Optimum conditions for sterilization of *Geobacillus stearothermophilus* spores using plasma-based ion implantation

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In recent years, substantial research into low-temperature sterilization techniques has been conducted. Plasma-based ion implantation (PBII) is a low-temperature, cost-effective technique for sterilizing medical equipment, containers, and foods. We sought to optimize the seterilization conditions for Geobacillus stearothermophilus spores, which are highly heat tolerant, using a PBII apparatus. G. stearothermophilus spore survivors decreased from 10^7 to 10^1 cfu/mL under the following conditions: O_2 atmosphere, 1 kHz frequency, 50 μs delay time, 20 μs pulse width, −12 kV pulse voltage, 240 V, RF power, and a 10 min exposure time. We were able, therefore, to achieve "6D" sterilization using low-temperature sterilization conditions.

Keywords – Geobacillus stearothermophilus, plasma-based ion implantation, sterilization, optimization.

Оптимални условия за стерилизация на спори на Geobacillus stearothermophilus чрез използването на плазмено-базирана йонна имплантация (Мотоко Хияма, Кожи Какугава, Аиа Якушижи, Кеничи Уатанбе, Рийота Матсуда, Йошинобу Тсучия, Такеши Танака). *През последните години се провежда съществено проучване на технологиите за нискотемпературна стерилизация. Плазмено-базираната йонна имплантация (ПБЙИ) е нискотемпературна, рентабилна технология за стерилизиране на медицинско оборудване, контейнери и храна. Стремим се да оптимизираме стерилизационните условия за спорите на Geobacillus staterothermophilu, които имат висока топлинна толерантност, чрез прилагане на ПБЙИ апаратура. При прилагане на следните условия: атмосферен кислород, 1 kHz честота, 50 μs закъснение, честота на пулса от 20 μs, напрежение на пулса от -12 kV, 240 V, RF мощност и 10 min излагане, оцелелите спори на G. Stearothermophilus намаляват от 107 до 101 cfu/mL. Заради това при използване на нискотемпературни стерилизиращи условия, успешно е достигнато „6D" ниво на стерилизиране.*

Introduction

Moist heating (boiling or retort treatment) is the most common method of sterilization in the food industry. However, excessive heating to ensure against microbial spoilage also degrades the quality of food products. Thus, we attempted to sterilize samples contaminated with *Geobacillus stearothermophilus*, a model species representing spore-forming bacteria, using a plasma-based ion implantation (PBII) method that does not use hydrogen peroxide.

Plasma-based ion implantation (PBII) is potentially applicable to rapidly sterilizing three-dimensional (3D) targets without using toxic gases. It is a low-temperature, cost-efficient technique for medical equipment, containers, and powdery foods [1 - 3]. However, the sterilization performance of conventional PBII, where the target is immersed in a radio frequency (RF) burst of externally generated inductively coupled plasma (ICP), is somewhat limited; 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. This shortcoming can be overcome with a self-ignited plasma, which can be generated around the target object 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 $10^4 – 10^5$ fold in just 5 min - much faster than can be achieved by exposure to an external RF plasma source at 222 kHz [4]. Our group also reported that thermotolerant spores of *Bacillus subtilis* were reduced from 10^7 to 10^2 cfu/mL after a 10 min exposure [5, 6].

Table 1

Experimental conditions for sterilization by PBII.

Items	Case 1	Case 2	Case 3	Case 4	Case 5
Supplied Gas	O_2	O_2	O_2	O_2	O_2
Gas pressure [Pa]	3	3	3	3	3
Frequency [kHz]	1	1	1	1	1
Delay time [μs]	30 ~ 70	50	50	50	50
Pulse width [μs]	5	5 ~ 25	5 ~ 25	5 ~ 25	20
Pulse voltage (peak) [kV]	−6	−6	−6	−6	−12 ~ −10
RF power [Va]	240	240	240	240	240
Exposure time [min]	10	10	15	20	10

This five-order sterilization effect is abbreviated as "5D."

G. stearothermophilus is a bacterium that causes flat-sour spoilage of canned foods. The species is classified as a thermophilic, facultative, anaerobic spore former [7]. Generally, the spores of this species are more thermotolerant than those of *B. subtilis*. We previously attempted sterilization by changing parameters including pulse voltage, RF power and treatment time and reported that the survival of spores of *G. stearothermophilus* decreased from 10^6 to 10^1 cfu/mL after a 40 min exposure under the effective conditions (pulse voltage: −6 kV and RF power: 240 VA) [8]. In the case of *B. subtilis*, similar effects were obtained after a 10 min treatment, as discussed above. Our group also reported that the survival of spore of *G. stearothermophilus* decreased from 10^7 to 10^2 cfu/mL under the following conditions: O_2 atmosphere, 1 kHz frequency, 50 μs delay time, 10 μs pulse width, −6 kV pulse voltage, 240 VA RF power and a 10 min exposure time [9].

In this study, we tried to further decrease *G. stearothermophilus* survival by changing parameters other than the frequency and RF power (e.g., pulse voltage, delay time, and pulse width and exposure time).

Materials and Methods

Spores of *G. stearothermophilus* NBRC13737 were used as the test specimens. *G. stearothermophilus* spores were grown on agar medium consisting of 5% HIPOLYPEPTON, 1% Bacto™ Yeast Extract, 7.5% $MgSO_4 \cdot 7H_2O$ and 7.5% agar (spore-forming medium) at 55°C for 14 days.

Cultured 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. Heated spores were washed three times by centrifugation (room temperature for 1 min at 12000 rpm) in phosphate buffer. After the final wash, the spores were heated at 80° C for 10 min. The spore concentration was determined and then diluted to 10^7 spores/mL with phosphate buffer.

Fig. 1 shows a 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 235 kHz.

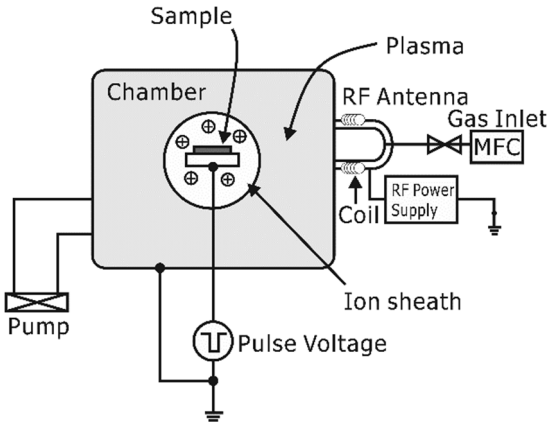

Fig. 1. Schematic diagram of the experimental apparatus.

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

One hundred μL of spore suspension and 300 μL of phosphate buffer were applied to a sterile glass dish, which was then sealed in a sterile paper bag. The sealed bag was placed into the chamber.

The target chamber was evacuated to a base pressure of 10 Pa and oxygen gas was 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 phosphate buffer was added to the treated glass 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. One hundred µL of each diluted spore solution was spread onto an agar plate and incubated conditions conducive to spore germination. Colony-forming units were counted to determine numbers of surviving spores. All data are expressed as the average ± standard deviation of triplet analyses.

Results and Discussion

The experiment evaluated the effectiveness of PBII using a self-ignited O_2 gas plasma for the destruction of *G. stearothermophilus* spores.

First, we conducted an experiment using Case 1 conditions (Table 1) to examine the effect of delay time. Delay time is the lag time before supplying a voltage after charging.

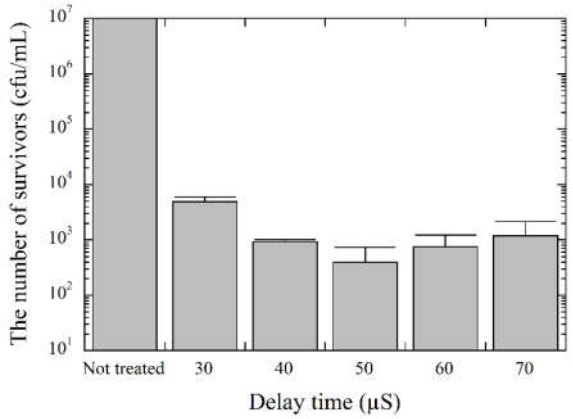

Fig. 2. Effect of delay time on the number of surviving spores.

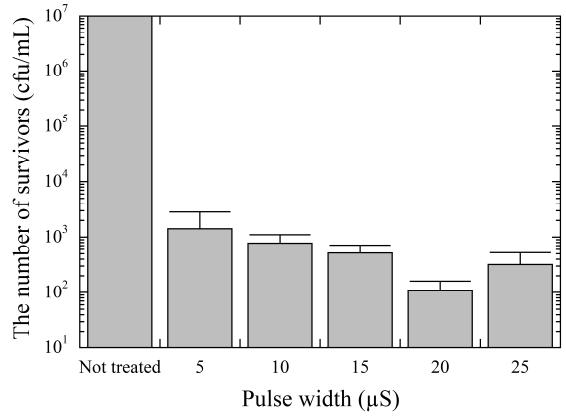

Fig. 3. Effect of pulse width on the number of surviving spores.

The number of survivors decreased when the delay time was shorter than 50 µs and increased when longer than 50 µs (Fig. 2). This suggests that 50 µs is the optimal value for delay time and this parameter was fixed to this value for the remaining experiments.

Next, we analyzed the effect of pulse width using Case 2 conditions. Pulse width is related to the speed of the ions.

The number of survivors decreased when the pulse width increased to 20 µs and then increased thereafter (Fig. 3). *G. stearothermophilus* survivors decreased from 10^7 to 10^2 cfu/mL ("5D") at a pulse width of 20 µs.

We speculated that if treatment time was extended, then higher degrees of sterilization might be achieved. Then therefore lengthened exposure time from 10 min to 15 min (Case 3) or 20 min (Case 4).

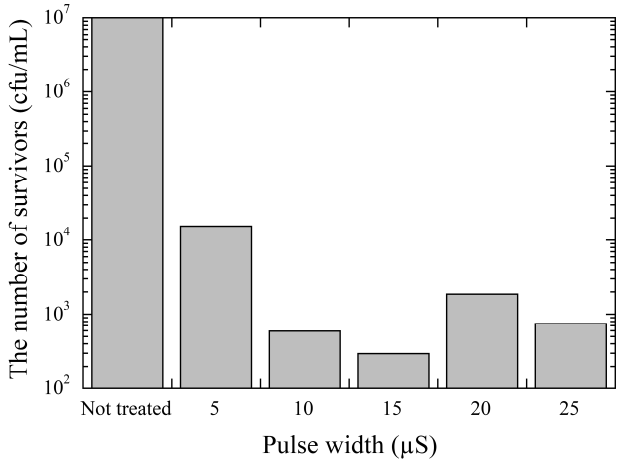

Fig. 4. Effect of pulse width on the number of surviving spores (15 min treatment).

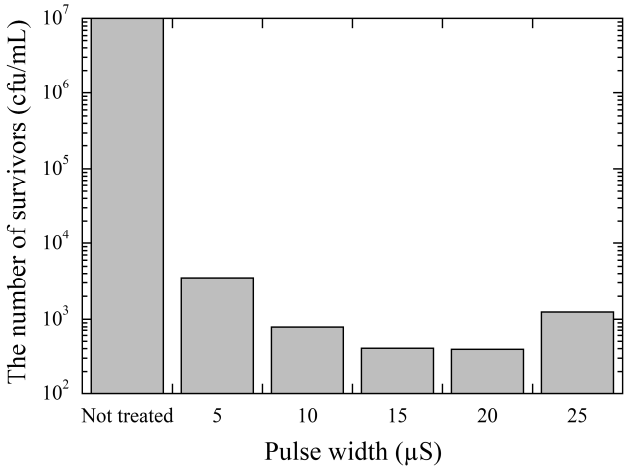

Fig. 5. Effect of pulse width on the number of surviving spores (20 min treatment).

Lengthening the incubation time had no effect on spore survival beyond 10 min (Figs. 4 and 5). This phenomenon may be related to the speed of the ions.

Positive ions are accelerated in the ion sheath, and when pulse width increases the ion sheath becomes thinner, possibly decreasing the speed of the ions that collide with the spore surfaces and increasing the survival rate.

These first four experiments allowed us to determine optimal conditions for delay time, pulse width and exposure time. Finally, we analyzed the effect of pulse voltage, using Case 5 conditions.

Under these conditions, *G. stearothermophilus* survival decreased from 10^7 to 10^1 cfu/mL ("6D") when the pulse voltage was −12 kV (Fig. 6).

In summary, *G. stearothermophilus* spore survival decreased from 10^7 to 10^1 cfu/mL after PBII treatment under the following conditions: 50 μS delay time, 20 μS pulse width and −12 kV pulse voltage.

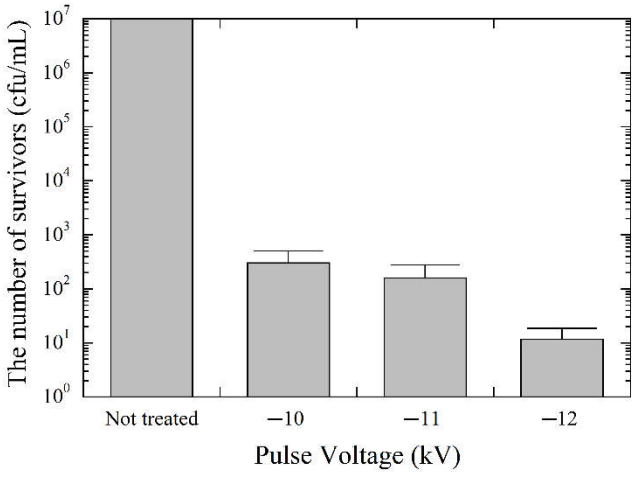

Fig. 6. Effect of pulse voltage width on the number of survivors.

Conclusions

We attempted to sterilize *G. stearothermophilus* spores, which are highly heat tolerant, using a PBII apparatus. *G. stearothermophilus* survivors decreased from 10^7 to 10^1 cfu/mL under the following conditions: O_2 atmosphere, 1 kHz frequency, 50 μs delay time, 20 μs pulse width, −12 kV pulse voltage, 240 VA RF power and a 10 min exposure time. The "6D" sterilization effect for the 10 min treatment is superior to that achieved for *B. subtilis* using optimized process conditions [5]. These results show that the PBII process combined with RF power is effective at sterilization of thermotolerant spores such as those of *G. stearothermophilus*. Thus, we have demonstrated the feasibility of developing a novel food sterilizing apparatus using low temperatures.

Acknowledgements

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