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Evaluation of Malolactic Bacteria Isolated from Oregon Wines†

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Oregon is a cool wine-producing region where grapes characteristically contain high concentrations of organic acids. To reduce the natural acidity and increase the microbiological stability and flavor complexity of the wine, malolactic fermentation is encouraged. In this study, strains of *Leuconostoc oenos* indigenous to Oregon wines were evaluated for their suitability to conduct malolactic fermentation in Oregon wines. Tests determined the malolactic activity of the Oregon isolates in comparison with commercial strains ML-34, PSU-1, MLT-kli, and ens 44-40 under various temperature and pH conditions. Sensitivities to sulfur dioxide, ethanol, and fumaric acid also were determined. Two Oregon strains, Er-1a and Ey-2d, were selected for commercial winemaking tests because they had greater malolactic activity under conditions of low pH (3.0) and low temperature (15 and 8°C), respectively.

Oregon's viticulture areas are similar in climate to the cooler wine-growing regions of Europe (2). Also, the Oregon wine industry, except for fruit wine production, is based on the use of European grape varieties of the species *Vitis vinifera*. Oregon musts and wines similarily have low pH and high titratable acidity values. The average pH of white musts from different locations in Oregon is 3.2, and the average titratable acidity (TA) as tartaric acid is 9.7 g/liter; for red musts, the average pH is 3.25 and the average TA is 9.5 g/liter (31).

An important factor affecting the quality of higher-acidity wines is the malolactic fermentation (MLF). In this fermentation, lactic acid bacteria (LAB) convert L-malic acid to L-lactic acid and CO_2 (8, 15, 22, 24, 25, 31). Malic acid constitutes between 0 and 50% of the wine acidity, the remainder being mostly tartaric acid (23). High malate concentration in musts and wines is associated with unripe grapes, since as the grapes ripen, the malic acid is metabolized in the grape berry and may disappear completely. In cool climates, however, grapes may not ripen completely and are then processed with high amounts of malate, resulting in high total acidity in the wine.

The conversion of the dicarboxylic malic acid to the monocarboxylic lactic acid with the release of CO_2 reduces the acidity of the wine; other bacterial metabolites further modify wine flavor (7). Induction of MLF offers microbiological stability by ensuring that the degradation of malic acid does not occur in the bottle, where the growth of LAB and the formation of CO_2 are considered spoilage. MLF occurs readily in high-pH wines if not actively discouraged by the use of inhibitory concentrations of SO_2 or sterilization by heat or filtration (31). In low-pH wines, spontaneous MLF by indigenous LAB has been unreliable and induction of MLF by inoculation with commercially available strains of *Leuconostoc oenos* has shown little success (19, 31).

With the assumption that LAB adapted to fermentation conditions and composition of Oregon wines might degrade malate more rapidly, Izuagbe et al. (14) isolated and characterized several strains of *L. oenos* from different wine samples. Fifteen of these isolates were compared in the present study with commercial strains of malolactic bacteria to determine whether or not any were better suited for induction of MLF under Oregon winemaking conditions of low pH and low temperature.

MATERIALS AND METHODS

Cultures. The malolactic strains used in this study were isolated from wines undergoing spontaneous MLF in two Oregon wineries. These strains, classified as *L. oenos* (9, 10; T. P. Dohman, M. S. thesis, Oregon State University, Corvallis, 1982), were Ey-1a, Ey-2a, Ey-2b, Ey-2c, Ey-2d, and Ey-4b, isolated from a 1978 Merlot; Ey-42, Ey-42a, and Ey-42b, isolated from a 1979 Chardonnay; Ey-c, isolated from a 1978 Pinot Noir; Er-1a, Er-1b, and Er-1c, isolated from a 1979 Pinot Noir; and Er-2, Er-2a, Er-3, and Er-4a, isolated from a 1978 Chardonnay.

L. oenos ML-34 (13) was obtained on a nutrient agar slant from R. E. Kunkee, University of California, Davis. *L. oenos* PSU-1 (4) was received as a freeze-dried preparation (Leucostart) from Tri Bio Laboratories, State College, Pa. It was rehydrated by following the manufacturer's instructions. Strain 44-40 (28) was obtained from BioLogicals Inc., Berkeley, Calif., as a freeze-dried preparation and rehydrated in the resuspension medium supplied by the manufacturer. Strain MLT-kli, a Swiss isolate, was on a nutrient agar slant made available by Microlife Technics, Sarasota, Fla. All cultures were maintained on MRV-8 agar slants (14).

Temperature trials. Wine used was prepared in the experimental winery of the Department of Food Science and Technology, Oregon State University. It was made from Pinot Noir grapes grown on the Agricultural Experiment Station at Medford, Oreg. Grape juice and wine were analyzed for degree Brix (soluble solids by refractometer, in grams per kilogram as sucrose), TA (in grams per liter as tartaric acid), volatile acidity (VA; by Cash steam distillation as grams per liter of acetic acid), pH, and alcohol (ebulliometer volume percent) (Table 1) (1).

The wine was dispensed (200 ml) into 250-ml Erlenmeyer flasks and autoclaved for 12 min at 121°C. After being cooled (25°C), the wine was analyzed for pH, TA, and VA (Table 1).

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TABLE 1. Analytical data on Pinor Noir grape must and new wine used in temperature trials

Sample	pH	TA	°Brix	% (vol/vol) alcohol	VA (g/liter)
Grapes	3.22	10.6	20.2		
New wine	3.49	8.0	0	10.9	0
Δ Wine[a]	3.34	8.0	0	0	0.023

[a] New wine heated (Δ) in an autoclave for 12 min at 121°C.

After being autoclaved, the wine contained no ethanol and no VA.

Erlenmeyer flasks with the uninoculated heated wines were placed into three constant temperature incubators at 20, 15, and 8°C overnight to equilibrate before inoculation. The bacterial cultures were grown at 30°C in MRV-8 broth (pH 5.5) in 16-mm loosely screw-capped glass tubes incubated under CO_2 (GasPak system; BBL Microbiology Systems, Cockeysville, Md.). After 35 h, the tubes had reached maximum turbidity and were refrigerated at 6°C. Approximately 20 h later, the wines were inoculated with 1% (vol/vol) of these cultures and placed back into the incubators. An uninoculated heated wine sample served as a control at each temperature.

Viable cells in the inoculated heated wine were determined by plating on MRV-8 medium at pH 4.6. Free and total sulfur dioxide and total soluble solids were determined by the methods given by Amerine and Ough (1).

Wines were periodically analyzed by paper chromatography (16) for the disappearance of malate and for the formation of lactate. Activity of the malolactic cultures was followed by measuring the size of the malate spot and recording its change. Variation in the size of the malate spot of duplicate samples was minimized by dividing its size (width times height) by the size of the malate spot of the uninoculated control. When the malate spot had disappeared on the paper chromatogram, samples were taken for plate count analysis as described above. Malolactic fermentation was considered complete when malate could no longer be detected on the paper chromatograph.

pH trials. Screw-capped (16 mm) tubes were filled with 10 ml of MRV-8 broth containing 2 g of L-malate per liter and adjusted to pH values ranging from 2.8 to 4.0 with 8 N tartaric acid. The media were sterilized by autoclaving (121°C for 15 min) and the desired pH values were verified before inoculation. Cultures were grown at 30°C in MRV-8 broth (pH 4.6) for 4 days to maximal cell density and inoculated at a rate of 1% (vol/vol). The concentration of L-malate was determined enzymatically (21). Malolactic fermentation was considered complete when the concentration of malate had reached less than 50 mg/liter.

Malolactic activity. On the basis of the results of the pH trials, comparative rates of MLF were calculated for each strain in a manner similar to that of Lafon-Lafourcade (17). Activity was expressed as the milligrams of malate degraded per log of viable cells per milliliter in 10 ml of medium in 24 h at 20°C. This expression was chosen in order to estimate the performance of a bacterial strain in wine, that is, how actively it would degrade the malic acid from a certain inoculum size. We were interested not in the amount of obtainable growth but in how actively a given culture would degrade the malic acid. Malate was determined enzymatically as described above for the pH trials.

Ethanol tolerance. The effect of various concentrations of ethanol on cell growth and malate metabolism was studied for strains ML-34 and Er-1a. The Chardonnay wine used had a pH of 3.03 and contained 9.4% (vol/vol) alcohol, 11.1 g of TA per liter, residual sugar (Clinitest; Ames Division, Miles Laboratories, Elkhart, Ind.), 5.6 g of malate per liter, and 8 mg of free and 13 mg of total SO_2 per liter. No attempt was made to sterilize the wine, since the experiment was meant to simulate a winery environment in which a commercial starter culture is inoculated into wines of different alcohol concentrations. The wine contained 9.4% (vol/vol) ethanol, and appropriate amounts of 95% (vol/vol) ethanol were added to achieve alcohol concentrations of 10, 12, and 14% (vol/vol). The pH of the wine was adjusted to 3.5 with 5 N NaOH. Sterile, 16-mm screw-capped glass tubes were filled completely with the wines to minimize the growth of aerobic spoilage organisms. Inocula were grown for 4 days at 30°C in 16-mm screw-capped glass tubes in MRV-8 broth (pH 4.6), and the wine was inoculated with 1% (vol/vol) ML-34 (1.5×10^9 CFU/ml) or 1% (vol/vol) Er-1a (1.2×10^9 CFU/ml). An uninoculated control was maintained for each treatment of the wine. The wines were incubated at 20°C. The number of viable cells was determined by plate count on MRV-8 medium (pH 4.6). Cycloheximide (70 mg/liter; Sigma Chemical Co., St. Louis, Mo.) was added to the plate count medium to inhibit yeast growth. The malic acid was determined enzymatically (21).

Fumarate and sulfur dioxide. The same wine used in the test of ethanol tolerance was used. It was divided into two lots; to one lot 30 mg of SO_2 (potassium metabisulfite) per liter was added, and the other lot was left as a control. The wine without added SO_2 contained 8 mg of free and 13 mg of total SO_2 per liter, and the wine with the added SO_2 contained 20 mg of free and 40 mg of total SO_2 per liter after 1 day of equilibration. Each lot was divided into three sublots to which 0, 0.5, or 1.5 g of fumaric acid per liter was added. The pH of the wine in all lots was then raised to 3.5 with 5 N sterile NaOH. Sterile, 16-mm screw-capped glass tubes were filled completely with the wines with the different treatments. One tube with each treatment was inoculated at 1% (vol/vol) with strain ML-34 (1.9×10^9 CFU/ml) or strain Er-1a (1.5×10^9 CFU/ml), producing three tubes for each inoculum. The inocula had been grown in MRV-8 broth (pH 4.6) at 30°C in 16-mm screw-capped glass tubes for 4 days. The wines were then incubated at 20°C. The number of viable cells was determined as described above.

Sulfur dioxide and pH. The same Chardonnay wine was used; 1 liter was adjusted to pH 3.3 with 1 N NaOH, and another liter was left at pH 3.03. To both wines, various additions of SO_2 were made. The final concentrations of free and total SO_2 were determined 24 h after the addition to allow equilibration of the two forms. The amounts of free and total SO_2 were determined by the method of Amerine and Ough (1). Since no difference in the SO_2-binding capacity of the wine at different pH values was observed, the same additions of SO_2 were made to both wines. Samples (64 ml) of each wine were put into screw-capped glass bottles, and SO_2 was added from a stock solution of potassium metabisulfite (17.7 g/liter). Additions of 20, 30, and 40 mg of SO_2 per liter resulted in 13, 20, and 26 mg of free and 30, 40, and 49 mg of total SO_2 per liter. No addition was made to one set of wine samples; it contained 8 mg of free and 13 mg of total SO_2 per liter.

Sterile, 20-mm screw-capped glass tubes were completely filled with the wines adjusted to the various pHs and SO_2 concentrations. After 24 h, one tube of each treatment was inoculated with 1% (vol/vol) each of ML-34 or Er-1a. The

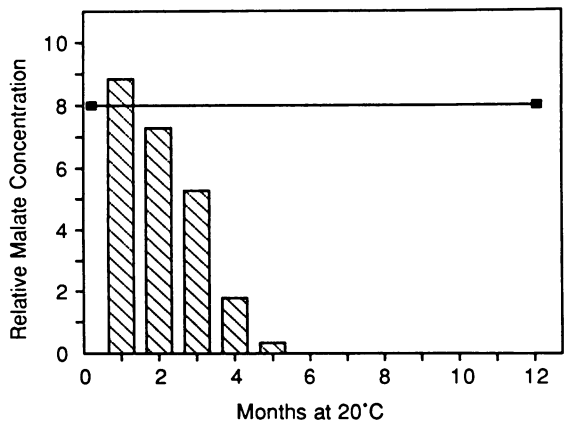

FIG. 1. Relative concentrations of malate in Pinot Noir fermented with *L. oenos* ML-34 at 20°C as determined by measuring the size of the malic acid spot on the paper chromatogram, dividing it by the size of the malic acid spot of the uninoculated control, and multiplying by 8 for graphical demonstration.

inocula had been grown in MRV-8 broth (pH 4.6) at 30°C for 3 days.

The number of viable cells was determined as described above. The concentration of malate was determined enzymatically (21).

RESULTS

Temperature trials. The initial cell density in the inoculated, heated wines was approximately 10^6 cells per ml, with the exception of ML-34, which contained 3.5×10^7 cells per ml, and Ey-2d, Ey-c, and Er-1a, containing 3×10^5 cells per ml. No viable cells were present in the uninoculated control at the beginning and end of the experiment. At the conclusion of each trial, viable cell counts in wine stored at 20°C ranged from 1.2×10^4 (Ey-2b) to 6.6×10^6 (Ey-42a) per ml; at 15°C, they ranged from <10 (PSU-1, ML-34, Ey-42, Er-2, and control) to 1.0×10^7 (Ey-42b) per ml; at 8°C, they ranged from <10 (PSU-1, ML-34, Ey-2a, Ey-42b, Er-2, and control) to 2.3×10^5 (Ey-2b) per ml.

Representative results of the relative malate utilization rates at 20, 15, and 8°C are given in Fig. 1 and 2. The fastest strains to complete MLF were Ey-2d, Ey-c, and Er-1a: each completed MLF within 21 days. These strains were followed by Ey-42 and Er-2 at 36 days, Ey-2b at 41 days, Ey-2a at 52 days, Ey-41 and PSU-1 at 83 days, Ey-42a at 87 days, ML-34 at 144 days, and Ey-42b, which had not completed MLF after 228 days when the trial was terminated.

At 15°C, the differences in temperature tolerance became very large. Three strains, PSU-1, ML-34, and Ey-42, did not show any malolactic activity within 12 months. Seven strains, Ey-2a, Ey-2d, Ey-42a, Ey-42b, Ey-41, Ey-c, and Er-2, completed MLF within this time, and two strains, Er-1a and Ey-2b, fermented the malate partially. Of the seven strains that completed MLF most quickly, Ey-2d finished at 145 days, followed by Ey-42b at 158 days, Ey-41 at 187 days, Er-2 at 225 days, Ey-42a at 256 days, Ey-2a at 309 days, and Ey-c at 340 days.

At 8°C, none of the strains completed MLF within 12 months; however, three strains, Ey-2d, Ey-2b, and Ey-42a, did ferment the malic acid to very low concentrations. Three other strains, Ey-c, Ey-42, and Ey-41, fermented small amounts of malic acid. All other strains were inactive at this temperature.

pH trials. The results of trials at pH 4.0, 3.5, 3.2, and 3.0 are given in Table 2. For the selection of strains suitable for MLF in Oregon low-pH wines, nine Oregon strains were evaluated in comparison to the four commercial strains PSU-1, ML-34, MLT-kli, and ens 44-40. It may be seen that, in contrast to the commercial strains, several isolates, especially Er-1a, performed better at pH 3.0 and 3.2. At pH 4.0, all strains grew rapidly to high cell densities, between 4 ×

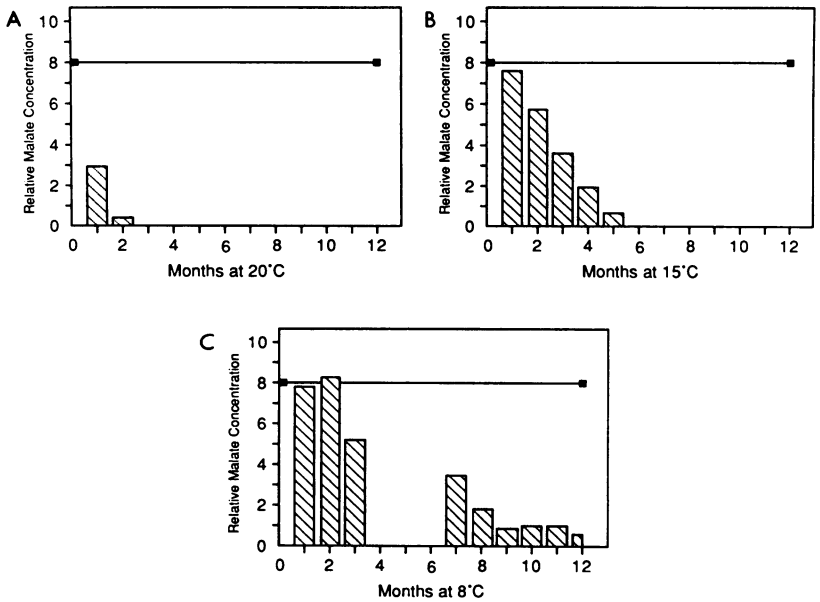

FIG. 2. Relative concentrations of malate in Pinot Noir fermented with *L. oenos* Ey-2d at 20°C (A), 15°C (B), or 8°C (C) as determined by measuring the approximate size of the malic acid spot on the paper chromatogram, dividing it by the approximate size of the malic acid spot of the uninoculated control, and multiplying by 8 for graphical demonstration.

TABLE 2. Malate concentration found when various *L. oenos* strains were incubated in MRV-8 broth at different pH values for the days indicated at 20°C

Strain	Malate concn (mg/liter) after no. of days											
	pH 4.0			pH 3.5			pH 3.2			pH 3.0		
	0	3	5	0	5	9	0	19	24	0	43	52
PSU-1	3,140	1,198	32	3,220	32	0	3,082	3,188	3,091	2,058	1,998	2,026
ML-34	3,214	976	28	3,117	3,043	1,104	3,139	3,114	3,191	2,141	1,308	2,219
MLT-kli	3,333	2,016	22				3,197	509	55	2,157	2,124	2,153
44-40	3,159	33	22	3,130	29	1	3,101	2,499	2,463	2,125	2,001	2,011
Er-1a	3,152	13	6	3,011	692	6	3,111	2,466	1,900	2,135	199	139
Er-1b	3,281	205	3	3,127	32	0	3,143	2,270	1,633	2,058	1,181	1,266
Er-1c	3,214	15	25	3,101	528	29	3,204	2,499	2,428	2,145	814	757
Er-3b	3,339	106	22	3,152	1,179	19	3,085	2,621	2,592	2,090	934	899
Er-4a	3,146	126	19	3,130	876	13	3,104	2,537	2,341	2,074	766	719
Ey-1a	3,243	1,191	25	3,069	934	6	3,291	2,362	2,360	2,109	2,099	2,017
Ey-2c	3,175	19	22	3,098	2,425	10	3,136	2,702	2,760	2,077	1,425	1,365
Ey-2d	3,201	1,117	0	3,133	3,078	2,805	2,921	1,938	1,616	2,096	2,026	2,143
Ey-4b	3,349	1,826	25	3,165	3,262	2,711	3,146	2,202	1,591	2,135	2,311	2,340

10^8 and 2×10^9 CFU/ml, and MLF was completed in most cultures before maximum cell density was reached after 3 days. At pH 3.5, growth of all strains was slower but maximum cell densities were similar to those obtained in media at pH 4.0. Also, the rate of malate degradation was slower. The rate of malate catabolism was fastest in the cultures inoculated with the highest cell density (PSU-1 and ens 44.40). The medium with pH 3.2 was inoculated with a large number of bacteria; all wines contained, after inoculation, approximately 10^7 CFU of viable cells per ml. At this pH, the cell density did not increase above the initial cell density. MLF was fastest in the cultures which maintained the highest density of viable cells ($>10^6$ CFU/ml). The number of viable cells of PSU-1 and ML-34 decreased rapidly after inoculation, and MLF did not commence. Only six Oregon isolates were able to metabolize malate at pH 3.0. These cultures contained the highest number of viable cells over the incubation period, with the exception of MLT-kli and Ey-2d.

At pH 3.2, strain MLT-kli was by far the most active of all strains, with a malolactic activity of 18.1. This strain nearly completed MLF within 24 days. Others which were more active than the majority of the group (average of all 13 strains was 5.67; standard deviation, 4.75) were strains Ey-4b, Ey-2d, and Er-1a. ML-34 and PSU-1 were less tolerant to low pH; they did not show any malolactic activity at pH 3.2 after 24 days.

At pH 3.0, the most active strain was Er-1a. Five other Oregon isolates also showed significant malolactic activities: Er-4a, Er-1c, Er-3b, Er-1b, and Ey-2c. These six strains also had some malolactic activity at pH 2.8 after 44 days (data not shown). Overall, the Oregon isolates degraded malate more actively at low pH than did the commercial strains used for comparison.

Ethanol tolerance. ML-34, Er-1a, and the indigenous bacterial flora in Chardonnay wine showed the same sensitivity towards ethanol. Their growth and concurrent degradation of malic acid were similarly inhibited with increasing ethanol concentration (data not shown). Malate degradation was complete within 44 days at 10% (vol/vol) ethanol, whereas it was 50% slower at 12% (vol/vol) and completely inhibited at 14% (vol/vol).

Following inoculation, the number of viable bacteria decreased rapidly by 2 to 4 logs with larger decreases at the higher ethanol concentrations. The cell numbers of ML-34 decreased less than the cell numbers of Er-1a at all three ethanol concentrations. However, following this initial decrease in cell numbers, Er-1a regained its viability more rapidly than ML-34 in all three wines.

The number of viable bacteria in the uninoculated control increased from less than log 2 CFU/ml at the start of the experiment to log 6.83 at 10% ethanol, log 6.11 at 12% (vol/vol) ethanol, and log 6.04 at 14% (vol/vol) ethanol by day 44. Thus at day 44 the number of viable cells in the uninoculated control wines were almost identical with that of the inoculated wines at 10 and 12% (vol/vol) ethanol. The rates of malic acid degradation were almost identical in the wine adjusted to 10% ethanol. At 12% ethanol, the malic acid degradation rates of the inoculated wines were faster than in the uninoculated control. At 14% (vol/vol) ethanol, however, the number of viable cells in the control wine was 1 log higher at days 44 and 59 than the number of viable cells in the same wine inoculated with ML-34 or Er-1a. At 14% ethanol, the malic acid degradation was fastest in the uninoculated wine which had the 10-fold-higher number of viable cells at day 44, when the degradation of malic acid started.

Fumarate and sulfur dioxide. The presence of 0.5 and 1.5 g of fumarate per liter in wine of pH 3.03 inhibited MLF by ML-34 and Er-1a for at least 70 days, at which point the experiment was stopped (Fig. 3A). The number of viable cells decreased in these wines from 5×10^7 CFU/ml at inoculation to less than 1×10^2 CFU/ml after day 31 (Fig. 3B). The cell numbers decreased more sharply for ML-34 than for Er-1a. In the wine with no fumarate added, both strains degraded all malate within 72 days. In the uninoculated control wines, with and without fumarate added, no significant reduction in the concentration of malic acid occurred over 72 days.

In the presence of 26 mg of free and 49 mg of total SO_2 per liter at pH 3.03 (30 mg of added SO_2 per liter, pH 3.03), MLF was inhibited in all wines with or without fumaric acid added (data not shown). The number of viable cells was reduced to 1×10^3 CFU/ml and less. In the presence of fumarate, the number of viable cells decreased to less than 10 CFU/ml by day 69; without the addition of fumarate, the number of viable cells decreased rapidly from log 1.6×10^7 CFU/ml to 1.6×10^4 CFU/ml by day 4; thereafter it slowly decreased to 2×10^3 CFU/ml by day 69 (data not shown). The number of viable cells for the wines inoculated with Er-1a, with 26 mg of free and 49 mg of total SO_2 per liter at pH 3.03, with or

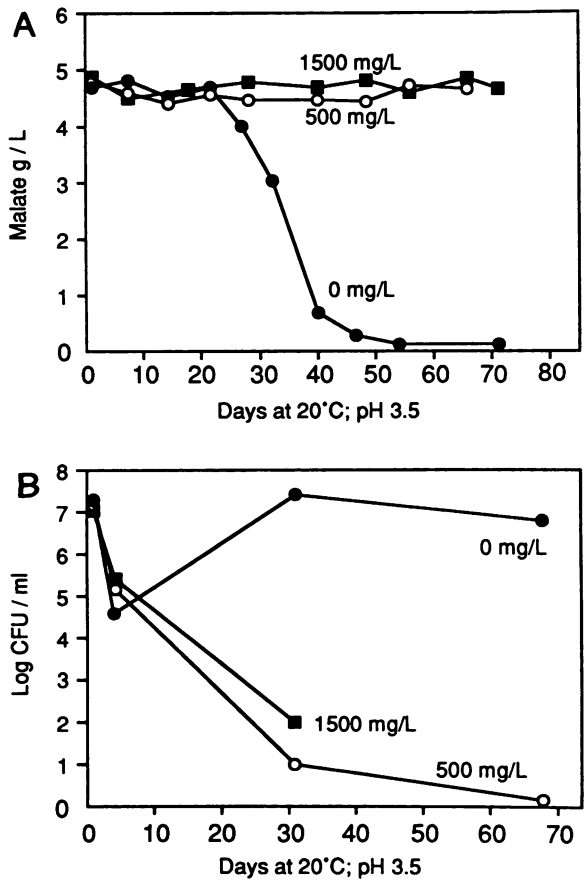

FIG. 3. Effect of three concentrations of fumaric acid on the rate of malate reduction (A) and number of viable cells (B) of *L. oenos* ML-34 in Chardonnay wine at 20°C, pH 3.5, 8 and 13 mg of free and total SO_2, respectively, per liter, and 9.7% (vol/vol) alcohol.

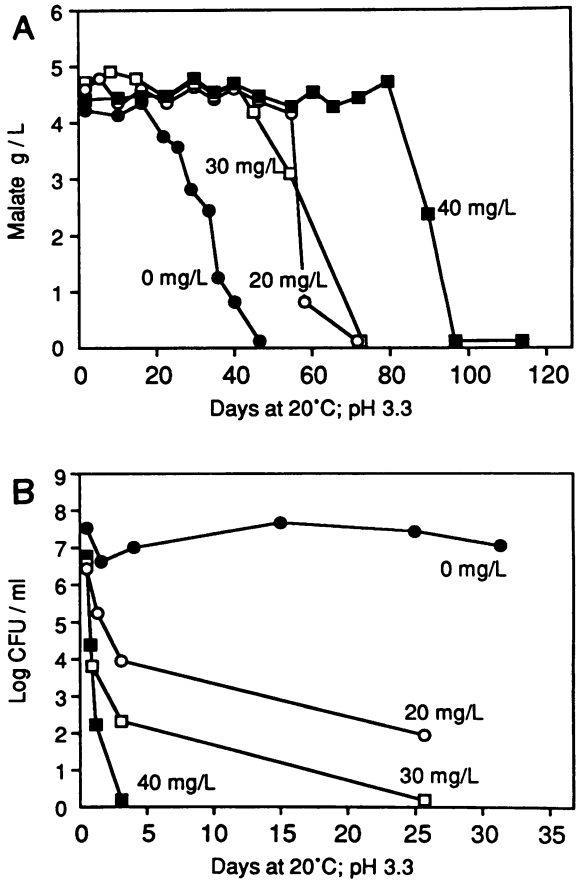

FIG. 4. Effect of three additions of SO_2 at pH 3.3 on the rate of malate reduction (A) and number of viable cells (B) of *L. oenos* ML-34 in Chardonnay wine at 20°C, 8 and 13 mg of free and total SO_2, respectively, per liter, and 9.7% alcohol. SO_2 additions were made to the wine 24 h before inoculation with the bacteria; the concentrations of free and total SO_2 in the wines at inoculation were 8 and 13, 13 and 30, 20 and 40, and 26 and 49 mg/liter for additions of 0, 20, 30, and 40 mg of SO_2, respectively, per liter.

without fumarate, also decreased dramatically, from 1.6×10^7 CFU/ml at inoculation to between 1×10^3 and 1×10^4 CFU/ml by day 4, and remained nearly constant until the end of the experiment at day 72. The number of viable cells in the wine with 30 mg of SO_2 per liter but no fumarate added started increasing after day 4 but never became high enough to degrade any large amounts of malic acid.

Sulfur dioxide and pH. At pH 3.3 in the Chardonnay wine with no additional SO_2 (8 mg of free and 13 mg of total SO_2 per liter), ML-34 completed MLF by day 46 (Fig. 4A). Er-1a completed MLF within 37 days. The addition of 20, 30, and 40 mg of SO_2 per liter 24 h before inoculation delayed MLF by both strains by between 60 and 95 days (Fig. 4A). Without the addition of SO_2, the number of viable cells was reduced between 0.5 log/ml for Er-1a and 1 log/ml for ML-34 in the wine without addition of SO_2. Additions of 20 and 30 mg of SO_2 per liter reduced the number of viable cells to approximately log 4 and log 2 CFU/ml, respectively; the addition of 40 mg of SO_2 per liter effectively eliminated all viable cells in the wine (Fig. 4B). After 60 to 90 days, MLF occurred in all wines except in the wine with 30 mg of added SO_2 per liter inoculated with Er-1a, where no MLF occurred within 120 days (Fig. 4).

DISCUSSION

All strains in the temperature trial had rather long fermentation times, considering that the wine in which they were studied was relatively high in pH and low in acidity and did not contain any alcohol or free SO_2 after being autoclaved. Autoclaving the wine might have altered its composition so that it became less fermentable; that is, nutrients and growth factors could have been destroyed or inhibitory substances could have been produced. Wines produced from heat-treated juice (thermovinification) have been found to be less suitable for growth of LAB than those produced without heat treatment (5, 15, 19). However, for this comparison we assumed that all bacteria tested were affected to the same degree.

At pH 4.0, near-optimal conditions for survival and growth of *L. oenos* allowed all strains to complete MLF within 3 to 5 days; also, at pH 3.5, most of the strains completed MLF within 9 days. For a successful inoculation which would initiate MLF within days after inoculation, it has been reported that a wine should be inoculated with a minimum of 10^6 CFU of viable bacteria per ml (3, 11, 20, 26, 29). Beelman and Gallander (3) found that an actively fermenting culture in wine contained more than 10^7 viable cells per ml. This effect, of a minimum number of viable cells for effectively conducting MLF, is also evident from the data reported here. The amount of malate degraded in a culture

depends on the number of viable cells and the metabolic activity of the individual cells. In all experiments, the arrest of MLF could be attributed to loss of viability of the bacterial culture. At cell densities below log 5 to log 6 CFU/ml, the amount of malate degraded can be so small that it is not detected in the samples taken. Because of logarithmic increases in the number of viable cells, the degradation of malate appears to be more rapid at cell densities above 10^6 CFU/ml.

For the selection of bacteria most suited to fermenting malate at low pH, the most important results are in the data for pH 3.2 and 3.0. Bacteria selected for induction of MLF in low-pH wines must be able to grow and ferment malate over the pH range from 2.8 to 3.5, which is typical for wines from cool climates (26, 29). In this range, pH 3.2 is a critical point below which it is very rare to see a spontaneous MLF and also very difficult to induce a MLF by bacterial inoculation (6, 12, 18). Several Oregon isolates were consistently more active at low pH than the commercial strains. Table 2 shows the superior ability of several Oregon isolates to ferment malic acid at low pHs of 3.2 and 3.0. The most active strain at these pHs was Er-1a.

Comparisons of the malolactic activity of individual strains indicate strain-specific differences in malate degradation. These differences might be due to different viability of the cells or to different activity of malate transporting and degrading systems. Strain ML-34 differs from Ey-2d and Ey-4b in that it degrades malic acid very rapidly once it begins MLF, while Ey-2d and Ey-4b are slower in degrading malic acid once they begin fermenting.

It was noticed that the Ey isolates were generally more cold tolerant than the Er isolates, while the Er isolates were more low-pH tolerant than the Ey isolates. The cellar temperature at winery EY, from which the Ey isolates were isolated, is normally lower than the cellar temperature at winery KE, from which the Er isolates were isolated. The consistantly lower cellar temperatures at winery EY apparently selected for bacteria that are more tolerant to low temperature. Information as to whether the wines at winery KE are usually lower in pH than the wines at winery EY was not available. Silver and Leighton (27, 28) also found that 0.4 to 0.7 g of fumarate per liter inhibited growth and MLF by *L. oenos* at pHs below 4.0.

The addition of 30 mg of SO_2 per liter 24 h before inoculation, giving 20 mg of free and 40 mg of total SO_2 per liter, was sufficient to inhibit MLF by both strains of *L. oenos*, with or without the addition of fumarate, which confirms other published data (31). The bactericidal effects of SO_2 and fumarate seemed to be additive for both strains. No spontaneous MLF occurred in any of the control wines, which does support the view that it is advantageous to inoculate wines, even those at pH 3.5, but with low concentrations of SO_2 (8 mg of free and 13 mg of total SO_2 per liter) and alcohol (9.7%) and at a favorable temperature.

The results of the SO_2-pH trial demonstrate (i) the increasing bactericidal effect of increasing concentrations of SO_2, (ii) the pH dependence of the bactericidal effect of SO_2, (iii) that the prevention of MLF by one addition of SO_2 is not permanent, and (iv) that strains ML-34 and Er-1a are equally sensitive to additions of SO_2. These observations are in agreement with previous reports (30), suggesting that most or all strains of *L. oenos* are equally sensitive to even small amounts of SO_2.

From the present work, strains Er-1a and Ey-2d were selected over ML-34, PSU-1, ens 44-40, and MLT-kli for commercial trials because of their improved MLF abilities under low temperatures and low pHs.

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