CHAPTER 11

# Inactivation of *Bacillus subtilis* Spores Using High Voltage Pulsed Electric Fields

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## ABSTRACT

A suspension of *Bacillus subtilis* spores was treated by pulsed electric fields (PEF) in a bench scale, continuous system. Structural changes in PEF-treated spores were revealed by scanning electron microscopy (SEM). The release of dipicolinic acid (DPA) from spores was monitored during the PEF treatment. More than 95% of the *B. subtilis* spores were inactivated at an electric field strength of 30 to 40 kV/cm for 2 to 3 milliseconds. PEF treatment was most lethal to *B. subtilis* spores at 36°C. SEM micrographs showed that PEF-treated spores had structural changes similar to those of thermally inactivated spores. The DPA release was correlated with spore inactivation by PEF treatment.

## INTRODUCTION

High voltage pulsed electric field (PEF) is a new technology that can be used to inactivate microorganisms without significant temperature increase. PEF has many advantages over conventional thermal preservation methods. Because food potentially can be pasteurized or sterilized by PEF at an ambient temperature, the color, texture, flavor, and nutrients of food are better preserved. Therefore, pulsed electric field pasteurization or sterilization is a potentially useful food processing technology (Zhang et al., 1995a).

Many researchers have demonstrated that PEF has lethal effects on microorganisms (Sale and Hamilton, 1967, 1968; Gupta and Murray, 1988; Mizuno and Hori, 1988; Jayaram et al., 1992; Zhang et al., 1994a, 1994b, 1995b).

Most of the earlier studies were carried out using only vegetative cells as target microorganisms. Because of their rigid structures, bacterial spores can survive harsh environments for a long period of time (Setlow, 1995). Limited studies have been done to inactivate bacterial spores by PEF. Some of these studies showed that bacterial spores are extremely resistant to PEF treatment (Hamilton and Sale, 1967; Yonemoto et al., 1993). Hamilton and Sale (1967) applied electric fields up to 30 kV/cm to spores of *Bacillus cereus* and *Bacillus polymyxa,* but the treatment did not affect viability of the spores. However, the authors reported that the electric fields had inactivation effects when the spores germinated. Yonemoto et al. (1993) treated spores of *Saccharomyces cerevisiae* and *Bacillus subtilis* with a pulsed electric field of 5.4 kV/cm. Their results indicated that 90% of yeast spores were inactivated by PEF, and that there was no viability change in bacterial spores. The scanning electron micrographs showed the PEF-treated yeast spores with many small holes on their surfaces, while structures of *B. subtilis* spores before and after PEF treatment were indistinguishable (Yonemoto et al., 1993). However, scanning electron micrographs revealed that there were some cracks on the surface of bacterial spores, and that the spores lost their viability after PEF treatment.

Dipicolinic acid (DPA), or pyridine 2,6-dicarboxylic acid, is a compound unique to bacterial spores and is involved in the thermal resistance of spores. When the integrity of the spore coats is compromised, DPA is released from the spore core into the surrounding environment. Release of DPA has been previously noted in *B. stearothermophilus* spores treated with ultrasonication (Palacios et al., 1991). PEF treatment damages spore coats and it is believed that DPA would be released from treated spores.

The purpose of this study was to evaluate the effectiveness of PEF treatment on the inactivation of *Bacillus subtilis* spores. The inactivation of spores was monitored by cell viability measurements, inspection of SEM micrographs, and assay for DPA release.

## MATERIALS AND METHODS

### PREPARATION OF *BACILLUS SUBTILIS* SPORES

*Bacillus subtilis* (OSU 872) spores were chosen as the target microorganisms. The vegetative cells of *B. subtilis,* at late logarithmic growth phase (9 to10 hr culture in tryptose broth, $O.D_{600} = 1.0$), were inoculated into bottle slants (containing nutrient agar + 0.03% $MgSO_4$) and incubated at 37°C for 4 days. The spores were collected from the slant surface with chilled, sterile distilled water. For a mass spore production, the collected spore suspension was heated at 80°C for 20 min. A portion of heated spores (1 mL) was transferred to a large

sporulation medium slant in a 150 mL dilution bottle, and incubated at 37°C for 4 to 6 days. Spores were washed off the agar surface with cold (ca. 0°C), sterile distilled water and combined in one flask. The spore suspension was centrifuged at 17,000 × g for 10 min at 4°C. There were two phases in the pellet. By microscopic examination, it was found that the upper phase contained spores with exosporium, and the lower phase contained matured spores. Therefore, the upper phase of the pellet was discarded. The remaining pellet was washed by resuspending into a saline solution followed by centrifugation at 17,000 × g for 10 min at 4°C. The washing procedure was repeated until a pure spore pellet was obtained. The final spore suspension was reconstituted with water to $10^8$ cfu/mL and stored at 4°C before use.

## PEF TREATMENT SYSTEM

A 15 kV high voltage power supply (Model 1450-4, Cober Electronics, Inc., Stamford, CT) was connected to a high voltage pulse generator (Model 2829, Cober Electronics, Inc., Stamford, CT) that can generate various waveform pulses. The pulse duration time and frequency were selected by a pulse trigger generator, and indicated by a two channel digital oscilloscope (Model TDS 320, Tektronix, Beaverton, OR). Since the square waveform is more effective than the exponential decay for inactivating microorganisms, as shown by Zhang et al. (1994b), the square waveform was selected for all the tests conducted in this study.

A continuous flow treatment chamber was designed to convert the high voltage pulses into high intensity pulsed electric fields. Inside the chamber, a converged electric field (valid treatment zone) formed between two liquid electrodes adjacent to the stainless steel electrodes. The two stainless steel electrodes were connected to the high voltage pulse generator and the ground, respectively. Two chambers, consisting of two 2 mm × 3 mm ($d \times l$) treatment zones, were connected in parallel in terms of the electric power connections. A schematic diagram of the PEF treatment system is illustrated in Figure 11.1.

Except where indicated, the treatment medium was an aqueous solution containing 3.42 mM NaCl and 1.14 mM L-alanine. The medium had conductivity of $1 \times 10^{-4}$ S/cm and pH of 6.8 at 22°C. A gear pump (Micropump Inc., Vancouver, WA) carried the treatment fluid through the system and controlled the flow rate of the fluid. A water bath (Neslab, Newington, NH) was used to control the system temperatures. A stainless steel coil was placed before the PEF treatment chamber and immersed in the waterbath to control the PEF treatment temperature. A digital thermometer (Fluke 52 K/J, Everett, WA) with two thermocouples was used to measure the sample temperatures at the inlet and outlet of the system. The outlet temperature of the fluid was defined as

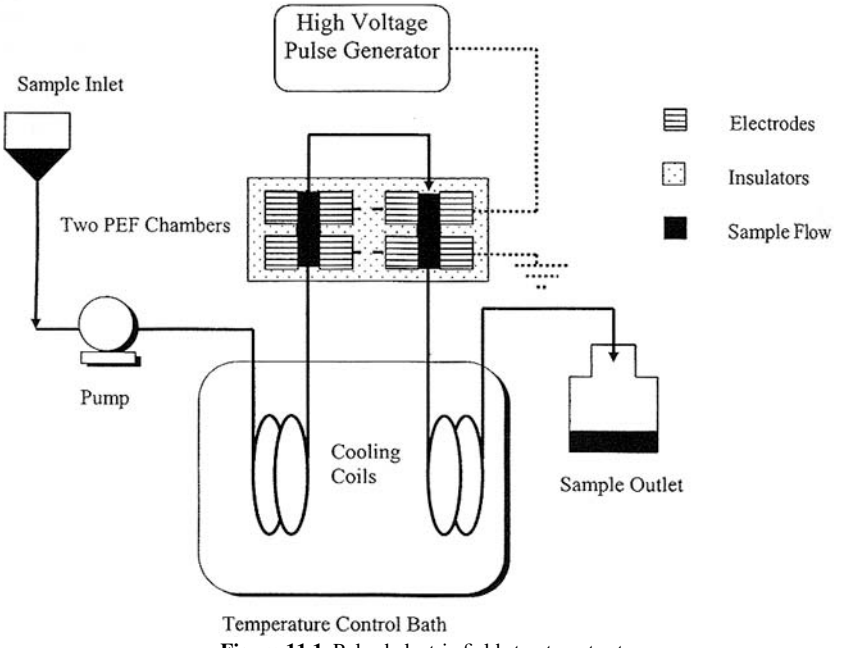

**Figure 11.1** Pulsed electric fields treatment setup.

the treatment temperature. Treatment time ($t$) was calculated by Equation (1),

$$t = n \bullet \tau_c \bullet f \bullet d/v \tag{1}$$

where $n$ is the number of treatment chambers, $\tau_c$ is effective pulse width (s), $f$ is repetition frequency (Hz), $d$ is distance between two electrodes (cm), and $v$ is the average velocity of flow inside treatment chamber (cm/s).

## TREATMENT OF *B. SUBTILIS* SPORES WITH PEF

A suspension of *B. subtilis* spores was prepared containing 3.42 mM NaCl and 1.14 mM L-alanine. The suspension was circulated immediately into the PEF treatment system. A control sample was taken at the inlet without PEF treatment. Samples also were taken at different treatment times.

## ENUMERATION OF *B. SUBTILIS* SPORES

Counts of *B. subtilis* with and without heat activation were determined. Spore suspension (5 mL) was heated at 80°C for 20 min. Heated or unheated suspension was serially diluted in 0.1% peptone water, and surface plated in duplicate onto tryptose agar. Plates containing 20 to 200 colonies were counted.

### EXAMINATION OF SPORES BY SCANNING ELECTRON MICROSCOPY

A PEF-treated spore suspension (35 mL) was centrifuged, and the pellet was spread onto a metal stub and dried in a laminar-flow hood for two hours. Untreated spores and thermally inactivated spores (121°C for 20 min) were also prepared for comparison. Samples were sputter coated and examined with a scanning electron microscope (JEOL JSM-820, Tokyo, Japan) at 20,000 × magnification.

### MEASUREMENT OF DIPICOLINIC ACID RELEASE

The amount of DPA released from spores during PEF treatment was assayed by the method of Janssen et al. (1958), as modified by Rotman and Fields (1968). PEF-treated spore suspension was centrifuged and DPA concentration was determined in the supernatants. To determine the amount of unreleased DPA, the spore pellet was resuspended in a cold saline solution (0.02% NaCl), autoclaved for 15 min at 121°C, and separated by centrifugation. The supernatant was then analyzed for the concentration of DPA released from the pellets by heat.

The colorimetric assay for quantitative measurement of DPA was rapid and yielded a reproducible standard curve. Often the concentration of DPA in the supernatant of PEF-treated spores was not easily detected using colorimetric assay. In these situations, the difference in DPA concentration in spore pellets from PEF-treated and untreated samples was used. These data were used to estimate the concentration of DPA released from the spores, due to PEF treatment, by comparing the DPA concentrations from the treated samples to that present initially. The data were normalized for graphic presentation.

## RESULTS AND DISCUSSION

### PULSE DURATION AND FREQUENCY

Increasing pulse duration time from 3 to 12 μs, and concomitantly decreasing frequency from 2000 to 500 Hz, resulted in survival rates of *B. subtilis* spores that were not significantly different ($P > 0.1$) (Figure 11.2). Inactivation of spores depended on total treatment time irrespective of pulse duration-frequency combination used.

### TREATMENT TEMPERATURE AND MEDIUM

The inactivation of spores by PEF was tested at 20, 30, 36, 40, and 50°C. Maximum inactivation occurred at 30 to 36°C (Figure 11.3). Sample temperature

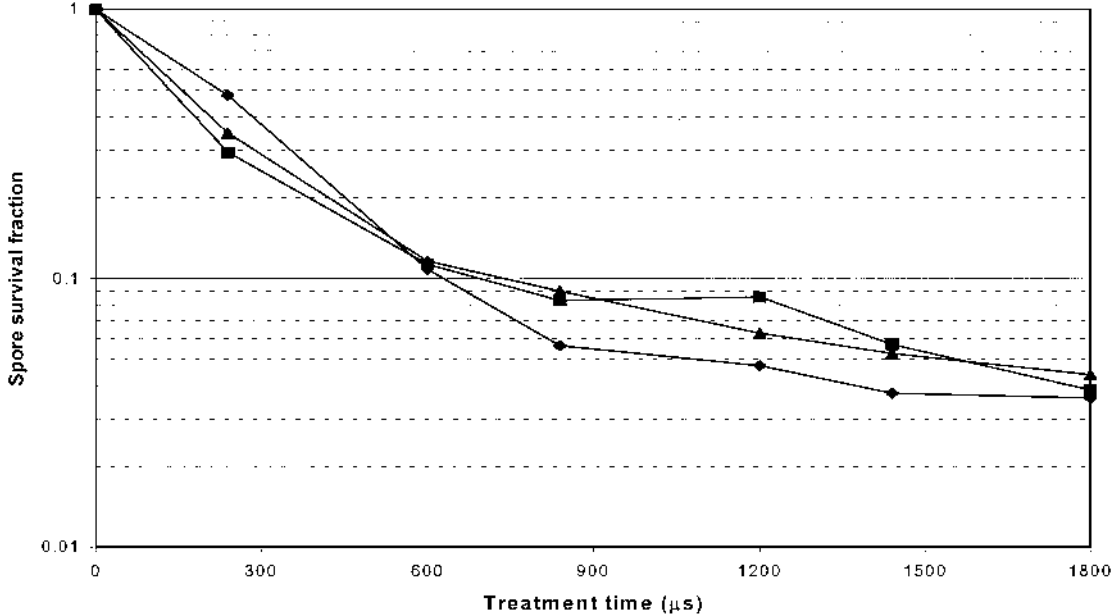


**Figure 11.2** Inactivation of *Bacillus subtilis* spores at 36°C, electric field of 30 kV/cm, and different pulse durations and frequencies: (♦) 3 μs, 2000 Hz; (■) 6 μs, 1000 Hz; (▲) 12 μs, 500 Hz.

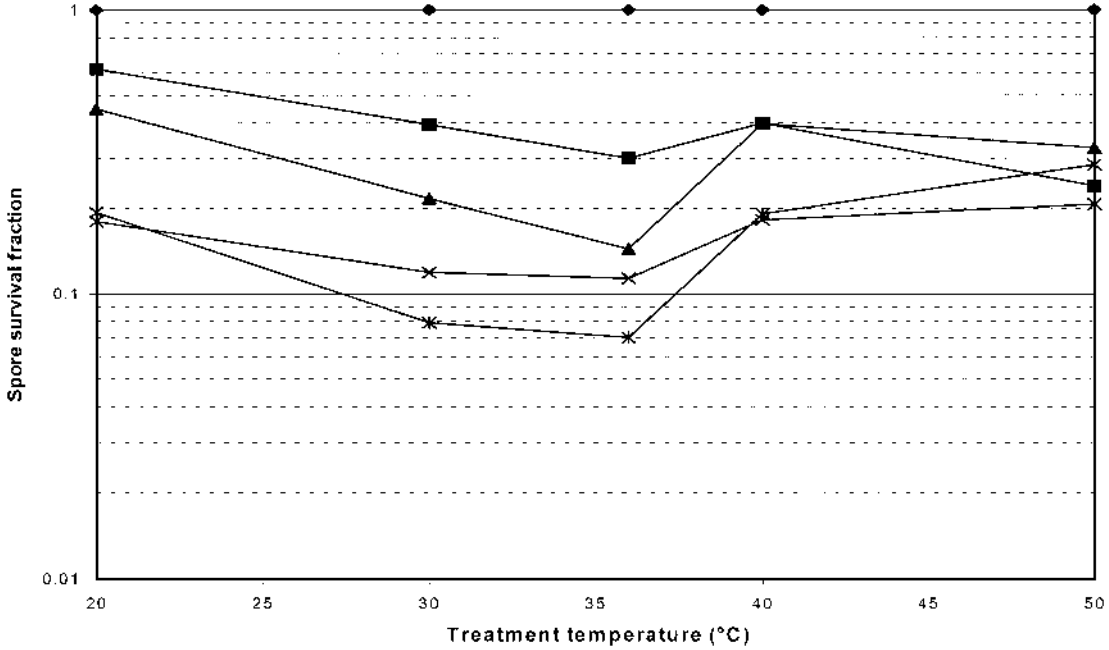


**Figure 11.3** Inactivation of *Bacillus subtilis* spores at a frequency of 1500 Hz, electric field of 30 kV/cm, pulse duration of 2 μs, and different temperatures and treatment times. Treatment time (μs): (♦) none; (■) 270; (▲) 540; (×) 1080; (∗) 1620.

during PEF treatment influences inactivation rates differently when vegetative cells, instead of bacterial spores, are used. Previous studies have shown that inactivation of vegetative cells increases with the increase in PEF treatment temperature (Dunn and Pearlman, 1987; Jayaram et al., 1993; Zhang et al., 1994a).

A germination control experiment (without PEF treatment) was performed at 36°C using a spore suspension similar to that used in PEF treatment experiments. Count of spores did not change appreciably during 48 min of incubation at 36°C; this indicates that spores did not germinate during the PEF treatment at that temperature.

Previous studies, however, indicate that germination of *Bacillus* spores was maximum at a temperature range from 30°C to 36°C (Levinson and Hyatt, 1970), and was inhibited at 49°C (Wax and Freese, 1968). Therefore, spores in the current study were at the most vulnerable state when they were exposed to the electric fields, since the treatment was done at temperatures most optimum for spore germination.

*B. subtilis* spores were suspended in an aqueous solution containing 3.42 mM NaCl with or without 1.14 mM L-alanine. Rate of inactivation was greater with alanine-containing medium than that containing NaCl only ($P < 0.01$) (Figure 11.4). L-alanine enhances germination of many strains of *B. subtilis* (Wax et al., 1967), but the amino acid did not cause the germination of the spores used in this experiment, as indicated earlier. However, combination of L-alanine and optimum temperature for spore germination may have caused physiological changes in spores, and increased their susceptibility to the PEF treatment.

## ELECTRIC FIELD INTENSITY AND TOTAL PEF TREATMENT TIME

Spores were treated with PEF at three electric fields (30, 37, and 40 kV/cm), while the other parameters (pulse frequency, pulse duration, and treatment temperature) were kept unchanged. Results (Figure 11.5) are consistent with what has been reported on vegetative cells by other researchers (Sale and Hamilton, 1967; Hulsheger and Niemann, 1980; Hulsheger et al., 1981; Matsumoto et al., 1991; Jayaram et al., 1992). As the electric field intensity increased, the inactivation rate of spores increased. The maximum PEF treatment (40 kV/cm for 3500 μs), caused 98% spore inactivation. Statistical analysis to compare different data points (Figure 11.5) suggests that the total PEF treatment time has a significant effect on the inactivation of bacterial spores ($P < 0.01$).

## SEM EVALUATION

Comparing the SEM micrographs (Figure 11.6) of PEF-treated and untreated *B. subtilis* spores revealed distinguishable structural differences between the two types of spores. Untreated spores have entirely smooth surfaces. After

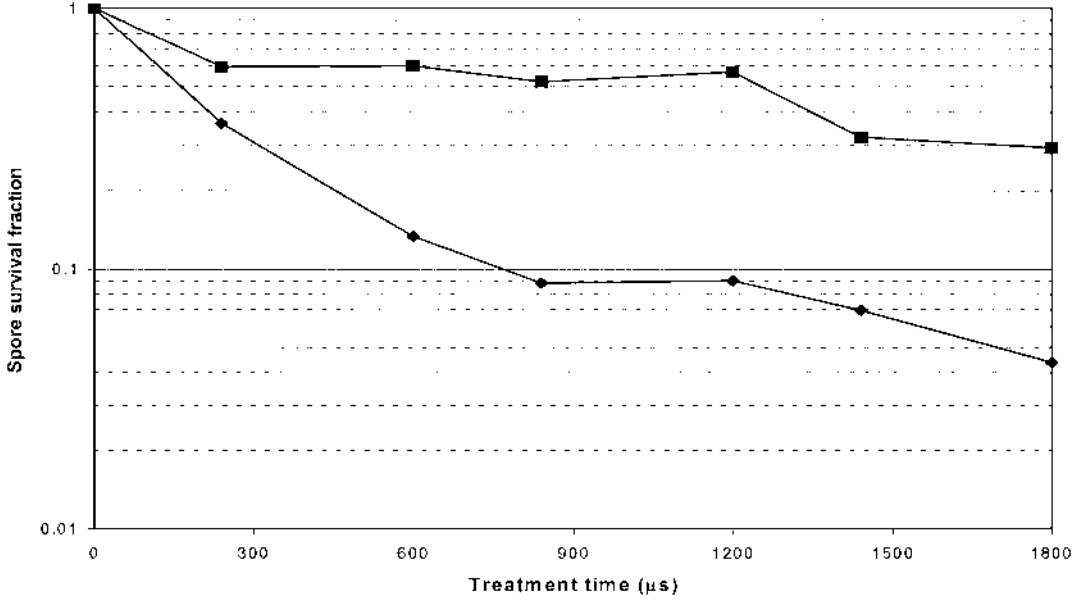


**Figure 11.4** Inactivation of *Bacillus subtilis* spores in two treatment media at 36°C, electric field of 30 kV/cm, frequency of 1000 Hz, and pulse duration of 6 μs: (♦) 0.02% NaCl + 0.01% L-alanine (ALA); (■) 0.02% NaCl solution.

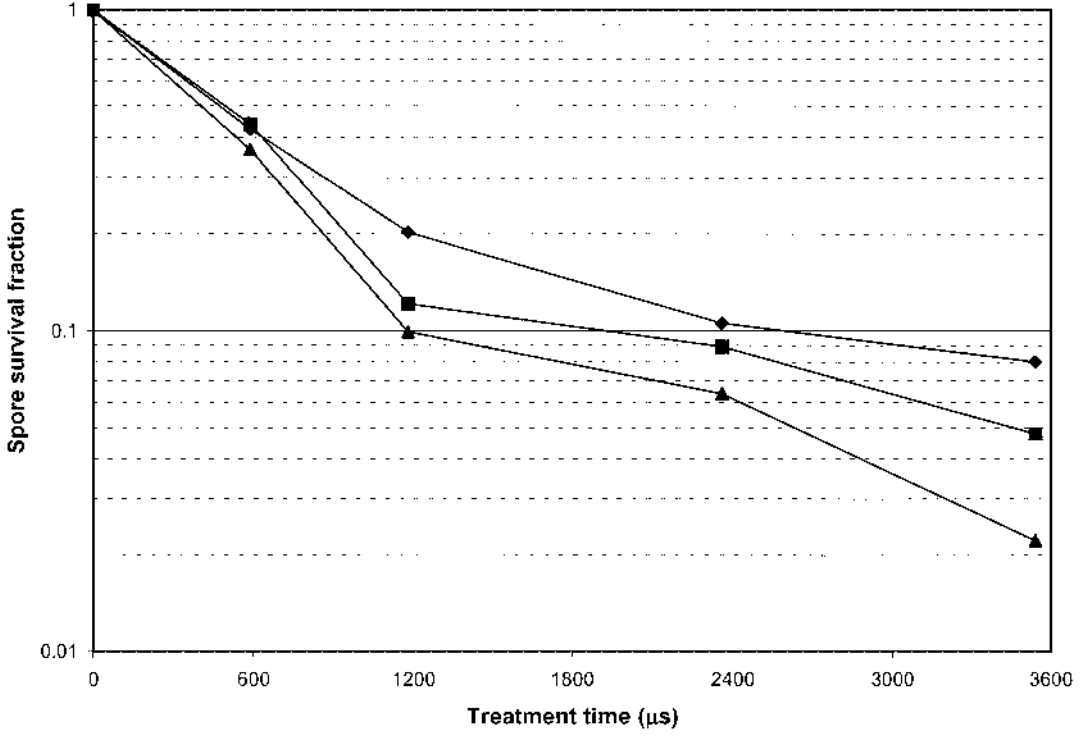


**Figure 11.5** Inactivation of *Bacillus subtilis* spores at 36°C, frequency of 2000 Hz, pulse duration of 6 μs, and different electric field strengths: (♦) 30 kV/cm; (■) 37 kV/cm; (▲) 40 kV/cm.

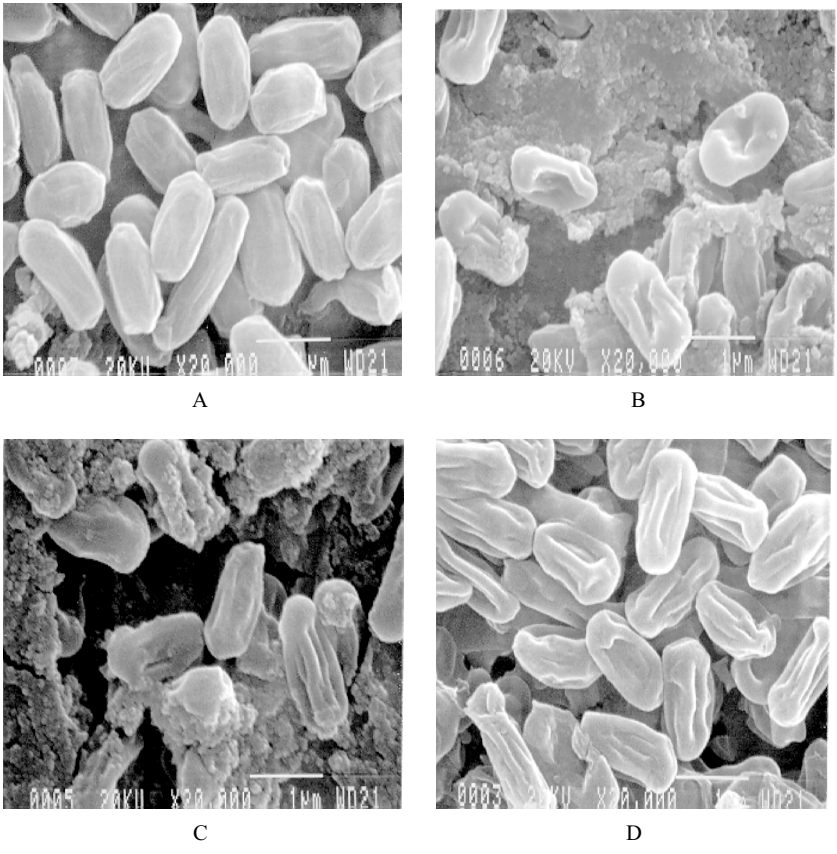


**Figure 11.6** Scanning electron micrograph of *Bacillus subtilis* spores: (a) untreated; (b) treated with 100 pulses, 6 μs/pulse, and 30 kV/cm electric field; (c) treated with 600 pulses, 6 μs/pulse, and 30 kV/cm electric field; (d) heated at 121°C for 20 minutes.

PEF treatment, the spores shrank and many wrinkles formed on their surfaces. The shrinkage and wrinkles may indicate that the spores were inactivated. The SEM micrograph of heat inactivated spores [Figure 6(d)] illustrates similar structural changes. Compared to spores that were treated with 100 pulses, spores treated with 600 pulses had more and deeper wrinkles, and greater similarity to thermally inactivated spores. Plate count data showed that the 100 pulse treated sample had more surviving spores than the 600 pulse treated sample. The differences of the structure and survivability of 100 and 600 pulse treated spores might be explained by the reversible and irreversible electric breakdown theory (Zimmermann, 1986). The less pulse treated spores are better able to recover and remain viable (reversible), while the more pulse treated spores are deeply damaged and lose their viability (irreversible).

## DPA RELEASE

PEF treatment caused a reduction in the number of viable spores, and the concomitant release of DPA (Figure 11.7). When the spore suspension was run through the PEF system in the absence of high voltage, the number of viable spores remained virtually unchanged after a time equivalent to 2000 μs of PEF treatment (Figure 11.7). By measuring the concentration of DPA remaining in the spore pellets of these samples, a small decline in DPA concentration in the spore pellets was observed between time 0 to 500 μs (equivalent run time), and the concentration remained unchanged from 500 to 1000 μs (equivalent run time) (Figure 11.7). These data suggest that there is little effect on the spore viability and DPA concentration when the spore suspension runs through the PEF system in the absence of high voltage.

With high voltage under the PEF conditions tested, a one-log reduction of viable spores was typically seen (Figure 11.7). During PEF treatment, the concentration of DPA in the spore pellet increased slightly after 500 μs, but generally decreased after 1000 and 2000 μs (Figure 11.7). After 500 μs of treatment, the spore coats may have sustained some damage, such as pores caused by the PEF treatment. While these pores may not penetrate all of the spore coats through

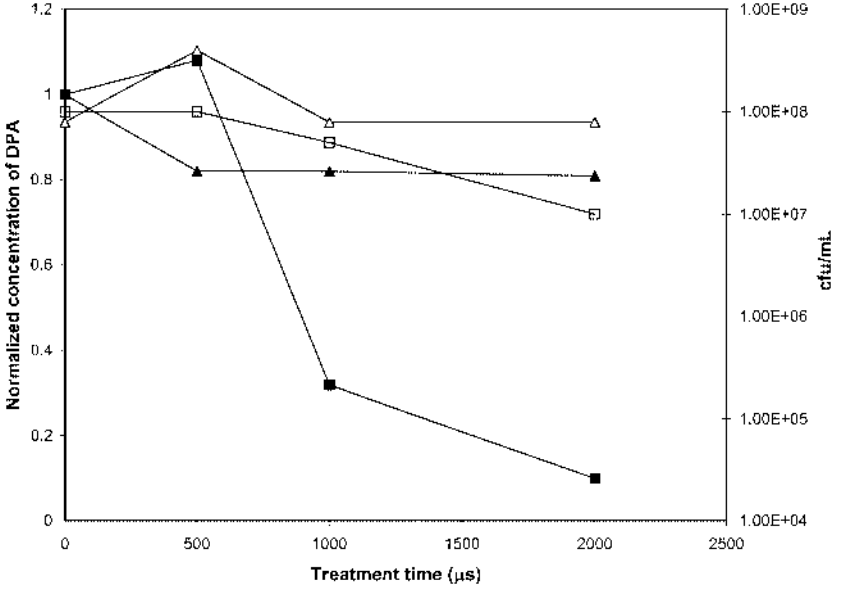


**Figure 11.7** The effect of PEF treatment on viable spore counts and DPA concentration. Spore count: (△) untreated, (□) PEF treated. Normalized concentration of DPA: (▲) untreated, (■) PEF treated.

to the spore core, the spore itself may become more porous. The increased porosity may allow the spore to adsorb some residual DPA that is present in the supernatant, thus resulting in pellet DPA that was greater at 500 μs than at pre-treatment. At greater treatment times, the holes caused by PEF treatment penetrate the spore coats, causing leakage of DPA from the spore core. Results indicate that PEF treatment at 2000 μs ($E = 35$ kV/cm) released 0.118 mM DPA per 10 mg dry weight spores, and decreased spore viability by 1.1 log. These data suggest that DPA is released from the spores, as a result of spore inactivation due to PEF treatment.

## CONCLUSIONS

This study shows that pulsed electric fields are effective in inactivating bacterial spores. Factors that affect bacterial spore inactivation by PEF treatment are identified. Increasing duration time from 3 to 12 μs, and concomitantly decreasing frequency from 2000 to 500 Hz, does not affect survival rate of spores. There is an optimum PEF treatment temperature (30°C to 36°C) for inactivation of bacterial spores. L-Alanine significantly enhances the bacterial spore inactivation by PEF. The inactivation rate of bacterial spores increases when the electric field strength and total PEF treatment time increase.

SEM micrographs suggest that the mechanism of PEF inactivating bacterial spores is related to reversible and irreversible dielectric breakdown. The structures of PEF-treated bacterial spores are distinguishable from those of the untreated ones. The PEF-treated bacterial spores have similar structural changes when compared to the thermally inactivated bacterial spores. DPA release correlates with spore inactivation from PEF treatment. The detection of DPA in the supernatant would probably be a more convenient method for indicating spore inactivation than SEM or cell viability.

## ACKNOWLEDGEMENT

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## REFERENCES

Dunn, J. E. and Pearlman, J. S. 1987. Methods and apparatus for extending the shelf life of fluid food products. U.S. Patent, 4,695,472.

Gupta, R. P. and Murray, W. 1988. Pulsed high electric field sterilization. *IEEE Pulsed Power Conference Record,* pp. 58–64.

Hamilton, W. A. and Sale, A. J. H. 1967. Effects of high electric fields on microorganisms. II. Mechanism of action of the lethal effect. *Biochimica et Biophysica Acta,* 148:789–800.

Hulsheger, H. and Niemann, E.-G. 1980. Lethal effects of high voltage pulses on *E. coli* K 12. *Radiation and Enviromental Biophysics,* 18:281–288.

Hulsheger, H., Potel, J., and Niemann, E. -G. 1981. Killing of bacteria with electric pulses of high voltage strength. *Radiation and Enviromental Biophysics,* 20:53–65.

Janssen, F. W., Lund, A. J., and Anderson, L. E. 1958. Colorimetric assay for dipicolinic acid in bacterial spores. *Science,* 127:26–27.

Jayaram, S., Castle, G. S. P., and Margritis, A. 1992. Kinetics of sterilization of *Lactobacillus brevis* cells by the application of high voltage pulses. *Biotechnology and Bioengineering,* 40:1412–1420.

Jayaram, S., Castle, G. S. P., and Argyrios, M. 1993. The effects of high field DC pulse and liquid medium conductivity on surviviability of *Lactobacillus brevis*. *Applied Microbiology and Biotechnology,* 40:117–122.

Levinson, H. S. and Hyatt, M. T. 1970. Effects of temperature on activation, germination, and outgrowth of *Bacillus magaterium* spores. *Journal of Bacteriology,* 101:58–64.

Matsumoto, Y., Satake, T., Shioji, N., and Sakuma, A. 1991. Inactivation of microorganisms by pulsed high voltage applications. *Conference Record of IEEE Industrial Applications Society Annual Meeting,* pp. 652–659.

Mizuno, A. and Hori, Y. 1988. Destruction of living cells by pulsed high-voltage applications. *Transaction of IEEE Industrial Applications,* 24:387–394.

Palacios, P., Burgos, J., Hoz, L., Sanz, B., and Ordoñez, J. A. 1991. Study of substances released by ultrasonic treatment form *Bacillus stearothermophilus* spores. *J. Appl. Bacteriol*. 71:445–451.

Rotman, Y. and Fields, M. L. 1968. A modified reagent for dipicolinic acid analysis. *Anal. Biochem*. 22:168.

Sale, A. J. H. and Hamilton, W. A. 1967. Effects of high electric fields on microorganisms. I. Killing of bacteria and yeasts, *Biochimica et Biophysica Acta,* 148:781–788.

Sale, A. J. H. and Hamilton, W. A. 1968. Effects of high electric fields on microorganisms. III. Lysis of erythrocytes and protoplasts. *Biochimica et Biophysica Acta,* 163:37–43.

Setlow, P. 1995. Mechanisms for the prevention of damage to DNA in spores of *Bacillus* species. *Annual Review of Microbiology,* 49:29–54.

Wax, R. and Freese, E. 1968. Initiation of the germination of *Bacillus subtilis* spore by a combination of compounds in place of L-alanine. *Journal of Bacteriology,* 95:433–438.

Wax, R., Freese, E., and Cashel, M. 1967. Separation of two functional roles of L-alanine in the initiation of *Bacillus subtitlis* spore germination. *Journal of Bacteriology,* 94:522–529.

Yonemoto, Y., Yamashita, T., Muraji, M., Tatebe, W., Ooshima, H., Kato, J., Kimura, A., and Murata, K. 1993. Resistance of yeast and bacterial spores to high voltage electric pulses. *Journal of Fermentation and Bioengineering,* 75:99–102.

Zhang, Q., Chang, F. J., Barbosa-Cánovas, G.V., and Swanson, B. G. 1994a. Inactivation of microorganisms in semisolid foods using high voltage pulsed electric fields. *Food Science and Technology (lwt),* 27:538.

Zhang, Q., Monsalve-González, A., Qin, B., Barbosa-Cánovas, G. V., and Swanson, B. G. 1994b. Inactivation of *Saccharomyces cerevisiae* in apple juice by square wave and exponential decay pulsed electric fields. *Journal of Food Processing and Engineering,* 17:469–478.

Zhang, Q., Barbosa-Cánovas, G. V., and Swanson, B. G. 1995a. Engineering aspects of pulsed electric field pasteurization. *Journal of Food Engineering,* 25:261–281.

Zhang, Q., Qin, B., Barbosa-Cánovas, G. V., and Swanson, B.G. 1995b. Inactivation of *E. coli* for food pasteurization by high intensity short duration pulsed electric fields. *Journal of Food Process and Preservation,* 19:103–118.

Zimmermann, U. 1986. Electric breakdown, electropermeabilization and electrofusion. *Review of Physiology Biochemistry Pharmacology,* 105:175–256.