Kinetics and Modeling of Lactic Acid Production by *Lactobacillus plantarum*

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Received 20 October 1993/Accepted 7 May 1994

An unstructured model was developed to describe bacterial growth, substrate utilization, and lactic acid production by *Lactobacillus plantarum* in cucumber juice. Significant lactic acid production occurred during growth, as well as stationary phases. The percentage of acid produced after growth ceased was a function of the medium composition. Up to 51% of the lactic acid was produced after growth ceased when NaCl was not present in the medium, whereas not more than 18% of the total lactic acid was produced after the growth ceased in presence of NaCl, probably because of an increase in the cell death rate. An equation relating the specific death rate and NaCl concentration was developed. With the kinetic model proposed by R. Luedeking and E. L. Piret (J. Biochem. Microbiol. Technol. Eng. 1:393–412, 1958) for lactic acid production rate, the growth-associated and non-growth-associated coefficients were determined as 51.9 (±4.2) mmol/g of cells and 7.2 (±0.9) mmol/g of cells h^{-1} respectively. The model was demonstrated for batch growth of *L. plantarum* in cucumber juice. Mathematical simulations were used to predict the influence of variations in death rate, proton concentration when growth ceased, and buffer capacity of the juice on the overall fermentation process.

Vegetable fermentations represent a dynamic ecosystem in which the type and rate of fermentation reflect the changing chemical and physical environment of the fermentation as it proceeds. Brined cucumbers traditionally are fermented by lactic acid bacteria (LAB) and other natural microflora in large wooden, fiberglass, or polyethylene tanks which are open to the atmosphere. Sugar and nutrients diffuse from the cucumber into the brine, while sodium chloride, acetic acid, and other brine solutes diffuse from the brine to the cucumber. These variables combine to exert selective effects on growth of the natural microflora and/or on the LAB starter culture (if added) during fermentation. Preservation of the fruit results from the transformation of all available fermentable sugars into lactic acid and other stable components and the accompanying reduction of the pH to below 4.0.

A minimum of 0.6% (66.7 mM) lactic acid has been recommended to ensure preservation (8). Excessive acidity levels, however, can adversely affect the texture (28) and flavor (5) of the fermented cucumbers. The addition of acetate buffers to the cover brine has been shown to ensure complete conversion of fermentable sugars to lactic acid by LAB (7, 11). The addition of a fermentative yeast, *Saccharomyces rosei*, has been suggested as a means of partial utilization of fermentable sugars to avoid excessive acid production by LAB (5). Although yeast and gas-forming bacteria can result in bloater damage to the cucumber, brines can be purged of the CO_2 produced to avoid this problem (9).

Lactobacillus plantarum has been suggested as a starter culture for cucumber fermentation because of its ability to predominate natural fermentations and its inability to produce CO_2 from hexoses (7). Although most naturally occurring *L. plantarum* strains produce CO_2 from malic acid, mutant strains that do not possess this ability have been isolated (6).

L. plantarum metabolizes hexose via the Embden-Meyerhof pathway to produce primarily lactic acid. The energy produced is used largely for cell division and maintenance requirements. Maintenance is the energy required for survival or for preservation of cell viability which is not directly related to or coupled with the synthesis of a new cell (1). Many studies have demonstrated uncoupled energy production by LAB (3, 12, 15, 17, 25, 26).

Understanding of such a complex system is aided by developing a mathematical model for an ideal process for cucumber fermentation. Mathematical models can be defined as either empirical or mechanistic. Empirical models to describe bacterial growth are normally sigmoidal expressions for cell concentration as a function of time, with the coefficients of the equation normally defined as polynomial functions of the variables of interest (pH, water activity, and temperature, etc.). Empirical models are used in food systems to predict the microbial safety or shelf life of products, detect critical points of the production and distribution process, and optimize production and distribution chains (31). In general, such models provide little indication of which variables influence growth, and they make no attempt to utilize knowledge about cellular metabolism and regulation (1).

When the objective of a model is fundamental understanding of the modeled system, a mechanistic model is called for. Food fermentation is a complex process in which different substrates and inhibitors are generally present. In many cases, a heterogeneous solid-liquid system exists and intraphase and interphase diffusion of solutes affects the course of the process. Such a system is the fermentation of whole cucumbers. Mechanistic models can play a central role in gaining such understanding. Mechanistic models have been formulated for production of antibiotics and alcohol for motor fuels, but models describing food microbial systems are rare in the literature.

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While simple mechanistic models for inhibition by a single environmental solute (e.g., antibiotic, pH, acid product, or ethanol, etc.) have been proposed, very few studies have attempted to model a product-inhibited fermentation involving multiple inhibition variables.

In the current study, an unstructured mechanistic model was developed to take into account the most important changes during the fermentation of cucumber juice (CJ). We chose a malate-utilizing strain of *L. plantarum* isolated from a commercial cucumber fermentation for the purpose of modeling cell growth, sugar utilization, and acid production during fermentation. The model may allow better understanding and control of cucumber fermentation by *L. plantarum*.

MATERIALS AND METHODS

Nomenclature. The following terms are used below: [Ac^-], dissociated acetic acid concentration (millimolar); [Ac_t], total acetic acid (HAc + Ac^-) concentration (millimolar); [H^+], hydrogen ion concentration (millimolar); [H_2Ma], undissociated malic acid concentration (millimolar); [HAc], undissociated acetic acid concentration (millimolar); [HLa], undissociated lactic acid concentration (millimolar); [Ic^+], mineral ion concentration associated with malate present in the cucumber composition (millimolar); [La^-], dissociated lactic acid concentration (millimolar); [$La_{t,Ma}$], total lactic acid concentration from malate utilization (millimolar); [$La_{t,s}$], total lactic acid concentration from hexose fermentation (millimolar); [La_t], total lactic acid (HLa + La^-) concentration (millimolar); [Ma^-], dissociated malic acid concentration (millimolar); [Ma_t], total malic acid (H_2Ma + HMa^- + Ma^{2-}) concentration (millimolar); [NaCl], sodium chloride (percent [weight/volume]); [S], hexose concentration (millimolar); X, cell concentration (grams per liter); X_o, initial cell concentration (grams per liter); X_t, total cell concentration (grams per liter); and X_v, viable cell concentration (grams per liter). Coefficients are as follows: μ, specific growth rate (per hour); μ_{max}, maximum specific growth rate (per hour); $[H^+]_{max}$, hydrogen ion concentration at which growth ceases (millimolar); k_d, specific death rate (per hour); K_i, dissociation constant, where i is Ac, La, or Ma; K_m, concentration of nutrient at which the specific growth rate has half its maximum value (millimolar); pK′, dissociation constant; z, ion net charge; $Y_{P/X}$, growth-associated coefficient (mmol product per gram of cells); $Y_{La/X}$, growth-associated coefficient (mmol lactic acid per gram of cells); $Y_{Ma/X}$, malic acid yield coefficient (mmol per gram of cells); $Y_{La/S}$, molar yield of lactic acid production from hexose; y_S, non-growth-associated coefficient (mmol lactic acid per gram of cells per hour); and y_{Ma}, malate utilization coefficient (mmol malate per gram of cells per hour).

Culture. *L. plantarum* MOP3 isolated previously (11) was stored in MRS broth (Difco Laboratories, Detroit, Mich.) containing 16% glycerol at −70°C. Isolated colonies from MRS agar streak plates were picked and grown twice at 30°C in CJ for 12 to 15 h. Inocula were diluted to an optical density (OD) at 630 nm of 0.4 to 0.5 and 1.0 volume was added to each growth medium studied to give an initial concentration of approximately 10^6 cells per ml.

Growth medium. CJ was prepared, centrifuged, diluted, and filter sterilized (0.22-μm-pore-size filter) according to the method of Passos et al. (22). Lactic acid, acetic acid, and NaCl were obtained from Aldrich Chemical Co., Inc. (Milwaukee, Wis.), and hydrochloric acid was obtained from Fisher Scientific (Pittsburgh, Pa.).

Fermentation conditions. Water-jacketed jars from Wheaton (Millville, N.J.), each with a 250-ml working volume, were used as batch or continuous culture growth systems. The growth medium was agitated with a magnetic stirrer, and the growth temperature was 30°C. Compressed N_2 was humidified and released into the headspace of the fermentor at a rate of 2.5 liters/h to ensure 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 to 2 h. For continuous culture, a Masterflex pump, 100-rpm microprocessor-controlled drive (Cole-Parmer Instrument Co., Chicago, Ill.), and tube #13 (silicone; Masterflex) were used to feed the fermentor in order to have a precise low flow rate (12.5 to 125 ml/h). 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 a flow rate equal to or greater than that of the feed pump. The different steady-state conditions were achieved through autoinhibition of *L. plantarum* by its production of lactic acid. Samples (2 ml) were periodically removed from the fermentor for pH and OD determination. When no further changes (<5%) were observed in these variables, three samples were taken at intervals of 2 to 8 h (depending on the specific growth rate) for chemical analyses. When no other chemical changes (<5%) were observed in such samples, steady-state conditions were assumed.

Death rate. Death rate experiments were performed in silicone-stoppered tubes (40-ml working volume) incubated at 30°C. Cells were grown in 50% CJ containing 2% NaCl and 10 mM acetic acid for 15 h at 30°C. The cells were harvested during the exponential growth phase, centrifuged, and concentrated 20×. An inoculum of 1 ml, giving an initial population of about 2×10^8 CFU/ml, was added to fresh CJ at different pHs, lactic and acetic acid concentrations, and NaCl concentrations. For 12 days, samples were removed aseptically by pipette from the 40-ml initial broth volume at intervals of 24 to 48 h, and OD, pH, viable cells, and lactic acid were monitored. First-order death rate constants (k_ds) were calculated from linear regression analysis of the logarithm of the viable cell count versus time.

Analytical methods. Cell growth was monitored by measurement of the OD of the medium in a 1.5-ml glass cuvette with a Novaspec II spectrophotometer (Pharmacia LKB Biotechnology, Piscataway, N.J.). The linear range extended to OD readings of 0.45. During growth, if the OD was higher than 0.30, the sample was diluted to within a range of 0.1 to 0.3 with distilled water. Standard curves were used to relate OD, dry weight (grams per liter), and cell number (CFU per milliliter). For dry weight determination, a 500-ml cell suspension (OD of around 0.8) was washed two times with an equal volume of sterile water and concentrated 50× by centrifugation and four samples of 3 ml each were dried to constant weight in a vacuum oven at 80°C. Viable cells were enumerated in MRS agar by using the same cell suspension used for dry weight. One unit of OD was equivalent to 0.264 g of cells per liter and 2.5×10^8 CFU/ml for dry weight and cell number, respectively. In the death rate experiment, viable cells were enumerated on MRS agar after proper dilution. Direct microscopic counts were determined with a Wild M20 phase-contrast microscope (Wild Heerbrugg Ltd., Heerbrugg, Switzerland). CFU per milliliter were determined at ×400 magnification with a Petroff-Hausser counting chamber (27). The number of cells per CFU was determined at ×1,000 magnification. Total microscopic counts were calculated by multiplication of CFU per milliliter by cells per CFU. NaCl was determined by titration with standard $AgNO_3$ using dichlorofluorescein as an indicator (10). All the other components were determined by high-pressure liquid

chromatography (HPLC). Broth samples were diluted twofold with distilled water, and 1.5 ml was 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.) at 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. When malic acid and fructose were present in the sample, a Dionex (Sunnyvale, Calif.) system was used with a conductivity detector for acid determination and a Dionex pulsed amperometric detector for sugar determination (19).

Model development. During fermentation of CJ by *L. plantarum*, the initial cells present (X_o) consume sugar (S) to produce new cells (X). Refer to "Nomenclature" above for definitions of designations used herein. Since this bacterium is homofermentative with respect to hexoses, lactic acid is the main product formed. Malic acid also is a natural component of CJ and is converted by *L. plantarum* to lactic acid and CO_2. These two reactions can be represented as follows: $[S] + [X_o] \rightarrow [X] + Y_{La_t/X}[X_v] + Y_{H^+/La^-}[La^-]$ and $[Ma_t] \rightarrow Y_{La_t/X}[X_v] + Y_{H^+Ma_t}[Ma^-] + Y_{CO_2/Ma_t}[Ma_t^-]$.

Assuming that these mathematical models describe the growth of *L. plantarum* during cucumber fermentation, a batch process, a mathematical representation of the specific growth rate as a function of the dynamic variables (pH, lactic acid, acetic acid, and NaCl), is necessary. This representation was previously defined (22). To account for the effect of substrate limitation on the specific growth rate, we included the Monod model, using a K_m value of 0.056 mM, which is considered an average value for bacteria growing in glucose (30), where

$$\mu = \frac{\mu_{max} \cdot S}{K_m + S} \tag{1}$$

For malic acid utilization, the apparent yield coefficient was previously defined (21) as follows: y_{Ma} (mmol/g of cells/h) = 27.6 (mmol/g of cells)μ + 0.09 (mmol/g of cells/h).

pH change. Since the specific growth rate equation is a function of the concentrations of hydrogen ion and the undissociated inhibitory form of acetic and lactic acid, an equation to predict pH change in the medium during fermentation was necessary. Using an approach similar to that used by Kuhn (16) and assuming malate as the more important buffer component of the CJ, equation 2 was proposed to describe the change in the medium pH.

$$\Sigma Z_i C_i = H^+ + Ic^+ - La^- - Ac^- - HMa^- - 2Ma^{2-} = 0 \tag{2}$$

where C_i is the concentration of each ionic species and z_i is its net charge. Since the malate is in the salt form, Ic^+ was added in the equation to represent the mineral cations associated with malate present in the CJ. Strong acids, and bases and salts such as NaCl, were considered to be fully dissociated (16) and were not included in the generalized equation 2. For weak acids, the dissociation step can be written as follows (24):

$$HA_{n-m+1}^{-(m-1)} \rightleftharpoons H^+ + HA_{n-m}^{-m} \tag{3}$$

where n is the total number of dissociation steps and m is the step number. Assuming that these reactions approach equilibrium quickly (4), the concentration of the ionic portion of a monoprotic weak acid such as lactic acid can be given as

$$La^- = \frac{K_{La} \cdot La_t}{H^+ + K_{La}} \tag{4}$$

Defining pK′ as $-\log(K_{La})$, the dissociation constant, K_{La}, can be calculated from equation 5:

$$pK' = pK - \frac{Z^2 \cdot 0.51\sqrt{\Gamma/2}}{1 + 1.6\sqrt{\Gamma/2}} \tag{5}$$

where pK is the dissociation acid constant (equal to 3.86 at infinite dilution) and $\Gamma/2$ is the ionic strength (29). The rate of proton production can be calculated by substituting ion concentrations (equation 3) into equation 2 and differentiating, yielding equation 13 below.

A set of ordinary differential equations was used to model the microbial growth and fermentation processes. The dynamic mechanistic model of microbial growth in a liquid-phase system consists of a set of ordinary differential equations having the general form

$$\frac{dX_i}{dt} = f_i(t, X_1, \ldots X_n) \tag{6}$$

where the functions f_1 on the right side are known. This is an initial-value type of model in which all of the Xs are given at some starting time ($t = 0$) and the values at subsequent times are determined by simultaneous integration of the system equations.

Thus, the following set of equations (equations 7 to 14) was used to represent growth and fermentation by *L. plantarum* in CJ:

$$\frac{d[X_t]}{dt} = \mu[X_v] \quad \text{(cell growth)} \tag{7}$$

$$\frac{[dX_v]}{dt} = (\mu - k_d)[X_v] \quad \text{(viable cell balance)} \tag{8}$$

$$\frac{d[La_{t,s}]}{dt} = Y_{La/X}(\mu[X_v]) + y_s[X_v] \quad \text{(lactate from hexose)} \tag{9}$$

$$\frac{d[Ma_t]}{dt} = -y_{Ma}[X_v] \quad \text{(malate consumption)} \tag{10}$$

$$\frac{d[La_{t,Ma}]}{dt} = -\frac{d[Ma_t]}{dt} \quad \text{(lactate from malate)} \tag{11}$$

$$\frac{d[La_t]}{dt} = \frac{d[La_{t,s}]}{dt} + \frac{d(La_{t,Ma}]}{dt} \quad \text{(total lactate production)} \tag{12}$$

$$\frac{d[S]}{dt} = -\left(\frac{1}{Y_{La/S}}\right)\frac{d[La_{t,s}]}{dt} \quad \text{(hexose utilization)} \quad (13)$$

$$\frac{d[H^+]}{dt} = \frac{\dfrac{K_{La}}{(K_{La} + [H^+])}\dfrac{d[La_t]}{dt} + \left(\dfrac{K_{Ma,1}[H^+] + 2K_{Ma,1}K_{Ma,2}}{D}\right)\dfrac{d[Ma_t]}{dt}}{1 + \dfrac{K_{Ac}[Ac_t]}{(K_{Ac} + [H^+])^2} + \dfrac{K_{La}[La_t]}{(K_{La} + [H^+])^2} - \dfrac{K_{Ma,1}[Ma_t]}{D} + \dfrac{2K_{Ma,1}[Ma_t]([H^+] + K_{Ma,2})(2[H^+] + K_{Ma,1})}{D^2}} \quad (14)$$

where

$$D = H^2 + K_{Ma,1}K_{Ma,2} + K_{Ma,1}H \quad (15)$$

Assuming that the specific growth rate (μ) of *L. plantarum* growing in CJ will be a function of product inhibition (hydrogen ion and undissociated lactic acid concentrations) and/or substrate limitation (hexose), variables that change in concentration in the course of the process, and that the initial growth rate will be a function of NaCl and undissociated acetic acid concentrations added in the juice, equation 16, proposed by Passos et al. (22), will be used.

$$\mu = \mu_o\left(\frac{[S]}{0.056 + [S]}\right)\left(1 - \frac{[H^+]}{[H^+]_{max}}\right)^{2.6}\left(1 - \frac{[HLa]}{69}\right)^{2.0} \quad (16)$$

where

$$\mu_o = 0.35\left(1 + \frac{1.5[HAc]}{5.8 + [HAc]}\right)$$

$$\left(1 - \frac{[HAc]}{150}\right)^{1.7}\left(1 + \frac{1.6[NACl]}{4.47 + [NaCl]}\right)\left(1 - \frac{[NaCl]}{11.8}\right) \quad (17)$$

Numerical solution. The dynamic model system of ordinary differential equations 7 to 14 was solved by the Fehlberg fourth-fifth-order Runge-Kutta method with change in the step size. A PC/486 computer using a Fortran 77 compiler in a Windows environment was used for these computations. A menu-driven program was written to ask for the input variables (initial values of cell mass; sugar, malate, lactate, acetate, and NaCl concentrations; pH; and the time of fermentation) and to generate tables with changes in time of total cells; viable cells; sugar, malate, and lactate concentrations; and pH. The value of $[H^+]_{max}$ in equation 16, previously defined (22), was chosen to best fit experimental data.

Sensitivity analyses and simulation. To illustrate the effect of parameter variations, simulations changing by ±20% the values of death rate constant and hydrogen ion concentration at which cell growth ceased ($[H^+]_{max}$) were run. The effect of increasing medium buffer capacity was tested by altering initial acetate concentrations and initial pH.

RESULTS

Data from typical batch growth of *L. plantarum* in CJ at 30°C are shown in Fig. 1. A clear exponential phase was observed until 10 h of growth. The pH increased slightly during the earliest exponential phase of growth, during which time malic acid was degraded. Subsequently (5 to 25 h), the pH rapidly decreased to about 3.2. The reduction of specific growth rate is due apparently to product inhibition. Cell growth ceased at about 24 h, as indicated by the total cell concentration (OD measurement). Lactic acid production continued after cell growth ceased and ended after about 100 h. The vertical broken line at 24 h is given to facilitate perception of conditions after cell growth ceased. After exponential growth halted, the viable cell number rapidly declined. Microscopic count of

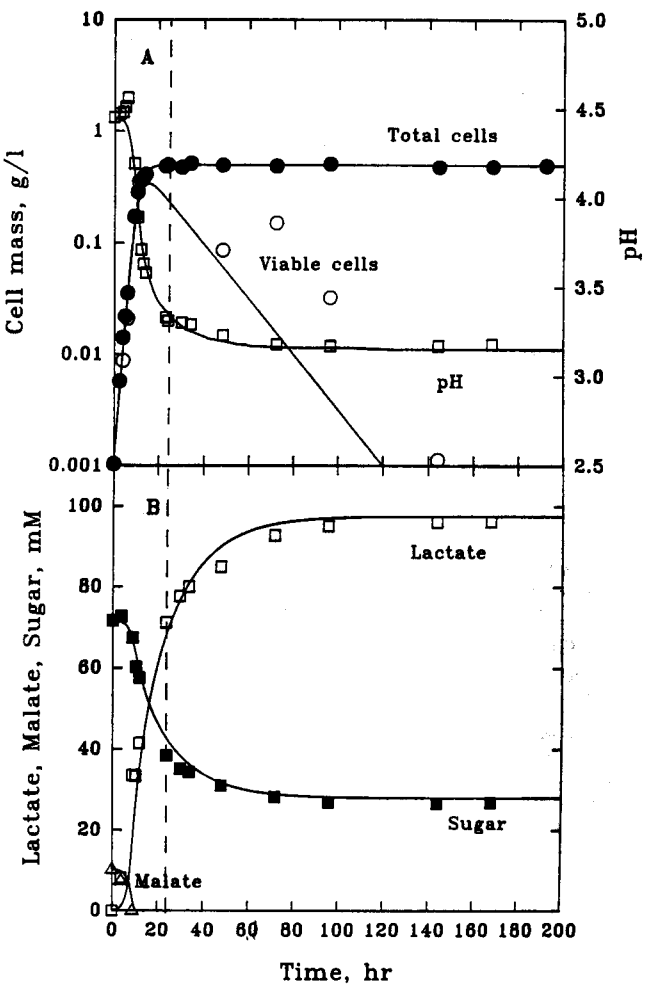

FIG. 1. Observed (symbols) and predicted (lines) changes in batch growth of *L. plantarum* MOP3 in CJ with 20 mM acetic acid and 3% NaCl at 30°C and uncontrolled pH. The vertical broken line traversing all curves at 24 h is given to facilitate perception of conditions after cell growth ceased.

TABLE 1. Effect of acetic acid on growth and total lactic acid production by *L. plantarum*[a]

Initial conditions		Conditions when growth ceased			Conditions when acid production ceased				
pH	[Ac_i] (mM)	pH	[La_t] (mM)	[Cell] (g/liter)	pH	[La_t] (mM)	[Sugar] (mM)	$Y_{La/S}$[b]	R_{La}[c] (%)
5.95	0.7	3.43	68.6	0.494	3.10	139.1	0.0	1.98	50.7
4.68	11.4	3.50	61.4	0.530	3.20	107.5	15.1	1.95	42.9
4.40	23.3	3.48	56.3	0.533	3.21	107.4	14.7	1.93	47.6
4.27	30.0	3.45	61.3	0.515	3.24	99.8	20.3	2.00	38.6
4.12	44.9	3.40	64.4	0.312	3.22	100.2	19.5	1.97	35.7
4.07	49.0	3.48	53.0	0.239	3.20	101.4	19.0	1.98	47.7
3.99	61.0	3.41	56.7	0.247	3.20	104.5	17.9	2.00	45.7

[a] The growth medium was CJ which contained 64.6 mM sugar (glucose and fructose) and 11.2 mM malic acid.
[b] Molar yield of lactic acid: ([La_t] − 11.2)/(64.6 − [residual sugar]).
[c] Lactic acid produced after cell growth ceased.

total cells presented a growth curve similar to that obtained by OD measurement, suggesting that cells were not lysing. No clumping of cells was observed microscopically.

Final conditions for batch growth of *L. plantarum* with different initial concentrations of acetic acid and NaCl are given in Tables 1 and 2. The measured values are divided into two groups: conditions when growth by *L. plantarum* ceased and conditions when acid production ceased. Acid production was assumed to be completed by 15 days since analyses after 20 days gave unchanged concentrations. About 36 to 51% of the lactic acid was produced after the growth ceased, and the final lactic acid concentrations (after 15 days) were 100 to 139 mM, depending on the acetic acid concentration (Table 1). Table 2 shows that while the lactic acid concentrations are similar to data in Table 1 when growth ceased, the final acid concentrations were a function of the initial NaCl levels; higher NaCl concentrations resulted in lower final lactic acid concentrations. Also, a lower proportion of the acid was produced after growth ceased, at higher NaCl concentration. In all 14 batch growth fermentations presented in Tables 1 and 2, viable cell numbers after 12 days of fermentation were equal to or less than 10^6 cells per ml (data not shown).

The molar yield of lactic acid produced from hexose (glucose and fructose) fermentation was measured as 1.96 (±0.04). For this calculation it was assumed that of the lactic acid present, 11.2 mM came from the very rapid malic acid conversion, and this amount was subtracted from the total concentration of lactic acid produced (an equimolar conversion of malic acid to lactic acid was assumed) to arrive at lactic acid produced from hexose. When cell growth ceased, the pH was 3.3 to 3.5 and the total lactic acid concentration was 53 to 73 mM, depending on the initial concentrations of NaCl and acetic acid in the medium. Glucose and fructose were degraded simultaneously but at different rates. At higher concentrations, the rate of glucose utilization was higher than that for fructose, but at reduced glucose concentrations the initial behavior was reversed, with the rate of fructose utilization being higher than that for glucose (data not shown). From the HPLC analyses, it was possible to conclude that *L. plantarum* remained homofermentative since no acetic acid or ethanol was produced during either batch or continuous growth.

Product formation. Figure 2 shows a linear relationship between the specific lactic acid production rate and specific growth rate in continuous culture using CJ as the feeding medium. The symbols represent each steady-state condition achieved in the fermentor. The solid line represents fitted values based on the model proposed by Luedeking and Piret (17), in which lactic acid production is a function of cell division and biomass concentration. The growth-associated coefficient, mathematically characterized by the slope of the curve, was 51.9 (±4.2) mmol/g of cells, and the non-growth-associated coefficient, the vertical intercept of the curve, was 7.2 (±0.9) mmol/g of cells h^{-1}. The parenthetic values represent the 95% confidence intervals for slope and intercept, respectively, of the regression line.

Death rate. The data in Fig. 3 represent the changes in total biomass concentration (OD) and viable cell concentration in CJ. Even at an initial pH as low as 3.2, some change in the total cell concentration was observed, with an average specific growth rate of 0.005 h^{-1}. At the same time, a reduction in the viable cell concentration was observed, suggesting a negative apparent specific growth rate ($\mu - k_d$), i.e., a death rate higher than the specific growth rate. Death rate constants (k_d) were calculated during the period when no significant growth was observed (75 to 300 h). No significant effect was detected as a function of either pH or acetic acid ($P \geq 0.05$), with an average

TABLE 2. Effect of NaCl on growth and total acid production by *L. plantarum*[a]

Initial conditions		Conditions when growth ceased			Conditions when acid production ceased				
pH	[NaCl] (%)	pH	[La_t] (mM)	[Cell] (g/liter)	pH	[La_t] (mM)	[Sugar] (mM)	$Y_{La/Sb}$	R_{La}[c] (%)
5.93	0.0	3.52	61.4	0.437	3.34	101.7	16.4	1.88	39.6
5.78	1.0	3.37	73.3	0.494	3.10	133.4	3.7	2.01	45.1
5.70	2.0	3.32	64.4	0.471	3.13	115.0	12.7	2.00	44.0
5.64	3.0	3.31	70.8	0.455	3.16	96.9	20.9	1.96	26.9
5.60	4.0	3.30	64.0	0.411	3.19	82.0	26.8	1.87	22.0
5.55	5.0	3.32	67.6	0.432	3.19	87.0	25.7	1.95	22.3
5.51	5.8	3.27	66.8	0.400	3.19	81.5	28.2	1.93	18.0

[a] The growth medium was CJ which contained 64.6 mM sugar (glucose and fructose) and 11.2 mM malic acid.
[b] Molar yield of lactic acid: ([La_t] − 11.2)/(64.6 − [residual sugar]).
[c] Lactic acid produced after cell growth ceased.

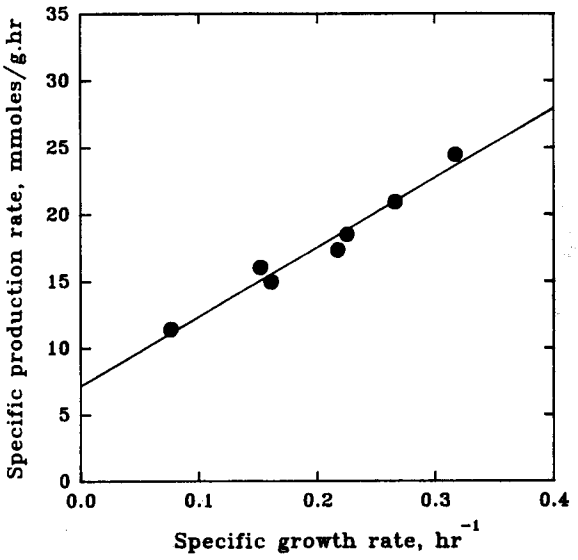

FIG. 2. Specific production rate of lactic acid versus specific growth rate in continuous culture at uncontrolled pH.

death rate constant of 0.031 (±0.005) h^{-1}. Lactic acid at a concentration of 60 mM slightly increased the k_d to 0.049 h^{-1}. The presence of 6% NaCl raised the k_d further, to 0.0911 h^{-1}. From the data, a linear relationship relating the death rate constant and NaCl concentration was suggested:

$$k_d = 0.024 + 0.011[\text{NaCl}] \quad (18)$$

Simulation and model demonstration. Figures 1, 4, and 5 illustrate three different experimental growth conditions tested for model validation, where symbols represent experimental values and the curves are calculated from the model. Figure 1 shows a batch culture in CJ to which 20 mM acetic acid and 3% NaCl were added. Figures 4 and 5 compare observed (symbols) and predicted (solid curves) values during growth of the bacterium in CJ containing 49 mM acetic acid (Fig. 4) or 20 mM acetic acid and 5.9% NaCl (Fig. 5). The death rate constant was predicted from equation 18. The value of $[H^+]$ in equation 16, previously defined as 0.427 mM (22), was changed to best fit experimental data. The values were 0.63 mM (pH 3.2), 0.427 mM (pH 3.37), and 0.562 mM (pH 3.25) in Fig. 1, 4, and 5, respectively. The model presents good agreement with the experimental values, except for the viable cell concentration in Fig. 1. For that variable, the model is close to experimental results during exponential growth but suggests values consistently lower than those observed after growth ceased. The equation used to predict pH change during the process was not sensitive enough to predict increases in the pH values during the initiation of the growth but gave reasonable values during most of the fermentation.

Figures 6 and 7 illustrate simulation of hypothetical conditions on the overall fermentation. Figure 6 illustrates the effect of change on the death rate constant (panels A and B) and on the value of $[H^+]_{max}$ (panels C and D) in CJ with 20 mM acetic acid and 3% NaCl. By using a death rate constant of 0.033 h^{-1} as predicted by equation 18 and a $[H^+]_{max}$ of 0.427 mM, as previously estimated by Passos et al. (22) to be where cell growth ceases, the predicted maximum cell concentration was 0.340 g/liter and the total lactic acid concentration was 97 mM. A ±20% hypothetical change of the value of this estimated death rate constant (±0.0066 h^{-1}) suggests a final lactic acid concentration of 85.4 or 114.3 mM, respectively. When the value of $[H^+]_{max}$ was changed ±20%, variations on the overall fermentation were observed for lactic acid and total cell concentration. Total lactic acid concentrations of 81 and 110.5 mM were predicted from maximum cell concentrations of 0.277 and 0.393 g/liter, respectively.

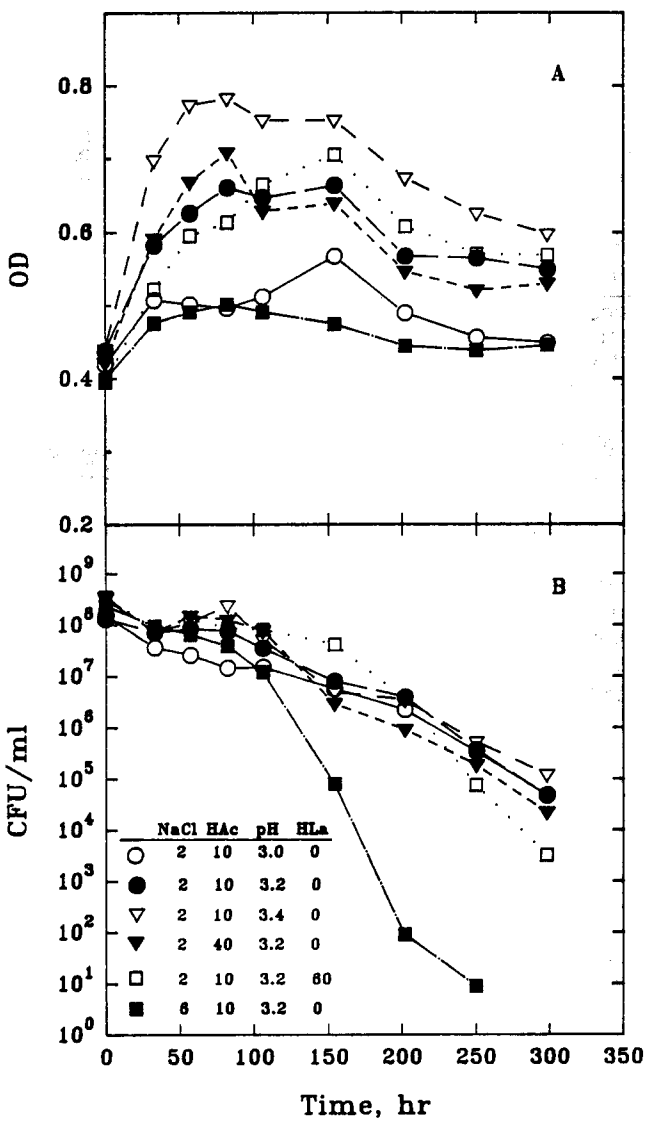

FIG. 3. Total cell concentration (A) and viable cell count (B) of *L. plantarum* in CJ at different initial pHs and lactate, acetate, and NaCl concentrations (millimolar, millimolar, and percent, respectively).

The influence of modification of buffer capacity of the CJ by adding acetic acid and correcting the initial pH of the fermentation is shown in Fig. 7. The calculated hypothetical addition of acetic acid in a concentration of 20 mM (Fig. 7A and B, broken curves) or 40 mM (Fig. 7C and D, broken curves) and correction of the initial pH to 4.76 predicted the culture to ferment all sugar present at 42 or 58 h, respectively. The hypothetical addition of acetic acid in the medium with no correction of the pH (solid curves) did not predict the cells to ferment all the sugar. The addition of 40 mM acetic acid

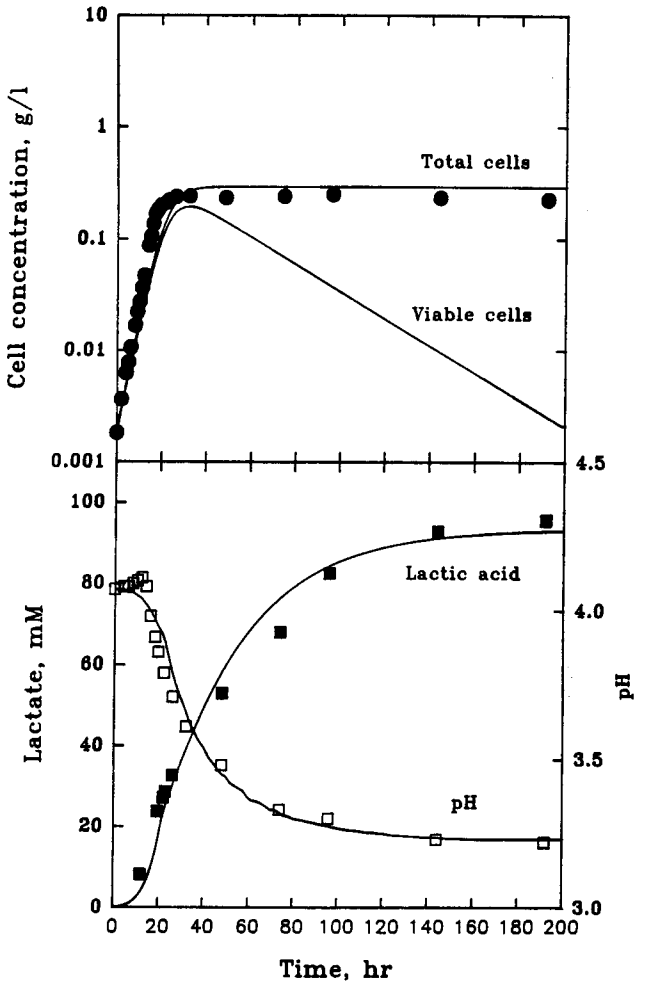

FIG. 4. Observed (symbols) and predicted (lines) changes in the lactic acid concentration, total and viable cell concentrations, and pH during batch growth of *L. plantarum* MOP3 in CJ at 30°C and uncontrolled pH. Initial condition: 49 mM acetic acid.

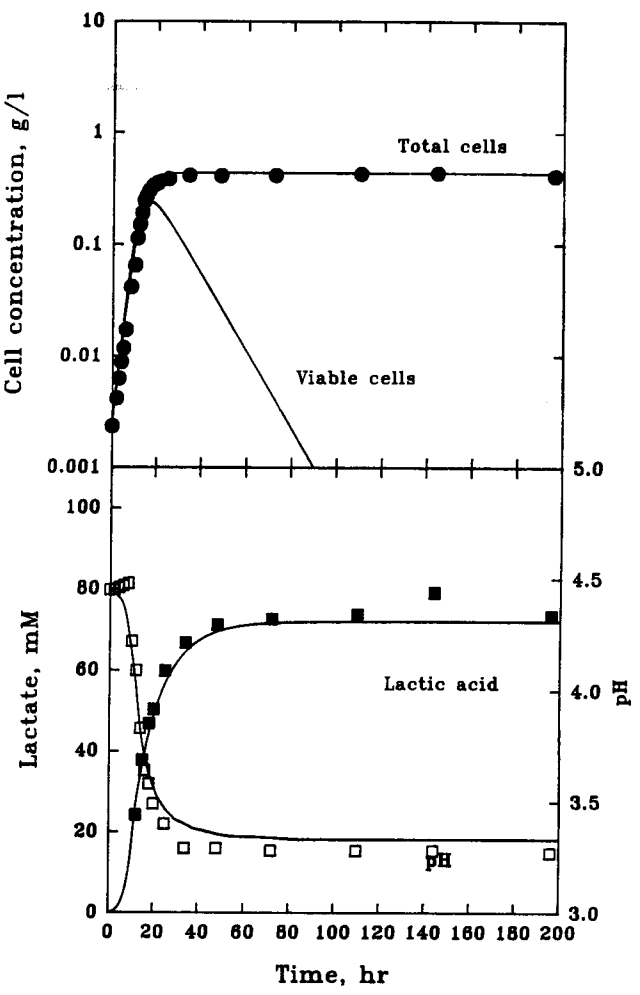

FIG. 5. Observed (symbols) and predicted (lines) changes in the lactic acid concentration, total and viable cell concentrations, and pH during batch growth of *L. plantarum* MOP3 in CJ at 30°C and uncontrolled pH. Initial conditions: 20 mM acetic acid and 5.9% NaCl.

increased the fermentation time and reduced the total lactic acid concentration produced compared with values when 20 mM acetic acid was added. In both simulations, growth ceased primarily because of hydrogen ion inhibition when the initial pH of the juice was not corrected. When the initial pH was adjusted to 4.76, the growth ceased because of sugar limitation. Thus, growth termination may be due to either acid inhibition or substrate limitation.

DISCUSSION

The coupled and uncoupled lactic acid production by *L. plantarum* was clearly observed in both continuous and batch culture. During the growth phase in continuous culture, lactic acid production was strongly related to both cell division and cell mass concentration, and thus the Luedeking and Piret model (17) represents the data well. This behavior was expected, since only lactic acid was produced during all the experiments, in agreement with a report by Giraud et al. (12), who grew *L. plantarum* in MRS medium. Bobillo and Marshall (2) grew different strains of *L. plantarum* in MRS medium and reported that in aerated cultures, acetic acid was produced in addition to lactate in a proportion depending on the acidity of the medium and NaCl concentration, with no acetate detected at pH 4.5 in the presence of salt. In anaerobic cultures, acetate was produced only in an alkaline environment of pH 7.5 or above.

In the present study, we measured a growth-associated coefficient of 51.9 (±4.2) mmol of lactate per g of cells and non-growth-associated coefficient of 7.2 (±0.9) mmol of lactate per g of cells h^{-1} from continuous culture data (Fig. 2). This LAB behavior has been shown by different authors.

For *L. plantarum*, ATP generation is closely coupled to lactic acid formation during growth in glucose and/or fructose. *L. plantarum* is homofermentative for hexoses, producing 2 mol of lactic acid per mol of hexose. In this and other anaerobic fermentations, microorganisms meet their ATP requirements for growth and maintenance by producing extracellular products (20), so the Luedeking and Piret model (17) is an expected kinetic representation for lactic acid production. Luedeking and Piret defined lactic acid production as a function of the energy necessary to form new bacterial protoplasm and the energy for the normal metabolic activity irrespective of growth. In their study, using *Lactobacillus delbrueckii* at constant pHs ranging from 6.0 to 4.5 in batch growth, they determined values

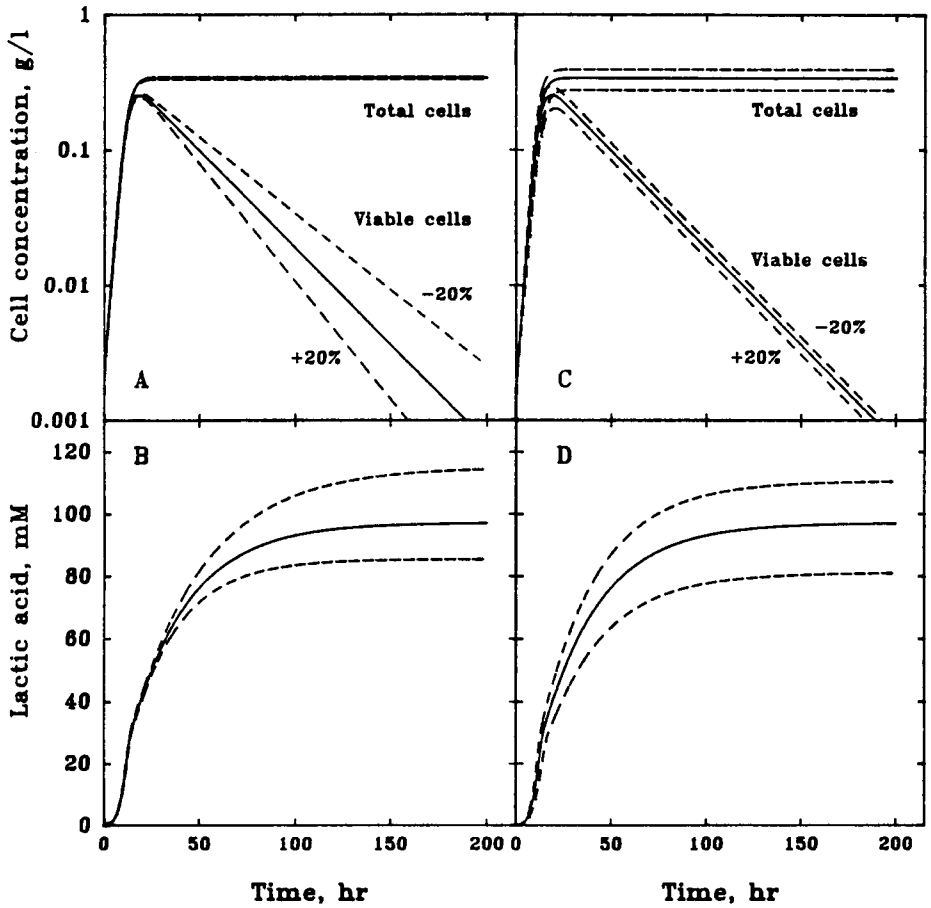

FIG. 6. Effect of change in either the death rate constant (A and B) or $[H^+]_{max}$ (C and D) on predicted lactic acid, total cell, and viable cell concentrations.

of the growth-associated coefficient to change from 24.4 mM lactic acid per OD unit at pH 6 to 39.4 mM/OD unit at pH 4.5 and the values of the non-growth-associated coefficient to change from 6.1 mM/OD unit/h at pH 6.0 to 1.2 mM/OD unit/h at pH 4.5.

Samuel and Lee (26), growing *L. plantarum* in crude extract from sweet sorghum supplemented with vetch juice, showed that after the stationary phase began, lactic acid continued to be produced. At a constant pH of 6.5, the stationary phase began at a lactic acid concentration of about 467 mM, but this concentration increased to about 667 mM when the substrate reached zero. Kemp et al. (15) applied the Luedeking and Piret model for lactic acid production by *L. plantarum* and found a growth-associated coefficient of 73.4 mmol/g of cells and a non-growth-associated parameter of 0.48 mmol/g of cells h^{-1}. The bacterium was grown at a constant pH of 6.0 in MRS broth. Breheny et al. (3) demonstrated the uncoupling of growth from acid production under conditions which inhibit growth of *Streptococcus cremoris*. When chloramphenicol, an inhibitor of protein synthesis in microorganisms, was added in a final concentration of 50 mg/liter to the growth culture, growth of the organism was immediately arrested while acid production continued at a reduced rate. With the same strain of *L. plantarum* used in this study (MOP3), a growth-associated coefficient of 73.0 or 58.3 mmol of lactate per g of cells was determined when YTA medium (yeast extract, Trypticase, ammonium sulfate, minerals, and glucose) was used, depending on the absence or presence of malate, respectively (21). This coefficient is thus a function not only of the bacterial strain but also of the medium. In the work by Luedeking and Piret (17) and Kemp et al. (15) the cause of the growth inhibition is not clear, but it was not $[H^+]$, since the pH was kept constant. In our study the main inhibition was high concentrations of H^+ and undissociated lactic acid. The measurement of maintenance energy is based on the idea that as the growth rate is decreased, the rate of metabolism of that carbon source approaches a positive limit, interpreted as the metabolism required to provide energy for needs not kinetically related to growth (18). While many measurements have confirmed the validity of the linear relation of specific product rate and specific growth rate, the interpretation of the relationship has been questioned (23). Hempfling and Mainzer (13) measured the maintenance coefficient of *Escherichia coli* in continuous culture limited by different carbon sources and reported nearly 25-fold variation. With our *L. plantarum* MOP3 strain in YTA medium, the value for the non-growth-associated coefficient was 3.5 or 1.5 mmol/g of cells h^{-1}, depending on the absence or presence of malate (21).

During batch growth, good agreement between the OD reading (experimental total cell concentration) and viable cell count (experimental viable cell concentration) observed during the initial exponential phase means that nearly all biomass was viable. After growth ceased, lactic acid productivity fell and is shown to be a function of viable cells only (cells able to divide

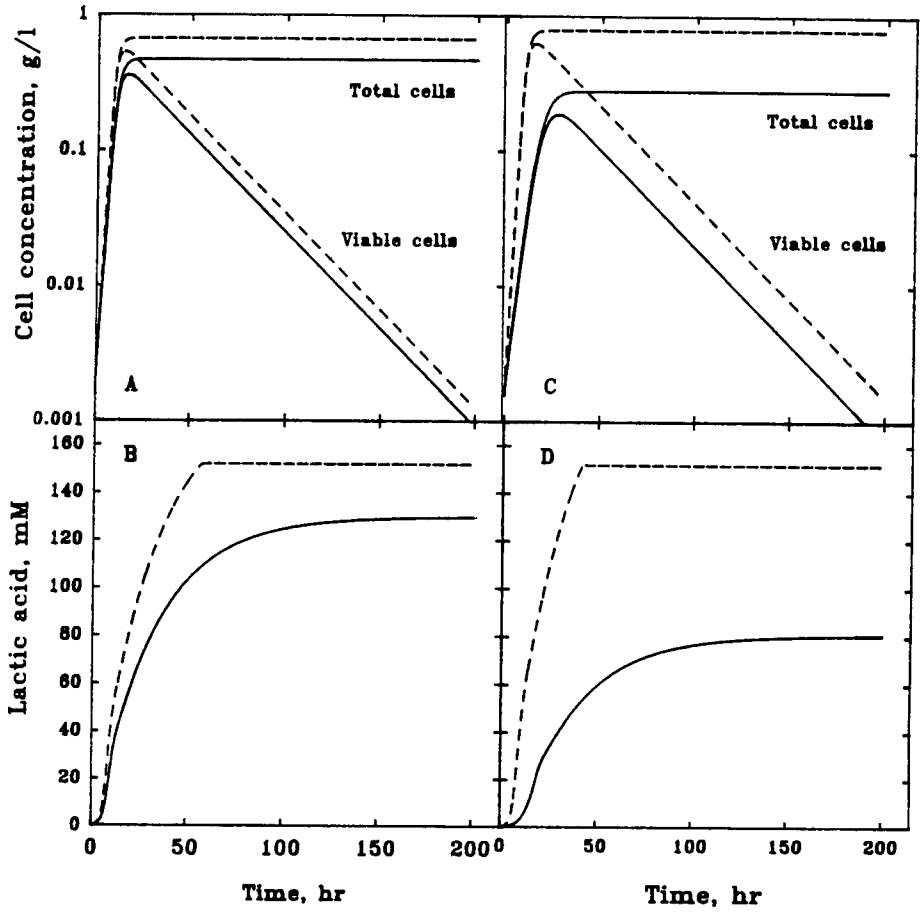

FIG. 7. Predicted effect of medium buffer capacity on lactic acid, total cell, and viable cell concentrations versus time. (A and B) Hypothetical addition of acetic acid at 20 mM with initial pH adjusted to 4.76 (broken lines) or unadjusted (solid lines); (C and D) hypothetical addition of acetic acid at 40 mM with initial pH adjusted to 4.76 (broken lines) or unadjusted (solid lines).

in fresh medium). During that phase, the concentration of cells determined by direct microscope count was constant and showed a close relationship with the OD reading (data not shown). The proposed model predicted the total biomass concentration (solid curve) well but gave a lag period between the predicted time for viable cell reduction (broken curve) and experimental viable cell data. It is not clear which component(s) of the model is responsible for this prediction discrepancy.

Little attention has been paid to the death phase of cell cultures, perhaps because the majority of fermentation processes modeled are halted before death begins (1). In the case of the present vegetable fermentation, metabolism after cell growth ceases also is important. A significant amount of lactic acid is produced after the growth ceases and some cell death has occurred, so the product formation kinetics during the death phase is important here.

We conclude that the final lactic acid concentration is a function of both the maximum cell concentration achieved during the growth phase and the death rate of the cells (in which NaCl seems to be the key variable). This information is consistent with the data in Table 2, suggesting that the low final concentration of lactic acid observed in the presence of higher concentrations of NaCl is due to the higher cell death rate constant. Henick-Kling (14) showed that all cultures of *L. plantarum* inoculated into wine of pH 4.0, 3.5, and 3.0 died after 1 to 2 days and that the death rate constant was a function of the initial pH of the wine and of the pH of the inoculum culture medium. The rate at which the cultures died was greatest for the culture which had the inoculum grown in grape juice at the highest initial pH; the effect of the initial pH of the starter culture was strongest in wine with a lower pH.

Figure 6 illustrates the important effect of the final phase of the fermentation process of LAB on the total lactic acid concentration, represented by change in the death rate constant (panels A and B) and the hydrogen ion concentration at cessation of growth (panels C and D). Buffering the CJ will greatly affect the process (Fig. 7), as has been shown with fermentation of whole cucumbers with 53 mM acetate at an initial pH of 4.6 (11).

Conclusions. A model is proposed to describe the growth of *L. plantarum* in anaerobic batch culture without pH control. It includes the effect of the main variables on the behavior of this system: hexose, lactate, acetate, NaCl, and hydrogen ion. It fits the data well for total biomass, lactate, malate, hexose, and hydrogen ion concentrations. However, an unexplained lag effect is noted for the cell death rate. Further work is intended to examine mixed-culture kinetics of *L. plantarum* and *S. rosei* and to refine cell death phase modeling. Otherwise, an acceptable model for CJ fermentation has been demonstrated.

ACKNOWLEDGMENTS

This investigation was supported in part by a research grant from Pickle Packers International, Inc., St. Charles, Ill. F. V. Passos received a scholarship from CNPq (Brazilian government agency) during the time this study was conducted.

We thank H. T. Tran for the help with the numerical solution of the system of equations, W. Stewart and K. Crowley for microbiological assistance, and R. L. Thompson for advice on HPLC analyses.

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