Oregon Wine Advisory Board
Research Progress Report

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The Use of Nisin and Nisin Resistant Strains of *Leuconostoc oenos* to Control Malolactic Fermentation and to Prevent the Growth of Spoilage Bacteria in Oregon Wines

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Objectives: To develop methodologies to allow winemakers to precisely control the malolactic fermentation and to prevent spoilage of wines by undesirable bacteria.

Progress

Abstract
Nisin, a bactericidal polypeptide, has the potential to inhibit spoilage bacteria and control rnatolactic fermentation in wines. Nisin activity has previously been observed to remain stable in white wines but to decrease in red wines. This decrease is believed to be directly proportional to the concentration of polyphenolic compounds in the wines. Six polyphenolic components of grapes and wine (gallic acid, catechin, myricetin, quercetin, malvidin 3,5-diglucoside, and crude grape tannin) were tested individually in a model wine system. Tannin resulted in the largest decrease in nisin activity. Grape tannin was further evaluated using a series of red wines ages for two, four, and six months before the addition of nisin. These studies showed a greater loss of nisin activity in the wine aged for six months that in the younger wines. Identical sets of wine, aged for two, four, and six months at either 4'C or 23'C before nisin was added, showed in each case that increased temperature resulted in an increased loss of nisin activity. This information may be valuable for winemakers who are considering the addition of fiisin to their wines to inhibit (or control) malolactic fermentation and the growth of spoilage lactic acid bacteria.

Introduction

Nisin, a bactericidal polypeptide produced by Lactococcus lactis , has been shown to be active against gram-positive bacteria, but not gram-negative bacteria or yeast (3). These antimicrobial properties have proven effective for inhibiting the lactic acid bacteria during winemaking (2)(11)(12). Adding nisin in combination with a nisin-resistant strain of Leuconostoc oenos may provide winemakers with a means of controlling the malolactic fermentation.

Nisin may have the potential to replace (or reduce) sulfiting agents traditionally added to wines to prevent the growth of spoilage lactic acid bacteria. This may be valuable since sulfites are believed to cause toxic responses in sensitive individuals (17)(20). As a result, the U.S. Food and Drug

Administration withdrew the GRAS (generally recognized as safe) status of sulfiting agents in 1986 and required their declaration on labels when concentrations present exceeded 10ppm (6).

Extensive studies have shown that nisin is a safe, non-toxic antimicrobial agent that is non-allergenic to humans (4)(7). It has been affirmed as GRAS by the U.S. Food and Drug Administration (Federal Register, April 6, 1988) for use in pasteurized chesse spreads as levels up to 250 Units/g to prevent the growth and toxin production of Clostridium botulinum. Nisin has not yet been approved for use in wines, however, petitions are currently pending.

Nisin was shown to retain activity in white wines but to decrease to less than 90% in red wines within four months (2). We hypothesize that this may be the result of nisin binding to polyphenolic compounds, which occur in red, but not white wines (15).

Although red wines prior to aging may contain up to 6000 mg/l of total phenols (14)(15), more typically, a young red light table wine might contain 1200 mg/L (15). Young white wines contain fewer, averaging about 200 mg/L (15). Tannin is the largest group of phenolic compounds found in red wine. Polymeric tannins form as a normal maturation reaction in wine (1)(9)(15)(18)(19) when phenolic compounds, such as anthocyanins, react with the monomeric tannins and polymerize. This creates dimers and trimers with molecular weights between 500 and 3000 (15)(23). These tannins, which may further polymerize to contain 8 to 14 flavonoid units, are capable of precipitating protein (8)(10)(15)(21)(22)(23)(24). Some winemakers, taking advantage of this reaction, use tannin as a fining agent to remove protein from wine. However, if tannin binds to the nisin polypeptide and inhibits its activity, this may create a problem for winemakers wanting to use nisin for control of malolactic fermentation. This tannin protein reaction may be useful, however, for elimination residual nisin, just as bentonite, another common fining agent, had already proven effective in nisin-removal from wines (2).

Although nisin in white wines should remain stable for the duration of the malolactic fermentation, the loss of nisin activity in red wines may present special problems. The purpose of this investigation was to explore the interactions between nisin and some of the components found in wines to better understand the factors which contribute to loss of nisin activity. For winemakers wanting to control malolactic fermentation, inhibit spoilage lactic acid bacteria, and reduce the use of sulfites, this information may be useful in determining when to introduce nisin, and what quantity to add.

Materials and Methods

Preparation of Wine Samples Pinot Noir and Cabernet Sauvignon 1990 harvest grapes were obtained from the Oregon State University vineyards. Grapes were crushed and destemmed, then free-run juices (with minimum skin contact) and juice left in contact with the skins (for maximum concentration of grape skin extractable components) were collected separately and made into wine. The new wines were racked and then frozen in air tight vessels until used.

Wine samples containing vary concentrations of pigments and polyphenols were obtained by serially diluting the fully pigmented wines in 25% increments with corresponding wine made from free-run juice with minimal pigment and phenolic content.

Identical sets of wine were places at different temperatures (4'C and 23'C) and allowed to age for two, four, and six months. Then 100 Units/ml of nisin was added to these wines, and samples were immediately assayed by agar well diffusion for nisin activity.

All wines were filter-sterilized by 0.2um membrane filters to prevent microbial contamination. Each

tube was completely filled and then sealed with parafilm to reduce oxidation. Samples were stored in the dark.

Model Wine System: A sterile base wine consisting of ethanol (13% v/v) and tartaric acid (2 g/L) was adjusted to pH 3.5 with KOH. Five flavonoid and one non-flavonoid wine components were filter-sterilized and added to aliquots of base wine at the levels given in Table 1.

Table 1. Concentration of compounds tested with nisin in a model wine system.

Phenolic Compound	Concentration (mg/L)
Myricetin [a]	50
Gallic acid [a]	30
Quercetin [a]	5
Catechin [a]	250
Grape Tannin [b]	200
Malvidin 3,5-diglucoside [a]	150

a. Sigma Chemical Co., St. Louis, MO.
b. GFSR, Santa Rosa, CA.

The amount of each component used in the model wine system was comparable to the level that might be found in a typical young red table wine (16). Nisin was added to each sample at a concentration of 110 Units/ml. All samples were shielded fom light, protected from oxidation, and maintained at a constant temperature (10'C).

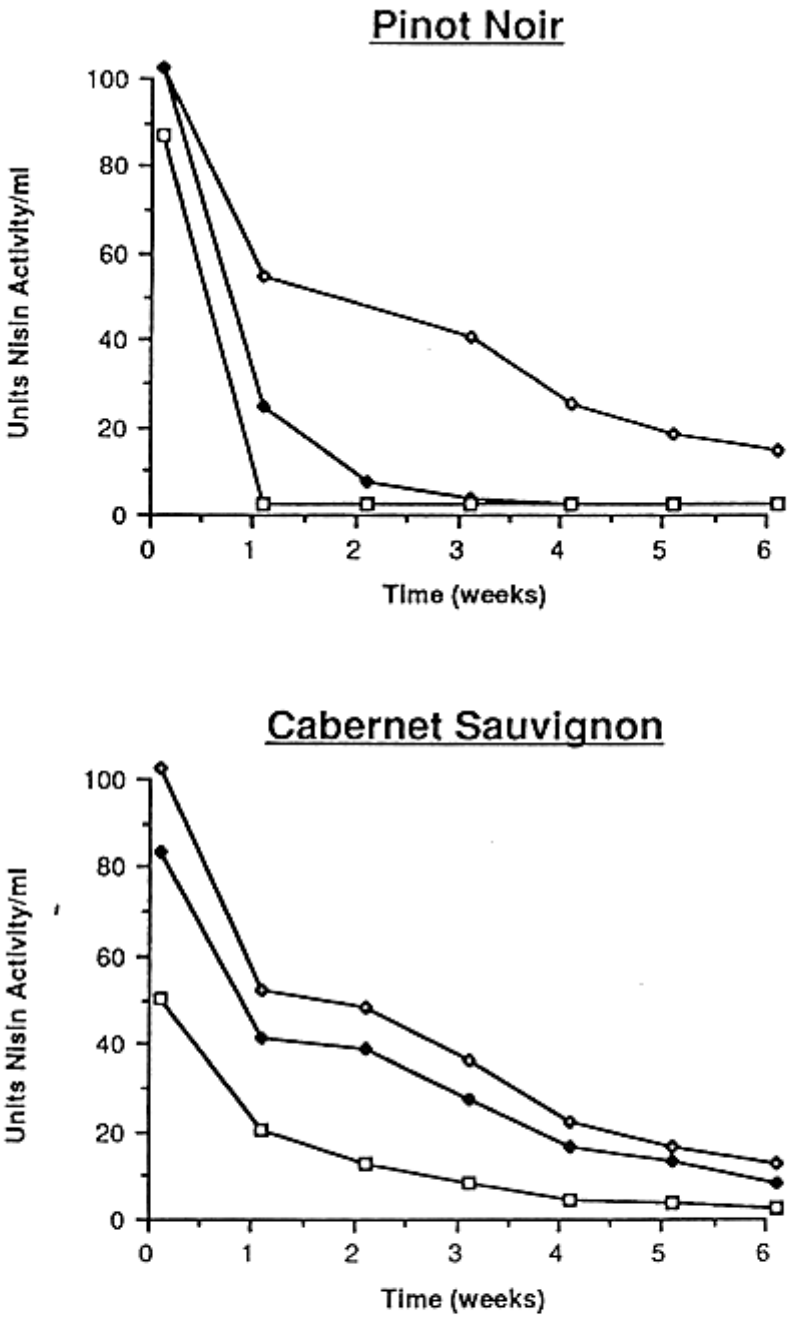
Nisin Assays: A Nisin preparation obtained from Aplin and Barrett Ltd. (Trowbridge, U.K) contained 37 x 10^6 international units/g (lot code 201H). A working stock solution of 10,000 Units/ml was prepared by solubilizing the nisin preparation in 0.01 N HCL (~pH 2.0) and storing frozen in the dark. Nisin activity was determined by agar well diffusion bioassay using Pediococcus pentosaceus FBB-61-2 as the sensitive indicator strain (2). Bioassay plates were prepared by adding a 0.1% inoculum of a log phase culture of the indicator to 50 ml of tempered MRS agar (Difco Laboratories, Detroit MI) that was then poured into petri plates. Wells were made (6.5 mm diameter).within the agar using a sterile brass cork borer. Standard nisin solutions and nisin-containing wine samples were added to wells in volumes up to 100 ul. For enhanced detection of nisin activity, well plates containing samples were allowed to prediffuse for up to 24-hours at 4'C prior to incubating at 37'C for outgrowth of the indicators.

Measurement of Wine Pigments: Wine samples were acidified with a pH 1.0 solution of 0.2 N KCl(1-part) and 0.2 N HCl(3-parts), and read spectrophotometrically at 520 nm. Anthocyanin concentrations were calculated from the mean value of duplicate samples according to Beers Law (C=Abs/e) using a 1% extinction coefficient of previously determined for Vitis vinifera (21).

Results and Discussion

Relationship between the quantity of wine pigment and nisin: Red wines, serially diluted with corresponding wines made from free-run grape juice before the addition of nisin, had lost a significant amount of nisin activity by the first week (Fig. 1). The wines containing maximum pigment concentration showed the most rapid decrease in nisin activity, while the wines made with minimal pigment lost nisin activity at a proportionally slower rate. Within a period of six weeks nisin activity was less than 10% of original in all sample.

It is likely that the components responsible for decreased nisin activity were derived from the grape skins, since the length of skin contact was the only difference between samples. Grape skins are known to contain high levels of polyphenolic compounds which are imparted to the wine during fermentation (5)(13). We believe the polyphenolic components from extracted grape skins may be responsible for the loss of nisin activity in red wines



◊ wine made with minimum grape skin contact

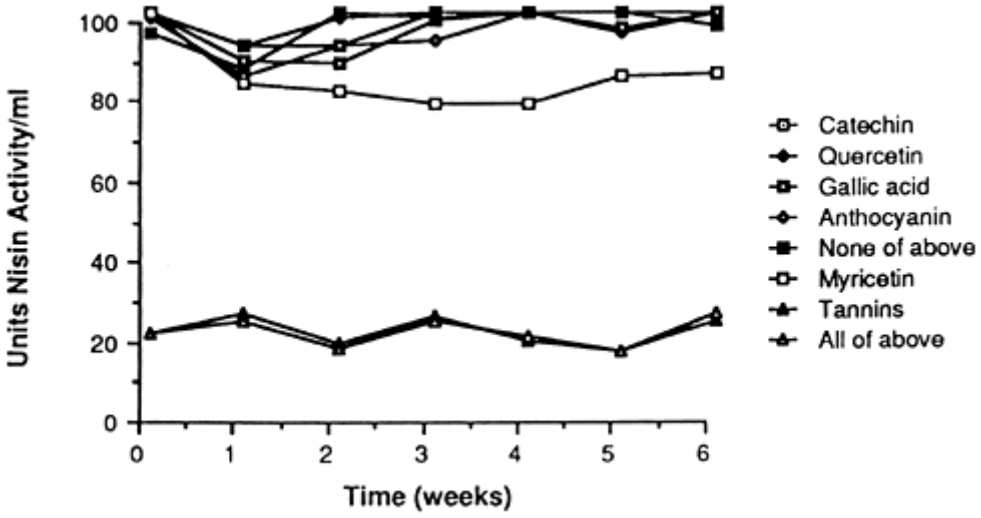
♦ 50% minimum plus 50% maximum grape skin contact

□ wine made with maximum grape skin contact

Fig. 1 The relationship between different concentrations of extractable grape skin components and nisin activity in red wines.

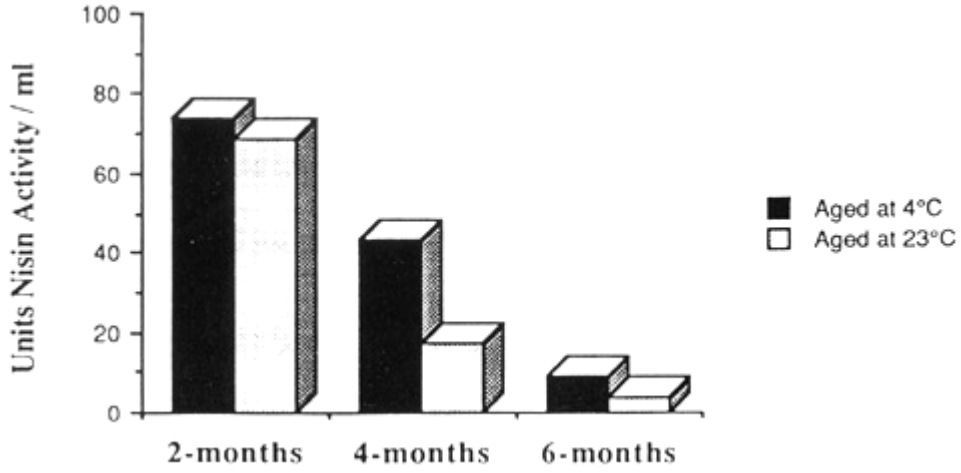
To test this hypothesis, six polyphenolic compounds found in red grapes and wine were examined

individually using the model wine. Anthocyanins were initially suspected of contributing to the loss of nisin activity. The model system revealed, however, that anthocyanins were not a significant factor (Fig. 2).



The flavonol myricetin showed a slight effect (20% loss of nisin activity in 6-weeks), however, this did not account for the large nisin losses that had been observed in red wines. No loss of nisin activity was seen with catechin, quercetin, or gallic acid over a six-week period. However, grape tannin caused an immediate 90% decrease in nisin levels when tested in model system.

Interactions between aged wines and nisin: As wine matures there is an increase in the ration of polymeric to monomeric tannins (1)(5)(9)(15)(19). When nisin was added to the wines aged for two, four, and siz months at 23'C, there was a greater loss of acrivity than with the corresponding wines aged at 4'C(Fig. 3). This effect was seen in both the Pinot Noir and Cabernet Sauvignon wines. When nisin was added to wines aged tow, four, and six months at a constant temperature (either 4'C or 23'C), the resulting decrease in nisin activity was significantly greater in the older wine samples (Fig.3).



This may indicate that the polymeric tannins found in the mature wines reacted more readily than the compounds prevalent in the younger wines. This was visually supported during the nisin bioassay by a precipitate which was present in the largest quantities in the sample wells of wines aged for six months.

Conclusions

This research focused on some of the interactions that occur in red wines which may result in a loss of nisin activity. Of six grape and wine polyphenolics tested, grape tannin was shown to cause the largest decrease in nisin activity. The storage temperature and the age of the wine at the time of nisin addition were also shown to be factors. Wines aged for six months before the addition of nisin retained less nisin activity than wines aged for two months. Wines aged at room temperature 23'C) before adding nisin showed a greater loss of nisin activity than wines aged at 4'C.

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