

Growth of *Leuconostoc mesenteroides* NRRL-B523 in an Alkaline Medium: Suboptimal pH Growth Inhibition of a Lactic Acid Bacterium

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Abstract: Bacterial profile modification (BPM), a form of tertiary oil recovery, diverts water from the water-flooded high-permeability zone into the oil-bearing low-permeability zone. During field use, exopolymer-producing bacteria plug the high-permeability zone only in the immediate vicinity of the injection point (the near-well bore region). For effective BPM the plug must penetrate far into the formation. Slowing the specific growth rate, lengthening the lag phase, and slowing the polymerization rate are techniques that can prolong the onset of biopolymer gelation and extend the depth of the biological plug. In batch experiments, the growth of *Leuconostoc mesenteroides* NRRL-B523 was inhibited by the synergistic effects of high substrate loading and an alkaline pH. Exponential growth was delayed up to 190 h. It was observed that cell division was significantly retarded until the medium pH, reduced by the acid by-products of fermentation, reached a critical value of 6.79 ± 0.06 . A mathematical model was developed to describe the relationship between specific growth rate, lag time, and medium pH. © 2004 Wiley Periodicals, Inc.

Keywords: *L. mesenteroides*; growth inhibition; bacterial kinetics; suboptimal pH; bacterial profile modification

INTRODUCTION

Water-flooding, a common oil-field recovery technique that can increase reservoir productivity after the completion of primary recovery, uses injected water to displace the remaining crude oil and sweep it toward the production well (Willhite, 1986). Primary and secondary techniques are able to remove up to 35% of the original volume of crude that is contained within the readily accessible high-permeability regions of an oil reservoir (Atlas and Bartha, 1998). Once

the high-permeability zones become water-flooded, tertiary recovery methods can potentially recover an additional 14% of the total oil volume (Atlas and Bartha, 1998). One such technique that relies on the in situ production of a flow-blocking polymer is termed bacterial profile modification (BPM) (Lappan and Fogler, 1994).

The successful implementation of BPM, a form of microbial-enhanced oil recovery (MEOR), requires the selective plugging of the water-flooded high-permeability zone of an oil reservoir. Once the high-permeability water-flooded zone is blocked by the production of bacteria and exopolymers that form a gel, water is injected into the low-permeability oil-containing zone, and oil is displaced from the reservoir. Although in situ bacterial growth and biopolymer production has been successfully performed in the laboratory, the rapid growth of bacteria near the injection well bore is an operational problem that leads to a decrease in the depth penetration of injected nutrients (Cusack et al., 1987; Jenneman et al., 1984; Robertson, 1998; Taylor and Jaffé, 1990). In a previous study it has been suggested that lengthening the lag phase and reducing the specific growth rate are two methods of increasing the depth penetration of injected nutrients, and thus improving BPM (Wolf and Fogler, 2001). Lengthening the lag phase and maintaining the bulk-phase cell concentrations at a low level will facilitate bacterial transport to greater depths within the water-flooded zone. To achieve lag times that provide a significant delay of biopolymer production, *L. mesenteroides* has been grown in media containing high sucrose concentrations (reduced water activity) and an alkaline pH. This study examines the time-varying change in medium pH and the existence of a medium pH (the critical pH) at which the bacterial population begins exponential growth.

The surface characteristics of the porous matrix, the initial porosity and permeability, and the pore size distribution affect the rate and extent of bacterial deposition (Harvey and Garabedian, 1991; Raiders et al., 1986). The bulk cell concentration also has a significant effect on the rate at which a

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porous matrix may be plugged by deposition (Jang et al., 1983; Sarkar et al., 1994). Although flow-induced deposition can lead to a decrease in permeability, it has also been shown that, without the production of exopolymers in a highly permeable matrix, there is no change in system permeability (Lappan and Fogler, 1992).

Bacterial growth and exopolymer formation are mechanisms that lead to the formation of a biofilm, a composite structure that can offer significant resistance to flow within a porous matrix (Wilderer and Characklis, 1989). Once formed, an exopolymer biofilm can reduce the permeability of a porous matrix to as much as 1/1000 of its initial value (Lappan and Fogler, 1992). Although extensive reduction of permeability is desirable for bacterial profile modification, it must be controlled so that near-well bore plugging is avoided. Once formed, localized sections of a biofilm can be partially removed by shearing, sloughing, abrasion, predation, or chemical disruption, with shear removal being the process most influenced by the local flow conditions within a porous matrix (Brading et al., 1995). Because porous matrix biofilms have been shown to last for as long as 2 years, bacterial plug placement is a process that needs to be controlled (Kim and Fogler, 2000).

Although it is desirable to have a fundamental understanding of the influence of pH and water activity (a_w), the water mole fraction, on bacterial metabolism, changes in cytoplasmic reaction networks, membrane transport properties, and chemical species transport constants are not fully quantifiable (Dilworth et al., 1999; Pichereau et al., 2000). Nevertheless, unstructured, unsegregated growth models have been shown to effectively describe the combined effects of a time-invariant suboptimal pH and water activity on specific growth rate (Cerf et al., 1996; Kalathenos et al., 1995; McMeekin et al., 1993; Presser et al., 1997). When growth medium properties are assumed to remain constant, predictive models can also accurately forecast lag times observed in batch reactors (Baranyi et al., 1993; McMeekin et al., 1993). When a growth parameter such as pH or a_w varies during the lag phase, the existing empirical models fail. For these reasons, a model that can account for changes in bacterial growth phase as a function of environmental conditions is necessary.

MATERIALS AND METHODS

Leuconostoc mesenteroides NRRL-B523 (ATCC 14935) was obtained from the American Type Culture Collection (Rockville, MD) and placed in a -80°C freezer for long-term storage. Every 30 days an aliquot was removed from the freezer, streaked onto agar plates prepared using Difco *Lactobacillus* MRS broth, grown for 18 h, and stored at 4°C . Streak plates were discarded after 30 days of 4°C storage.

L. mesenteroides inoculum was prepared by transferring one colony from the streak plate into media containing 7.9 g/L glucose, 7.9 g/L fructose, and 10 g/L Difco yeast extract solution diluted in a mineral salt solution. The min-

eral salt solution contained 0.067 M sodium acetate, 0.075 M sodium chloride, 0.061 M ammonium chloride, 6.1×10^{-3} M $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, 3.1×10^{-3} M ascorbic acid, 4.1×10^{-4} M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 4.0×10^{-5} M $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, and 4.0×10^{-5} M $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$. After 22 h of growth at 30°C , 10 mL of inoculum was aseptically transferred to a 1000-mL side-arm Erlenmeyer flask containing 550 mL of the desired growth medium. The initial cell concentration was $7.5 \times 10^6 \pm 0.1 \times 10^6$ cells/mL. The growth medium contained 10 g/L Difco yeast extract, varying concentrations of sucrose, and varying concentrations of sodium hydroxide dissolved in the mineral water solution just described. All experiments used an identical lot of yeast extract.

The medium pH was measured using a Corning Model 476156 semi-micro pH combination electrode and an Oakton Model 510 benchtop pH meter. The pH probe was calibrated twice a day with pH 4.01, 7.00, and 10.01 buffers. When carefully calibrated, the pH probe is accurate to ± 0.02 pH unit (Harris, 1987). The medium initial pH value was adjusted using 4 M sodium hydroxide and 1 M HCl; the medium pH value was not controlled after inoculation. Throughout this study, the term "initial pH" refers to the growth medium pH value immediately after inoculation.

Batch kinetics reactions were carried out in screw-top glass Erlenmeyer flasks fitted with a side-arm capped by a septum. Before inoculation, the batch reactors and media were autoclaved at 121°C for 15 min to ensure sterility. The contents of the batch reactor were unaltered during the course of the reaction except for the withdrawal of liquid samples and accompanying carbon dioxide gas dissolved in the growth medium. All batch reactions were conducted at a constant 30°C with constant mixing using a 1.5-in. stir bar rotating at 120 rpm. Sealed batch reactors provided anaerobic growth conditions. During the reaction period, liquid samples were aseptically taken from the reactor upon inoculation and at intervals ranging from 10 min to 24 h. The total cell count was measured using a Model ZF Coulter Counter fitted with a 30- μm diameter aperture tube. Viable colonies were enumerated using the spread plate technique, with sterile phosphate-buffered saline solution used as a diluent.

The cell concentration data from batch growth experiments were fit to the modified Gompertz equation using the Levenberg–Marquardt method (Press et al., 1992; Zwietering et al., 1990). The modified Gompertz equation is as follows:

$$y = A \cdot \exp \left[-\exp \left\{ \frac{\mu \cdot e}{A} (\lambda - t) + 1 \right\} \right] \quad (1)$$

where $y = \ln(N/N_0)$, $A = \ln(N_\infty/N_0)$, $e = 2.718$, t denotes the time in units of hours, μ denotes the specific growth rate in units of 1/h, and λ denotes the length of lag time in units of hours. N is the cell concentration at any time during the experiment in units of cells/mL, N_0 is the initial cell concentration in units of cells/mL, and N_∞ is the stationary-phase cell concentration in units of cells/mL.

Water activity is calculated using Raoult's law. Assuming an ideal solution of nonelectrolytes, the water activity of a solution containing n_w moles of solvent and n_s moles of solute is given as:

$$a_w = \frac{n_w}{n_w + n_s} \quad (2)$$

where water is the solvent and sucrose is the solute.

RESULTS AND DISCUSSION

To understand and model metabolic responses to an adverse medium, *L. mesenteroides* was grown in a variety of conditions in which two parameters were varied: a_w and pH. In a previous study, it was determined that increasing the sucrose concentration, and thus a lower a_w , reduced the specific growth rate and increased the lag time in *L. mesenteroides* batch fermentations (Wolf and Fogler, 2001). It was found that the change in lag time and specific growth rate increased linearly with decreasing a_w and that, in combination with a suboptimal medium pH, the lag time increased by an order of magnitude. Although bacteria are able to grow at approximately the same rate for a wide range of medium conditions (optimum conditions), a growth medium is considered suboptimal when there is a large deviation from the optimum growth dynamics (Pichereau, 2000). This study focuses on the suboptimal growth regime.

Suboptimal Water Activity Effects

To identify the region of suboptimal water activity, the sucrose concentration was varied while the initial pH was maintained near the maximum tolerable value. The maximum tolerable pH was determined to be 8.2 in earlier studies (Wolf and Fogler, 2001). Sucrose concentrations that induced lag times of >100 h are indicative of a suboptimal growth regime (Fig. 1). To facilitate comparisons with current research, sucrose concentration has been

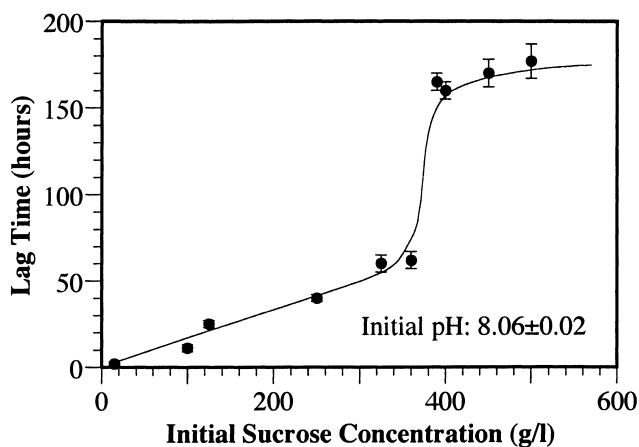


Figure 1. Initial sucrose concentration controls lag time. Error bars indicate 95% confidence intervals.

transformed into water activity for the discussion of the present study's results.

The measured molality of each sucrose solution was used to calculate the corresponding initial a_w using Eq. (2). An initial pH of 8.06 was chosen because of its proximity to the maximum tolerable pH. Other pH values were tested (data not shown), but 8.06 afforded significant and repeatable results. The results, shown in Figure 2, indicate that there is a critical a_w of 0.973 below which the lag time increases discontinuously from 60 to 160 h. The specific growth rate measured during the exponential growth phase (Fig. 2) decreases in a linear fashion.

Although a small discontinuity in the specific growth rate has also been observed in *Staphylococcus aureus* by Scott (1953), an accompanying change in lag times has not been reported. These results indicate that there is a change in bacterial metabolism when at $a_w < 0.973$. The long lag times observed at $a_w < 0.973$ indicate that *L. mesenteroides* may be actively accumulating a compatible solute to combat the sucrose-induced osmotic gradient across the cellular membrane (Gouffi et al., 1998). Because intracellular solute accumulation is a prerequisite for exponential growth in a medium with a high osmolarity, protein synthesis may be delayed until the intracellular conditions have been adjusted (Christian and Hall, 1972). The increased osmotic stress experienced by *L. mesenteroides* may also lead to a change in gene expression; new metabolic pathways require energy that otherwise would be used to combat an adverse osmolarity (Pichereau et al., 2000).

Suboptimal pH Effects

To determine the effects of a suboptimal pH on *L. mesenteroides* growth, the initial a_w was adjusted to a constant value of 0.960 while the initial pH was varied. An initial a_w value of 0.960 was chosen because it afforded a significant and repeatable increase in lag time over a range of pH values (Wolf and Fogler, 2001). From previous studies on *L. mesenteroides*, it was determined that a medium pH of >7.5 was suboptimal because of an exponential increase

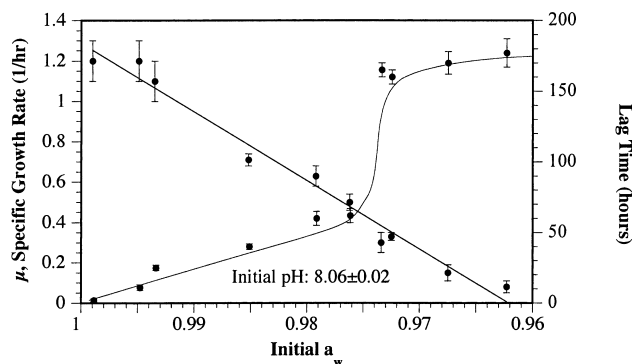


Figure 2. The specific growth rate varies linearly as a function of a_w in conjunction with a discontinuity in lag time. Error bars indicate 95% confidence intervals.

in lag time (Wolf and Fogler, 2001). This phenomenon has been examined in more detail in this study. In the suboptimal pH range, the pH begins to drop from its initial value as the bacteria produce lactic acid (Holzapfel and Schillinger, 1981) and carbon dioxide that dissolves and is protonated to form carbonic acid. The following was found: regardless of the initial suboptimal pH, *L. mesenteroides* transitioned into the exponential growth phase when the solution pH was 6.79 with a 95% confidence interval of 0.06 pH unit (Figs. 3 and 4). The lag phase duration increased with increasing initial pH because of the additional acid production required to reduce the solution pH to 6.79. This transition pH, herein designated the critical pH, is a novel finding.

Batch Growth Model

Because of the inhibitory conditions of the growth medium used in this study, it was shown that the transition to the exponential phase can be delayed for up to 200 h; however, cell growth was detected as the solution pH decreased toward a value of 6.79. According to Cooper's strict definition of the bacterial growth cycle, the lag phase is the pause in the bacterial growth cycle before mitosis begins (Cooper, 1991). The period of slow growth observed in this study does not strictly satisfy Cooper's definition of the lag phase; however, to simplify our discussion, we have chosen to describe this slow-growth stage as a lag phase.

After *L. mesenteroides* was inoculated into a medium with a suboptimal pH there was a linear increase in the cell concentration and an exponential increase in acid concentration (Fig. 5). Viable cell counts (data not shown) confirmed there was no cell death occurring during the lag phase. Furthermore, it was found that the specific growth rate during the lag phase decreased exponentially with respect to the initial, suboptimal medium pH (Fig. 6). The following combined mass balance and rate law equation

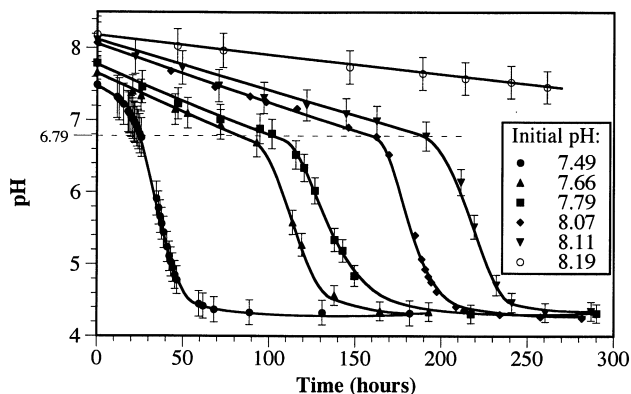


Figure 3. Medium pH. Experiments were performed in duplicate and were in good agreement. A single representative experiment is shown for each condition. Error bars indicate 95% confidence intervals for triplicate pH measurement.

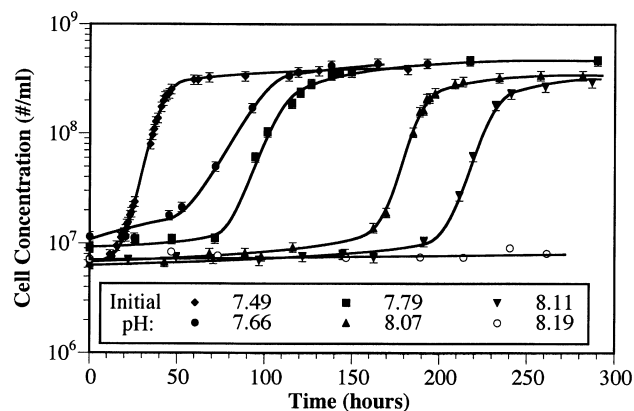


Figure 4. Bacterial growth at suboptimal pH. Experiments were performed in duplicate and were in good agreement. A single representative experiment is shown for each condition. Error bars indicate 95% confidence intervals for triplicate cell concentration measurement.

accounts for growth during the lag phase based on experimental observation (Fig. 6):

$$\frac{dC_x}{dt} = k_1 C_x \quad (3)$$

with the empirical relationship $k_1 = 2.13 \times 10^{15} \exp(-5.15 \text{pH}_0) \text{h}^{-1}$, where C_x = cell concentration (cells/mL), t = time (h), pH_0 = pH measured at experiment start time.

During the lag phase, slow growth was accompanied by a slow rate of acid production that was observed to be growth-associated. The following acid generation rate equation is based on experimental observation:

$$\frac{dC_{H^+}}{dt} = k_2 C_{H^+} \quad (4)$$

where C_{H^+} is acid concentration ($[H^+]$ /mL), and $k_2 = (0.012 + 2.97k_1) \text{h}^{-1}$.

Unlike predictive models that attempt to describe the lag phase using an adjustment function (Baranyi et al., 1993), this model predicts the transition from lag phase to exponential growth phase with an empirically determined

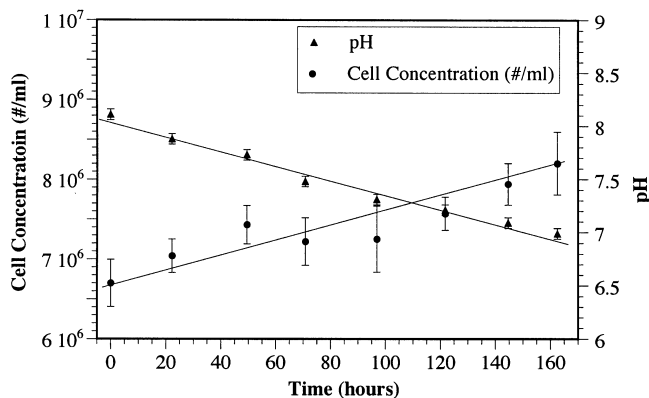


Figure 5. Lag phase growth for a typical batch reaction. Error bars indicate 95% confidence intervals.

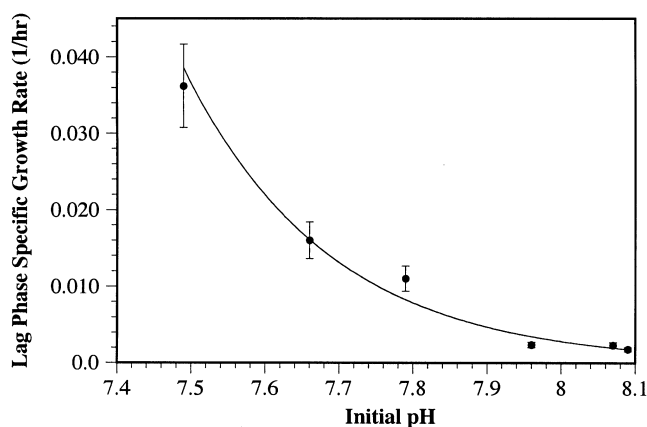


Figure 6. Lag phase specific growth rate decreases exponentially with increasing initial pH. Experiments were performed in duplicate. Error bars indicate 95% confidence intervals.

growth parameter. Growth during the exponential growth phase and the subsequent stationary phase was assumed to follow logistic growth kinetics (Fogler, 1999), where the maximum cell concentration varies according to the initial cell concentration, and was determined from experiments:

$$r_x = \frac{dC_x}{dt} = \mu C_x \left(1 - \frac{C_x}{C_{xmax}}\right)^n, \quad (5)$$

with $n = 1$

where $C_{xmax} = 52.46 C_{xinitial}$.

The specific growth rate observed during the exponential growth phase was determined from a series of batch experiments conducted in a previous study. The substrate-inhibited specific growth rate law and rate constants were determined previously by Wolf and Fogler (2001):

$$\mu = \frac{\mu_{max} \cdot C_S \cdot e^{[-(C_S/K_i)^2]}}{K_m + C_S} \quad (6)$$

where $K_i = 389 \text{ g/L}$, $\mu_{max} = 1.39 \text{ h}^{-1}$, and $K_m = 1.29 \text{ g/L}$.

The production of H^+ during the exponential growth phase was growth-associated. Regardless of the initial pH, the rate of production of metabolites was a linear function of the instantaneous specific growth rate:

$$\frac{dC_{H^+}}{dt} = k_3 \frac{dC_x}{dt} \quad (7)$$

with $k_3 = 1.17 \times 10^{-13} [H^+]/\text{mL}$.

Table I. Suboptimal pH growth model results.

Initial pH	Observed lag time (h)	Model lag time (h)
7.66	40.1 ± 10.2	34
7.79	71.8 ± 4.3	64
7.90	110.0 ± 12.0	101
8.10	195.5 ± 15.0	188

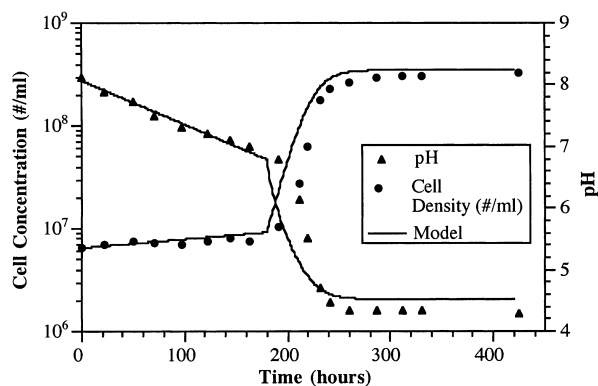


Figure 7. Model predictions for cell concentration and pH for an initial pH of 8.10 and an initial cell concentration of 6.4×10^6 cells/mL.

The rate of substrate consumption is given by the following relation:

$$\frac{dC_S}{dt} = -\frac{1}{Y_{XS}} \frac{dC_x}{dt} \quad (8)$$

where $Y_{XS} = 4.9 \times 10^6$ cells/g sucrose.

The sucrose yield was calculated from experimentally determined cell and sucrose concentrations measured during the exponential growth phase.

The batch growth model, consisting of Eqs. (3)–(8), predicted lag times for a variety of initial suboptimal pH values. By varying the initial pH and keeping all other parameters constant, the model predicts pH and cell concentration curves as a function of time. Within the suboptimal pH range, the predictive model and experimental lag times are in agreement, as can be seen in Table I. Shown in Figure 7 is a representative comparison between the model and experimental data for an initial pH of 8.10 and an initial bacterial concentration of 6.4×10^6 cells/mL.

CONCLUSIONS

Although the lag phase is often a variable feature of the bacterial growth curve, this study has shown the lag phase can be controlled with precision. When inoculated into an alkaline growth medium with a low a_w , *L. mesenteroides* can ameliorate the medium through acid production. There is a critical pH of 6.79 ± 0.06 at which exponential growth began, regardless of the initial suboptimal pH. The duration of the lag phase can be predicted if *L. mesenteroides* is grown in a medium with an initial a_w of 0.960: the batch growth model has verified that cell concentration, solution pH, and lag times can be predicted when the medium conditions change as a function of time. Because *L. mesenteroides* has effective mechanisms to compensate for unfavorable growth conditions, it may be possible to observe a critical pH phenomenon in other acidophiles inoculated into an alkaline medium.

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