APPLIED MICROBIOLOGY, Jan. 1968, p. 69-72

Vol. 16, No. 1
Printed in U.S.A.

# Continuous Production of *Clostridium tetani* Toxin

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Received for publication 26 July 1967

The continuous production of *Clostridium tetani* toxin has been carried out in a 1-liter stirred culture vessel for as long as 65 days. Toxin production of approximately 120 flocculating units per ml was maintained with a dilution rate of 0.125 $hr^{-1}$, a temperature of 34 C, a *p*H of 7.4, and the addition to the medium of 0.1 g of potassium chloride per liter. The average minimal lethal intraperitoneal dose of the toxin in mice was approximately $10^6$ per ml.

The production of tetanus toxin was greatly facilitated by the isolation of the highly toxigenic Harvard strain and the use of a suitable medium for this strain, as reported by Mueller and Miller (8, 9). However, the conventional method of toxin production (in cotton-stoppered glass vessels of a rather small volume), which has been used for many years, is cumbersome and uneconomical. The dialysis culture techniques of Feeney et al. (1) and Koch and Kaplan (6) produce high yields of toxin, but attempts to scale up the volume in this production process have encountered great technical difficulties. Hepple (2) and Nielsen (11) reported on the successful use of large stirred tanks for single-batch tetanus toxin production, and Thompson (15) developed a semicontinuous system for large-scale toxin production. Thompson also attempted a completely continuous process, but he reported that the latter procedure gave a low toxin yield compared with his semicontinuous method and that his strain appeared to lose toxigenicity during long-term cultivation. Another unsuccessful attempt to produce tetanus toxin in continuous culture was made by Nielsen (10).

## MATERIALS AND METHODS

A modification of the 1-liter fermentor described by Holme and Zacharias (3) was used. The modifications consist of a magnetic transmission for stirring fastened to the bottom of the vessel and inlets for the supply of medium, acid, and alkali as described by Holmström and Hedén (4).

The temperature was controlled by a regulating thermometer which, when the temperature was lower than a preset value, gave an impulse to a transistorized relay that regulated a heating tape (Electrothermal, P.V.C. Insulated Waterproof Pipe Heater, 240 v, 44 w, No. HP 4400) wrapped round the fermentor. No cooling device was introduced in this vessel. The *p*H control system has been described by Holmström and Hedén (4); it was, however, modified to control two hose-pressure pumps, one of which was used for acid additions and the other for alkali additions. When the *p*H deflected from a set value, an impulse from the regulating instrument set off the appropriate pump, which continued to operate until the *p*H had been adjusted.

*Cultivation. Clostridium tetani* strain 107 was received from Dr. Steiskal (Institute of Sera and Vaccines, Prague, Czechoslovakia). This is a derivative of the Harvard strain of Mueller and Miller. When grown batch-wise, this strain produced an amount of toxin corresponding to 50 to 70 flocculating units per ml.

The culture was stored in a lyophilized state. Inoculum was grown in thioglycolate broth (Thioglycolate medium, Brewer, Oxoid Ltd., England). The medium used for toxin production was basically the same as that described by Latham et al. (7); the concentration of all ingredients was, however, reduced by half and Tryptone T (Oxoid Ltd., England) was substituted for N-Z-case (8, 9).

The composition of the culture medium (LL2), per liter of deionized water, was as follows: glucose, 5.0 g, sodium chloride, 1.25 g; magnesium sulfate, 0.05 g; cystine, 0.063 g; Tryptone T, 12.5 g; ferrichloride, 16 mg; thiamine, 0.13 mg; riboflavine, 0.13 mg; pyridoxine, 0.13 mg; calcium pantothenate, 0.5 mg; uracil, 0.63 mg; nicotinic acid, 0.13 mg; biotin, 1.3 $\mu$g; and vitamin $B_{12}$, 0.025 $\mu$g.

All ingredeints were dissolved in deionized water, and the *p*H was adjusted to 7.4 by the addition of 1 M sodium hydroxide. After autoclaving, 0.025 g of streptomycin (Strepsulfat, Kabi, Sweden) per liter was added. The *p*H was controlled by 1 M solutions of acetic acid and sodium hydroxide. Commercial nitrogen (99.8% $N_2$) was used for gassing the cultures.

The *p*H electrode was sterilized chemically for 4 hr in a solution containing 75% ethyl alcohol, 0.5% concentrated sulfuric acid, and distilled water. The media and all other equipment were autoclaved. After sterilization and assembly of the system, the culture vessel, containing 800 ml of medium, was inoculated with 20 ml of an 18-hr bacterial culture. The fermentation was run as a batch culture for 4 days before the continuous-flow system was started.

*Analytical methods.* A chilled sample was collected each day. The dilution rate ($D$) was calculated from the amount of culture collected during 1 hr ($F$) and the volume of the culture ($V$), according to the formula: $D = F/V$, $hr^{-1}$. A 60-ml amount of the collected sample was drawn off, divided into equal halves, and centrifuged. Bacterial cells were washed twice with distilled water and dried at 105 C for dry weight determinations. Supernatant fluids were filtered through a membrane filter (0.45 $\mu$; Millipore Corp., Bedford, Mass.). Glucose was analyzed by the method of Hultman (5) and Redei and Nagy (14). Estimations of flocculating units (Lf/ml) were carried out on the supernatant fluids by the method of Ramon (12). An antiserum containing 900 Lf/ml (kindly supplied by the State Bacteriological Laboratories, Sweden) was used as a standard. The samples containing a considerable amount of toxin and the corresponding antisera were diluted with a Sörensen buffer at *p*H 7.2. The minimal lethal dose (MLD/ml) in mice was determined intraperitoneally.

The *p*H of the samples was measured and compared with values given by the *p*H control system. If agreement was not obtained, the *p*H control system was adjusted.

Each sample was streaked on blood-agar and nutrient agar and incubated aerobically for 24 hr at 37 C. A Gram-stained sample was examined microscopically. If no growth was obtained on the plates and the stained sample contained only rods, the culture was regarded as pure.

## Results and Discussion

Several successful continuous cultures were run with *C. tetani* for 28 to 65 days; each produced an amount of tetanus toxin corresponding to 65 to 70 Lf/ml (average flocculating time, 10 min) and reached a titer of about $10^6$ MLD/ml (Table 1). The influence of *p*H on glucose consumption and production of toxin was investigated in continuous culture (Table 2). No flocculating units were detected in the culture fluid until the glucose was consumed. The optimal *p*H for toxin production was 7.8. A *p*H of 7.4 is, however, recommended, since at 7.8 the culture fluid turned very dark from an iron-containing precipitate which interfered with dry weight determinations. It is also possible to adjust the *p*H of the samples to 6.0, at which level the precipitate dissolves without loss of flocculating activity.

TABLE 1. *Tetanus toxin production in continuous cultures of Clostridium tetani strain 107*[a]

| Expt no. | Duration of continuous cultivation | Avg toxin | Avg minimal lethal dose |
|---|---|---|---|
| | *days* | *Lf/ml* | *Mld/ml* |
| 1 | 30 | 65 | $6 \times 10^5$ |
| 2 | 28 | 65 | $8 \times 10^5$ |
| 3 | 45 | 70 | $2 \times 10^6$ |
| 4 | 56 | 70 | $3 \times 10^6$ |
| 5 | 65 | 70 | $1 \times 10^6$ |

[a] Dilution rate was maintained at about 0.045 $hr^{-1}$, *p*H at 7.4, and temperature at 37 C. Toxin titer (Lf/ml) was established by flocculation with tetanus antiserum.

TABLE 2. *Influence of pH on residual glucose and flocculating units in the supernatant fluid of a continuous culture of Clostridium tetani strain 107*[a]

| *p*H | Residual glucose | Avg toxin |
|---|---|---|
| | *g/liter* | *Lf/ml* |
| 5.9 | 2.6 | 0 |
| 6.2 | 0 | 40 |
| 7.0 | 0 | 60 |
| 7.8 | 0 | 80 |
| 8.8 | 0 | 60 |
| 9.5 | 2.0 | 0 |

[a] Dilution rate was maintained at 0.074 $hr^{-1}$ and temperature at 37 C. The *p*H was altered every 5th day. Average flocculation time was 12 min.

When the *p*H of the continuous culture stabilized itself at the same value as that of the medium pumped into the culture, no regulation of *p*H was needed. To determine whether the lack of toxin production at the lower and the higher *p*H values (Table 2) was due to inhibition of the production of toxin by the bacteria or to destruction of the released toxin, culture fluid containing 60 Lf/ml was divided into three equal portions. The *p*H of one was adjusted to 5.9 with acetic acid, and the second and third portions were adjusted to *p*H 7.8 and 9.5, respectively, with sodium hydroxide. Solutions were kept for 36 hr under the same conditions as the unadjusted culture fluid. No decrease in flocculating units was detected. It was therefore assumed that either (i) an enzyme system connected with toxin production within the bacterial cell was impaired by higher or lower *p*H values, or (ii) the release system (membranes, etc.) was affected. Experiments designed to elucidate this phenomenon, if possible, are now in progress.

The influence of temperature on toxin production was also studied in continuous culture. In the temperature range of 32 to 39 C, the optimal production of flocculating units (80 Lf/ml) occurred at 34 C (Table 3). The flocculating reaction also appeared more distinct with toxins produced at the lower temperatures (32 to 34 C). The average flocculating time was 8 min, and the temperature was altered every 6th day.

The effect of dilution rates on dry weight, glucose consumption, and toxin production was

determined in continuous culture. Between the rates of 0.030 and 0.125 $hr^{-1}$, dry weight varied slightly, no glucose was detected in the culture fluid, and flocculation was about 70 to 75 Lf/ml. The average flocculating time was 12 min, and the dilution rate was changed after a quantity of medium, corresponding to at least five times the volume of the culture, had passed through the the fermentor (Table 4). Above a dilution rate of 0.125 $hr^{-1}$, glucose appeared in the culture fluid and no flocculating units were found (Table 4). It would seem, from this and other experiments, that toxin was not released from bacteria grown in continuous culture until glucose had become a limiting factor. Experiments with 10 and 2.5 g of glucose per liter have, however, shown the same pattern of toxin release, and no changes in toxin production have been detected.

TABLE 3. *Effect of temperature on production of tetanus toxin in the culture supernatant fluid collected from a continuous culture of Clostridium tetani strain 107*[a]

| Temp | Avg toxin |
|---|---|
| *C* | *Lf/ml* |
| 32 | 65 |
| 34 | 80 |
| 37 | 65 |
| 39 | 45 |

[a] Dilution rate was maintained at 0.045 $hr^{-1}$ and *p*H at 7.4. The temperature was altered every 6th day. Average flocculation time was 8 min.

TABLE 4. *Influence of the dilution rate on dry weight, residual glucose, and tetanus toxin production in the culture fluid collected from a continuous culture of Clostridium tetani strain 107*[a]

| Dilution rate | Dry wt | Residual glucose | Avg toxin |
|---|---|---|---|
| $hr^{-1}$ | *g/liter* | *g/liter* | *Lf/ml* |
| 0.030 | 0.72 | 0.0 | 70 |
| 0.045 | 0.71 | 0.0 | 75 |
| 0.065 | 0.66 | 0.0 | 70 |
| 0.074 | 0.70 | 0.0 | 75 |
| 0.084 | 0.68 | 0.0 | 75 |
| 0.108 | 0.69 | 0.0 | 70 |
| 0.113 | 0.67 | 0.0 | 75 |
| 0.125 | 0.65 | 0.0 | 75 |
| 0.150 | 0.60 | 2.8 | 0 |
| 0.169 | 0.55 | 2.9 | 0 |

[a] The *p*H of the culture was maintained at 7.4 and temperature at 37 C. The dilution rate was changed after a quantity of medium, corresponding to at least five times the volume of the culture, had passed through the fermentor. Average flocculation time was 12 min.

TABLE 5. *Influence of the addition of potassium chloride and calcium chloride on dry weight and tetanus toxin production in a continuous culture of Clostridium tetani strain 107*[a]

| Medium | Dry weight | Avg toxin |
|---|---|---|
| | *g/liter* | *Lf/ml* |
| LL2 | 0.37 | 70 |
| LL2 + 0.1 g of KCl/liter | 0.37 | 130 |
| LL2 | 0.39 | 70 |
| LL2 + 0.15 g of $CaCl_2$ | 0.36 | 40 |
| LL2 | 0.37 | 70 |

[a] Dilution rate was maintained at 0.065 $hr^{-1}$, temperature at 37 C, and *p*H at 7.4. The medium was changed every 4th day. Average flocculation time was 10 min.

Å. Bovallius found that the addition of potassium chloride to the medium increased enzyme production in culture fluids of a *Cytophaga* sp. (*personal communication*), and it is a well-known fact that potassium ions increase the permeability of cell membranes and that calcium ions have the opposite effect. The influence of potassium and calcium choride upon the continuous culture of *C. tetani* was, therefore, investigated in medium LL2 (Table 5). The dilution rate was maintained at 0.065 $hr^{-1}$, the temperature at 37 C, and the *p*H at 7.4; the medium was changed every 4th day. The addition of 0.1 g of potassium chloride per liter almost doubled the amount of flocculating units (130 Lf/ml; average flocculating time, 10 min) in the supernatant fluid, whereas the addition of 0.15 g of calcium chloride per liter caused a considerable decrease (40 Lf/ml). In Raynaud's (16) extraction experiments with tetanus toxin, potassium chloride was inferior to sodium chloride as an extraction agent. An investigation of the extraction ability of different chloride solutions is currently underway.

To confirm the effectiveness of potassium chloride in increasing the yields of tetanus toxin, a continuous culture was run for 30 days at a dilution rate of 0.125 $hr^{-1}$ with a temperature of 34 C, a *p*H of 7.4, and the addition to the medium of 0.1 g of potassium chloride per liter. Glucose consumption, flocculating units, and minimal lethal doses were recorded. When toxin appeared in the culture fluid, no glucose was detected. The flocculating units obtained were 120 $\pm$ 10 Lf/ml, and the average minimal lethal dose per milliliter in mice was just below $10^6$. In a 1-liter fermentor, the daily production of tetanus toxin was calculated to be $3.6 \times 10^5$ Lf.

AC KNOWLEDGMENTS

We are indebted to Henrik Markkula (Research Institute of National Defence, Sweden) for valuable

discussions and to Berndt Malmgren (Karolinska Institutet, Sweden), who so kindly placed laboratory facilities at our disposal at the beginning of this investigation. We should also like to express our gratitude to K. Ullberg-Olsson (Statens Bakteriologiska Laboratorium, Sweden), who was most generous with her advice and assistance.

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