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Malolactic Fermentation:

A Review of Current Practices, Problems, and Research at OSU

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Introduction

Malolactic fermentations are important in the production of quality table wines in cool climate winegrowing areas world-wide. The fermentation is encouraged to lower the acidity by converting malic to lactic acid, to increase aroma and flavor complexity, and to increase biological stability (1,2,3). Traditionally malolactic fermentations occur sporadically from growth of indigenous lactic acid bacteria generally after completion of alcoholic fermentation. Characteristic microflora may be established in individual vineyard areas and in wineries on processing equipment and in used cooperage. Although low numbers of malolactic bacteria have been detected on winegrapes at harvest, significant levels only arise during processing and fermentation (1,2,3,4,5,6,7).

The types of lactic acid bacteria found in musts and wines include *Pediococcus*, *Lactobacillus,* and *Leuconostoc* species. Of these only certain strains of *Leuconostoc oenos* are considered to produce wines with desirable aromas and flavors. In low pH musts, typical of cool winegrowing regions, most indigenous bacteria die during yeast fermentation with the exception of *L. oenos* strains which under the proper conditions of low $S0_2$ (less than 15-20 mg/L 'free') and moderate temperature (15-18^{o} C) can begin vigorous growth towards the end of yeast fermentation and conduct a malolactic fermentation. In high pH musts, however, typical of warmer winegrowing regions, certain strains of *Pediococcus* and *Lactobacillus* have also been associated with malolactic fermentations and with the production of undesirable odors and flavors. 'Lactic' spoilage is believed to be due to excessive levels of lactic acid, acetic acid, diacetyl, and acrolein formed by degradation of substrates such as glucose, glycerol, and tartaric acid. Such wines have been described as having ,mousey,' 'sweaty,' 'sauerkrauty,' 'vinegary,' and 'buttery' off odors and bitter aftertastes (3,4,5,6,7,19).

Although it is generally assumed that wines are 'stable' to further growth of lactic acid bacteria following complete degradation of malic acid, it has recently been reported that wine can support the growth of a succession of species of lactic acid bacteria. In this manner *L. oenos* could complete a malolactic fermentation and be followed by growth of a 'spoilage' lactic acid bacteria stimulated by the raise in pH and the remaining nutrients in the wine (6,19). The stability and quality of wines after malolactic fermentation may be dependent on subsequent processing including addition of $S0_2$ and filtration.

Relying on the indigenous microflora to complete a timely and desirable malolactic fermentation can be risky, even in low pH musts and wines. Even when desirable bacteria are established in a winery, the

onset of the fermentation may take several months and often occurs in some barrels and tanks and not others. Cross inoculation with several percent of wine undergoing the fermentation is often necessary to get uniform results.

The state of the art today is to inoculate with pure cultures when a malolactic fermentation is desired. A moderate addition Of $S0_2$ is added at crush (25-50 mg/L) to inhibit growth of 'wild' bacteria and a pure culture of *Leuconostoc oenos* is added, generally during the alcoholic fermentation (1,2,5,6,8).

Some authors have recommended that malolactic fermentation not be encouraged until all the fermentable sugars are utilized by yeast to avoid any possibility of 'lactic' spoilage from bacterial degradation of glucose. However, it has recently been demonstrated that strains of *L. oenos* can conduct malolactic fermentation in low pH musts without attacking sugar (3,5,19,23).

Isolation and characterization of Oregon malolactic bacteria:

Oregon winemakers have been inoculating with malolactic cultures since the early 1970's in order to encourage the fermentation in Pinot noir and in many Chardonnay wines. Several commercially available bacteria have been used, including ML-34 and PSU-1, with mixed results. In low pH musts and wines, especially white wines, difficulty in inducing malolactic fermentations using these cultures has often been encountered. ML-34 was isolated from a red vinifera wine in Napa Valley and has proven very successful under California conditions (1,2). PSU-1 was isolated from American French hybrid wine in Pennsylvania and has proven more successful under eastern winemaking conditions than ML-34. A wide range in heterogeneity apparently exists in the *Leuconostoc oenos* species and environmental adaptation may be critical to successful fermentation within a given winegrowing region (1,2,5,6,7,8,9). It is perhaps not surprising that these organisms tend to be most successful when used in the winegrowing regions from which they were isolated.

During the mid 1970's, David Lett at The Eyrie Vineyards and Dick Erath at Knudsen-Erath Winery observed desirable malolactic fermentations occurring in uninoculated wines at low pH and cool cellar temperatures, presumably due to the growth of indigenous bacteria established in their wineries. Subsequently, OSU enologists in the Department of Food Science and Technology and Dr. Sandine's group in the Department of Microbiology began a cooperative research project with OSU Agriculture Experiment Station funds to isolate and characterize malolactic bacteria from Oregon wines to find strains better adapted to our winemaking conditions.

Twenty-three strains of lactic acid bacteria were isolated from the two wineries from 1978 Pinot noir, Chardonnay and Merlot wines. Twelve of these strains were fully characterized as *Leuconostoc oenos* and described as gram positive cocci occurring in pairs and chains and as facultative anaerobes capable of producing lactic acid and CO_2 from malic acid at pH values less than 4 in the presence of 10% ethanol (10,11). Ten of the strains were then evaluated with commercially available strains for ability to ferment malic acid at different pH's and temperatures. Two promising isolates, Er1a a and Ey2d, were selected for their ability to ferment malic acid in defined media at pH 3.2 more rapidly than ML-34 and PSU-1. In addition, Er1a a had the lowest pH tolerance and fermented malic acid at pH 3.0, and Ey2d had the lowest temperature tolerance and fermented malic acid slowly at 8^oC (460^oF) where the other strains tested were inactive (12,13,14).

The effects of alcohol, $S0_2$, and fumaric acid were also evaluated and found to be similar for all strains tested. Higher alcohol levels delayed but did not prevent the onset of malolactic fermentation in inoculated wines (levels up to 14% were tested). Addition of 20 mg/L of $S0_2$ greatly reduced the number

of viable cells in a white wine with pH 3.3 which had been inoculated with the bacteria strains. Addition of 30 mg/L of $S0_2$ reduced the viable cell level to near zero within one month. Addition of 500 mg/L of fumaric acid inhibited bacterial growth in inoculated wines in the absence of inhibiting levels of $S0_2$. In practice only 1 to 2 pounds of fumaric acid per thousand gallons of wine (120 to 240 mg/L) is used due to the difficulty of solubilizing the acid in wine, and it is usually added in conjunction with the addition of 20 to 30 mg/L of 'free' $S0_2$ to ensure inhibition of malolactic fermentation (1,2).

Evaluation of Oregon strains in experimental wines

During the 1981 vintage we compared the performance of the Oregon strains with that of commercially available strains in Oregon wines at our experimental winery in the Department of Food Science (12,13,14). The wines in the trials were chosen with pH's typical of the range encountered in Oregon wines.

A Pinot noir wine with an initial pH of 3.46 was inoculated 10^6 cells/ml with Er1a, Ey2d, PSU-1 and ML-34. All four strains completed the fermentation rapidly within 31 days at 18^oC. The uninoculated control with an initial viable cell count of less than 100 cells/ml also completed the fermentation in 56 days, approximately twice the time of the inoculated wines (Fig. 1).

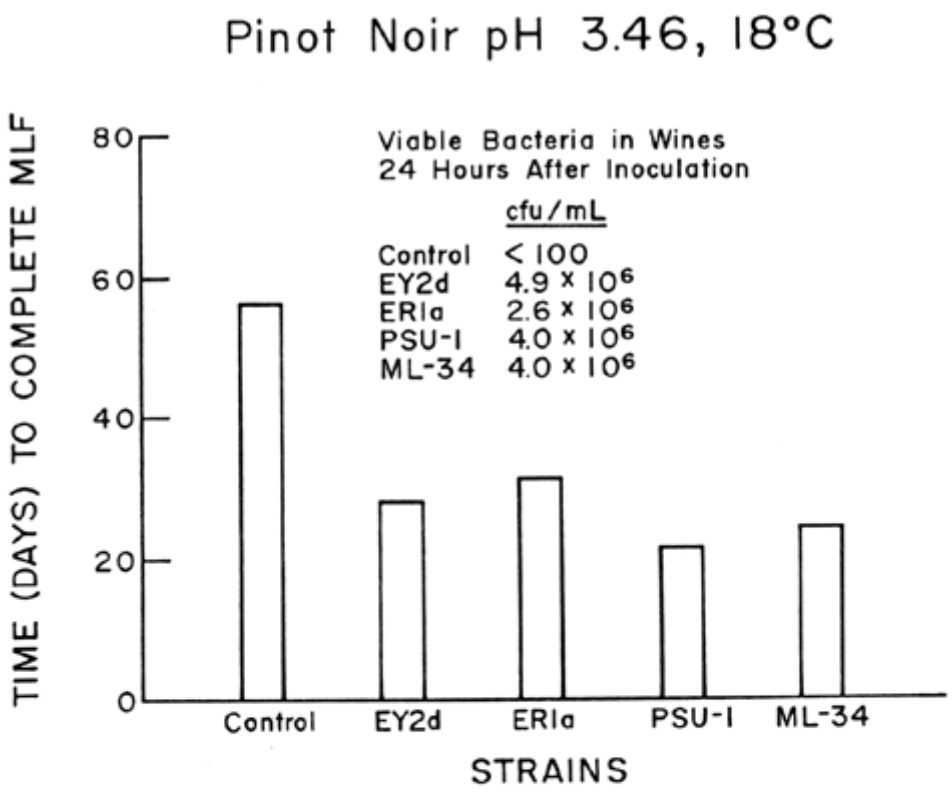


Fig. 1.

Effect of pH on malolactic strains in different experimental wines[1] *at 18° C.*

[1]*Wine composition at inoculation:* Pinot noir: *alcohol 12.1%, pH 3.46, TA 5.8 g/L, malate 1.7 g/L, free SO_2 8 mg/L, total SO_2 13 mg/L;* Pinot blanc: *alcohol 8.7%, pH 3.24, TA 9.7 g/L, malate 4.9 g/L, free SO_2 3 mg/L, total SO_2 13 mg/L;* Chardonnay: *alcohol 9.4%, pH 3.06, TA 11.1 g/L, malate 5.6 g/L, free SO_2 8 mg/L, total SO_2 13 mg/L.*

A Pinot blanc wine with an initial pH of 3.24 was inoculated similarly with the same strains and MLT (a Swiss-Austrian strain Microlife Technics, Florida) and BB44-40 (Bio-Logicals, CA). Strains Er1a and Ey2d completed malolactic fermentation the most rapidly in 36 and 63 days, respectively, followed by MLT in 71 days, PSU-1 in 88 days, ML-34 in 98 days, and BB44-40 in 105 days. The uninoculated control with an initial viable cell count of less than 100 cells/ml also completed the fermentation in 112 days (Fig. 1).

A Chardonnay wine with an initial pH of 3.06 was inoculated similarly with the same strains as the Pinot blanc wine. Again, Er1a a and Ey2d completed the fermentation the most rapidly in 60 and 125 days, respectively, while all the other strains required 183 days or longer. The uninoculated control with an initial viable cell count of less than 100 cells/ml also completed the fermentation in 198 days (Fig. 1).

At moderate pH (e.g. Pinot noir at pH 3.46) all the strains tested performed well; however, at low pH

(Pinot blanc pH 3.24 and Chardonnay at pH 3.06) the Oregon isolates Er1a a and Ey2d outperformed the commercial strains tested. Er1a a was the most pH tolerant and completed the fermentations in approximately half the time required by Ey2d. Both Oregon strains, however, were better adapted and able to maintain high viable cell counts after inoculation in low pH wine (Fig. 2) while all the commercial strains tested suffered significant losses resulting in delayed fermentations.

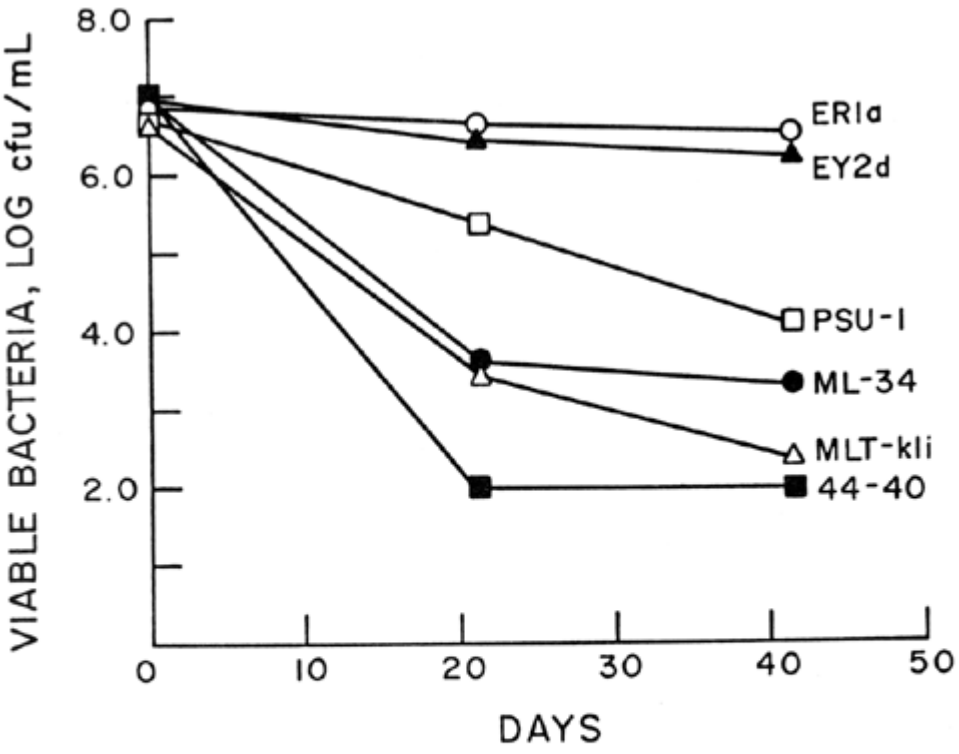


Fig. 2.

Viable bacteria in Chardonnay[1] after inoculation.

[1]Wine composition at inoculation: alcohol 9.4%, pH 3.06, TA 11.1 g/L, free SO_2 8 mg/L, total SO_2 13 mg/L.

During our trials we consistently observed that the number of viable cells surviving in wine following inoculation must be on the order of 10^5 to 10^6 cells/ml for the fermentation to occur with a minimal initial lag period. Malolactic fermentation does not occur appreciably during the initial rapid growth phase of the bacteria but only after the cell density approaches the high levels found during the stationary phase of growth (3,6,8).

The Oregon strains and the commercial strains all produced good quality, sound wines. In sensory evaluations by OSU enologists and Oregon winemakers no obvious qualitative differences were observed. The wines are currently being evaluated by a trained panel at OSU for detection of any significant differences (see Trained Panel Evaluation of Pinot Noir Fermented with Different Strains of Malolactic Bacteria, Mina McDaniel).

Commercial Winery Trials

Encouraged by the success of our experimental winery trials, we began selected industry trials in 1982 with the close cooperation of several Oregon wineries and support from the Oregon Table Wine Research Advisory Board (TWRAB). Through careful monitoring of starter cultures and inoculated wines we developed procedures for successful commercial application of the Oregon strains under our winery conditions (13,14).

Pure cultures of Er1a and Ey2d were first grown in enriched nutrient broth to very high cell densities. Grape juice media (GJM) consisting of juice and water (1:1) adjusted to pH 3.6 and with .01% yeast extract added were inoculated with the broth cultures. These juice cultures contained on the order of 109 cells/ml after incubation for two weeks at 26^{o}C. Starter cultures were prepared in the wineries by inoculating 50 to 250 gallon lots of fermenting wine 2% by volume with GJM cultures. The fermenting wines used were prepared from must with low S02 (less than 20 ml/L 'free'), with .01% added yeast extract, and with calcium carbonate added when necessary to adjust the pH to 3.3-3.4. The musts were then inoculated with the desired yeast strain followed by the GJM bacterial cultures one to two days after onset of the yeast fermentation. These winery starters contained on the order of 10^8 viable bacteria cells/ml after two weeks at 16^{o}C in the presence of a similar level of wine yeast. Large volumes of wines were inoculated 0.5 to 2% by volume with the winery starter cultures and were found to contain on the order of 10^5 to 10^6 cells/ml following the inoculation.

Chardonnay wines inoculated with Ey2d during yeast fermentation completed malolactic fermentation in an average of 56 days at 8 to 13^{o}C. Pinot noir wines inoculated similarly with Er1a a completed the fermentation in an average of 38 days at 14^{o}C. Some inoculated wines failed to undergo malolactic fermentations but these were usually found to have excess levels of $S0_2$ or to have been subjected to long periods of cold temperatures. In some cases, however, the reasons for failure were not so obvious.

Dr. Sandine's laboratory in the Department of Microbiology began searching for the presence of bacteriophages (viruses) which could infect and inactivate malolactic bacteria in Oregon wines (16). One group of researchers in Switzerland had reported the isolation of bacteriophages from wine and that their presence was associated with abnormal malolactic fermentations (17). No phages were found in the Oregon wines screened; however, electron microscopy did show the presence of bacteriophages in one five-gallon lot of winery starter culture. This particular lot of juice had been inoculated with a pure GJM culture of Er1a and wine yeast. A normal malolactic fermentation ensued and part of the lot was used successfully to inoculate larger starter volumes. Some time later fresh juice was added to the old culture but the bacteria present did not renew malolactic activity. Reinoculation with Er1a a also failed to promptly induce the fermentation. Photographs of phages were obtained but attempts to isolate the viruses failed.

A bacteriophage culture (Lco 23) was obtained from Switzerland and was shown to be able to infect Er1a a but not Ey2d, PSU-1, ML-34 or MLT using spot plate assays and double agar layer methods. The phage was inhibited, however, by the presence of alcohol and was rendered non-infectious by addition of low pH Pinot noir and Chardonnay juice (16). Bacteriophages specific for *L. oenos* have recently been isolated from red wines in Australia (25). The phages did not infect all strains of L. oenos tested and they were found to survive in wines with pH values above 3.5 and to be inactivated in wines with lower pH. The phages were also inactivated by addition of $S0_2$ or bentonite.

The fact that bacteriophages were not found in Oregon wines may be due to their inability to survive at low pH. Phage infection of malolactic bacteria in Oregon wines does not appear to be a problem. A potential problem may exist, however, in winery starter culture preparation when the pH is high enough to allow phage survival, as may have been the case in the one starter lot where presence of phage was

demonstrated.

During our commercial winery trials there was also some evidence to suggest that certain yeast strains were affecting malolactic fermentation. Subsequently, in trials in Dr. Sandine's laboratory, *S. bayanus* California Champagne strain (UCD 505), *S. bayanus* Pasteur Champagne strain (UCD 595), and *S. cerevisiae* Montrachet strain (UCD 522) were paired with *L. oenos* strains Er1a a, Ey2d, and ML-34 in yeast extract fortified grape juice to observe the effect of the yeast strains on the growth of the bacteria. In general, all the yeast strains appeared to stimulate growth of ML-34. Ey2d appeared to be unaffected by Montrachet and Pasteur Champagne strains but negatively affected by California Champagne strain. Er1a on the other hand appeared negatively affected by Montrachet strain, stimulated by California Champagne strain, and unaffected by Pasteur Champagne strain (20). This preliminary evidence suggested that in fact certain wine yeasts may stimulate, inhibit, or have a neutral effect upon specific malolactic bacteria strains. Recently interactions of this type have also been reported in the literature (18). Continued research to clarify the microbial ecology of wine yeasts and malolactic bacteria is crucial to the successful commercial application of the new Oregon strains.

Production of the Oregon strains for commercial use

Research was also conducted in Dr. Sandine's laboratory to develop methods for direct inoculation of commercial wines with Er1a and Ey2d using freeze dried and frozen concentrates. So far the best results in survival of viable cells has been obtained in concentrates frozen at -40^{o}C in the presence of 15% glycerol (20).

Since 1983, OSU has provided the Oregon wine industry with pure GJM cultures of Er1a a and Ey2d for general use in commercial wines and has also provided the technical services necessary for their successful application. Most of the Oregon wineries who use malolactic fermentations are now using these strains with good success. We will continue to provide the Oregon wine industry with the malolactic bacteria cultures until commercial cultures are available.

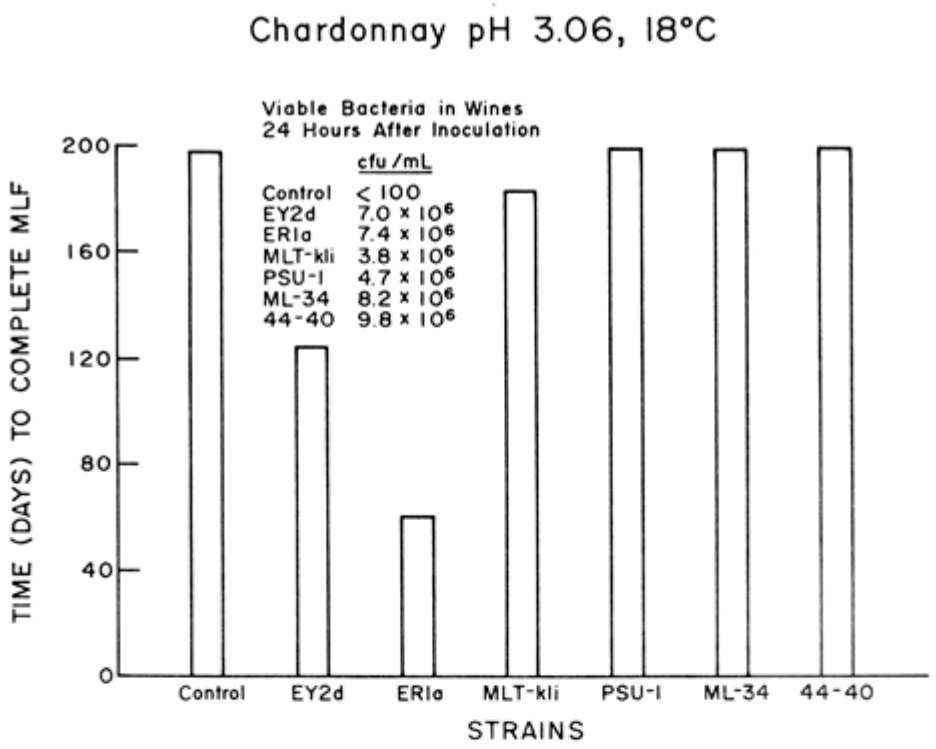
As of October 15, 1985 a U.S. patent was issued on the organisms (#4,547,373) and the cultures are available for general distribution from the American Type Culture Collection in Maryland (ATCC #39401-02) subject to licensing by OSU for any commercial production. Currently, three companies have expressed interest in commercially producing the cultures and have been communicating with OSU scientists involved, and Kim Smith, the OSU patent officer.

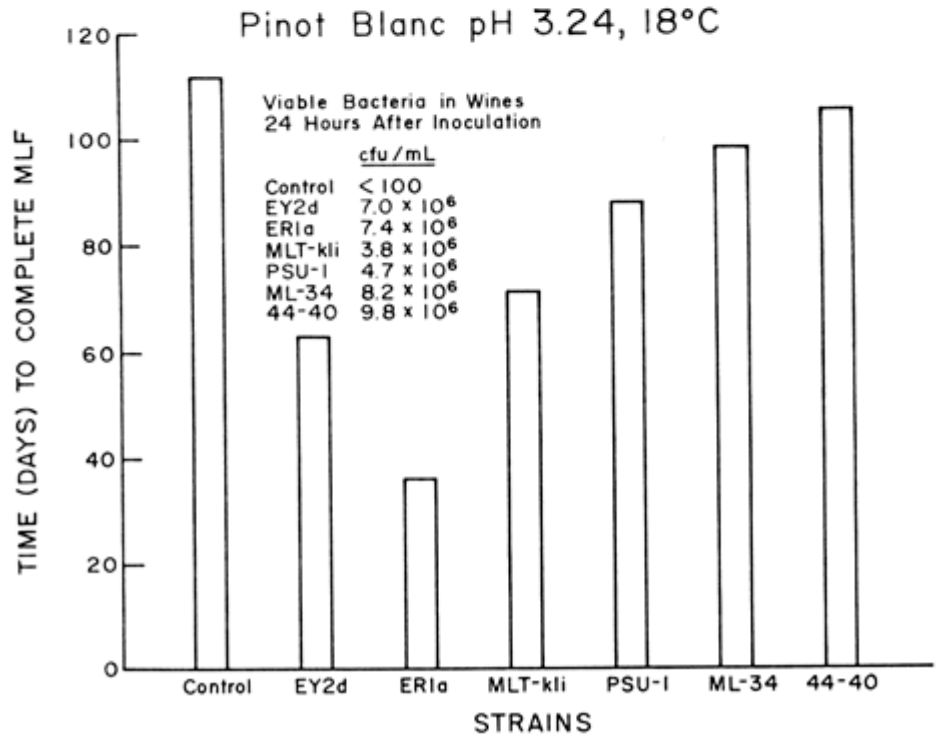
Current malolactic research at OSU

More research is needed in order to understand the microbial ecology of lactic acid bacteria in Oregon wines to better control desirable malolactic fermentations. The Wine Advisory Board is currently funding new research for this purpose in Dr. Sandine's laboratory in the Department of Microbiology in close collaboration with enologists and sensory scientists in the Department of Food Science. A graduate research assistantship has been funded by WAB and has been awarded to Dick Avedovech who is working on this project for his Ph.D. thesis.

The objectives of the research are to develop selective plating techniques for isolating lactic acid bacteria from musts and wines during different stages of vinification. Various bacteria of the *Leuconostoc*, *Pediococcus,* and *Lactobacillus* genera will be isolated, characterized, and compared with Er1a a, Ey2d, and ML-34 for their ability to degrade malic acid. The optimal conditions for their growth will be determined including the effect of alcohol, $S0_2$, pH, and temperature. Different strains of wine yeasts will be screened for their stimulatory or inhibitory effect on the bacteria strains, and the yeasts

will also be evaluated for their own ability to metabolize malic acid during fermentation. Chemical analysis will be done on musts and wines before and after fermentation with the malolactic bacteria strains in order to evaluate the environmental and nutritional conditions necessary for their growth. Sensory analysis of the wines will then be done by a trained panel in order to evaluate each bacteria strain for the production of desirable and undesirable aromas and flavors.





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