Effect of Temperature on Growth and Alpha Toxin Production by *Clostridium perfringens*

Y. PARK and E. M. MIKOLAJCIEK*

Department of Food Science and Nutrition, Ohio Agricultural Research and Development Center, Columbus, Ohio 43210

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**ABSTRACT**

Growth and alpha toxin production by a strain of *Clostridium perfringens* was determined in Thioglycollate medium, beef broth with ground beef, and beef broth with ground beef and soy protein. Incubation temperatures ranged from 15 to 50 C. In Thioglycollate medium, maximum alpha toxin production occurred at 35 C and was 40 times greater than that observed at 45 C. However, generation time and maximum population were approximately the same at 35 and 45 C. At 15 C, a 3 log cycle reduction in viable counts occurred within 6 h. Irrespective of incubation temperature, alpha toxin levels in Thioglycollate medium declined as the incubation period was extended beyond the stationary growth phase. In the beef broth with ground meat system which was studied at 35 C only, the organism grew slower and produced less toxin than in Thioglycollate medium. The amount of alpha toxin detected was influenced to a greater extent by the incubation time and temperature, the holding time beyond the stationary growth phase, and the growth medium than by the temperature and substrate on estimation of numbers of *C. perfringens*.

It has been suggested that quantification of alpha toxin produced by *Clostridium perfringens* can be used as an index of growth of the organism (8). Results obtained by the alpha toxin procedure may be influenced by several factors. For example, a wide variation has been demonstrated between strains of *C. perfringens* with respect to alpha toxin production (6,11,15). Temperature and substrate are known to influence biological activity of all microorganisms and would also likely affect the growth and alpha toxin production of *C. perfringens*.

This paper reports on the effects of incubation time and temperature as well as on effect of the nature of the substrate on estimation of numbers of *C. perfringens* by alpha toxin assay.

**MATERIALS AND METHODS**

**Test organism**

The strain (OSU 1) of *C. perfringens* used in the experiment was obtained from the Department of Animal Science, The Ohio State University, Columbus, Ohio. The organism was propagated in Thioglycollate medium.

**Test media**

The test media included Brewer's Thioglycollate medium (Difco) and beef broth (RJR Foods, Inc., Winston-Salem, North Carolina) with added ground beef or a mixture of ground beef and Promine-D (Central Soya, Chicago, Illinois). The beef broth was blended with 20% ground beef, or 14% ground beef and 6% Promine-D and sterilized (121 C for 15 min) in the blender jars. These media were blended well before use and distributed into previously sterilized 50-ml screw­ capped Erlenmeyer flasks. No attempt was made to control final pH of the beef/soy protein systems.

**Measurement of growth and alpha toxin production**

One tenth milliliter of an overnight broth culture of *C. perfringens* was inoculated into 40 ml of the test medium contained in an Erlenmeyer flask. For the Thioglycollate medium, incubation was carried out at 15, 26, 35, and 50 C in a temperature-controlled water bath. However, when the beef broth with ground meat and/or soy protein were used as the test media, optimum incubation temperature (35 C) was used. Samples were taken at selected time intervals in sterile test tubes (12 x 100 mm) for the measurement of viable counts, production and pH. The viable cell population was estimated by pour plates prepared with SFP agar (7) without addition of Polymyxin B sulfate and Kanamycin sulfate. Alpha toxin production was measured by the Hemolysin Indicator (HI) plate test of Duncan and Harmon (5) with modifications suggested by Park and Mikolajcik (2). The pH was measured using a PHM 62 Standard pH Meter (Radiometer, Copenhagen).

Growth curves were constructed by plotting the logarithm of the colony-forming units (CFU) versus incubation time. The generation time was calculated by the formula: Gt = t/n = t/(3.3 log b/a) where Gt (generation time) is equal to the time, t = the elapsed time between measurement of a, the initial population, and b, the final population, divided by the number of generations. n (number of generations being equal to 3.3 log b/a.

**RESULTS AND DISCUSSION**

Growth and alpha toxin production in Thioglycollate medium.

Results for cell growth, toxin production and pH changes at 25, 35, 45 and 50 C are shown in Fig. 1-4. Data at 15 C are not presented because the organisms failed to grow and exhibited a two-log reduction in counts within 6 h of incubation.

In general, increases in alpha toxin activity closely paralleled population increases and a population of at least 400,000/ml was required before alpha toxin activity could be detected. These data are in agreement with those found by Harmon and Kautter (7) who showed that there is a relationship between *C. perfringens* population in a food sample and alpha toxin activity. It was observed that the incubation temperature strongly influenced the rate of alpha toxin production and maximum yield of alpha toxin. The optimum temperature for alpha toxin production was 35 C (Fig. 2). The rate of alpha toxin production was closely related to the growth rate,
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irrespective of the incubation temperature. However, the maximum yield of alpha toxin did not correlate well with the growth rate. For example, the maximum yield of alpha toxin at 35 C was 40 times greater than at 45 C, although the growth rate at 35 C was practically the same as that at 45 C.

The relationship between the viable count of C. perfringens on SFP base agar and the amount of alpha toxin produced at various incubation temperatures is presented in Fig. 5. At a population level of $10^9$, C. perfringens produced approximately $1.0 \times 10^2$ unit of alpha toxin/ml at 35 C and $6.5 \times 10^3$ unit/ml at 45 C.

The optimum temperature for C. perfringens on the basis of minimum generation time was found to be 35 to 45 C. Rey et al. (14) observed the optimum growth temperature for this organism being between 30 and 40 C; Breed et al. (1), 35 and 37 C; Collee et al. (4) and

Smith and Holdeman (20), 43 and 45 C. Although our strain had a generation time of 19 min., others have reported generation times of 9.5-12.5 min (16), 8.5 min (2) or 24-32 min (19). Apparently, generation time at optimum temperature is subject to such variables as strain, growth media, age, size of inoculum, pH and redox potential among others. At 45 C (Fig. 3), the growth was almost as fast as at 35 C, i.e. the difference in generation times between 35 C and 45 C was almost negligible (Table 1). Even at 50 C (Fig. 4), the growth was fairly rapid. At 25 C (Fig. 1), the growth was considerably slower than at 35 C. The time required to reach the stationary phase ranged from 4 to 5 h at 35, 45 and 50 C, in contrast to 22 h at 25 C.

At different incubation temperatures, the maximum viable population reached at the end of logarithmic growth phase also varied. It was approximately $2.2 \times 10^8$
organisms per ml at 25, 35 and 45 C and 1.3 x 10^7 organisms per ml at 50 C. In general, after the stationary phase the higher the incubation temperature, the more rapid the decline phase. The number of organisms developing in a food system is important because it is generally accepted that 100,000-1,000,000 C. perfringens/ml are required for an infective dose. Others (9,10) suggest the total dose to be 100 million organisms.

Stability of the alpha toxin was apparently dependent on the temperature and time of incubation. Generally, the destruction rate of alpha toxin closely followed first order kinetics. At the incubation temperatures examined, alpha toxin was most unstable at 35 C. At all incubation temperatures, the decline of alpha toxin activity occurred at the end of the exponential growth phase and the longer the incubation time after the stationary phase of growth had been attained, the more alpha toxin was destroyed. Shemanova et al. (18) demonstrated that the rapid decline of phospholipase C activity in the culture supernatant fluid coincided with the appearance of maximum proteolytic activity. However, Nord et al. (11) detected little proteolytic activity and doubted that proteolytic degradation was responsible for the rapid loss of enzyme activity. This discrepancy still needs to be resolved.

In general, pH of the medium dropped when maximum growth and alpha toxin production were attained (Fig. 1-4). The optimum pH for alpha toxin activity and production was reported to be approximately 7.0-7.2 by Pivnick et al. (13).

**Growth and alpha toxin production in beef broth with ground beef and Promine-D**

Before proceeding to the study of alpha toxin production by C. perfringens in beef broth with ground beef and Promine-D, recovery of known amounts of alpha toxin suspended in beef broth was determined. The recovery was essentially 100%, i.e. quantification of alpha toxin using beef broth as diluent yielded practically the equivalent hemolytic zone diameter as that observed when Thioglycollate medium was the diluent.

When C. perfringens was inoculated into beef broth with 20% ground beef and incubated at 35 C (Fig. 6), the organism grew slower (generation time, 156 min) and produced less alpha toxin (.008 unit/ml) per the same number of organisms than when it was grown in Thioglycollate medium (Gt, 19.3 min; .224 unit/ml). However, the final population level was slightly higher in the beef broth/ground beef system than in the Thioglycollate medium.

The increased use of soy protein extenders for meat products prompted the study of the effect of soy protein isolates on growth and alpha toxin production of C. perfringens. When 6% Promine-D was blended with 14% ground beef in beef broth under conditions where anaerobiosis was not controlled, viable counts of C. perfringens decreased two log cycles within 24 h (Fig. 6). No alpha toxin was detected. Busta and Schroder (3), using Trypticase (BBL) in Thioglycollate medium as a protein control, demonstrated that some proteins had stimulative effects on growth of C. perfringens, whereas...
others were inhibitory. They postulated that a modification in protein availability through manufacturing processes of soy protein and the presence or absence of some inhibitory factors might play a part in its effect on growth. Schroder and Busta (16) also have reported that under actual meat loaf conditions, addition of soy protein to beef did not affect the growth of C. perfringens.

In Fig. 7, the relationship between substrate, population levels and production of alpha toxin is shown. At a population level of 107, C. perfringens produced approximately $7.1 \times 10^{-3}$ unit of alpha toxin/ml in Thioglycollate medium and $4.0 \times 10^{-4}$ unit/ml in beef broth blended with 20% ground beef.

Thus the amount of alpha toxin produced in any food sample may be influenced by the nature of the substrate as well as the time and temperature at which it is being held. However, Harmon and Kautter (8) have maintained that the type of food associated with the different C. perfringens food poisoning outbreaks appeared to have little effect on population estimates based on the quantification of alpha toxin.

Overall, the results have demonstrated that if population levels of C. perfringens are to be estimated from the amount of alpha toxin detected in a food system, prior knowledge of incubation time and temperature, storage time and composition of the food is essential.

**REFERENCES**


