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MALO-LACTIC BACTERIA; FERMENTATION

PROPERTIES AND STORAGE STABILITY

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Abstract approved: Dr. William E. Sandine

Gram positive cocci isolated from Oregon wines were characterized and their relative malate fermentation abilities compared. They were heterofermentative, catalase negative, and facultatively anaerobic. Glucose, fructose, cellobiose, maltose, ribose, trehalose, salicin and esculin were fermented by all strains. Arabinose was fermented weakly but lactose, raffinose, sucrose, xylose, rhamnose, mannose and glycerol were not fermented. Dextran was not produced from sucrose nor ammonia from arginine.

The isolates grew well in a tomato/vegetable juice medium and fermented malate to lactate as detected by

chromatography. Optimum growth temperatures ranged between 28 and 31°C and generation times between 5.2 and 18.7 were observed at pH 5.5. The pH optimum ranged between 4.5 and 5.75. Growth was obtained in artificial media containing 14% ethanol and at pH values lower than 3.3. These malo-lactic bacteria were considered to be strains of Leuconostoc oenos.

Malate fermentation rates indicated that these isolates effected a more rapid and complete malo-lactic fermentation than reference Leuconostoc strains. This was found in both artificial media at pH 3.5 and in Pinot Noir wine, pH 3.45. Levels of 30 ppm free sulfur dioxide and 0.6 g/L fumaric acid were observed to inhibit growth.

Stability of cryopreserved cultures were studied over extended storage. Following 3 months storage of frozen concentrates at -20 and -40°C, survival rates of 70 to 80% were achieved using a modified Rogosa medium plus 15% glycerol. Following 2 months storage of lyophilized concentrates at room temperature, survival rates of 35 to 60% were achieved in milk (11% solids).

CHARACTERIZATION OF OREGON-DERIVED MALO-LACTIC BACTERIA; FERMENTATION PROPERTIES AND STORAGE STABILITY

by

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Typed by Kathleen Dohman for Thomas Philip Dohman

To my wife, Kathy, whose patience, assistance and love have contributed greatly to this accomplishment, my sincere and heartfelt thanks.

I believe that, by the exercise of science as well as of other intellectual pursuits, mankind grows continuously into some higher form and that in some mysterious way it is in the process of transcending itself. While my own contribution to this upward trend will of course be very small, it has an immense value nevertheless, because it becomes part of a spirtual structure that is endlessly emerging from amorphous matter. Science is not only an effort to gather knowledge and develop techniques for achieving mastery over nature. As Aristotle wrote two thousand years ago in his Ethics, science is above all the search for understanding. Aristotle's words still convey today the very spirit of the scientific way of life. While it may never be possible to reach absolute truth, nevertheless each one of us adds a small stone to the structure of knowledge, and from all these efforts there emerges a certain grandeur.

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INTRODUCTION

Varietal wines produced in the northwestern United States are generally of the high acid type. Grapes grown in cooler climates are usually lower in sugar and higher in acid than grapes grown in warmer climates and may result in the production of wines with harsh tastes (Beelman and Gallander, 1979). For example, titratable acidities and sugar content of Oregon musts may range from 0.93 to 1.62, expressed as g of tartaric acid/100 ml and 15.7 to 22.6 °Brix, respectively (Yang, 1973). Such values for California musts may range from 0.56 to 1.01, expressed as g of tartaric acid/100ml and 20.0 to 24.1 °Brix (Amerine and Winkler, 1963). These differences may be due to grape varieties, cultural practices, soil and climate, all factors known to influence the quality of wines produced in various regions (Beelman and Gallander, 1979; Rankine et al., 1971).

Attempts to improve the acid qualities of Oregon wines have met with limited successs. Amelioration of the wine is not a viable alternative since it may result in a dilution of the aroma, bouquet, flavor, body and

color of the wine (Kluba and Beelman, 1975). Fermentation of wines by Schizosaccharmyces pombe, a yeast capable of alcoholic fermentation and organic acid degradation, was tested but its use was never accepted for various reasons (Yang, 1973). Despite the doubts that a malo-lactic fermentation could take place in wines of such high acid and low pH (Yang, 1973; Castino et al., 1975) its spontaneous, albeit irregular, occurrence in Oregon wines proved otherwise.

Malo-lactic fermentation, the conversion of L-malic acid to L-lactic acid and carbon dioxide, occurs in wine as the result of the metabolic activity of certain strains of lactic acid bacteria. These bacteria may originally be associated with either the grapes, winery equipment and environment, or the cooperage (Kunkee, 1967). The fermentation has been suggested as a valuable means of reducing acidity in wines made in cool viticultural areas such as the eastern United States (Beelman and Gallander, 1970, 1979; Rice, 1965; Rice and Mattick, 1970). The main effects of the fermentation are an increase in pH, reduction in the titratable acidity, an increase in biological stability and an apparent effect on sensory quality and flavor complexity of the resultant wine (Kunkee, 1967; Rankine, 1972).

Pure culture inoculation with various malo-lactic bacteria, particularly Leuconostoc oenos ML34, has been attempted in laboratory experiments and commercially in

California and other places (Webb and Ingraham, 1960; Tchelistcheff et al., 1971; Rankine, 1977; Beelman et al., 1980). Use of such a pure culture would be desirable for at least two reasons: (1) it would enable the winemaker to stimulate a malo-lactic fermentation in a rapid and predictable manner, (2) it would provide assurance that a dependable identifiable strain is dominant in the fermentation, thus insuring elimination of possible deleterious flavors and odors by undesirable microflora (Beelman et al., 1980; Pilone et al., 1966).

Three genera of bacteria have been identified as malo-lactic bacteria and include Lactobacillus, Pediococcus, and Leuconostoc (Ingraham et al., 1960; Kunkee, 1974; Rankine,1977; Beelman and Gallander, 1979). Of these genera, the Leuconostoc spp. have been determined to initiate the fermentation most frequently and have generally been found to be the most desirable (Fornachon, 1957, 1965; Kunkee, 1967, 1974; Rankine 1977).

Studies have been conducted concerning the use of pure culture inoculation with Leuconostoc oenos ML34 to induce malo-lactic fermentation in high acid eastern wines and have often met with failure (Baretto De Menzes et al., 1972; Beelman and Gallander, 1970; Gallander, 1974). Use of L. oenos ML34 has been practiced to some extent in Oregon wineries but the organism appears ill suited to the low temperature and high acid conditions

prevalent during Oregon wine production.

A logical alternative in this situation was to isolate malo-lactic bacteria indigenous to Oregon wines for use in pure culture stimulation of this secondary fermentation. The development of such pure culture inoculation methodologies with regional or world-wide application is hardly new (Fornachon, 1965; Kunkee and Pilone, 1972; Beelman et al., 1977; Chalfon et al., 1977). Our intent was to isolate bacteria from wines active in causing a malo-lactic fermentation, characterize them as to identity and ability to ferment malate with subsequent selection of the most active strains for concentration and inoculation on a commercial scale. Since it has been shown that the wine leuconostocs are better adapted to low temperature and pH conditions and are more successful in effecting a malo-lactic fermentation (Kunkee, 1974), we concentrated on isolating these malo-lactic bacteria. The effects of temperature, alcohol concentration, pH, sulfur dioxide and fumaric acid on growth and malate metabolism were also determined. Growth and malate reduction in various artificial media and two experimental wine samples were compared with reference isolates obtained from other investigators.

Finally, pure cultures of active strains were grown, concentrated and preserved in various artificial media with and without cryoprotectant additives by

freezing at -20 and -40°C and by lyophilization. Stability in these preserved states was measured in order to determine the most beneficial and practical means of supplying these cultures to the wine industry.

MATERIALS AND METHODS

Source of Bacteria

Aseptic sampling of wines was performed using Whirl Pak bags (VWR Scientific). Samples were taken from both oak casks and stainless steel fermentor tanks at two cooperating Oregon wineries in the northwestern Willamette Valley. Samples of Pinot Noir, Chardonnay and Merlot wines at various stages of yeast and malo-lactic fermentation were obtained from Knudsen Erath Winery, Dundee, Oregon, courtesy of Dick Erath. Samples of Pinot Noir, Merlot and Chardonnay wines also at various stages of yeast and malo-lactic fermentation were obtained from Eyrie Vineyards, McMinnville, Oregon, courtesy of David Lett.

Additionally, reference strains of commercial malo-lactic bacteria were obtained to compare with the isolates found in Oregon wines. Leuconostoc oenos, ML 34 was obtained from the culture collection at University of California, Davis. Leuco-Start, a freeze-dried preparation of PSU-1 from Tri-Bio Laboratories, State College, Pennsylvania was also obtained for this study.

In the later stages of this study, two other commercial malo-lactic isolates became available for our use. A European culture, MLT-kli, was provided courtesy of Microlife Technics, Sarasota, Florida. The final

isolate used for limited comparison was ens 44-40, made available through Biologicals, Santa Rosa, California.

Culture Media

Medium A

This was basically a modified Rogosa medium (Ingraham et al, 1960; Pilone and Kunkee, 1972). It consisted of 2.0% Tryptone (Bacto), 0.5% yeast extract (Yeast Products Inc.), 0.5% peptone (Bacto), 0.5% glucose (Sigma), 0.3% fructose (Sigma), 0.2% L-malic acid (Sigma), and 0.005% Tween 80 (Baker). The medium base was a 1:4 dilution of tomato juice (S & W brand) which was initially centrifuged in a Beckman Model J2-21 centrifuge at 10,000 x g for 15 min to remove tomato pulp. The supernatant was filtered through analytical filter papers (Schleicher & Schuell #597) and then filtered again through glass microfiber filters (Whatman GFA). This resulted in a medium which did not exhibit sedimentation. The pH of the medium was adjusted to 5.5 with 6N NaOH using a Corning 125 digital pH meter. The same medium was used for plating agar by the addition of 12 g/L Davis agar.

For initial isolation of strains from wine samples, an appropriate aliquot of filter-sterilized cycloheximide solution (Sigma) was added just prior to pouring to achieve a concentration of approximately 50 ppm in the agar medium.

Medium B

Same as Medium A except it lacked tomato juice, glucose and fructose and was used as a basal broth.

Medium C

Same as Medium A except that it was made using V-8 Juice (Campbell's) instead of tomato juice as a base. It was found to reduce the time necessary to obtain satisfactory growth of our isolates (Izuagbe, 1981).

Medium D

Essentially a modified Phase 4 medium (Galloway West Co., Fond du Lac, WI., patent no. 4,282,255).

Medium E

A grape juice medium containing 0.5% Proteose Peptone (Difco), 0.5% Tryptone (Bacto), 0.5% yeast extract (Yeast Products Inc.), 0.2% L-malic acid (Sigma), 0.2% ammonium citrate, dibasic (Baker), 0.5% sodium acetate (Mallinckrodt), 0.1% Tween 80 (Baker), 0.5% potassium phosphate, dibasic (Mallinckrodt), 0.05% magnesium sulfate, 7 hydrate (Mallinckrodt), 0.02% manganese chloride, 4 hydrate (Mallinckrodt), 0.005% iron sulfate, 4 hydrate (Mallinckrodt) dissolved in 1000 ml of white grape juice (Welches). The pH was adjusted to 5.5 with 6N NaOH.

Medium F

LBS, Lactobacillus Selective Medium (Rogosa et al., 1951) adjusted to pH 5.5, using glacial acetic acid.

Medium G

A grape juice medium made from 1:1 dilution of white grape juice (frozen, then thawed) plus 0.05% yeast extract (YPI) and adjusted to pH 3.5 using 6N NaOH.

Medium H

Phase 4 bulk starter medium, used only for freezing and lyophilizing trials (Galloway West Co., Fond du Lac, WI., patent no. 4, 282, 255).

Medium I

Nonfat dry milk (11% solids) used only for freezing and lyophilizing trials.

Media A, B, C, E, F and G were sterilized by autoclaving at 121°C, 15 psi for 15 min. Medium D was autoclaved as two separate components and combined. Medium H was steam sterilized for 30 min and Medium I was autoclaved at 121°C, 15 psi for 12 min.

Isolation of Bacteria

Bacteria were isolated by making pour plates of

0.5 ml aliquots of diluted wine samples in Medium A treated with 50 mg/L cycloheximide (Sigma). This medium was used as initial isolation medium to prevent yeast and mold contamination of slow-growing malo-lactic organisms. Samples contaminated with large numbers of wine yeasts (as evidenced by obvious turbidity) were initially subjected to a sterile centrifuge treatment. Centrifugation at 1000 RPM for 15 min removed most of the yeast cells. The clarified supernatant was then plated in similar fashion.

Plates were incubated under carbon dioxide tension of approximately 8% at 28-30°C for 3 to 4 days. Typical lactic acid bacterial colonies developed that were elliptical in shape and creamy white in color. Microscopic examination showed them to be gram positive cocci in pairs and chains. Representative isolated colonies of varying size and color hue were removed aseptically from the agar in small blocks (approximately 64 cubic mm). These were then suspended and disrupted in sterile screw-capped tubes containing 10 ml of Medium A broth. Following growth at 30°C for 3 to 4 days, cultures were streaked on Medium A plates for isolation. When satisfied that distinct pure colonies had developed, they were again examined, inoculated as stab cultures in solidified Medium A and stored at 4°C. Cultures were routinely incubated either in the Gas Pak Carbon Dioxide System (BBL) or in a controlled environment carbon

dioxide incubator (National Appliance Co.) at 30°C for 3-4 days. Stab cultures were transferred every 3-4 months to maintain viabilty. In all, 28 strains were isolated for further study.

Throughout this study, all viable cell counts have been made using a micro-drop technique. The technique used was one that was developed for lactic streptococci and their phages (Willrett, 1982). It is well documented that Leuconostoc oenos is capable of forming long chains of cells (Beelman et al., 1980; Pilone and Kunkee, 1972). Such chains have been observed with regularity in gram stains of our cultures. Long chains containing numerous cells may produce only one macrocolony; therefore, the possibility of erroneously low counts is quite real. For enumeration, 1.0 ml of sample was aseptically blended with a Waring blender in chilled 0.1% (w/v) peptone (Bacto) diluent (99 ml) at high speed for 60 sec to break up the chains of cocci (Martley, 1972). The blended sample was then serially diluted in sterile 0.1% peptone. Each dilution was dispensed in four separate 0.025 ml micro-drops with an Oxford Micro-Doser repetitive pipette onto pre-dried (48 hour ambient temperature) plates of solidified Medium A or C at pH 5.5. Cell counts were determined by averaging the colony counts of the four drops and multiplying by the appropriate dilution factor. Accuracy of counts is thereby increased and the need for duplicate plating is avoided. Plates were always

incubated at 30°C under carbon dioxide tension for 3-5 days before enumeration.

Characterization of Bacteria

Isolates were characterized according to a number of parameters, based on the characterization techniques of previous investigators (Kunkee, 1967; Garvie, 1967 and Pilone and Kunkee, 1972). Isolates were first gram stained, checked for presence of catalase and surface growth on agar stab cultures. These are considered presumptive tests for malo-lactic bacteria.

Following these presumptive tests, all of the 28 isolates were checked for their relative ability to metabolize malic acid. Only those isolates determined to be active in decarboxylating L-malate were characterized further.

Cultures were then checked for dextran production from sucrose, ammonia production from arginine and production of lactic acid from glucose. These were determined according to accepted methods (Garvie, 1967; Pilone and Kunkee, 1972).

Carbohydrate Fermentation

Initial attempts to determine carbohydrate fermentation patterns met with equivocal results. The use of the Minitek Differentiation System (BBL) involving sugar-impregnated discs gave conflicting patterns. The

method described by Garvie (1967) produced extremely few positive results. The ultimate patterns of fermentation were elucidated by the following method. Cultures were grown in 100 ml of Medium A and centrifuged at 7000 RPM for 10 min. The pellet was washed in 100 ml of 0.1% peptone water and spun again. The pellet was resuspended in 10 ml of 0.1% peptone water for use as inoculum. Screw-capped tubes containing 5 ml of Medium B plus 0.5% of the membrane-filtered sugar to be tested received a 2% culture inoculum. Incubation was at 30°C. Sugars tested included L-arabinose, D-arabinose, cellobiose, fructose, glucose, lactose, maltose, mannitol, raffinose, ribose, sucrose, trehalose, xylose, galactose, glycerol, rhamnose, D-mannose, L-sorbose, salicin, and esculin. Due to limited solubility, salicin and esculin were prepared in 0.2% concentration. Uninoculated media were used as negative controls.

A specific growth rate was determined for each carbohydrate. Spectrophotometric analysis was performed at periodic intervals for 96 hours using a Perkin-Elmer 35 Spectrophotometer at 600 nm. The specific growth rate, k, was determined using the formula:

$$k = \frac{2.303\ (\log b - \log a)}{t}$$

where a and b are the optical density readings at two sampling times during logarithmic growth, and t is the time elapsed. Where necessary, cultures were diluted to

remain within an absorbance range of approximately 0 to 0.4, the range within which Beer's Law is functional.

The specific growth rates thus determined were compared to the growth rate for glucose as carbon source. All growth was expressed as a percentage growth rate compared to glucose, which was considered to be 100%.

Growth Rate in Various Media

In order to determine the best growth medium, the growth rate was ascertained for two isolates in four test media. Media A, D, E, and F were employed. A 4 day broth culture of organisms grown in Medium A was used as inoculum. A 2% inoculum was added to a 50 ml volume of test media. Cultures were incubated at 30°C for 4 days on a Multi-MagneStir (Lab-Line Scientific, Inc.) at its lowest setting. Viable cell counts were determined daily for 4 days on solidified Medium A.

Optimum Growth Temperature

A Thermocon temperature gradient incubator (Model TN-3, Scientific Industries, Inc.) was used to determine temperature optima for six selected isolates. A temperature gradient of 5 to 36°C was established in the incubator. The L-shaped test tubes were each filled with 15 ml of Medium A, sterilized by autoclaving and placed in the incubator for 2 hours to allow a temperature gradient to form. Tubes were inoculated at a rate of

approximately 1% from a 72-hour broth culture in Medium A. Growth was measured spectrophotometrically at 600nm for 72 hours. Specific growth rates (k) per hour were determined using the formula previously described. Generation times (g) were calculated from k values with the equation, g = .693/k. Optimal growth temperatures were determined through inspection of graphs constructed by plotting generation time against temperature.

Optimum pH

Eight of our isolates and four reference strains were grown at different pH values in Medium C ranging from 2.9 to 7.0. Growth was followed spectrophotometrically for 3-4 days at 600 nm. The pH was adjusted with either 8N tartaric acid or 6N NaOH. Uninoculated media served as controls and blanks.

Growth at 10%, 12%, and 14% Ethanol

Small screw-capped tubes containing Medium G, pH 3.5, and appropriate volumes of 95% ethanol added to achieve 10, 12 and 14% ETOH (v/v) were used. Broth cultures in log phase were used as inoculum at the rate of 2%. Tubes containing the same medium with no added ethanol were used as controls. Optical densities were determined spectrophotometrically at 600 nm.

Utilization of Organic Acids

The ability to utilize two organic acids, L-malate and citrate, in the presence of a fermentable carbohydrate was tested. Isolates were grown 4 days in Medium B plus 0.5% added glucose and 0.2% of the organic acid to be tested. Uninoculated media served as controls. Disappearance of the appropriate spot on a paper chromatogram was considered positive utilization of the acid. (Paper chromatography method - see below).

Effect of Added Malate on Growth

The effect of several concentrations of malic acid on the growth of four isolates was determined by the addition of 0.10, 0.15, 0.20, 0.25, and 0.30% malate plus 0.5% glucose to Medium B. Readings were taken in a spectrophotometer at 600 nm after growth for 3 days.

Detection of Malo-Lactic Activity

Paper Chromatography

This method was used to qualitatively detect the conversion of malic acid to lactic acid. A butanol:water:formic acid solution plus bromcresol green was used to develop the chromatogram (Kunkee, 1968, 1974). Whatman #1 chromatographic paper measuring 20 by 28 cm was commonly used. Spots of samples to be tested using 10 microliter pipettes were made 3 cm from the

bottom edge. After drying, the paper was stapled to form a free-standing cylinder. The paper was placed with spots toward the bottom into a chromatographic jar containing about 70 ml of the solvent described above. These chromatograms were generally run for 8 hours or overnight; the paper was removed and allowed to dry under a solvent hood. Presence of organic acids was indicated by yellow spots against a blue-green background. Absence of malate, coupled with the appearance of a lactate spot, was indicative of a positive malo-lactic fermentation.

Respirometry

A semi-quantitative method used to measure malate reduction was respirometric analysis. An eight-channel Gilson Differential Respirometer (Gilson Medical Electronics, Inc.) was used to determine carbon dioxide production from malate. This involved adding 1.0 ml of L-malate solution (2%) to 0.8 ml of sodium acetate buffer (pH 5.0) plus 0.1 ml of 1% nicotinamide adenine dinucleotide (NAD, Sigma) solution and 0.1 ml of 1% manganese chloride (Sigma) solution. This was considered the reaction mixture and was placed in the main vessel chamber. One ml of washed, resting cells was usually placed in one side arm and 1 ml of 6N HCl was placed in the other. The reaction was started by tipping the cells into the reaction mixture. The rate of evolution was determined from 90-minute plots of carbon dioxide

evolution in microliters versus time in 15-min intervals. After the 90 min reading, the side arm containing the HCl was tipped in to the main reaction chamber. This served to liberate any remaining carbon dioxide from the solution and gave the final gas volume. A control was included which contained all of the solutions with no added cells. The number of microliters were read directly from the dial micrometers on each channel. These were corrected for volume of gas at standard temperature and pressure by using the formula:

$$\text{Correction factor} = \frac{273\ (P_b)}{(t + 273)(760)}$$

where P_b = operating pressure (mmHg)

and t = operating temperature in °C

Since t was 30°C and P_b was almost always 762.0, a correction factor of 0.90 was always used.

Since the malate decarboxylating activity of our isolates and reference strains could vary depending on the mass of cells present, the amount of carbon dioxide produced per mg dry cell weight was determined.

Enzymatic Method

This quantitative method (McCloskey, 1980) was utilized when it was necessary or desirable to measure precise amounts of malate present in a medium or wine. It was advantageous that the sample volume was only 25

microliters in this assay; therefore malate levels could be tested for a period of time with no appreciable change of volume in the sample. The assay consisted of combining 25 microliters of sample and 3.0 ml of a glycine-glutamate buffer, pH 9.8, plus NAD in a 1 cm cuvette. After mixing, the absorbance of this mixture was read at 340 nm in an Update Gilford 2000 spectrophotometer and noted as E_1. The assay reaction was begun by adding 25 microliters of an enzyme solution containing 1250 and 450 IU/ml of malate dehydrogenase (Calbiochem) and glutamate oxaloacetate transaminase (Sigma) respectively. The mixture was agitated and incubated at 28-30°C for 8-10 min. The final absorbance was noted as E_2. Calculations of the malate level remaining in the experimental sample were made from the following equation:

$$\text{Sample malic acid in mg/L (ppm)} = \triangle E(\text{Sample}) \times F$$

where F is the factor determined by performing the assay on a set of standards with known concentrations of malic acid. The factor of 3220 was determined for our system.

Relative Malate-Reducing Ability

In order to reduce the number of strains in our collection for further study, a malate assay (enzymatic method) was performed in Medium C and E, pH 5.5 at 22°C. Only those strains capable of a fairly rapid and complete

reduction of malic acid in the sample continued in the final analyses.

Determinants of Malate Fermentation

pH Effect on Growth and Malate Reduction

In order to demonstrate the efficacy of our isolates in reducing malate at pH levels approximating those of Oregon wines, cultures were subjected to a series of trials at decreasing pH values in Medium C. The pH was adjusted with 8N tartaric acid solution or 6N NaOH solution. Cell numbers were determined at regular intervals in these same trials to determine cell population growth, survival or destruction. Malate levels were determined by enzyme assay.

Sulfur Dioxide Tolerance

Since residual sulfur dioxide may be present in a new wine at levels ranging from 0-30 ppm, depending on the type of wine and initial treatment level, it was necessary to determine the level at which cell inhibition occurs. Appropriate aliquots of a 0.1% sodium metabisulfite solution were added to Medium G, pH 3.5, to obtain levels ranging from 5 to 30 ppm free sulfur dioxide. These samples were allowed to equilibrate for 24 hours. Sulfur dioxide levels were determined using the Ripper titration method (Amerine and Ough, 1980). Growth was determined spectrophotometrically at 600 nm

and cultures were incubated at 22°C. Cultures in media with no added sulfur dioxide were included as baseline controls.

Inhibition by Fumaric Acid

The addition of fumaric acid to inhibit the malo-lactic fermentation is still practiced by some wineries where the fermentation is considered undesirable (Pilone, 1975; Cofran and Meyer, 1970). It was of some interest, therefore, to determine the vulnerability of our strains to this toxic additive. Tubes of Medium G, pH 3.5, were prepared with 0.3 g/L, 0.6 g/L and 1.2 g/L fumaric acid. Tubes with no added fumaric acid were included as baseline controls. Growth was determined by a spectrophotometer at 600 nm; incubation was at 30°C.

Malate Reduction and Growth in New Wine

Two samples of new wine from cooperating wineries served as small-scale trials for our isolates. Both wines were sterile-filtered to remove contaminating organisms from them. The samples of Pinot Noir were inoculated at a rate of 1%. Samples were checked at regular intervals for malate levels and viable cells. The titratable acidity, pH and presence of malic acid in the samples was determined prior to the trials and also at the completion of the experiment. Control samples were included (no inoculum) and all samples were

incubated at 22°C.

Bacterial Survival of Freezing or Freeze-Drying and Subsequent Storage

In order to make these malo-lactic bacteria available for commercial use, it was necessary to determine the best method of storage for concentrated cell suspensions. Four of our isolates that had proven promising in laboratory scale experiments were chosen to be tested.

Tables 8 through 12 in the Results section give the complete format of cultures and media used, additives employed, storage temperatures and percent survival for each set of conditions.

Frozen Concentrates

Cultures were prepared as 3 day broth cultures grown in 100 ml of Medium C at 30°C. Cells were harvested by centrifugation at 7000 RPM for 10 min. Pellets were then resuspended in 50 ml of the medium to be tested. These suspensions were then sterile blended for 15 sec in order to make the cell concentration homogeneous. The suspension was then dispensed into sterile 5 ml polypropylene cryotubes (Vangard International Inc.) in 3-ml volumes and frozen at -20 and -40°C. Initial viable cell counts were performed using 1.0 ml of the concentrated cell suspension. Viable cell counts were made at 2-week intervals for 3 months.

Samples to be plated were quick-thawed at 30°C for 30 min in a serological water bath (Scientific Products).

Freeze-Dried Concentrates

Cultures were prepared and harvested as for frozen concentrates. Concentrated cell suspensions were made using various media and blended for homogeneity. Initial viable cell counts were performed using 1.0 ml of the concentrated cell suspension. The suspension was then dispensed into sterile, 3-ml long-necked, glass lyophilizer ampules (Wheaton Scientific) in 2 ml volumes. The contents were quick-frozen by gentle agitation in an acetone/dry ice bath. Samples were immediately placed on the vacuum tree of a lyophyilizer (Virtis, Model 10-145 MRBA) and connected to vacuum. The lyophilizer condensor was maintained at -50°C and the vacuum was maintained at 30-50 microns pressure. Samples were allowed to run overnight, approximately 16 hours. Ampules were removed without loss of vacuum by flame-sealing the vial with a two-pronged gas torch. Individual sealed ampules were checked for adequate vacuum using a high frequency vacuum tester (Electro-Technic Co.). Ampules with insufficient vacuum were discarded.

All ampules containing freeze-dried bacteria were stored at room temperature and pressure. Viable cell counts were determined at 2-week intervals for 2 months. Samples were always rehydrated with 1.0 ml of Medium C

using sterile syringes. Since the rehydration volume was half the initial volume, the resultant counts were divided by 2 to give the actual count per ml.

RESULTS

Through the isolation techniques employed, 28 isolates were obtained. Eighteen isolates active in the malo-lactic fermentation (MLF) were further characterized (Table 1). Activity was initially determined by growth in Medium A. Disappearance of the malic acid spot after 1 week was considered a positive MLF.

In order to provide for the fastidious requirements of wine leuconostocs, a complex medium containing tomato juice or vegetable juice supplemented with glucose and fructose was generally used for cultivation of our isolates. It has been demonstrated that such organisms grow best in media containing these substances (Amachi, 1969, 1975; Garvie and Mabbit, 1967; Ingraham et al., 1960; Kunkee, 1967; Radler, 1975; Yoshizumi, 1975). Our experience confirms these findings.

Characterization of Bacteria

All active strains isolated were characterized as summarized in Table 2. Carbohydrate fermentation patterns were quite similar among the isolates; four representative isolates are compared in Table 3. All of the strains fermented glucose, fructose, maltose, ribose, cellobiose, trehalose, salicin and esculin. Arabinose was utilized by all to some extent, sorbose was utilized by some strains. Mannitol, raffinose, xylose and sucrose

Table 1. Number of strains isolated from each wine variety

Wine variety	No. of isolates	No. active in MLF
Pinot Noir	20	14
Merlot	6	3
Chardonnay	2	1

Table 2. Characterization profile of 18 isolates

Morphology	Cocci, pairs and chains
Gram reaction	+
Heterofermentative	+
Facultative anaerobes	+
Gas from glucose	+
Catalase reaction	-
Growth on agar stab surface	+/-
Dextran from sucrose	-
Ammonia from arginine	-
Lactic acid from glucose	+

Table 3. Carbon and energy sources utilized by 4 isolates

Carbon Source	% Growth rate compared to glucose			
	Er-1a	Er-1c	Ey-1a	Ey-2d
L-arabinose	57	35	33	31
Cellobiose	115	103	87	93
Fructose	109	105	106	103
Glucose	100	100	100	100
Lactose	0	0	0	0
Maltose	98	102	108	106
Mannitol	0	25	0	15
Raffinose	0	0	0	5
Salicin	85	90	77	72
Ribose	111	107	105	96
Sucrose	0	9	0	0
Trehalose	87	101	103	112
Xylose	5	0	3	0
D-arabinose	39	47	81	42
Galactose	110	0	0	0
Glycerol	0	0	0	0
Rhamnose	0	0	0	0
D-mannose	0	0	0	0
L-sorbose	89	11	5	5
Esculin	93	98	85	89

were weakly utilized by some strains. Galactose was readily used by one strain. Lactose, glycerol, rhamnose and mannose were not utilized by any of the strains. All 18 of the isolates utilized malate and citrate in the presence of a fermentable carbohydrate (glucose).

These general characteristics are similar to those of L. oenos as described by previous investigators (Garvie, 1967; Pilone and Kunkee, 1972; Beelman et al, 1977) as well as Bergey's Manual of Determinative Bacteriology (Buchanan et al., 1974).

Growth Rate in Various Media

Inspection of Figures 1 through 4 show the greatest increase in cell numbers over time in Medium A, the modified Rogosa medium. Beginning with approximately 1×10^6 CFU/ml at time 0, cell numbers reach approximatelly 3×10^8 CFU/ml at 96 hours, an increase of greater than 0.5 log units per day. Adequate growth is obtained in Medium E. Media D and F appear not to be desirable growth media for our isolates.

Optimum Growth Temperature

Figures 5 through 10 show the temperature optima for six selected strains. In all cases, the optimum lies between 28 and 30°C. There is some variation in the exactness of the optima. Some, like Ey-1a and Ey-2c had a sharply defined optimum. Others, most notably Er-1a

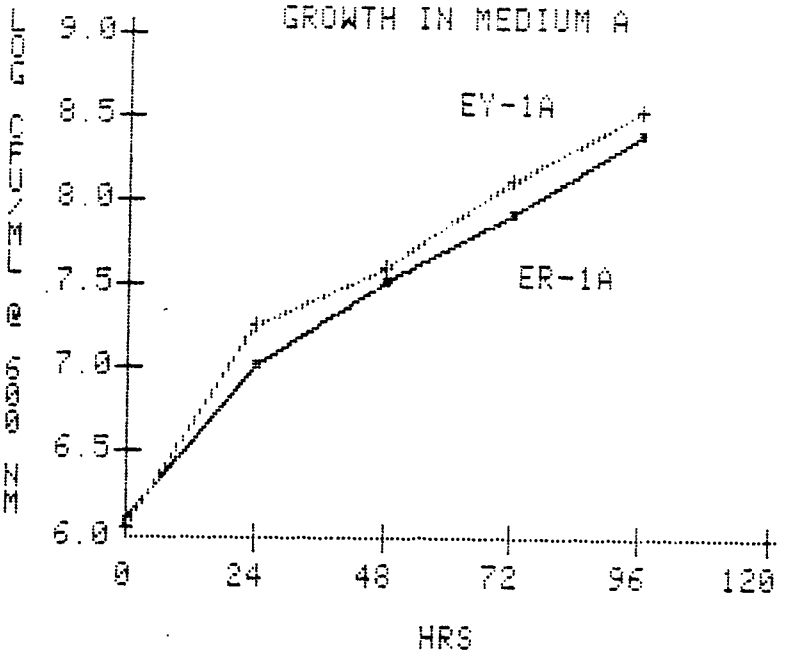

Figure 1. Cell counts of 2 isolates in Medium A

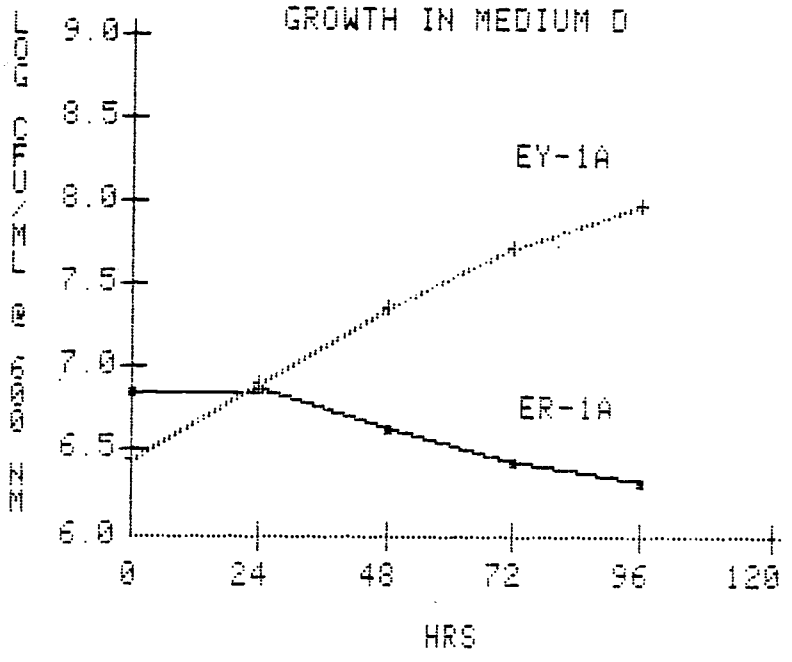

Figure 2. Cell counts of 2 isolates in Medium D

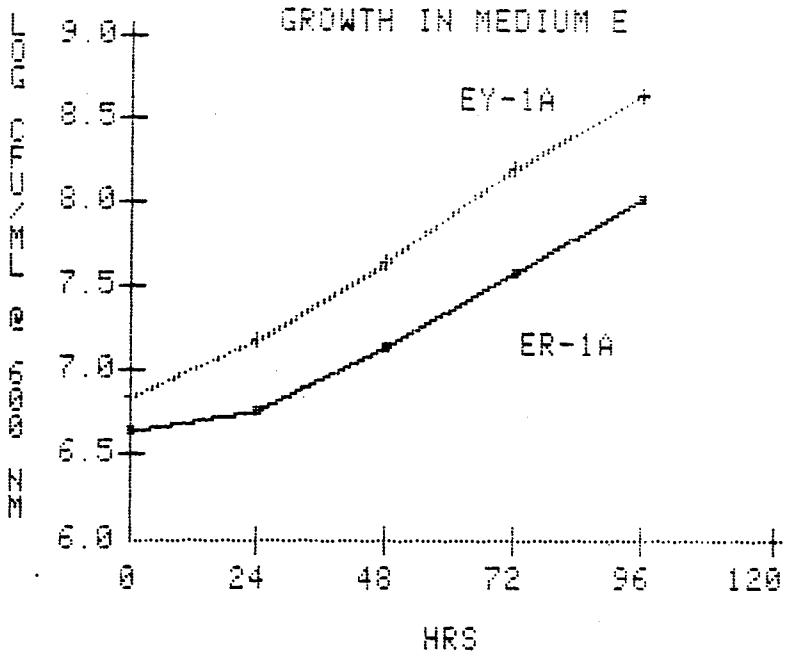

Figure 3. Cell counts of 2 isolates in Medium E

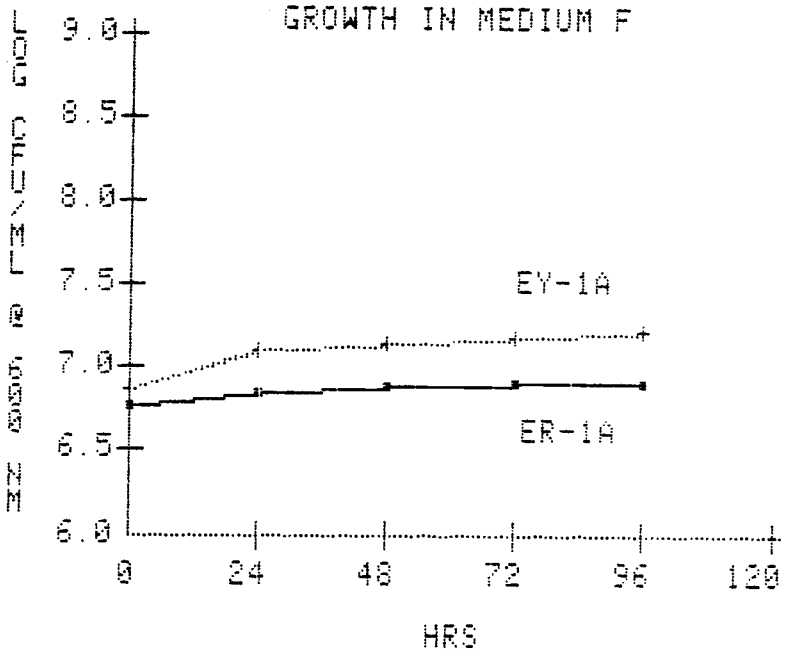

Figure 4. Cell counts of 2 isolates in Medium F

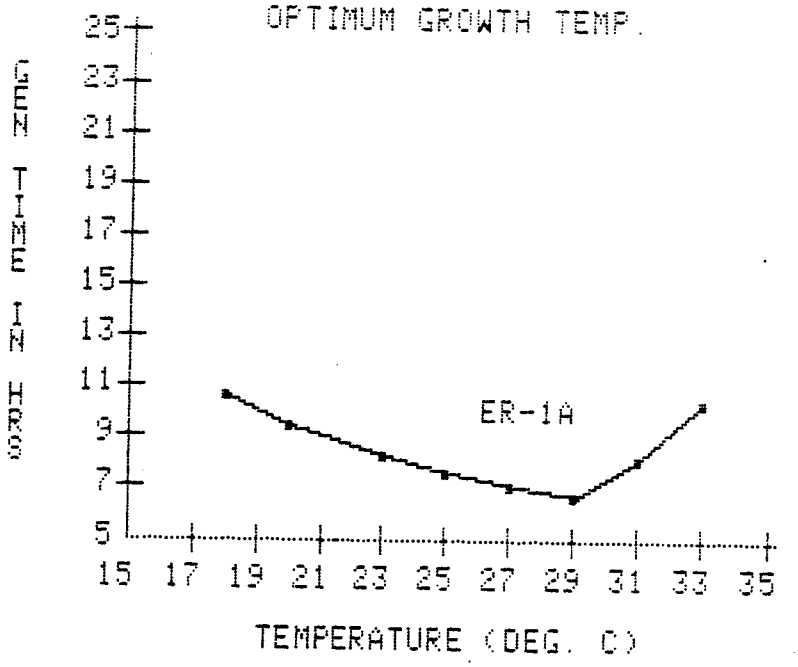

Figure 5. Temperature optimum of strain Er-1a

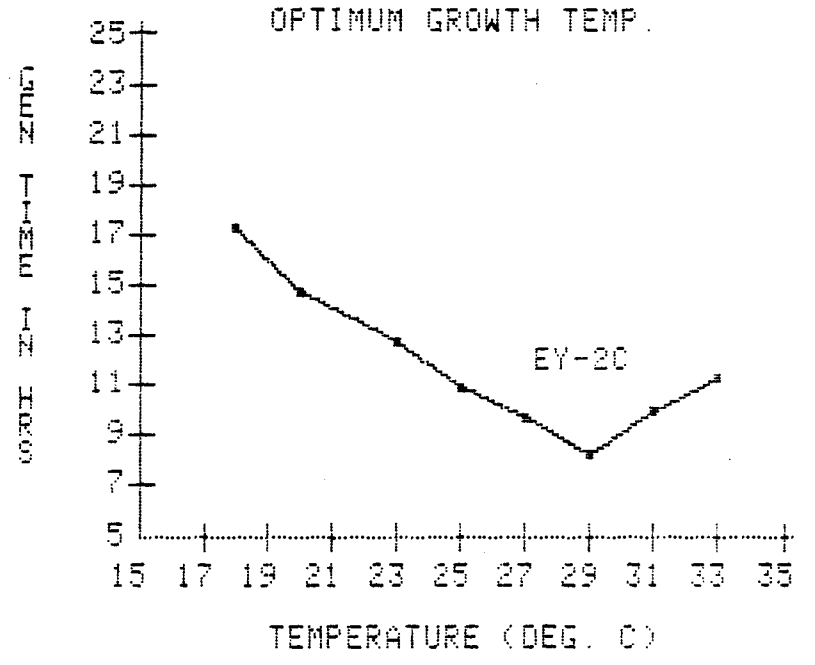

Figure 6. Temperature optimum of strain Ey-2c

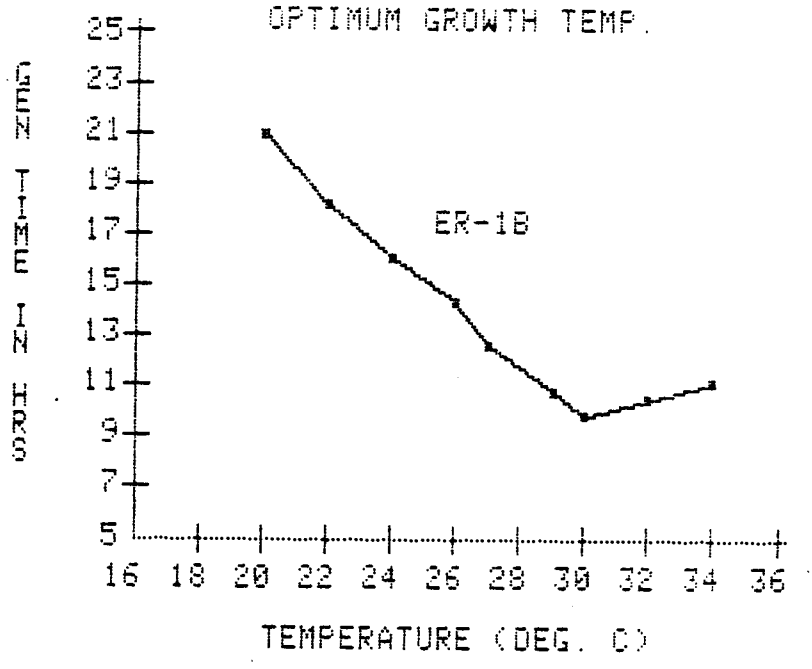

Figure 7. Temperature optimum of strain Er-1b

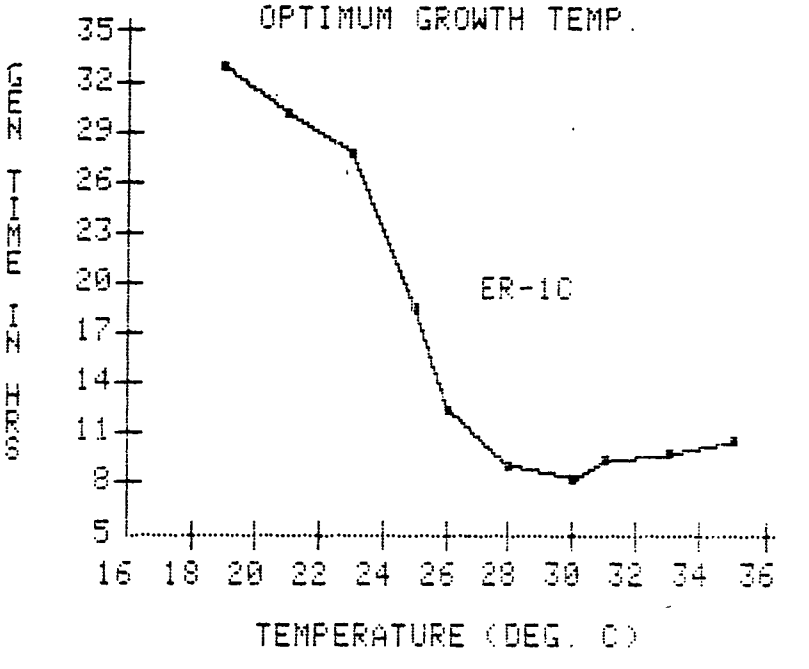

Figure 8. Temperature optimum of strain Er-1c

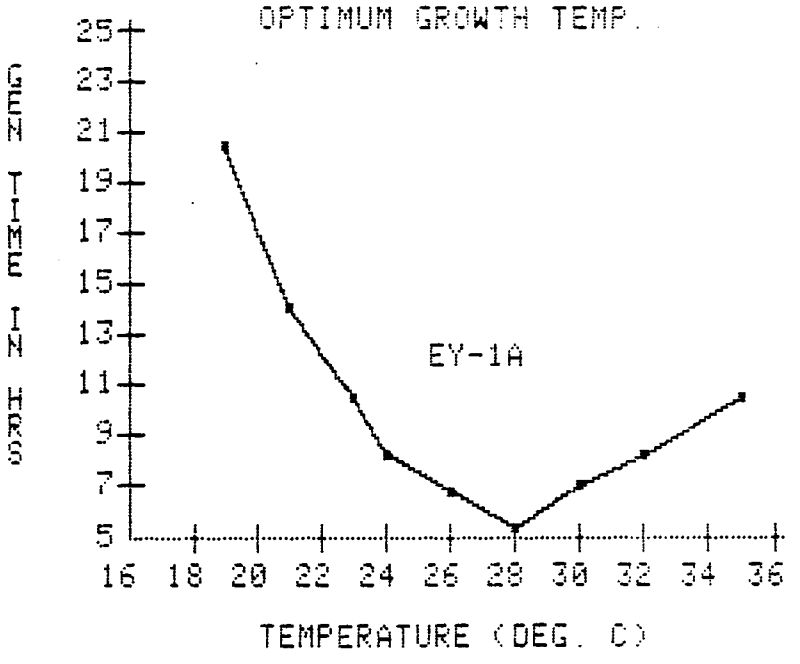

Figure 9. Temperature optimum of strain Ey-1a

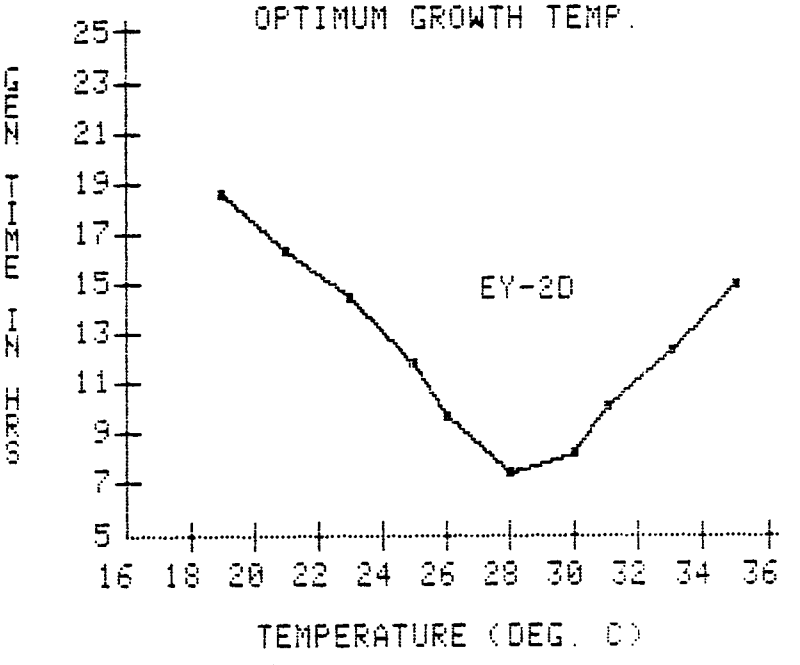

Figure 10. Temperature optimum of strain Ey-2d

have a less well defined optimum. The rate of increase in generation time below and above the optimum varies among strains. For strain Er-1a, the rate of increase in generation time as the temperature decreases is by far the lowest of all strains tested. Even at 18°C, the generation time is only 10.8 hours.

Optimum pH

Inspection of Figures 11 through 16 demonstrates the slight variability of optimum pH for 8 of our isolates and 4 reference strains. Some strains exhibit a sharp optimum, between pH 5.0 and 5.5, as evidenced by PSU-1, MLT-k1i, Ey-2c, and Ey-4b. Other strains exhibit a relatively broad optimum ranging between pH 4.5 and 5.75, as in the graphs of Er-1a, Er-1b, Er-1c, Ey-1a; Er-3b and Ey-2d have optima which are intermediate by comparison. Strain ML34 appears to exhibit the largest range, having a broad optimum between pH 4.0 and 5.5.

Growth at 10%, 12%, and 14% Ethanol

Figures 17 through 20 depict the effect that varying concentrations of ethanol had on 4 of our isolates. Strains Er-1a, Er-1c and Ey-1a all demonstrate a peak of growth at approximately 8 days followed by a decrease in optical density. One notable exception was strain Ey-2d which, at 10% ethanol, grew at nearly the same rate as the control. All strains exhibited a

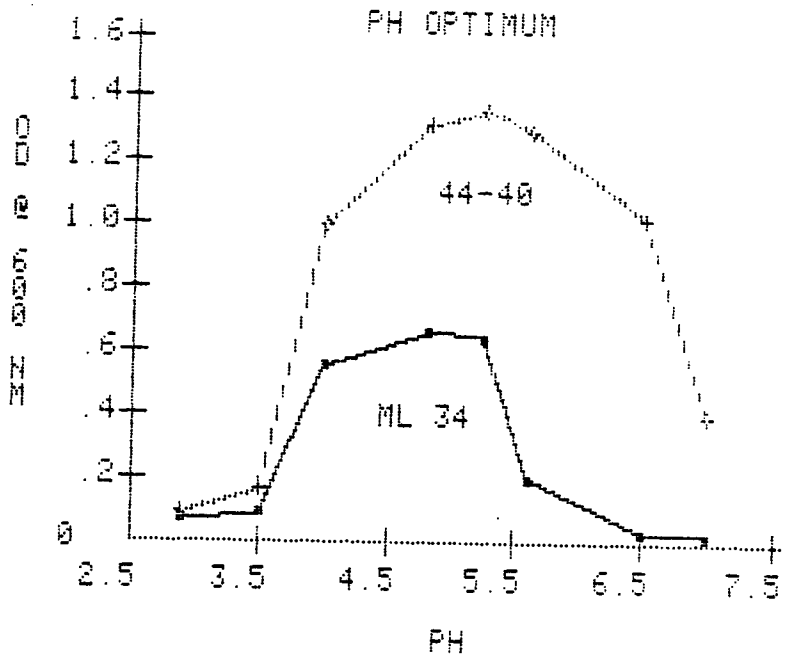

Figure 11. pH Optimum of reference strains 44-40 and ML34

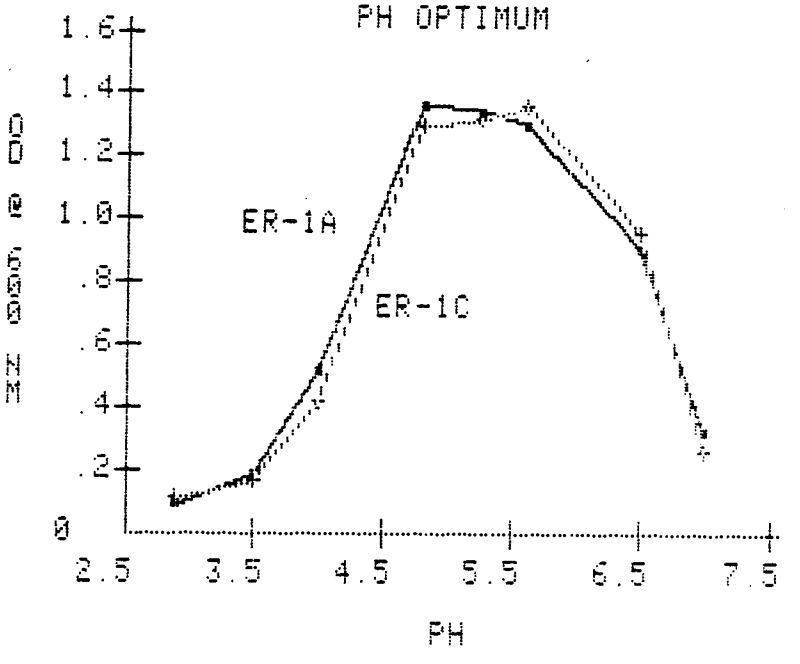

Figure 12. pH Optimum of strains Er-1a and Er-1c

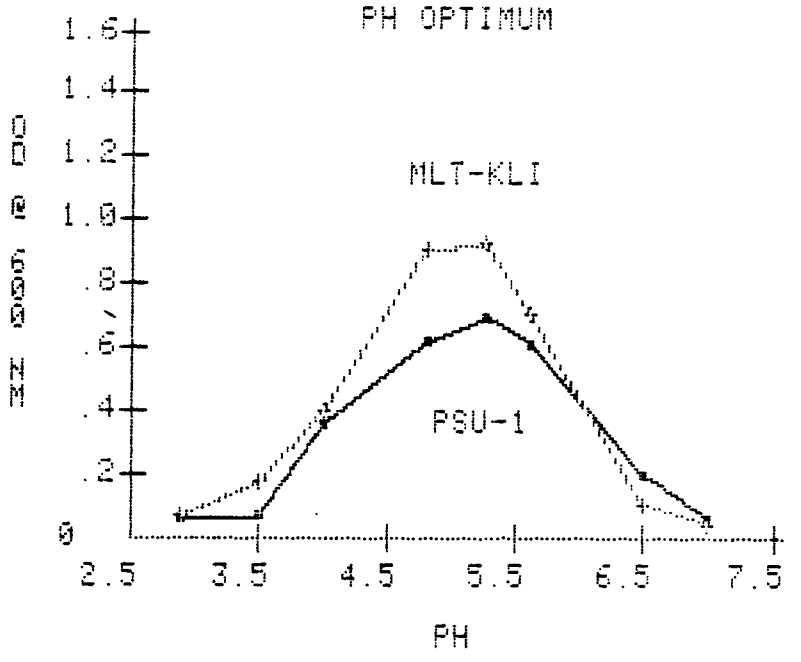

Figure 13. pH Optimum of reference strains MLT-kli and PSU-1

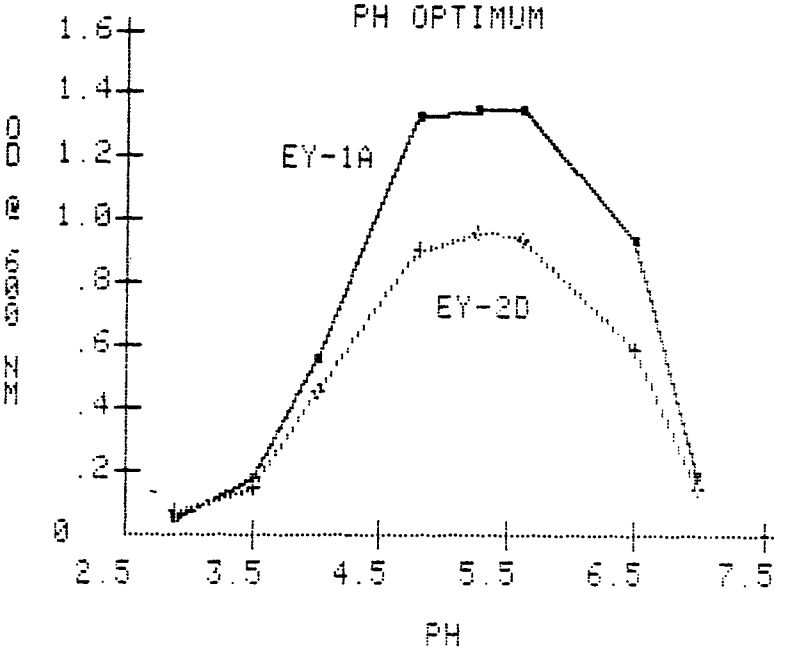

Figure 14. pH Optimum of strains Ey-1a and Ey-2d

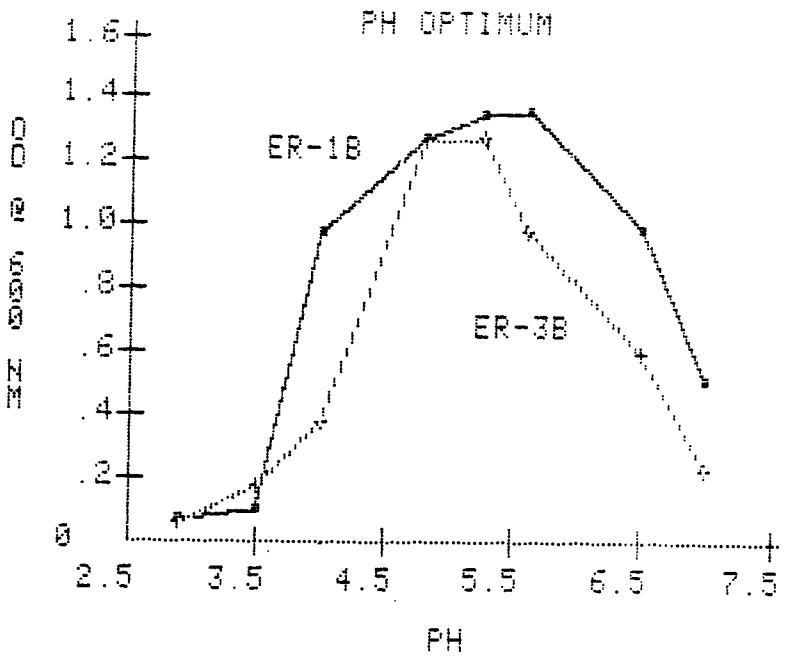

Figure 15. pH Optimum of strains Er-1b and Er-3b

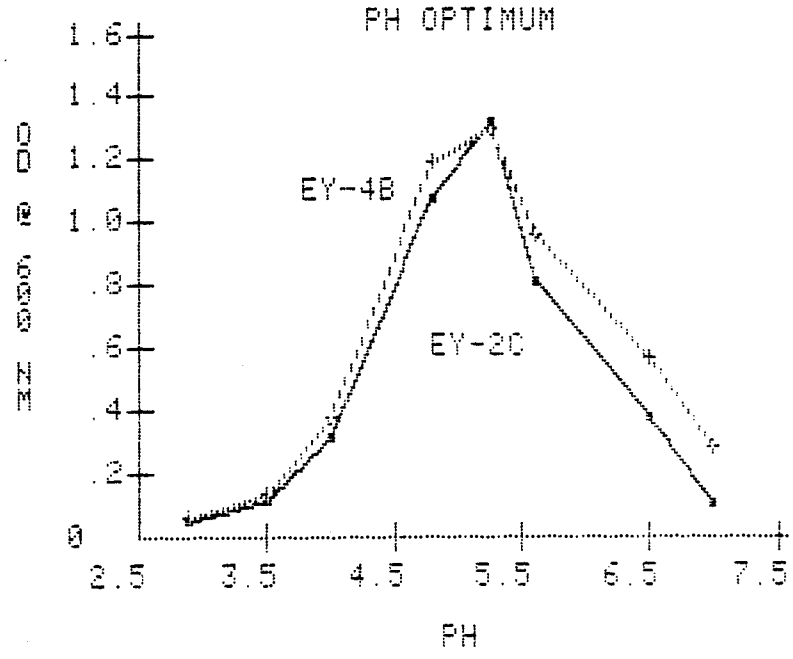

Figure 16. pH Optimum of strains Ey-4b and Ey-2c

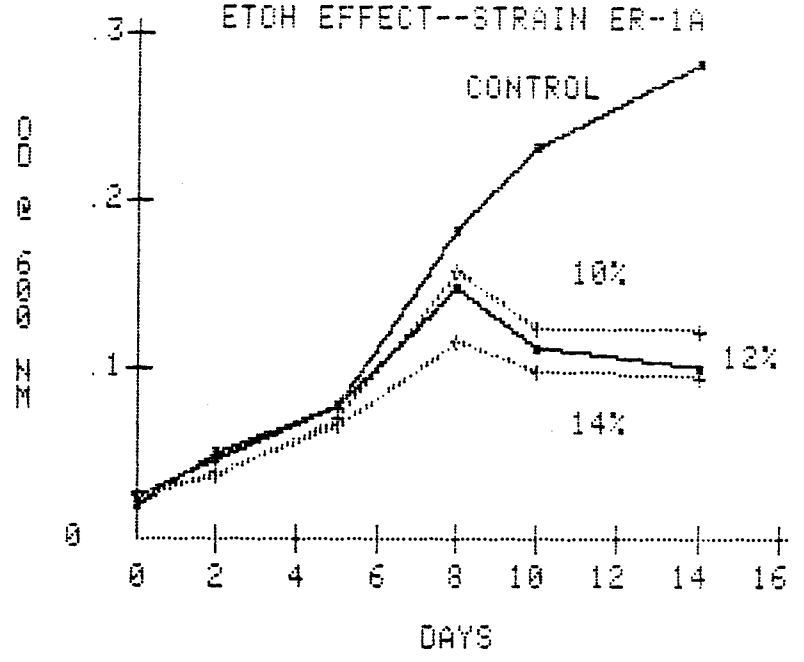

Figure 17. Effect of various ethanol levels on growth of strain Er-1a

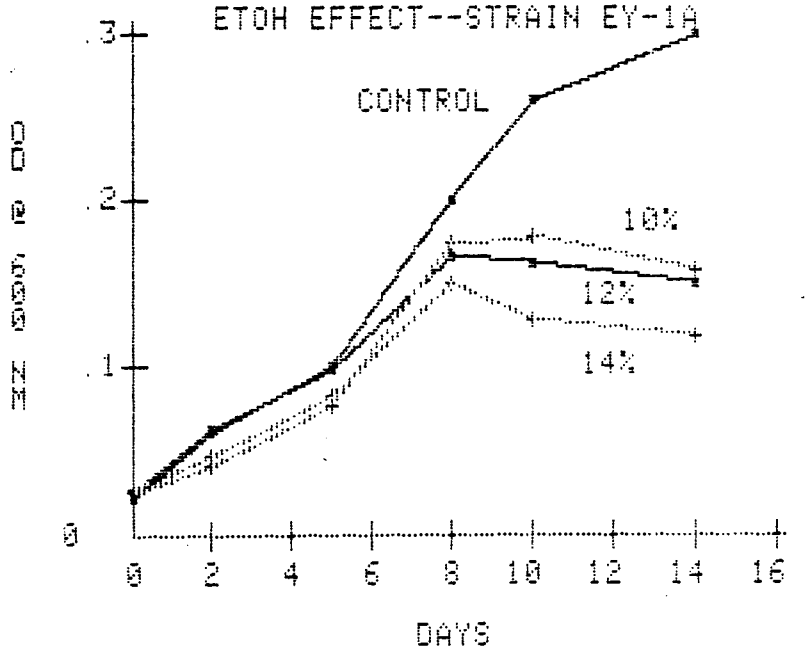

Figure 18. Effect of various ethanol levels on growth of strain Ey-1a

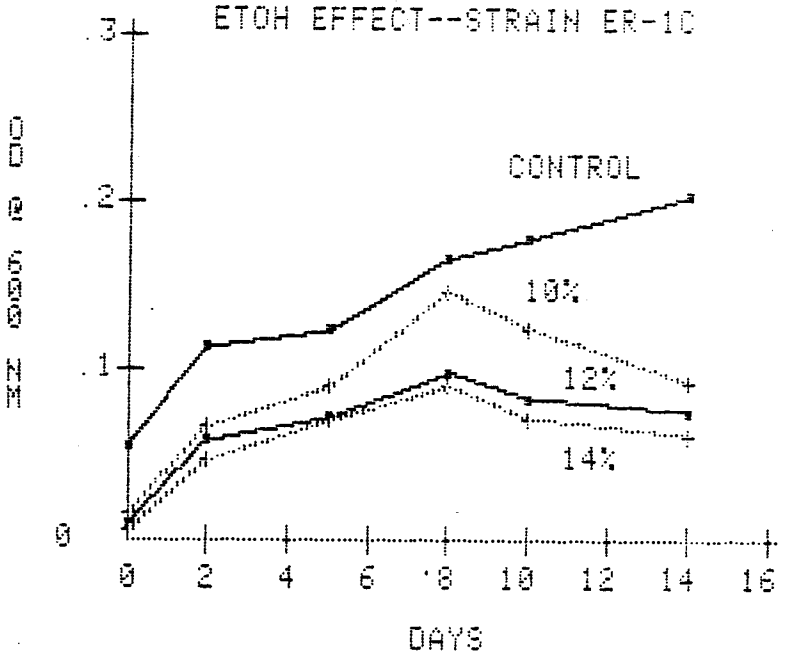

Figure 19. Effect of various ethanol levels on growth of strain Er-1c

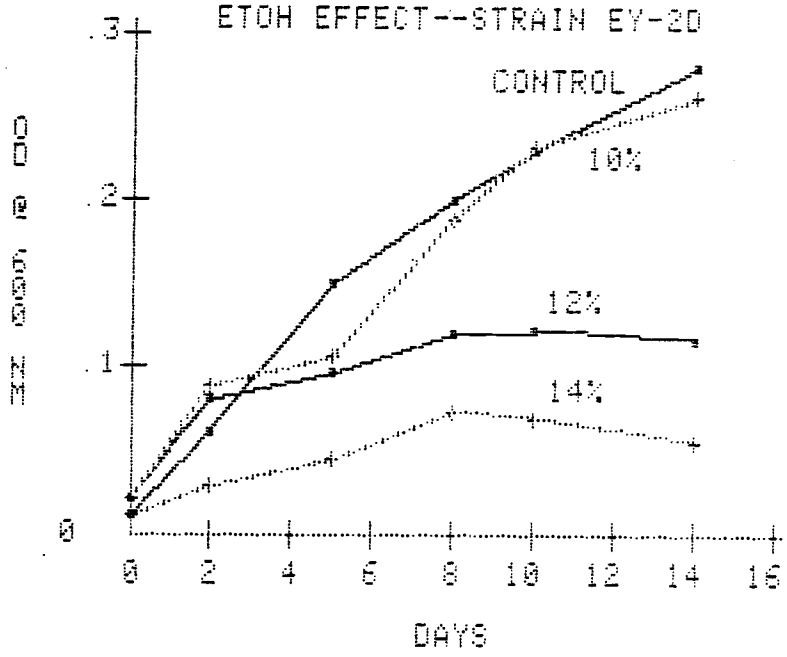

Figure 20. Effect of various ethanol levels on growth of strain Ey-2d

proportionate decrease in growth as the alcohol level increased.

Effect of Added Malate on Growth

Figure 21 shows the effect of increasing malate levels on the growth of four isolates compared with the reference strain PSU-1. While the reference strain appeared to grow better under these conditions (pH 5.5 and 30°C), there was little increase in optical density above 0.2% malate and no increase above 0.25%.

Detection of Malo-Lactic Activity

Paper Chromatography

All 28 strains were checked for their ability to convert malic acid to lactic acid. Cultures were grown in tubes of Medium A for 1 week. Paper chromatograms were performed at the completion of the experiment. Disappearance of the malic spot and subsequent appearance of the lactic spot were accepted as proof of MLF activity. In all, 18 strains were shown to completely metabolize the malic acid present and were retained for further study.

Respirometry

The respirometric analysis of MLF activity in 10 of our strains and two reference strains is shown in Table 4. One strain, Ey-2d, was able to produce carbon

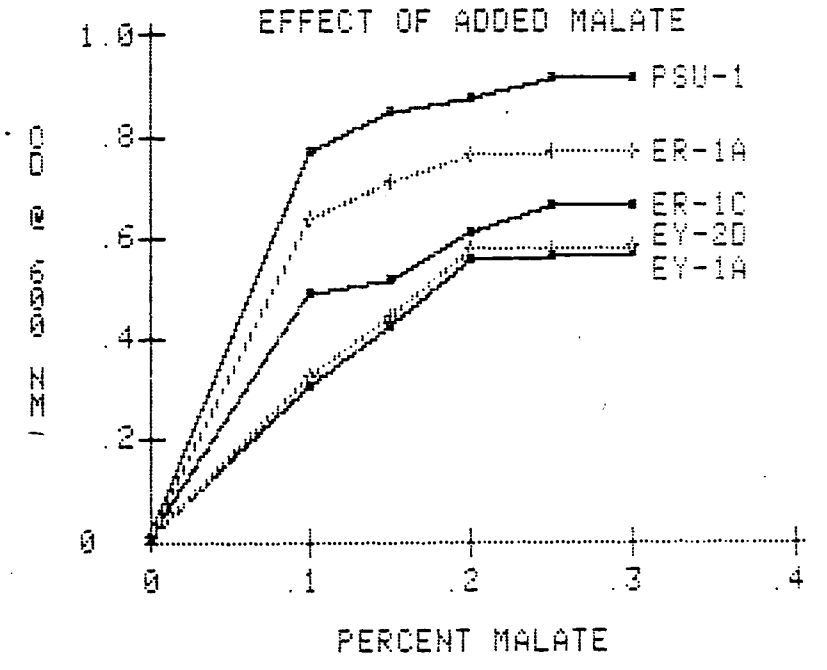

Figure 21. Effect of added malate on growth of various strains

dioxide at a rate greater than ML34. Several strains performed better than or comparable to the reference strain PSU-1. Eight strains not shown produced very little carbon dioxide under these conditions. Strains which produced less than 4.0 ul of gas/min/mg dry weight were not considered active in the decarboxylation of malic acid.

As depicted in Table 4, the abilities of the isolates to decarboxylate malate with respect to their dry weights were compared. The malo-lactic activity of these organisms did not appear to be proportional to the dry weight determination. It appears that the capacity to decarboxylate malic acid is dependent on individual strain characteristics rather than the mass of cells present.

Relative Malate-Reducing Ability

An enzymatic essay was performed periodically on all 18 strains growing in two different media. As shown in Table 5, the malate fermentation rate, expressed as ppm malate reduced per day was compared for these strains. A number of our isolates are again shown to be marginal in their MLF activity. However, an equal number appear to be quite active. A reduction in malate level of 300 ppm/day in Medium C and 500 ppm/day in Medium E was arbitrarily used to exclude 9 organisms from further study. The higher rates for malate reduction in Medium E

Table 4. Comparison of carbon dioxide evolution[a] versus cellular dry weight of isolates and reference strains

Strain	Dry wt. (mg/ml)	ulCO2/min[b]	ulCO2/min/mg
Er-1a	0.38	5.44	14.3
Er-1b	0.21	1.66	7.9
Er-1c	0.57	4.56	8.0
Er-3b	0.86	3.83	4.5
Er-4a	1.12	7.52	6.7
Ey-1a	1.32	11.80	8.9
Ey-1c	0.18	0.59	3.3
Ey-2a	1.44	6.20	4.3
Ey-2c	0.75	8.42	11.2
Ey-2d	0.54	12.93	23.8
ML34	0.48	10.86	22.5
PSU-1	0.69	5.55	8.1

[a] Values are averages of duplicate trials

[b] Amount of CO2 produced per minute using the respirometer

Table 5. Relative malate fermentation rates of 18 isolates and 2 reference strains

Strain	Medium C (ppm/day)	Medium E (ppm/day)
PSU-1	419	395
ML-34	146	327
Er-1a	440	544
Er-1b	370	509
Er-1c	417	530
Er-1d	32	230
Er-1e	86	194
Er-3b	416	534
Er-3c	102	209
Er-3d	244	159
Er-3e	193	262
Er-4a	398	523
Er-4c	155	533
Er-4d	278	518
Ey-1a	407	528
Ey-1c	238	452
Ey-2a	121	314
Ey-2c	296	537
Ey-2d	413	547
Ey-4b	380	536

are due in part to a higher initial level of malic acid (3000 ppm vs. 2000 ppm) in the medium.

Determinants of Malate Fermentation

pH Effect on Growth and Malate Reduction

Inspection of Figures 22 through 25 reveal little difference between two of our isolates and two of the reference srains at pH 4.0. All 4 strains exhibit adequate growth without any delay and effectively reduce the malate present. Strain Er-1a appears to be the most efficient on both counts. At pH 3.5, differences become more pronounced as observed in Figures 26 through 29. Growth is adequate for 3 strains; ML34 experiences a noticeable lag in growth. Malate reduction follows a similar pattern, exhibiting delayed reduction in the case of ML34. At pH 3.0, Figures 30 through 33, all cultures experience a decrease in cell numbers with ML34 showing the greatest decrease and Er-1a showing the smallest decrease. Malate levels follow the predictable course of an inverse function of cell numbers. ML34 experiences nearly a complete loss of viable cells with no subsequent MLF activity. Er-1a shows the highest cell survival and the most rapid and complete MLF activity. At pH 2.8, Figures 34 through 37, the pattern is repeated, with only strains Er-1a and Ey-2d exhibiting a detectable decrease in malate levels, despite continued loss of viable cells.

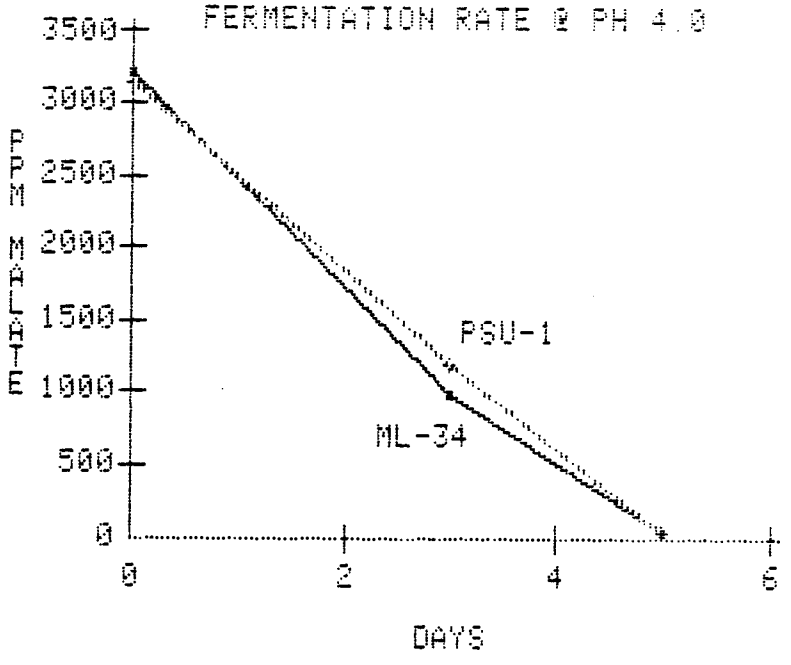

Figure 22. Malate fermentation rate at pH 4.0 of strains PSU-1 and ML34

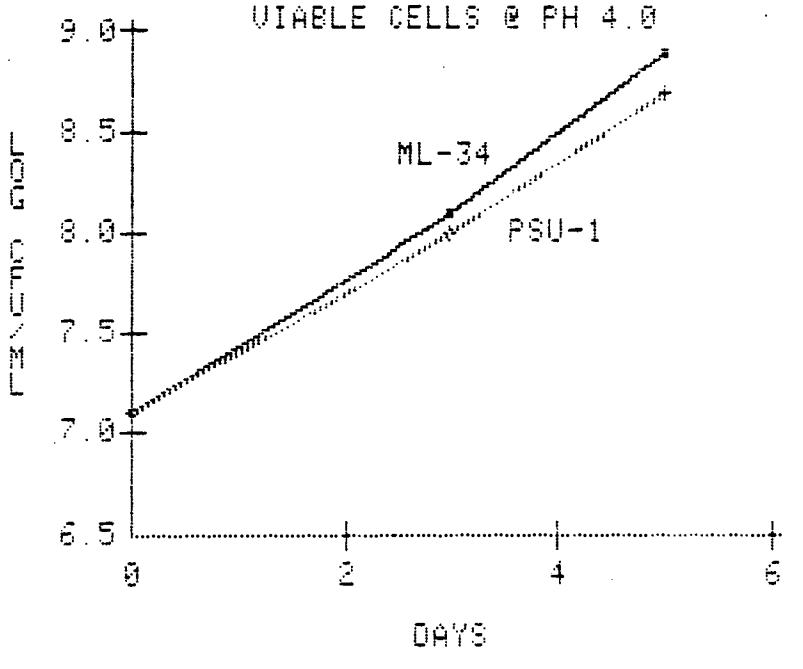

Figure 23. Viable cell counts at pH 4.0 of strains PSU-1 and ML34

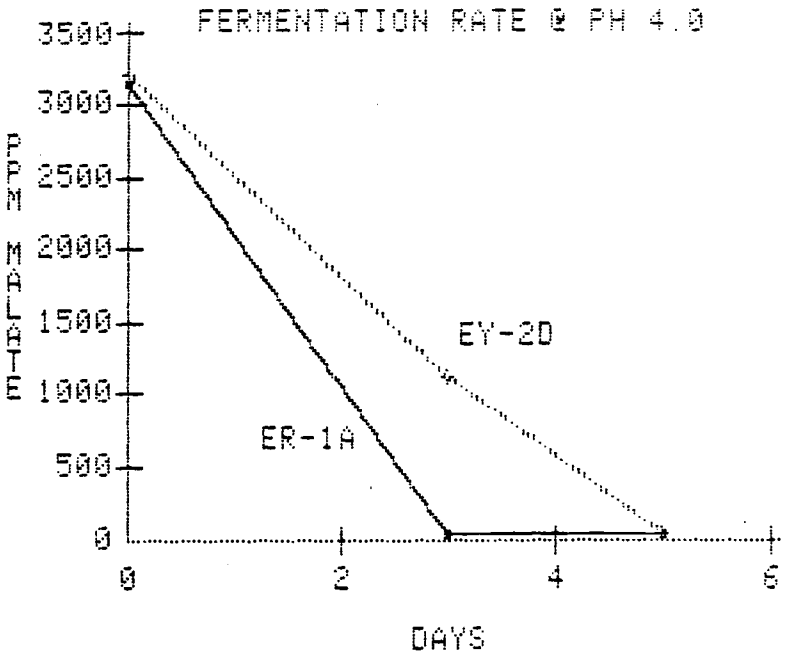

Figure 24. Malate fermentation rate at pH 4.0 of strains Er-1a and Ey-2d

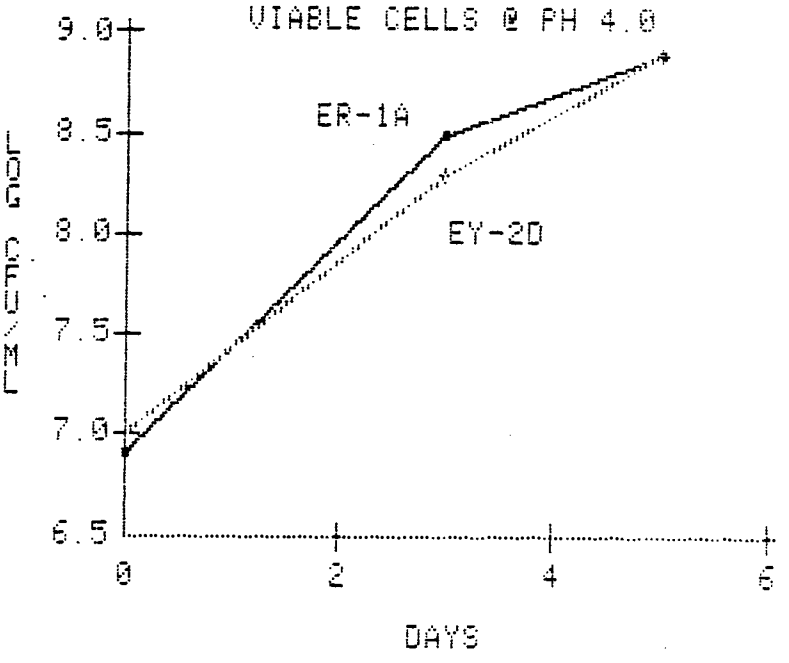

Figure 25. Viable cell counts at pH 4.0 of strains Er-1a and Ey-2d

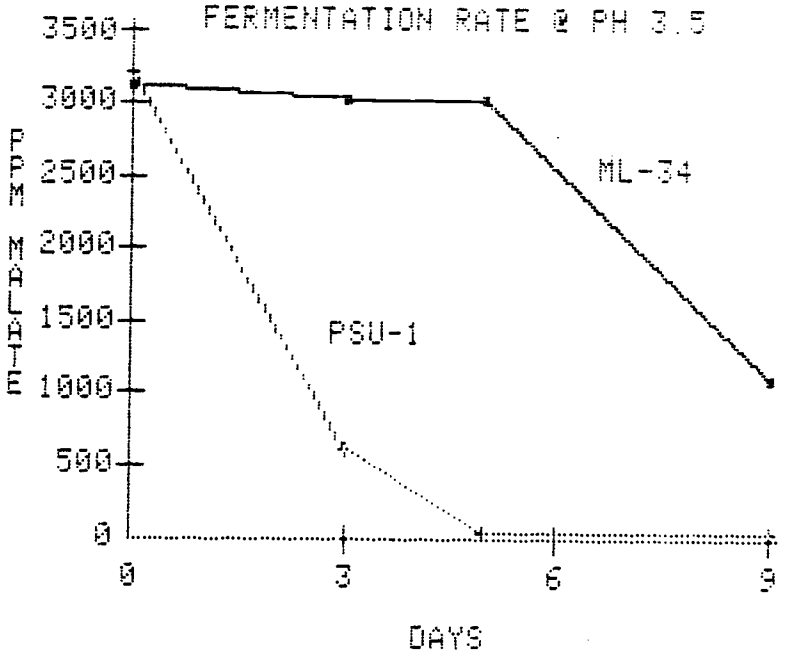

Figure 26. Malate fermentation rate at pH 3.5 of strains PSU-1 and ML34

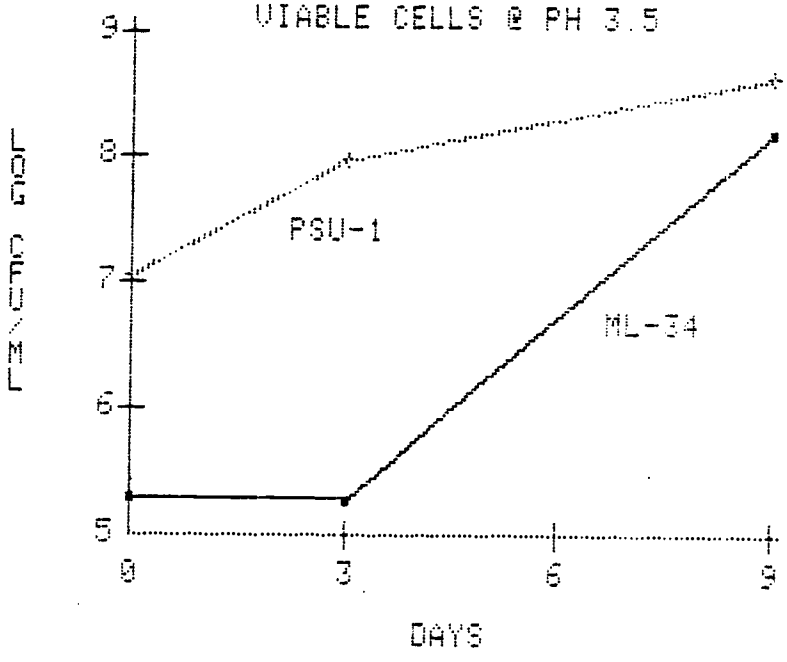

Figure 27. Viable cell counts at pH 3.5 of strains PSU-1 and ML34

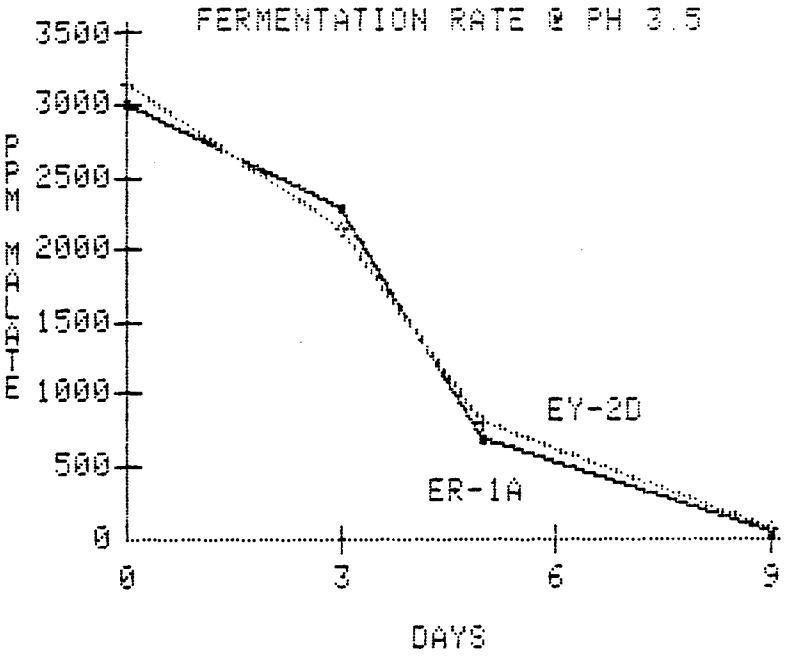

Figure 28. Malate fermentation rate at pH 3.5 of strains Er-1a and Ey-2d

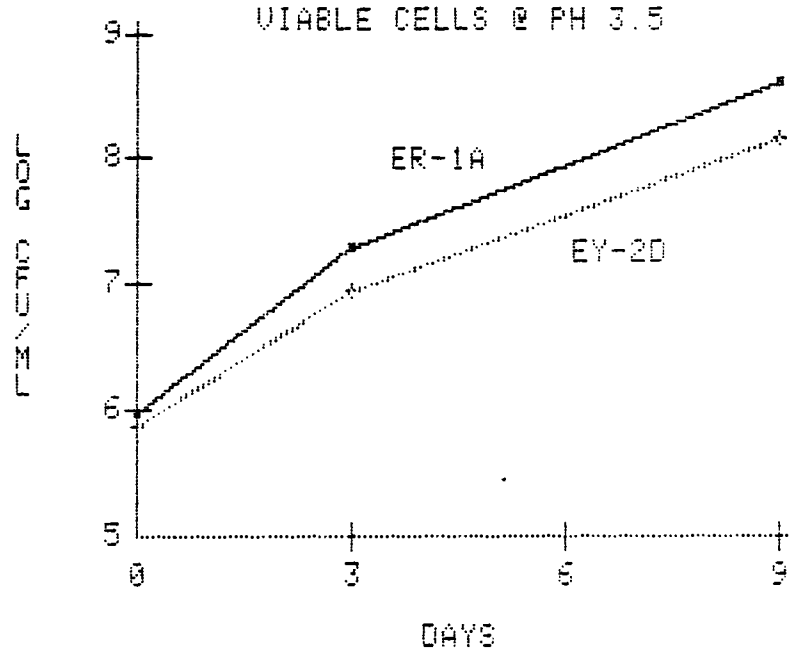

Figure 29. Viable cell counts at pH 3.5 of strains Er-1a and Ey-2d

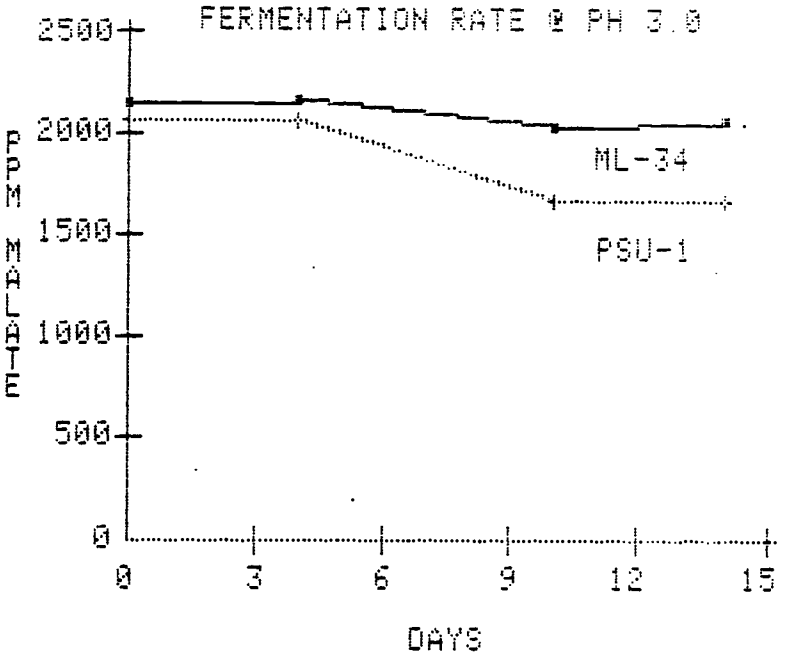

Figure 30. Malate fermentation rate at pH 3.0 of strains PSU-1 and ML34

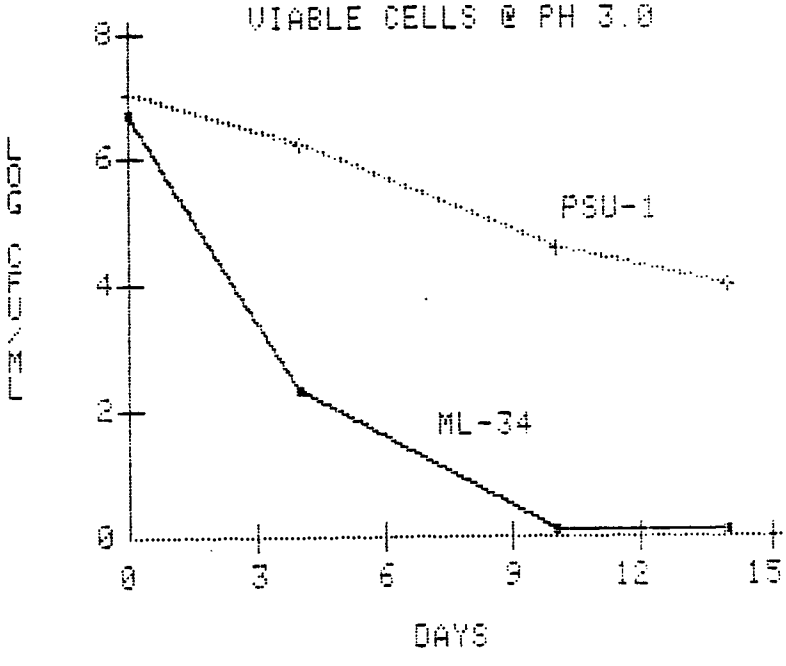

Figure 31. Viable cell counts at pH 3.0 of strains PSU-1 and ML34

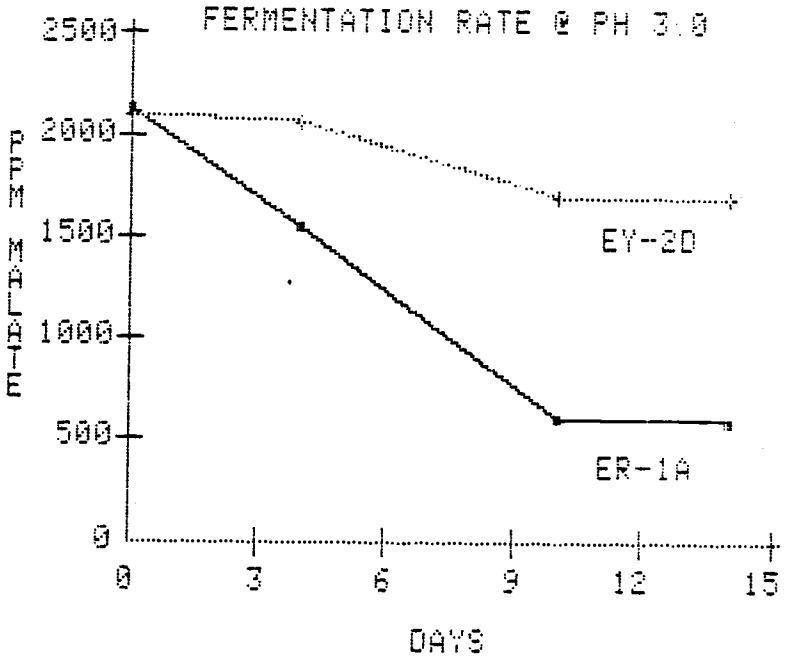

Figure 32. Malate fermentation rate at pH 3.0 of strains Er-1a and Ey-2d

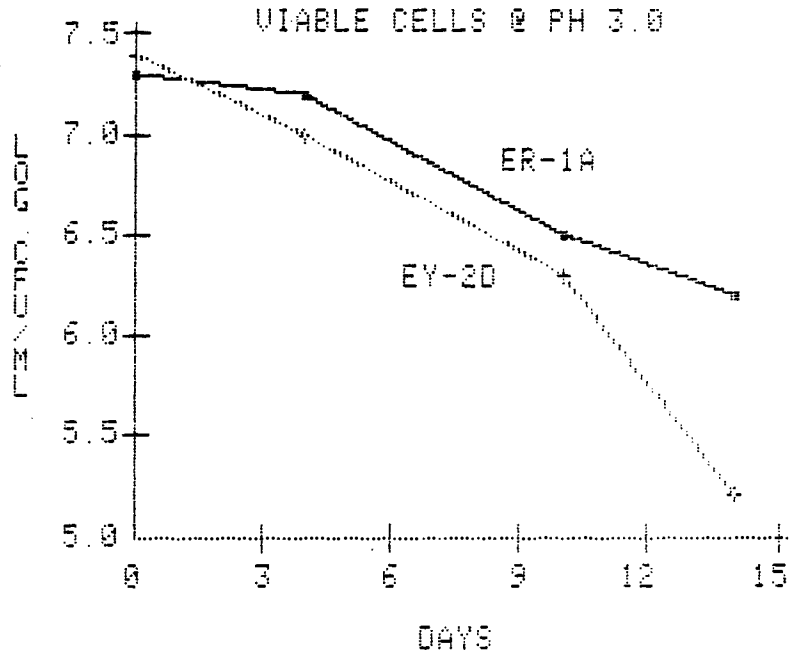

Figure 33. Viable cell counts at pH 3.0 of strains Er-1a and Ey-2d

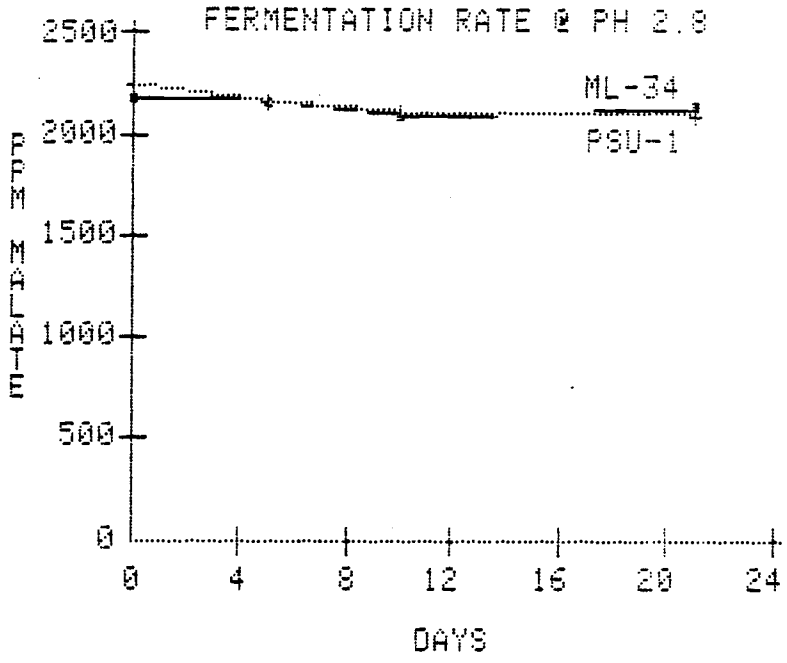

Figure 34. Malate fermentation rate at pH 2.8 of strains PSU-1 and ML34

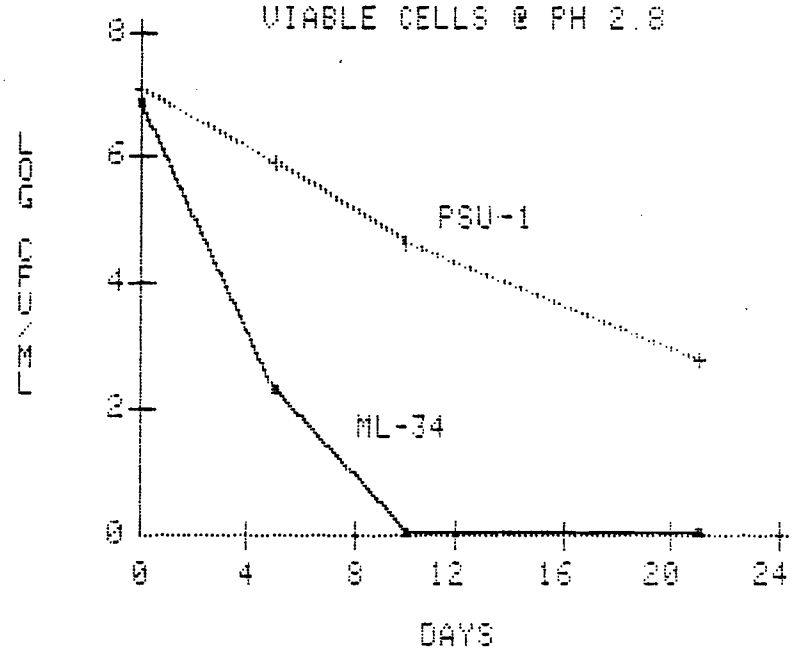

Figure 35. Viable cell counts at pH 2.8 of strains PSU-1 and ML34

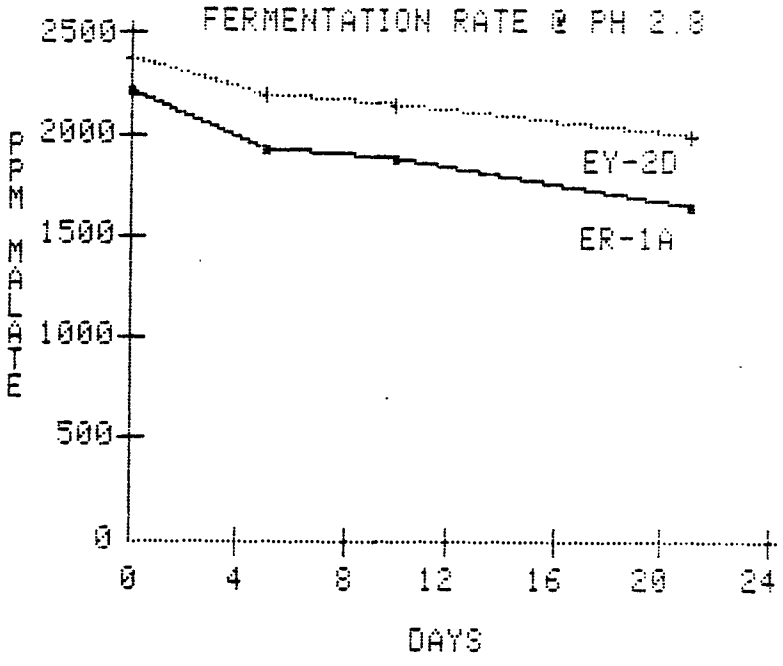

Figure 36. Malate fermentation rate at pH 2.8 of strains Er-1a and Ey-2d

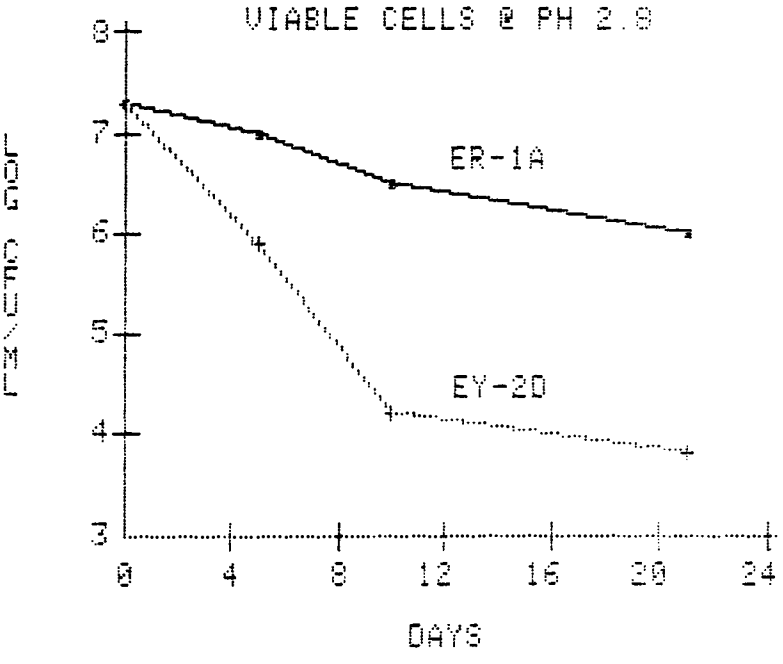

Figure 37. Viable cell counts at pH 2.8 of strains Er-1a and Ey-2d

Sulfur Dioxide Tolerance

Tolerance to various levels of free sulfur dioxide was compared for two isolates versus two reference strains. In Figures 38 and 39 the reference strains demonstrate a fairly consistent inhibition of growth at increasing levels of free sulfur dioxide. The two isolates, Er-1a and Ey-2d, both show reduced growth but moderate tolerance to free sulfur dioxide up to 20 ppm. At 30 ppm free sulfur dioxide inhibition is nearly complete for all strains tested.

Inhibition by Fumaric Acid

Fumaric acid was employed at levels comparable to that used commercially in some wineries. Figures 40 and 41 demonstrate rather graphically the inhibitory effect of fumaric acid, even at relatively low concentrations. There appears to be little, if any, difference between the bactericidal effect of fumarate on our isolates versus the reference strain PSU-1. Reference strain 44-40 demonstrated slightly greater tolerance at 0.6 g/L fumarate, but it too was completely inhibited at 1.2 g/L.

Malate Reduction in New Wine

Figures 42 through 45 show the fermentation rates of four selected isolates compared to the four reference

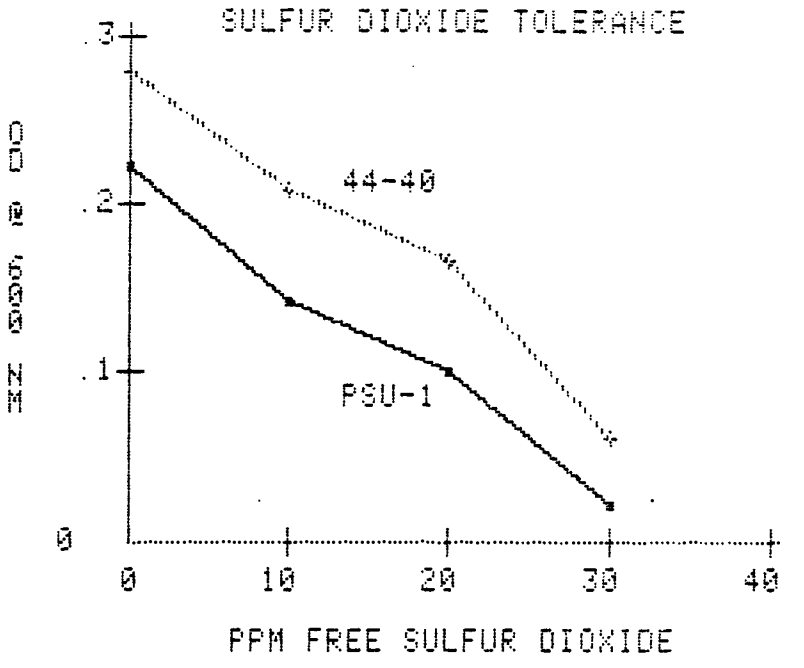

Figure 38. Sulfur dioxide inhibition on growth of strains 44-40 and PSU-1

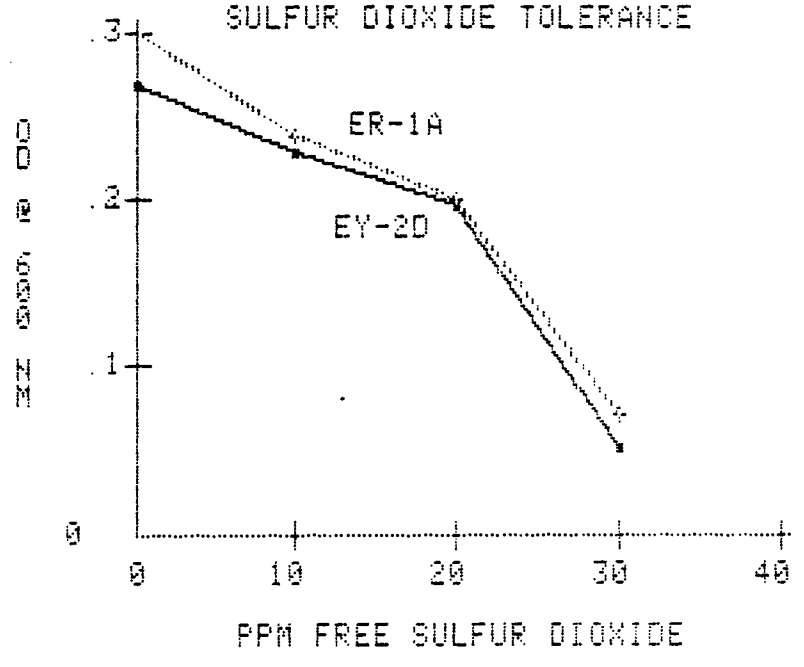

Figure 39. Sulfur dioxide inhibition on growth of strains Er-1a and Ey-2d

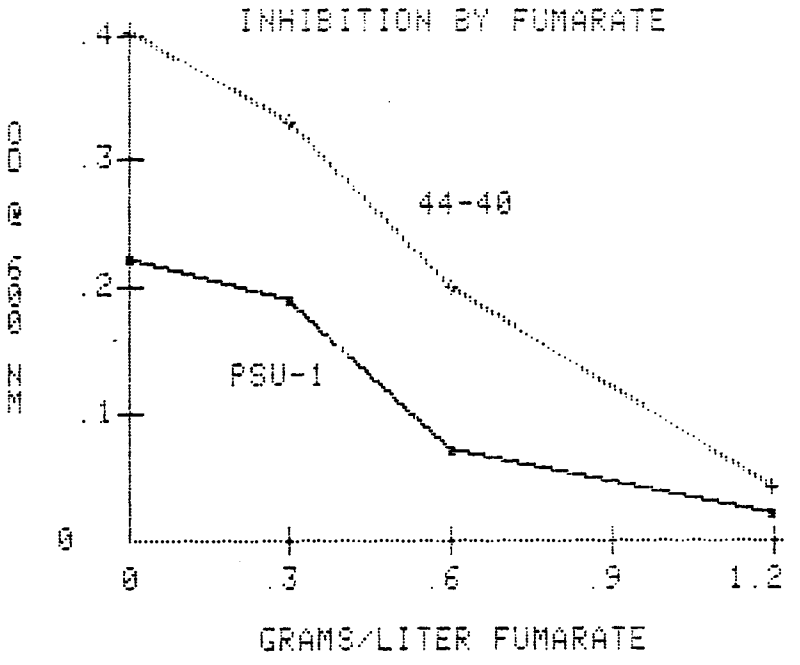

Figure 40. Fumaric acid inhibition on growth of strains 44-40 and PSU-1

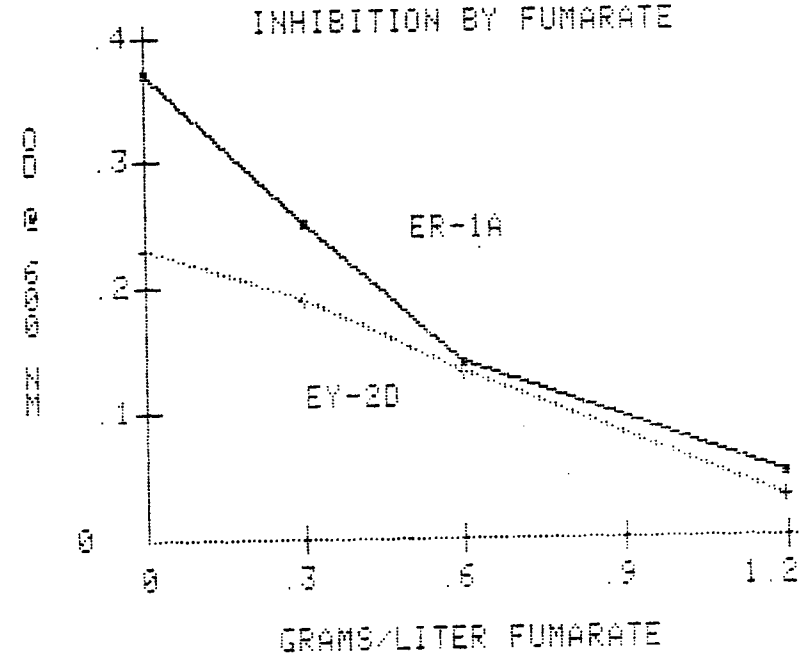

Figure 41. Fumaric acid inhibition on growth of strains Er-1a and Ey-2d

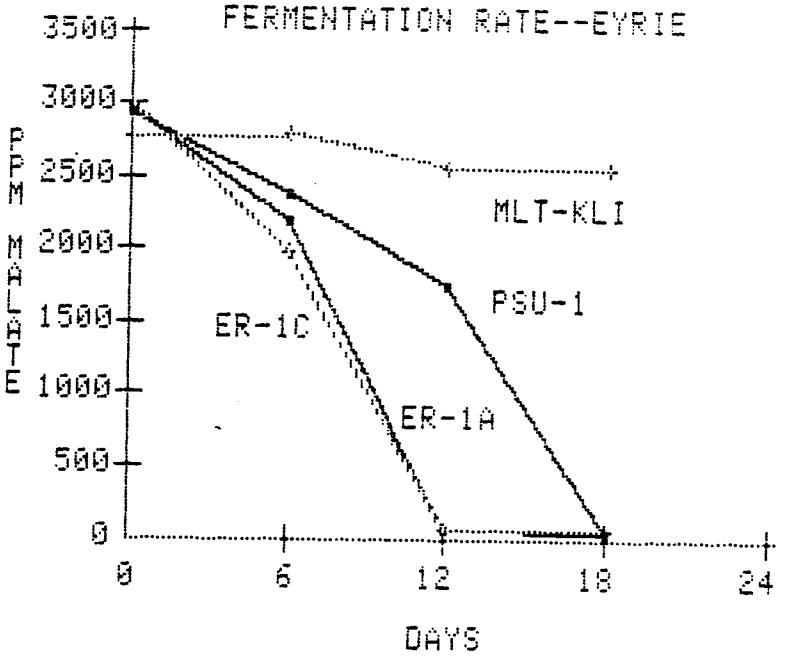

Figure 42. Malate fermentation rate of various strains in 1981 Pinot Noir

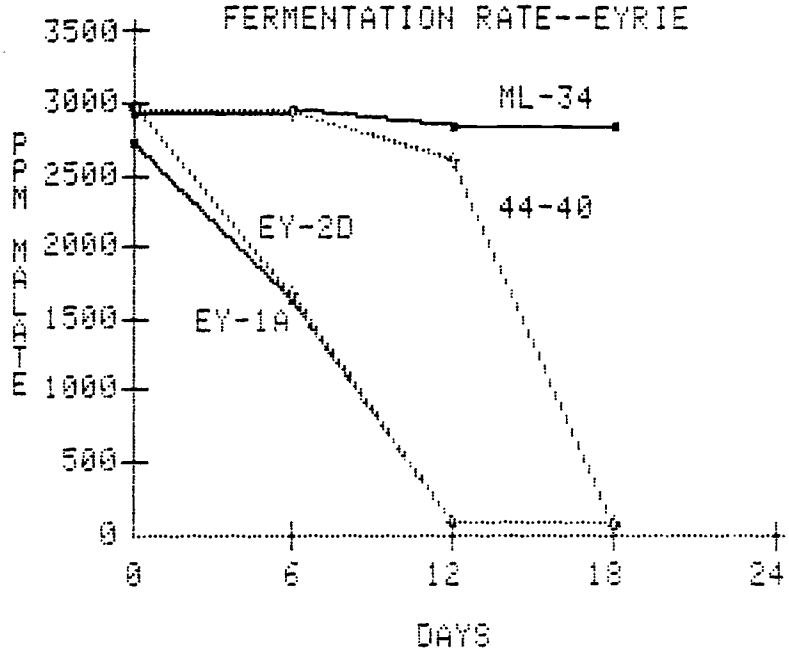

Figure 43. Malate fermentation rate of various strains in 1981 Pinot Noir

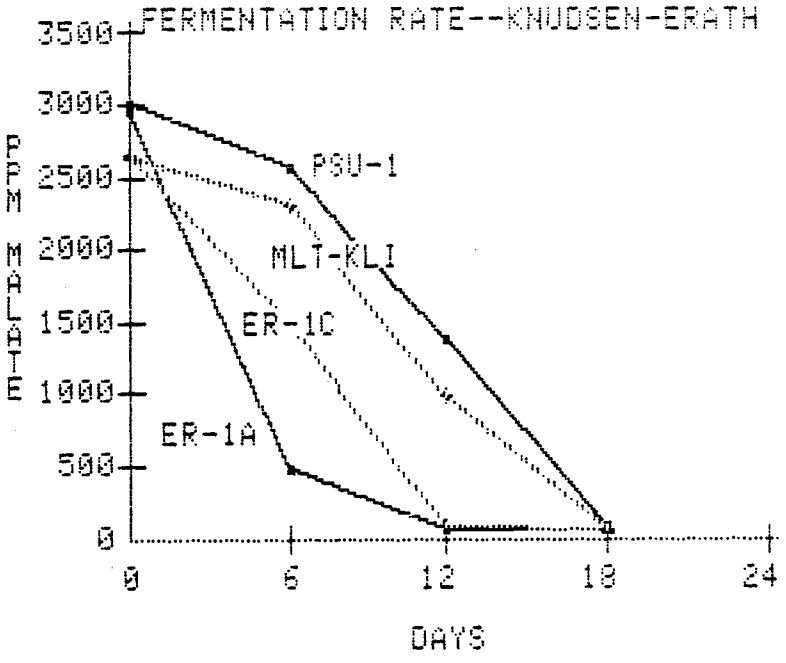

Figure 44. Malate fermentation rate of various strains in 1981 Pinot Noir

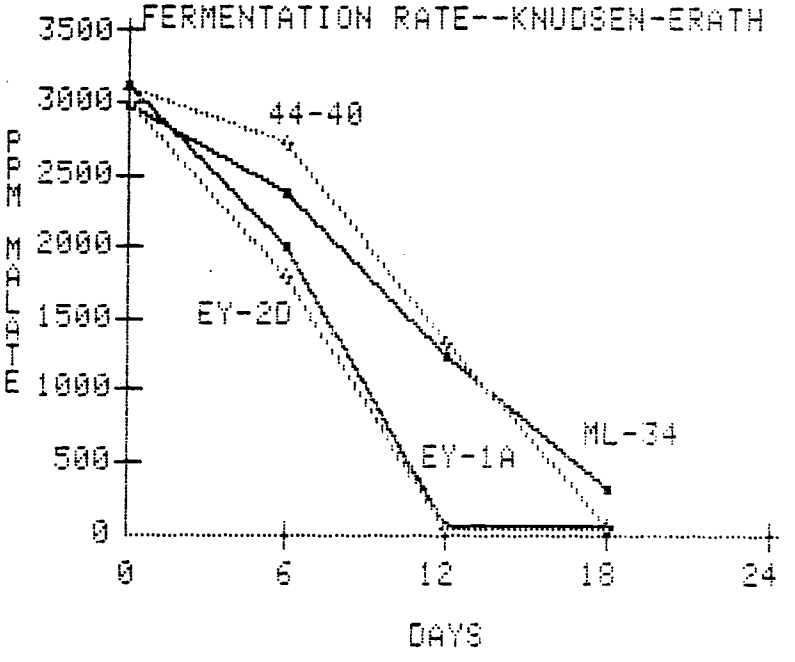

Figure 45. Malate fermentation rate of various strains in 1981 Pinot Noir

strains in two Pinot Noir wines. Malate levels were originally determined to be approximately 3000 ppm. In both cases, the four Oregon isolates brought about a more rapid reduction of the malate present in 30% less time. In the case of the Eyrie Pinot Noir, neither ML34 nor MLT-kli were able to appreciably reduce the malate level even after nearly 3 weeks time.

The effect, in terms of pH elevation and reduction of titratable acidity in these trials is illustrated in Tables 6 and 7. The initial levels were determined at zero time and the samples and control were determined at the completion of the experiment. A paper chromatogram was done to confirm the presence or absence of malate. Again, Oregon isolates Er-1a and Ey-2d, showed the greatest pH increase of nearly 0.3 units and the greatest reduction of titratable acidity of nearly 0.2 units.

Bacterial Survival of Freezing or Freeze-Drying and Subsequent Storage

Frozen Concentrates

Figures 46 through 49 show the decrease in viable cells after freezing and storage for 3 months of two of the four cultures tested. All cultures show some loss of viable cells but there are distinct differences in the degree of protection afforded by each freezing medium.

Tables 8 through 11 give complete information for all four isolates and most importantly list the percent survival of each strain in each medium type. In each

Table 6. pH and T.A.[a] changes of Eyrie 1981 Pinot Noir following 3 week fermentation

	pH	T.A.	Malate spot[b]
Initial	3.46	.75	+
Control	3.49	.74	+
PSU-1	3.69	.60	-
MLT-kli	3.66	.68	+
ML34	3.52	.72	+
44-40	3.72	.63	-
Er-1a	3.72	.60	-
Er-1c	3.74	.62	-
Ey-1a	3.75	.60	-
Ey-2d	3.75	.59	-

[a]Titratable acidity expressed as grams tartaric acid/100ml

[b]Presence of malate detected by paper chromatography method

Table 7. pH and T.A.[a] changes of Knudsen-Erath 1981 Pinot Noir following 3 week fermentation

	pH	T.A.	Malate spot[b]
Initial	3.57	.74	+
Control	3.59	.74	+
PSU-1	3.82	.60	-
MLT-kli	3.82	.59	-
ML34	3.80	.61	+/-
44-40	3.82	.60	-
Er-1a	3.86	.57	-
Er-1c	3.84	.58	-
Ey-1a	3.81	.59	-
Ey-2d	3.84	.58	-

[a]Titratable acidity expressed as grams tartaric acid/100ml

[b]Presence of malate detected by paper chromatography method

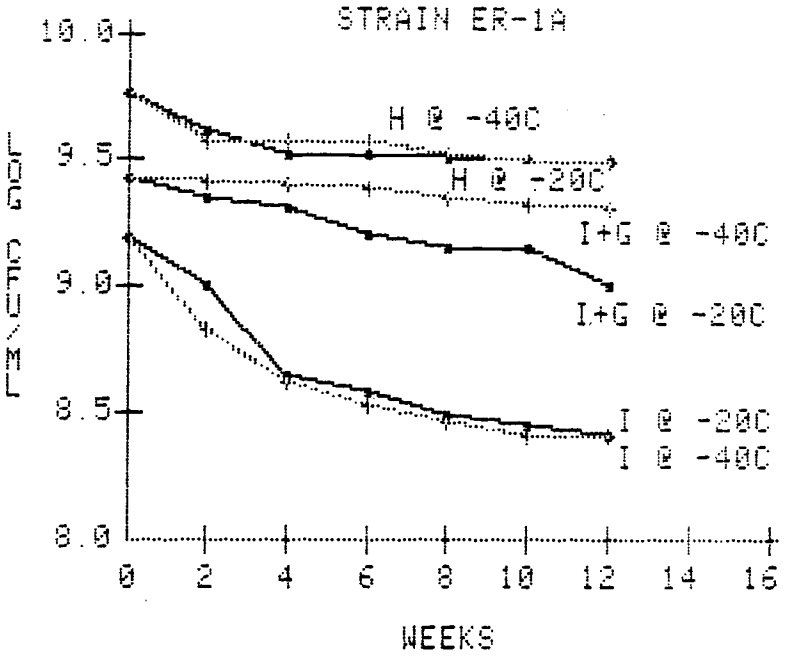

Figure 46. Viable cell counts of strain Er-1a following freezing in Media H and I (G-15% glycerol)

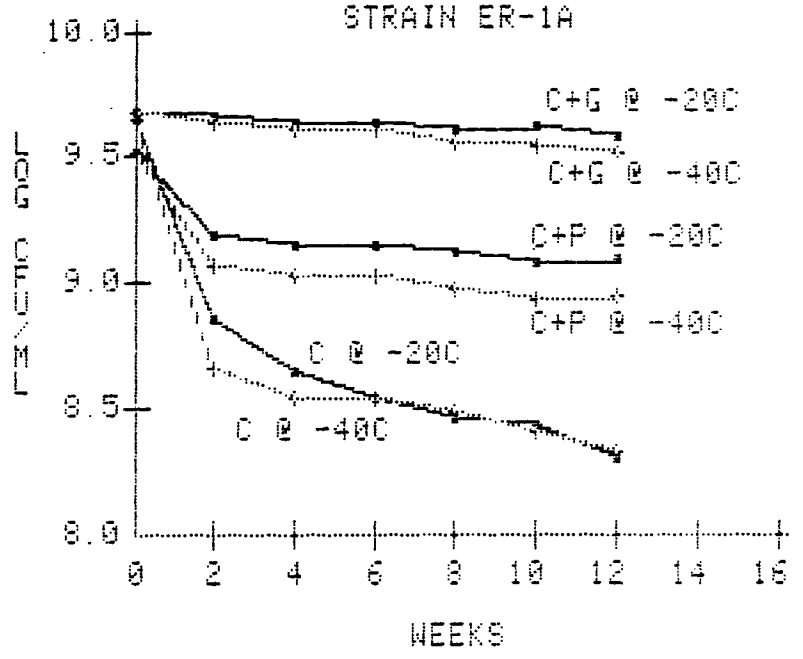

Figure 47. Viable cell counts of strain Er-1a following freezing in Medium C (G-15% glycerol, P-0.1% MgPO4)

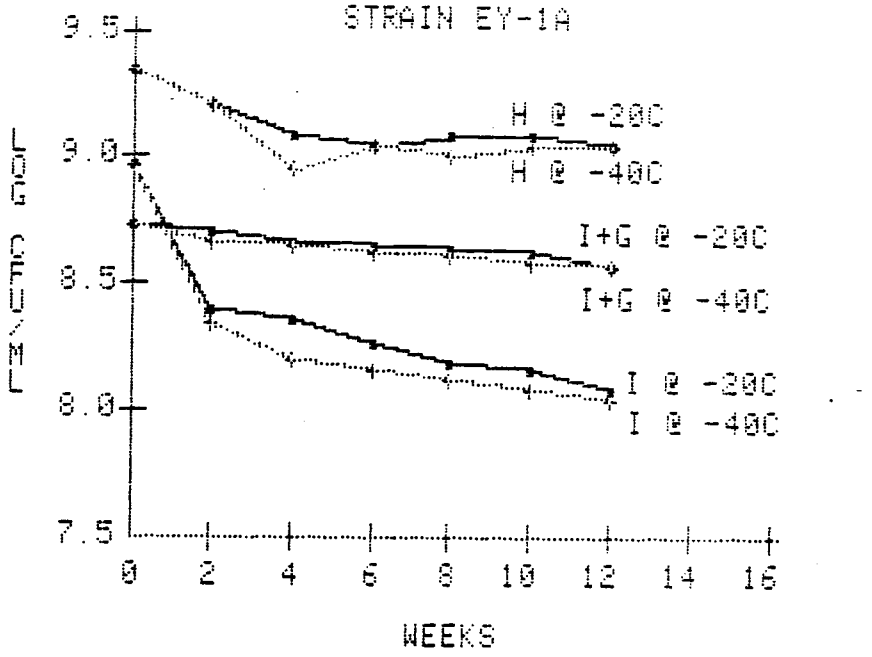

Figure 48. Viable cell counts of strain Ey-1a following freezing in Media H and I (G-15% glycerol)

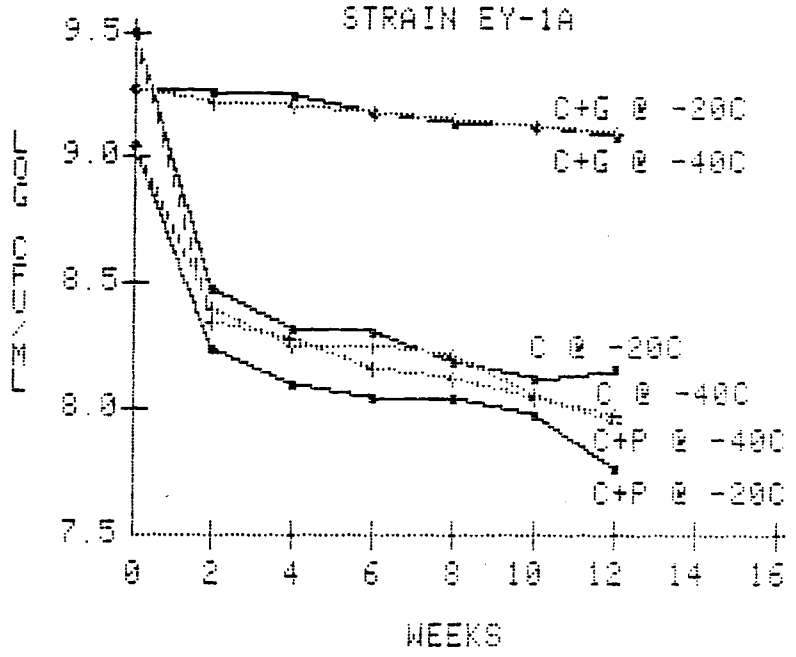

Figure 49. Viable cell counts of strain Ey-1a following freezing in Medium C (G-15% glycerol, P-0.1% MgPO4)

Table 8. Preservation media, conditions, and % survival of strain Er-1a following 3-months of frozen storage

Medium	Additive	Storage Temp. (°C)	% Survival
MRV-8[a]	None	-20	4
		-40	5
MRV-8	15% Gly[b]	-20	81
		-40	71
MRV-8	0.1% MP[c]	-20	37
		-40	27
Phase 4	None	-20	55
		-40	55
Milk[d]	None	-20	17
		-40	17
Milk	15% Gly	-20	37
		-40	74

[a] Medium C

[b] Glycerol added as cryoprotectant

[c] Magnesium phosphate added as cryoprotectant

[d] Medium I

Table 9. Preservation media, conditions, and % survival of strain Er-1c following 3 months of frozen storage

Medium	Additive	Storage Temp. (°C)	% Survival
MRV-8[a]	None	-20	2
		-40	1
MRV-8	15% Gly[b]	-20	60
		-40	74
MRV-8	0.1% MP[c]	-20	13
		-40	10
Phase 4	None	-20	35
		-40	56
Milk[d]	None	-20	9
		-40	13
Milk	15% Gly	-20	62
		-40	56

[a] Medium C

[b] Glycerol added as cryoprotectant

[c] Magnesium phosphate added as cryoprotectant

[d] Medium I

Table 10. Preservation media, conditions, and % survival of strain Ey-1a following 3 months of frozen storage

Medium	Additive	Storage Temp. (°C)	% Survival
MRV-8[a]	None	-20	4
		-40	3
MRV-8	15% Gly[b]	-20	65
		-40	68
MRV-8	0.1% MP[c]	-20	5
		-40	8
Phase 4	None	-20	50
		-40	50
Milk[d]	None	-20	13
		-40	12
Milk	15% Gly	-20	55
		-40	55

[a] Medium C

[b] Glycerol added as cryoprotectant

[c] Magnesium phosphate added as cryoprotectant

[d] Medium I

Table 11. Preservation media, conditions, and % survival of strain Ey-2d following 3 months of frozen storage

Medium	Additive	Storage Temp. (°C)	% Survival
MRV-8[a]	None	-20	3
		-40	2
MRV-8	15% Gly[b]	-20	59
		-40	69
MRV-8	0.1% MP[c]	-20	10
		-40	12
Phase 4	None	-20	34
		-40	32
Milk[d]	None	-20	31
		-40	36
Milk	15% Gly	-20	37
		-40	65

[a] Medium C

[b] Glycerol added as cryoprotectant

[c] Magnesium phosphate added as cryoprotectant

[d] Medium I

case, the medium affording superior protection in terms of viable cells preserved is Medium C plus 15% glycerol. The data regarding optimum freezing temperature is much less consistent. For Medium C plus glycerol, it appears that -40°C is more advantageous in most cases, although in one case -20°C proved better. Differences in survival rate between the two temperatures usually are not greater than 14%. In instances where there is a great difference, as in milk plus glycerol, the increased survival rate is clearly at the higher temperature.

Freeze-Dried Concentrates

Figures 50 through 53 show the decrease in viable cells of two cultures after lyophilization and storage at room temperature for 2 months. In general, there is a greater decrease in viable cells initially in these preparations than in the frozen concentrates suggesting that the freeze-drying process is more injurious to these cultures. Table 12 gives the percent survival of the four strains tested in the various media. With this method, milk appears to offer the most protection against injury although, on the average, greater than 50% loss of viable cells was recorded. An interesting finding was that in three of the four media employed for freeze-drying, cell survival was actually greater than in the frozen cultures following extended storage.

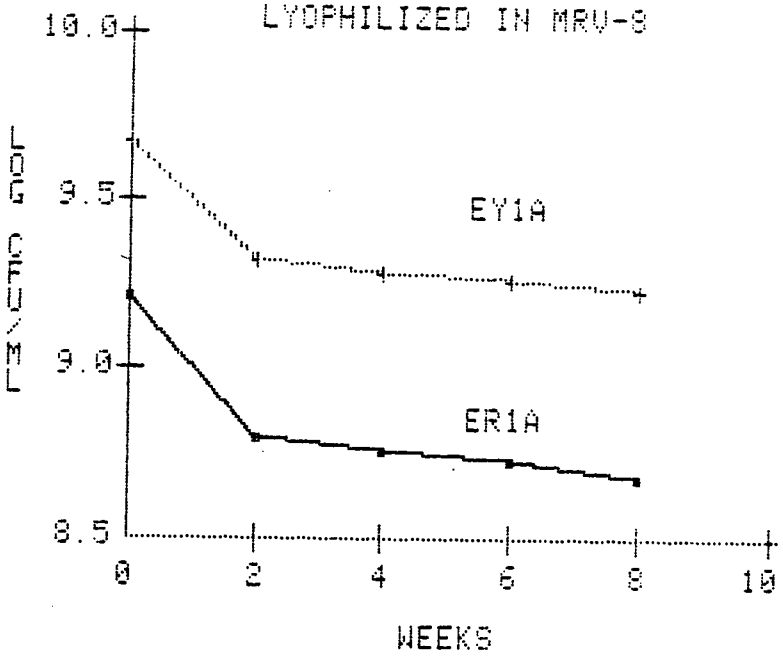

Figure 50. Viable cell counts of strains Er-1a and Ey-1a following lyophilization in Medium C

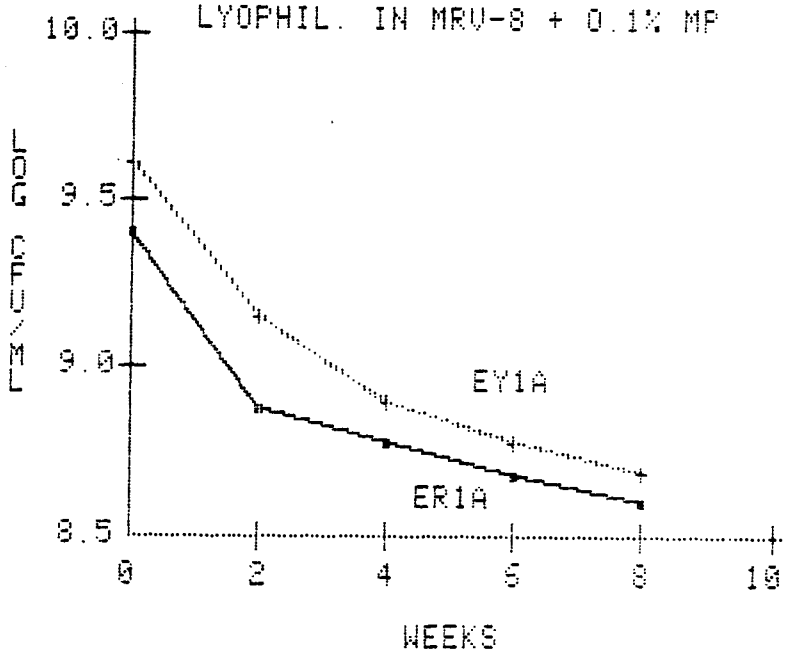

Figure 51. Viable cell counts of strains Er-1a and Ey-1a following lyophilization in Medium C plus 0.1% MgFO4

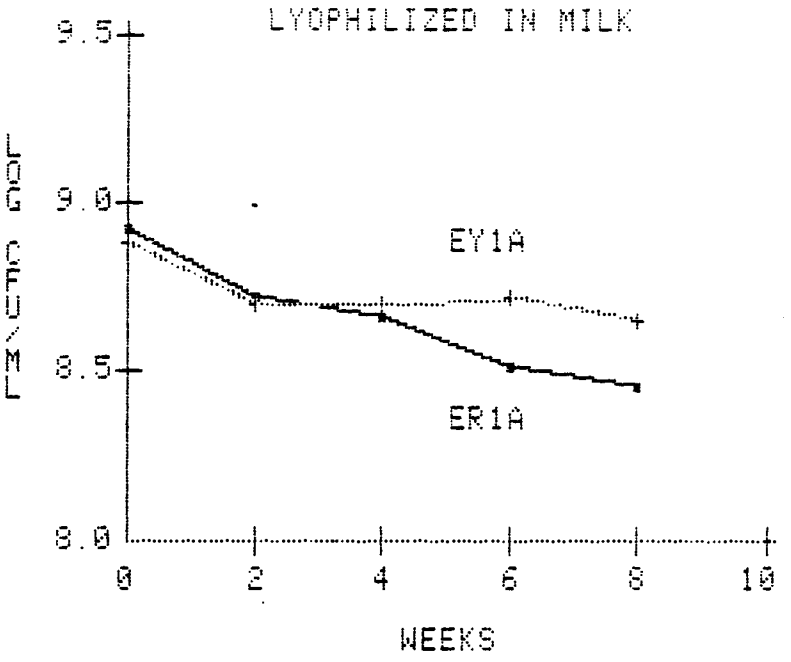

Figure 52. Viable cell counts of strains Er-1a and Ey-1a following lyophilization in Medium I

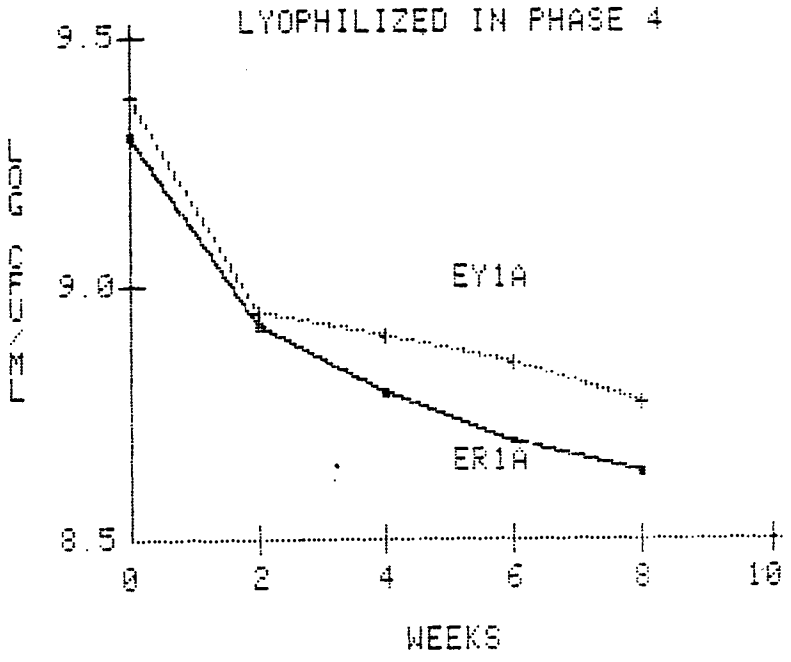

Figure 53. Viable cell counts of strains Er-1a and Ey-1a following lyophilization in Medium H

Table 12. Preservation media and % survival of strains Er-1a, Er-1c, Ey-1a and Ey-2d following 2 months of lyophilized storage at room temperature

Medium	Additive	% Survival			
		Er-1a	Er-1c	Ey-1a	Ey-2d
MRV-8[a]	None	29	32	36	32
MRV-8	0.1% MP[b]	15	19	12	14
Phase 4	None	21	22	25	20
Milk[c]	None	34	50	59	47

[a] Medium C

[b] Magnesium phosphate added as cryoprotectant

[c] Medium I

DISCUSSION

A gradual evolution in the practice of winemaking has culminated in substantial control of microbial parameters during the alcoholic fermentation. The advent and use of pure yeast starter cultures, many of which are now available in a dry active form, has eliminated to a large degree a major source of wine quality problems.

The secondary bacterial fermentation or malo-lactic fermentation (MLF), occurs spontaneously and naturally in many wines. Though initially thought to be detrimental to wine quality, it has progressed in the eyes of winemakers and enologists alike. It is now considered desirable if it occurs during the normal vinification process since it provides bacteriological stability and contributes to the flavor complexity of the wine, as pointed out earlier. Several workers have indicated that defined methods for control of the MLF are of extreme importance in the production of premium wines (Beelman et al., 1977; Pilone et al., 1966; Rankine, 1977).

Oregon wines, produced in a region with a generally cool climate noted for its high acid grapes, have developed a history of sporadic malo-lactic fermentation. It has therefore been necessary to develop some dependable method of inducing the fermentation. Efforts have been made by Oregon winemakers to optimize

those conditions believed to favor the development of spontaneous or natural malo-lactic fermentations (Castino et al., 1975). Racking off from the yeast lees was delayed a short time, judicious use of potassium metabisulfite in the sulfuring process, and storage in cooperage believed to harbor a malo-lactic microflora were all attempted in hopes of stimulating the fermentation (Kunkee, 1974). Attempts also included inoculation of new wine with aliquots of wine from the previous year which had undergone a complete MLF. The combination of these efforts met with limited success.

Reports regarding the use of Leuconostoc oenos in pure culture inoculations of wines elsewhere led to increased interest in this organism for use in this region. However, strains of this organism frequently failed to initiate a fermentation in regions such as Oregon, California and New York, as mentioned previously. The only logical alternative was to isolate from Oregon wines similar strains that are better adapted to the particular conditions prevailing, evaluate them for efficacy in effecting a rapid and complete fermentation and employ them by pure culture methodology. A profile of these bacteria, including tolerance to low pH, sulfur dioxide and ethanol levels would prove of great importance in choosing the best strains for use. Moreover, development of methods to preserve these cultures and make them readily available for use would

provide a tremendous service to the anxious winemakers.

In this study, we have attempted to provide a characterization of the malo-lactic isolates from Oregon wines, compare their malo-lactic abilities under various conditions and determine their tolerance to certain inhibitory substances or conditions commonly found in new wine. We have also attempted to devise the best method for providing these cultures to the industry in a stable and practical form.

There have been reports that red wines are generally more favorable to malo-lactic bacteria than white wines, presumably because of the higher sulfur dioxide concentration and lower pH generally associated with white wines (Fornachon, 1957; Kunkee, 1967, 1974). Our results show a preponderance of strains isolated from two red wines (Pinot Noir and Merlot) while only a few were obtained from a white wine (Chardonnay).

The strain characteristics described in the Results section and in Tables 2 and 3 show good correlation to the characteristics of Leuconostoc oenos as published in the 8th edition of Bergey's Manual (Buchanan et al., 1974). They are also similar to the properties used in separating L. oenos from other non-acidophilic leuconostocs (Garvie, 1967). The results also showed similarity with the characters published for L. oenos ML34 (Pilone and Kunkee, 1972), L. oenos PSU-1 (Beelman et al., 1977), and those of several strains of

L. oenos A-9, A-10 and A-11 (Chalfan et al., 1977).

The results with regard to morphology, catalase and gram reaction, gas production from glucose (heterofermentation), formation of dextran from sucrose and facultative anaerobic nature are in general agreement with the findings of the above workers. Our isolates also grew in media with pH values much less than 4.0 and in the presence of 10% ethanol as found by previous investigators (Garvie, 1967, 1980; Pilone and Kunkee, 1972; Chalfan et al., 1977). There is also general agreement regarding lack of ammonia production from arginine although two strains have been reported with this capability (Garvie, 1980). Citrate and malate were found to be metabolized in the presence of fermentable carbohydrate as reported earlier (Pilone and Kunkee, 1972; Beelman et al., 1977).

Varying sugar fermentation patterns for L. oenos have been presented by various workers. There is, however, a general consensus that glucose, fructose, ribose, trehalose, cellobiose, salicin and esculin are fermented while lactose, sucrose, rhamnose and raffinose are not. The results presented here agree with these findings to a large extent. Additionally, some investigators have found that maltose was not fermented by this organism (Garvie, 1967; Pilone and Kunkee, 1972) while others report that their strains do ferment maltose (Chalfan et al., 1977; Beelman et al., 1977 and Silver

and Leighton, 1981). Most of the Oregon isolates utilized maltose quite readily.

The utilization of pentoses by L. oenos strains has not been completely resolved. Some workers have found that xylose and/or arabinose may be utilized by some strains (Garvie, 1967; Silver and Leighton, 1981) while others did not observe utilization of either (Pilone and Kunkee, 1972; Beelman et al., 1977; Chalfan et al., 1977). It has been suggested that those wine leuconostocs which fermented either xylose or arabinose be named L. oenos while those not fermenting either be called L. gracile. Most of our strains fermented L- and D-arabinose while xylose was fermented only very weakly; this would place them in the L. oenos group. One of our isolates, Er-1a, utilized galactose quite readily which had not previously been reported. The variation in sugar utilization is of little surprise as it has been stated that L. oenos is hardly a homogenous group (Garvie, 1967, 1980).

A brief and simple comparison of growth of our isolates was conducted to determine the medium of choice. The following media were used: a modified Rogosa medium used by previous investigators (Ingraham et al., 1960; Pilone and Kunkee, 1972), a grape juice medium which was formulated in our lab, a modified Phase 4 medium (Galloway West Co., Fond du Lac, WI.) and Lactobacillus Selective Medium (Rogosa et al., 1951). Viable cell

counts indicated that the modified Rogosa medium was superior as predicted by previous studies (Amachi, 1969, 1975; Ingraham et al., 1960; Kunkee, 1967). The grape juice medium also supported adequate growth as might be expected since the growth factor for malo-lactic bacteria (TJF) is commonly present in grape juices (Amachi, 1975; Yoshizumi, 1975).

The effects of temperature on growth and metabolic systems of malo-lactic bacteria are quite significant. It has been reported that strains of L. oenos grow at a temperature range of 10 to 35°C and that optimum growth temperature was in the range of 18 to 24°C (Garvie, 1974). Other investigators did not find any growth at 10°C and found the best growth rate between 25 and 37°C (Chalfan et al., 1977). Other reports have largely been somewhere between 12 and 32°C (Beelman et al., 1977; Silver and Leighton, 1981). The optimum growth temperatures of our strains generally ranged between 28 and 31°C. More significantly, one of our strains, Er-1a, did not exhibit such a rapid increase of generation time as the temperature decreased. Since ambient storage temperatures for wine may range from 5 to 20°C, this finding may prove extremely valuable.

The determination of optimum pH for growth seemed reasonably important both for strain propagation as well as a means for determining which strains might perform better in wine samples. Some investigators have reported

the pH optimum to be between 4.2 and 4.8 (Garvie, 1974; Lafon-Lafourcade, 1975). Others reported the optimum pH to lie between 4.8 and 5.6 (Pilone and Kunkee, 1976; Beelman et al., 1977). In our trials, all strains had an optimum pH within the range of 4.0 and 5.8. Some strains exhibited a rather broad optimum, particularly ML34 and 44-40, two of the reference strains, while others particularly Ey-2c and Ey-4b had very sharp optimums near 5.4. These variations may be explained by the fact that the pH threshold of growth is characteristic of the bacterial strain and of the composition of the medium (Lafon-Lafourcade, 1975). In general, it may be said that these isolates grow well between 22 and 32°C and pH values below 6.0.

The possibility of growth inhibition of our strains at various ethanol levels was tested in Medium G at pH 3.5 in order to more closely approximate wine conditions. Such inhibition has been described by other investigators in other media at pH 4.5 and at levels of ethanol ranging from 10% to 16% (Beelman et al., 1977; Silver and Leighton, 1981). In our trials, all strains grew well with no added ethanol and exhibited proportionate decreases in growth as the alcohol level increased. One notable exception was strain Ey-2d which showed minimal inhibition at 10% with marked inhibition at 12% and 14% ethanol. All of the strains grew to some extent at all three levels of ethanol tested.

Since malic acid has been added to media used in the isolation and cultivation of malo-lactic bacteria (Ingraham et al., 1960; Silver and Leighton, 1981), the appropriate level of addition was determined for several isolates. Growth increases were noted up to approximately 0.2% malic acid with no further increase at all seen beyond 0.25%.

A comparison of the carbon dioxide evolution from malate solutions in a respirometer was made and thus a profile of malate-decarboxylating activity could be determined for our strains. The results showed that at least five of the Oregon isolates were more active in the decarboxylation than one of the reference strains, PSU-1. Earlier mention that the number of cells present was the most important factor in effecting a rapid MLF (Rice and Mattick, 1970) proved not to be the case. By determining a value of amount of carbon dioxide per minute per mg dry weight, we were able to demonstrate that the malo-lactic ability was more likely dependent on the individual strain and its enzymatic capacity to effect the conversion.

Relative malate-reducing ability was compared for our strains in an attempt to reduce the number of strains to only those most active in MLF. The malate fermentation rate ranged from 32 to 547 ppm malate per day. Growth occurred in all tubes and yet the amount of malate reduction was quite variable, again emphasizing

individual strain characteristics.

The pH of a wine is known to be an important determinant of malo-lactic fermentation (Beelman and Gallander, 1979; Bousbouras and Kunkee, 1971; Lafon-Lafourcade, 1975). Indeed, by at least one author, it is considered the single most important factor influencing a successful or unsuccessful malo-lactic fermentation (Castino et al., 1975). Therefore, a careful analysis of our isolates versus reference strains was performed at pH 4.0, 3.5, 3.0 and 2.8. Viable cell numbers and fermentation rate by enzyme assay were determined at regular intervals. These fermentation trials were extremely important in demonstrating that several of our strains were much more active at the lower pH than reference strains tested. These experiments seem to suggest a practical limit of pH 2.9 to 3.0 for a rapid and complete MLF by our strains.

The inhibitory effects of sulfur dioxide, free and bound, on malo-lactic organisms has been well discussed and demonstrated (Kunkee, 1967; Fornachon, 1963; Rankine, 1970; Lafon-Lafourcade, 1975; Castino et al., 1975). Indeed, by some it is considered the most determining factor for MLF and is sometimes excluded until the bacterial fermentation is complete (Rankine et al., 1970). In another case, delayed racking without sulfur dioxide addition greatly stimulated MLF in South African wines (Van Wyk, 1976). Tolerance to levels of free

sulfur dioxide, which is believed to have the greatest bactericidal effect (Fornachon, 1963) was found to be slightly greater for some of our isolates than reference strains tested. At 10 and 20 ppm free sulfur dioxide, there is moderate inhibition of growth compared to the controls which had no added sulfur dioxide but growth was occurring nonetheless. At 30 ppm, inhibition was nearly complete for all strains tested. A level of 20 ppm appears to be a limit before sizable inhibition occurs. This result might support a similar finding that, at levels less than 20 ppm free sulfur dioxide, malo-lactic fermentations proceeded only with slight delay (Rice, 1974).

The effect of fumaric acid addition to young table wines in delaying or preventing malo-lactic fermentation was first observed by investigators in 1969 (Peterson, 1969; Tchelistcheff et al., 1977). Since then, others have made similar findings regarding its toxic effect on malo-lactic bacteria (Cofran and Meyer, 1970; Pilone et al., 1974; Pilone 1975). It is believed that fumaric acid prevents malo-lactic fermentation in wine by interfering with the ability of lactic acid bacteria to synthesize nucleic acid precursors (Silver and Leighton, 1980). It is particularly inhibitory to malo-lactic fermentation at pH levels below 4.0 (Pilone, 1975). Whatever, the mechanism may be, it is shown in our experiments to be effectively inhibitory at

concentrations as low as 0.6 g/L for all strains tested. This would indicate a slightly higher sensitivity to this substance than previously reported (Cofran and Meyer, 1970; Tchelistcheff et al., 1971). An exception was reference strain 44-40 which appeared to be somewhat more resistant to this fumarate effect.

While this study did not actually involve much analysis of actual wine samples, an attempt was made to compare malate fermentation rates and reduction of acid levels in two new wines. The results are encouraging insomuch as they indicate an effective MLF may be stimulated in less time by selected Oregon leuconostoc isolates than by any commercial strains available. In fact, these wines at approximate pH 3.5 and titratable acidity of .75 g/L are certainly not the most severe test by any means. It is strongly suspected from experimental results, that an even greater differential of malo-lactic activity will be observed when the pH and titratable acidity level of wines tested become less conducive to a rapid MLF. It may well prove that for more resistant wines, growth in media plus 20% wine may allow better adaptation to subsequent growth in wine (Hayman and Monk, 1982).

The preservation of bacterial cells by freezing or freeze-drying is a process that seems fraught with many uncertainties. Part of the difficulty arises because the information in most studies is not sufficiently complete

to determine the degree to which differences in response to low temperature result from inherent differences among organisms or from differences in the procedures followed in effecting low-temperature exposure (Mazur, 1960). That rather lengthy observation on the state of cryopreservation holds true to some extent even today. However, some generalities seem to have emerged.

Many investigators seem to have agreed at least as to the importance of cooling and warming rates on the survival of cryopreserved bacteria (Mazur, 1966; Litvan, 1972; Calcott et al., 1976). The rate of removal of cellular water corresponding to the cooling rate and permeability of the cell wall determines whether water in a cell will freeze internally or externally (Nei, 1973; Mazur, 1966). When cells are subjected to intracellular ice formation, they may lose their original semipermeability and become subject to death (Nei, 1973). On the other hand, if the cooling rate is too slow, there can be death or injury due to dehydration (Mazur, 1966; Litvan, 1972). It appears that an intermediate freezing velocity renders optimal survival since it is fast enough to minimize dehydration but slow enough to avoid cell wall damage.

The rate of warming of frozen cultures is also important, particularly when intracellular ice has formed. Evidence seems to point to the greatest recovery of viable cells following rapid thawing (Gibson et al.,

1966; Johannsen, 1972). Freezing of our cultures at -20°C and -40°C could probably be expressed as slow and intermediate rates of freezing. Generally speaking, the intermediate rate resulted in a somewhat higher percent survival of the strains tested.

The incorporation of various cryoprotectants in media of varying types has been successful in increasing survival rates of many genera of bacteria. Some of the most successful adjuncts employed are glycerol at various levels (Bauman and Reinbold, 1966; Gibson et al., 1966; Johannsen, 1972; Stadhouders et al., 1971), milk and its products (Lattuada and Foster, 1963; Gilliland et al., 1970), yeast extract (Bauman and Reinbold, 1966; Johannsen, 1972), malic acid (Gibson et al., 1966; Johannsen, 1972) and DMSO (Bauman and Reinbold, 1966; Stadhouders et al., 1971). The presence of peptides in freezing and recovery media, perhaps to aid in RNA resynthesis, was found to increase viable numbers following freezing (Morichi and Irie, 1973). The relationship of cellular components, particularly fatty acids, to the stability of lactic acid bacteria to freezing has been observed (Smittle et al., 1974; Gilliland and Speck 1974; Goldberg and Eschar, 1977). In fact, the death rate of Lactobacillus bulgaricus NCS 1 was decreased from 100% to 5% when cells were grown in medium containing Tween 80 (Smittle et al., 1972). In another study, a malo-lactic organism used in Israeli

wines, Lactobacillus sp. A-12, was frozen in a tomato juice medium similar to our Medium A. Cell survival was increased from 20% to 50% when the cultures were grown in the tomato juice medium plus Tween 80 or oleic acid. This is a marked improvement over the survival rate of our strains in Medium C although their storage period was much shorter (2 weeks versus 3 months) (Goldberg and Eschar, 1977). In the above cases, there was a close correlation between the cellular content of certain long chain fatty acids and cell resistance to freeze damage. Much of the lipid material in gram positive microorganisms is associated with the cell wall. Thus, if Tween 80 were metabolized, it could play a significant role in developing cell membranes whose integrity is maintained during frozen storage (Smittle et al., 1972).

Medium C contains, among other things, peptone, carbohydrates, malic acid, yeast extact and Tween 80. Its use as a freezing menstrum ws attempted, along with added glycerol and magnesium phosphate, which is believed to have some cryoprotectant qualities (Pat. No. 4,282,255). Medium C alone did not perform adequately as a freezing medium, perhaps due to to the fact that its cryoprotectant additives were in meager concentrations. However, in the case of Medium C plus 15% glycerol adequate cryoprotection was achieved particularly at 40°C. Cell survival was nearly 70% for all four organisms tested. Greater than 55% survival was obtained

in milk plus 15% glycerol whereas milk alone was generally not as protective. Use of Phase 4 provided roughly comparable cryoprotection for our isolates as noted for lactic streptococci (Shigeno, 1982, unpublished data).

Freeze-drying has always proved more lethal than freeze-thawing (Greaves et al., 1967). Perhaps the fact that freeze-drying is a step beyond freezing, with a chance of killing or damaging cells during drying as well as during freezing, accounts for this (Cahalan, 1978). In general, all of the aforementioned effects of freezing hold true for freeze-drying of organisms.

Studies have been done to measure the effects of residual moisture content on the survival of freeze-dried bacteria during storage. It appears as though there are upper and lower limits as to the residual moisture content for maximal survival. It has been shown that for freeze-dried bacteria stored in vacuum, the higher the residual moisture content, the lower the survival rate (Nei et al., 1966). Further studies have shown that survival of freeze-dried cells of 18% fraction water was 80%, almost the same as that achieved by freezing only. On further drying from 18% to 0% water, the cell survival decreased as the residual moisture was reduced, suggesting that the removal of unfreezable water causes cell death (Nei, 1973).

Many of the additives employed in freezing are

also employed in freeze-drying of bacteria. High concentrations of peptone have been shown to be effective in freeze drying (Greaves et al., 1967) as well as the use of milk as suspending medium (Sinha et al., 1974). Limited protection by hexoses and other reducing sugars was demonstrated while excellent protection was afforded by meso-inositol, a non-reducing sugar (Redway and Lapage, 1974).

Our results with freeze-dryng of the leuconostoc isolates seem to indicate clearly the superiority of milk (NFDM, 11% solids) in cell preservation. These results appear to be in agreement with data shown for the freeze-drying of Leuconostoc citrovorum and Leuconostoc dextranicum, related species of the same genus. After storage for 2 months at 30°C, there was about 65% survival of these organisms (Sinha et al., 1974). This was a decrease of about 25-30% over time since immediately after freeze-drying the % survival was nearly 90%. It may be that reactive groups in the cell wall may exhibit considerable activity at room temperatures or above, resulting in continuing cell damage (Greaves et al., 1967). While in our study cell numbers were not determined immediately after freeze-drying, the large decrease in cell numbers at 2 weeks, followed by smaller rates of decrease would seem to indicate that the freeze drying process was itself the most lethal. It is interesting to note, however, that those organisms

freeze-dried in milk did not experience this sharp initial drop in cell numbers, which might serve to confirm the previous findings.

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