Spore-forming bacteria sterilization using plasma-based ion implantation (2nd report)

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A main purpose of sterilization is to kill the harmful microorganisms. The spore-forming bacteria which are present in foods frequently cause food spoilage. Although the vegetative cells of those bacteria are sterilized easily, the spores need harsher conditions to be inactivated. In this study, the authors tried to sterilize Bacillus stearothermophilus and Clostridium sporogenes which produce thermotolerant spore using PBII sterilization technique with a harmless gas at a low temperature.

Стерилизация на споро-образуващи бактерии с използване на плазма-базирана йонна имплантация (Втора статия) (Кожи Какугава, Кай Саито, Кения Хаяши, Казухиро Шимоно, Хиромитсу Ногучи, Йошинобу Тсучия, Такеши Танака). *Основна цел на стерилизация е да убие вредните микроорганизми. Спорообразуващите бактерии, които присъстват в храните, често причиняват развалянето им. Въпреки, че растителните клетки на тези бактерии се стерилизират лесно, са необходими строги условия, за да се инактивират спорите. В това изследване, авторите се опитаха да се стерилизират Bacillus Stearothermophilus и Clostridium sporogenes, които произвеждат терморезистентни спори, с помощта на плазмена йонна имплантация (PBII) за стерилизация с безвреден газ при ниска температура.*

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 thermotolerant 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. Some foods (ex. powdery spice) are difficult to sterilize by using thermal sterilization methods. So it is expected to develop the newly sterilization equipment which is able to sterilize such as powdery 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 without the use of toxic gases in a short time. 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] and also reported that thermotolerant spores of *B. subtilis* reduced from 10^7 cfu/ml to 10^2 cfu/ml for 10 min exposure [5]. Additionally, this sterilization effect was often abbreviated “5D”.

In this study, the authors selected *B. stearothermophilus* and *C. sporogenes* as test strains.

The spores of these strains are more thermotolerant than those of *B. subtilis*. Generally *B. stearothermophilus* is known as bacteria which caused flat-sour spoilage of canned foods. This is classified as thermophilic facultative anaerobic spore former [6]. This type of spoilage is not a health hazard but the product is inedible. And *C. sporogenes* is a kind of an obligate anaerobic spore former. *C. sporogenes* is phenotypically similar to *C. botulinum*, but it lacks the capability to produce the neurotoxin botulinum that causes human disease. So spores of this strain are often used in commercial thermal challenge studies in place of *C. botulinum* [6]. Based on the above thing, the authors investigated the inactivating behavior of *B. stearothermophilus* spores and *C. sporogenes* spores using plasma source ion implantation.

Materials and Methods

Spores of *B. stearothermophilus* NBRC 12550 and *C. sporogenes* NBRC 16411 were used as the test specimen. *B. stearothermophilus* spores were grown on agar medium consisted of 5% polypeptone, 0.1% yeast extract, 0.5% $MgSO_4$ and 1.5% agar (spore forming medium) at 55°C for 7 days. *C. sporogenes* spores were grown on agar medium consisted of 1% yeast extract, 0.125% K_2HPO_4, 0.1% soluble starch, 0.05% sodium mercaptoacetate, 2% agar (spore forming medium) at 37°C for 7 days under anaerobic condition.

The cultured spores were harvested adding a small amount of phosphate buffer (pH 7.2) and heated at 65°C for 30 min to eliminate the vegetative cells. Heated spores were washed three times by

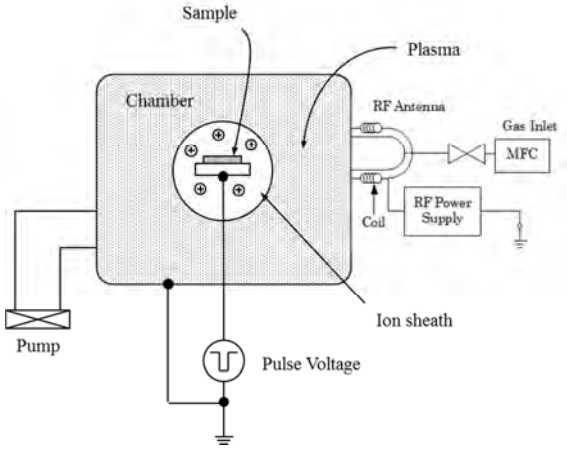

Fig.1. Schematic diagram of experimental apparatus

centrifugation in phosphate buffer (pH 7.2). 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 10^6 spores/ml with phosphate buffer (pH 7.2).

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 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 glass dish (15mm x Φ90 mm) and the sterile glass dish 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.

Table 1

Experiment conditions of sterilization by PBII

		Case 1	Case 2
Gas		O_2	O_2
Gas Pressure	[Pa]	3	3
Pulse Width	[ms]	6.3	6.3
Pulse Rate	[pulses/s]	1000	1000
Pulse voltage(peak)	[kV]	-6 - 0	-6
Pulse current(peak)	[A]	4	4
RF Power	[W]	0 - 240	240
Exposure time	[min]	10	10 - 40

After exposure to plasma and ion bombardment, 100 μl of phosphate buffer (pH 7.2) was added to treated glass plate. After pipetting, this phosphate buffer (pH 7.2) was added into 900 μl of phosphate buffer (pH 7.2). The dilution series was made by using

this solution and 100 µl of the diluted spore solution was spread on spore forming agar plate. After incubation under an adequate condition for each spore, colony forming units were counted to determine the numbers of survivors. All data are expressed as average ± standard deviation of triplet analysis.

Results and Discussion

This experiment evaluated the effectiveness of

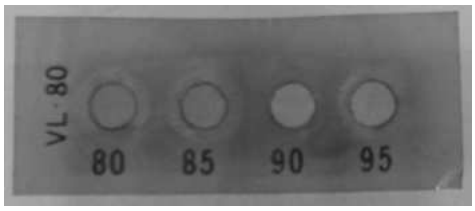

Fig.2. Result of the temperature measurement.

plasma-based ion implantation using a self-ignited O_2 gas plasma against *B.* stearothermophilus spores and *C. sporogenes* spores. First of all, the authors measured the temperature of the surface of glass plate

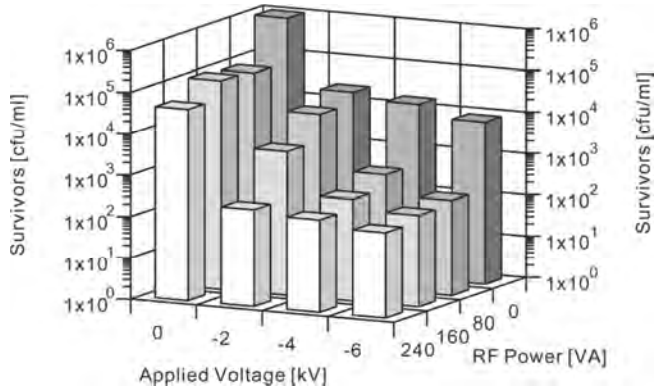

Fig.3. Result of sterilization treatment for B. stearothermophilus by PBII.

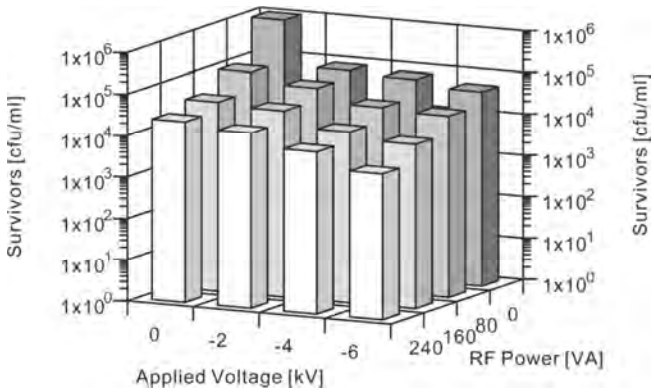

Fig.4. Result of sterilization treatment for C. sporogenes by PBII.

by using THERMOLABEL (NiGK Corporation) under sterilization treatment as shown in Table 1. The result of Case 1 is shown in Fig. 2 as an example.

If the surface temperature of a label becomes more than an assignment temperature which shows number written on a label, a discoloration area should change from a pale yellow to a black irreversibly. As shown in Fig. 1, all area after sterilization treatment did not change black. From the above result, the temperature in a PBII apparatus has verified being kept 80ºC or less.

The authors tried to case 1 experiment to examine the influence of pulse voltage and rf power. The result is shown in Fig. 3 and Fig. 4.

The previous data show that the energy of incident ions is more important for sterilization than the ion dose [5, 7]. 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. 3 and Fig. 4 because the sample bag was burned in fact. In follow-on experiments, we decided the range of pulse voltage from -6 kV to 0 kV.

As shown in Fig. 3 and Fig. 4, the number of survivors had decreased when the pulse voltage and RF power were raised. This phenomena might be similar to our previous data [5, 7]. It is known that the spores of *B. stearothermophilus* and *C. sporogenes* do not die at the temperature below 100ºC under normal pressure [8, 9]. The phenomenon of this experiment that the spores died even at temperature under 80 ºC

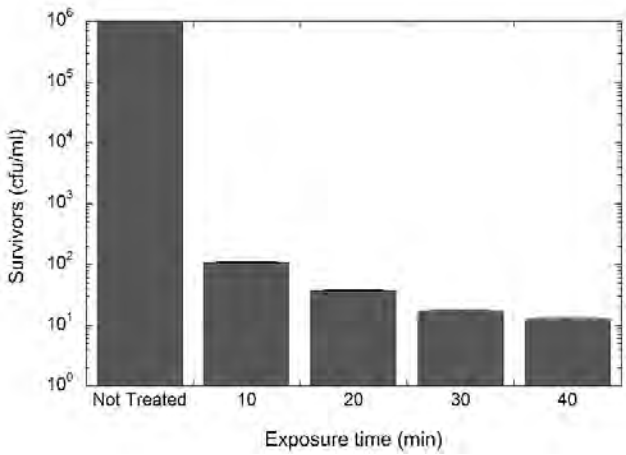

Fig.5. Influence of exposure time on inactivation of B. stearothermophilus spores.

shows that plasma contributed mainly to the death.

By the way, the killing effect of *B. subtilis* spores using PBII was 5D after 10 min sterilization [10]. On the other hand, that of *B. stearothermophilus* was 4D and that of *C. sporogenes* was 3D under same condition of *B. subtilis* spores treatment respectively. The authors therefore tried to extend the exposure

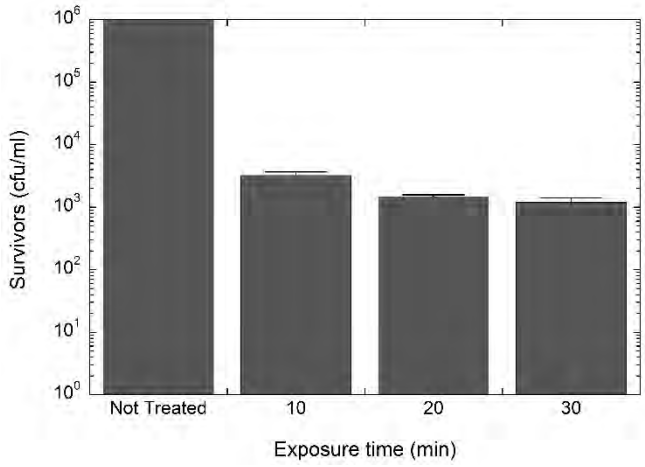

Fig.6. Influence of exposure time on inactivation of C. sporogenes spores.

time according to the condition of case 2. The result is described as Fig. 5 and Fig. 6.

As shown in Fig. 5, the killing effect of *B. stearothermophilus* is 5D after 40 min exposure. The survivors did not decrease over 50 min exposure (data not shown).

As shown in Fig. 6, the killing effect of *C. sporogenes* is 3D after 30 min exposure. The survivors did not decrease over 40 min exposure (data not shown).

In this research, the authors tried to sterilize *B. stearothermophilus* spores and *C. sporogenes* spores using PBII apparatus. The survivors of *B. stearothermophilus* decreased from 10^6 cfu/ml to 10^1 cfu/ml under the most effective condition (pulse voltage: -6 kV, exposure time: 40 min, pulse burst rf plasma: 240 VA) and the survivors of *C. sporogenes* decreased from 10^6 cfu/ml to 10^3 cfu/ml under the most effective condition (pulse voltage: -6 kV, exposure time: 30 min, pulse burst rf plasma: 240 VA).

The authors showed that PBII treatment without hydrogen peroxide under 80ºC might be able to sterilize the thermotolerant spores. However the ability of the killing effect is insufficient against that of *B. subtilis*. The sterilization using PBII may be caused by collision of ions and radicals, which are produced under a high-pressure pulse against the spores on the base. And the physical damage of spore surface observed by SEM after PBII treatment (data not shown).

By the way, $D_{121\,^{\circ}C}$ value of *B. subtilis* is 0.5 min, that of *B. stearothermophilus* is 2.6 min and that of *C. sporogenes* is 1.05 min [8, 9]. Generally, it is considered that a thermotolerance is proportional to the hardness of a spore. So in this case, a hardness of *B. stearothermophilus* spore should be higher than that of *C. sporogenes* spore.

However, in the sterilization treatment using PBII, *C. sporogenes* was more difficult than *B. stearothermophilus* as shown in Fig. 5 and Fig. 6. These results have suggested that not only the hardness of a spore but the composition of cell wall of a spore might be related to thermal resistance. We would like to advance the investigation about the relationship between cell-wall compositions and sterilization conditions.

Conclusion

The authors tried to sterilize *B. stearothermophilus* spores and *C. sporogenes* spores, which were highly heat tolerance using PBII apparatus. The survivors of *B. stearothermophilus* decreased from 10^6 cfu/ml to 10^1 cfu/ml at this time. And the survivors of *C. sporogenes* decreased from 10^6 cfu/ml to 10^3 cfu/ml. We showed the possibility that thermotolerant spores were able to sterilize using PBII apparatus without hydrogen peroxide at a low temperature. It is thought that this result shows the possibility of the development of a novel food sterilizer.

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