# Effectiveness of Using HHO Gas for PBII Treatment in Sterilizing *Escherichia Coli*

K. Kakugawa[1,2], K. Nosaki[1], S. Umemoto[2], T. Noda[1], and T. Tanaka[3]

***Abstract*—**Plasma-based ion implantation (PBII) is a surface modification technique that uses a negative high-voltage pulse in plasma to implant ions uniformly onto a sample. This technique is performed by narrowing the width of the ion sheath formed near the sample and aligning the shape of the sheath closely to the sample, particularly for complexly shaped samples. Our investigation focuses on the application potential of PBII in sterilizing microorganisms. In particular, we aimed to determine the most suitable gas species for sterilization using PBII on Escherichia coli, a type of microorganism that can contaminate food. Our findings demonstrate that nitrogen or oxygen gas could increase the sterilization effect with an increase in applied voltage. The provided conductive stainless-steel is used as the Petri dish for sample fixation. By contrast, sterilization becomes more effective when HHO gas, which is produced by water electrolysis and is non-hazardous compared with peroxide gas, is utilized compared with other gases, even at a low applied voltage of −2 kV. Therefore, the use of HHO gas in PBII could enable milder sterilization conditions than previously attainable when sterilizing foodstuffs.

***Keywords*—***Escherichia coli*, Plasma-based ion implantation, Sterilization.

## I. Introduction

Contemporary food production requires processing methods that ensure safety from chemical contamination and minimal impairment of flavor and functionality. Microbial spoilage of food is a significant hazard from a safety standpoint. Thus, sterilization is commonly used to eradicate microorganisms that cause foodborne illnesses. At present, food sterilization is categorized as either heat sterilization or non-heat sterilization. In general, heat sterilization methods, such as boiling or retort sterilization, are utilized to kill harmful microorganisms. Nonetheless, excessive heating can deteriorate the quality of food. On the contrary, non-heat sterilization methods can eliminate microbes without diminishing the quality of the food, which leads to a range of innovative sterilization technologies, such as ultra-high pressure treatment, electrolysis, and plasma treatment[1]–[3]. This study focuses on plasma treatment. Plasma sterilization systems utilizing hydrogen peroxide have been implemented in the medical field. However, the application of such systems is hindered because of the high purchase cost of hydrogen peroxide and exposure of medical personnel to hydrogen peroxide, which is harmful to the human body.

In this study, plasma-based ion implantation (PBII) can uniformly implant ions into a sample by introducing a negative voltage to the sample in the plasma, which then attracts and accelerates ions in the plasma around the sample. PBII is used for modifying the surface of materials with complex shapes, such as automotive and precision engineering components, as well as medical materials, such as artificial bones and blood vessels[4]–[6]. Moreover, PBII does not use harmful gases. We believe that ion implantation into the cell wall may be a feasible method for sterilizing microorganisms. Thus, we aim to develop PBII into a low-temperature, short-time sterilization technique[7].

Our previous research has shown that PBII treatment reduces *Geobacillus stearothermophilus* spores from $10^7$ CFU/mL to $10^1$ CFU/mL[8],[9]. Therefore, a 6D sterilizing effect was achieved on *G. stearothermophilus* spores under the following conditions: $O_2$ atmosphere, frequency of 1 kHz, delay time of 50 μs, pulse width of 10 μs, applied voltage of −12 kV, RF output of −240 VA, and irradiation time of 10 min. Although these conditions were effective against heat-resistant spore bacteria, they were deemed too harsh for future use as a food sterilization device. Thus, we are searching for milder sterilization conditions. Previous research has demonstrated that the sterilizing effect is easily influenced by the Petri dish material used to secure the sample and the gases utilized at low applied voltages[10]. Therefore, apart from nitrogen and oxygen gases, we investigated the HHO gas obtained through water hydrolysis to identify the most effective gas for sterilization under low applied voltage conditions.

## II. Materials and Methods

### A. Experimental Apparatus

Fig. 1 shows a schematic diagram of the PBII apparatus[11]. The chamber, measuring 450 mm in height, 590 mm in width, and 470 mm in depth, is electrically grounded. The RF antenna of the ICP source is a wound inside the upper lid, with one end grounded. The antenna is a five-turn copper coil, approximately 250 mm in diameter, which operates at 240 kHz. The sample is positioned on a stainless-steel electrode supported by an insulated stainless-steel rod at the center of the vacuum chamber.

Manuscript received Apr. 27, 2023.

This work was supported by JSPS KAKENHI Grant Number JP22K05504.

[1]Department of Food Sciences and Biotechnology, Faculty of Life Sciences, Hiroshima Institute of Technology, Hiroshima, Japan.

[2]Major in Biological and Biomedical Engineering, Graduate School of Science and Technology, Hiroshima Institute of Technology, Hiroshima, Japan.

[3]Major in Electrical and Electronic Engineering, Graduate School of Science and Technology, Hiroshima Institute of Technology, Hiroshima, Japan.

Corresponding Author is Koji Kakugawa

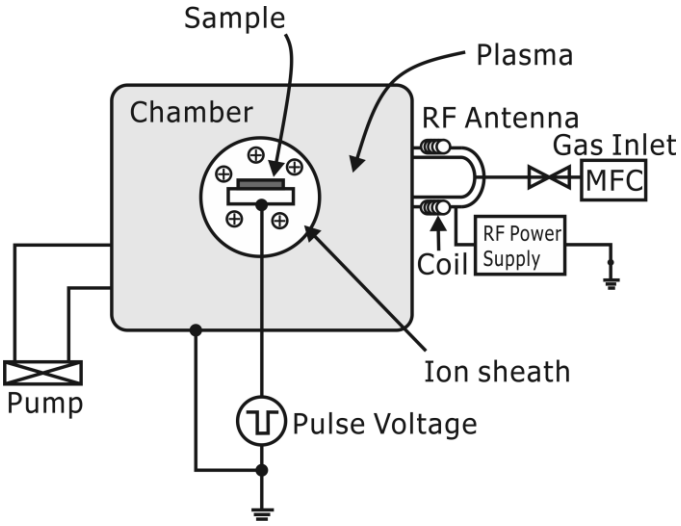


Fig. 1 Schematic diagram of the PBII apparatus

### B. Sample Preparation

*Escherichia coli* NBRC3972 were used as the test organism in this study. The bacteria were cultured on a Luria–Bertani (LB) agar medium at 37°C for 24 h and then collected by suspending them using a platinum ear after adding a small amount of phosphate buffer (pH 7.2). The bacterial concentration was adjusted to $10^8$ CFU/mL with phosphate buffer.

### C. Plasma Treatment

One hundred microliters of the bacterial suspension and 300 µL of phosphate buffer were added to a sterile Petri dish, spread, and air dried. The treated Petri dish was transferred to the sterilization chamber. Glass or stainless-steel Petri dishes were used. The chamber was evacuated to achieve a base pressure of 20 Pa, following which a carrier gas was introduced to attain a pressure of 200 Pa. This process was repeated three times. Then, the gas pressure was stabilized at 5 Pa before generating plasma. The carrier gas used in the experiment comprised nitrogen, oxygen, or HHO gas, with the latter being a hydrogen and oxygen mixture (2:1) generated by using a SUN WELDER SW-134 (Sun well Co. Ltd). Sterilization involved the following operational parameters: frequency of 50 Hz, delay time of 50 µs, pulse width of 10 µs, applied voltage ranging from −2 to −4 kV, RF output of 48 VA, and treatment time of 10 min. Following plasma treatment, 1 mL of phosphate buffer was added to the Petri dish containing the treated bacteria, and the suspension was transferred to sterile microtubes via a micropipette. Dilution series was subsequently prepared using phosphate buffer, and one hundred µL of each dilution was added to the LB medium, followed by the pour plate method at 37°C for 24 h. Upon completion of the culture, the number of viable cells was determined by counting the colony-forming units. The data were analyzed in triplicate and expressed as mean ± standard deviation.

## III. RESULTS AND DISCUSSION

### A. PBII treatment with $N_2$ gas

Fig. 2 shows the results of PBII treatment using nitrogen gas.

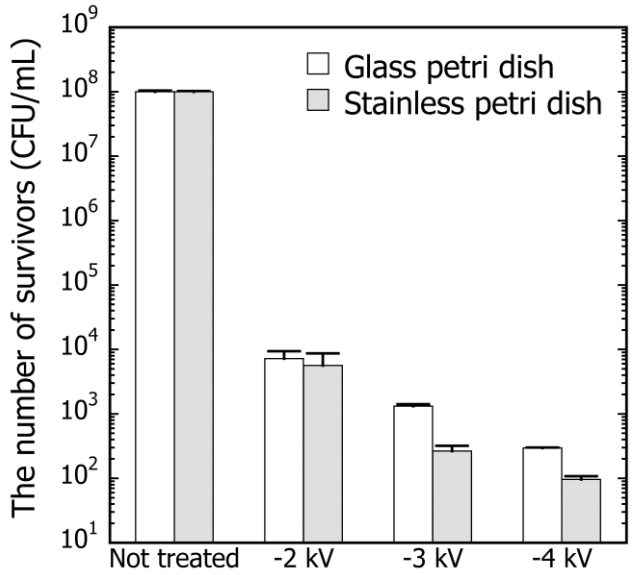


Fig. 2 Sterilization effect of *E. coli* treated by nitrogen gas plasma

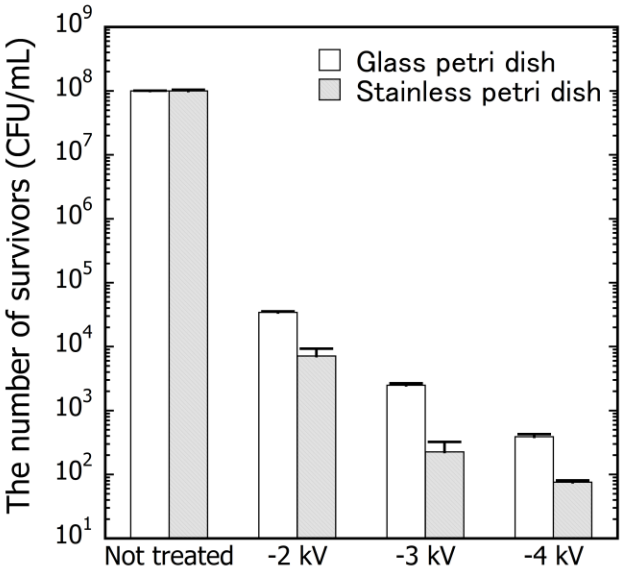


Fig. 3 Sterilization effect of *E. coli* treated by oxygen gas plasma

When utilizing nitrogen gas, no difference in the sterilization effect was observed between glass and stainless-steel Petri dishes at an applied voltage of −2 kV. However, at −3 kV, the sterilization effect of the glass Petri dish was 4.9 D, whereas that of the stainless-steel Petri dish was 5.6 D, indicating that the sterilization effect of the stainless-steel Petri dish was approximately 0.7 D superior. In addition, the sterilization effect of the stainless-steel Petri dish was better at an applied voltage of −4 kV, whereas the sterilization effect increased to 6.0 D.

### *B. PBII treatment with $O_2$ gas*

Subsequently, Fig. 3 shows the results of PBII treatment using oxygen gas. When utilizing oxygen gas, the sterilization effect of the stainless-steel Petri dish was more significant at −2 kV. At −4 kV, the sterilization effect was 6.1 D greater than that of the stainless-steel Petri dish. The difference in the sterilizing effect between oxygen gas and nitrogen gas at the same applied voltage could be due to the generation of highly reactive molecular species, such as oxygen radicals, when oxygen gas is used, resulting in a stronger sterilization effect caused by the chemical action[12].

### *C. PBII treatment with HHO gas*

Finally, the results of using HHO gas are shown in Fig. 4.

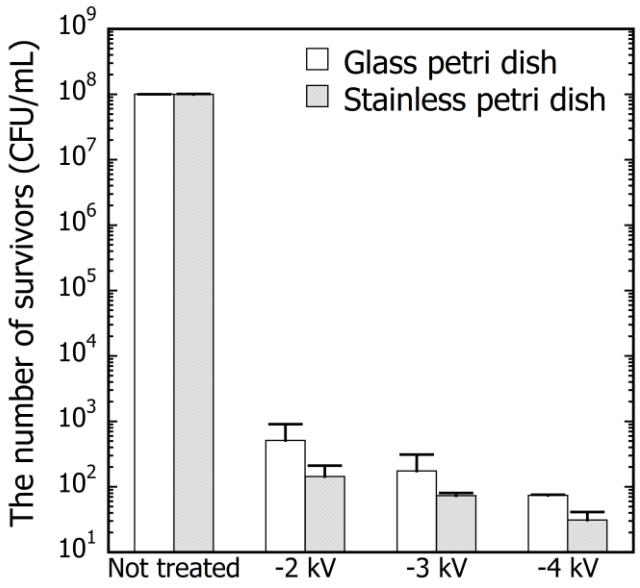


Fig.4 Sterilization effect of *E. coli* treated by HHO gas plasma

The sterilizing effect of HHO gas was 5.3 D for the glass Petri dish and 5.8 D for the stainless-steel Petri dish at an applied voltage of −2 kV, which was higher than that of nitrogen or oxygen gas. At an applied voltage of −4 kV, the sterilization effect was 6.1 D for the glass Petri dish and 6.5 D for the stainless-steel Petri dish.

Thus, the outcomes have demonstrated that regardless of the gas used, the sterilization effect of the stainless-steel Petri dish surpasses that of the glass Petri dish. This finding can be attributed to the high conductivity of the stainless-steel compared with glass, which allows for efficient high-voltage application in the gas phase and facilitates ion acceleration. However, previous ion density computations have shown that the difference in the conductivity of the sample-fixing Petri dish is nullified by the conductive breakdown when the applied voltage is increased to −5 kV[10]. We are examining the difference in the sterilizing effect in the lower voltage range. We have confirmed that using a stainless-steel Petri dish for sterilization in the lower voltage range is effective.

Thus, for the sake of discussion, the outcomes of the stainless-steel Petri dish are summarized in detail in Fig. 5.

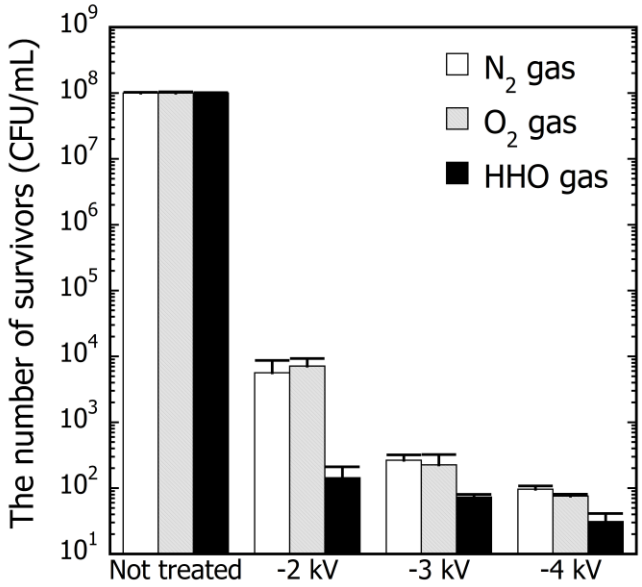


Fig.5 Effect of different gas species used in PBII treatment on the sterilization of *Escherichia coli*

As shown in Fig. 5, when sterilizing at a low-voltage range, HHO gas is considered more effective than nitrogen or oxygen gas because HHO gas generates more diverse molecular species than the other gases tested. Thus, HHO gas can cause chemical damage before the applied voltage is raised to cause physical collisions. We have not been able to recognize the molecular species generated when HHO gas is used.

Therefore, in the future, we must identify the molecular species generated by HHO gas to clarify the source of the damage. HHO gas is produced from water, and it is considered safe for food products. It is also thought to be the most effective gas for sterilizing food materials because it can easily enhance the sterilization effect at lower voltages compared with nitrogen or oxygen gases. Furthermore, we must determine the conditions under which HHO gas can be used to sterilize *G. stearothermophilus*, a heat-resistant spore bacterium.

## IV. CONCLUSION

The PBII technique was used to treat *E. coli*, a Gram-negative bacterium, using nitrogen, oxygen, and HHO gases. The use of stainless-steel Petri dishes during experiments resulted in a higher sterilization effect for all gases as compared with glass Petri dishes. Remarkably, the sterilizing effect of HHO gas was found to be sufficient (5.8 D) even under a low applied voltage (−2 kV). We posit that the sterilizing effect of PBII treatment is due to the synergistic interplay between the physical collision of ions and the chemical reaction of plasma species with the bacterial cells. Notably, the chemical effect of HHO gas is stronger, thereby leading to a better sterilization effect at a lower applied voltage compared with the other gases tested. In the future, the application of HHO gas to *G. stearothermophilus*, a thermotolerant spore bacterium that is difficult to kill, will be investigated.

## REFERENCES


[1] P. T. Jacobs, S.-M. Lin, in: R. L. Clough, S. W. Shalaby (Eds.), Irradiation of polymers : fundamentals and technological applications, American Chemical Society, Washington, DC, 1996, pp. 216-239.

[2] K. Kelly-Wintenberg, A. Hodge, T. C. Montie, L. Deleanu, D. Sherman, J. Reece Roth, P. Tsai, L. Wadsworth, "Use of a one atmosphere uniform glow discharge plasma to kill a broad spectrum of microorganisms", *Journal of Vacuum Science & Technology A*, Vol. 17, pp. 1539-1544 (1999). https://doi.org/10.1116/1.581849

[3] S. Moreau, M. Moisan, M. Tabrizian, J. Barbeau, J. Pelletier, A. Ricard, L. H. Yahia, "Using the flowing afterglow of a plasma to inactivate *Bacillus subtilis* spores: Influence of the operating conditions", *Journal of Applied Physics*, Vol. 88, pp. 1166-1174 (2000). https://doi.org/10.1063/1.373792

[4] S. Mandl, R. Sader, G. Thorwarth, D. Krause, H. F. Zeilhofer, H. H. Horch, B. Rauschenbach, "Investigation on plasma immersion ion implantation treated medical implants", *Biomol Eng*, Vol. 19, pp. 129-132 (2002). https://doi.org/10.1016/S1389-0344(02)00025-4

[5] S. Han, Y. Lee, H. Kim, G.-h. Kim, J. Lee, J.-H. Yoon, G. Kim, "Polymer surface modification by plasma source ion implantation", *Surface and Coatings Technology*, Vol. 93, pp. 261-264 (1997). https://doi.org/10.1016/S0257-8972(97)00057-1

[6] D. R. McKenzie, K. Newton-McGee, P. Ruch, M. M. Bilek, B. K. Gan, "Modification of polymers by plasma-based ion implantation for biomedical applications", *Surface and Coatings Technology*, Vol. 186, pp. 239-244 (2004). https://doi.org/10.1016/j.surfcoat.2004.04.093

[7] M. Yoshida, T. Tanaka, S. Watanabe, T. Takagi, M. Shinohara, S. Fujii, "Experimental study on a new sterilization process using plasma source ion implantation with $N_2$ gas", *Journal of Vacuum Science ; Technology A*, Vol. 21, pp. 1230-1236 (2003). https://doi.org/10.1116/1.1562183

[8] K. Kakugawa, A. Yamashiro, K. Saitoh, T. Mizutane, T. Kano, T. Tanaka, The effect of pulse width on the sterilization of *Bacillus stearothermophilus* spores using plasma-based ion implantation, Bali, pp.84-90, 2017.

[9] M. Hiyama, K. Kakugawa, A. Yakushiji, K. Watanabe, R. Matsuda, Y. Tsuchiya, TanakaT., "Optimum conditions for sterilization of *Geobacillus stearothermophilus* spores using plasma-based ion implantation", *ELECTROTECHNICA & ELECTRONICA*, Vol. 53, pp. 199-203 (2018).

[10] K. Kakugawa, S. Umemoto, T. Noda, K. Nosaki, T. Tanaka, K. Vutova, "Effect of the conductivity of the petri dish placed on the electrode on the surface layer of the spores in the PBII method", *ELECTROTECHNICA & ELECTRONICA*, Vol. 57, pp. 41-45 (2022).

[11] N. Fujimura, K. Shimono, M. Kubo, H. Noguchi, H. Toyota, K. Kakugawa, T. Tanaka, "Generation of Oxygen Plasma Using Plasma-based Ion Implantation and its Application to Sterilization of Spore-forming Bacteria. (in Japanese)", *IEEJ Transactions on Fundamentals and Materials*, Vol. 135, pp. 373-378 (2015). https://doi.org/10.1541/ieejfms.135.373

[12] H. Shintani, A. Sakudo, P. Burke, G. McDonnell, "Gas plasma sterilization of microorganisms and mechanisms of action", *Exp Ther Med*, Vol. 1, pp. 731-738 (2010). https://doi.org/10.3892/etm.2010.136


Koji Kakugawa is a currently a Dean and a Professor at Department of Food Sciences and Biotechnology, Faculty of Life Sciences, Hiroshima Institute of Technology. His current research interests include sterilization technology including developing new food sterilization equipment using plasma technology as well as researching microorganisms that enrich our lives, such as lactic acid bacteria, actinomycetes, and yeast.