

EFFECT OF CULTIVATION PARAMETERS ON BACTERIAL DENSITY AND TOXIN PRODUCTION OF A *CLOSTRIDIUM PERFRINGENS* TYPE C STRAIN

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Abstract

The purpose of the current study was to investigate the effect of certain cultivation parameters, such as pH adjustment, stirring and nutrient addition, on the bacterial density and toxin production of a *Clostridium perfringens* type C culture. Bacterial cultures were grown simultaneously on a 30-litre Bionet bioreactor and an industrial 500-litre bioreactor. Both experiments were performed using the same culture media, the same nutrients and the same inoculum and during the same timeframe. Differences in bioreactor equipment, such as homogenisation systems and pH measuring equipment, resulted in different final cultivation outcomes. The toxicity of the experimental Bionet culture was determined at 3200 LD₅₀, while the industrial bioreactor culture tested at only 800 LD₅₀ toxicity.

Key words: beta toxin, immunogenic, *Clostridium perfringens*, cultivation, vaccine.

INTRODUCTION

Clostridium perfringens is an anaerobic, Gram-positive, spore-forming bacterium that, compared to other anaerobes, is relatively resistant in the presence of oxygen (McClane & Robertson, 2013). It is a major pathogen of both humans and animals, its virulence being largely due to secreted toxins but also to its very rapid doubling time, which allows it to quickly reach pathogenic burdens in the infected material (Gohari et al., 2021).

Clostridium perfringens type C (ClpC) is the etiological agent of necrotising enteritis in domestic animals, such as goats, pigs, cattle, horses, and humans. In small ruminants, the infection causes haemorrhagic enteritis in lambs and an acute enterotoxaemia, known as “struck”, manifesting as sudden death in adult sheep (Diab et al., 2016). In piglets, calves, foals, and kids, type C strains produce haemorrhagic and necrotic enterotoxaemia. In poultry, ClpC strains can cause necrotic enteritis, resulting in increased mortality and the risk of contamination of products intended for human consumption (Caraman et al., 2024). Piglets are

especially susceptible to the infection, which can result in mortality rates of 30 to 50% (Fisher et al., 2006).

C. perfringens is a rapidly multiplying bacterium and a prolific toxin producer (Anwar et al., 2019). Virulence factors of ClpC strains include beta toxin, alpha toxin and also other toxins such as perfringolysin O, beta2 toxin, and/or enterotoxin (Rood, 1998). The ability to sporulate grants them increased resistance in the environment, making them impossible to eradicate (Zaragoza et al., 2019). With the increasing concern regarding antibiotic resistance among bacterial strains in the intensive farming system (Șandru et al., 2023), active immunisation with toxoid vaccines remains the main protective method against infection (Zaragoza et al., 2019). Type C outbreaks determine massive economic losses and mortality rates that can reach 100% in unvaccinated herds (Nagahama et al., 2015). The efficacy of anaerobic vaccines depends largely on the toxin yield of the bacterial culture. Toxin production by ClpC depends on the presence of a peptide source, pH control, and the presence of nutrients in the form of fermentable

carbohydrates, such as glucose, fructose or dextrin, which have been shown to increase culture growth and toxin production (Ducan, 1979; Esmailnejad-Ahranjani, 2023).

The purpose of the current study was to investigate the efficacy of two different cultivation settings, a 30-litre Bionet bioreactor and a 500-litre industrial bioreactor, in regard to bacterial density and toxin production, for a vaccine strain of ClpC.

MATERIALS AND METHODS

The subject bacterial strain, *Clostridium perfringens* type C - Stamatin, belonged to the Seed Bank of Pasteur Institute, and the experiment was carried out within the Antibacterial Vaccines Department of the Pasteur Institute, in Bucharest. The culture media utilised in the experiment were prepared in-house. The inoculum was prepared using VF culture medium, containing *Clostridium perfringens* sporulation broth, liver hydrolysate and yeast extract, all three ingredients in atomised powdered form, to which L-cystine, glucose and liquid yeast extract (*Saccharomyces karlsbergensis*, neutralized to pH 7.2-7.4 and clarified by filtration) were added. The pH of the medium was adjusted to 7.6 using NaOH solution. The culture medium (*C. perfringens* medium) for the bioreactor cultivation was prepared after an original recipe, containing liver meat glucose cysteine broth, cooked meat medium broth, e.t. medium, *Clostridium perfringens* sporulation broth, casein yeast peptone, liver hydrolysate, yeast extract, liver extract, glucose, l-cysteine and purified water. The culture medium was sterilised by filtration with 0.2 µm pore size Millipore filters.

The lyophilised bacterial strain was reconstituted by adding 2 mL of sterile PBS solution and homogenising by repeated pipetting. The inoculum was obtained by seeding VF broth tubes with 0.5 mL reconstituted strain. The seeded tubes were incubated for 6 hours at 37°C under anaerobic conditions. The cultures obtained on VF medium were transferred into glass bottles containing 400 mL *C. perfringens* medium and incubated following the same protocol. In preparation of the experiment, the bioreactors were sterilised, filled with the filtered culture

medium - 20 L of medium for the Bionet bioreactor and 450 L for the industrial bioreactor, and brought to 37°C. Four sterile glass bottles containing the inoculum, 50% glucose solution, 10N NaOH solution and liquid yeast extract were attached to each bioreactor.

Bionet cultivation. Anaerobic conditions during cultivation were ensured by introducing a gas mixture containing 85% nitrogen, 5% carbon dioxide and 10% hydrogen into the bioreactor at a pressure of 300 bar and stirring at 400 rpm for 30 minutes at a flow rate of 5 L per minute. Before inoculation, the temperature of the culture medium was brought to 36±2°C. When the set temperature was reached, yeast extract and glucose were added to the medium. Stirring was stopped and the medium was inoculated with the prepared inoculum. Throughout cultivation, the pH and temperature of the culture were monitored in real time. The Bionet cultivation parameters were as follows: temperature 37°C, homogenisation 200 rpm, pH 7.4. After inoculation, visible growth was detected at 2 hours p.i. At this time, the bioreactor began the automatic pH regulation. Nutrient addition was performed manually to support the growth of the culture, as required. Samples were collected at 1 h, 2 h, 3 h, 4 h, 5 h, and 6 h p.i.

Industrial bioreactor cultivation. The culture medium was brought to 37°C and the pH was adjusted to 7.4 using 10N NaOH solution. The same mixture of gases was used to create anaerobic conditions. Yeast extract and glucose solution were added and homogenised with the medium, after which the inoculum was added. Samples were collected every hour, and the pH was measured and adjusted manually. Homogenisation was performed using the bioreactor's vibration system, and nutrients were also added manually during cultivation. Samples for the comparison experiment were collected at the same times p.i. as the Bionet cultivation (1 h, 2 h, 3 h, 4 h, 5 h, and 6 h).

The collected samples from both cultivations were stored on ice until processing. Gram-stained slides were prepared from both cultures to microscopically assess the morphology of the bacilli. The cultures were seeded onto Columbia blood agar and Sabouraud agar and incubated at 37°C to ensure bacterial purity and fungal sterility.

To determine the differences between the experimental cultivations, the samples from each cultivation were tested for bacterial density and toxicity. Bacterial density was determined by inoculating 10-fold dilutions of the culture samples into plates with melted TSC agar. The plates were incubated for 24 h at 37°C in anaerobiosis, after which the resulting colonies were counted.

The ClpC toxin was prepared by culture centrifugation (4000 rpm, 30 minutes, 4°C) and supernatant filtration through 0.8, 0.45, 0.22 and 0.1 µm pore size Millipore filters. To determine the median lethal dose, dilutions in peptone water were made from whole toxins. The potency of bacterial toxins from each collected sample was tested on *BALB/c* mice of 21-24 grams. Each mouse received an i.v. injection of 0.5 ml of the supernatant dilution. The toxin dilutions used in the assay were 1/100, 1/200, 1/400, 1/800, and 1/1600. As negative controls, 3 mice received i.v. injections of sterile peptone water. The supernatant toxicity titre was calculated as twice the reciprocal of the highest dilution that induced mortality of at least 2 out of 3 mice within 72 hours of administration.

RESULTS AND DISCUSSIONS

The microscopic examination of the samples revealed Gram-positive rods, short, thick, with rounded ends, isolated or grouped in clusters (Figure 1). On blood agar, the colonies appeared smooth, greyish, with intense haemolysis.

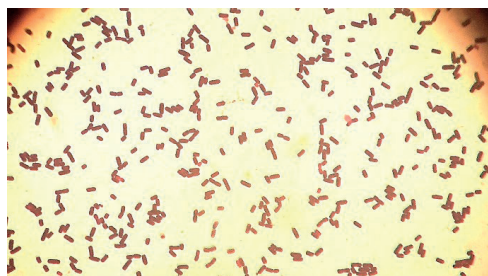


Figure 1. *Clostridium perfringens* type C, microscopic aspect

Culture parameters for the Bionet cultivation are presented in Table 1.

The density of the bacterial cultures obtained from the Bionet cultivation sampling was as follows: sample 1 collected 1-hour p.i. had a

bacterial density of 4.5×10^2 , sample 2 collected at 2 hours p.i. - 5×10^3 , sample 3 (3 hours p.i.) - 7.8×10^4 , sample 4 (4 hours p.i.) - 9.2×10^5 , sample 5 (5 hours p.i.) - 3.4×10^7 , and sample 6 (6 hours p.i.) - 3.8×10^8 (Table 2).

Table 1. Cultivation parameters for Bionet culture experiment

	pH	Stirring	NaOH	Nutrients
1 h	7.40	-	-	-
2 h	7.20	191 rpm	120 mL	-
3 h	7.35	198 rpm	150 mL	G - 40 mL Y - 40 mL
4 h	7.25	198 rpm	158 mL	-
5 h	7.28	199 rpm	170 mL	-
6 h	7.20	199 rpm	-	-

Legend: 1 h, ..., 6 h - hours post-inoculation, rpm - rotation per minute, G - Glucose 40%, Y - Yeast extract.

The industrial bioreactor cultivation was performed at the same time as the Bionet cultivation, using the same batches of culture media and nutrients. pH measurements and adjustments were performed manually on an hourly basis.

The industrial bioreactor samples had slightly lower bacterial density: sample 1 (1-hour p.i.) - 1.25×10^2 , sample 2 (2 hours p.i.) - 1.95×10^2 , sample 3 (3 hours p.i.) - 7×10^3 , sample 4 (4 hours p.i.) - 9.4×10^4 , sample 5 (5 hours p.i.) - 5.3×10^5 , and sample 6 (6 hours p.i.) - 3×10^7 (Table 2).

Table 2. Bacterial density of the samples collected from the experimental cultivations

Sample	Bacterial density - Bionet cultivation	Bacterial density - Industrial cultivation
1	4.5×10^2 CFU/mL	1.25×10^2 CFU/mL
2	5×10^3 CFU/mL	1.95×10^2 CFU/mL
3	7.8×10^4 CFU/mL	7×10^3 CFU/mL
4	9.2×10^5 CFU/mL	9.4×10^4 CFU/mL
5	3.4×10^7 CFU/mL	5.3×10^5 CFU/mL
6	3.8×10^8 CFU/mL	3×10^7 CFU/mL

The mouse intravenous injection model was used to assess the lethality of the culture supernatants obtained from each collected sample. Sterile filtered culture supernatants were used to prepare dilutions in peptone water, which were injected into the tail vein of Balb/C mice.

The supernatant toxicity evaluation revealed superior results for the Bionet cultivation,

reaching a maximum toxin titre of 3200 LD₅₀ within 5 hours of cultivation (Table 3). The industrial bioreactor cultivation only yielded a titre of 800 LD₅₀ on samples collected 5-, and 6-hours p.i. (Table 4). The negative control group survived, without exhibiting any clinical signs of illness, after being administered peptone water.

Table 3. Toxicity test results for the Bionet culture (number of dead mice within 72 hours of administration)

Sample	Toxin dilutions				
	1/100	1/200	1/400	1/800	1/1600
1	-	-	-	-	-
2	-	-	-	-	-
3	3	3	2	1	-
4	3	3	3	1	-
5	3	3	3	3	2
6	3	3	3	2	2

The results of the Bionet experiment show that samples collected after 5 and 6 hours of cultivation had the highest toxicity levels. It also shows a direct correlation between the increasing bacterial density and the rise in toxicity levels, with the exception of the last sample, where LD₅₀ remained the same as sample 5, despite having a higher CFU/ml than sample 5.

Table 4. Toxicity test results for the industrial culture (number of dead mice within 72 hours of administration)

Sample	Toxin dilutions				
	1/100	1/200	1/400	1/800	1/1600
1	2	1	-	-	-
2	3	3	2	-	-
3	3	3	1	-	-
4	3	3	2	-	-
5	3	3	2	-	-
6	3	3	3	1	-

Supernatants obtained from the industrial culture samples had a rather stable level of toxicity, showing little to no correlation with the increase of the bacterial density or the fluctuation of the pH levels. For decades, researchers have investigated the optimum cultivation techniques for *C. perfringens* strains in order to increase bacterial density and toxin production. It has been reported in literature that toxin production of *C. perfringens* strains varies depending on a

number of factors, such as the bacterial strain, the culture medium, pH, heat treatment, etc. (Saito, 1990). Studies have shown the importance of the nutrients contained by the culture medium regarding toxin production, proving that different media provide different toxicity outcomes when used to cultivate the same bacterial strain (Fernandez-Miyakawa et al., 2007). In regard to the optimum homogenisation technique during cultivation, Esmailnejad-Ahranjani et al. (2023) have found that increasing the stirring rate to 300 rpm decreased the lag time from 60 min to 30 min; however, enhancing it further had the opposite effect, increasing the lag time back to 60 min. In industrial conditions, due to the large volume of culture, stirring plays an important role, as it has been documented that an uneven distribution of a culture in a bioreactor may cause gradients in biomass, substrate and pH, which may lead to a decrease in productivity (Esmailnejad-Ahranjani et al., 2022; Madian et al., 2022). Maintaining a stable pH throughout cultivation is a crucial factor in determining the toxicity of the culture. Researchers have demonstrated that pH level is one of the factors which have a remarkable effect on *Clostridium* toxin activity (Ahmed et al., 2022; Khoshanabadi et al., 2022; Tariq et al., 2022). This may explain the results obtained in the current study, specifically the lower toxicity of the culture obtained in the industrial bioreactor which had a vibration homogenising system, compared with the Bionet culture, obtained with a constant stirring at 200 rpm.

CONCLUSIONS

Clostridium perfringens type C cultures were grown simultaneously in a 30-litre Bionet bench scale bioreactor and a 500-litre industrial bioreactor, using the same bacterial strain and culture medium, to assess the difference in bacterial density and toxin production. Samples were collected each hour post-inoculation and tested for bacterial density by incorporating 10-fold dilutions into melted TSC agar and for toxicity using the mouse intravenous injection model. Results showed that the Bionet culture had a final bacterial density of 3.8x10⁸ CFU/mL, while the industrial culture obtained only 3x10⁷ CFU/mL. Toxicity evaluation of the bionet

experiment showed a maximum toxin titer of 3200 LD₅₀ within 5 hours of cultivation. The industrial cultivation had a toxin titre of 800 LD₅₀ on samples collected 5-, and 6-hours p.i. Further research is necessary in order to optimise cultivation parameters for a more successful scaling-up process.

REFERENCES

- Ahmed, M., Yehia, M., & Ahmed, H. E. (2022). Factors affecting on production of *Clostridium novyi* type (B) Alpha Toxin. *Journal of Applied Veterinary Sciences*, 7(2), 53–57.
- Anwar, Z., Regan, S. B., & Linden, J. (2019). Enrichment and detection of *Clostridium perfringens* toxinotypes in retail food samples. *JoVE (Journal of Visualized Experiments)*, (152), e59931. doi:10.3791/59931
- Caraman, M., Chiselita, N., Chiselita, O., Petcu, I., & Mashner, O. (2024). The curative efficiency of the CMP-3 in avian clostridiosis. *Scientific Works. Series C. Veterinary Medicine*, 70(1), 74–81.
- Diab, S. S. (2016). *Diseases produced by Clostridium perfringens type C*. In: *Clostridial Diseases of Animals* (ed. F. A. Uzal, J. G. Songer, J. F. Prescott & M. R. Popoff), 143–155. Ames, IA, USA: Wiley-Blackwell.
- Duncan, C. L. (1979). Effect of carbohydrates and control of culture pH on beta toxin production by *Clostridium perfringens* type C. *Microbiology and immunology*, 23(5), 313–318. doi:10.1111/j.1348-0421.1979.tb00468.x
- Esmailnejad-Ahranjani, P., & Hajimoradi, M. (2022). Optimization of industrial-scale centrifugal separation of biological products: comparing the performance of tubular and disc stack centrifuges. *Biochemical Engineering Journal*, 178, 108281.
- Esmailnejad-Ahranjani, P., Majidi, B., Paradise, A., & Hasanzadeh, M. (2023). Optimization and scale-up of *Clostridium perfringens* type D culture and epsilon-toxin production: Effects of stirring, glucose and pH adjustment. *Toxicon*, 234, 107302.
- Fernandez-Miyakawa, M. E., Marcellino, R., & Uzal F. A., (2007). *Clostridium perfringens* type A toxin media. *Journal of Veterinary Diagnostic Investigation*, 19(2), 184–186. doi:10.1177/104063870701900208
- Fisher, D. J., Fernandez-Miyakawa, M. E., Sayeed, S., Poon, R., Adams, V., Rood, J. I., Uzal, F.A., & McClane, B. A. (2006). Dissecting the contributions of *Clostridium perfringens* type C toxins to lethality in the mouse intravenous injection model. *Infection and immunity*, 74(9), 5200–5210.
- Khoshanabadi, R. J., Fathi Najafi, M., Heravi, M. M., & Bozorgmehr, M. R. (2022). Purification and characterization of beta toxin of *Clostridium perfringens* type B. *Egyptian Academic Journal of Biological Sciences. C, Physiology and Molecular Biology*, 14(1), 201–212.
- Madian, H. R., Hamouda, H. I., & Hosny, M. (2022). Statistical optimization of bioethanol production from giant reed hydrolysate by *Candida tropicalis* using Taguchi design. *Journal of Biotechnology*, 360, 71–78.
- McClane, B. A., Robertson, S. L., & Li, J. (2013). *Clostridium perfringens*. In: *Food Microbiology: fundamentals and Frontiers*. 4th ed. (ed. M. P. Doyle, R. L., Buchanan), 465–489, Washington D.C., USA: ASM press.
- Mehdizadeh Gohari, I., A. Navarro, M., Li, J., Shrestha, A., Uzal, F., & A. McClane, B. (2021). Pathogenicity and virulence of *Clostridium perfringens*. *Virulence*, 12(1), 723–753.
- Rood, J.I. (1998). Virulence genes of *Clostridium perfringens*. *Annual review of microbiology*, 52(1), 333–360.
- Nagahama, M., Ochi, S., Oda, M., Miyamoto, K., Takehara, M., & Kobayashi, K. (2015). Recent insights into *Clostridium perfringens* beta-toxin. *Toxins*, 7(2), 396–406.
- Saito, M. (1990). Production of enterotoxin by *Clostridium perfringens* derived from humans, animals, foods, and the natural environment in Japan. *Journal of food protection*, 53(2), 115–118.
- Şandru, C. D., Olah, D., Duca, G., Cerbu, C., Vasii, A., Pall, E., Văleanu, C., & Spînu, M. (2023). Comparative levels of antibiotic resistance in pigs raised under different technologies. *Scientific Works. Series C, Veterinary Medicine*, 69(2), 69–71.
- Tariq, M., Anjum, A. A., Sheikh, A. A., Awan, A. R., Sattar, M. M. K., Ali, T., & Nawaz, M. (2022). Physical and chemical factors affecting biomass and alpha toxin production of *Clostridium perfringens* toxinotype A. *JAPS: Journal of Animal & Plant Sciences*, 32(6), 1731–1743.
- Zaragoza, N. E., Orellana, C. A., Moonen, G. A., Moutafis, G., & Marcellin, E. (2019). Vaccine production to protect animals against pathogenic clostridia. *Toxins*, 11(9)