Stimulatory Effect of Malo-Lactic Fermentation on the Growth Rate of Leuconostoc oenos

GORDON J. PILONE and RALPH E. KUNKEE*

Department of Viticulture and Enology, University of California, Davis, California 95616

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Although L-malic acid is not an energy source for the malo-lactic organism Leuconostoc oenos (L. citrovorum) ML 34, the growth rate of the organism was found to be greatly increased by malo-lactic fermentation (the decarboxylation of L-malic acid to L-lactic acid). The stimulation was especially striking at the low pH (below pH 4) of wine, the natural habitat of this bacterium. The stimulation of growth did not result from changes in pH that accompany malo-lactic fermentation. Thus, these results suggest a biological function of malo-lactic fermentation.

Most lactic acid bacteria found in wine can carry out the malo-lactic fermentation, the degradation of L(-)-malic acid to L(+)-lactic acid and carbon dioxide. The enzymatic basis for this reaction was first studied by Ochoa and Korkes and co-workers in Lactobacillus arabinosus (L. plantarum) (3–5). The reaction seemed to be an oxidative decarboxylation of L-malic acid to pyruvic acid catalyzed by Mn²⁺ and an nicotinamide adenine dinucleotide (NAD)-specific "malic" enzyme [EC 1.1.1.39 (cf. 10)], with subsequent reduction of pyruvic acid to L-lactic acid by L-lactate dehydrogenase (EC 1.1.1.27) and reduced nicotinamide adenine dinucleotide. Pyruvic acid itself was found only in small amounts in the reaction residual and thus, it was thought to be bound in a complex involving the two enzymes (3). If malic acid were completely converted to lactic acid, the energy of the reaction would not be biologically available since the reaction does not involve formation of high energy phosphate, and there is no net change in the redox state of the coenzyme (7, 10, 11). There is some doubt as to whether the reaction is complete. Stoichiometric studies have shown a wide range of values (75 to 106%) of recovery of lactic acid from malic acid (cf. 7, 14, 11). If the reaction is not complete, some energy might be made available from the intermediary pyruvic acid formed. However, only small increases in cell yields have been reported from L-malic acid and that only when the acid is degraded in the presence of fermentable carbohydrate (6, 11, 12).

Thus, the biological function, if any, of the malo-lactic fermentation is obscure. Besides a role in energy formation, several suggestions as to the benefit of the fermentation for the organism have been presented (7), including the indirect effect of the resulting increase in pH. We pursued previous work that showed an interrelationship between malo-lactic and carbohydrate fermentations (11). The results presented here show that malo-lactic fermentation in Leuconostoc oenos ML 34 does benefit the organism by stimulating the growth rate, especially at low pH, the pH of wine, its natural habitat. This increase in growth rate did not result from the change in pH that accompanies malo-lactic fermentation.

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MATERIALS AND METHODS

Organism. The malo-lactic bacterium L. oenos ML 34 formerly named L. citrovorum ML 34 (cf. 11) was from our culture collection and was maintained as previously described (11).

Culture media. Modified Rogosa medium as previously described (11) was used. It contained 2% tryptone (Difco Manufacturing Co., Detroit, Mich.), 0.5% yeast extract (Difco), 0.5% peptone (Difco), 0.1% liver fraction 1 N.F. XI (Wilson Laboratories, Chicago, III.), and 0.005% Tween-80 (Nutritional Biochemicals, Cleveland, Ohio). Glucose (J. T. Baker, Phillipsburg, N.J.) (0.5%) was added as carbohydrate source. When malic acid was used, 0.2 g of L-malic acid (Nutritional Biochemicals)/100 ml was added. All media were brought to the indicated pH with KOH or phosphoric acid and sterilized by being autoclaved.

Growth rate measurements. Fifteen-milliliter volumes of medium with or without malic acid, final

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1 Present address: The Christian Brothers, Mont La Salle Vineyards, Box 420, Napa, CA 94558.
pH as indicated in the text, in screw-capped culture tubes (20 by 150 mm) were inoculated with 0.15 ml of L. oenos ML 34 (the inoculum was grown without malic acid and was in the stationary phase of growth) and incubated at 25°C. The optical density readings were measured periodically in a Bausch and Lomb spectrophotometer at 600 nm. (The tubes were marked and centered in the same position for each reading.) Two or three inoculated samples and one uninoculated sample (as control) were used for each pH level. Small increases in optical density readings occurred in the controls initially at pH's below 4, but the readings became stable before detectable growth occurred in the inoculated samples.

For the high pH media (above pH 4.5), the specific growth rates, $k$, were calculated from the generation times ($g$) which were determined from straight-line portions of plots of the logarithm of the optical densities versus time ($k = 0.693/g$). At low pH, specific growth rates were difficult to determine because of the slowness and small amount of growth. Thus, comparisons of growth were made quantitatively from drawings of the growth curves.

Cellular dry weight. Cultures were centrifuged (20,000 x g, 10 min) and the pellets were washed twice with one-half volumes of distilled water. After the final centrifugation, the cells were washed into preweighed aluminum weighing pans, with use of small amount of distilled water, and dried at 60°C in a vacuum oven to a constant weight. As blank controls, the dry weight of the particulate material from noninoculated media was determined by the same method of washing and drying, and the blank value was then subtracted from the corresponding growth medium value.

Cell concentration. The concentration of cells was determined microscopically with the use of a Levy-Hauser counting chamber. The values were reported as chains of cells per milliliter, the chains consisting mostly of two cells with 10 to 20% of the chains containing four cells or more.

Paper chromatography. Paper chromatographic analysis for qualitative detection of malic and lactic acids was the n-butanol:formic acid:water:bromo-cresol green system described by Kunkee (8).

pH. pH adjustments and determinations were made with the use of the expanded scale of a radiometer pH meter model 26 calibrated with standard buffer solutions of pH 4.01 and 7.00 (Coleman Instruments Division, Oak Brook, Ill.).

Organic acids. In addition to L-malic acid, the following organic acids were used: L-aspartic and D-malic (Calbiochem, La Jolla, Calif.); citric and fumaric (Eastman Kodak, Rochester, N. Y.); α-ketoglutaric, oxaloacetic, and pyruvic (Nutritional Biochemicals); citric, DL-lactic, and succinic (J. T. Baker); and L-tartaric (Mallinckrodt, St. Louis, Mo.).

RESULTS

High pH levels. Specific growth rates at 25°C of L. oenos ML 34 growing with and without L-malic acid at several initial pH levels (4.5 to 6.0) are shown in Fig. 1. The optimal pH for cells growing in the modified Rogosa medium without the addition of L-malic acid was in the range of pH 5.4 to 5.6 with a specific growth rate of $k = 0.0182/h$ ($g = 38 h$). In the presence of 0.2% L-malic acid, the optimal pH range was 4.8 to 5.2 with a specific growth rate which was almost doubled, $k = 0.0315/h$ ($g = 22 h$).

The increase in growth rate with L-malic acid was not the result of external pH change which accompanies malo-lactic fermentation (see Discussion), since the growth rates of the control cultures were never as great as the cultures containing L-malic acid regardless of initial pH. Moreover, in the control sample at the optimal pH, any change in pH resulting from malo-lactic fermentation would bring about a decreased growth rate rather than an increased rate as is seen in Fig. 1.

Low pH levels. The natural habitat of L. oenos ML 34 is wine, which usually has a pH below 4. Thus, the effect of L-malic acid on growth at pH lower than 4 was also tested. At these low pH levels there was very little growth and it was difficult to obtain straight-line relationships between increases in optical density
and time. Although growth rates were not calculated (see Materials and Methods), one can see from Fig. 2 that L-malic acid brought about a striking stimulation of growth. At the initial pH of 3.65, in the control, one doubling of growth was obtained in about 5 days (120 h), whereas in the presence of 0.2% L-malic acid, four doublings were found at this time. The stimulations occurred at all pH levels tested between pH 3.5 and 3.9 (data not shown). At pH 3.5 there was virtually no growth in the control, and L-malic acid exerted an all or none effect. Also in Fig. 2 is plotted the growth of a control with an initial pH of 3.85. This initial pH is higher than the final pH of the culture shown with L-malic acid; the growth with L-malic acid is still more rapid than the control growth at a higher pH. Thus, the stimulation of growth rate by malo-lactic fermentation cannot be explained by the increase in pH. The stimulation at low pH seems to be greater than the maximum found at high pH. Although the mechanism of the stimulation is unknown, this observation could be explained by optimal enzyme activity at high pH and optimal penetration of the non-ionized substrate at low pH.

To show that these increases in optical densities truly represented increases in growth, the experiments at low pH were repeated to determine increases in cellular dry weights and cell concentrations. Larger (500 ml) volumes of media were prepared at two different pH levels, with and without L-malic acid, and with a higher pH without malic acid as a control. After 5 days (120 h) of incubation after inoculation with L. oenos ML 34, the cellular dry weights and cell concentrations were determined as given in Materials and Methods. The results (Table 1) supported the above conclusions: the increases in optical density in presence of L-malic acid were accompanied by increases in cellular dry weights and cell concentrations. Again, increases were not caused by the change in pH accompanying malo-lactic fermentation.

Several other organic acids were also tested at pH 3.5 and 5.6 and found to have no stimulatory effect. These acids included: L-aspartic, fumaric, α-ketoglutaric, DL-lactic, D-malic, oxaloacetic, pyruvic, succinic and L-tartaric. Citric acid, which has been shown to stimulate the growth of some leuconostocs at pH 5.4 (see Discussion), apparently by providing pyruvic acid as an energy source or a hydrogen acceptor, gave stimulation of growth of L. oenos ML 34 at low pH (pH 3.6).

**DISCUSSION**

The role of the malo-lactic reaction in the metabolism of malo-lactic bacteria has been questioned (7). If the reaction does not provide energy, is there any beneficial effect of it for the organism? During the fermentation, the escape of carbon dioxide resulting from the decarboxylation of L-malic acid brings about a loss of acidity and an increase in pH. This increase in pH may play a beneficial indirect role for the organism. For example, Radler (13) has reported a stimulation in yield of cell mass from malo-lactic fermentation, but this resulted from the utilization of carbohydrate from the increase in pH that accompanied the fermentation. We were careful to control this indirect effect in the work reported here and in previous work which showed a slight increase in cell

**Table 1. Growth of L. oenos ML 34 in the presence of L-malic acid after 120 h of incubation**

<table>
<thead>
<tr>
<th>L-Malic acid</th>
<th>Initial pH</th>
<th>Final pH</th>
<th>Increase in optical density (600 nm)</th>
<th>Dry wt (µg/ml)</th>
<th>Final cell concn</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.61</td>
<td>3.62</td>
<td>0.004</td>
<td>-a</td>
<td>5.0 × 10⁴/ml</td>
</tr>
<tr>
<td>0.2%</td>
<td>3.62</td>
<td>3.70</td>
<td>0.060</td>
<td>8.42</td>
<td>7.8 × 10⁴</td>
</tr>
<tr>
<td>0</td>
<td>3.66</td>
<td>3.67</td>
<td>0.008</td>
<td>-a</td>
<td>1.5 × 10⁴</td>
</tr>
<tr>
<td>0.2</td>
<td>3.65</td>
<td>3.79</td>
<td>0.083</td>
<td>20.0</td>
<td>1.9 × 10⁴</td>
</tr>
<tr>
<td>0</td>
<td>3.84</td>
<td>3.96</td>
<td>0.005</td>
<td>-a</td>
<td>2.0 × 10⁴</td>
</tr>
</tbody>
</table>

a Difference between blank and experimental values negligible.
mass from L-malic acid (11). Malo-lactic fermentation markedly stimulated the growth rate of L. oenos ML 34 and the stimulation was much greater than could be accounted for by the change in pH. At the optimal pH, the change in pH would have tended to decrease the growth rate rather than increase it.

The stimulation of growth is especially interesting at pH levels below 4.0. Although the organism occurs naturally in wine, wine must be considered a hostile environment because of the low pH, lack of nutrients, high concentrations of ethanol and sulfur dioxide, and the usual low temperature of storage. However, wine also contains L-malic acid. The results reported here of a beneficial effect of malo-lactic fermentation give a suggestion as to why most lactic acid bacteria isolated from wine are capable of degrading L-malic acid.

The mechanism for growth rate stimulation by malo-lactic fermentation is as yet unknown. Harvey and Collins (2) have shown an increase in growth rate of Streptococcus diacetilactis in a lactose medium when citric acid degradation occurred. They attributed the increase in growth rate as the result of metabolism of the pyruvic acid formed from citric acid degradation. The acetyl-coenzyme A formed from the pyruvic acid was in part incorporated into cellular lipid material. In a later study, Collins and Bruhn (1) showed that in this organism the rate of growth was limited by the rate of acetyl-coenzyme A synthesis used for production of cellular lipid material and that the rate of acetyl-coenzyme A synthesis from pyruvic acid is higher than the rate from acetate. The stimulatory effect of malo-lactic fermentation on the growth rate of L. oenos ML 34 reported here may be similar since some free pyruvic acid results from L-malic acid degradation (9).

LITERATURE CITED