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F. V. Passos · H. P. Fleming · H. M. Hassan · R. F. McFeeters

Effect of malic acid on the growth kinetics of *Lactobacillus plantarum*

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Abstract The fermentation kinetics of *Lactobacillus plantarum* were studied in a specially designed broth formulated from commercially available, dehydrated components (yeast extract, trypticase, ammonium sulfate) in batch and continuous culture. During batch growth in the absence of malic acid, the specific growth rate was 0.20 h^{-1}. Malic acid in the medium, at 2 mM or 10 mM, increased the specific growth rate of *L. plantarum* to 0.34 h^{-1}. An increase in the maximum cell yield due to malic acid also was observed. Malic acid in the medium (12 mM) reduced the non-growth-associated (maintenance energy) coefficient and increased the biomass yield in continuous culture, based on calculations from the Luedeking and Piret model. The biomass yield coefficient was estimated as 27.4 mg or 34.3 mg cells $mmol^{-1}$ hexose in the absence or presence of malic acid, respectively. The maintenance coefficient was estimated as 3.5 mmol or 1.5 mmol hexose mg^{-1} cell h^{-1} in the absence or presence of malic acid. These results clearly demonstrate the energy-sparing effect of malic acid on the growth- and non-growth-associated energy requirements for *L. plantarum*. The quantitative energy-sparing effect of malic acid on *L. plantarum* has heretofore not been reported, to our knowledge.

F. V. Passos (✉) · H. P. Fleming · H. M. Hassan · R. F. McFeeters
United States Department of Agriculture,
Agricultural Research Service,
and North Carolina Agricultural Research Service,
Department of Food Science, N.C. State University,
Raleigh, NC 27695-7624, USA
e-mail: fvpassos@ufv.br
Tel.: +1-919-5152979
Fax: +1-919-8564361

Present address:
F. V. Passos, Department of Food Science,
Universidade Federal de Viçosa,
MG 36570-000 Viçosa, Brazil

Present address:
H. P. Fleming, USDA-ARS and North Carolina Agricultural Research Service, Department of Food Science,
N.C. State University, Raleigh, NC 27695-7624, USA

Present address:
H. M. Hassan, Department of Microbiology,
N.C. State University, Raleigh, NC 27695-7622, USA

Present address:
R. F. McFeeters, USDA-ARS and North Carolina Agricultural Research Service, Department of Food Science,
N.C. State University, Raleigh, NC 27695-7624, USA

Introduction

The effects of malic acid in the growth medium of lactic acid bacteria are not fully understood. Pilone and Kunkee (1972) noted the fact that all lactic acid bacteria isolated from wine are capable of degrading L-malic acid, suggesting a beneficial effect to the bacteria by malolactic fermentation. It was suggested that the reaction stimulates growth of *Leuconostoc oenos* and increases biomass yield (Pilone and Kunkee 1972; Salou et al. 1991). It was hypothesized that decarboxylation of malate gives an energetic advantage by increasing the internal pH of the cell (Henick-Kling 1986) or perhaps through a stimulated utilization of carbon sources (Pilone and Kunkee 1972). Salou et al. (1991) showed that addition of malic acid to the medium resulted in faster growth of *L. oenos*, up to 75 mM, whereas a slight inhibitory effect was observed above this value. Champagne et al. (1989) found that 37 mM malic acid in apple juice inhibited growth of *L. oenos*. Caspritz and Radler (1983) isolated and studied the malolactic enzyme from *Lactobacillus plantarum* and showed that this enzyme catalyzes the NAD^+- and manganese-dependent conversion of L-malate to CO_2 and L-lactate. The stoichiometry of the conversion of L-malate to L-lactate and CO_2 was always 1:1 in studies using *L. plantarum* in wine fermentation (Henick-Kling 1986).

The objective of this work was to study the effect of malate utilization on growth kinetics and cell yield of *L. plantarum* MOP-3, a strain isolated from a cucumber fermentation, during batch and continuous culture in YTA (yeast extract, trypticase, ammonium sulfate) medium. An

equation to describe malate utilization rate by *L. plantarum* was sought. This equation will be important in the effort to develop a mathematical model to describe and simulate the fermentation of whole cucumbers.

Materials and methods

Culture

L. plantarum MOP-3, isolated from fermenting cucumbers (Fleming et al. 1988), was stored at −70 °C in MRS broth (Difco) containing 16% glycerol. Isolated colonies from MRS agar streak-plates were picked and grown twice at 30 °C in cucumber juice for 12–15 h. Inocula were diluted to an optical density at 630 nm (OD_{630}) of 0.4–0.5 and 1.0% (v/v) was added to each growth medium studied to give an initial concentration of around 10^6 cells ml^{-1}.

Growth media

YTA, prepared from dehydrated components, contained yeast extract (5.0 g l^{-1}; Difco Laboratories, Detroit, Mich.), trypticase peptone (8.0 g l^{-1}; BBL Microbiology Systems, Cockeysville, Md.), and ammonium sulfate (5.0 g l^{-1}; Sigma Chemical Co., St. Louis, Mo.). The broth was adjusted to the desired pH with HCl or NH_3OH and autoclaved for 15 min. Glucose (Sigma) was autoclaved separately and added in the medium to give a final concentration of 50 mM or 100 mM, depending on the experiment. Magnesium sulfate and manganese sulfate (Sigma) also were autoclaved separately and added to the medium to give final concentrations of 0.5 g l^{-1} and 10 mM, respectively. The initial pH of this solution was also adjusted to the desired pH with HCl or NH_3OH. The chemicals used in the study were hydrochloric acid (Aldrich Chemical Co., Milwaukee, Wis.), ammonium hydroxide (Fisher Scientific, Pittsburgh, Pa.), and L(−)malic acid (Sigma).

Fermentation conditions

Water-jacketed jars from Wheaton (Millville, N.J.), with 250 ml working volume, were used as batch or continuous culture growth systems. The growth medium was held at 30 °C and was agitated by magnetic stirrer. Compressed N_2 was humidified and released into the headspace of the fermentor at a rate of 2.5 l h^{-1} to assure anaerobic conditions in all the experiments. During batch growth, samples were removed aseptically by syringe from the 200 ml initial broth volume at intervals of 1–2 h. For continuous culture, a Masterflex pump, 100 rpm microprocessor-controlled drive (Cole Parmer, Chicago, Ill.), and size 13 silicone tubing (Masterflex) were used to feed the fermenter in order to have the desired flow rate (12.5–125.0 ml h^{-1}). Another pump (600 rpm, Masterflex) was used for exit of the medium. The volume of liquid was held constant at 200 ml by positioning the exit tube above this level and controlling the exit pump to an equal or greater flow rate than the feed pump. The different steady-state conditions were achieved by lactic acid inhibition, produced by *L. plantarum*. Samples (2 ml) were periodically removed from the fermentor for pH and OD_{630} determination. After at least eight full residence times, when no further changes were observed in these parameters, three additional samples were taken at intervals of 2–8 h (depending on the specific growth rate) for chemical analyses to further confirm steady-state conditions. If no chemical changes were observed (<5%), steady-state conditions were assumed.

Analytical methods

Cell growth was followed by measurement of the OD_{630} of the medium in a 1.5-ml glass cuvette, using a Novaspec II spectrophotometer (Pharmacia). The linear range extended to OD_{630} readings of 0.45. During growth, if the OD_{630} was higher than 0.30, the sample was diluted using distilled water to within a range of 0.1–0.3. Standard curves were used to relate OD_{630}, dry weight (g l^{-1}) and cell number [colony-forming units (CFU) ml^{-1}]. For dry weight determination, 500 ml of cell suspension (OD_{630} ca. 0.8) was washed two times with an equal volume of sterile water, concentrated 50× by centrifugation, and four samples of 3 ml each were dried in pre-weighed aluminum dishes (A. H. Thomas Co., Philadelphia, Pa.) to constant weight in a vacuum oven at 80 °C.

Viable cells were enumerated in MRS agar, using the same cell suspension used for dry weight. A change of 1 unit at OD_{630} was equivalent to 0.264 g l^{-1} and 2.5×10^8 CFU/ml, for dry weight and cell number, respectively. In the death-rate experiment, viable cells were enumerated by surface-streaking on MRS agar after proper dilution.

All chemical components were determined using HPLC. Broth samples were diluted two-fold using distilled water and 1.5 ml was then centrifuged at 12,000 *g* (centrifuge model 5415; Eppendorf, Westburg, N.Y.). Glucose, lactic acid, and acetic acid were analyzed with an Aminex HPX-87H column (Bio-Rad Laboratories, Richmond, Calif.) heated to 65 °C, a differential refractometer (Waters Associates, Milford, Mass.) and an integrator (Shimadzu Corp., Columbia, Md.). The column was eluted with 0.01 M H_2SO_4 at a flow rate of 0.7 ml min^{-1} (Lazaro et al. 1989). When malic acid and fructose were both present in the sample, a Dionex system (Dionex Corp., Sunnyvale, Calif.) was used with a conductivity detector for acid determination and a Dionex pulsed amperometric detector for sugar determination (McFeeters 1993).

For batch cultures, the specific growth rate (μ) was calculated from linear regression analysis of the initial linear portion of the growth curve, plotted as the log of cell concentration versus time. For continuous culture, μ was calculated by assuming that, at steady-state condition, μ was equal to the dilution rate and the dilution rate was equal to the flow rate (flux), divided by total liquid volume in the fermentor.

The growth-associated coefficient ($Y_{P/X}$) and the non-growth-associated coefficient (Y_m) for lactic acid production were mathematically represented (Luedeking and Piret 1958; Pirt 1982):

$$\frac{1}{X}\frac{dP}{dt} = Y_{P/X}\cdot\mu + Y_m \quad (1)$$

The specific production rate of lactic acid (left side of Eq. 1) in continuous culture was calculated at each steady-state condition by dividing the concentration of lactic acid at that point by the cell concentration and the residence time. In the graph of specific production rate versus specific growth rate (Fig. 2), $Y_{P/X}$ is the slope of the curve, and Y_m is the intercept.

The specific utilization rate of malate in continuous culture was calculated at each steady-state condition as for lactic acid. The specific consumption rate of malate in continuous culture was calculated at each steady-state condition by dividing the concentration of malate consumed (initial concentration of malate subtracted by the concentration at steady-state condition) by the cell concentration and the residence time. In the graph of specific consumption rate versus specific growth rate (Fig. 3), the growth-associated coefficient ($Y_{M/X}$) is mathematically represented by the slope of the curve and Y_m is the intercept of the curve (Luedeking and Piret 1958; Pirt 1982), or:

$$\frac{1}{X}\frac{\Delta M}{\Delta t} = Y_{M/X}\cdot\mu + Y_m \quad (2)$$

Results

At pH 4.1, the presence of malate in the medium, at 2 mM or 10 mM, increased the specific growth rate from 0.20 h^{-1} to 0.35 h^{-1} and 0.33 h^{-1}, respectively, measured

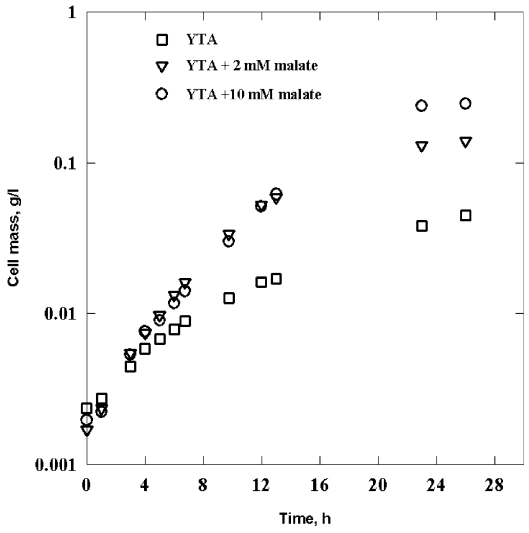

Fig. 1 Effect of the presence of 2 mM or 10 mM malate on the growth rate of *L. plantarum* during batch growth at initial pH 4.1. Malic acid was completely depleted after 8 h or 14 h, when 2 mM or 10 mM malate were added to the medium (*YTA*), respectively

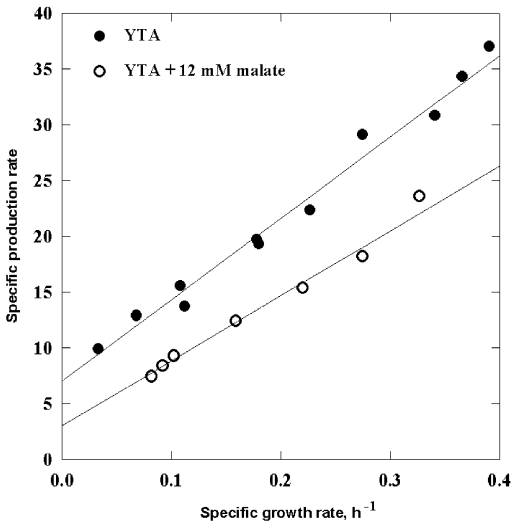

Fig. 2 Effect of presence of malate on the specific production rate of lactate during continuous culture at different specific growth rates

Table 1 Effect of malate concentration on the kinetics of *Lactobacillus plantarum* MOP-3 in continuous culture fed with YTA medium (see Materials and methods), at a constant specific growth rate of 0.086 h^{-1} and 30 mM glucose

Feed conditions Malate (mM)	Steady-state conditions					
	Sugar (mM)	Malate (mM)	pH	Cell (g l^{-1})	Lactate (mM)	$Y_{X/S}$ (g mol^{-1})
0.0	11.4	0.0	3.6	0.195	37.1	10.5
12.3	5.6	0.0	3.9	0.570	61.4	23.5
31.5	0.7	2.1	4.1	0.750	90.1	25.6

during the period of malate utilization (Fig. 1). Malic acid was completely depleted after 8 h when 2 mM malate was added to the medium or after 14 h when 10 mM malate was added to the medium. Besides stimulating growth, the presence of malate increased the maximum cell concentration, measured after 26 h of growth (Fig. 1). Steady-state conditions for growth of *L. plantarum* in continuous culture at 0.0, 12.3 and 31.5 mM malate are given in Table 1. Increase in the malate concentration of the feed medium increased the biomass yield and the lactic acid production from glucose. There was no significant difference for the apparent biomass yield ($Y_{X/S}$) estimated when 12.3 mM or 31.5 mM malate was added to the feed broth (23.5 g or 25.6 g cells mol^{-1} glucose, respectively), but the yield was significantly lower when no malate was used (10.5 g cell mol^{-1} glucose). In all three runs, the specific growth rate was limited apparently by product inhibition (hydrogen ion, uncharged lactic acid), since excess carbohydrate was available at steady-state conditions.

Fig. 2 illustrates the relationship between specific lactic acid production rate and specific growth rate of *L. plantarum* in continuous culture in the presence and absence of malic acid. Lactic acid production was calculated only from glucose metabolism. The filled circles represent each steady-state condition achieved in the fermentor when no malate was added and the open circles when 12 mM malate was added to the YTA medium. The solid lines represent the model proposed by Luedeking and Piret (1958), where lactic acid production is a function of cell division and biomass concentration. The growth-associated coefficient is mathematically represented by the slope of the curve and the non-growth-associated coefficient (maintenance energy) is the intercept of the curve (Luedeking and Piret 1958; Pirt 1982). The growth-associated coefficient was 73.0±2.5 mmol lactate g^{-1} cell when no malate was present in the feed medium and 58.3±2.3 mmol lactate g^{-1} cell when malate was present. The non-growth-associated coefficient was 7.0±0.7 mmol lactate g^{-1} cell h^{-1} when malate was not present and 3.0±0.6 mmol lactate g^{-1} cell h^{-1} when malate was present. From these coefficients, it is possible to estimate other coefficients summarized in Table 2. The biomass yield ($Y_{X/S}$, mg cell $mmol^{-1}$ glucose) and the maintenance coefficient (Y_m, mmol glucose g^{-1} cell h^{-1}) were calculated assuming a conversion factor of 2 mol lactate mol^{-1} glucose fermented. ATP yield (Y_{ATP}, mmol ATP g^{-1} cells) was estimated assuming a conversion rate of 2 mol ATP mol^{-1} glucose fermented.

Fig. 3 presents the specific consumption rate of malic acid against the specific growth rate in continuous culture. The plot suggested a linear relationship between con-

Table 2 Effect of malate (12 mM) on the growth-associated ($Y_{X/S}$) and non-growth associated (Y_m) coefficients for *L. plantarum* MOP-3 in YTA medium, defined in continuous culture. Data are calculated from Fig. 2. *Glucose* coefficients are calculated considering the total lactic acid concentration from glucose only. *Glucose + malic* coefficients are calculated considering the total lactic acid concentration from glucose plus malate utilization. *YTAM* YTA plus 12 mM malate

Metabolism coefficients	Glucose		Glucose + malic YTAM
	YTA	YTAM	
$Y_{P/X}$ (mmole g^{-1})	73.0±2.5	58.3±2.3	96.8±2.6
Y_p (mmole g^{-1} h^{-1})	7.0±0.7	3.0±0.6	0.9±0.64
r^2	0.988	0.991	0.996
$Y_{X/S}$ (mg $mmol^{-1}$)	27.4	34.3	
Y_m (mmol g^{-1} h^{-1})	3.5	1.5	
Y_{ATP} (mg $mmol^{-1}$)	13.7	17.2	

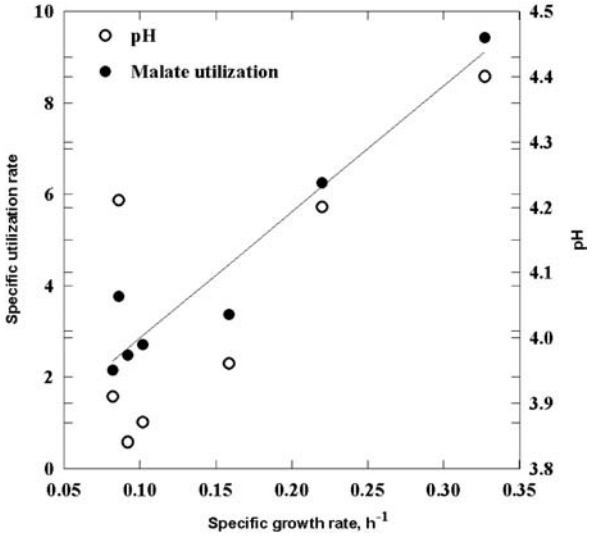

Fig. 3 Specific utilization rate of malate versus specific growth rate achieved during continuous culture. Open circles represent pH during each steady-state condition achieved

sumption rate of malate and specific growth rate of the cell. A linear regression of the data gave an intercept of 0.09±0.061 mmol malate g^{-1} cell h^{-1} and a slope of 27.6±3.5 mmol malate g^{-1} cell. The open circles represent the pH at each steady-state achieved. Since each steady-state condition was achieved by product inhibition, pH values were different for each specific growth rate.

Discussion

Other authors reported the stimulatory effect of malate on growth of lactic acid bacteria. Pilone and Kunkee (1972) showed that the specific growth rate of *Leuconostoc oenos* was greatly increased by malolactic fermentation, especially at pH below 4.0. They consider that, since the stimulation of growth did not result from changes in pH that accompany malolactic fermentation, a biological function of malolactic fermentation was indicated. Salou et al. (1991), working with *L. oenos*, showed that the biomass yield from glucose was higher during the malate consumption phase than when glucose alone was consumed, in accord with the present study (batch data, Table 1). They suggested that the malolactic reaction must generate some energetic advantage, such as an increase in the proton motive force and the internal pH of the cells. During batch growth at constant pH 5.0, the authors estimated an apparent biomass yield of 10.6 mg or 19.8 mg cell $mmol^{-1}$ glucose in the absence or presence of malate (9 mM or 37 mM), respectively; and the specific malic consumption rate changed from 6.1 mol to 19.4 mmol malate g^{-1} cell h^{-1}, depending on the malate concentration added in the medium.

The ATP yield was 73.0 mmol ATP g^{-1} dry cells when malate was not present in the YTA medium and 58.1 mmol ATP g^{-1} dry cells when malate was present. This means that 12 mM malate in the medium makes the cell spend 15 mM ATP less to produce 1 g dry cells, i.e. a 20% reduction in the ATP required. The stimulatory effect of malic acid on the growth of *Lactobacillus plantarum* was observed not only during batch growth (Fig. 1), but also during continuous culture. For the same specific growth rate (0.086 h^{-1}), the lactic acid concentrations were 61.4 mM and 90.1 mM and the pHs 3.9 and 4.1 when in the presence of 12.3 mM or 31.5 mM malate (Table 1). When malic acid was not present, the steady-state conditions were 37.1 mM lactate and pH 3.6. This is in accord with Henick-Kling (1986), who suggested that the decarboxylation of malic can give the cell an energetic advantage by increasing internal pH, even though the uptake of malate by *L. plantarum* requires energy. He assumed that malic acid is transported actively through the membrane in the undissociated form (H_2Ma) and lactic acid is also transported out in the undissociated form (HLa).

No change was observed in the apparent biomass yield when 12.3 mM or 31.5 mM malate was used during continuous culture growth (Table 1), supporting the previous report by Salou et al. (1991), who suggested that malate probably was not used as a carbon source.

From the data collected in continuous culture, it is not clear whether differences in the rate of malate degradation were due to differences in medium pH or specific growth rate of the cells (Fig. 3). The plot of specific malate utilization rate against specific growth rate was linear during continuous culture and steady-state conditions achieved by product inhibition, or with different pH values for each specific growth rate. This suggests that the non-growth-associated consumption rate and the growth-associated consumption rate, 0.09±0.061 mmol malate g^{-1} cell h^{-1} and 27.6±3.5 mmol malate g^{-1} cell, respectively, were independent of the pH value. The fact that pH also changes at each growth rate limits conclusions on cause and effect relationships. Naourin et al. (1989) studied malolactic enzyme activity in *Lactobacillus* sp. 89 and showed high activity in the pH range 3.5–5.0, giving a specific activity of 18 mmol g^{-1} cell h^{-1} in that pH range.

Henick-Kling (1986) reported that malolactic activity in *L. plantarum* was fully induced by the mid-exponential stage of growth and decreased after the cells reached the stationary phase. That author also showed that the optimal pH for the two enzymes that govern the system are different, about pH 3.0–4.5 for the transport system and about pH 6.3 for the malolactic enzyme. Several authors (Bousbouras and Kinkee 1971; Henick-Kling 1986; Wibowo et al. 1985) showed that the malolactic fermentation in wine is completed more rapidly at higher medium pH values. Henick-Kling (1986) suggested that, since malate transport requires energy, the rate of malolactic fermentation is governed by the rate of carbohydrate metabolism and growth. Growth rate is highest at high pH and, therefore, it was suggested that the energy necessary for cell growth and transport of malate is more readily available at high pH. Malic acid utilization rate is probably the summation of various factors: activity of the malate transport system, activity of the malolactic enzyme inside the cell, the utilization rate of sugar and energy available, and the malic acid concentration in the medium.

Finally, we conclude that the presence of malic acid stimulates the growth of *L. plantarum*, as evidenced by results from growth in batch and continuous culture. A lower maintenance coefficient was observed when malate was present in the feed medium of continuous culture. Malate increases the biomass yield by buffering the medium and by reducing the maintenance coefficient. For a more conclusive kinetic model of malate utilization, additional studies are needed. Effects of pH, malic acid concentration, and carbohydrate concentration individually and in combination need to be determined.

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