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Toxin Production by *Clostridium botulinum* Type A Under Various Fermentation Conditions

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The time of appearance and the quantity of toxin produced by the Hall strain of *Clostridium botulinum* type A were examined under various conditions. A 70-liter fermentor and a complex medium consisting of 2% casein hydrolysate and 1% yeast extract plus an appropriate concentration of glucose were employed. Optimal conditions for toxin production were as follows: a nitrogen overlay at a rate of 5 liters/min, an agitation rate of 50 rpm, a temperature of 35°C, and an initial glucose concentration of 1.0% with the pH uncontrolled. Under these conditions, the maximum toxin concentration (6.3×10^5 mouse median lethal doses/ml) was attained within 24 h. Cell lysis was apparently not required to obtain maximum toxin concentrations under the fermentation conditions described.

The botulinum toxoid currently in use for human immunization is pentavalent (types A-E) and was produced in the late 1950s. At that time, full knowledge of the neurotoxin was not available. For type A, the preparation contains only about 10% neurotoxoid (6), and similar values are to be expected for the other types. This toxoid produces sustained measurable antibody titers only after a series of four injections over a period of 1 year. Mild side reactions, including itching, tenderness, redness, heat, and swelling at the site of injection, are common (9).

In an effort to produce a highly purified neurotoxoid from all seven types of *Clostridium botulinum*, we have been investigating the growth and nutritional conditions required by the organism for the maximum synthesis of high-potency toxins. For these studies, we have employed a fermentor system, in contrast to previous workers who have used statically grown cultures (2-4). The advantages of the fermentor include precise, continuous measurement and control of temperature, pH, oxidation-reduction potential (Eh), and rates of agitation and sparging. In the present investigation, studies were conducted to determine the time of appearance and the quantity of toxin produced by the Hall strain of *C. botulinum* type A when cultured under various conditions. A preliminary report of portions of this study has been presented (L. S. Siegel and J. F. Metzger, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, P14, p. 213).

MATERIALS AND METHODS

Microorganism. The microorganism used was the Hall strain of *C. botulinum* type A, obtained from Charles L. Hatheway, Center for Disease Control, Atlanta, Ga.

Growth medium. The medium employed in all fermentor studies consisted of 2.0% casein hydrolysate (N-Z amine, NAK; Humko Sheffield Chemical Co., Memphis, Tenn.) and 1.0% yeast extract (Difco Laboratories, Detroit, Mich.), adjusted to pH 7.3 before being autoclaved for 30 min. After this mixture had cooled, an appropriate concentration of filter-sterilized glucose was added aseptically. This complex medium was used because such media have been reported to support approximately 10-fold more toxin production than chemically defined media (5). The addition of 0.1% thioglycolate to the medium did not affect growth or toxin production.

Preparation of inocula. Stock inocula were prepared by freezing 2.0-ml portions of 24-h cultures grown in cooked meat medium (Difco) and storing them at −70°C. A separate stock culture was used for each experiment. The inoculum for the fermentor was prepared by inoculating a thawed stock culture into 100 ml of cooked meat medium and, after 24 h of incubation, decanting the liquid portion into 2 liters of fermentor medium. After 14 to 18 h of incubation, this culture was used to inoculate the fermentor. Cultures used as inocula were grown under static conditions at a temperature corresponding to that of the fermentor (usually 35°C). No special precautions were employed to achieve anaerobic conditions.

Cultivation. A 70-liter jacketed fermentor (model IF 70; New Brunswick Scientific Co., New Brunswick, N. J.), operated with a liquid volume of 50 liters, was used in these studies. The fermentor is equipped with an exhaust air incinerator (model CN-10, New Brunswick Scientific Co.), as well as instrumentation for sterilization and for control of temperature, gas flow, agitation, Eh, pH, and foaming. The temperature, Eh and pH were continuously measured and recorded. The pH was regulated in certain experiments by the automatic addition of 4 N NaOH via the pH controller.

In all fermentor studies, automatic foam control was achieved by use of a commercial antifoam preparation (Antifoam 60; Harwick, Inc., Akron, Ohio). Fermentations were sampled for the determination of culture growth and toxin production via a sampling port on the side of the tank.

Determination of bacterial growth. Bacterial growth was followed by measuring the optical density of the culture or an appropriate dilution at 540 nm with a Coleman Junior II spectrophotometer (model 6/20; Coleman Instruments Division, Oak Brook, Ill.). Readings were made against a blank of a corresponding dilution of sterile medium.

Toxin assay. At timed intervals, the concentration of toxin in the culture fluid was determined. Removal of cells from the culture fluid by use of membrane filtration (Nalgene filter unit, 0.20-μm porosity) was precluded by the apparent adsorption of the toxin to the filters. Therefore, culture samples were centrifuged at 10,000 × *g* for 10 min at 4°C. The supernatant was decanted, and the pellet was discarded. Samples were tested immediately or were stored at 4°C. For assay, samples were diluted in cold gel-phosphate buffer (0.2% gelatin, 0.4% dibasic sodium phosphate, pH 6.2), and 0.5-ml volumes of appropriate serial 10-fold dilutions were injected intraperitoneally into male Swiss mice weighing 16 to 22 g; four mice were used per dilution. After 4 days of observation for deaths, the median lethal dose (LD_{50}) per milliliter was calculated by the method of Reed and Muench (11).

RESULTS

Relationship between bacterial growth and appearance of toxin. The time course of growth and appearance of toxin in the culture fluid is presented in Fig. 1. Fermentation was at 35°C with an agitation rate of 50 rpm and a nitrogen overlay (5 liters/min). The initial glucose concentration was 0.5%.

Growth, as measured by optical density, was exponential for about 6 h. During this period the mean generation time was 76 min. The Eh decreased from the time of inoculation, reaching a minimum at 5 h, and then rose during the course of the experiment. The pH declined to 5.4 and did not increase from this value during the 72-h time period.

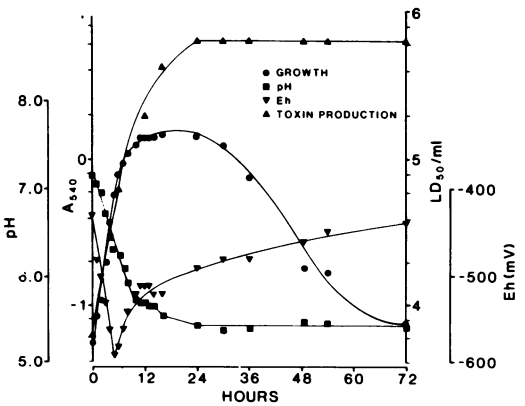

FIG. 1. *Growth, cultural conditions and toxin production of C. botulinum type A, Hall strain. The initial glucose concentration was 0.5%. Fermentation was at 35°C with an agitation rate of 50 rpm and a nitrogen overlay (5 liters/min).*

The amount of toxin in the culture fluid rose steadily during the first 24 h to a maximum of 6.3×10^5 mouse LD_{50}/ml. Continued incubation for up to 72 h did not further increase the toxin concentration. When the growth curve and the toxin curve are examined together, it is evident that the concentration of toxin increased during the logarithmic and stationary phases of growth and that the maximum toxin value was attained before the cells had lysed to an appreciable extent.

Rates of agitation and nitrogen sparging or overlay. The effects of different rates of nitrogen sparging of the culture versus a nitrogen overlay, in conjunction with the rate of agitation, on culture growth and toxin production were examined (Fig. 2). The initial glucose concentration was 0.5%, and the temperature was 35°C. In all studies, maximum growth was obtained in 16 h, and lysis was essentially complete in 72 h. With the nitrogen overlay and agitation of 50 rpm, the maximum toxin concentrations (6.3×10^5 LD_{50}/ml) were reached in 24 h, and prolonged fermentation, up to 126 h, did not increase toxicity. When nitrogen sparging at 5 liters/min and agitation of 50 rpm, or 10 liters/min and agitation of 100 rpm, were used, the appearance of toxin was delayed, and toxin values subsequently decreased during prolonged incubation. Sparging with CO_2 at a rate of 1 liter/min gave results similar to those obtained with nitrogen sparging at 5 liters/min (data not shown).

Glucose concentration. The growth curves for fermentations in which glucose concentrations of 1.5, 1.0, 0.5, and 0.25% were used, as well as for no added carbohydrate, are depicted in Fig. 3. All fermentations were at 35°C with an agitation rate of 50 rpm and nitrogen sparging at 5 liters/min. Growth was dependent on glucose concentration up to 1.0%. Significant lysis occurred only with 0.5% glucose. During growth under these conditions, the pH of the culture medium was monitored (Fig. 4). Maximum acidity (pH 5.5) developed in 15 h in cultures supplemented with 0.5, 1.0, and 1.5% glucose. However, with 0.25% glucose, the pH decreased only to 6.1 and then increased to 7.4 at 35 h. In the absence of carbohydrate, the pH fell to 6.5 and subsequently increased to 7.7 at 36 h.

The amount of toxin in the culture fluid (Fig. 5) was identical in cultures supplemented with 1.0 and 1.5% glucose, with the maximum concen-

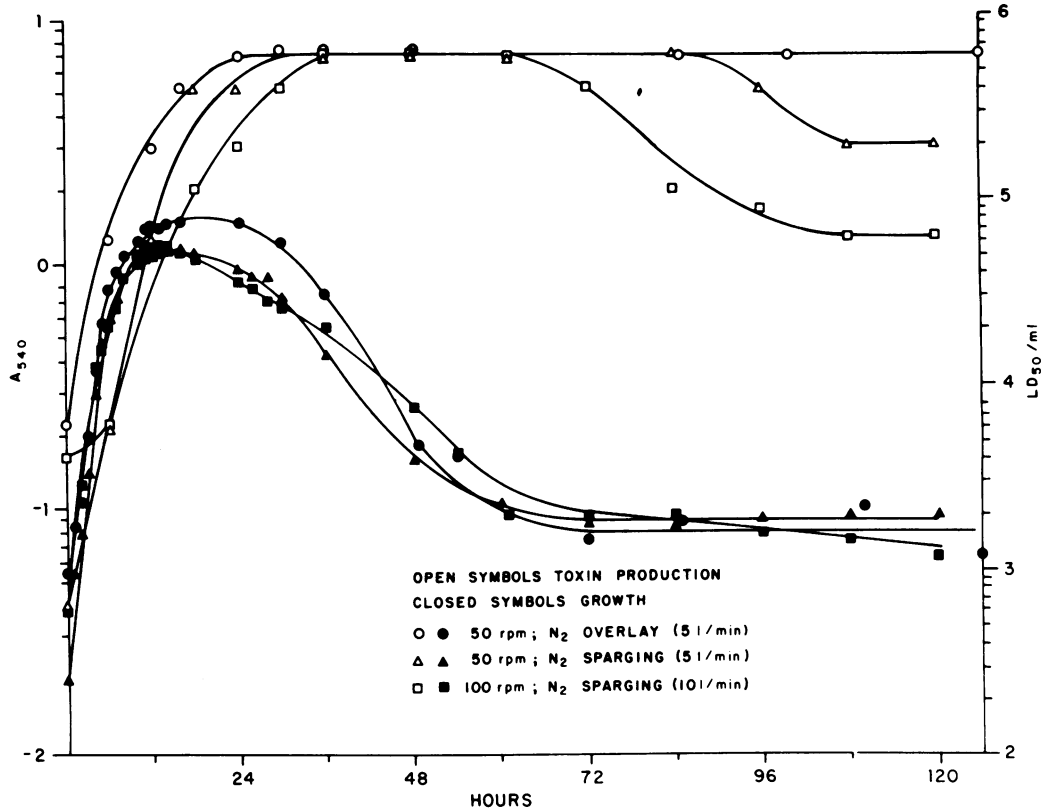

FIG. 2. *Effect of agitation and nitrogen sparging or overlay on growth and toxin production by C. botulinum type A, Hall strain. The initial glucose concentration was 0.5% and the temperature was 35°C.*

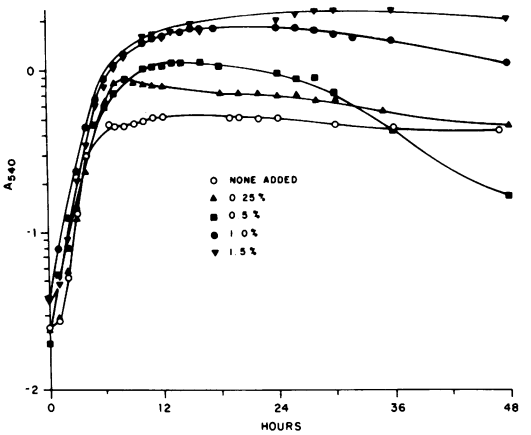

FIG. 3. *Effect of glucose concentration on growth of C. botulinum type A, Hall strain. Fermentations were all at 35°C with an agitation rate of 50 rpm and nitrogen sparging at 5 liters/min.*

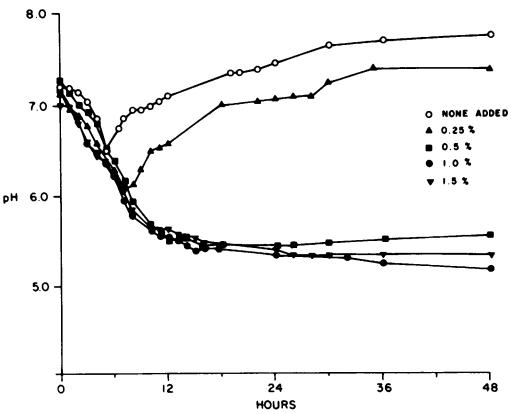

FIG. 4. *Effect of glucose concentration on the pH of the culture medium during growth of C. botulinum type A, Hall strain.*

trations occurring in 24 h. When 0.5% glucose was used, maximum concentrations occurred in 30 h. In cultures supplemented with 0.25% glucose and in those with no additional carbohydrate, considerably less toxin was produced, and this was apparently inactivated.

After 8 h of growth in medium initially supplemented with 1.0% glucose, an addition of glucose was made to the culture to yield a fur-

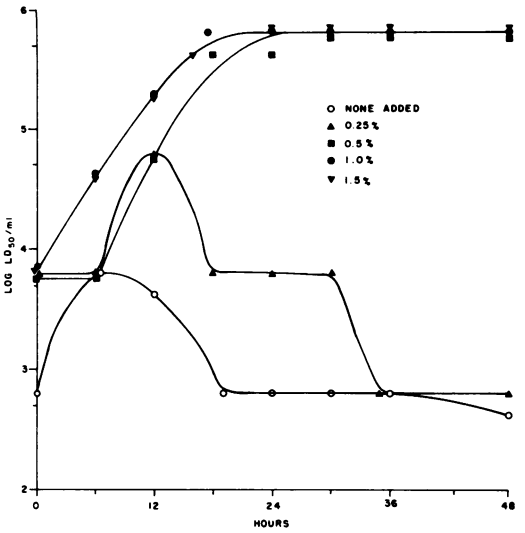

FIG. 5. *Effect of glucose concentration on toxin production by C. botulinum type A, Hall strain.*

ther 1.0% glucose. This procedure did not increase toxin concentrations beyond those obtained with 1.0% glucose only (data not shown).

pH control. The possibility of increasing toxin yield by controlling the pH of the culture was also investigated (Fig. 6). The pH (which after inoculation was 7.1) was uncontrolled until pH 6.0 was reached, which occurred after approximately 8 h of growth. The pH was then maintained at 6.0 for the duration of the experiment. Fermentations with and without pH control were at 35°C with an agitation rate of 50 rpm and nitrogen sparging at 5 liters/min. The initial glucose concentration was 1.0%. The growth rate was unaffected by pH control, and, as shown in Fig. 6, concentrations of toxin were not increased beyond 6.3×10^5 LD_{50}/ml. However, these conditions did serve to maintain maximum toxin concentrations with time.

Temperature. The effect of temperature on growth and toxin production was examined in the range from 30 to 45°C. In all studies, the agitation rate was 50 rpm with a nitrogen overlay (5 liters/min). The initial glucose concentration was 0.5%. Growth occurred at all temperatures tested, but 40°C was apparently optimal (Fig. 7A). However, of the temperatures tested, the optimum for toxin production was 35°C (Fig. 7B), with maximum toxin concentrations attained in 24 h. Incubation at 45°C dramatically reduced toxin production.

DISCUSSION

For the production of quantities of toxin, strains of *C. botulinum* type A have previously been grown in static cultures incubated for 4 or 5 days (1, 8, 12, 13). In the studies reported here, in which a fermentor system was used, maximum yields of toxin (6.3×10^5 mouse LD_{50}/ml) were attained within 24 h. Fermentor conditions for optimal toxin production were as follows: a nitrogen overlay at 5 liters/min with an agitation rate of 50 rpm (Fig. 2) at 35°C (Fig. 7B) without pH control (Fig. 6).

The nutritional requirements of *C. botulinum* type A for toxin production have been examined in static cultures (4, 10) but remain to be investigated in a fermentor system. In the studies described here, a medium composed of 2.0% casein hydrolysate and 1.0% yeast extract was used, and maximum toxin yields were attained in 24 h in cultures supplemented with 1.0 and 1.5% glucose and in 30 h with 0.5% glucose (Fig. 5). In cultures containing 0.25% glucose and in those to which no additional carbohydrate was added, considerably less toxin was produced, and it was apparently inactivated. This inactivation is most probably due to the rise in pH as shown in Fig. 4, since the toxin is reportedly unstable at pH above 6.8 (3). In contrast, Bonventre and Kempe (2) reported that glucose concentrations as low as 0.1% allowed for maximum toxin production if the incubation of the cultures was sufficiently prolonged for complete autolysis to occur. They also noted that growth of *C. botu-*

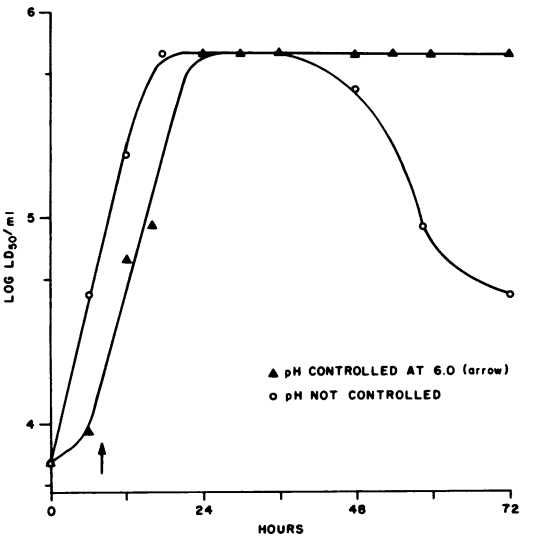

FIG. 6. *Effect of pH control on toxin production by C. botulinum type A, Hall strain. The initial glucose concentration was 1.0%. Both fermentations were at 35°C with an agitation rate of 50 rpm and nitrogen sparging at 5 liters/min. After 8 h of growth (arrow), the pH was controlled at 6.0. In the control experiment the pH was not controlled.*

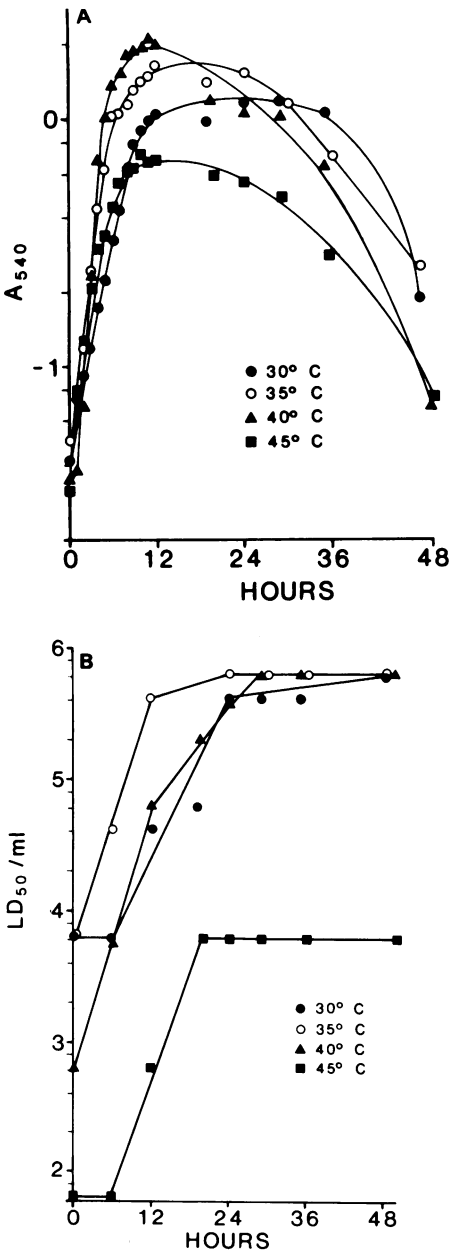

FIG. 7. *Effect of temperature on growth and toxin production of C. botulinum type A, Hall strain. The initial glucose concentration was 0.5%. The agitation rate was 50 rpm with a nitrogen overlay at 5 liters/min. (A) Growth. (B) Toxin production.*

linum in the absence of carbohydrate reduced the toxicity of the culture fluid by 1,000-fold (2), whereas in the present study the omission of glucose from the medium decreased toxicity by a factor of 100 (Fig. 5). Of the concentrations of glucose tested, significant lysis occurred only with 0.5% (Fig. 3). Bowers and Williams have reported that the lysis of strain 62A of type A occurred earlier and to a greater extent in the presence of 0.5% glucose than with 1.0 or 1.5% glucose (7).

Previous investigators have maintained that autolysis is the mechanism by which toxin is liberated from *C. botulinum* type A (3, 4). However, cell lysis is apparently not required to obtain maximum toxin concentrations in the culture fluid when this organism is grown under the fermentation conditions described. As shown in Fig. 1, toxin values reached the maximum before the culture had lysed to an appreciable extent. Autolysis occurred by 72 h, but prolonged incubation for up to 126 h did not further increase toxicity beyond the levels at 24 h (Fig. 2).

Discrepancies between the data obtained with static cultures and with the fermentor may be due to difficulties in obtaining representative samples with static cultures. Mixing of static cultures in preparation for sampling may introduce sufficient oxygen to inhibit the growth of *C. botulinum* (3, 10). In addition, variations in toxin production may be attributed to differences in medium composition. Some inconsistencies may also be ascribed to strain differences.

ACKNOWLEDGMENTS

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