Journal of Food Protection, Vol. 78, No. 4, 2015, Pages 728–737
doi:10.4315/0362-028X.JFP-14-478

Effects of Acetic Acid and Arginine on pH Elevation and Growth of *Bacillus licheniformis* in an Acidified Cucumber Juice Medium†

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MS 14-478: Received 29 September 2014/Accepted 3 December 2014

ABSTRACT

Bacillus licheniformis has been shown to cause pH elevation in tomato products having an initial pH below 4.6 and metabiotic effects that can lead to the growth of pathogenic bacteria. Because of this, the organism poses a potential risk to acidified vegetable products; however, little is known about the growth and metabolism of this organism in these products. To clarify the mechanisms of pH change and growth of *B. licheniformis* in vegetable broth under acidic conditions, a cucumber juice medium representative of a noninhibitory vegetable broth was used to monitor changes in pH, cell growth, and catabolism of sugars and amino acids. For initial pH values between pH 4.1 to 6.0, pH changes resulted from both fermentation of sugar (lowering pH) and ammonia production (raising pH). An initial pH elevation occurred, with starting pH values of pH 4.1 to 4.9 under both aerobic and anaerobic conditions, and was apparently mediated by the arginine deiminase reaction of *B. licheniformis*. This initial pH elevation was prevented if 5 mM or greater acetic acid was present in the brine at the same pH. In laboratory media, under favorable conditions for growth, data indicated that growth of the organism was inhibited at pH 4.6 with protonated acetic acid concentrations of 10 to 20 mM, corresponding to 25 to 50 mM total acetic acid; however, growth inhibition required greater than 300 mM citric acid (10-fold excess of the amount in processed tomato products) products under similar conditions. The data indicate that growth and pH increase by *B. licheniformis* may be inhibited by the acetic acid present in most commercial acidified vegetable products but not by the citric acid in many tomato products.

Bacillus licheniformis is a gram-positive, spore-forming bacterium that is widely distributed in soil, on vegetables, fruits, and spices. *B. licheniformis* has been shown to be capable of growing at pH values below 4.6 and is considered as a member of the acid-tolerant bacillus group *(16).* Acid-tolerant bacillus strains are generally considered to cause not only economic spoilage of acidic foods but may contribute to foodborne disease through metabiosis, because these bacteria are able to elevate the pH of some acidic foods, particularly tomato products. If the pH rises above 4.6, this presents an opportunity for the growth of microorganisms of public health significance, such as *Clostridium botulinum*. It is reported that *B. licheniformis* could elevate the pH of a model system for improperly canned tomatoes (with oxygen present) from pH 4.4 to values close to and above neutrality, creating an environment suitable for growth and toxin production of coinoculated *C. botulinum (23).* Rodriguez et al. reported metabiotic effects of *B. licheniformis* and *Bacillus subtilis* in tomato juice, potentially allowing the growth of *C. botulinum (26).* The pH of tomato juice was elevated to pH 8.0 or greater. These studies indicate that pH elevating bacteria may be a potential risk for acidified vegetable processing.

Acid-tolerant bacilli can cause pH change in foods by the production of organic acids (primarily lactic acid) due to the fermentation of sugar and the degradation of proteins and amino acids during growth *(25).* Changes in environmental pH may be influenced by factors, such as oxygen supply, initial pH, buffering, ionic strength and temperature, fermentation of available sugars, ammonia production, and decarboxylation reactions (effectively removing protons from solution). It is known that proteolysis and utilization of released amino acids by bacilli leads to the formation of ammonia, which is responsible for increasing pH during growth *(1, 24).*

The arginine (Arg) deiminase pathway (ADI) has been found to be a common mechanism for bacteria to raise pH in acidic media and has been identified in a variety of microorganisms, including *Mycoplasma, Halobacterium,* and *Pseudomonas* species, as well as lactic acid bacteria *(10, 11).* Genes encoding ADI enzymes have been found in

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Bacillus species *(8, 17)*. It was reported that the internal pH of *Bacillus cereus* was maintained at higher values when acid shocked in presence of glutamate, Arg, or lysine, enhancing the survival rate *(28)*. That study also showed transcription of the Arg deiminase gene (*arcA*) in *B. cereus* was upregulated sixfold during acid adaptation. The ADI pathway was functional in *Bacillus* cultures with low levels of oxygen *(8)*. However, those studies were performed with pH values near neutral, and the effects of pH activity of these pathways in *B. licheniformis* under conditions representative of acidified vegetable products has not studied.

Fermented and acidified vegetables are popular worldwide. Acidified vegetables, such as cucumbers, peppers, and others, contain sugars and amino acids that are present in the brines *(13)*. These nutrients may potentially allow for the growth of spoilage bacteria in acidified vegetable products, depending on processing and storage conditions, including pasteurization, salt and acid concentration, and pH values. *B. licheniformis* is a spore-forming microorganism and is widely distributed in soil and even has been proposed as a biological control agent for vegetables or fruits *(18, 19, 29, 31)*. Spores of *B. licheniformis* can survive typical pasteurization treatments for acidified vegetables (74°C for 15 min) *(12)*. Recently, the U.S. Food and Drug Administration has proposed processing conditions to kill *Bacillus* spores in a draft guidance document for the acidified foods industry *(32)*.

To investigate the potential for *B. licheniformis* to raise pH in acidified vegetables, the pH and biochemical changes due to *B. licheniformis* growth in acidified cucumber juice under aerobic and anaerobic conditions were characterized. Because we were interested in determining growth for *B. licheniformis* under conditions representative of commercial acidified vegetables packaged in sealed (anaerobic) jars, brines were not preincubated anaerobically to remove residual dissolved oxygen prior to anaerobic incubation. These conditions, therefore, represented a worst-case scenario, being as permissive as possible for allowing growth of acid-tolerant bacilli, which favor aerobic growth. Using a dilute cucumber juice medium (CJ; 10% cucumber juice) under acid conditions, we measured Arg catabolism, cell growth, and the catabolism of other amino acids and sugars. Cucumber juice was chosen as an ideal medium for these studies because it contains no known antimicrobial compounds and has been used as a representative vegetable broth for vegetable products in other studies of the safety of acidified foods *(6, 20)*. Previous study has indicated that among *Bacillus* and *Alicyclobacillus* species, *B. licheniformis* is the primary organism of concern for acidified vegetable products *(22)*. Although anaerobic acidified vegetable products with pH values below 4.6 were the target of the study, a range of pH and oxygen and acid conditions were included in the study to allow a better characterization of conditions leading to pH changes during growth.

MATERIALS AND METHODS

Bacterial strain and culture conditions. *B. licheniformis* strains B412, B413, B501 were obtained from a U.S. Department of Agriculture, Agricultural Research Service culture collection (Bacterial Foodborne Pathogens and Mycology Research Unit, Peoria, IL); strains B426, B427, and B428 were obtained from the Grocery Manufacturers Association (Washington, DC). B412 was used for most studies, as described in the following. Cells were prepared by growth in tryptic soy broth (TSB; BBL, BD, Franklin Lakes, NJ) and on tryptic soy agar (TSA; BBL, BD). Cells were grown at 37°C in 100 ml of TSB medium in a 250-ml Erlenmeyer flask adjusted to pH 5.5 with hydrochloric acid (HCl) and shaken at 150 rpm for 12 h. The cells were collected by centrifugation at 5,000 × *g* for 5 min at 20°C, and the cell pellets were suspended in 0.85% saline (pH 5.5) and washed twice. The cells were finally suspended in 10 ml of 0.85% saline, with a cell concentration of approximately 10^9 CFU/ml, and used as an inoculum with appropriate dilution for the experiments described in the following. Bromocresol purple was added (0.002%, wt/vol, from a 0.3% bromocresol stock in ethanol) to acidified TSA or TSB as a pH indicator, with the initial pH adjusted to pH 4.9 with HCl to determine if a pH rise had occurred. Cell concentrations were determined by plating on TSB agar using a spiral plater (Autoplate 4000, Spiral Biotech, Norwood, MA) and an automated plate reader (Q-count, Spiral Biotech). CJ was prepared from fresh, size 2B pickling cucumbers (approximately 3.5 to 3.8 cm in diameter) obtained from commercial sources. Cucumbers were washed, blended with an equal volume of deionized water, centrifuged, and the resulting clear supernatant filtered using a 0.45-μm-pore-size filter, as described *(5)*.

The defined medium (DM) was prepared with 17 amino acids (listed according to the standardized International Union of Pure and Applied Chemistry), including Asp, Thr, Ser, Glu, Gly, Ala, Cys, Val, Met, Ile, Leu, Tyr, Phe, Lys, His, Trp, Pro, (0.5 mmol of each) into 1 liter of salt solution containing 0.5 g NH_4Cl, 9.52 g of KH_2PO_4, 0.142 g of Na_2SO_4, 0.085 g of $NaNO_3$, 0.075 g of KCl, 2.460 g of $MgCl_2 \cdot 6H_2O$, 1.08 mg of $FeCl_3 \cdot 6H_2O$, 0.079 g of $MnCl_2 \cdot 4H_2O$, and 0.111 g of $CaCl_2$ and adjusted to pH 4.9, followed by filter sterilization by using a 0.45-μm-pore-size filter. DM was made, excluding amide amino acids, asparagine and glutamine. DM was finally supplemented or not (as indicated in the following) with 0.5 mM Arg.

Environmental effects on growth, metabolism, and pH. The pH change of broth cultures was monitored by centrifugation of 0.8 ml of cultures at 10,000 × *g* in a microcentrifuge for 10 min and then directly measuring pH of supernatants using pH meter (Accumet AR25, Fisher Scientific, Pittsburgh, PA). For studying the effects of oxygen and initial cell count on pH elevation of *B. licheniformis* in CJ, 10% (vol/vol) of CJ medium was adjusted to pH 4.1, 4.3, 4.6, 4.9 with HCl, and filter sterilized. CJ (5 ml) at pH 4.3 or 4.9 was inoculated with each of five dilutions, to give approximately 10^3 to 10^7 CFU/ml, and incubated aerobically and anaerobically at 37°C for 8 days. The aerobic cultures were grown with shaking at 150 rpm. Anaerobic conditions were defined by incubation of cultures in an anaerobic chamber (Coy Laboratory Products Inc., Grass Lake, MI). To study the effects of amino acid catabolism on the initial pH elevation caused by *B. licheniformis,* 40 μl (0.5% inoculum) of the cell suspension was inoculated into 8 ml of DM (with and without Arg, as described in the following) and incubated aerobically and anaerobically at 37°C for 3 days. Samples collected at 0, 2.5, 5.0, 7.5, 21, 46, and 72 h were used for pH determination and amino acids analysis. To study of the effects of Arg on the growth and metabolism of *B. licheniformis* in acidified CJ, the CJ was diluted (10%, vol/vol) and adjusted to pH 4.1, 4.3, 4.6, 4.9, 5.2, and 6.0. The CJ was supplemented with 0, 25, and 100 μg/ml Arg and inoculated with 10, 2.0, 1.0, 0.5, 0.2, 0.1, 0.02, and 0.01% (10^8 to 10^5 CFU/ml) of the cell suspension

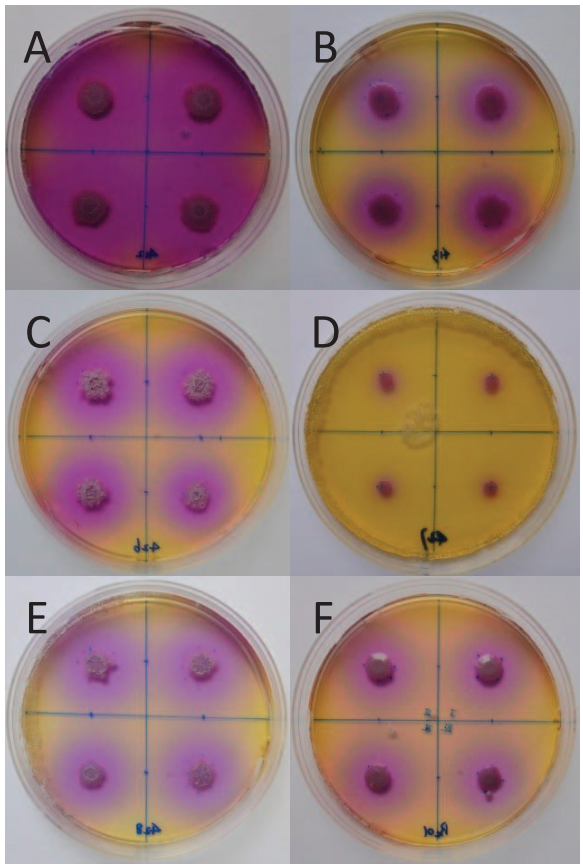

FIGURE 1. *Increase in pH by different* Bacillus *strains using acidified TSB agar (initial pH 4.9), supplemented with 0.003% bromocresol purple: (A) B412, (B) B413, (C) B426, (D) B427, (E) B428, and (F) B501.*

(prepared as described previously) and aerobically grown with 8-ml volumes in 15-ml loosely capped test tubes at 37°C for 2 weeks. To study the effects of acetic acid on the growth of *B. licheniformis* in CJ, 1 M sodium acetate buffer was added to a final concentration of 0.01 and 0.005 M, and then inoculated with 1.0, 0.5, and 0.1% (10^7 to 10^6 CFU/ml) of cell suspension and aerobically incubated at 37°C for 2 weeks, as described above. The cultures were initially sampled at 0, 2.5, 5.0, and 7.5 h and then every 1 to 3 days.

Growth rates for bacterial cultures in the presence of acetic or citric acid were characterized using an automated microtiter plate reader (ELx808, Bioteck Instruments, Inc. Winooski, VT). Cell cultures (5 ml) of B426, B427, B412, B413, and B501 were grown overnight at 37°C in TSB, harvested by centrifugation (2,300 × *g*, 10 min, 10°; Sorvall SS 34 rotor, Thermo Fisher Scientific, Waltham, MA) resuspended in an equal volume of 0.85% sodium chloride, and equal volumes were combined into a single cocktail. Cells were diluted into TSB to give an initial optical density (OD) of approximately 0.05 at 630 nm (corresponding to an initial cell concentration of approximately 10^6 CFU/ml) in 200 μl of TSB using flat-bottomed microtiter plates (Fisher Scientific). TSB contained 25, 50, 75, or 100 mM acetic acid or 75, 150, 225, and 300 mM citric acid at four different pH values: 4.6, 5, 6, and 7. Controls included TSB plus acid solutions with no cells and TSB with no added organic acid at each pH (adjusted with HCl). Growth was monitored hourly for 24 or 48 h at 37°C in the microtiter plate reader, using automated reading after shaking to evenly suspend cells. Data was exported to a spreadsheet file and imported to MATLAB (www.mathworks.com). Growth rates were calculated using a custom MATLAB algorithm, to sequentially process growth curves, essentially as described *(7)*.

To estimate the kinetic parameters for pH change, a modified Gompertz equation *(14, 15)* was used for determining the rate of pH change using the SigmaPlot software (version 10, Systat Software Inc., Chicago, IL). The change in pH (ΔpH) for cell cultures was calculated as the change in pH value from the starting value to the value at the sampling time. The initial rate of change for pH (k, h^{-1}) was estimated by linear regression of for ΔpH versus time during first 7.5 h of cell growth.

Biochemical analysis. For biochemical analysis to determine organic acids, sugars, and amino acids in growth medium samples, 1-ml volumes of culture supernatants were centrifuged at 10,000 × *g* for 10 min and filtered through a 0.45-μm syringe filter prior to analysis. Sugars and organic acids were determined by high-performance liquid chromatography using a Thermo Separation Products HPLC system (ThermoQuest Inc., San Jose, CA), consisting of a model P1000 pump, an SCM100 solvent degasser, and an AS3000 autosampler. A Bio-Rad HPX-87H column (300 by 7.8 mm; Bio-Rad, Hercules, CA) was used with a differential refractometer (Waters model 410, Millipore, Milford, MA) and a UV detector (UV6000LP, Thermo Separation Products, San Jose, CA) for detection of the analytes. Operating conditions of the system included a sample tray at 6°C, a column at 65°C, and 0.03 N sulfuric acid as the eluent with a flow rate of 0.9 ml/min. The UV6000 detector was set to 210 nm at a rate of 1 Hz for data collection, and 2 Hz was used for refractive index data. ChromQuest version 4.1 chromatography software was used to control the system and analyze the data, using the peak heights for quantification, based on standard solutions with four different concentrations. The lower detection limit for organic acids and sugars was around 0.1 mM.

For the analysis of the free amino acids present, 0.5 ml of sample was centrifuged and diluted into 0.02 N HCl to a final volume of 1 ml *(2)* then analyzed using a Hitachi Model L-8900 Analyzer (Hitachi High Technologies, Dallas, TX). The analyzer was fitted with an analytical column (model 2622SC PF; 40-mm length, 6.0-mm inside diameter; Hitachi High Technologies) with a guard column. Separation of the amino acids was done by using a gradient of borate buffers (PF type, Hitachi High Technologies) and a temperature gradient of 30 to 70°C, according to the user manual supplied with the instrument, with additional changes provided by Hitachi personnel (Shinichi Otaka, personal communication). Postcolumn derivatization was performed by the instrument using ninhydrin. The instrument used visible detection at wavelengths of 570 and 440 nm. Standard curves of amino acids were prepared by making serial dilutions of amino acid standard mixture (Fluka, St. Louis, MO) containing 2.5 μmol of 18 individual amino acids in 0.02 N HCl. The range of the standard curves was 1 to 5 nM.

Statistical analysis. Statistical analysis was carried out by using the *t* test program of SigmaPlot software (version 10, Systat Software Inc.), and results were considered significant at $P < 0.05$. Experiments were done with at least three independent replications for statistical analysis.

RESULTS AND DISCUSSION

Characterization of *B. licheniformis* growth and pH elevation in cucumber juice. *B. licheniformis* strain B412 showed the strongest alkaline-producing capacity on acidified TSB agar (pH 4.9) compared with other *Bacillus*

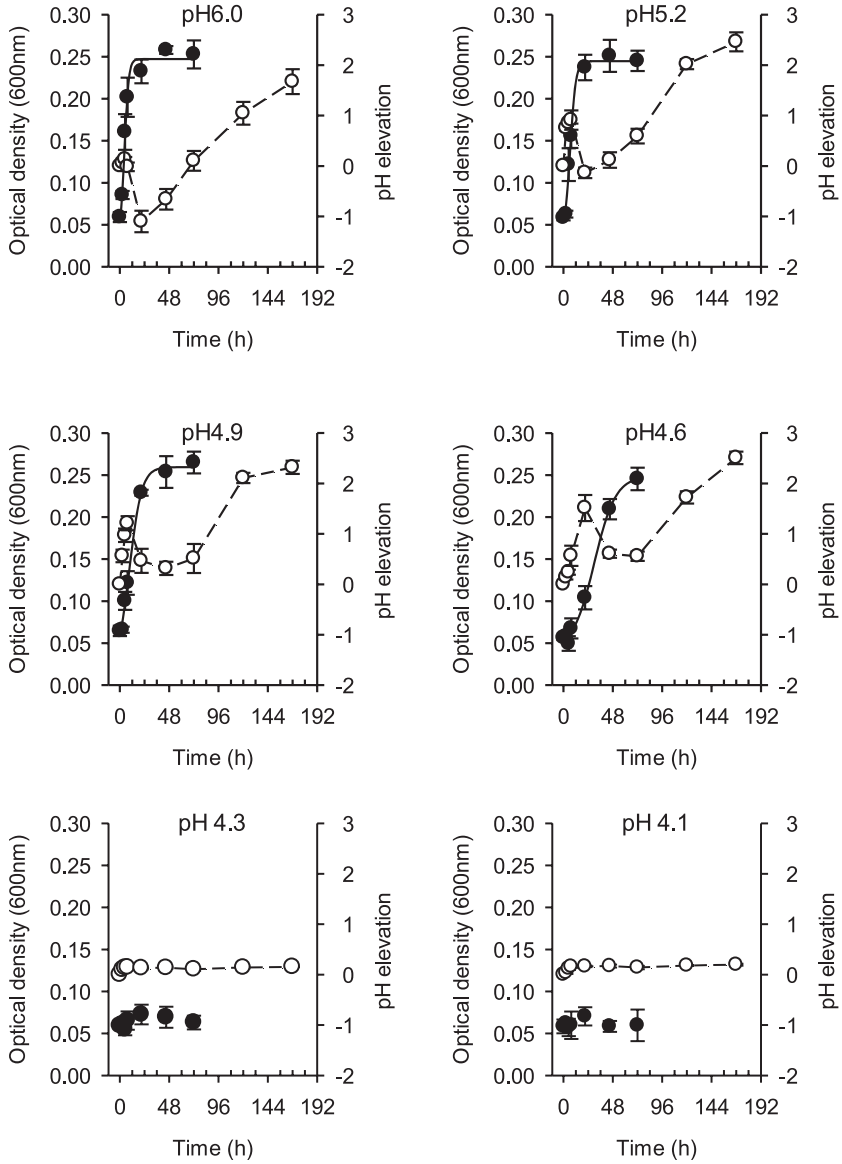

FIGURE 2. *Characterization of growth and pH elevation of* B. licheniformis *B412 in CJ under aerobic conditions. The data point of OD values (●) and pH elevation values (○) were determined for cultures incubated at 37°C with initial cell density of 10^6 CFU/ml. Values correspond to the mean of three replicates ± standard deviation.*

spp. strains (Fig. 1). *B. licheniformis* B412 was, therefore, selected for further analysis of pH changes in CJ medium. Previous studies with 13 *Bacillus* strains (*B. licheniformis, Bacillus coagulans,* and *Alicyclobacillus acidocaldarius*) had shown that *B. licheniformis* B412 was most able to grow and raise pH under anaerobic incubation conditions *(22, 33)*. B412 was incubated aerobically and anaerobically at 37°C in CJ. Initial experiments showed that 50% CJ, representative of brined cucumber sugar concentrations of 1 to 2% glucose and fructose *(13),* resulted only in pH decrease due to fermentation of sugar (data not shown); therefore, subsequent studies were carried out with diluted CJ to reduce sugar concentrations. For dilute CJ (10%) with an initial pH of 4.3 to 6.0, the data showed that *B. licheniformis* B412 can grow and cause pH elevation under both aerobic and anaerobic incubation conditions but with different patterns (Figs. 2 and 3) and kinetic parameters (Table 1) for the change in OD and rates of pH elevation (ΔpH).

Growth of *B. licheniformis* B412 in 10% CJ was dependent on pH, but, in general, grew better when incubated under aerobic than anaerobic conditions. The maximum cell density (N_{max}) 0.25 OD was achieved when cells were incubated aerobically (Table 1 and Fig. 2). The maximum specific growth rate (μ) was 0.2 (±0.02) h^{-1} (doubling time of about 3.5 h) for aerobic conditions at pH 6.0, and decreased to 0.05 h^{-1} (doubling time of approximately 14 h), as the initial pH decreased to 4.6. The patterns of pH change could be divided into three stages. The first stage was defined as 0 to 7.5 h, pH increased 1 to 1.5 units (for initial pH of 4.6 to 5.2). The initial pH elevation rate was 0.3 units per h at an initial for pH 5.2, and declined to 0.074 unit h^{-1} at pH 4.6. The pH change had a positive correlation with μ for treatments with an initial pH of 4.6 to 5.2, with an *R*-squared value of 0.99 (regression not shown). However, at a pH of 6.0, the initial pH elevation was smaller, possibly due to acid production during the initial growth stage. The second stage occurred during late log phase, in which the pH decreased by 1.2 units, for an initial pH of 6.0 and 0.5 for pH 4.6, presumably due to acid accumulation. The third stage was represented by pH elevation, which occurred under aerobic conditions during

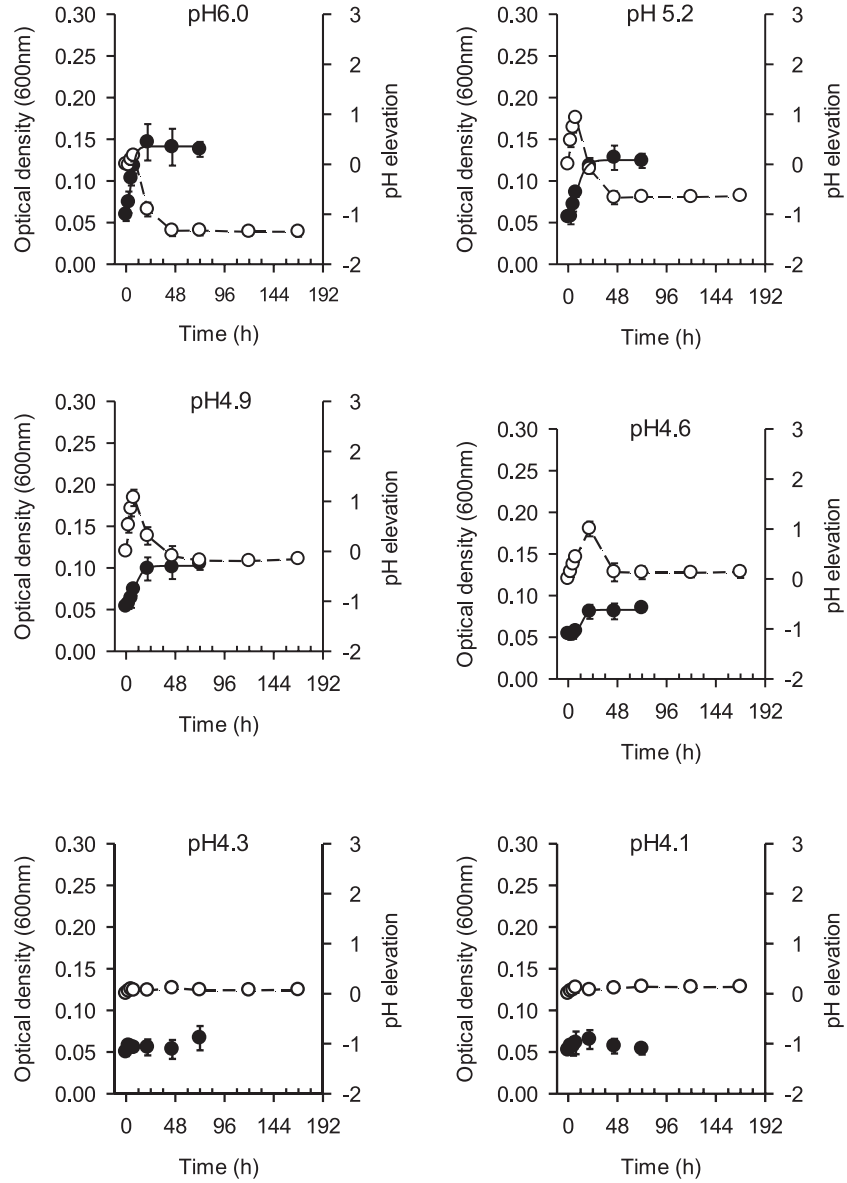

FIGURE 3. *Characterization of growth and pH elevation of* B. licheniformis *B412 in CJ anaerobic conditions. The data point of OD values (●) and pH elevation values (○) were determined for cultures incubated at 37°C with initial cell density of 10^6 CFU/ml. Values correspond to the mean of three replicates ± standard deviation.*

the stationary phase, but was not evident under anaerobic conditions. For aerobic samples, the pH increased slowly by 2 to 3 pH units to a final pH at 350 h of pH 8.2 to 8.4.

Under anaerobic incubation conditions in 10% CJ, *B. licheniformis* B412 growth was slower than was observed for aerobic incubation, with a μ of 0.11 h^{-1} at pH 6, and the N_{max} value decreased from 0.142 (at pH 6.0) to 0.09 (pH 4.6) (Table 1 and Fig. 3). The initial changes in pH were similar to that of aerobic incubation, and pH elevation also occurred at during the lag phase and early log phase of anaerobic growth. The initial pH elevation rate was 0.22 unit h^{-1} (at pH 5.2) and declined at 0.047 unit h^{-1}, as pH decreased to 4.6, similar to the aerobic growth and pH data. The specific growth rate correlated positively with the initial pH rise (*R*-squared of 0.94). However, the second pH elevation did not occur, and the changes in pH were kept at 0 to −1.3 units until the end of the experiment (350 h). The final sugar consumption was varied between 27.5% (pH 4.6) and 81.4% (pH 6.0), which positively correlated with N_{max}, pH, μ, and initial pH value. No obvious changes in OD or pH were observed with initial pH values of 4.3 and 4.1 during either aerobic or anaerobic incubations, with less than 10 mM sugar (glucose and fructose) consumed (Table 1).

Mechanism of pH increase by *B. licheniformis* B412 in acidified cucumber juice. To investigate the mechanism(s) of pH change in dilute CJ by B412, biochemical analysis to determine sugar utilization, acid production, and changes in the amino acid concentrations of CJ were carried out (Fig. 4). Sugar utilization occurred both anaerobically and aerobically (Fig. 4A). Under anaerobic conditions, lactic acid increased by 3 to 4 mM (Fig. 4B), corresponding to a similar decrease in glucose and fructose. Interestingly, under aerobic conditions, lactic acid decreased to undetectable levels after 160 h (approximately 6.5 days), while the acetic acid concentration increased by 4 mM, and sugars were reduced to undetectable levels (by 100 h; Fig. 4A). Under anaerobic conditions, lactic acid continued to increase for 160 h to 3 mM. By 160 h, acetic acid and sugars decreased to undetectable levels. During the initial pH elevation, the total amino acids in the aerobic and

TABLE 1. *Growth parameters for* Bacillus licheniformis *B412 in 10% CJ at 30°C*

Oxygen	Initial pH	Growth parameters[a]				pH elevation		Sugar consumption (%)[b]
		μ (h^{-1})	SE	λ (h)	N_{max} (OD_{600})	Initial pH elevation rate (unit h^{-1})[c]	Final pH[d]	
Aerobic	6	0.1989	0.0191	0.2317	0.258	—	8.4	100
	5.2	0.1819	0.0246	1.7523	0.2511	0.3	8.3	100
	4.9	0.1216	0.0175	2.0331	0.265	0.2	8.4	100
	4.6	0.0456	0.0057	3.8425	0.2454	0.07	8.2	100
	4.3	ND[e]	ND	ND	ND	0.02	4.4	9.1
	4.1	ND	ND	ND	ND	0.02	4.3	8.4
Anaerobic	6	0.1108	0.0206	0.2195	0.1464	—	4.7	81.4
	5.2	0.0827	0.018	2.3703	0.1278	0.22	4.6	73.1
	4.9	0.0529	0.0205	1.5966	0.1052	0.17	4.8	63.6
	4.6	0.0251	0.0042	4.3988	0.0849	0.05	4.8	27.3
	4.3	ND	ND	ND	ND	0.02	4.5	8
	4.1	ND	ND	ND	ND	0.01	4.2	7.5

[a] Growth parameters: μ, specific growth rate; SE, standard error for the estimated specific growth rate; λ, estimated lag time; N_{max}, mean maximum OD_{600} (optical density at 600 nm).
[b] Sugar consumption: the percentage of initial sugar utilized.
[c] pH elevation rate: the rate of pH increase for the initial pH rise.
[d] Final pH: the pH value after 72 h of growth.
[e] ND, not determined.

anaerobic cultures decreased similarly, and ammonia concentration increased rapidly to 1.4 and 1.0 mM at 7.5 h, respectively (Fig. 4C). However, the decrease in the total amino acid concentration and the ammonia production after 21 h in aerobic cultures was faster than that in anaerobic cultures (Fig. 4D), which correlated with the pH elevation seen under aerobic but not anaerobic conditions. The amino acid measurements also showed an immediate drop in Arg for both aerobic and anaerobic cells, to undetectable levels after the first measurement (Fig. 4D). This drop corresponded to an equimolar increase in ornithine (Orn; approximately 0.06 mM). These data are consistent with the hypothesis that Arg metabolism was concurrent with glucose and fructose catabolism at the initial stage growth in cucumber juice, and the production of Orn and ammonia was by the ADI pathway *(8, 21)*. Subsequent sugar metabolism resulted in the accumulation of organic acids and a decrease in pH. The mechanism of the second pH elevation remains to be investigated but was presumably due to aerobic degradation of amino acids.

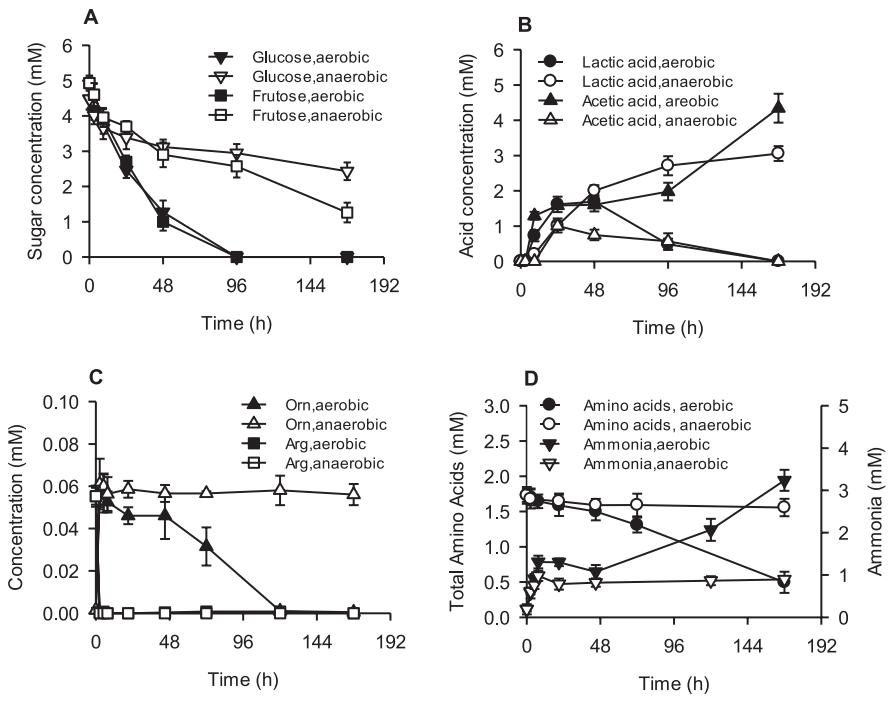

FIGURE 4. *Sugar and amino acid catabolism of* B. licheniformis *B412 in 10% CJ at pH 4.9 under aerobic and anaerobic conditions. Changes in sugar and organic acid concentrations (A and B) and in Orn and Arg and the total amino acid and ammonia concentrations (C and D) for both aerobic (solid points) and anaerobic (empty points) cultures. Data points represent the mean of three replicates ± standard deviation.*

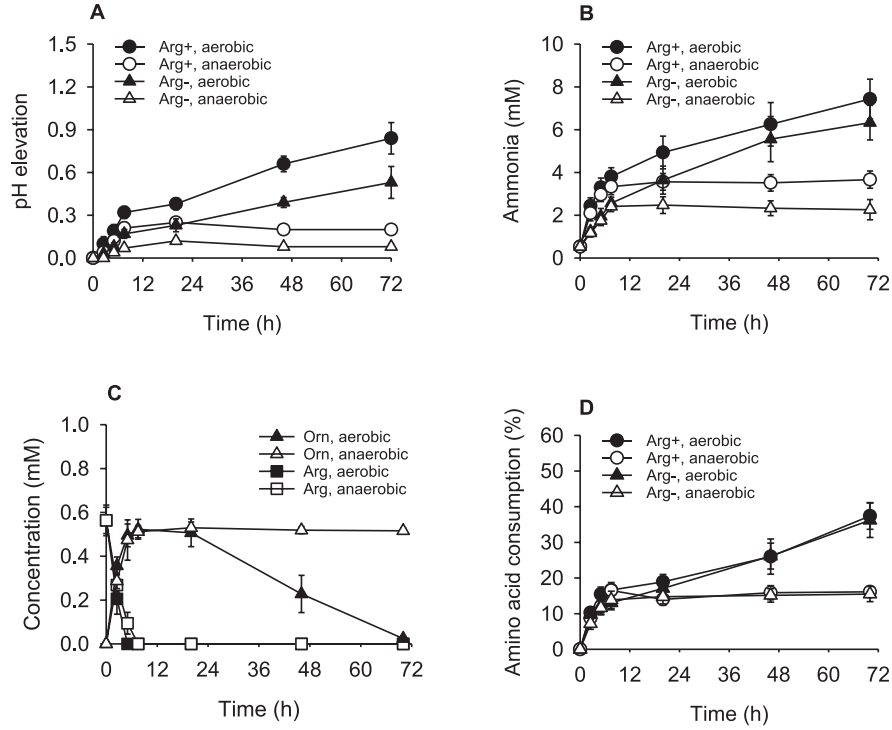

FIGURE 5. *The effect of Arg on* Bacillus licheniformis *B412 in 10% CJ at pH 4.9 under aerobic and anaerobic conditions (A, B, and D) and its effect on pH elevation in the defined medium (C, pH 4.9), showing Arg and Orn production without sugar under both aerobic (solid points) and anaerobic (empty points) conditions.*

Metabolism of *B. licheniformis* B412 in the defined medium without sugar. To investigate the effects of amino acid metabolism on the initial pH change, cells were inoculated into DM (approximately 3×10^6 CFU/ml) with and without Arg. The addition of Arg significantly increased the pH and ammonia concentrations ($P < 0.05$) after the onset of growth. The data showed the Arg treatment affected the initial pH rise and ammonia production but not subsequent changes (Fig. 5A and 5B, respectively). The accumulation of Orn in DM with and without Arg followed the same trend as was seen with the 10% CJ (Figs. 4C and 5C), with Orn remaining in the medium during anaerobic incubation, but not under aerobic incubation. Under both aerobic and anaerobic conditions Arg was immediately utilized with an equimolar production of Orn. At 72 h, total amino acid concentrations were not significantly affected by presence or absence of Arg ($P < 0.05$), although consumption was generally less under anaerobic conditions. These data confirm the findings from studies with CJ that the Arg was primarily utilized during first few hours of incubation via the ADI pathway, resulting in ammonia and Orn production.

TABLE 2. *Effect of inoculum level and pH on growth and metabolism of* B. licheniformis *B412 in 10% CJ at 30°C*

Initial pH[a]	Inoculum level (log CFU/ml)[b]	No added Arg		Added Arg	
		Final pH	% sugar utilization	Final pH	% sugar utilization
4.3	7.55	8.2	100	8.3	100
	7.15	8.1	100	8.3	100
	6.85	4.5	9.1	8.2	100
	6.55	4.4	8.7	8.2	100
	6.15	4.4	8.5	4.5	10.3
	5.85	4.4	8.4	4.4	8.5
	5.15	4.4	5.8	4.4	7.6
	4.85	4.4	4.8	4.4	4.3
4.6	7.55	8.2	100	8.5	100
	7.15	8.1	100	8.3	100
	6.85	8.2	100	8.4	100
	6.55	8	100	8.3	100
	6.15	4.6	8.9	8.3	100
	5.85	4.6	8.2	8.2	100
	5.15	4.6	6.3	8.1	100
	4.85	4.6	6	8.2	100

[a] Initial pH value of CJ.
[b] Inoculum level of *B. licheniformis* B412.

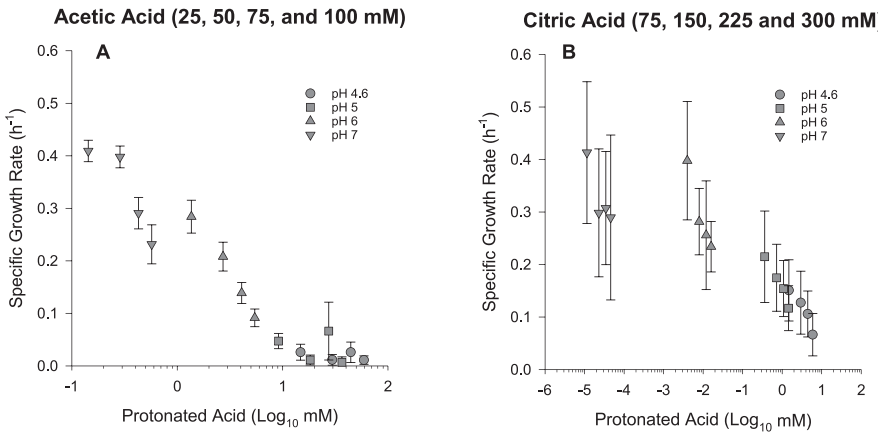

FIGURE 6. *Effect of protonated acetic and citric acids on the growth of* B. licheniformis *in TSB. The specific growth rate for the* B. licheniformis *strain cocktail was plotted against (A) acetic acid concentrations of 25, 50, 75, and 100 mM, or (B) citric acid concentrations of 75, 150, 225, and 300 mM. The data show acid concentrations as log of the protonated acid concentration for each pH: down triangle, pH 7; up triangle, pH 6; square, pH 5; and circle, pH 4.6. The error bars indicate the standard error for two or three independent trials.*

Factors effecting pH rise and sugar utilization. To study the effects of initial cell count and Arg concentration on pH elevation, 10% CJ (with and without 0.6 mM Arg) at pH 4.6 and 4.3 were inoculated with eight different inoculum levels of *B. licheniformis* from 4.85 to 7.55 log CFU/ml (Table 2). For initial cell counts of less than 7 log CFU/ml, less than 10 mM sugar was used, and pH increase was limited 0.2 pH units or less at pH 4.3. However, with the addition of 0.6 mM Arg, pH increases of approximately 4 pH units and complete sugar utilization was seen for inoculum levels of 6.6 log CFU/ml or greater. At pH 4.6, a similar trend was seen. Without added Arg, treatments with cell numbers less than below 6.6 log resulted in no change in pH and less than 10% utilization of sugar. However, for treatments with 0.6 mM added Arg, complete sugar utilization was observed, regardless of inoculation level. These data indicate that supplementing the dilute CJ with Arg can reduce the number of vegetative cells needed to initiate growth, pH elevation, and sugar metabolism at pH 4.3 and 4.6.

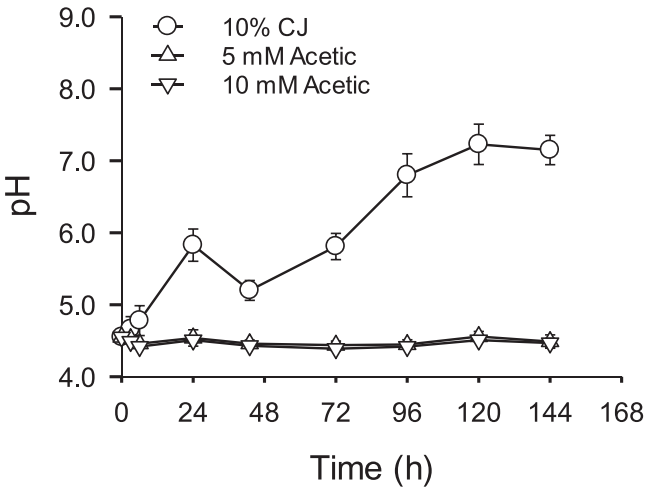

FIGURE 7. *The effect of acetic acid on pH changes in 10% CJ. The pH changes caused by* B. licheniformis *under aerobic conditions at pH 4.6 in the 10% CJ 0 mM (open circles), 5 mM (up triangle), or 10 mM (down triangle) acetic acid to pH 4.6.*

Effects of acetic and citric acid on growth of a *B. lichenifomis* stain cocktail in TSB. To determine the effect of pH and acetic acid on the growth of *B. licheniformis,* we used a laboratory medium under favorable conditions for growth in a rich medium, TSB, aerobic and at neutral pH. For each pH, a trend of decreasing growth rate with increasing protonated acid concentration was observed (Fig. 6). Protonated acetic acid concentrations of 10 to 20 mM were present at pH 4.6 and pH 5, and the combination of pH and acid was sufficient to effectively inhibit growth (Fig. 6A). One data point (Fig. 6A) at pH 5 (for 75 mM acetic acid) had a higher mean growth rate and was apparently due to a single contaminated microtiter plate well (data not shown). To show effects on growth with citric acid, higher concentrations of acid were needed (Fig. 6B). Interestingly, regardless of acid concentration or pH, growth was not inhibited by citric acid, even for pH 4.6 and 300 mM total citric acid, corresponding to approximately 6 mM fully protonated acid (for triprotic citric acid). The lowest growth rate achieved was with 300 mM citric acid and a pH of 4.6, was 0.07 ± 0.04 μ h^{-1} (approximately 4-day doubling time). The variation in growth rate measured data for citric acid was greater than was observed for acetic acid. The reason for this difference is unclear, although it is likely that citric acid may be consumed by cells during growth under permissive conditions, possibly altering the growth rate. Although bacillus is known to use citrate as a carbon source *(9),* at low pH (4.6), citrate was inhibitory compared with growth at pH 7, which resulted in growth rates of 0.41 ± 0.13 μ h^{-1} for 75 mM citric acid at pH 7 to 0.29 ± 0.16 μ h^{-1} for 300 mM citric acid. Further research will be necessary to define the effects of citrate on the growth of *B. licheniformis.*

Acetate (200 mM) at neutral pH (approximately 1 mM protonated acid) can inhibit *B. subtilis* growth by inhibiting the transport of nutrients, including amino acids in whole cells and membrane vesicles *(30).* We found that pH elevation and cell growth at pH 4.6 was inhibited by 5 or 10 mM acetic acid, corresponding to approximately 3 and 6 mM protonated acetic acid, respectively (Fig. 7). Interestingly, 3 to 6 mM acetic acid did allow growth in TSB. It is possible that Arg uptake was inhibited, metabolism was inhibited, or both by the added acid, which

would result in a decrease in internal pH, and also result in the accumulation of acid anion within the cell *(27)*. Both reduced internal pH and increased anion concentration could decrease metabolic activity by *B. licheniformis*. Biochemical analysis at 72 h confirmed that Arg and sugars were not used (data not shown), and cell growth was not apparent. These data are supportive of the hypothesis that pH rise resulting from Arg catabolism and ammonia production were required for growth with an initial pH of 4.6 in 10% CJ and growth and pH rise.

Our data showed that growth of *B. licheniformis* in dilute 10% CJ at pH 4.6 or higher resulted in a multiphase pH change. The data indicate that the initial pH elevation was due to ammonia production from the ADI pathway of *B. licheniformis,* followed by pH decrease due to fermentation of sugar and lactic acid production. The second pH elevation occurred during stationary phase, upon depletion of sugars, and resulted in the accumulation of ammonia from the catabolism of additional amino acids. Under favorable growth conditions (TSB medium, 37°C), the growth rates of *B. licheniformis* were inversely related to protonated acetic acid concentrations, and a concentration of 10 to 20 mM protonated acid was sufficient to inhibit growth and metabolism of Arg. Most pickled vegetable products use acetic acid as the primary acidulent, typically at concentrations of 0.5% (83.3 mM) or greater and therefore have protonated acid concentrations of greater than 40 mM for pH values at or below pH 4.6, which was sufficient to inhibit growth of *B. licheniformis*. Interestingly, citric acid, which is present in tomato products at 20 to 40 mM *(3, 4),* was unable to inhibit growth of *B. licheniformis* with total acid concentrations of up to 300 mM, from pH 4.6 to pH 7 in TSB at 30°C. These data could explain why bacilli have been found to grow in tomato products and raise pH *(23, 26),* but spoilage of acidified vegetables by spore-forming bacilli has not been observed.

ACKNOWLEDGMENTS

This research was supported in part by Pickle Packers International, Inc. (Washington, DC) and by the National Natural Scientific Foundation of China (Grant 30901047), Jiangsu Natural Scientific Foundation project (BK2009191), and grant (08KJD550003) from the Department of Education of Jiangsu Province. Support was also received from the USDA Cooperative State Research, Education, and Extension service project 2006-01240. We thank Mrs. Sandra Parker for her assistance in editing the manuscript. We thank Dr. Glenn Black, Grocery Manufacturers Association, Washington, DC, for helpful discussions and supplying *Bacillus* cultures.

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