Influence of pH on Organic Acid Production by *Clostridium sporogenes* in Test Tube and Fermentor Cultures

The influence of pH on the growth parameters of and the organic acids produced by *Clostridium sporogenes* 3121 cultured in test tubes and fermentors at 35°C was examined. Specific growth rates in the fermentor maintained at a constant pH ranged from 0.20 h^{-1} at pH 5.00 to 0.86 h^{-1} at pH 6.50. Acetic acid was the primary organic acid in supernatants of 24-h cultures; total organic acid levels were 2.0 to 22.0 μmol/ml. Supernatants from pH 5.00 and 5.50 cultures had total organic acid levels less than one-third of those found at pH 6.00 to 7.00. The specific growth rates of the test tube cultures ranged from 0.51 h^{-1} at pH 5.00 to 0.95 h^{-1} at pH 6.50. The pH of the medium did not affect the average total organic acid content (51.5 μmol/ml) but did affect the distribution of the organic acids, which included formic, acetic, propionic, butyric, 3-(*p*-hydroxyphenyl)propionic, and 3-phenylpropionic acids. Butyric acid levels were lower, but formic and propionic acid levels were higher, at pH 5.00 than at other pHs.

Clostridium botulinum growth in foods usually results in overt spoilage due to the production of gas, volatile organic acids, and proteolytic enzymes. Failure to find signs of spoilage is not, however, sufficient grounds on which to assume that toxin is absent (23). Toxin can be produced before the product becomes organoleptically objectionable in cured meats with a brine concentration of 6 to 7% salt (12). An average of 25 cases of botulism per year (4) indicates that *C. botulinum* can produce toxin without making the food totally unpalatable.

Cultural conditions influence gas and protease production by *C. botulinum* (17). The inhibition of gas production by a low pH suggested an influence of pH on botulinal physiology and on the production of other microbial metabolites. Many studies have been done on the composition of fermentation products with the aim of developing tools for species identification (8, 9, 13) or presumptive indicators of botulinal growth (1, 15). Although medium composition is a major determinant of which organic acids are produced (1, 25), there are, to our knowledge, no reports on the influence of extrinsic factors such as pH on organic acid production. The objectives of this study were to determine the influence of pH on *C. sporogenes* growth parameters and organic acid production. This species was used as a model for *C. botulinum* because the fermentation products of *C. botulinum* toxigenic strains are indistinguishable from those of atoxigenic strains or of *C. sporogenes* strains cultured in the same medium (15). The use of *C. sporogenes* eliminated the rigorous safety precautions required by studies of *C. botulinum*. In addition to investigating the physiology of *C. sporogenes* at constant pH in a fermentor, we conducted parallel studies in which only the initial pH was defined, since growth without pH control more closely approximate microbial growth in foods.

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MATERIALS AND METHODS

Organism. Inocula of *C. sporogenes* 3121 were prepared by using 0.1 ml of a cooked-meat medium stock culture to inoculate 20 ml of supplemented Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) (see below) which was incubated anaerobically at 35°C for 18 h. After centrifugation (10,000 × *g*, 10 min), the cells were resuspended in an equal volume of fresh medium, of which 0.2 ml was used to inoculate the Hungate tubes and 10 ml was used to inoculate the fermentor.

Medium preparation. Trypticase soy broth was supplemented with 0.5 g of cysteine-hydrochloride (Sigma Chemical Co., St. Louis, Mo.), 1 mg of resazurin (Difco Laboratories, Detroit, Mich.) and 2.5 g of glucose per liter to give a final glucose concentration of 5 g/liter. The medium was heated to boiling temperature and cooled to room temperature before the pH was adjusted with 1.5 N HCl or NaOH. This adjustment minimized pH changes caused by autoclaving. All media were prepared as one batch. Media for fermentor runs were sterilized (121°C, 15 min) in flasks and aseptically dispensed into the fermentor vessel. Media for the test tube experiments were adjusted to the proper pH and dispensed (10 ml per tube) into Hungate tubes (Belco Glass, Inc., Vineland, N.J.) before sterilization.

Test tube cultures. Media in Hungate tubes were equilibrated to 35°C in a water bath, inoculated through the septum cap, and checked periodically for changes in A_{610} with a spectrophotometer (Spectronic 710; Bausch & Lomb, Inc., Rochester N.Y.). Three tubes of medium at each pH were inoculated, and the results were averaged. After 24 h, the contents of the triplicate tubes were pooled and centrifuged (10,000 × *g*, 10 min, 5°C), and the supernatants were frozen in a dry ice acetone bath.

Fermentor cultures. The fermentor used in these studies was of our own design. The fermentor vessel was a tall-form, 1,000-ml Pyrex beaker. The headpiece was a no. 15 rubber bung with ports for acid and base addition, nitrogen sparge and overlay lines, a filter-protected vent, a pH probe, and sampling. A 35-mm-diameter, magnetic "star bar" was used

TABLE 1. Growth data for *C. sporogenes* 3121 cultured in test tubes and a fermentor

Culture type and initial pH of medium	Lag period (h)	Final A_{610}	Final pH	Residual glucose (%)[a]	Specific growth rate $(h)^{-1}$
Fermentor					
7.00	0.5	1.7	7.12	0	0.71
6.50	0.5	1.8	6.48	0	0.86
6.00	1	3.1	5.97	0	0.50
5.50	2	2.9	5.55	0	0.46
5.00	>8	0.6	5.06	0.25	0.20
Test tube					
7.00	0	1.9	5.40	0.1	0.89
6.50	0	1.8	5.35	0.1	0.95
6.00	0	1.6	5.25	0.1	0.81
5.50	0	1.5	5.09	0.1–0.25	0.81
5.00	2	1.2	4.96	0.25	0.51

[a] Initial glucose was 0.5%.

for agitation. The vessel was sterilized (121°C, 15 min) while empty, and the medium was added aseptically.

A pencil-thin combination pH electrode (Broadly James Corp., Santa Ana, Calif.) was sterilized by immersion for 30 min in a solution of 70% ethanol, rinsed with sterile distilled water, and inserted into the vessel. The pH was controlled by a Chemcadet pH meter-controller (model 5625-00; Cole-Parmer Instrument Co., Chicago, Ill.) by the addition of 0.5 N HCl or NaOH. The fermentor vessel was placed in a transparent bath containing water heated by a circulating heater (Fisher Scientific Co., Pittsburgh, Pa.). The fermentor and bath were placed on a magnetic stirrer (model 310; Fisher Scientific Co.).

The fermentor was sparged with nitrogen (500 ml/h) for 18 h and equilibrated to 35°C before inoculation. Stirring took place at 250 rpm. Immediately after inoculation, the gas input was switched from sparge to overlay to minimize the stripping off of volatiles. Samples were drawn every 0.5 h for absorbance readings. The sample pH was determined with a second pH meter to check for drift in the pH control unit. Samples for fermentation product analysis were taken at 24 h of incubation and processed in the same fashion as the test tube cultures.

Organic acid standards. Short-chain organic acids (Aldrich Chemical Co., Metuchen, N.J.) were prepared as standard solutions by dissolving them in the high-pressure liquid chromatographic mobile phase.

Analysis procedures. The culture supernatants were thawed and their short-chain organic acids were extracted in ethyl ether by the procedure of Guerrant et al. (13). A total of 10 μl of the extracts was injected for chromatographic analysis. Residual glucose in the culture supernatants was determined semiquantitatively with Diastix reagent strips (Miles Laboratories, Inc., Elkhart, Inc.).

High-pressure liquid chromatography. Chromatographic separations were carried out on a system (Waters Associates, Inc., Milford, Mass.) which included a model 6000A solvent delivery system, a model U6K injector, a model 450 variable-wavelength detector adjusted to a wavelength of 210 nm and to a sensitivity of 0.4 absorbance units (full scale), and a model HP-3390A reporting integrator (Hewlett-Packard Co., Avondale, Pa.). The instrument was equipped with an Aminex HPX-87H ion exclusion column (300 by 7.8 mm) for organic acids (Bio-Rad Laboratories, Richmond, Calif.). Retention times were determined for pyruvic, succinic, fumaric, formic, lactic, acetic, propionic, isobutyric, mercaptopropionic, butyric, isovaleric, valeric, isocaproic, cresol, phenylacetic, and caproic acids and 3-(*p*-hydroxyphenyl)propionate (3-phφp) and 3-phenylpropionate (3-φp). The mobile phase was 5.0% (vol/vol) acetonitrile in 0.007 N H_2SO_4, with a flow rate of 0.6 ml/min and a temperature of 60°C.

RESULTS

Growth. In the fermentor studies, exponential growth occurred after a lag period that increased with decreasing pH (Table 1). Final cell densities were comparable at pH 7.00 and 6.50 but higher at pH 6.00 and 5.50. At pH 5.00, the cell density was the lowest of all the cultures in this study. The final culture pHs were generally within 0.05 U of the target value. The exception occurred at pH 7.00, where electrode fouling during the latter stages of the fermentation caused a 0.12-U drift. The fastest growth occurred at pH 6.50, with substantially lower specific growth rates at pH 6.00 and 5.50. The growth rate was very slow at pH 5.00.

Test tube cultures grew exponentially without an appreciable lag period, except at pH 5.00, where there was a 2-h lag. Final cell densities decreased with decreasing pHs, but the changes were not as marked as those observed in the fermentor. Although the initial pH of the media covered a 2-U range, the final pH ranged from 4.96 to 5.40. Growth rates were highest at pH 6.50, but the effect of pH on specific growth rates was not dramatic in the range of 5.50 to 7.00. The cells cultured at pH 5.00 had a lower specific growth rate.

Organic acid production. The method of Guerrant et al. (13) was not completely satisfactory because, in our hands, formic acid was not resolved from fumaric acid. In addition, the sample analysis time exceeded 80 min. Operating the column at 60°C resulted in baseline separation of fumaric and formic acids and allowed runs to be completed in 49 min. Fumaric, formic, acetic, propionic, isobutyric, and butyric acids and 3-phφp and 3-φp were detected in the fermentation broths. An additional nine unidentified peaks occurred with regularity. However, the area of the unidentified peaks represented less than 20% of the total integrated area in chromatograms from the fermentor samples. Less than 9% of the peak area was unidentified in chromatograms from the test tube supernatants. The uninoculated medium contained trace amounts of isobutyric acid and 3-φp. Acetic acid was present at 11.2 μmol/ml. This value was subtracted from the amount of acetic acid found in the culture supernatants to give corrected values for production of acetic acid (Tables 2 and 3).

The response factor of the high-pressure liquid chromatographic detector for simple organic acids, such as formic, acetic, propionic, and butyric acids, was 1.3×10^{-7} to $1.5 \times$

TABLE 2. Organic acids produced by *C. sporogenes* 3121 fermentor cultures in Trypticase soy broth with pH control

pH	Concn (μmol/ml)						
	Formate	Acetate	Propionate	Butyrate	3-phφp	3-φp	Total
7.00	0.7	12.8	ND[a]	ND	1.3	3.1	17.9
6.50	0.8	8.8	ND	5.0	1.1	2.9	19.2
6.00	0.7	8.1	ND	8.6	1.4	3.0	22.0
5.50	0.8	Nil	ND	3.6	0.4	0.8	5.5
5.00	0.5	Nil	ND	0.5	0.2	0.8	2.0

[a] ND, Not detected.
[b] Nil, Detected at levels comparable to those in uninoculated medium.

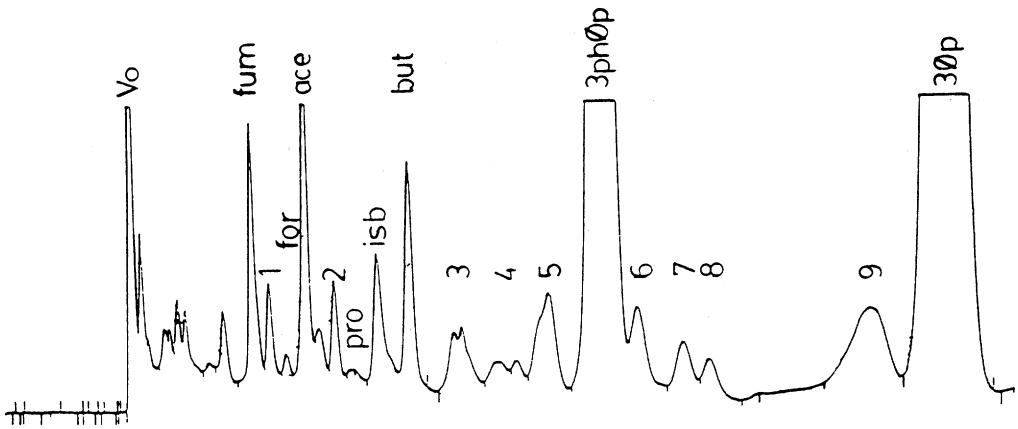

FIG. 1. High-pressure liquid chromatograms of organic acids produced by test tube culture of *C. sporogenes* 3121 in supplemented Trypticase soy broth with an initial pH of 7.00. Abbreviations: Vo, void volume; fum, fumarate; for, formate; ace, acetate; pro, propionate; isb, isobutyrate; but, butyrate.

10^{-7} μmol per unit area integrated; 4.7×10^{-10} μmole per unit area for the unsaturated dicarboxylic acid fumaric acid; and 1.0×10^{-10} μM per unit area for phenyl-containing compounds such as 3-phφp and 3-φp. Thus, although the chromatogram of the pH 7.00 test tube sample had many major peaks (Fig. 1), quantitatively, acetic and butyric acids were the major organic acids (Table 3). Fumaric acid was detected in all of the fermentation broths, but in quantities of less than 0.001 μmol/ml. Neither lactic acid nor mercaptopropionic acid was detected in any of the fermentation broths.

pH did have an affect on both the nature and amount of organic acids produced in the fermentor. The amount of formic acid was fairly constant (0.8 μmol/ml) at pH 7.00 to 5.50 (Table 3). The decrease at pH 5.00 was probably due to the lower cell density (Table 1). Acetic acid production was high at pH 6.00, 6.50, and 7.00 but negligible at pH 5.5 and 5.0. Concentrations of butyric acid inreased from an undetectable level of pH 7.00 to 8.6 μmol/ml at pH 6.00 and then decreased at lower pHs. Butyric acid was, however, the major organic acid identified at pH 5.50. The total levels of identifiable organic acids were comparable at the neutral pHs but markedly lower at pH 5.50 and 5.00. It is quite likely that volatilization influenced the amount of acids in the sparged fermentors, especially since pH did not affect total acids in the closed test tubes.

Formic, acetic, propionic, isobutyric, and butyric acids and 3-phφp and 3-φp were detected in supernatants of test tube cultures at all pHs examined. The total concentration of identifiable organic acids was similar under all pH conditions (Table 3). Acetic acid was produced in quantities of >10 μmol/ml in all of the fermentation broths. Shifts in fermentation products occurred with changing pH. The butyric acid level was high between pH 7.00 and 6.00 but fell at pH 5.00. In contrast, levels of formic and propionic acids, which were low at pH 7.00 to 6.00, increased at pH 5.50 and 5.00. pH-dependent changes in unidentified compounds that eluted between 3-phφp and 3-φp were reproducibly observed. These compounds probably represented phenolic compounds present at low levels in the fermentation broth.

TABLE 3. Organic acids produced by *C. sporogenes* 3121 test tube cultures in Trypticase soy broth with the indicated initial pH

pH	Concn (μmol/ml)						
	Formate	Acetate	Propionate	Butyrate	3-phφp	3-φp	Total
7.00	3.9	18.0	1.5	16.4	5.1	7.6	52.5
6.50	2.5	11.7	1.3	16.8	5.5	13.4	51.2
6.00	2.8	12.2	2.5	17.8	5.2	8.3	48.0
5.50	4.9	15.3	4.1	13.8	5.3	11.2	54.6
5.00	10.8	17.0	7.1	1.9	4.7	10.1	51.6

DISCUSSION

In fermentor and test tube cultures, growth was most rapid at pH 6.50. Fujioka and Frank (10) found growth of strain PA 3679 to be optimum at pH 7.0 to 7.5 in a chemically defined medium. Pang et al. (20) reported a specific growth rate of 0.5 h^{-h} for *C. sporogenes*. This was only slightly affected by pH over the range of 6.0 to 7.0. The maximum specific growth rate in this study was higher, probably because incubation took place at 35°C rather than 30°C. *C. botulinum* has been reported to grow most rapidly between pH 6.5 and 7.0 (3, 18). Thus, our observations on the effect of pH were not unexpected. What was unexpected was that test tube cultures grew faster than fermentor cultures, especially at low pH. Whereas the growth rate of *C. sporogenes* in test tubes at pH 5.00 was 0.5 h^{-1} (higher than that observed by Pang et al. [20] under optimal conditions at 30°C), the specific growth rate in the fermentor was only 0.2 h^{-1}.

The highest final cell densities occurred in the pH 6.00 and 5.50 fermentor cultures. At these pHs and above, all of the glucose in the medium was consumed. The lower cell densities at pH 7.00 and 6.50 were probably due to cell lysis, which is pH dependent in *C. botulinum* (18). In test tube cultures, glucose utilization was incomplete; final cell densities were lower than those in the fermentor.

Because the closed system with uncontrolled pH appeared to be a better model than a fermentor for examining the physiology of a food-borne microbe, only the test tube culture results will be discussed further. Despite the widely recognized influence of medium on fermentation products (1, 25), the chromatograms of *C. sporogenes* supernatants (Fig. 1) were qualitatively similar to those obtained by Guerrant et al. (13) for *Peptostreptococcus anaerobius* cultured in peptone-yeast extract-glucose medium.

The organic acids detected in this study were formic, acetic, propionic, and butyric acids and 3-phφp and 3-φp. These are products of glucose (14, 24, 26) and amino acid (2,

11, 16, 19, 21, 22) catabolism. Other investigators have reported that butyric (1, 16, 25), isovaleric (9, 25), 4-methylvaleric, 3-methylbutyric, 2-methylpropionic (1), isobutyric, isocaproic (25), pyruvic, *n*-caproic (9), aminovaleric (16), and lactic (5) acids could be detected in supernatants of *C. sporogenes* or *C. botulinum* grown in various media. Production of formic acid has not previously been reported for *C. sporogenes* or *C. botulinum*. The increased level of formic acid at low pH is consistent with our earlier observation that gas production is inhibited at pH 5.0 and is probably due to inhibition of formate lyase under acidic conditions. Production of 3-ϕp in *P. anaerobius* and *C. difficile* and 3-phϕp in *P. anaerobius* has been reported elsewhere (13). These organic acids are products of the Stickland reaction when tyrosine or phenylalanine are substrates.

The data presented are indicative only of the metabolic status of cells at the time (in this case, early stationary phase) of sampling. Day and Costilow (6) have observed that, upon sporulation, acetic acid levels decrease and valeric acid begins to accumulate. Because the Stickland degradation of proline and alanine furnishes the exogenous energy source for forespore maturation (7), higher levels of propionic and aminovaleric acids might also be found in sporulating cultures.

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