

Interaction of pH and NaCl on Culture Density of *Clostridium botulinum* 62A

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Clostridium botulinum 62A growth rates declined with decreasing pH and increasing salt levels. Lysis rates, however, were affected only by pH. Due to competition between growth and lysis rates, an accurate assessment of interactive effects was obtained only when optical density determinations were made at multiple intervals.

Ever since a report by Dozier in 1924 (6) which stated that *Clostridium botulinum* cannot grow below pH 4.87, tremendous emphasis has been placed on determining the growth-limiting pH for this organism (8–10, 12). Recent reports (14, 17, 19) of specialized circumstances of toxin production at pH < 4.6 have stimulated additional research. Because of the adverse consequences associated with *C. botulinum* growth in foods, research has focused on growth-limiting conditions rather than on the physiology of the organism under subinhibitory conditions. Even though it is known that combinations of factors such as pH and NaCl levels are more effective when used together than when used separately (7, 13), information on these interactions is more limited and usually presented in the context of cured meats. Riemann et al. (15) have gathered data from many publications which show that the pH required to inhibit *C. botulinum* increases with increasing NaCl concentration. Studies on the interaction of water activity and pH (2) have shown that, in the absence of NaCl, the growth-limiting pH in reinforced clostridial medium is 5.3, but that at 4.8 and 6.5% NaCl, this increases to pH 6.0 and 7.0, respectively. Roberts and Ingram (16) demonstrated the interactions of pH, NaCl, and nitrite on growth of *C. botulinum*.

The purpose of this research was to study characteristics of cell growth and lysis in the inhibitory, but nonlimiting, regions and to reexamine the combined effect of pH and NaCl in determining growth-limiting conditions.

The basal medium used was botulinum assay medium (BAM) (11). NaCl was added at concentrations of 0, 2, 3, 4, and 6%. The pH of each medium was adjusted to 7.00, 6.50, 6.00, 5.50, and 5.00 with 1 N HCl. pH was measured with a digital meter (model PAX 9000; Sargent Welch) equipped with slope control and a combination

electrode standardized against buffers at pH 4.00 and 7.00. The media were dispensed into flint glass, screw-cap test tubes (5 ml per 13- by 100-mm tube) and autoclaved at 121°C for 15 min. The pH of media checked after autoclaving remained within 0.1 pH unit of the target value. Experimental procedures were carried out in an anaerobic chamber (1) where media were equilibrated before use.

Inocula were prepared from *C. botulinum* 62A which has intermediate pH sensitivity (3). Heat-shocked spores were produced as previously described (11) and inoculated into duplicate tubes of each medium at 10^6 spores per tube. Vegetative cells from a 72-h culture in BAM (pH 7.0, 0% NaCl) were washed, diluted with 0.1% peptone water, and inoculated into duplicate tubes of each medium at 10^4 or 10^6 cells per tube. Inocula levels were based on direct counts with a Petroff-Hauser counting chamber (Hauser Scientific, Blue Bell, Pa.). The inoculated media were incubated at 30°C for at least 30 days. Cell density (absorbance) was determined at 610 nm in a colorimeter (model 7453; Markson Science, Inc., Del Mar, Calif.) against a blank of uninoculated medium.

The effect of pH and NaCl on culture density was clearest in cultures inoculated with 10^4 vegetative cells. These results are shown in Fig. 1 where absorbance is represented by the height of a block placed in a four-by-four NaCl-pH matrix. At day 1, the optical density was greatest at a high pH and a low NaCl concentration, as expected. However, at increased incubation times, growth commenced at progressively more inhibitory combinations of pH and NaCl, whereas the cell density under the previously optimal conditions decreased. Thus, if cultures had been examined only at 7 days of incubation (Fig. 1d), it would have been erroneously concluded that optimal growth conditions were at pH 7, 4%

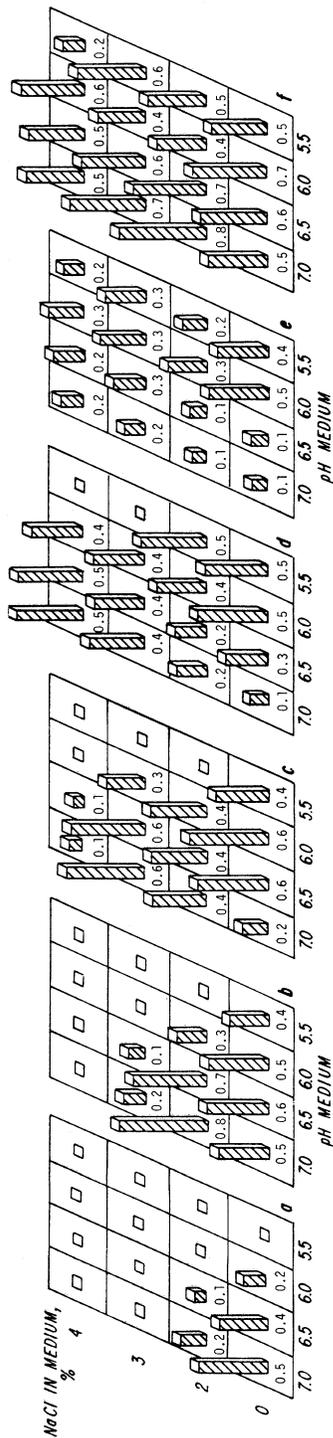


FIG. 1. The optical density at 610 nm of BAM cultures inoculated with 10^4 vegetative cells of *C. botulinum* 62A after 1, 2, 4, 7, and 14 (a, b, c, d, and e, respectively) days of incubation at 30°C is represented by the height of the block on the pH-salt grid. Grid f represents the maximum optical density obtained during 30 days of incubation. Numerical values for absorbance are given at the base of each block.

NaCl; pH 6, 0% NaCl; and pH 5.5, 2% NaCl.

The interaction of pH and NaCl was also noted. Decreasing the pH in the absence of salt or adding salt to media at pH 7.0 were only moderately inhibitory, but the combined effect inhibited growth for more than 7 days. In this respect, salt combined with low pH was particularly effective (Fig. 1c and d). Growth occurred in media at pH 5.0, but only in the absence of added salt (Table 1). Growth did not occur at pH 4.8 (data not shown). Salt at a concentration of 6% inhibited the growth of *C. botulinum* at all pH levels for the entire 30-day incubation period. Extended incubation (85 days) did not alter these results. Although there was inhibition of growth under most pH-NaCl combinations at some time during the experiment, with the exception of pH 5.5, 4% salt, there was no marked effect on the maximum cell density (Fig. 1f). The use of 10^6 spores or 10^6 vegetative cells as inocula resulted in data qualitatively similar to those in Fig. 1.

Quantitative data on growth and lysis rates (Table 1) help explain the changing skylines in Fig. 1. Cell growth rates decreased with decreasing pH but were not affected by up to 3% salt. The addition of 4% NaCl slowed the growth rates, with this effect becoming more pronounced at a lower pH. Lysis rates did not follow first-order kinetics but were empirically determined to be linear ($r = -0.96$ to -0.99) when plotted as absorbance (A) versus log(days). The slopes of these curves were used to calculate the lysis rates [ΔA per log(day)] presented in Table 1. The lysis rates decreased with decreasing pH. The extent of lysis also decreased with declining pH. NaCl per se did not affect either. The lag phase was extended by 4% salt, with decreasing pH increasing the lag period even further. Thus, under permissive conditions, cultures started growth early, grew rapidly, and then lysed rapidly at a time when cultures under adverse conditions were just beginning to grow. As time went on, the cultures grown under adverse conditions continued growth with relatively little lysis. At incubation periods beyond 7 days, these cultures had the highest cell densities.

Both the limiting pH and the inhibition of growth reported here were consistent with literature reports. Growth ratios for pH 5.5 versus 7.0 were 3.0 to 4.5, when calculated by the method of Blocher et al., who reported values of 2.2 to 3.8 at pH 5.63 and a slightly higher incubation temperature (3). Hauschild and Hilsheimer (8) found no toxin in caviar at a salt concentration of $>5.56\%$ or $\text{pH} < 5.0$ and demonstrated that increasing the NaCl concentration from 3.95 to 4.67% increased the limiting pH from 5.2 to 5.6. Interactions reported here were

TABLE 1. Effect of NaCl and pH on lag period, growth rate, lysis rate, and extent of lysis in BAM inoculated with 10^6 vegetative cells of *C. botulinum*

NaCl (%)	pH	Lag period (days)	Growth rate (ΔA days ⁻¹)	Lysis rate [ΔA (log days ⁻¹)]	Extent of lysis (%)
0	7.0	<1	>0.67	-0.56	86
	6.5	<1	0.32	-0.43	88
	6.0	<1	0.30	-0.36	52
	5.5	<1	0.11	-0.25	43
	5.0	2	0.03	-0.03	14
2	7.0	<1	0.67	-0.69	88
	6.5	<1	0.34	-0.70	92
	6.0	<1	0.19	-0.59	79
	5.5	<1	0.16	-0.29	42
	5.0	>85	NA ^a	NA	NA
3	7.0	<1	0.59	-0.72	93
	6.5	<1	0.30	-0.53	87
	6.0	<1	0.25	-0.50	64
	5.5	2	0.16	-0.21	36
	5.0	>85	NA	NA	NA
4	7.0	2	0.37	-0.76	86
	6.5	2	0.20	-0.60	75
	6.0	3	0.18	-0.40	52
	5.5	7	0.05	-0.33	29
	5.0	>85	NA	NA	NA

^a NA, Not applicable.

of a similar magnitude. Reports setting the growth-limiting NaCl concentration at 6.5% (15) or 7.0% (18) at pH 7.0 contrast with the inhibition at 6% reported here. These differences may be due to the strains and substrates used or interactions with other uncontrolled variables such as redox potential. For example, Smoot and Pierson (18) found that 6% NaCl inhibits growth at $E_{h7} = -60$ mV, but that at $E_{h7} = -140$ mV, 7% NaCl is required.

Autolysis of *C. botulinum* cultures has previously been examined as a mechanism of toxin release (5). My observations on the effect of pH on lysis in media without NaCl were similar to those of Bonventre and Kempe (4). The rate of autolysis decreased with decreasing pH in their study but over a shorter time frame than reported here. This temporal difference is probably due to their use of a large log-phase inoculum.

In summary, this report has shown significant interactions of pH and NaCl on the culture density of *C. botulinum* cultures. The combination of NaCl and pH which produced the maximum culture density depended on when the culture density was measured because the length of the lag period, the growth and lysis rates, and the extent of cell lysis were all a function of the pH or salt concentration of the media or both. Frequent observation of cell density may be

important in the study of other inhibitory systems where no a priori assumptions about lysis rates can be made.

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