# Spore-Forming Bacteria Sterilization Using Plasma-Based Ion Implantation

**Koji Kakugawa, Masatoshi Kubo, Hiromitsu Noguchi, Kazuhiro Shimono, Nobuyuki Fujimura, Yoshinobu Tsuchiya, Takeshi Tanaka**

*A main purpose of sterilization is to kill the harmful microorganisms. In food industry, the representatives of microorganisms which are difficult to sterilize are spore-forming bacteria. Because these bacteria are extremely heat resistant. So it is no exaggeration to say high-pressure / high temperature sterilization method is the only method to sterilize spore-forming bacteria. By the way, plasma sterilization method is used to sterilize the medical equipment. However the present plasma sterilization method cannot be used for food because of containing hydrogen peroxide.*

*In this study, the authors tried to sterilize Bacillus subtilis which is model bacteria of spore-forming bacteria using plasma-based ion implantation (PBII) method which doesn't use hydrogen peroxide. The result of experimentation, the living spore reduced from $1x10^8$ cfu/ml to $7.8x10^4$ cfu/ml with 20 min exposure. The authors showed that PBII treatment without hydrogen peroxide might be able to sterilize the heat tolerance spores.*

***Стерилизация на споро-образуващи бактерии с използване на плазма-базирана йонна имплантация (К. Какугава, М. Кубо, Х. Погучи, К. Шимоно, Н. Фуджимура, Й. Цучия, Т. Танака).*** *Главната цел на стерилизацията е да убие вредните микроорганизми. В хранителната индустрия, представители на микроорганизмите, които е трудно да бъдат стерилизирани са споро-образуващите бактерии, защото тези бактерии са екстремно устойчиви при нагряване. Така не е преувеличено да се каже, че методите на стерилизация с високо-налягане и висока температура са единствени за стерилизиране на споро-образуващи бактерии. Впрочем, плазмен метод на стерилизация се използва за стерилизиране на медицински инструменти. Обаче, сегашните плазмени-стерилизационни методи не могат да се използват за храни, защото се използва водороден пероксид.*

*В тази работа авторите опитват да стерилизират Бацилиус сибтилис, който е моделна бактерия на споро-образуващи бактерии, с йонна имплантация, базирана на плазма, при който не се използва водороден пероксид. Резултатите от експериментите представят, че след 20 минутно експониране живеещите спори намаляват от $1x10^8$ cfu/ml до $7.8x10^4$ cfu/ml. Авторите показват, че с йонна имплантация, базирана на плазма без използване на водороден пероксид е възможно да се стерилизират издръжливи на нагряване спори.*

## Introduction

In most of the food industries, food products have been sterilized by moist heating (boiling or retort treatment). However, the excessive thermal conditions for ensuring the safety of microbial spoilage degrade the quality of products. Spores of *Bacillus* genus produce highly thermotolerance spores, and those spores often spoil heat sterilized foods. *Bacillus* genus is widely distributed in nature, and commonly associated with a variety of food products. Spore formation ability allows these bacteria to survive in the environment and provides them with resistance to pasteurization treatments. Therefore, many studies on thermal sterilization of foods have conducted.

A very diverse range of pasteurized food products is now available to the consumer and with world-wide preferences for more highly spiced and flavored foods, these products are likely to contain many different ingredients which could be contaminated with *Bacillus* species. However, powdery spices are difficult to sterilize by using thermal sterilization methods. So it is expected to develop the newly

sterilization equipment which is able to sterilize the powdered spice.

By the way, plasma-based ion implantation (PBII) is potentially applicable for the sterilization of three-dimensional targets as a low-temperature, cost-efficient technique for medical equipment, containers and also powdery foods[1-3]. PBII can be used to sterilize three-dimensional shapes in a short time, without the use of toxic gases. However, the sterilization performance in conventional PBII, where the target is immersed in an rf burst inductively coupled plasma (ICP) that is generated externally, is somewhat limited in that certain areas may be shadowed from exposure due to the shape of the object, and the ion penetration depth may not be adequate for complete sterilization. These shortcomings can be overcome through the use of a self-ignited plasma (SIP), which can be generated around the workpiece as a transient ion sheath by applying a pulsed dc voltage to the target. Our group previously reported that PBII reduces *Bacillus pumilus* vegetative cells by $10^4$–$10^5$ times in just 5 min, much faster than can be achieved by exposure to an external rf plasma source at 222 kHz[4].

In this study, the authors investigated the inactivating behavior of *B. subtilis* spores which were highly thermotolerance using plasma source ion implantation.

**Materials and Methods**

Spores of *B. subtilis* NITE 13719 were used as the test specimen. *B. subtilis* spores were grown on agar medium consisted of 1% polypeptone, 0.2 % yeast extract, 0.1 % $MgSO_4$ and 1.5 % agar (spore forming medium) at 35 °C for 5 days. The spores were harvested adding a small amount of saline solution and heated at 65 °C for 30 min to eliminate the vegetative cells. Heated spores were washed three times by centrifugation in saline solution. After the final wash treatment, the spores were heated at 65 °C for 30 min. The spore population of the suspension was determined by diluting the suspension and the concentration of spore suspension was diluted to $10^8$ spores/ml with saline solution.

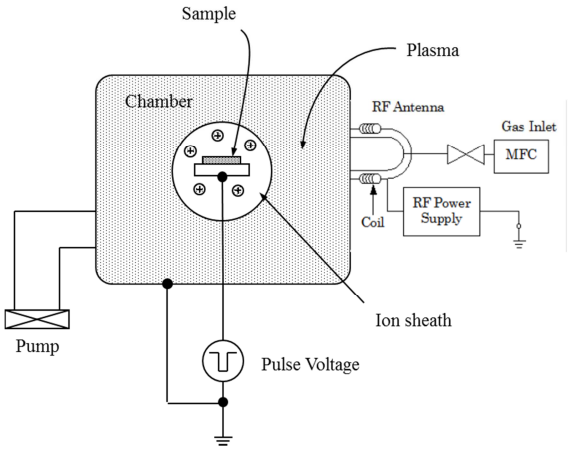


*Fig.1. Schematic diagram of experimental apparatus*

Figure 1 shows a schematic diagram of the PBII apparatus. The chamber is electrically grounded and has dimensions of 450 mm in height, 590 mm in width, and 470 mm in depth. The rf antenna for the inductively coupled plasma (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 235 kHz.

In the experiment, the sample was placed on a stainless-steel electrode supported by an insulated stainless-steel rod at the center of the vacuum chamber. A negative pulsed voltage was applied to the electrode during sterilization.

A pulsed negative voltage of up to -4 kV with a pulse width of 6.3 μs was applied to the target by a high-voltage pulse modulator with a maximum current of approximately 8 A. A rf discharge of input power 160 W was applied in some cases to investigate the effect of ordinary external plasma with respect to

**Table.1.**

*Experiment conditions of parameter on this experiment*

| | | Case 1 | Case 2 | Case 3 | Case 4 |
|---|---|---|---|---|---|
| Gas | | $O_2$ | $O_2$ | $O_2$ | $O_2$ |
| Gas Pressure | [Pa] | 4 | 3 | 3 | 3 |
| Pulse Width | [ms] | 6.3 | 6.3 | 6.3 | 6.3 |
| Pulse Rate | [pulses/s] | 1000 | 1000 | 1000 | 1000 |
| Pulse voltage(peak) | [kV] | -8 - -1 | -6 - -2 | -6 | -4 |
| Pulse current(peak) | [A] | 4 | 4 | 4 | 4 |
| RF Power | [W] | - | 160 | 160 | 160 |
| Exposure time | [min] | 10 | 10 | 5-20 | 5-20 |

sterilization. The distance between the rf antenna and the target was about 200 mm, and no arcing or surface charging was observed for the samples employed.

100 µl of spore suspension applied on the sterile paper cup and the cup was sealed in sterile paper bag. A sealed bag was inserted into the chamber.

The target chamber was evacuated to a base pressure of 10 Pa and the oxygen gas is injected to a pressure of 1 kPa. This procedure was repeated three times. Finally, gas pressure during plasma generation was maintained at 3 Pa. A summary of the experimental sterilization conditions is shown in Table 1.

After exposure to plasma and ion bombardment, 100 µl of saline solution was added to treated paper cup. After pipetting, this saline solution was added into 900 µl of saline solution. The dilution series was made by using this solution and 100 µl of the diluted spore solution was spreaded on spore forming agar plate. After incubation at 37 °C for 12 hr, colony forming units were counted to determine the numbers of survivors of *B. subtilis* spore. All data are expressed as average ± standard deviation of triplet analysis.

**Results and Discussion**

This experiment evaluated the effectiveness of plasma-based ion implantation using a self-ignited $O_2$ gas plasma againt *B. subtilis* spores. First of all, the Authors tried to case 1 experiment to examine the influence of pulse voltage. The result is shown in Fig.

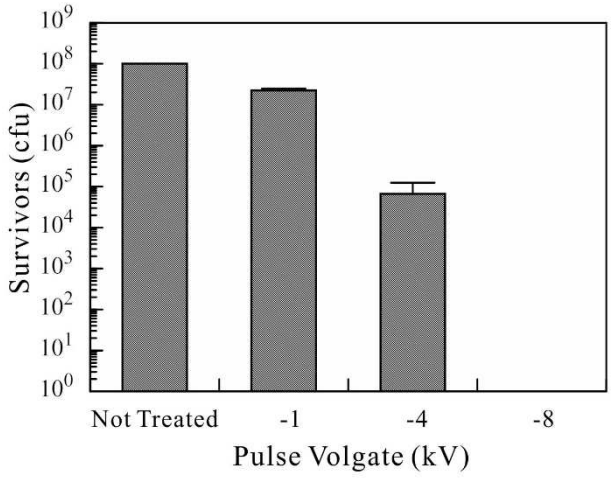


*Fig.2.Influence of Pulse Voltage*

2.

As shown in Fig.2, the number of survivors had decreased when the pulse voltage was raised. This phenomena might be similar to our previous data[5]. The previous data show that the energy of incident ions is more important for sterilization than the ion dose[5]. However a strong energy of ions caused to burn a hole on the surface of a sample bag at this time. So the data of -8 kV was not shown in Fig.1 because the sample bag was burned. And this means that food materials might also be burned. In further experiments, we decided the range of pulse voltage from -6 kV to 0 kV.

Next, the authors analyzed the influence of pulse burst rf plasma according to the condition of case 2. The result is shown in Fig.3.

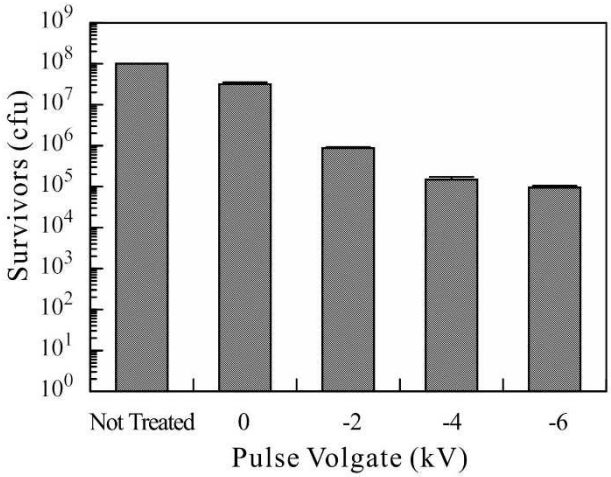


*Fig.3. Influence of pulse burst rf plasma*

As shown in Fig.3, the higher pulse voltage, the fewer survivors under the condition of adding pulse rf plasma. However, Comparison between Fig.2 and Fig.

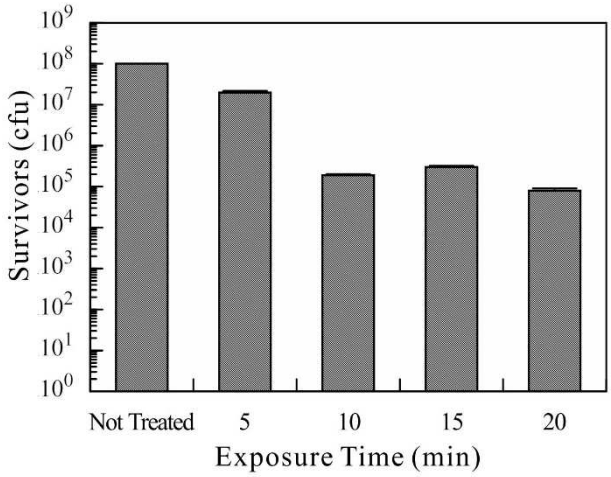


*Fig.4. Influence of exposure time*

3, a significant difference of survivors was unable to confirm. So next experiment whose condition described as case 3, the authors try to extend the exposure time. The result is described as Fig.4.

As shown in Fig.4, the survivors seem not to have

changed over 10 min exposure. The authors thought that these data were inaccurate. Because the sealed bag burned and there was a possibility that the sterile condition was not maintained. In addition, it is thought that the application to powdery foods is impossible in this condition because those foods may burn. So the authors changed pulse voltage to -4 kV (case 4). The result is shown in Fig.5.

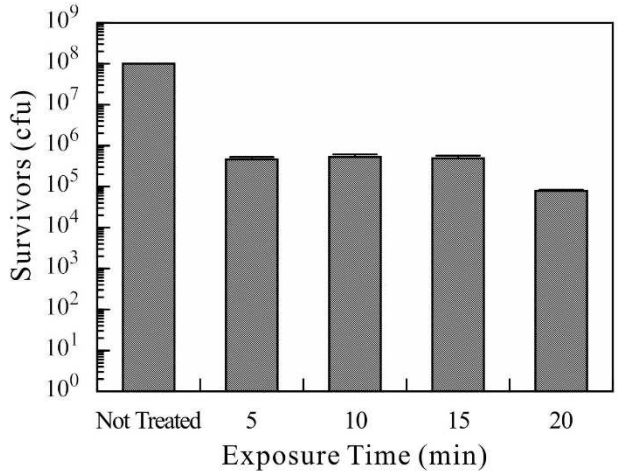


*Fig.5 Influence of exposure time under the low pulse voltage condition*

As shown in Fig.5, most effective exposure time was 20 min and the survivors decreased from $1x10^8$ cfu/ml to $7.8x10^4$ cfu/ml.

In this research, the authors tried to sterilize *B. subtilis* spores, which were highly heat tolerance, using PBII apparatus. The survivors of *B. subtilis* decreased from $1x10^8$ cfu/ml to $7.8x10^4$ cfu/ml under the most effective condition (pulse voltage : -4KV, exposure time : 20min, pulse burst rf plasma : bombardment). The authors showed that PBII treatment without hydrogen peroxide might be able to sterilize the heat tolerance spores. However the ability of this condition is insufficient, because the survival curve shows a plateau as shown in Fig.5. The sterilization using PBII method may be caused by collision of ions and radicals, which are produced under a high-pressure pulse against the spores on the base. So when the density of ions and radicals are increased, the efficiency of sterilization would be expected to be improved. In this experiment, despite under the bombardment of pulse burst rf plasma, the spores did not decrease to the value below $10^4$ cfu/ml. This may be caused by using paper cup as sample carriers. There is a possibility that the fiber in the paper cup influences the irradiation efficiency of the plasma. This phenomenon might be improved to change a paper cup to a glass plate. Including this problem, our research is being continued to sterilize the heat tolerance spore completely.

## Conclusion

The authors tried to sterilize *B. subtilis* spores, which were highly heat tolerance using PBII apparatus. The survivors of *B. subtilis* decreased from $1x10^8$ cfu/ml to $7.8x10^4$ cfu/ml at this time. We showed the possibility that the thermotolerance spores were able to sterilize using PBII apparatus without hydrogen peroxide. It is thought that this result shows the possibility of the development of a novel spice sterilizer.

## REFERENCES

[1] K. Kelly-Wintenberg, A. Hodge, T.C. Montie, L. Deleanu, D. Sherman, J. Reece Roth, P. Tsai, L. Wadsworth, Journal of Vacuum Science & Technology A 17 (1999) 1539-1544.

[2] 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.

[3] S. Moreau, M. Moisan, M. Tabrizian, J. Barbeau, J. Pelletier, A. Ricard, L.H. Yahia, Journal of Applied Physics 88 (2000) 1166-1174.

[4] M. Yoshida, T. Tanaka, S. Watanabe, T. Takagi, M. Shinohara, S. Fujii, Journal of Vacuum Science ; Technology A 21 (2003) 1230-1236.

[5] T. Tanaka, T. Hironaka, S. Hayashi, I. Koyama, ELECTROTECHNICA & ELECTRONICA 5-6 (2012) 268-272

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