

Medium for Toxin Production by *Clostridium perfringens* in Continuous Culture¹

GRACE CHOU

Department of Microbiology, University of Hong Kong, Hong Kong

Received for publication 16 February 1971

A tryptone-salts medium for continuous growth and alpha toxin production of *Clostridium perfringens* type A in an adapted chemostat is described. In such steady-state cultures, fermentative and biochemical activity of *C. perfringens* remained unchanged. Toxigenic ability to produce alpha, theta, and nu toxins was preserved.

For cultivation of *Clostridium perfringens* in continuous culture, a particle-free fluid medium that supports growth and promotes toxin production is essential. The presence of any particles such as minute pieces of meat obstructs the continuous flow of medium smoothly metered into the culture vessel. In this paper, the finding of a simple medium that serves all the purposes as described is reported.

MATERIALS AND METHODS

Organism. *C. perfringens* type A S107 (NCTC 8237) obtained from the National Collection of Type Cultures, Colindale, London, was used.

Media. In preliminary studies, luxuriant growth of *C. perfringens* was obtained in 1% glucose-meat infusion broth, but alpha-toxin titers were poor in the absence of meat particles. Consequently, the merits of two clear peptone media, tryptone-salts medium and proteose-salts medium, were studied. Both media were initially tested for growth and alpha toxin of *C. perfringens* in static batch cultures. Tryptone-salts medium was subsequently chosen for use in continuous cultivation.

Tryptone-salts medium contained 50 g of tryptone (Oxoid L42), 50 ml of salt solution (see below), and 1.5 ml of 10% calcium chloride in 1 liter of distilled water. For proteose peptone-salts medium, 50 g of proteose peptone (Oxoid 146) was substituted for tryptone.

A 50-g amount of tryptone or proteose peptone was dissolved in 1 liter of distilled water, and 1.5 ml of 0.1% calcium chloride solution was added. After boiling, the peptone solution was filtered through Whatman no. 1 paper. The reaction was adjusted to pH 7.4 and the filtration was repeated.

A 50-ml volume of salt solution was added to each liter of peptone base. Salt solution was prepared by dissolving 10.0 g of Na₂HPO₄, 0.2 g of KH₂PO₄, and 0.08 g of MgSO₄ · 7H₂O per 100 ml of distilled water. The medium was readjusted to pH 7.8 and autoclaved for 30 min at 115 C.

Growth measurement. Growth was measured turbidimetrically in a nephelometer (Evans Electro-selenium Ltd.) with uninoculated medium as a blank.

Cultural characteristics of *C. perfringens*. Procedures used for testing biochemical activities were those described by Willis (9).

Tests for toxigenicity. Alpha, theta, and nu toxigenicity of *C. perfringens* were detected from plate growth on appropriate media. Presence of alpha toxin was judged by lecithinase C activity on lactose-egg yolk agar medium (10); theta toxin was judged by hemolysis produced on horse blood agar and nu toxin by clear zones surrounding colonies on deoxyribonuclease agar (Oxoid CM321) flooded with hydrochloric acid.

Alpha toxin was assayed by a modified tube method of van Heyningen (8). Five drops of crude toxin from a standard dropper were transferred into test tubes (80 by 10 mm), followed by five drops of the respective antitoxin subunits (1.0, 0.9, 0.8, 0.7, 0.65, 0.55, 0.5, 0.45, 0.4, 0.35, 0.3, 0.2, and 0.1) added to each tube except the control. After shaking, the tubes were incubated in a water bath for 30 min at 37 C. Then five drops of egg yolk-saline solution were delivered into all tubes which were shaken and reincubated for an additional 2 hr at 37 C. The tube that contained the highest dilution of antitoxin and still showing turbidity was the end point expressed as the antitoxin combining power (Lb). One Lb unit is defined as the minimal concentration of toxin which, when mixed with one International Unit of antitoxin, will still produce turbidity in the egg yolk solution indicator.

Chemostat. Problems of regulated fluid consumption, medium storage and replenishment, maintenance of a homogeneous culture with facilities for disposal of the effluent, and sterilization of equipment were considered for the construction of a suitable chemostat (Fig. 1). This model for small scale operation functioned efficiently for at least 152 hr. The self-regulatory property of the continuous flow system is evident with the establishment of *C. perfringens* steady-state populations in 0.1% glucose-meat infusion broth at two dilution rates of 0.3 and 0.5 hr⁻¹ (Fig. 2).

Metering of the required nutrients was achieved by

¹ This paper is a portion of a dissertation submitted by G. Chou to the University of Hong Kong for a Master of Science degree.

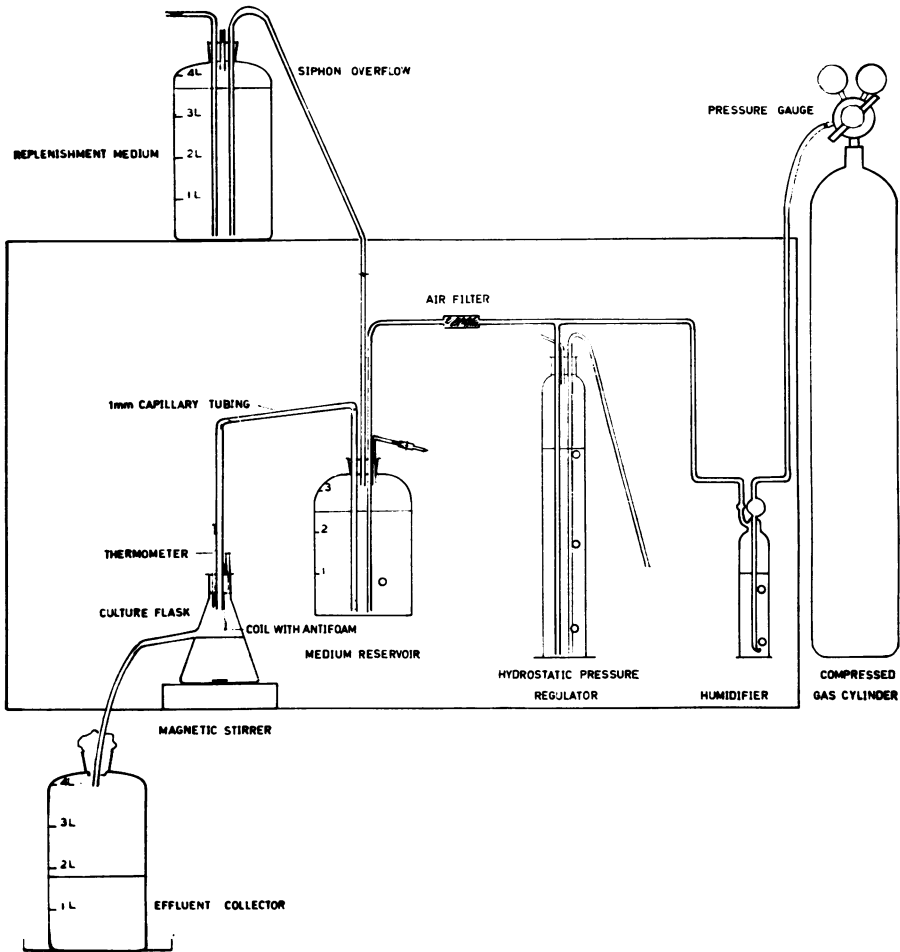


FIG. 1. Diagrammatic representation of chemostatic components for continuous flow culture in an improvised model for small scale operation.

passing nitrogen gas through a humidifier into a hydrostatic pressure regulator with an outlet to the atmosphere. Through a bypass, the saturated gas passed into an air filter to enter the reservoir holding sterile tryptone medium. The gas escaped through the medium into the atmosphere through a tapering air outlet with a screw adjustment.

In a chemostat either the flow rate or the culture volume are permissible variables. Of the two alternatives, change of flow rate is more practicable than change of culture volume. A 250-ml Erlenmeyer flask with a sloping side arm was modified as a cultivator, holding set volumes of 240 or 250 ml of cultural suspension.

Mixing of the bacteria with the inflowing medium was accomplished by a magnetic stirrer. Effluent flowing through polyethylene tubing connected to the side arm of the culture flask passed into a receiving vessel. Alternately, it was received in 25-ml Universal bottles held in packed ice for sampling.

Reserve tryptone-salts medium was stored in 4-liter

recharging bottles fitted with a siphon trap for replenishment of fresh sterile medium into the emptied-medium reservoir bottle. The whole glassware assembly, with the exception of the recharging and discard bottles, was placed in an incubating cabinet.

Method of operation. The assembled equipment was allowed to equilibrate overnight at 37 C.

A young actively growing *C. perfringens* culture (3 ml) in Robertson's cooked meat broth was used as the starter culture. It was added through the barrel of a hypodermic syringe used as an inoculating port inserted into the stopper of the cultivator. A 0.5-ml amount of 0.1% thioglycolic acid accelerated the growth initiation. Silicone antifoam held in a wire coil was suspended above the surface of the inoculated medium to reduce frothing.

Growth proceeded as a batch culture before chemostat operations were started with the admittance of nitrogen and the adjustment of the flow rate.

Static culture. Growth and alpha toxin production of *C. perfringens* type A in static culture were tested

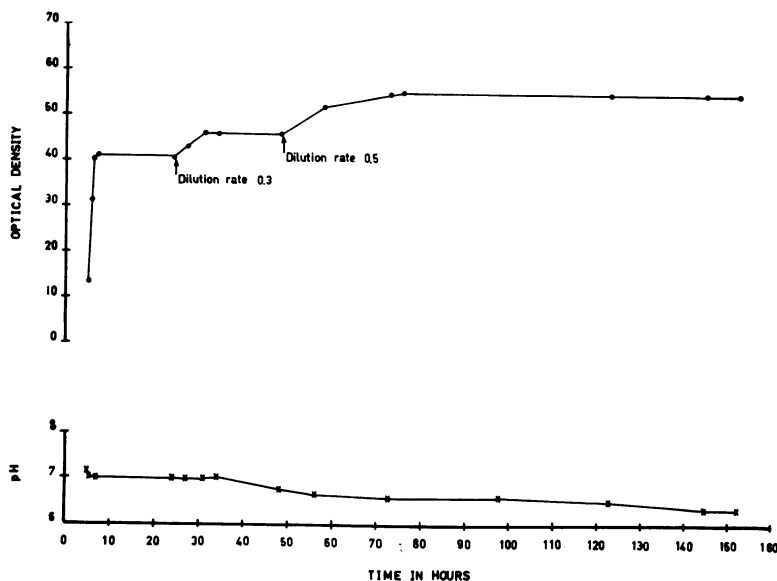


FIG. 2. The establishment of *Clostridium perfringens* type A S107 steady-state populations at dilution rates of 0.3 and 0.5 hr^{-1} with pH values in 0.1% glucose-meat infusion broth.

in two media, viz. 1% glucose-tryptone-salts medium and 1% glucose-proteose peptone-salts medium. A 2-hr seed culture was inoculated to a 300-ml amount of each medium incubated at 37 C. After visible turbidity, 10-ml samples were removed at regular intervals and tested for growth and alpha-toxin titer.

Continuous culture. Pure culture of *C. perfringens* type A was cultivated in 0.1% glucose-tryptone-salts medium. With the attainment of steady-state growth levels, the microscopic appearance, fermentative biochemical properties, and toxigenicity of the strain during continuous cultivation were examined. The strain recovered from the effluent was subcultured in 1% glucose-Robertson's cooked meat medium for titration of alpha toxin together with a stock strain as control.

RESULTS AND DISCUSSION

The amount of growth and alpha-toxin titers produced by *C. perfringens* type A S107 in both 1% glucose-tryptone-salts medium and in 1% glucose-proteose peptone-salts medium in static cultures are plotted in Fig. 3. Tryptone medium supported greater amounts of growth.

In continuous cultivation, 0.1% glucose-tryptone-salts medium supported steady-state growth of *C. perfringens*. Growth levels attained for each adjustment of flow rates from dilution rates of 0.1 to 1.1 hr^{-1} are shown in Fig. 4.

The effluent from three dilution rates, 0.2, 1.1, and 0.1 hr^{-1} , respectively, were plated onto Willis and Hobbs medium (10) incubated at 37 C for 18 hr in an anaerobic jar. On subculture, colonies from the faster growing cells removed

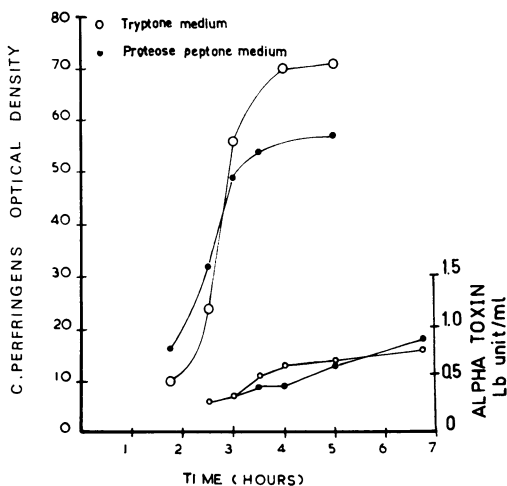


FIG. 3. Growth and alpha-toxin titer of *Clostridium perfringens* type A S107 in 1% glucose-tryptone and 1% glucose-proteose peptone media.

from the chemostat were lactose-positive, circular, entire, smooth, convex, and shiny, whereas the colonial appearance of cells previously adopted to growth at much slower rates of 0.1 and 0.2 hr^{-1} was characteristically irregular and of fernleaf appearance, flat with a central elevation and radial striations.

The principle of steady-state growth inherent in a chemostat is again illustrated with the growth of *C. perfringens* in 0.2% glucose-tryp-

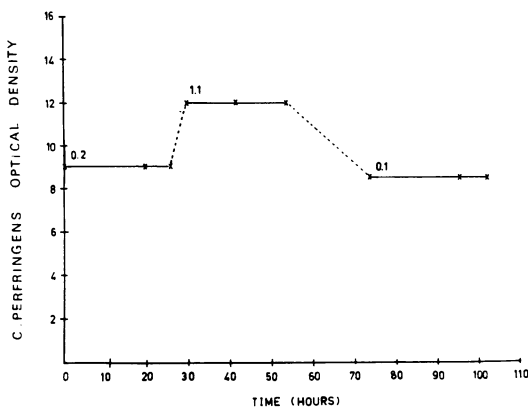


FIG. 4. Steady-state population of *Clostridium perfringens* type A S107 in 0.1% glucose-tryptone-salts medium at dilution rates 0.2, 1.1, and 0.1 hr^{-1} , respectively.

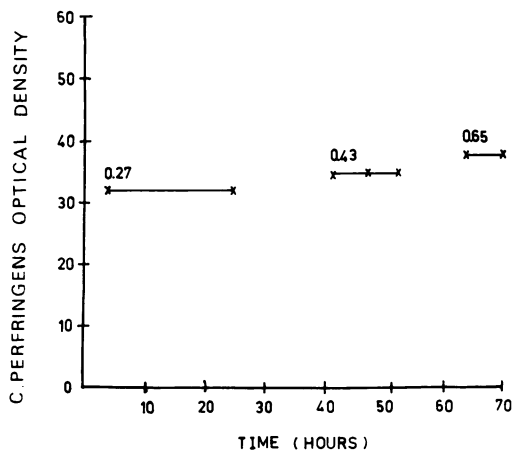


FIG. 5. Steady-state population levels of *Clostridium perfringens* type A S107 at dilution rates 0.27, 0.43, and 0.65 hr^{-1} , respectively, in 0.2% glucose-tryptone-salts medium.

tone-salts medium and is represented by plots at dilution rates of 0.27, 0.43, and 0.65 hr^{-1} (Fig. 5).

Microscopically, the cells were typical gram-positive rods with rounded ends, occurring singly, in small groups in angular arrangement, or in chains of four to six rods.

Biochemical properties of *C. perfringens* were retained irrespective of the concentration of nutrients available. Glucose, lactose, maltose, and sucrose were attacked within 24 hr, and inositol was attacked in 48 hr with acid and gas production. Salicin, xylose, arabinose, mannitol, and dulcitol were not fermented. Activities tested included gelatin liquefaction, nitrate reduction, indole production, and fermentation of litmus

milk. *C. perfringens* liquified gelatin, reduced nitrate to nitrite, and exhibited typical stormy clot formation. Indole was not produced.

The alpha, theta, and nu toxins were evident throughout from examination of plate colonies. Titters of alpha toxin assayed from effluent filtrates were consistently in the region of 0.1 to 0.2 Lb unit/ml. This strain, when subcultured in 1% glucose-Robertson's cooked meat medium, was fully toxigenic with alpha titers (1.0 to 1.3 Lb unit/ml) and equal to that of the reference strain.

The toxigenic-promoting factors in meat have been attributed to be present in enzymatic digests of meats (7, 8) and casein (1) but absent in meat infusions. On this basis, proteose peptone, an enzymatic digest of fresh meat, and tryptone, a tryptic hydrolysate of casein, were the choice. Adams, Hendee, and Pappenheimer (2) reported that casein was a good nitrogen source in toxin production. Jayko and Lichstein (3) found higher yields from enzymatic hydrolyzed casein rather than from acid-hydrolyzed casein. The best yields have come from a beef pancreatic digest of beef heart (4) and a peptone preparation prepared by peptic digestion of pork (5). However, the former was highly cumbersome and time-consuming to prepare when compared with the latter. The tryptone-salts medium in this study was a modification of the latter.

A laboratory chemostat used in this study is inexpensive and simple to construct. The design was adapted from the original model of Novick and Szilard (6). The inherent character in chemostat work is that bacterial populations at any exponential growth rate can be accurately controlled through the flow rate of the incoming medium containing one limiting growth factor. The choice of glucose as the limiting substrate was based upon preliminary findings that low concentrations were directly proportional to the growth rate of *C. perfringens* in tryptone-salts medium.

In this medium, *C. perfringens* established steady-state populations for both very fast and very slow rates of utilization of growth medium. With a 240-ml culture volume and a slow dilution rate of 0.1 hr^{-1} , the flow rate was 24 ml/hr compared with 264 ml/hr at a dilution rate of 1.1 hr^{-1} . At this fast flow rate, 6.5 liters of tryptone-salts medium was consumed daily. The practical lower limit of operation was at dilution rate of 0.1 hr^{-1} . Below this figure, a less accurate control was apparent.

ACKNOWLEDGMENT

This investigation was supported by a research grant from the University of Hong Kong.

LITERATURE CITED

1. Adams, M. H., and E. D. Hendee. 1945. Methods for the production of the alpha and theta toxins of *Clostridium welchii*. *J. Immunol.* 51:249-256.
2. Adams, M. H., E. D. Hendee, and A. M. Pappenheimer. 1947. Factors involved in production of *Clostridium welchii* alpha toxin. *J. Exp. Med.* 85:701-713.
3. Jayko, L. G., and H. C. Lichstein. 1959. Nutritional factors concerned with growth and lecithinase production by *Clostridium perfringens*. *J. Infec. Dis.* 104:142-151.
4. Logan, M. A., A. A. Tytell, I. S. Danielson, and A. M. Griner. 1945. Production of *Clostridium perfringens* alpha toxin. *J. Immunol.* 51:317-328.
5. Murata, R., T. Yamada, and S. Kameyama. 1956. Production of alpha toxin of *Clostridium perfringens*. I. Preparation of the reproducible peptone medium for the production of the alpha toxin of high potency. *Jap. J. Med. Sci. Biol.* 9:81-91.
6. Novick, A., and L. Szilard. 1950. Description of the chemostat. *Science* 112:715-716.
7. Rogers, H. J., and B. C. J. G. Knight. 1946. The recognition of material present in horse muscle affecting the formation of alpha toxin by a strain of *Clostridium welchii*. *Biochem. J.* 40:400-406.
8. van Heyningen, W. E. 1948. The biochemistry of the gas gangrene toxins. 3. Development of a medium suitable for the large scale production of the toxins of *Clostridium welchii* type A. *Biochem. J.* 42:127-130.
9. Willis, A. T. 1964. *In Anaerobic bacteriology in clinical medicine*, 2nd ed. Butterworths, London.
10. Willis, A. T., and G. Hobbs. 1959. Some new media for the isolation and identification of clostridia. *J. Pathol. Bacteriol.* 77:511-521.