Effect of the conductivity of the petri dish placed on the electrode on the surface layer of the spores in the PBII method

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Plasma-based ion implantation (PBII) method is a technique that allows uniform ion implantation to a sample by applying a negative voltage to the model to draw and accelerate ions in the ion sheath existing around the piece. We are researching to apply the PBII method as a new pasteurization technology. This study investigated how the different conductivity of Petri dishes used for sample processing affects the cell surface layer of Geobacillus stearothermophilus spores. The PBII treatment conditions were as follows: gas pressure: 5 Pa, treatment time: 10 min, gas type: nitrogen, applied voltage: -3 kV to -5 kV, RF: 60 VA, petri dish material: glass or stainless steel. After PBII treatment, the spore surface was observed using a scanning electron microscope. When spores were observed by scanning electron microscopy, it was found that the spore surface condition was different between the glass petri dish and the stainless steel petri dish. The sterilization effectiveness and ion density calculations at -3 kV showed significantly higher with the stainless steel petri dish. On the other hand, when the applied voltage was -5 kV, they were no longer quite different. It is suggested that this difference is due to differences in conductivity resulting from the material of the petri dish. Therefore, it was found that it is better to use a Petri dish having good conductivity to improve the sterilization effect using a lower applied voltage condition.

Keywords – Geobacillus stearothermophilus, non-thermal sterilization, Plasma-based ion implantation, Thermotolerant spore.

Introduction

Modern food production requires processing that is safe, free of chemical contamination, and with minimal loss of palatability and functionality. From the standpoint of safety, the alteration of food by microorganisms is a significant problem. Heat sterilization is generally used to prevent food spoilage caused by microorganisms. However, among microorganisms, spore bacteria are known to be challenging to sterilize because they produce spores with high heat resistance. When contamination with spores is suspected, pressurized thermal sterilization is used. However, pressure heat treatment may damage the quality of food products. In addition, since pressurized heat treatment is a wet process, spices and other ingredients are difficult to sterilize, even though they are often contaminated with spores.

For this reason, non-heat sterilization techniques have been attracting attention, and various new sterilization technologies have been developed. One example is ultrahigh-pressure, electrolytic, and plasma treatment [1 - 3]. As for the plasma treatment we focus on, the medical field has practiced plasma sterilization equipment using hydrogen peroxide. However, it is known that there are two problems: higher running costs due to the high cost of purchasing hydrogen peroxide and exposure of medical personnel to hydrogen peroxide, which is harmful to humans.

The plasma-based ion implantation (PBII) method is a technique that can uniformly implant ions into a sample by applying a negative voltage to the sample in plasma to draw out and accelerate the ions in the plasma around the sample. The PBII method is used for surface modification of three-dimensional shaped materials such as engineering components in the automotive and precision machinery industries and medical materials such as artificial bones and blood vessels [4 - 6]. Currently, we are researching to develop

the PBII method as a technology capable of low-temperature, short-time sterilization [7]. The PBII method does not require water during the sterilization process. Therefore, it has the potential to be used for the sterilization of powdered foods such as spices, which are difficult to sterilize using conventional sterilization methods.

We have been trying to sterilize *Geobacillus stearothermophilus*, which is known as a heat-resistant spore-generating bacterium and a sterilization indicator bacterium for plasma sterilization treatment, using the PBII method [8, 9]. Although 6D sterilization effects have been obtained in previous studies, the RF output, Etc., is too strong for developing practical machines for food sterilization.

For this reason, it is necessary to find conditions that allow sterilization at a lower output than before without affecting the food material. Therefore, this study closely examined the conditions under which damage can be done to the spores.

Materials and Methods

A schematic diagram of the PBII experimental apparatus used in our study is shown in Fig. 1.

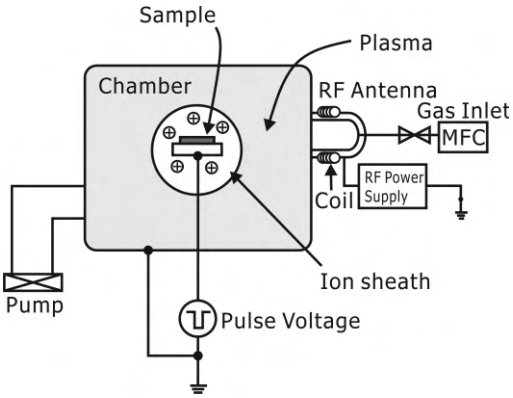


Fig.1. Schematic diagram of the PBII apparatus.

The chamber is electrically grounded and is 450 mm height, 590 mm wide, and 470 mm deep. The RF antenna for the ICP source is wound on the inside of the upper lid, with one end grounded. The antenna itself is a 5-turn copper coil, approximately 250 mm in diameter and operating at 240 kHz.

The sample was placed on a stainless-steel electrode supported by an insulated stainless-steel rod at the center of the vacuum chamber.

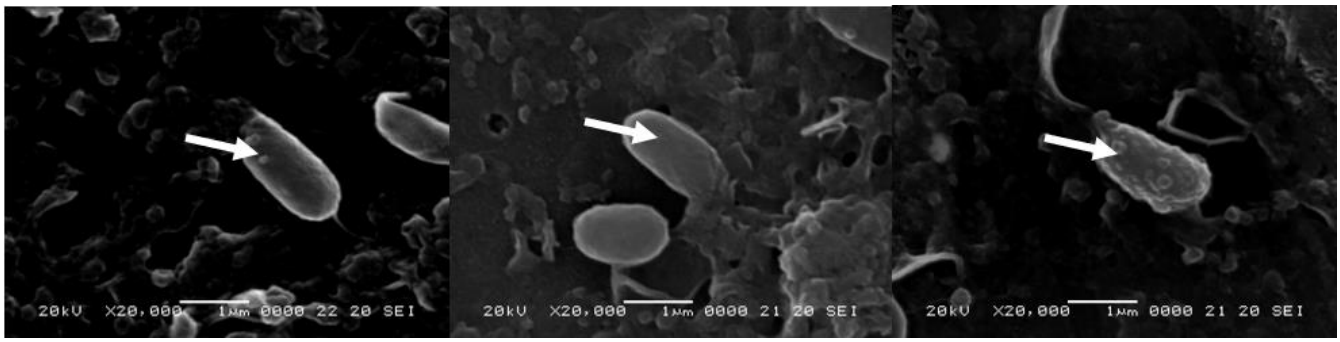
Spores of *G. stearothermophilus* NBRC12550 were used as the test specimens. In the Nutrient Agar medium, *G. stearothermophilus* vegetative cells were cultured at 55 °C for 48 hours. The cells were then inoculated into TYEA medium for spore formation and incubated at 55 °C for 10 days [10]. After confirming spore formation under a microscope, the spores were harvested by adding a small amount of phosphate buffer (pH 7.2) and heating at 80°C for 10 min to eliminate vegetative cells. In phosphate buffer, heated spores were washed three times by centrifugation (room temperature for 1 min at 12000 rpm). After the final wash, the spores were heated at 80 °C for 10 min. The spore concentration was determined and then diluted to 107 spores/mL with phosphate buffer. After adding 300 μL of phosphate buffer to 100 μL of the adjusted spore suspension, the mixture was firmly suspended using a vortex. The suspension was spread uniformly on a stainless steel plate (SUS304, φ 60 mm) or glass plate (soda-lime-silica glass, φ 60 mm) and dried up using a desiccator for 90 minutes. The treated plate was placed into the chamber. The target chamber was evacuated to a base pressure of 20 Pa, and nitrogen gas was injected to a pressure of 200 Pa. This procedure was repeated three times. Finally, gas pressure during plasma generation was maintained at 5 Pa. A summary of the experimental sterilization conditions is shown in Table 1.

Table 1

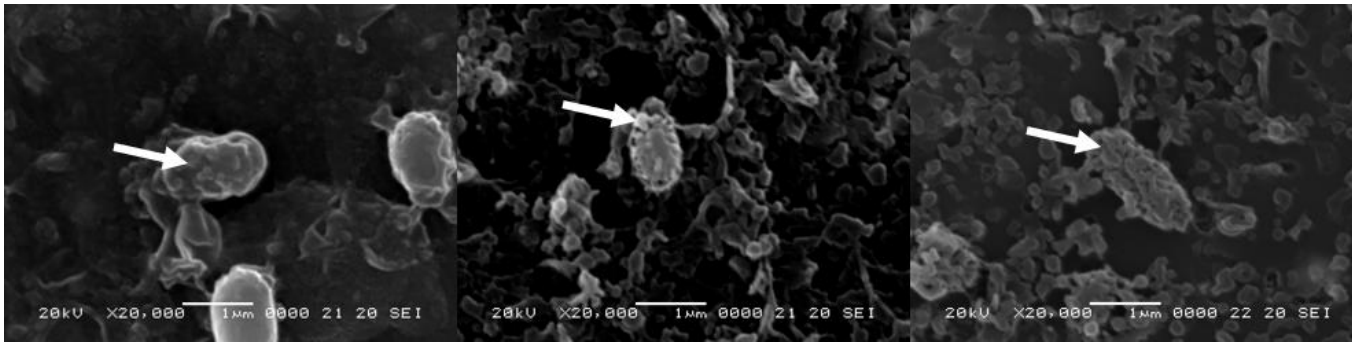
Experimental conditions for sterilization by PBII

Items	Value
Process Gas	N_2
Gas Pressure	5 Pa
RF Power	48 VA
Frequency	500Hz
Pulse width	10 μs
Delay time	50 μs
Exposure time	10 min
Pulse voltage (peak)	-3, -4, -5 kV

After exposure to plasma and ion bombardment, 100 μL of phosphate buffer was added to the treated plates. After removal by pipetting, the suspension was added to 900 μL of phosphate buffer, and a dilution series was produced with more phosphate buffer. 100 μL of each diluted spore solution was spread onto an agar plate and incubated in conditions conducive to spore germination. Colony-forming units were counted to determine the number of surviving spores. All data are expressed as the average ± standard deviation of triplet analyzes.



(a) Applied voltage: -3kV *(b) Applied voltage: -4kV* *(c) Applied voltage: -5kV*



(d) Applied voltage: -3kV *(e) Applied voltage: -4kv* *(f)Applied voltage : -5kV*

Fig.2. SEM photomicrographs of PBII-treated spores of G. stearothermophilus (a) – (c): Spores fixed on grass petri dish, (d)-(f): Spore fixed on stainless petri dish.

For electron microscopic observation of the spores, the samples were freeze-dried and sputtered with gold in a sputter coater (JFC-1600, JEOL, Japan). The sputtered samples were observed using Scanning electron microscopy (JSM-5610, JEOL, Japan).

The ion density was calculated based on the following formula[11].

$$n_i = \frac{1}{eR_1A}\sqrt{\frac{mV_p}{2e}} \quad (1)$$

n_i: ion density (m^{-3}), e: electron charge (C)

R_1: ion sheath resistance (Ω), A: target area (m^2)

M: ion species mass (kg)

V_p: target applied voltage (kV)

Results

We investigated the material of the petri dish for spore fixation, which is thought to affect the sterilization effect when sterilizing using the PBII method.

PBII treatment was performed on *G. stearothermophilus* spores under the conditions shown in Table 1. In this experiment, the applied voltage varied from -3kV, -4kV, and -5kV. The results of electron microscopy observation of treated spores are shown in Fig. 2.

As shown in Fig. 2(a) – (c), when a glass plate is used, the surface of the spores seems to be scraped off with each high voltage applied. On the other hand, when a stainless steel plate is used, it is considered that the time for which the high voltage is applied is longer, and the ion energy is increased compared to the case where the glass plate is used. Therefore, as shown in Fig. 2(d) – (f), besides the surface of the spores being scraped, there are holes in them. And as the applied voltage increases, the spores' damage appears to increase.

Next, the sterilization rate of spores was measured. The results are shown in Fig. 3.

As shown in Fig. 3, at the applied voltage of -3 kV, the sterilization effect is higher with stainless steel Petri dishes than with glass Petri dishes, but at -5 kV, there is no difference. This result may be because the use of stainless steel created holes in the surface of the spores, which caused leakage of the contents and facilitated their death. On the other hand, when a glass petri dish

was used, the surface of the spores was scraped, but the surface was not scraped enough to kill them.

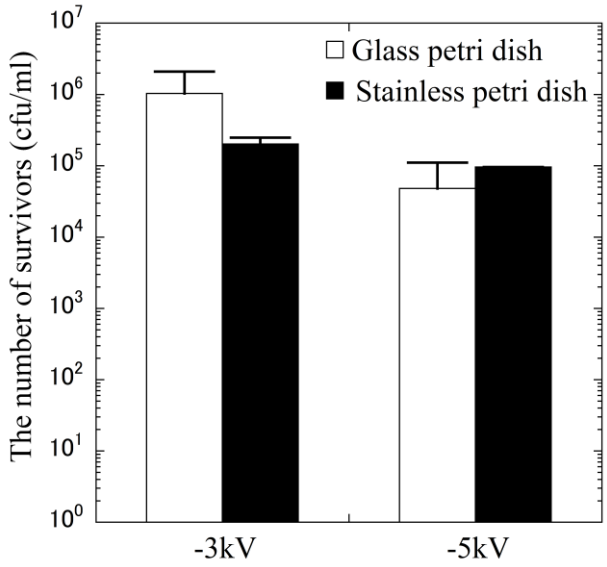


Fig.3. Influence of the material of petri dish on the sterilization effect.

For the -5 kV treatment, when glass Petri dishes were used, the degree of surface abrasion was more significant, and the number of dead spores was thought to have increased all at once. It is considered stainless steel to have a longer acceleration time and higher energy of ions than glass. Therefore, it is thought that stainless steel may cause holes in the material.

Finally, to verify the above discussion, the ion density was calculated. The results are shown in Fig. 4.

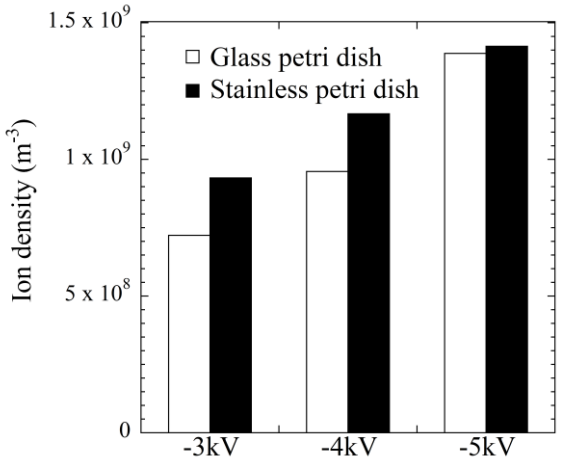


Fig.4. Relationship between petri dish material and ion density.

As shown in Fig. 4, the ion density is lower in glass up to -3 kV and becomes comparable at -5 kV. This result can be attributed to the fact that glass is dielectric. At an applied voltage of -3 kV, the dielectric reduces the voltage applied to the sample surface. When the applied voltage is increased, the charge-up due to secondary electron emission cannot be suppressed by the current through the glass substrate, and the voltage applied to the gas phase is reduced compared to stainless steel. However, it is suggested that increasing the voltage to -5 kV increases the plasma density in the gas phase, increasing ions irradiating the dielectric surface, which increases conductivity and, consequently, decreases the resistivity of the derivative. Therefore, it is suggested that the ion density values are about the same for stainless steel and glass and that the sterilization effect is also about the same. In conclusion, when the applied voltage is increased, there seems to be no significant difference between glass and metal as the material of the petri dish used for sample fixation.

We are considering using the PBII system as a food sterilization device, and we would like to use as low an applied voltage as possible for future food sterilization. Therefore, when using PBII treatment for foods, it would be desirable to use a sample dish made of metal such as stainless that has a high sterilization effect even at low voltage because charge-up is unlikely to occur.

Conclusion

When using the PBII method for sterilization, we examined the material of the stage for fixing samples, which is thought to affect the sterilization effect. The damage to the surface of *G. stearothermophilus* spores was different when stainless steel Petri dishes were used than when glass Petri dishes were used. When glass Petri dishes were used, the entire surface of the spores appeared to be scraped. On the other hand, when a stainless steel petri dish was used, the surface of the spores appeared to be perforated. At -3 kV, the sterilization effect of the stainless steel petri dish was better than that of the glass petri dish. However, when the applied voltage was increased to -5 kV, there was no significant difference in the sterilization effect. To investigate the cause of this phenomenon, we calculated the ion density. When the applied voltage was -3 kV, the ion density of glass was lower than that of stainless steel. However, there was no difference when the applied voltage was increased to -5 kV. This phenomenon is suggested to be caused by the fact that stainless steel can easily apply voltage to the gas phase at lower voltages. These are thought to be due to the difference in conductivity between glass and stainless steel. Therefore, it was found that in the PBII treatment, if the applied voltage is increased, it is possible to sterilize the spores regardless of the material to which the sample is fixed. It was also found that when

treatment is performed at lower voltages, it is better to use metallic materials such as stainless steel for sample fixation.

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