Inactivation of *Enterobacter aerogenes* in carboxymethyl cellulose solution using intense pulsed electric fields (iPEF) combined with moderate thermal treatment

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ABSTRACT

This paper describes low-temperature sterilization of *Enterobacter aerogenes* in carboxymethyl cellulose solution using intense pulsed electric fields (iPEF) combined with moderate thermal energy. The bacterial suspension was exposed to moderate temperatures of up to 55ºC for 2 minutes after tens of 530 ns-long, 50 kV/cm pulses in a single-pass continuous flow system. Suspension temperatures at the entrance and exit of the iPEF exposure chamber were maintained at 40ºC by means of an electrode cooling system. The iPEF combined with subsequent thermal energy of 55ºC reduced bacterial population by 6.6 Log_{10} cycles or more, compared with a reduction of only 2.1 Log_{10} cycles by iPEF without heat treatment. Sterilization effects increased with increasing thermal treatment temperature and pulse number. Results obtained after culturing the iPEF-exposed bacteria in NaCl rich agar, which hinders the reorganization of the damaged membrane, implies that even bacteria surviving the iPEF exposure are damaged to some extent though may later recover. Bacteria were made vulnerable to subsequent thermal treatment by iPEF-induced membrane damage. This indicates that moderate thermal stress after iPEF exposure increases sterilization effects.

Index Terms — **liquid sterilization, pulsed electric field, thermal energy, Blumlein generator**

1 INTRODUCTION

One third of those living in industrialized countries may be affected by foodborne illnesses each year [1] occurring in liquid food products such as liquid whole eggs, fruit juice, and milk. Thermal treatment and chemical substances have been used for bacteriostasis, bacteria elimination, and sterilization treatment method for food [2]. Temperatures exceeding 60ºC are required for conventional thermal treatment, whereas most proteins are denatured at temperatures over 57ºC [3]; thus, a low-temperature sterilization method has long been sought.

Food sterilization involves three factors: efficiency (including treatment speed and energy consumption), efficacy, and, to possible extent, avoidance of effects on food taste. For example, two types of low temperature pasteurization methods are generally used for milk, one at temperatures ranging between 62 and 65ºC and duration of 30 minutes (LTLT pasteurization); the other at 72ºC and 15 seconds (HTST pasteurization). LTLT pasteurization is the simplest method and is able to maintain flavor, texture and nutritional content. However, this process suffers from low efficiency as it is a batch type requiring long processing time. HTST pasteurization is now the most common method, especially for higher volume processing. Although this method is faster and more energy efficient, the higher temperature may affect flavor [4]. Pasteurized milk by both methods still presents risk of foodborne illness [5]. Thermal treatment combined with chemical additives is also utilized as a low-temperature treatment; however, the use of chemical compounds is unpopular with consumers.

Low-temperature sterilization technologies for liquids are desirable due to efficiency, efficacy, and lack of influence on taste. Several methods such as UV, electron beams, high pressure, and pulse electric fields have long been studied, with some implemented in the food industry. While UV sterilization technology is appropriate for surface sterilization of food or

medical instruments and has been implemented in the some countries, its liquid permeability is insufficiently deep for practical use on liquid foods [6]. Electron beam sterilization is appropriate for surface sterilization and can control penetration power by changing accelerating voltage such as the use of soft electrons, or electrons with energies of 300 keV or lower, which uses low energy for sterilization [7]. However, creating penetration power sufficient to sterilize liquid requires a large amount of power with, for example, 10 MeV required to affect to a depth of 5 cm in liquid [8]; large-sized accelerators are required to provide such high energy to an electron beam. Most high pressure gas sterilization systems use pressures exceeding 300 MPa, vastly increasing equipment costs and scale. Also, high-pressure sterilization utilizes batch processing, leading to low efficiency [9]. To avoid these problems with efficiency and efficacy, PEF (pulsed electronic field) sterilization has been proposed. PEF technology is simple and low-cost; however, its sterilization effects remain insufficient for practical use.

Hamilton and Sale reported the first experiment of liquid sterilization using PEF in 1967 [10-12], and many experiments have since been conducted. However, as energy requirements for PEF sterilization remain large, PEF sterilization technology has yet to be put into practical use in the food industry [13]. Attempts have been made to increase sterilization strength of PEF to lower its energy requirements, including combinations with additional technologies such as high pressure [14], chemical additives [15, 16], and thermal energy [17, 18]. For example, Pataro et al. reported that PEF treatment under pressurized conditions reduced bacterial population by 2.4 Log-cycles [19], and use of an emulsifier and ethylenediaminetetraacetic acid (EDTA) as chelating agents of metal ions was found to significantly enhance the sterilization effect of PEF treatment [13]. Of significance to this study, in 2007, Amiali et al. reported that the combination of thermal and PEF treatments killed bacteria synergistically [20]. Their study demonstrated that PEF treatment (210 μs, 30 kV/cm) at 40ºC resulted in a reduction of 4.8 Log_{10} cycles, whereas PEF that at 20 ºC was only 0.8 Log_{10} cycles. The greater efficacy of PEF combined with thermal treatment may be that a number of bacteria survive PEF exposure in a damaged condition and were thus vulnerable to subsequent, relatively low-level heat. Although their experiment did not result in sterilization strength sufficient for practical use in the food industry, their results promise stronger sterilization effects by optimization of PEF and thermal treatments.

This paper describes sterilization effects of intense pulsed electric field (iPEF) combined with subsequent thermal treatment. Our study used a 530 ns-long, 50 kV/cm rectangular pulse to cause significant damage to the bacterial membrane. Thermal treatment temperature was ranged up to 55ºC, a level sufficient to expose bacteria to thermal stress but insufficient to denature the food ingredient proteins in the liquid food sterilized. We used *Enterobacter aerogenes* as target bacteria and Carboxymethyl cellulose solution as a bacterial suspension to simulate liquid whole egg. The bacterial suspension was treated in a single-pass continuous flow system including the iPEF exposure chamber and pre- and post-treatment heaters. Finally, we discuss mechanisms of the enhanced killing effect owing to exposure to moderate temperature after the iPEF treatment on the basis of investigation of iPEF-induced membrane damage and recovery from this damage.

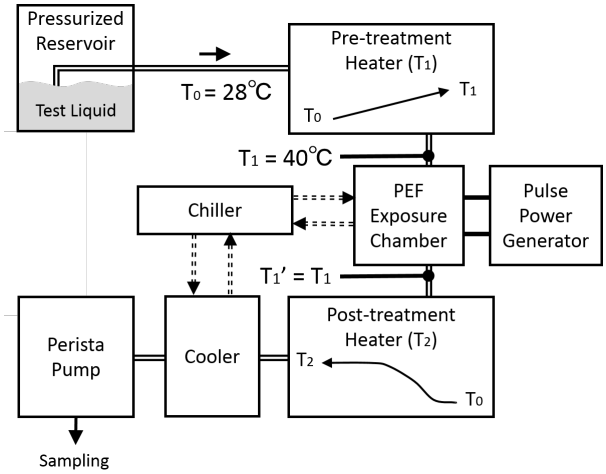

Figure 1. General view of flow sterilization system.

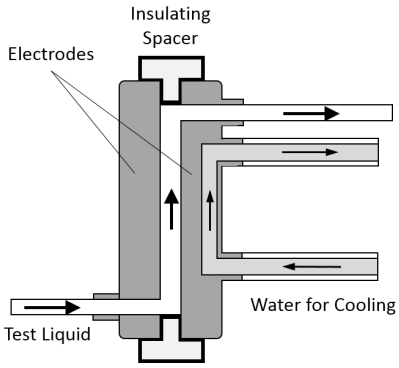

Figure 2. Cross sectional view of the iPEF exposure chamber. Electrodes are connected to the pulsed power generator.

2 MATERIALS

2.1 BACTERIA AND SUSPENDING MEDIUM

Enterobacter aerogenes (ATCC 13048) were used as target bacteria to evaluate sterilization strength. This bacterium is a Gram-negative, rod-shaped microorganism of the *Enterobacteriaceae* family. Carboxymethyl cellulose (CMC) solution containing 0.25% sodium chloride and 1.5% CMC was used as bacterial suspension medium. Viscosity and electrical conductivity of the synthetic solution were 15.5 mPas and 6.7 mS/cm, respectively, both of which are comparable to those of liquid whole egg. Bacteria were suspended in a culture medium containing casein-peptone 5.0 g/L, yeast extract 2.5 g/L, glucose 1.0 g/L and agar 14.0 g/L and incubated at 32ºC for 24 hours before they were used in the test. The bacterium concentration of the CMC solution for the test was 10^7 CFU/mL.

2.2 IPEF EXPOSURE CHAMBER AND TREATMENT SYSTEM

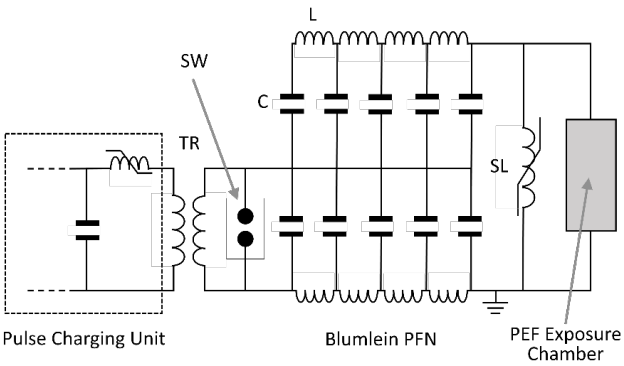

Figure 3. Equivalent circuit of pulsed power generator based on Blumlein-type pulse-forming network (BPFN). A pulse-charging unit repetitively delivers pulsed energy to BPFN through a transformer.

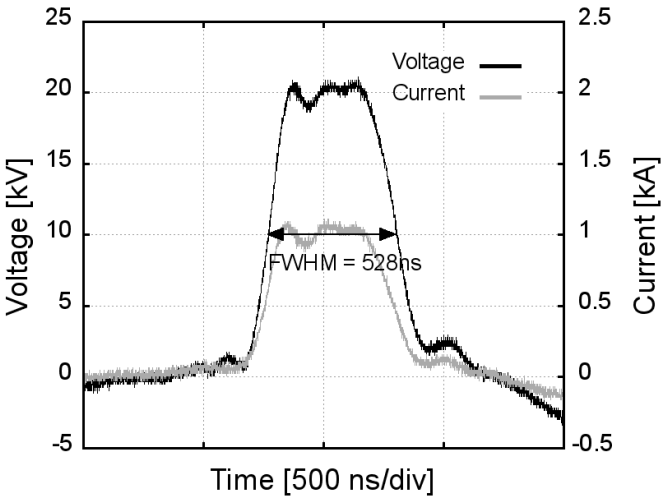

Figure 4. Waveform of the pulse voltage and current applied to the iPEF exposure chamber.

Figure 1 shows a block diagram of the continuous flow single pass treatment system. The bacterial suspension medium flowed from the reservoir to the pre-treatment heater, where it was warmed up to 40ºC for 2 minutes. The medium was exposed to tens of iPEF pulses while passing through the iPEF exposure chamber before flowing into the post-treatment heater, where it was heated to a prescribed temperature within 2 minutes and maintained there for an additional 2 minutes. Subsequently, the suspension was rapidly cooled to 4ºC for sampling after passing through a Perista pump (AC2120, ATTO) to control the system flow rate. The system was pressurized at 0.3 MPa to prevent electrical breakdown at the iPEF exposure chamber. The pulse repetition frequency was fixed at 1 Hz; pulse number was controlled by varying the liquid flow rate between 1.37 and 3.20 mL/min. A laminar flow was expected in the experimental condition. The temperatures in the pre- and the post-treatment heaters defined as T_1 and T_2 were controlled independently with accuracy of 1ºC. In this study, T_1 was fixed at 40ºC and T_2 was variously set at 28, 45, 50 or 55ºC. Figure 2 shows a section view of the iPEF exposure chamber, consisting of 4 mm-spaced parallel stainless steel electrodes (SUS316) and a polyethersulfone spacer. Chamber dimensions were 6 mm in width and 35 mm in height, forming a capacity of 0.8 ml. Resistance of the exposure chamber with the flowing CMC solution was approximately 20 Ω. Temperatures of the suspension at the inlet (T_1) and outlet (T_1') of the exposure chamber were monitored using fiber-optic thermometers (FL-2000, Anritsu). Electrodes were directly cooled by a circulating system for T_1' to be equal to T_1 in all pulse conditions.

2.3 PULSE GENERATOR

Figure 3 shows our 6-staged Blumlein-type pulse-forming network (BPFN), which was designed and built to generate submicrosecond-long mono-polar pulse to the 20 Ω load. The inductor L and capacitor C were 300 nH and 2.6 nF, respectively, producing an output impedance of 21 Ω and pulse duration of 530 ns. This pulse duration was chosen to be as long as possible to apply strong electrical stress to the bacterial membrane unless breakdown through the bacterial suspension occurs under the intense field of 50 kV/cm. The BPFN was powered by a pulse-charging unit through transformer TR. A pressurized spark gap switch SW closed automatically when the BPFN was fully charged. A saturable inductor SL with a troidal FINEMET core (FT-3H, Hitachi Metals) functioned as a low impedance while the capacitors were charged, becoming high impedance when SW was closed. Figure 4 shows a waveform of the voltage pulse applied to the iPEF exposure chamber detected using a voltage probe (P6015, Tektronix). The current waveform, detected using a current monitor (Model 110, Pearson), was identical to the voltage. In this study, the voltage amplitude was fixed at 20 kV, corresponding to the field strength of 50 kV/cm. Voltage amplitude deviation for the repetitive operation was 3%. Rise and fall times were 130 and 200 ns, respectively. The dissipation energy per pulse was 11 J, 80% of which contributed to the effective electric field exceeding 90% of the maximum.

2.4 EVALUATION OF STERILIZATION EFFECT

Treatment sterilization strength was evaluated by colony counting method. Bacterial suspension was appropriately diluted by phosphate buffered saline (PBS) before being smeared onto nutritious agar media to allow for surviving bacteria to form colonies. Two types of culture agar media were used: one standard, the other with basically the same content as standard but additionally containing 5% sodium chloride. After incubation at 32ºC for 24 hours, colony forming units (CFU) on the agar were counted.

2.5 MICROSCOPIC FLUORESCENT IMAGING OF IPEF EXPOSED BACTERIA

To detect bacteria with damaged membranes caused by iPEF, microscopic and the fluorescent dye propedium iodide (PI), which fluoresces in red when intercalating to DNA, was used. Since PI is not permeable to intact membranes, only bacteria with damaged membranes fluoresce. PI was

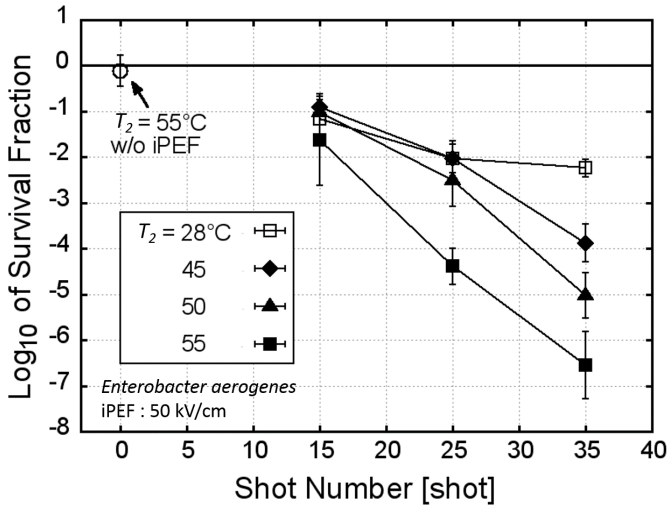

Figure 5. Influence of shot number and temperature of post-treatment exchanger (T_2) on inactivation of *Enterobacter aerogenes* by iPEF and thermal in CMC solution.

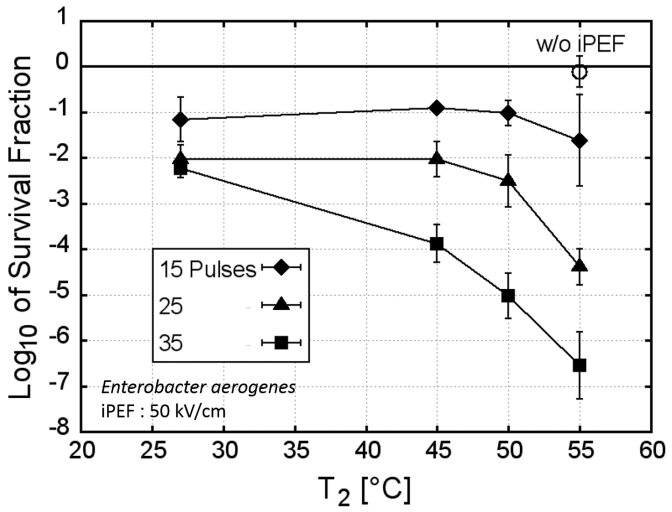

Figure 6. Influence of temperature of post-treatment exchanger (T_2) and shot number on inactivation of *Enterobacter aerogenes* by iPEF and thermal in CMC solution.

suspended in a CMC solution including bacteria which were exposed to iPEF with no thermal treatment in the flow system and sampled.

3 RESULTS

3.1 STERILIZATION IN THE CONTINUOUS FLOW SYSTEM

Figure 5 shows sterilization effects as a function of pulse number and temperature of post-treatment heater T_2. All experiments were performed more than three times on different days. Error bars in the figures indicate standard deviation. The circle indicates the result of thermal treatment at T_1=55ºC without iPEF exposure, indicating that sole thermal treatment only slightly reduced the bacterial population (0.1 Log_{10} cycle); this compares with a reduction of 2.1 Log_{10} cycles after exposure to 35 pulses of 50 kV/cm with no thermal treatment. When thermal treatment was subsequently added to the iPEF exposure, sterilization strength increased significantly. The strength increased both with an increase in pulse number N and temperature in the subsequent thermal treatment T_2. At T_2=55ºC, sterilization strength linearly increased with pulse number, whereas treatment with no thermal treatment seems to saturate against pulse number under the same tendency most experiments have previously reported [17, 21, 22]. A sterilization strength of 6.6 Log_{10} cycle was achieved at the condition of T_2=55 and N=35. In this experiment, energy expenditure for the sterilization strength exceeding 6.6 was 340 kJ/L. If a 10 kW pulse generator were used for treatment, the processing rate would be approximately 100 L/hour, still insufficient by a factor of 10 for practical use in the food industry.

Figure 6 shows dependence of sterilization strength on post-treatment temperature, T_2, for pulse numbers of 15, 25 and 35. As an overall tendency, bacteria exposed to larger number of the pulses are more sensitive to the temperature. The sterilization effect is strengthened by increasing the temperature in the range more than the certain value, which we define as threshold temperature for heat resistance T_{hr}, whereas it does not depend on the temperature in the range less than T_{hr}. Approximate value of T_{hr} for N=15, 25 and 35, which can be deduced from extension of the curve in Fig. 6, is 50ºC, 48ºC, and 40ºC, respectively, being lowered by an increase in the number of the pulses.

3.2 MICROSCOPIC FLUORESCENT OBSERVATION

According to previous studies [11, 23-26], exposure to an intense pulsed electric field causes defects in the function and structure of the cell membrane, leading to an increase in permeability. While this phenomenon is temporary when the damage is relatively small, when large, it is irreversibly fatal to the cell. Generally, a cell membrane works as a dielectric film by interrupting a current flow under an external electric field, leading to the enhancement of electric field on the membrane. Exposure of bacteria to the pulsed field of 50 kV/cm is expected to produce a voltage of 5 V across the cell membrane. The field strength on a membrane whose thickness is 6-8 nm exceeds 3 MV/cm, which is sufficiently large to damage the membrane structure due to electrical and/or mechanical breakdown [27].

Figure 7 shows microscopic images of bacteria to demonstrate membrane damage induced by a 50 kV/cm iPEF; 7(a) and 7(c) show bright images of bacteria,while 7(b) and 7(d) show fluorescent images (negatives) of 7(a) and 7(b) before and after exposure to 35 pulses, respectively. The number of fluorescent (i.e., dead) bacteria increased drastically after iPEF exposure, indicating that bacterial membranes were damaged by exposure to tens of iPEF. This membrane damage not only results in an increase in

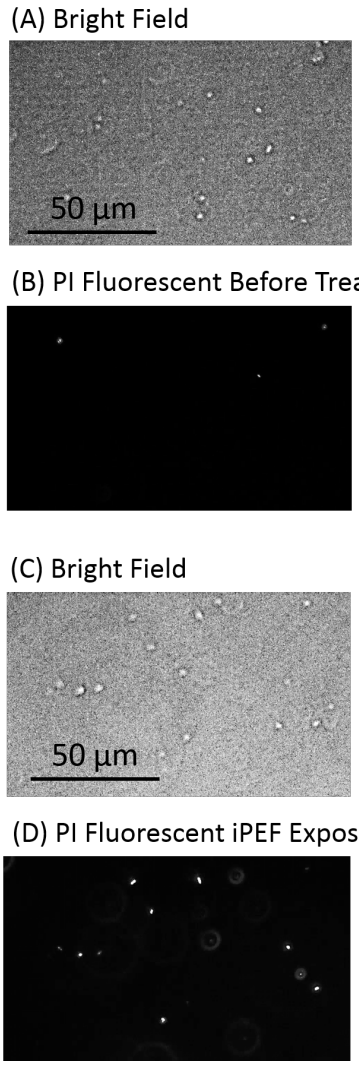

Figure 7. Microscopic and biochemical analysis using PI of damage by iPEF to cell membranes. (A) and (B) show before treatment from Bright image and fluorescent image respectively, (C) and (D) show after treatment. Points indicate damaging cells.
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permeability but also, most probably, promotes heat penetration to the bacterium.

4 DISCUSSION

We discuss why iPEF treatment in the absence of thermal treatment is relatively ineffective and discuss how subsequent thermal treatment enhances the sterilization effect of iPEF exposure. Our experiment shows exposure to iPEF with no thermal treatment killed 99% of bacteria, with 1% surviving. Electroporation caused by strong electric fields is well-known to physically damage the cell membrane, resulting in an increase in permeability for substances including ions. Bacterial cell membranes shield the interior from surrounding conditions to maintain appropriate conditions for optimal physiological activity. On the basis of our experimental result

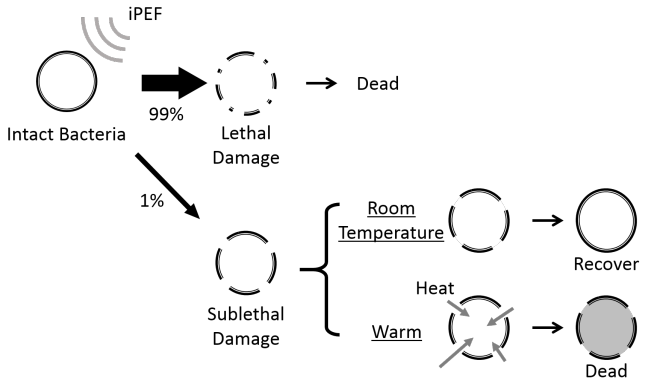

Figure 8. Hypothesis of the mechanism of enhanced killing effect of the additional thermal energy to the intense iPEF exposure.

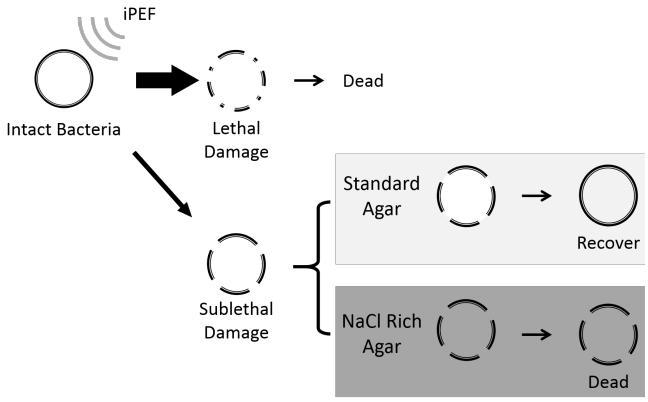

Figure 9. Experimental scheme to investigate membrane injury caused by iPEF pulses and the self-recovery from the injury, by means of culturing iPEF exposed bacteria in two different agar media, standard and sodium chloride rich ones.

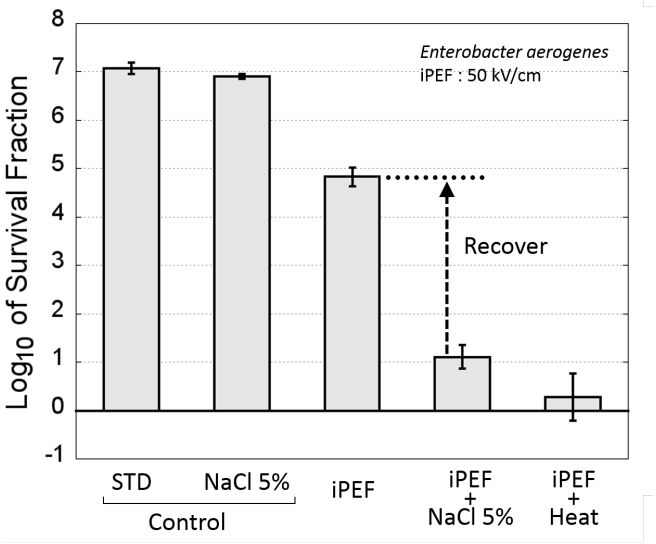

Figure 10. The result of measuring the sublethal injury bacillus and comparing the number of CFU: sham (STD); iPEF treatment only and cultured on non-selective medium (iPEF); iPEF treatment only and cultured on selective medium (iPEF+NaCl 5%); iPEF treatment combined 55ºC of thermal effect (iPEF+Heat). All data show the average of more than three trials.

as shown in Figs. 5 and 7, we have reached the following hypothesis as illustrated in Fig. 8. First, self-recovery of the damaged membrane suppresses the sterilization effect of iPEF. The exposure to tens of iPEF pulses is likely to injure all bacteria to some extent. Bacteria with relatively minor membrane damage were reorganized with no thermal treatment to resume their physiological activity, whereas bacteria with severe membrane damage died. The recovery of these bacteria indicates that iPEF treatment in the absence of thermal treatment is relatively ineffective. Secondly, subsequent thermal energy interferes with reorganization of the damaged membrane and penetrates into the interior to raise intracellular temperature, resulting in deactivation of physiological activity. Membranes physically damaged by iPEF exposure are likely to be permeable not only by ions but also by heat. In summary, ineffectiveness of iPEF treatment at the room temperature is caused by bacterial recovery, and the effectiveness of combined treatment is caused by bacteria whose damage due to exposure to iPEF made them vulnerable to subsequent thermal treatment.

In order to demonstrate our hypothesis, we carried out an additional experiments as shown in Fig. 9 using two kinds of nutrient agar media, a standard one and one including 5% sodium chloride which in this paper is called NaCl rich agar. It is known that sodium chloride hinders the repair of injured membranes, which is why injured bacteria do not form colonies in NaCl rich agar [20, 28]. Therefore, comparing colonies formed in the standard and in the NaCl rich agar media allowed us to obtain a portion of bacteria which had recovered from membrane injury caused by iPEF exposure with no thermal treatment. According to our hypothesis, the number of colonies formed in the NaCl rich agar is expected to be the same as the result of the combination of iPEF and the subsequent thermal treatment. Figure 10 shows survival fractions of bacteria exposed to iPEF and subsequently cultured in a NaCl rich medium along with those exposed to iPEF and to both iPEF and subsequent thermal energy and then cultured in a standard medium. Toxicity of the culture medium including 5% NaCl on intact bacteria is negligible. For the bacteria exposed to iPEF with no thermal treatment, the number of surviving bacteria cultured in a NaCl rich medium is much less than that in a standard medium and the same level as that for the combination of iPEF and the subsequent thermal treatments. This result indicates iPEF treatment with no thermal treatment can damage almost all (approximately 9,000,000/10,000,000) bacterial membrane, but most (approximately 100,000/9,000,000) bacteria can self-recover from membrane damage. On the other hand, iPEF treatment followed by thermal treatment can kill bacteria with membrane damage, indicated by the fact that the number of surviving bacteria cultured in a NaCl rich medium is the same level as iPEF treatment at high temperature. This result agrees with our hypothesis that most bacteria exposed to iPEF with no thermal treatment recovered from membrane damage, and that effectiveness of combined treatment is caused by bacteria whose damage due to exposure to iPEF left them vulnerable to subsequent thermal treatment.

As for heat vulnerability of bacterial membrane exposed to the pulses, a reasonable hypothesis is that larger number of the pulse is likely to cause more severe damage to the membrane, resulting in vulnerability to subsequent thermal treatment. This hypothesis is supported by the experimental result that, as shown in Fig. 6, larger number of the pulses makes bacteria more sensitive to the subsequent treatment temperature and lowers the threshold temperature for the heat resistance capability of bacteria.

5 CONCLUSION

This paper shows a synergistic effect of intense pulsed electric field and subsequent thermal exposure at moderate temperatures of up to 55ºC on sterilization of *Enterobacter aerogenes* in a synthetic suspending medium. The experiment demonstrated a sterilization strength of 6.6 $\log_{10}$ cycles achieved under conditions of 35 iPEF pulses and temperature of 55ºC. Our experiment results support our hypothesis that the relative ineffectiveness of iPEF treatment with no thermal treatment is due to recovery of the bacterial membrane, while subsequent thermal treatment exploits this vulnerability.

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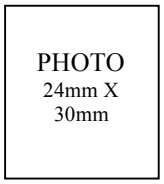

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