeze-dried preparation in a pH 7.2 phosphate buffer just prior to addition.

Grape Juice

Chardonnay grapes were picked from the Oregon State University vineyard during fall 1999, crushed and pressed, adjusted to 40ppm total SO_2 (using potassium metabisulfite) and frozen. Before use the juice was thawed and diluted to 21^0B using distilled water. pH was adjusted to 3.8 using $KHCO_3$, and H_2O_2 was added to lower the SO_2 content to 3ppm total (7). The juice was sterile filtered using a 0.45μ membrane filter.

Inhibition in peptone water

Acetobacter pasteurianus was grown at 27^{0}C to *ca* 10^8 CFU/mL and then spun down and washed in a phosphate buffer. The bacteria were then resuspended in fresh buffer and diluted to concentrations ranging from 10^3 to 10^7 CFU/mL in 0.1% peptone water. Native lysozyme was added to one treatment, PUL to the second and plain buffer to the third; both types of lysozyme were added to give a final concentration of 200ppm. The bacteria were then incubated at 30^{0}C for 2 hours. At the end of the exposure time the treatments were serially diluted and spread on agar pour-plates and incubated for 72 hours before enumeration.

Inhibition in growth conditions

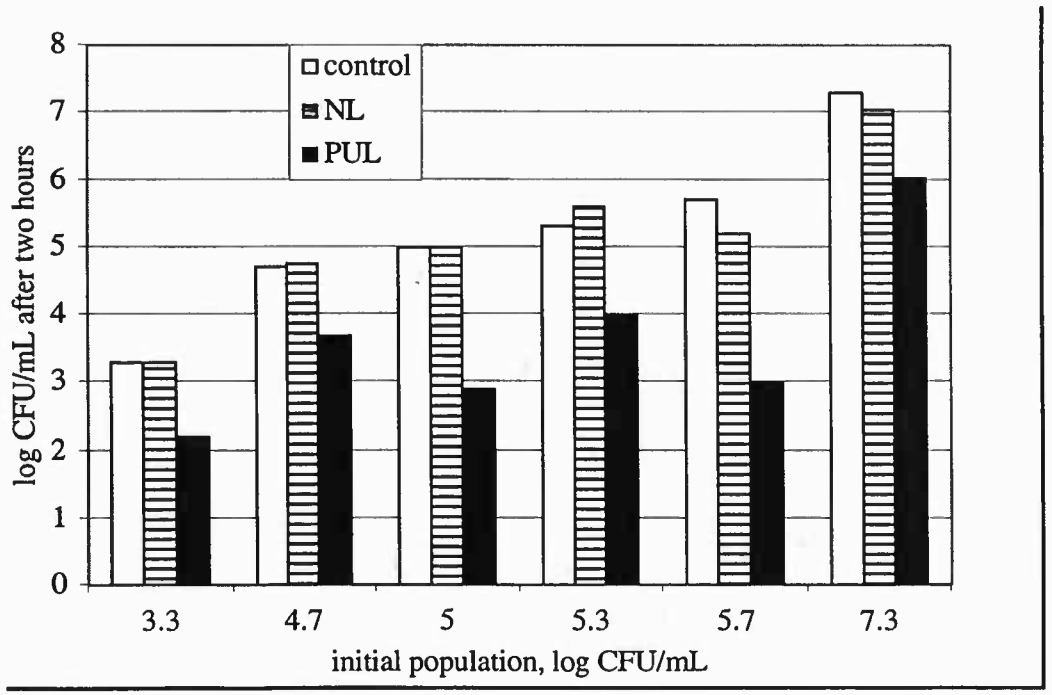
Bacteria were grown in a 1:1 mixture of yeast extract broth and sterile Chardonnay juice for 3 days. The bacteria were then transferred to three flasks containing the same medium to give an initial population of *ca* 10^5 CFU/mL. Native lysozyme and PUL were added aseptically to two of the flasks to give a concentration of 250ppm. The flasks were then incubated at 30^{0}C. Bacterial populations were monitored daily as described in the previous section.

Results and discussion

Inhibition of A. pasteurianus in peptone water

In contrast to NL, which showed no inhibition, PUL had a significant bactericidal effect against *A. pasteurianus.* After 2 hours of exposure to 200ppm of PUL bacterial populations were reduced by more than 95% compared with control (see figure1). The addition of similar levels of NL had small and variable effects on the survival of the bacteria that averaged to a reduction by 6%. This is in agreement with the findings of Ibrahim et al.(20,22) that unlike NL, PUL has an antimicrobial effect against Gram-negative bacteria.

Figure 1: The effect of NL and PUL (200ppm) on different initial populations of *A. pasteurianus* after 2 hours of exposure.



Inhibition of A. pasteurianus in growth conditions

Neither NL nor PUL caused any inhibition of *A. pasteurianus* when added (at 250ppm) to a 1:1 mixture of yeast-extract broth and Chardonnay juice. All three treatments reached maximal populations within 2 days (see appendix B); similar trends were observed in broth and in a wine-broth mixture. The absence of any antimicrobial effect in the broth-juice environment may be due to the bacteria's ability to overcome the PUL's effect during growth or due to the unfolded enzyme's instability. Little is known about the stability of this form of the enzyme and it is possible that the pH and ionic strength of the juice may have caused a conformational change resulting in the loss of its activity against Gram-negative bacteria. Furthermore, observations made by Ibrahim et al. (20,22) as well as during this study indicate that PUL has a strong tendency to aggregate and it is possible that constituents found in the juice as well as in the yeast-extract may have associated with the PUL and caused its precipitation or inactivation. Finally, one more possibility is that since the amount of PUL is limited, the effects of its inhibition may be masked by the rapidly growing bacterial population.

Conclusion

In peptone water, 200ppm of PUL caused more than a three-log reduction in populations of *A. pasteurianus* within two hours; a similar concentration of NL had no inhibitory effect. This supports the findings of Ibrahim et al. (20,22) that PUL has a bactericidal effect on Gram-negative bacteria. However, the same levels of PUL in a mixture of broth and juice, had no inhibitory effect. The findings of this study verify the existence of an antimicrobial effect of PUL against Gram-negative bacteria and for the first time against the wine spoilage organism *A. pasteurianus*. However, application of PUL as an antimicrobial in winemaking or other food industries may be impossible due to its inability to exert its effect in such environments, either due to its instability or to its limited capacity.

LYSOZYME INHIBITION OF LACTIC ACID BACTERIA IN FERMENTING GRAPE JUICE

Abstract

To provide a possible alternative to the use of sulfur dioxide in winemaking, and address the issue of stuck wine fermentations, I studied the efficacy of native lysozyme (NL) and partially unfolded lysozyme (PUL) as antimicrobials in grape juice. *Lactobacillus kunkeei*, a bacterium associated with the induction of stuck fermentations and the production of high levels of volatile acidity (VA), was added to Chardonnay and Pinot Noir juices in combination with the two forms of lysozyme; the juice was inoculated 48 hours later with yeast strain EC1118. In both juices, addition of 250ppm of either NL or PUL reduced populations of *L. kunkeei* to undetectable levels within 24 hours while in grape juices that contained no lysozyme, populations reached 10^9 CFU/mL after two days; grape juices supporting these populations of *L. kunkeei* developed 2-3g/L VA, up to 14 times higher than the control treatment or those containing lysozyme. No significant differences were noticed between the control, the NL, and the PUL treatments. Pinot Noir juice fermented in the presence of *L. kunkeei* had a reduced alcohol content (ca 11.5% compared to ca 12.7% in the control and lysozyme treatments) and VA levels exceeding 2g/L. In Chardonnay, though much more variability was observed, the addition of either form of the enzyme always kept VA levels comparable to those developed in the control while the treatments containing *L. kunkeei* and no lysozyme, developed between 3 and 14 times more VA.

Introduction

Stuck and sluggish fermentations continue to be a source of economic loss to the wine industry (1,2). Among the various factors that may contribute to the stress on yeast and cause a stuck fermentation, the growth of bacteria during fermentation warrants more attention. Lactic acid bacteria (LAB) such as *Lactobacillus kunkeei* and certain strains of *Oenococcus oeni* have been shown to be able to cause stuck fermentations while producing high levels of acetic acid and possibly other inhibitory substances (7,15). The proliferation of acetic acid bacteria (AAB) during alcoholic fermentation has also been demonstrated to cause stuck fermentations (5). The growth of such bacteria, even when limited in extent and duration, can nevertheless produce enough inhibitory metabolites to act together with other factors to possibly cause a stuck fermentation or produce an otherwise unacceptable wine.

In most cases such problems can be avoided by the addition of sulfur dioxide (SO_2); however, public concerns have led winemakers to try to minimize the use of SO_2 (9,11,41). Not only is SO_2 toxic to certain individuals but it can also become a stress factor in stuck and sluggish fermentations as it can be toxic to yeast and cause nutrient depletion (2,3,9). In these scenarios and others, such as organic wines, where the permitted level cannot exceed 100ppm, or in high pH wines where its efficacy is greatly reduced, an alternative antimicrobial is needed.

Lysozyme is a bacteriolytic enzyme commercially produced from hen egg white that has been shown to be effective in the control of maololactic fermentation and the protection against microbial spoilage of wine by LAB (12,13,14). Since it targets the bacterial cell wall, lysozyme has limited activity against Gram-negative bacteria (such as AAB), which are protected by an outer membrane (15,25,34). However, recent studies have shown that by means of a simple heat treatment, lysozyme's activity can be altered to include Gram-negative bacteria as well (20,22). A heat treatment of 20 minutes at pH 6 and 80^{0}C yields a partially unfolded form of lysozyme that is inhibitory towards both Gram-positive and Gram-negative bacteria

(20,22). Such a form of the enzyme can therefore be used to inhibit both LAB and AAB in wine and can thus become an effective substitute for the antimicrobial role of SO_2 in winemaking.

The objectives of this study were to examine the efficacy of NL and PUL in protecting wine fermentations from the proliferation of LAB and its possible consequences.

Materials and Methods

Microorganisms and media

Lactobacillus kunkeei was received from Professor Charlie G. Edwards (Washington State University, Pullman, WA) and yeast strain Prise de mousse (EC1118) was obtained from Lallemand (Montréal, Canada). The bacteria were cultured in modified Rogosa medium (appendix D) and enumerated using MRS agar (Difco, Detroit, MI) to which 50ppm of Nystatin (Sigma, St. Louis, MO) were added to prevent the growth of yeast and molds.

Lysozyme

Native chicken lysozyme was obtained from Fordras (Lugano, Switzerland) while partially unfolded lysozyme was a gift from Professor Hisham Ibrahim (Yamaguchi University, Japan). Both were rehydrated from a freeze-dried preparation in a pH 7.2 phosphate buffer just prior to addition to grape juice.

Grape Juice

Chardonnay grapes were picked from the Oregon State University vineyard during fall 1999, crushed and pressed, adjusted to 40ppm total SO_2 (using potassium metabisulfite) and frozen. Before use the juice was thawed and diluted to 21°B using distilled water. pH was adjusted to 3.8 using $KHCO_3$, and H_2O_2 was added to lower the SO_2 content to 3ppm total (7). Indigenous yeast populations were reduced by adding 300ppm dimethyl dicarbonate (Miles Inc., Pittsburgh, PA).

Pinot Noir was picked at the Oregon State University vineyard during fall 1999, crushed, adjusted to 50ppm total SO_2 (using potassium metabisulfite) and frozen. Before use the must was thawed and the juice was pressed off the skins; the juice was then adjusted to 22°B and pH 3.8 as described above. To avoid an early onset of fermentation, the indigenous yeast populations were reduced to less than 1 CFU/mL by heating the juice for 10 minutes at 55°C.

Inhibition of bacteria during fermentation

Lactobacillus kunkeei was grown for three days at 30°C and then transferred (0.1ml) into 50ml of fresh medium and incubated once again for three days. Twenty-five mls was taken, spun down, washed in a pH7.2 phosphate buffer and spun again. The bacterial pellet was resuspended in a phosphate buffer and inoculated into the juice to give an initial population of *ca* 10^5 CFU/mL. Inoculation of yeast was two days after the addition of bacteria. Yeasts were rehydrated in 40°C phosphate buffer (pH 7.2) for 20 minutes (0.1g yeast in 10mL buffer) before dispensing into the juice to give an initial population of *ca* 10^5 CFU/mL.

Fermentations were conducted in aseptic jars fitted with air locks, each containing 100mL of juice. Periodic sampling was done aseptically for 11 days at which point wines were analyzed.

Chemical analysis of wine

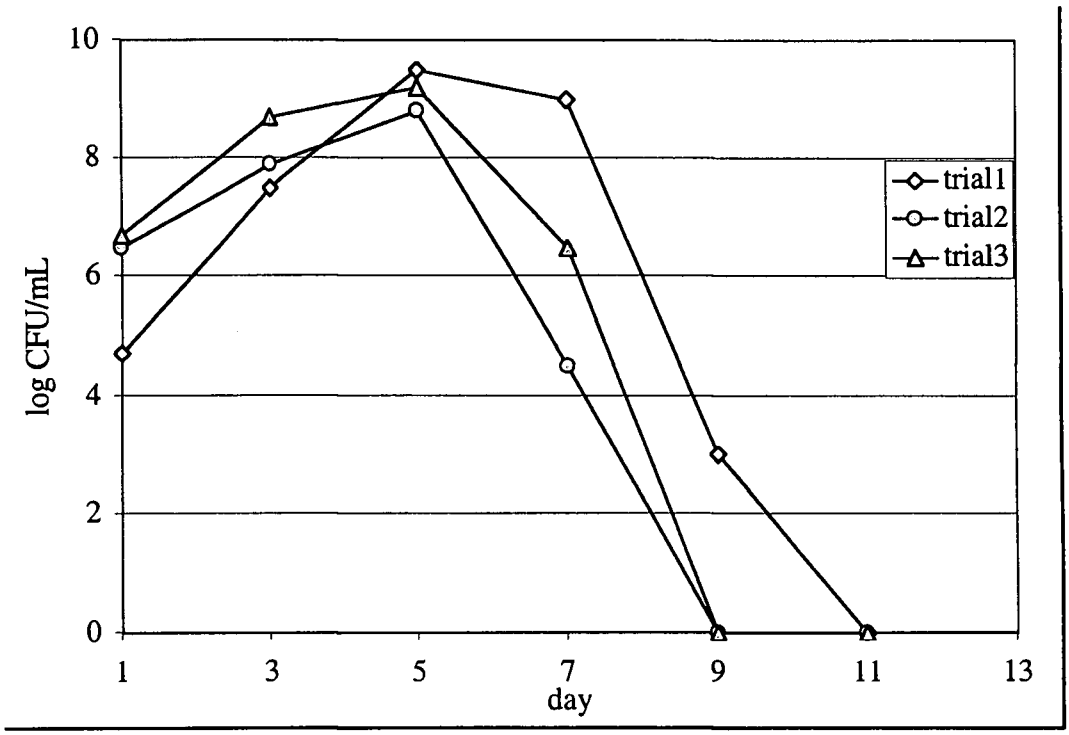
Alcohol was determined by ebulliometry, titratable acidity by titration with NaOH, volatile acidity using a cash still, and sulfur dioxide using the Ripper method (41). Residual sugar concentration was determined using the DNS method (38)

Results and Discussion

The addition of either NL or PUL reduced bacterial populations of *L. kunkeei* to undetectable levels within 24 hours. No resurgence in bacterial populations was detected during the 10 days following inoculation. In grape juices free from lysozyme, the bacterium reached levels of *ca* 10^9 CFU/mL and persisted for up to 10 days (see figure 2). The bacteria survived for longer periods in Chardonnay fermentations, probably due to the slower fermentation rate. Wines supporting large populations of *L. kunkeei* showed elevated levels of VA and TA; the amount of VA produced did not account for the observed increase in TA, suggesting that other acids were produced in the process as well.

Pinot Noir juice inoculated with *L. kunkeei* and yeast, but containing no lysozyme, developed more than 2g/L VA, about 10 times more than juices fermented with just yeast or those protected by lysozyme. In addition to high VA levels, these fermentations reached a final alcohol level of 11.2-11.9% compared with 12.3-12.9% in the fermentations protected by lysozyme. The differences in residual sugar concentrations did not reflect the differences in alcohol, suggesting that these sugars were metabolized by the bacteria. Wines protected by either form of lysozyme showed no significant differences compared with the control (see table p.24).

Figure 2: Growth of *L. kunkeei* in Pinot Noir, yeast added at day 3.



Chemical analysis of three trials of Pinot Noir fermented with just yeast, with yeast and *L. kunkeei* and with yeast, *L. kunkeei* and 250ppm NL.

	Just Yeast			*L. Kunkeei* + Lysozyme			*L. Kunkeei*		
	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3
Alcohol*	12.7	12.4	13.2	12.7	12.3	12.9	11.9	11.2	11.8
pH	3.6	3.6	3.5	3.7	3.6	3.5	3.6	3.5	3.4
TA	8.4	7.3	8.3	8.2	6.8	8.3	11.1	9.7	11.9
VA	0.2	0.2	0.3	0.3	0.2	0.3	2.0	2.1	2.6
RS	0.1	0.1	0.2	0.2	0.1	0.1	0.1	0.3	0.3

*Alcohol expressed as % by volume, titratable acidity (TA) as grams of tartaric acid per liter, volatile acidity (VA) as grams of acetic acid per liter, and residual sugar (RS) as % glucose by weight.

In Chardonnay fermentations, a greater variability was experienced between trials in terms of alcohol and residual sugar levels reached. However, a few trends were consistent throughout all trials: In fermentations containing either form of the enzyme, populations of *L. kunkeei* were always reduced to less than 10 CFU/mL within 24 hours of inoculation; VA levels of wines containing *L. kunkeei* but no lysozyme were as high as 3.5g/L and were always at least three times higher than those of either the control or the ones protected by lysozyme (see appendix A). As was the case with the Pinot Noir fermentations, no differences were detected between the action of NL and that of PUL in terms of bacterial inhibition or of the chemical composition of the finished wines.

Conclusion

Native lysozyme can prevent the proliferation of *L. kunkeei* in grape juice and can reduce initial loads of 10^6 CFU/mL to less than 10 CFU/mL within 24 hours. Its use can minimize production of VA and possibly other inhibitory substances. The same is true for both forms of the enzyme and in both Chardonnay and Pinot Noir juices. In terms of the enzyme's effect on the yeast or the other parameters of the wine, no differences were detected between the control and the wines protected by either form of the enzyme. Our findings suggest that lysozyme can be used effectively to ward off grape juice spoilage by *L. kunkeei* (and possibly other LAB) and thus prevent production of high levels of VA and prevent the development of a stuck fermentation.

CONCLUSION

The growth of *L. kunkeei* during wine fermentation can result in wines that are low in alcohol and high in VA and may possibly lead to a stuck fermentation. I have shown that both NL and PUL can be used to eliminate initial populations and suppress subsequent growth of *L. kunkeei* in grape juice and thus prevent such problems.

I have observed that PUL also has a bactericidal effect against *A. pasteurianus*. However, this effect was only observed in a simple peptone water system and not in a mixture of broth and grape juice. Native lysozyme did not cause inhibition of *A. pasteurianus* in either peptone water or a broth-juice mixture. These findings support those of Ibrahim et al. (20,22) that PUL, unlike NL, has an antimicrobial effect against Gram-negative bacteria. On the other hand our findings suggest that the application of PUL in more complex systems such as foods and beverages, may not be practical due to its inability to cause inhibition in such environments.

Therefore, both NL and PUL can be used to prevent the growth of *L. kunkeei* (and possibly other LAB) in fermenting must and thus reduce the risk of stuck fermentations. Partially unfolded lysozyme can also inhibit the growth of *A. pasteurianus* but only in peptone water; it is therefore not a suitable substitute for SO_2 in preventing the growth of AAB in fermenting must.

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APPENDICES

Appendix A: Growth of *L. kunkeei* in Fermenting Chardonnay Juice.

Figure A1: Growth of *L. kunkeei* in fermenting Chardonnay juice (yeast added at day 3); trial 1.

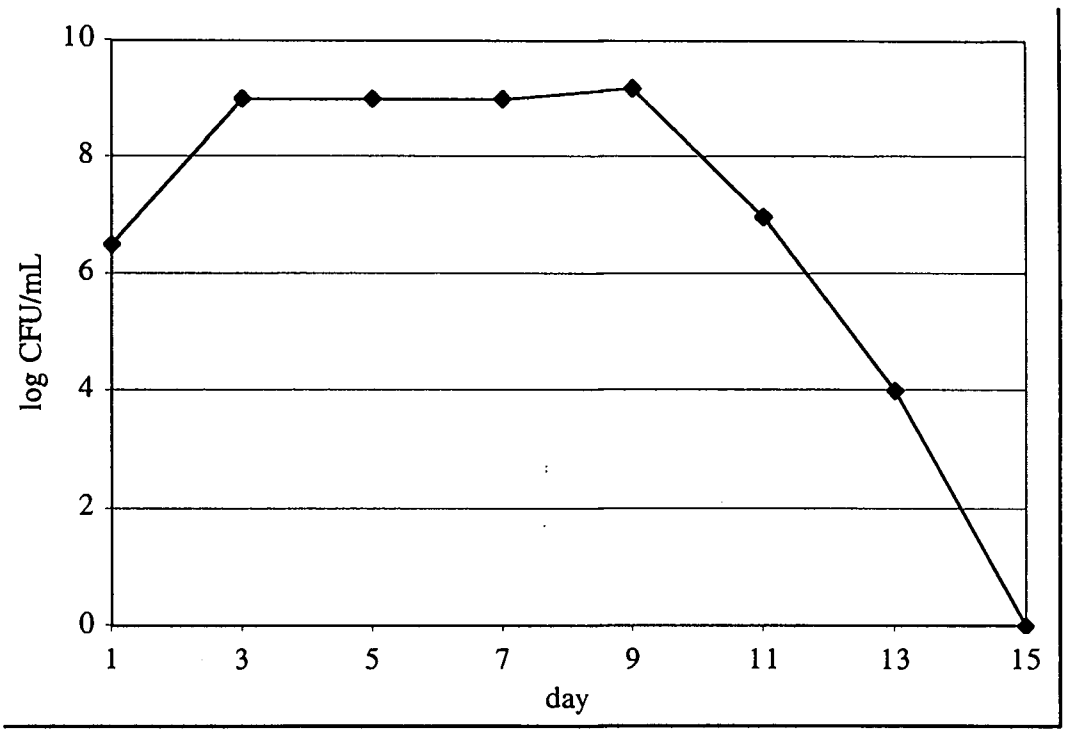


Table A1: Chemical analysis of Chardonnay fermented with yeast (Y), yeast, *L. kunkeei* and 250ppm lysozyme (KYL), and yeast and *L. kunkeei* (KY), trial 1.

	Y	KYL	KY
Alcohol*	11.5	11.5	11.3
TA	8.4	8.3	9.6
VA	0.10	0.10	1.40
RS	0.2	1.2	0.2

*Alcohol expressed as % by volume, titratable acidity (TA) as grams of tartaric acid per liter, volatile acidity (VA) as grams of acetic acid per liter, and residual sugar (RS) as % glucose by weight.

Figure A2: Growth of *L. kunkeei* in fermenting Chardonnay juice (yeast added at day 3); trial 2.

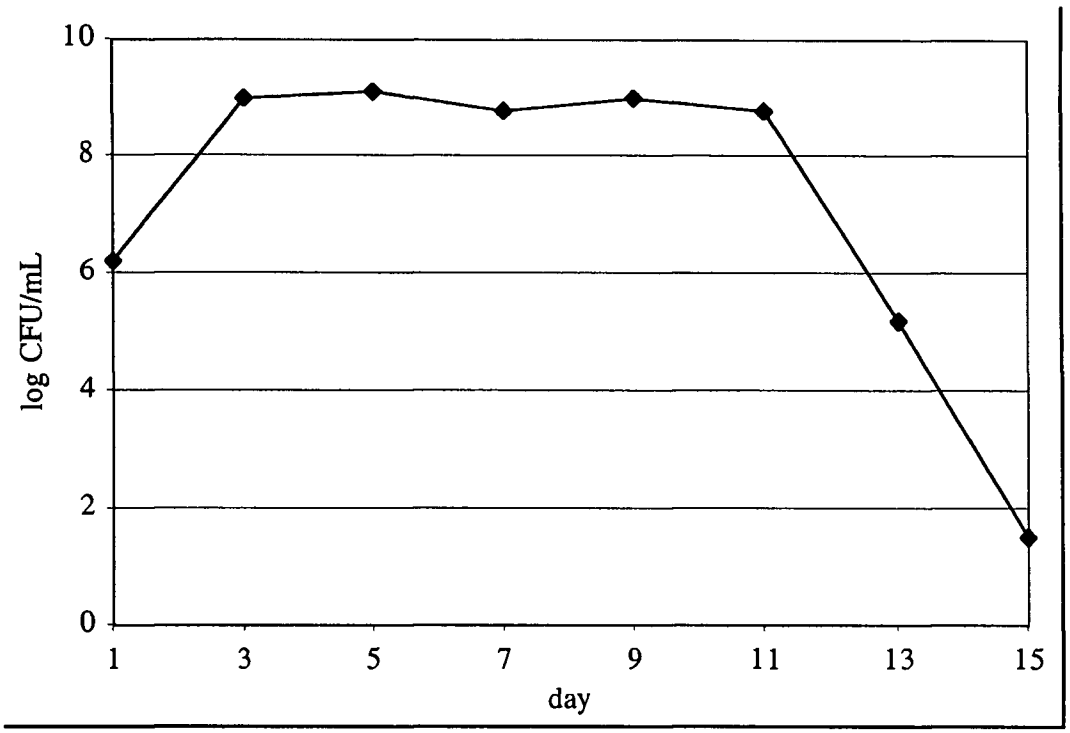


Table A2: Chemical analysis of Chardonnay fermented with yeast (Y), yeast, *L. kunkeei* and 250ppm lysozyme (KYL), and yeast and *L. kunkeei* (KY), trial 2.

	Y	KYL	KY
Alcohol	9.9	11.3	11.4
TA	5.6	7.1	8.9
VA	0.1	0.1	0.5
RS	4.5	1.8	0.1

*Alcohol expressed as % by volume, titratable acidity (TA) as grams of tartaric acid per liter, volatile acidity (VA) as grams of acetic acid per liter, and residual sugar (RS) as % glucose by weight.

Figure A3: Growth of *L. kunkeei* in fermenting Chardonnay juice (yeast added at day 3); trial 3.

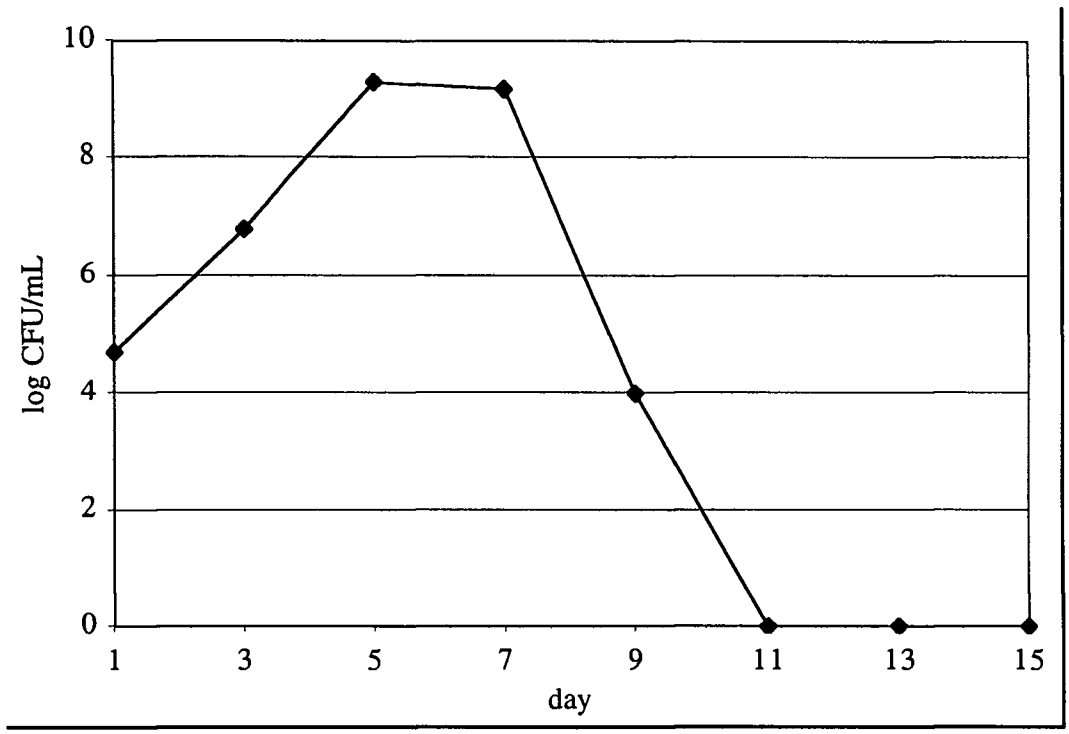


Table A3: Chemical analysis of Chardonnay fermented with yeast (Y), yeast, *L. kunkeei* and 250ppm lysozyme (KYL), and yeast and *L. kunkeei* (KY), trial 3.

	Y	KYL	KY
Alcohol	13.0	12.9	12.4
pH	3.6	3.6	3.6
TA	8.0	7.8	8.7
VA	0.19	0.18	1.24
RS	0.11	0.14	0.26

*Alcohol expressed as % by volume, titratable acidity (TA) as grams of tartaric acid per liter, volatile acidity (VA) as grams of acetic acid per liter, and residual sugar (RS) as % glucose by weight.

Figure A4: Growth of *L. kunkeei* in fermenting Chardonnay juice (yeast added at day 3); trial 4.

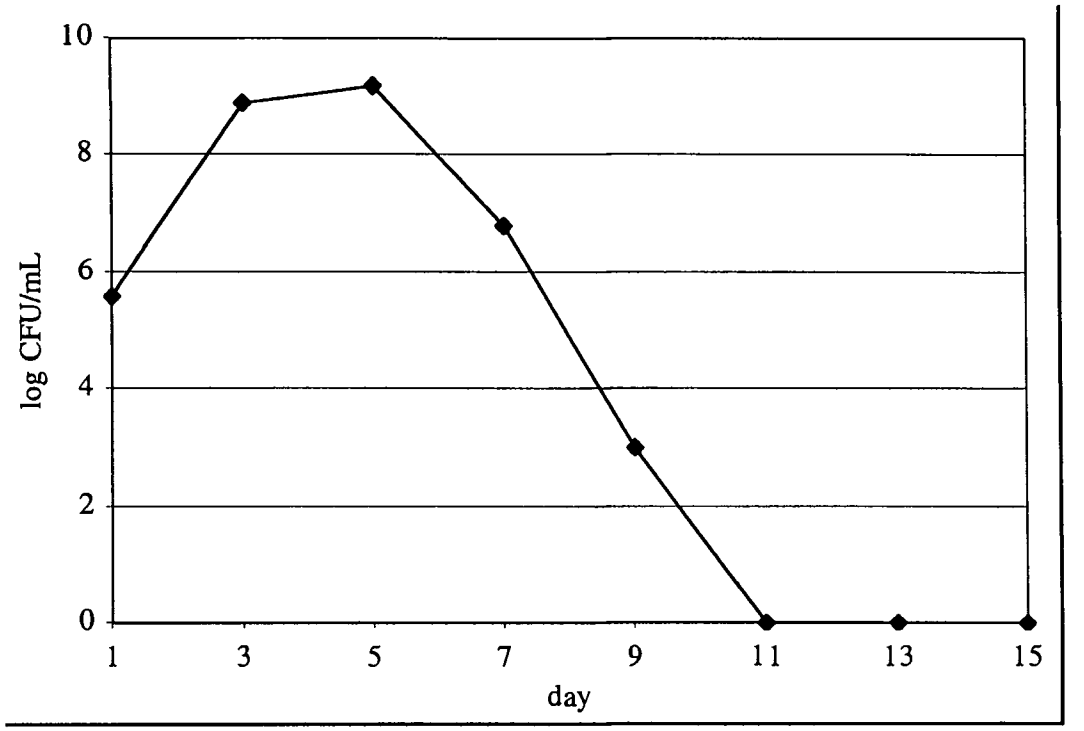


Table A4: Chemical analysis of Chardonnay fermented with yeast (Y), yeast, *L. kunkeei* and 250ppm lysozyme (KYL), and yeast and *L. kunkeei* (KY), trial 4.

	Y	KYL	KY
Alcohol	7.95	7.7	9.8
pH	3.66	3.69	3.4
TA	6.7	6.6	11.9
VA	0.84	0.81	2.55
RS	6.27	6.27	3.26

*Alcohol expressed as % by volume, titratable acidity (TA) as grams of tartaric acid per liter, volatile acidity (VA) as grams of acetic acid per liter, and residual sugar (RS) as % glucose by weight.

Figure A5: Growth of *L. kunkeei* in fermenting Chardonnay juice (yeast added at day 3); trial 5.

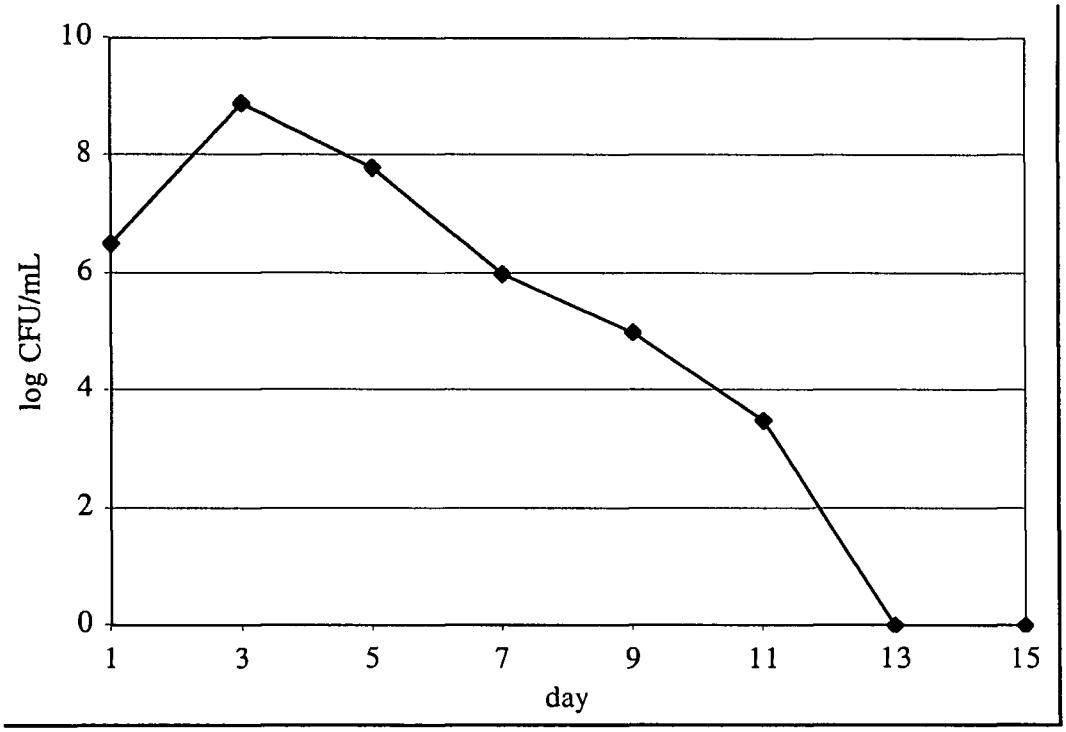


Table A5: Chemical analysis of Chardonnay fermented with yeast (Y), yeast, *L. kunkeei* and 250ppm PUL (KYL), and yeast and *L. kunkeei* (KY), trial 5.

	Y	KYL	KY
Alcohol*	8.5	8.72	9.15
pH	3.8	3.8	3.5
TA	6.8	6.9	13.5
VA	0.90	0.87	3.30
RS	6.16	5.85	3.48

*Alcohol expressed as % by volume, titratable acidity (TA) as grams of tartaric acid per liter, volatile acidity (VA) as grams of acetic acid per liter, and residual sugar (RS) as % glucose by weight.

Appendix B: Growth of *A. pasteurianus* in Broth Mixtures.

Figure B1: Growth of *A. pasteurianus* in a juice-broth mixture in the presence of NL and PUL

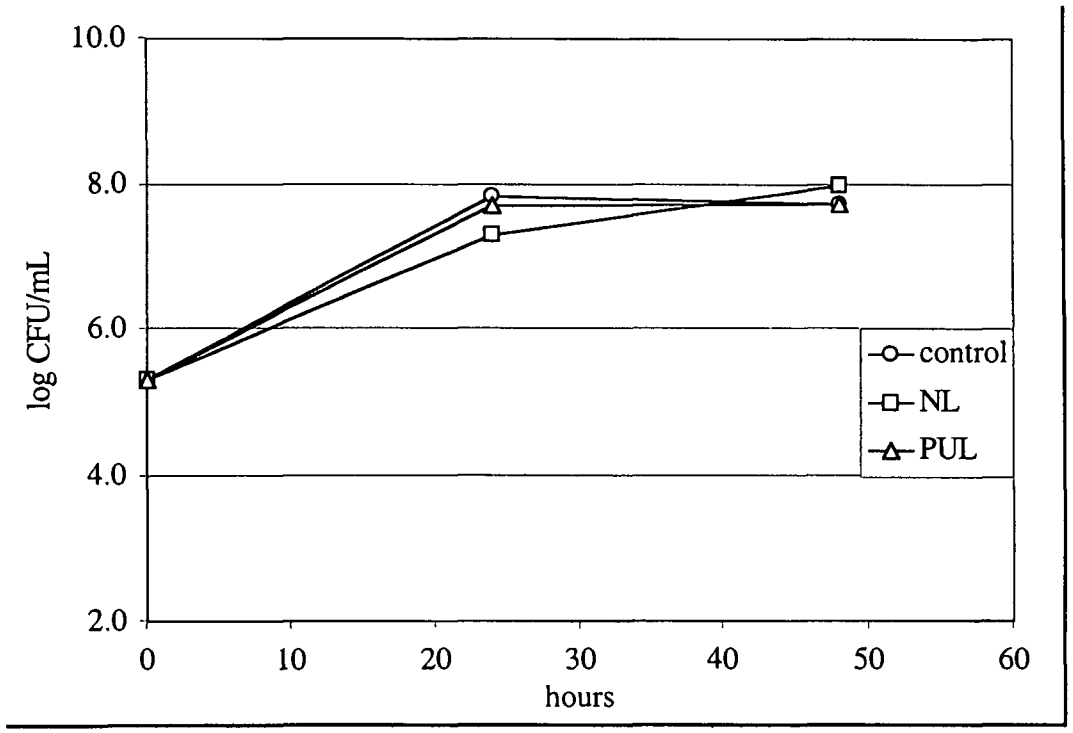
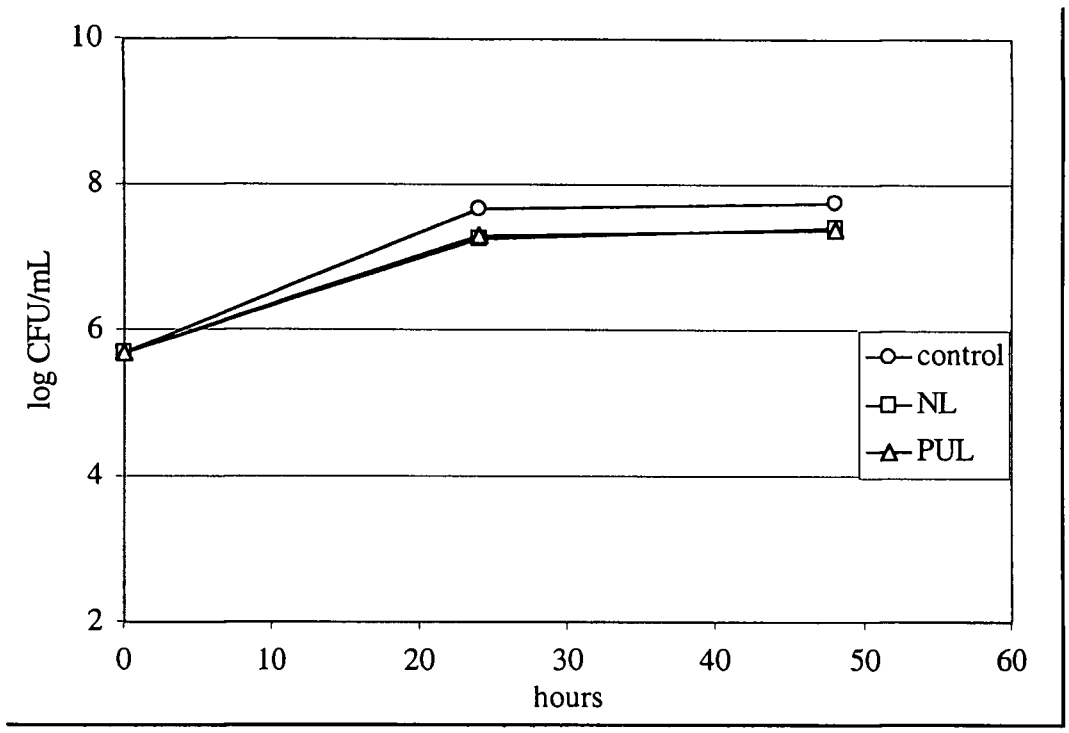


Figure B2: Growth of *A. pasteurianus* in a wine-broth mixture in the presence of NL and PUL



Appendix C: Heat Instability of Native Lysozyme in Wine

Haze formation

Six commercial wines (Chardonnay and Sauvignon Blanc) were mixed with lysozyme to give 4 different concentrations and then left at room temperature overnight. The wines were then heated in a water bath either for six hours at 80°C or for 48 hours at 55°C. At the end of the heating period the wines were cooled to room temperature. Haze was measured using a DRT100B Turbidimeter (HF Instruments, Fort Myers, FL).

Figure C1: Haze formation (in NTUs) in six commercial white wines with four different lysozyme levels (0, 50, 100 and 312ppm) after a heat treatment of 80°C for six hours (values exceeding 100NTUs appear on bars).

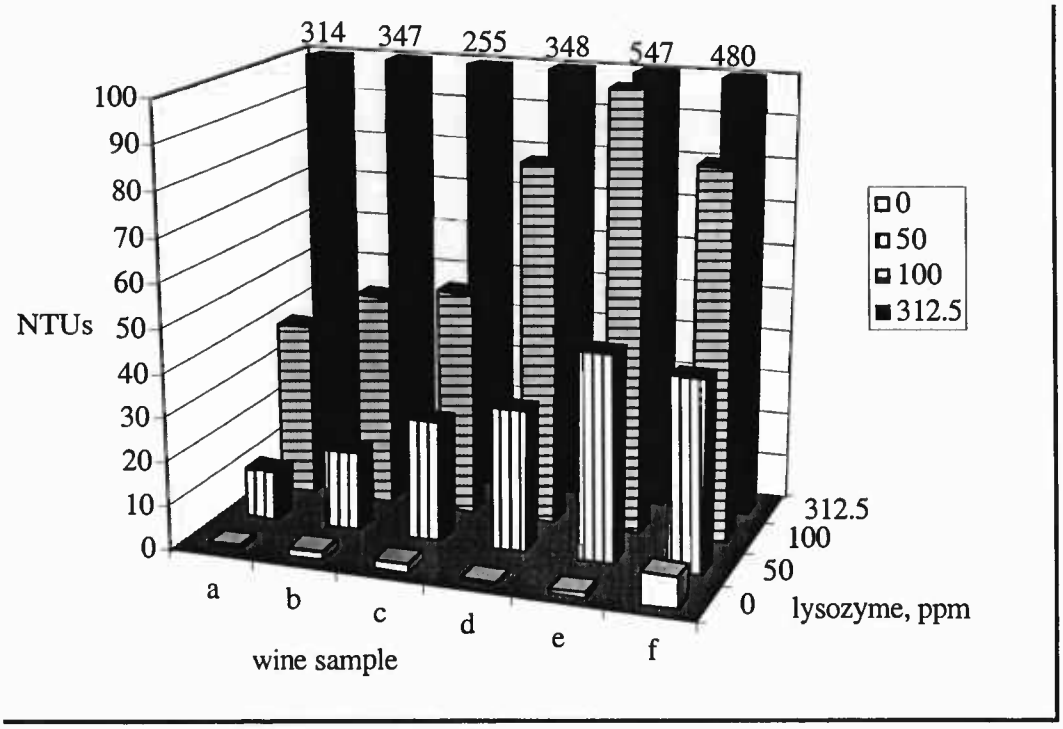
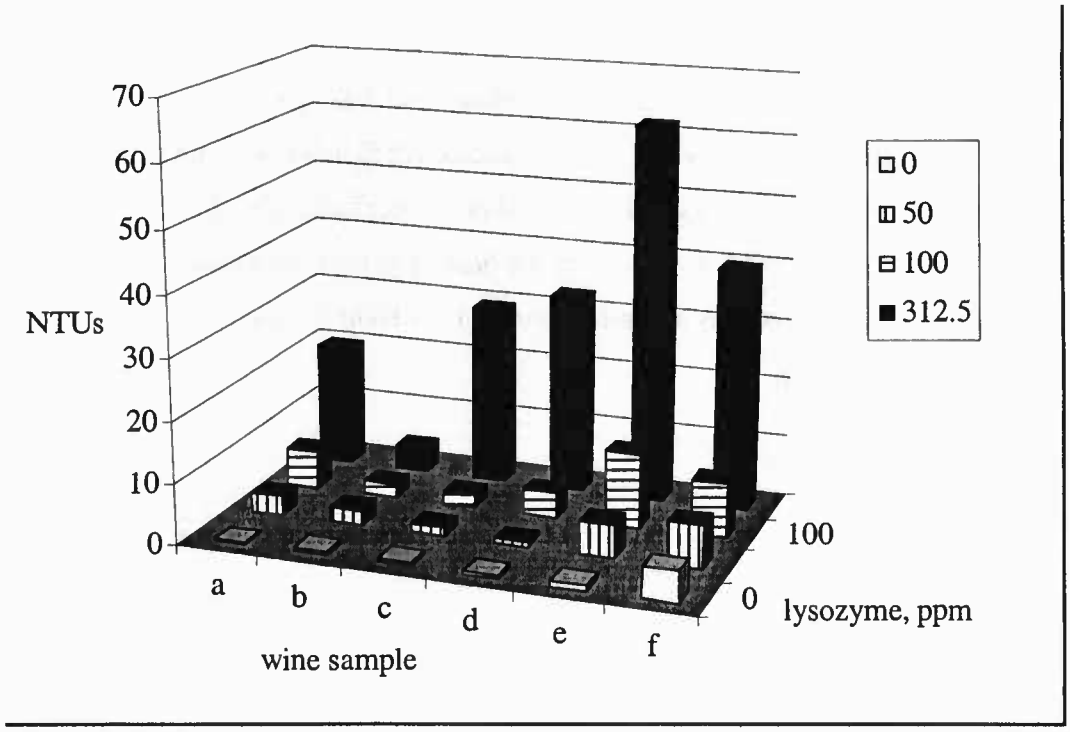


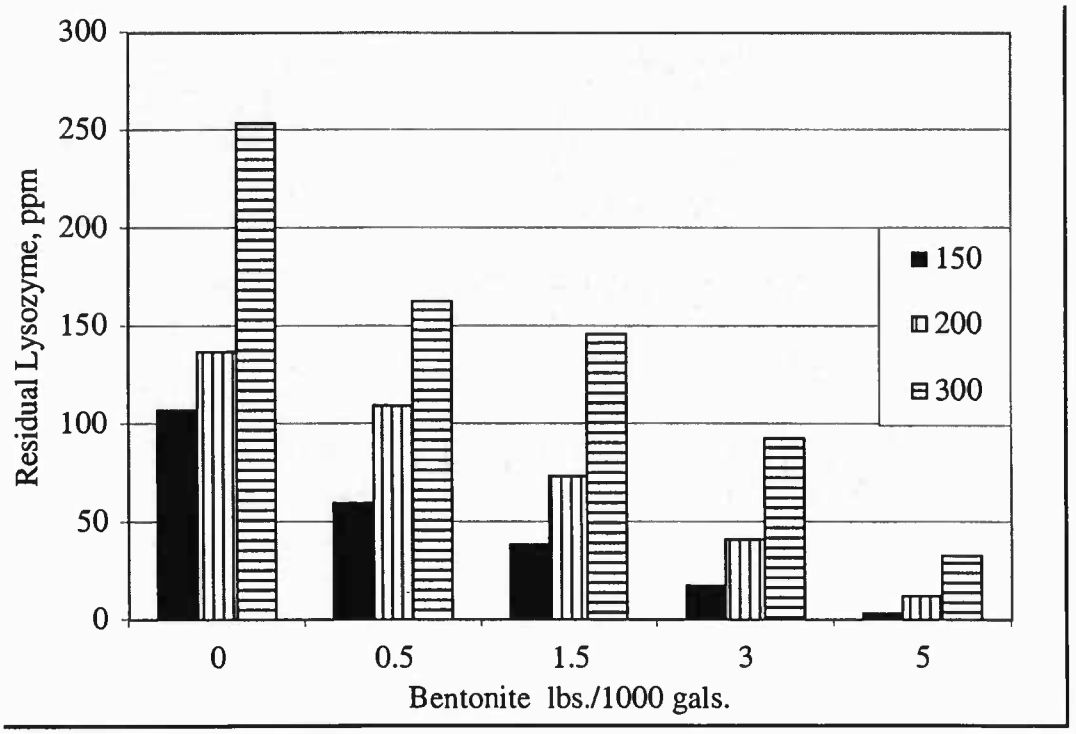
Figure C2: Haze formation (in NTUs) in six commercial white wines with four different lysozyme levels (0, 50, 100 and 312ppm) after a heat treatment of 55^0C for 48 hours.



Removal of lysozyme with Bentonite.

Chardonnay wine was made in a commercial California winery and was treated with different levels of lysozyme. Bentonite fining trials were performed and the wines were then analysed in our laboratory for residual lysozyme activity using the turbidimetric assay (37).

Figure C3: Residual lysozyme concentrations in a chardonnay wine with three initial levels of lysozyme (150, 250, and 300ppm) after treatment with bentonite.



Appendix D: Recipe for Modified Rogosa Medium.

(C. Edwards, personal communication, 2001)

For 1L of medium :

1. Mix100mL of distilled water with 1g of beef extract for 30 minutes then filter through Whatman filter paper #1.
2. Weigh 20g of tryptone, 5g of peptone, 5g of glucose and 5g of yeast extract and mix with 700mL of distilled water.
3. Add 1mL of a 5% Tween 80 solution, 200mL Martinelli apple juice and the filtrate obtained at step 1.
4. Adjust the pH to 4.5 using 50% H_3PO_4.
5. Autoclave for 21 minutes at 121^{0}C.