

Effect of Fermentation Conditions on Toxin Production by *Clostridium botulinum* Type B

LYNN S. SIEGEL* AND JOSEPH F. METZGER

United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Maryland 21701

To obtain high yields of toxin for the preparation of purified neurotoxoids, we examined the time of appearance and the quantity of toxin produced by the Bean strain of *Clostridium botulinum* type B under various conditions by using a fermentor system. The medium employed consisted of 2.0% casein hydrolysate and 1.5% yeast extract plus an appropriate concentration of glucose. The maximum toxin concentration (4×10^6 to 5×10^6 mouse median lethal doses per ml) was attained within 48 h under the following fermentation conditions: an initial glucose concentration of 0.5 or 1.0%, a temperature of 35°C, a nitrogen overlay at a rate of 5 liters/min, and an agitation rate of 50 rpm.

There are eight types of *Clostridium botulinum*, designated A, B, C_α, C_β, D, E, F, and G, each producing an immunologically distinct neurotoxin. Because the symptoms of botulism are caused solely by the toxin, immunity to the toxin protects against the disease.

The toxoid currently employed for human immunization against botulism is derived from Formalin-inactivated types A, B, C, D, and E toxins and was manufactured under contract to the U.S. Army in 1958. This product elicits sustained measurable antibody levels only after a series of four injections over a period of one year (8). Mild side reactions (itching, tenderness, redness, heat, and swelling at the site of injection) are common (6). For type A, the preparation contains only about 10% neurotoxoid (3); similar low percentages of purity are likely for the other types. A new product, derived from highly purified neurotoxins and including types F and G, is required.

To prepare a purified neurotoxoid suitable for human immunization, methods must be developed to prepare large quantities of toxin. Therefore, the growth and nutritional conditions required by the organism for the maximum production of toxin are being investigated using a fermentor system. This paper describes the results of such studies with the Bean strain of *C. botulinum* type B.

(Portions of these data were presented at the Annual Meeting of the American Society for Microbiology, May 11-16, 1980 [L. S. Siegel and J. F. Metzger, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, P7, p. 190]).

MATERIALS AND METHODS

The Bean strain of *C. botulinum* type B was obtained from Charles L. Hatheway, Center for Disease Control, Atlanta, Ga.

A complex medium was used in all fermentor studies because such media have been reported to support approximately 10-fold more toxin production than do chemically defined media (2). The medium employed consisted of 2.0% casein hydrolysate (N-Z amine, NAK; Humko Sheffield Chemical Co., Memphis, Tenn.) and 1.5% yeast extract (Difco Laboratories, Detroit, Mich.) and was adjusted to pH 7.0 before being autoclaved for 30 min. After this mixture had cooled, an appropriate concentration of filter-sterilized glucose was added aseptically.

C. botulinum type B was grown in a 70-liter jacketed fermentor (model IF 70; New Brunswick Scientific Co., New Brunswick, N.J.), operated with a liquid volume of 50 liters. The methods used in the preparation of inocula, cultivation with the fermentor system, and determination of bacterial growth have been described (10).

The concentration of toxin in the culture fluid was determined at timed intervals by using the mouse bioassay (10). Male Swiss mice were injected intraperitoneally with 0.5 ml of serial twofold dilutions of the sample; four mice were used per dilution. After 4 days of observation for deaths, the median lethal dose (LD₅₀) per milliliter was calculated by the method of Reed and Muench (9).

RESULTS

Bacterial growth and appearance of toxin. Growth of *C. botulinum* type B and appearance of toxin in the culture fluid were examined over a time course of 120 h (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 5 h (mean generation time, 64 min). Maximum growth was obtained in 12 h. The culture then lysed, followed by a resumption of growth, and a long stationary phase. The oxidation-reduction potential (E_h), recorded at the pH of the culture, decreased from the time

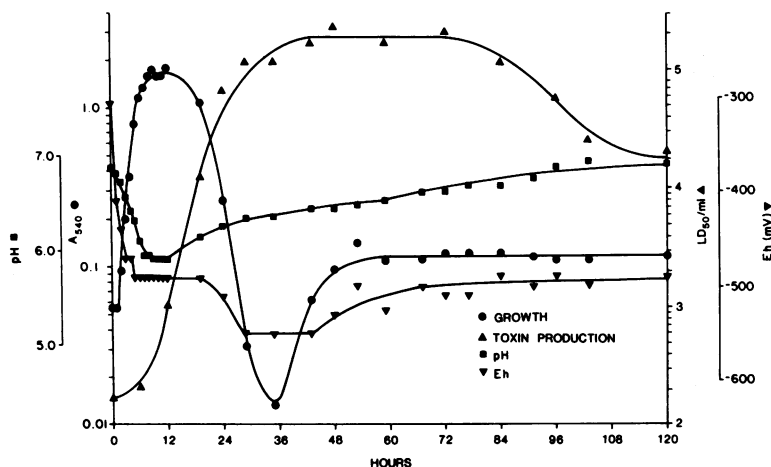


FIG. 1. Growth, toxin production, and cultural conditions of *C. botulinum* type B, Bean strain. Fermentation was at 35°C with a nitrogen overlay (5 liters/min) and an agitation rate of 50 rpm. The initial glucose concentration was 0.5%.

of inoculation until 5 h, remained constant from 5 to 19 h, and then declined to reach a minimum at 29 h. It remained at this minimum value until 43 h and then increased during the course of the experiment. The pH, which was not regulated, declined to a minimum of 5.90 at 9 h. After 12 h, the pH rose gradually for the remainder of the time period studied.

The amount of toxin in the culture fluid increased during the first 48 h to a maximum of 2.5×10^5 mouse LD₅₀/ml. Toxin accumulation was not augmented by continued incubation. As shown in Fig. 1, the toxin concentration in the culture fluid decreased with time after 72 h. When the growth and toxin curves are examined together, it is evident that the maximum concentration of toxin was attained after lysis of the cells had occurred (Fig. 1).

Nitrogen sparging or overlay and carbon dioxide sparging. The effects of sparging with nitrogen or with CO₂ versus a nitrogen overlay on culture growth and toxin production are depicted in Fig. 2. In these studies, the agitation rate was 50 rpm, the temperature was 35°C, and the initial glucose concentration was 0.5%. Maximum growth was obtained within 12 h in all cases, followed by lysis at various rates (Fig. 2A). The maximum toxin concentrations (4×10^5 LD₅₀/ml) were reached in 48 h by using the nitrogen overlay at a rate of 5 liters/min (Fig. 2B). Sparging of the culture with nitrogen at 5 liters/min or with CO₂ at 1 liter/min resulted in decreased toxin accumulation (Fig. 2B).

Temperature. The effect of temperatures in the range from 25 to 40°C on growth and toxin production was determined (Fig. 3). In all fermentations, the initial glucose concentration was

0.5%. The agitation rate was 50 rpm with a nitrogen overlay (5 liters/min). Growth occurred at all temperatures tested, but 40°C was optimal (Fig. 3A). However, of the temperatures tested, the optimum for toxin production was 35°C, with maximum toxin concentrations being attained in 48 h (Fig. 3B). Toxin production was curtailed when an incubation temperature of 25 or 40°C was employed.

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 that for no added carbohydrate, are shown in Fig. 4A. In all studies, the agitation rate was 50 rpm, with a nitrogen overlay at 5 liters/min and a temperature of 35°C. With 0.25, 0.5, and 1.0% glucose, significant lysis of the culture occurred. Increasing the glucose concentration delayed the time at which lysis of the culture began.

Toxin production was similar in cultures supplemented with 0.5 and 1.0% glucose, with maximum toxin concentrations occurring in 48 h (Fig. 4B). Cultures supplemented with 0.25 and 1.5% glucose produced less toxin. In the absence of added carbohydrate, only low concentrations of toxin were obtained.

DISCUSSION

In the course of these studies, the following optimum conditions were established for the production of toxin by the Bean strain of *C. botulinum* type B cultivated in a fermentor system: (i) a nitrogen overlay at a rate of 5 liters/min with an agitation rate of 50 rpm (Fig. 2); (ii) a temperature of 35°C (Fig. 3); and (iii) a glucose concentration of 0.5 or 1.0% (Fig. 4). Under these conditions, the maximum toxin concentration (4

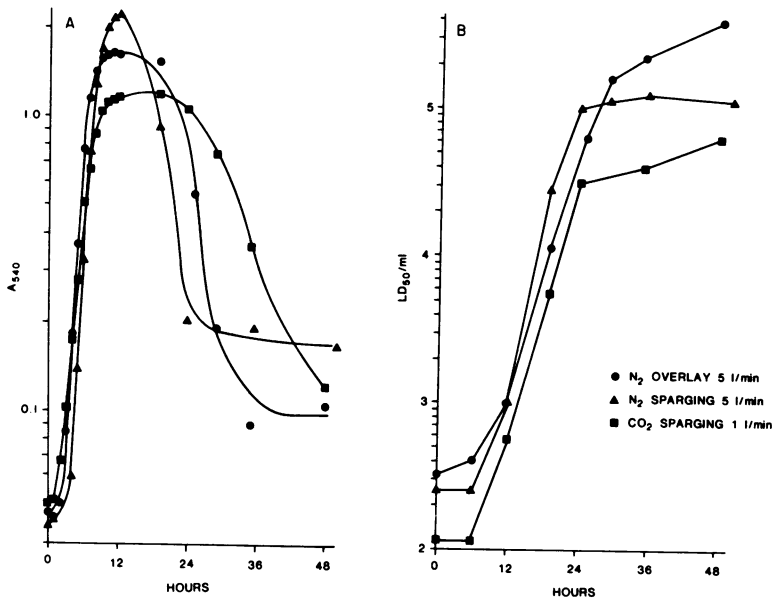


FIG. 2. Effect of nitrogen sparging or overlay and carbon dioxide sparging on growth and toxin production by *C. botulinum* type B, Bean strain. Fermentations were all at 35°C with an agitation rate of 50 rpm and an initial glucose concentration of 0.5%. (A) Growth; (B) toxin production.

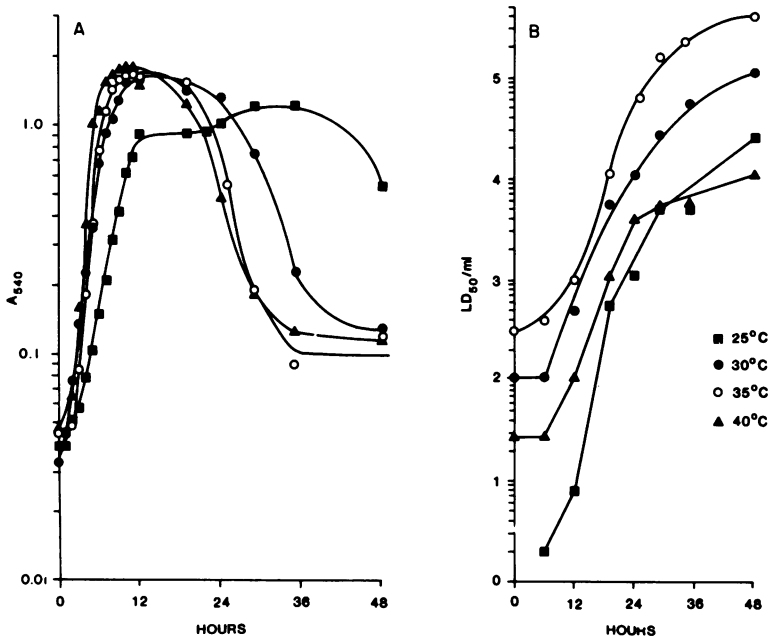


FIG. 3. Effect of temperature on growth and toxin production by *C. botulinum* type B, Bean strain. The agitation rate was 50 rpm with a nitrogen overlay (5 liters/min). The initial glucose concentration was 0.5%. (Cultures used as inocula were grown under static conditions at a temperature corresponding to that of the fermentor.) (A) Growth; (B) toxin production.

$\times 10^5$ to 5×10^5 mouse LD₅₀/ml) was attained within 48 h. In preliminary studies, the Okra strain of type B produced 1×10^6 to 2×10^6

LD₅₀/ml in 30 h when cultivated in this manner. Previously, strains of type B were grown in static cultures for toxin production, with incubation

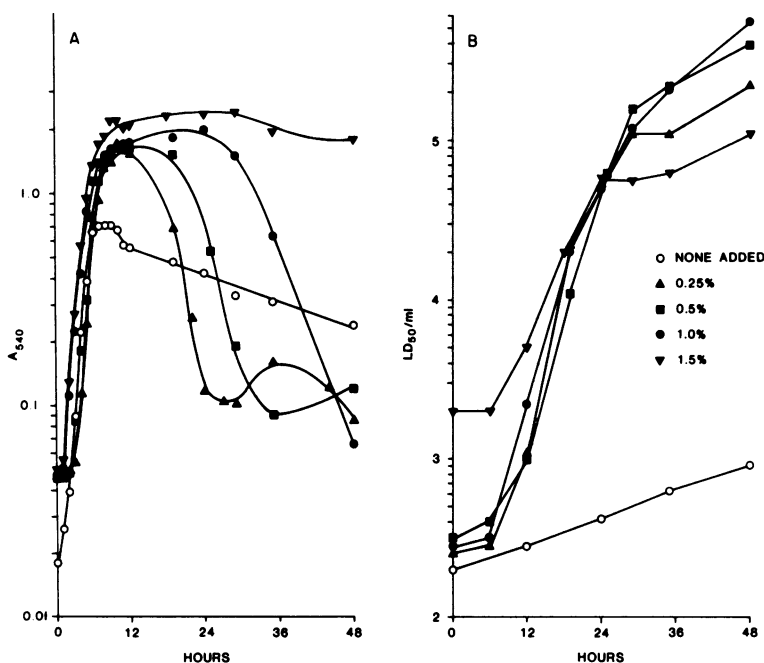


FIG. 4. Effect of glucose concentration on growth and toxin production by *C. botulinum* type B, Bean strain. All fermentations were at 35°C with an agitation rate of 50 rpm and nitrogen sparging at 5 liters/min. (A) Growth; (B) toxin production.

times of from 2 to 3 days up to 10 days reportedly being necessary to obtain maximum toxin concentrations (1, 4, 5, 7).

High yields of toxin (6.3×10^5 mouse LD_{50}/ml) have been obtained with the Hall strain of type A cultivated in a fermentor system (10). The conditions required by this strain for maximum toxin production (10) are identical to those reported here for the Bean strain of type B. With type A (Hall), the maximum toxin concentration was attained in 24 h (10). In contrast, 48 h of incubation is required for the highest toxin yields with type B.

ACKNOWLEDGMENTS

We thank Lisa Heiges and Sam Kulinski for expert technical assistance.

LITERATURE CITED

1. Beers, W. H., and E. Reich. 1969. Isolation and characterization of *Clostridium botulinum* type B toxin. *J. Biol. Chem.* **244**:4473-4479.
2. Boroff, D. A., and B. R. DasGupta. 1971. Botulinum toxin, p. 1-68. In S. Kadis, T. C. Montie, and S. J. Ajl (ed.), *Microbial toxins*, vol. IIA. Academic Press, Inc., New York.
3. Boroff, D. A., H. P. Meloche, and B. R. DasGupta. 1970. Amino acid analysis of the isolated and purified components from crystalline toxin of *Clostridium botulinum* type A. *Infect. Immun.* **2**:679-680.
4. DasGupta, B. R., and H. Sugiyama. 1976. Molecular forms of neurotoxins in proteolytic *Clostridium botulinum* type B cultures. *Infect. Immun.* **14**:680-686.
5. Duff, J. T., J. Klerer, R. H. Bibler, D. E. Moore, C. Gottfried, and G. G. Wright. 1957. Studies on immunity to toxins of *Clostridium botulinum*. II. Production and purification of type B toxin for toxoid. *J. Bacteriol.* **73**:597-601.
6. Flock, M. A., M. A. Cardella, and N. F. Gearinger. 1963. Studies on immunity to toxins of *Clostridium botulinum*. IX. Immunologic response of man to purified pentavalent ABCDE botulinum toxoid. *J. Immunol.* **90**:697-702.
7. Lamanna, C., and H. N. Glassman. 1947. The isolation of type B botulinum toxin. *J. Bacteriol.* **54**:575-584.
8. Metzger, J. F., and G. E. Lewis, Jr. 1979. Human-derived immune globulins for the treatment of botulism. *Rev. Infect. Dis.* **1**:689-690.
9. Reed, L. J., and H. Muench. 1938. A simple method of estimating fifty percent endpoints. *Am. J. Hyg.* **27**:493-497.
10. Siegel, L. S., and J. F. Metzger. 1979. Toxin production by *Clostridium botulinum* type A under various fermentation conditions. *Appl. Environ. Microbiol.* **38**:606-611.