Production of Pullorum Antigen by Continuous Submerged Culture

By SHOZO TANAKA

Biological Products Division, National Institute of Animal Health

The pullorum disease is an infectious disease of the avian species caused by Salmonella pullorum. The disease is most commonly spread by egg transmission. Therefore, the test and slaughter method based on the detection and elimination of the carrier chickens has been successfully carried out in order to eradicate the disease in Japan.

The preparation method of antigen must be standardized accurately to obtain the constant quality of products because the process to prepare antigen and the cultural conditions influence the specific agglutinability and occurrence of non-pullorum reactions.

Recently in Japan, the statistical assay method which employs the national standard anti-pullorum serum has been established.[4),8),9)]

Until, 1968, the antigen for the whole blood test was produced from the culture on the glycerin agar or YCC agar so a large amount of equipment and labor are necessary for preparation.

In general, there are some properties of the continuous culture method which make it attractive for the preparation of biological products,[1),2),5),6),10)] namely, large quantities can be produced in comparatively small vessels and a more homogeneous product is able to be obtained than the batch process or the conventional culture method.

Moreover, advantageously, this method makes it possible to increase a scope to study the influence of environmental conditions on the formation of antigenic substances in the bacterial cells.[3)]

Since 1962, efforts have been made to resolve some fundamental problems for the application of continuous cultivation.[7)]

However, a number of problems remained to be elucidated. One of them was the improvement and establishment of the apparatus to evaluate the optimal culture conditions and the other was to investigate the optimal conditions to harvest bacterial cells for the preparation of antigen.

This paper is concerned with a preparation method of pullorum antigen using the continuous culture and the operation of apparatus developed.

Continuous culture apparatus

The apparatus, being constructed as shown in Fig. 1, is composed of Jar fermentor, equipment for measuring and automatic controlling, and reservoirs for the medium and cultured cells.

1) Jar fermentor

The culture vessel (10 L volume) was made from Pyrex section with stainless steel upper cover plate and base plate with water jacket (Marubishi Laboratory Equipment Co., Ltd., Tokyo, Japan). Special inlet and outlet ports were fixed in the upper cover plate to allow the entry and exit of all control and supply equipment. Agitator shaft was fixed at the center of the upper cover plate through a bearing case and sealing housing. Two disk-turbine type impellers and a foam-breaking impeller were fitted to the agitation shaft.

2) Controlling and recording system

Automatic controlling and recording de-

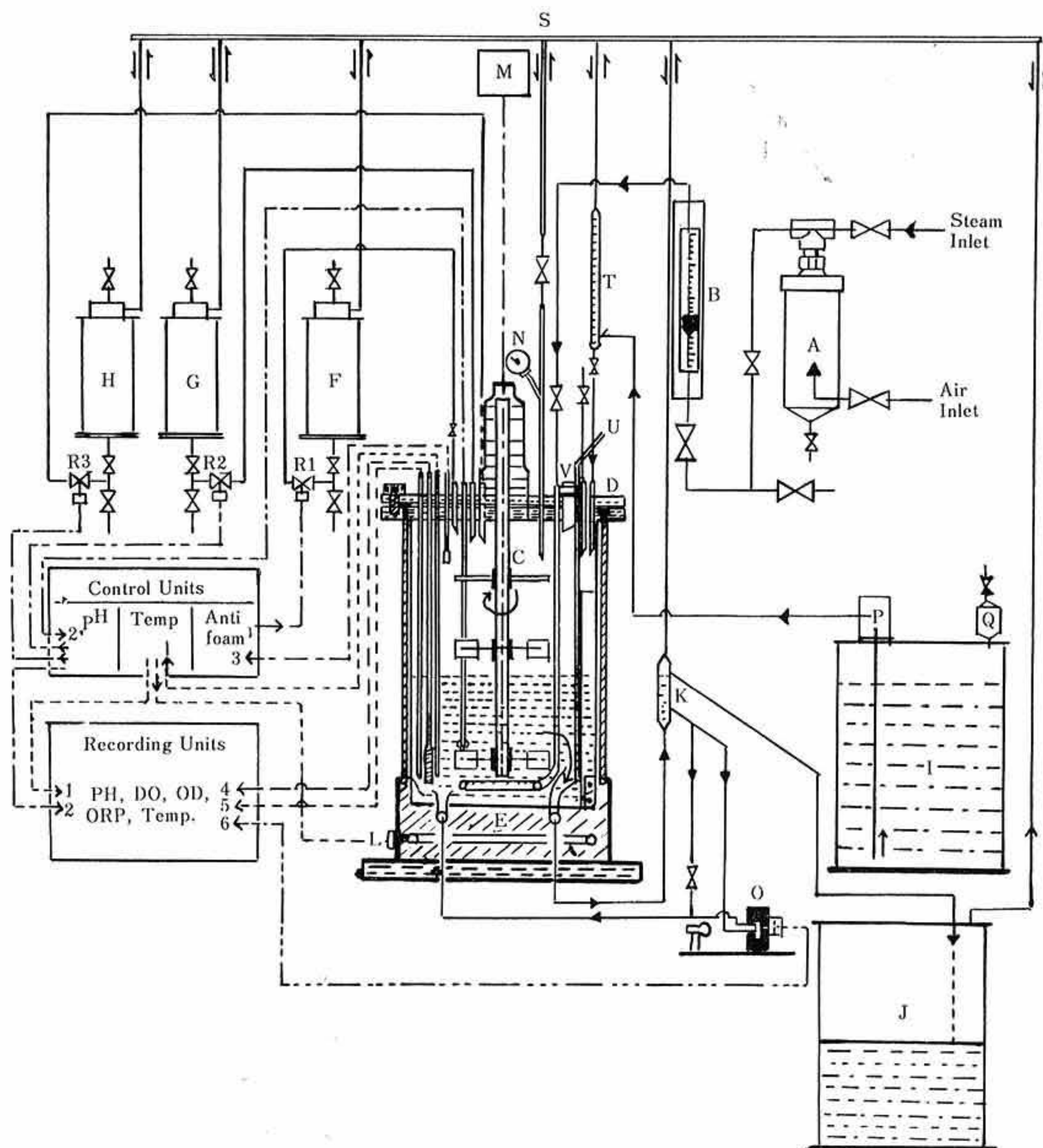

Fig. 1. Diagram of the continuous culture apparatus

A: Air filter; B: Air flow-meter; C: Agitater shaft & impeller; D: Upper cover; E: Water jacket; F: Anti-foamer agent tank; G: Acid tank; H: Alkali tank; I: Medium tank; J: Harvesting tank; K: Leveller; L: Heater; M: Mortor & variable peed sulley; N: Pressre gauge; O: Cell concentration (O, D) detector; P: Media supply pump; Q: Filter; R_1, R_2, R_3: Control valve; S: Air pressure balancing line; T: Medium caribration chamber; U: Sampling port; V: Seeding port

1, 1: Thermocouple, automation thermocontroller & recording units; 2, 2: pH electrode, automatic pH controller & recording units; 3: Anti-foamer electrode & automatic anti-foamer; 4: Dissolved oxygen (D. O) electrode & D. O. recorning units; 5: Oxidation-reduction potential (OPR) electrode & OPR recording units; 6: Optical density (O. D) detector & O. D recording units

vices of pH, dissolved oxygen (D.O) and oxidation-reduction potential (OPR), composed of each electrode and electric units, were equipped. Total bacterial concentration was also determined photometrically and recorded by means of an equipment made by the authors. Automatic anti-foamer and temperature controller were also equipped.

3) Medium supplying and harvesting system

Capacities of 30–50 L of the medium tank were connected via the medium supply pump and flow-metering chamber to the culture vessels by neoprene tube. Culture harvesting system consisted of the harvesting tank and leveller. A part of the culture suspension passed through the leveller was circulated via the O.D detector, and the other part of the suspension was collected into the harvesting tank by over flowing.

Other materials

Culture medium: From the results of the comparative experiment with various compositions, a formula shown in Table 1 was considered to be most suitable. The medium consistents with exception of glucose were dissolved in hot-distilled water. After adjusting pH at 7.2, the medium was sterilized in the autoclave or by filtrating through a filer press.

Table 1. Composition of the medium

Trypton (Eiken)*[1]	17 gm
Soy pepton (Eiken)	3 gm
Yeast extract (Difco)	3 gm
Dipotassium phosyhate	2.5 gm
Sodium chloride	5 gm
Dextrose*[2]	7.5 gm
Distilled water	1,000 ml

*[1] Eiken Chemical Co. Ltd., Tokyo
*[2] Separately dissolved and sterilized in autoclave

pH controlling solution: One normal solution of sodium hydroxide and hydrochloric acid was used for the controlling of the optimal pH.

Anti-foam agent: Two per cent solution of silicone anti-foam KM 70 (Shinetsu Chemical Co., Ltd., Tokyo, Japan) was effective as an anti-foam agent.

Operational technique

1) Sterilization

Sterilization of the culture vessel fitted with electrode and tanks was carried out by steaming directly into the culture vessel through the flow-meter and air-filter of the aeration line. At the same time, all of the inlet and outlet ports of the culture vessel also were sterilized by steaming. The tanks for medium reserving and cultures harvesting were sterilized in the autoclave. After sterilization and construction of the apparatus 4 L of the medium was pumped into the culture vessel, and sterility test was carried out with incubating at 37°C overnight with agitation (500 rev./min) and supplying air at 10 L/min with 0.2 kg/cm^2 of pressure within the culture apparatus.

2) Starting the culture

Seed suspension of Salmonella pullorum (strain 9–25) grown on Tryptosoy agar (Eiken chemical) was inoculated into the culture vessel, so as to provide an initial viable cell of approximately one billion per ml. The culture was grown as a batch method until the end of the exponential growth. Samples were taken off twice a day at every morning and night for purity and viable cell counting tests. Fresh medium was then supplied at constant rate and some volume of the culture suspension concurrently with the supply of fresh medium was overflowed into the harvesting tank continuously.

Some aspects of continuous culture

1) Comparison of cell yield within several batch operations

Comparative experiments were conducted to confirm the relationship between yields and some cultural conditions in batch operation. As a result, it was found that excess

glucose, and insufficient yeast extract and aeration might be one of the important factors to reduce the yield, which might be caused by irreversible decrease of pH value.

2) Cultural conditions and cell yield in continuous culture

When the cultures were grown under several conditions at dilution rate less than 0.75, cell yields were almost constant but depend upon glucose concentration and dilution rate primarily, as shown in Table 2.

Table 2. Yields in continuous culture with various conditions

No. of exper.	Basal medium	% of Glucose	Dilution rate (hr^{-1})	pH in steady state	Cell yields (gm)	Agglutinabl-lity	Final products (ml)
46-2	TS Broth +0.3% YE*	0.50	0.25	7.3—7.5 not controlled	906	Slightry high	14,400
45-1	〃	〃	0.30	7.4—7.5 not controlled	1,300	Slightry high	21,080
46-1	〃	0.70	0.30	7.1—7.3 not controlled	1,110	Good	17,980
46-3	〃	0.75	0.30	7.1—7.2 not controlled	1,653	Good	27,040
47-1	〃	〃	0.33	7.0—7.1 not controlled	1,475	Good	23,790
45-2	〃	〃	0.50	controlled at 7.0	1,140	Good	18,415
45-1	〃	〃	0.75	controlled at 7.1	1,510	Slightryl ow	24,350
46-4	TS Broth +0.5% YE*	0.80	0.25	7.4—7.5 not controlled	2,175	Slightry low	34,840

* Yeast extract (Difco)
Each experiment employed 100 liters of medium

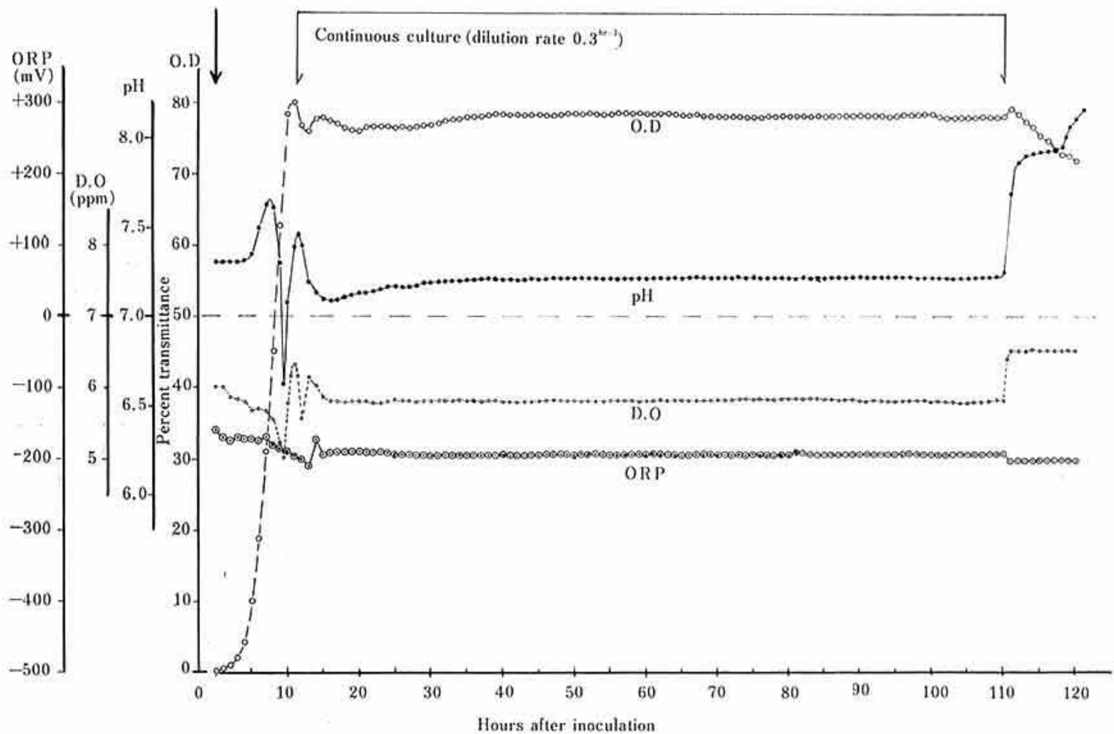

Fig. 2. Continuous culture of Salmonella pullorum st. 9-25

The result of the continuous culture in Tryptosoy broth supplemented with 0.7 per cent of glucose is shown in Fig. 2. Continuous culture was started at the 11th hour after the inoculation of the seed culture and the steady state was obtained about 3 hours after starting the continuous culture.

About 1.5–2.0 per cent (wet weight) of cell mass were obtained in these suitable conditions, and at the present time colonial mutation (S→R) has not been observed in these conditions.

3) Antigenicity of cells

Remarkable difference of antigenicity was observed in cells obtained from batch operation and continuous cultures. Furthermore, the cells in batch operation had apparently lower agglutinability than those from conventional slant culture, while various degrees of agglutinability were recognized in cells obtained from the continuous culture which depends upon the culture condition.

At present, the factors influencing agglutinability were dilution rate and time lapse in the continuous culture. The continuous culture cells, which were run with dilution rate of 0.1, 0.25, 0.5 and 0.75 respectively, were examined for their agglutinabilities against the anti-pullorum serum at 12 hours intervals. Gradual increases in agglutinability were noted in the cells obtained after long-term operation of continuous culture. And this trend was enhanced in the lower dilution rate. But their significance has not been known yet.

4) Preparation of pullorum antigen using cells obtained in continuous culture

Preparation method of the pullorum antigen for the whole blood test is shown in Fig. 3. Because of the different agglutinabilities in cells harvested daily in the intervals, cultured suspension must be handled as sublot separately. After examining the agglutinability of each sublot against standard anti-pullorum serum, the antigen for practical use was prepared by blending of adequate amount of sublots.

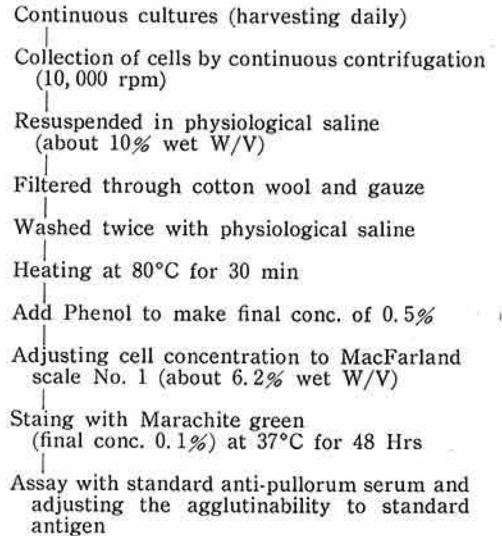

Fig. 3. Procedure for preparation of the pullorum antigen for whole blood test

As shown in Table 2, in the continuous culture employed 100 liters of Tryptosoy broth supplemented with 0.75 per cent glucose, and maintained dilution rate at 0.3–0.5, 1.1–1.5 kg of wet weight of cell masses were harvested and about 18,000–27,000 ml of final antigen were prepared with these cell masses.

References

1) Boice, K. J. & Edgar, A. W.: Production of freeze-dried Brucella abortus st. 19 vaccine using cells produced by continuous culture. *J. Appl. Bact.*, 29, 401–408 (1966).
2) Collins, J. M., Boice, K. J. & Edgar, A. W.: Preparation of a living vaccine from continuous culture produced cells. *Symp. Ser. Immunobiol. Stand.* 3, 171–176 (1966).
3) Formal, S. B., Baron, L. S. & Spilma, W. P.: The virulence and immunogenicity of *Salmonella typhosa* grown in continuous culture. *J. Bacteriol.*, 73, 168–175 (1956).
4) Hatakeyama, H. et al.: Standardization of pullorum antigen by a sequential trial method. *Bull. Nat. Inst. Anim. Hlth*, No. 66, 22–32 (1973). [In Japanese, English summary: *Nat. Inst. Anim. Hlth. Quart.*, 13, 103 (1973)].
5) Holme, T.: Controlled growth of bacteria by continuous culture. *Symp. Ser. Immunobiol. Stand.*, 3, 167–170 (1966).

6) Nielsen, K. E.: Growth and toxin production by *Clostridium tetani*. *Symp. Ser. Immunobiol. Stand.*, 3, 207–216 (1966).
7) Tanaka, S. et al.: Production of pullorum antigen by submerged culture I. Conditions of the batch culture. *Bull. Nat. Inst. Anim. Hlth.*, No. 58, 31–38 (1969). [In Japanese, English summary: *Nat. Inst. Anim. Hlth. Quart.*, 9, 117–118 (1969)].
8) Tanaka, S. et al.: Studies on the standard antiserum and assay method for the pullorum antigen. *Bull. Nat. Inst. Anim. Hlth.*, No. 66, 13–21 (1973). [In Japanese, English summary: *Nat. Inst. Anim. Hlth. Quart.*, 13, 102–103 (1973)].
9) Tanaka, S. et al.: Standardization of pullorum antigen: Comparison of non-pullorum reaction between antigens produced from slant agar culture and continuous submerged cultivation. *Bull. Nat. Inst. Anim. Hlth.*, No. 67, 16–23 (1973). [In Japanese, English summary: *Nat. Inst. Anim. Hlth. Quart.*, 13, 232 (1973)].
10) Veress, A. Füvessy, I. & Böszörmenyi, J.: Continuous cultivation of *Streptococcus pyogenes* in an automatically controlled system. *Ann. Immunol. Hung.*, 7, 63–67 (1964).