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Pulsed electric field inactivation of diarrhoeagenic *Bacillus cereus* through irreversible electroporation

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N.J. ROWAN, S.J. MACGREGOR, J.G. ANDERSON, R.A. FOURACRE AND O. FARISH. 2000. The physical effects of high-intensity pulsed electric fields (PEF) on the inactivation of diarrhoeagenic *Bacillus cereus* cells suspended in 0·1% peptone water were examined by transmission electron microscopy (TEM). The levels of PEF-induced microbial cell death were determined by enumeration on tryptone soy yeast extract agar and *Bacillus cereus*-selective agar plates. Following exposure to lethal levels of PEF, TEM investigation revealed irreversible cell membrane rupture at a number of locations, with the apparent leakage of intracellular contents. This study provides a clearer understanding of the mechanism of PEF-induced cellular damage, information that is essential for the further optimization of this emerging food-processing technology.

INTRODUCTION

The increasing incidence of reported food-borne illnesses associated with microbial enteropathogens continues to be an increasing problem (Slutsker *et al.* 1998; Rowan 1999). These events have raised major concerns over food safety problems and their impact on public health. Consequently, the control of microbial contamination of foodstuffs has been given the highest priority in these countries. Also, there is growing consumer preference for minimally processed, fresh food produce, and this has increased interest in non-thermal techniques for food safety and food preservation (Gould 1996; Barbosa-Cánovas 1997).

The potential use of high-intensity, pulsed-power techniques, such as pulsed electric field (PEF) treatment, for food processing applications is currently receiving considerable attention since inactivation of micro-organisms takes place under reduced temperature conditions (Gould 1996; Qin *et al.* 1996; Wouters *et al.* 1999). The advantages of such an electrotechnology include the potential retention of fresh food characteristics and organoleptic qualities such as flavour, aroma and texture (Castro *et al.* 1993). Preliminary studies suggest that such treatment represents a promising non-thermal technique that may supplement or replace conventional pasteurization methods (Castro *et al.* 1993; Qin *et al.* 1995, 1996; Liu *et al.* 1997; Wouters *et al.* 1999). PEF treatment involves the application of pulsed electric fields, with a magnitude usually greater than 20 kV cm^{-1}, for short durations (500 ns to 4 μs), to liquid foods or to appropriate solid foods that can be pumped. A number of recent studies have shown that a range of spoilage and pathogenic micro-organisms are susceptible to PEF, with reductions of $\geq 10^6$ organisms ml^{-1} being reported in both laboratory-based culture media and in certain liquid foodstuffs (Castro *et al.* 1993; Qin *et al.* 1996; Wouters *et al.* 1999).

Although this treatment method has significant potential, the underlying mechanisms of microbial inactivation have still to be fully elucidated (Gould 1996; Schoenbach 1997; Wouters *et al.* 1999). The most commonly suggested theory is that of severe electroporation (i.e. the formation of pores in cell membranes by the action of high voltage electric fields), where local instabilities in the membranes of treated micro-organisms are formed by electromechanical compression and electrical field-induced tension (Wouters *et al.* 1999). PEF inactivation via electroporation is believed to be associated with the migration and accumulation of charge across the cell membrane under the action of an applied electric field. It is generally considered that the critical membrane potential, induced by the electric field, that causes microbial inactivation is about 1 V (Sitzmann 1996). At this level it is thought that the permeability of the membrane increases such that cell death occurs (Schoenbach 1997). The breakdown of biological membranes has been extensively studied with such model systems as liposomes, planar bilayers and phospholipid vesicles (Qin *et al.* 1996). There are a number of theories relating to electroporation,

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including dielectric breakdown, transmembrane potential-induced compression of the bilayer or cell membrane, induced changes in the viscoelastic properties of the cell membrane, rearrangement of the fluid mosaic arrangements of the lipids and membranes in the cell membrane; structural defects in the membranes, and colloidal osmotic swelling (Qin *et al.* 1996). The objective of the present study was to assess, by means of transmission electron microscopy, the nature and extent of cellular damage caused by the application of lethal levels of high-intensity pulsed-electric fields, to determine whether PEF treatment results in the formation of irreparable pores in the cell membrane of *Bacillus cereus*.

MATERIALS AND METHODS

Bacterial strains and growth media

The diarrhoeagenic strain of *Bacillus cereus* (NCTC 11145, National Collection of Type Cultures, Colindale, UK) was used in this study. Cells were initially grown in 50-ml tryptone soy broth supplemented with 0·6% (w/v) yeast extract (TSYEB) for 24 h at 30 °C with agitation (125 rev min^{-1}). A 1-ml sample of a 10^{-5} dilution was transferred to fresh 50-ml TSYEB (giving a starting cell population of $\sim 10^3$ cells ml^{-1}), and incubated at 125 rev min^{-1} for 6 h at 30 °C to obtain vegetative cells in the mid-exponential growing phase. Cells were pelleted by centrifugation (3000 *g* for 10 min at 4 °C), washed twice in phosphate-buffered saline (PBS) and resuspended in 0·1% (w/v) peptone water to a cell density of $\sim 10^8$ cells ml^{-1} before transfer to the PEF treatment chamber. The absence of bacterial endospores was confirmed by spore staining of triplicate samples and by heating at 80 °C for 15 min prior to enumeration on TSYEA plates. Stored bacteria were kept at −70 °C in PBS with 20% glycerol (v/v) until used.

Pulsed electric field (PEF) treatment of *Bacillus cereus*

The test cells, suspended in 0·1% peptone water, were subjected to high intensity pulsed electric fields. A 100-kV Glassman high voltage DC generator was used to charge a coaxial cable Blumlein pulse generator through a charging resistance of 10 MΩ. The coaxial cable generator was constructed from 100 m of URM67, 40-kV cable. The high voltage output pulse from the generator was 500 ns in duration, and the generator had an output impedance of 100 Ω, a switching impedance of 50 Ω, and an open circuit gain of 2. The generator was wound inductively on a 30-cm diameter former in order to minimize secondary transmission line losses. The electrical circuit layout is shown schematically in Fig. 1 and the charging circuit was arranged to

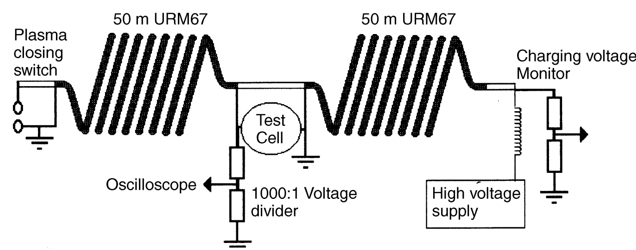

Fig. 1 The electrical circuit layout for PEF treatment

ensure that the generator charging currents did not flow through the test cells.

The pulse generator was charged from one end of the cable, and was fired by switching the inner conductor to ground at the opposite end. Although both sides of the test chamber were earthed, the output from the cable generator, which was connected to one side of the test chamber, was transiently decoupled from earth during application of the voltage pulse. The Blumlein generator was fired by a triggered corona stabilized switch. For the present study, the pulse repetition frequency was limited to a maximum value of 5–10 pulses per second in order to ensure that there were no thermal inactivation effects associated with the energy dissipation in the test chamber. The output pulse from the generator was monitored throughout the experiments with a 1000 : 1, Tektronix high voltage probe. The voltage pulse profile used for PEF treatment, measured across the test cell, is shown in Fig. 2.

Test samples of *Bacillus cereus* were subjected to a pulsed electric field at a level of ~ 30 kV cm^{-1} in a uniform-field static test cell. The volume of the test cell was 30 ml and samples of a predetermined cell number were subjected to a range of pulses from 500 to 6000 pulses. A forced air cooling system was used to maintain the test chamber temperature between 25 and 30 °C. A thermocouple was employed throughout the studies in order to verify the temperature of the treated liquid. The levels of microbial inactivation and cell integrity (examined by transmission electron microscopy) were assessed post PEF-treatment. Surviving organisms were enumerated with both the pour and spiral plating techniques on TSYEA and on *Bacillus cereus*-selective agar (BCSA, Oxoid) plates.

Transmission electron microscopy

Bacillus cereus cells treated by PEF were washed twice with PBS and resuspended in sterile-distilled water before application to formvar-coated grids. After the grid was dried, one drop of a solution containing 3% (v/v) tungstopho-

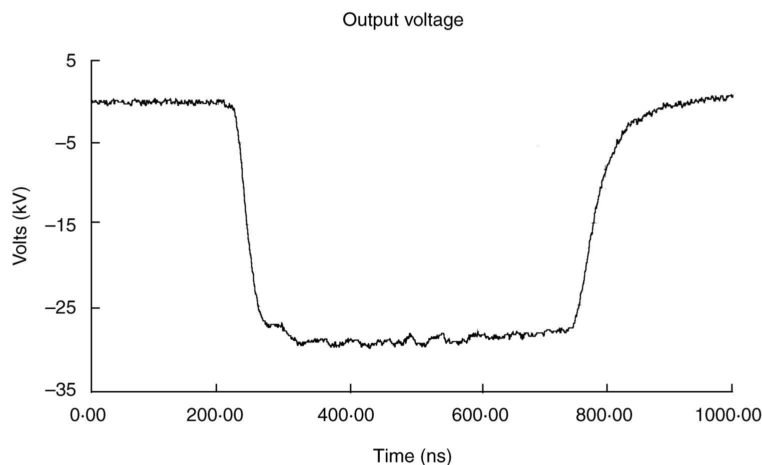

Fig. 2 Measured output pulse waveform applied to the test cell for PEF treatment

sphoric acid and 0·3% (v/v) sucrose (pH 6·8–7·4) was added. The solution was removed after 30–60 s, and the grid was dried and examined on a Zeiss 902 transmission electron microscope.

Statistical analysis

All of the experiments in this study were performed in triplicate and results are reported as averages. Significant differences in the PEF treatment results were calculated at the 95% confidence interval using analysis of variance (one way) with Minitab software Release 11 (Mintab Inc., State College, PA, USA).

RESULTS AND DISCUSSION

The results described in Table 1 show the effect of applying up to 6000 pulses to populations of *B. cereus* with cell survivors enumerated on TSYEA and BCSA plates. A 5·5–6 log order reduction was achieved following the maximum treatment of 6000 pulses. In both sets of cell survivor data, the inactivation was similar and the population reduction increased with the greater the number of pulses applied. The BCSA enumerated data is also observed to be consistently lower than that of the TSYEA data for treatments of 1000 pulses and more. This difference in recovery is possibly due to the added stress of dyes and antibiotics in the selective plating medium (Rowan and Anderson 1998; Rowan 1999).

Transmission electron microscopy (TEM) of untreated (Fig. 3a) and PEF-treated (Fig. 3b) *B. cereus* cells was carried out after 6000 pulses at 30 kV cm^{-1}. TEM results from PEF-treated cells revealed that there was rupture of the cell wall (CW) with irreversible electroporation of the cell membrane with leakage of the intracellular contents or cellular debris (CD) (Fig. 3b). It is significant that PEF-induced pore formation (PF) in the cell envelope was observed at several locations along the length of this rod-shaped bacterium, as well as occurring at the extremities of the rod. In addition to the loss of cytoplasmic and nuclear material by disintegration of the cell envelope (indicated in Fig. 3b as CD). This shrinkage pattern (indicated by → in Fig. 3b) suggests leakage of cytoplasmic and nuclear material from the cell. It is clear that not all PEF-treated bacteria have had their cell wall ruptured, as evident by the presence of an intact *B. cereus* (IBC) cell in this figure, although this should not be interpreted as indicating a viable cell. This observation is in general agreement with

Table 1 Survival data for PEF-treated *Bacillus cereus* cells enumerated on TSYEA and BCSA plates

Number of PEF pulses	Number of cell survivors*		Significance $P < 0{\cdot}050$
TSYEA†	BCSA†		
0	8·69 (±0·2)	8·55 (±0·2)	No
500	7·15 (±0·2)	6·91 (±0·3)	No
1000	6·21 (±0·3)	5·75 (±0·2)	Yes
3000	5·61 (±0·3)	4·89 (±0·3)	Yes
6000	3·25 (±0·3)	2·44 (±0·4)	Yes

*Measured as $\log_{10}$ cfu ml^{-1}, where counts were averages of three replicate trials.
†Numbers in parenthesis refer to variation about the mean.

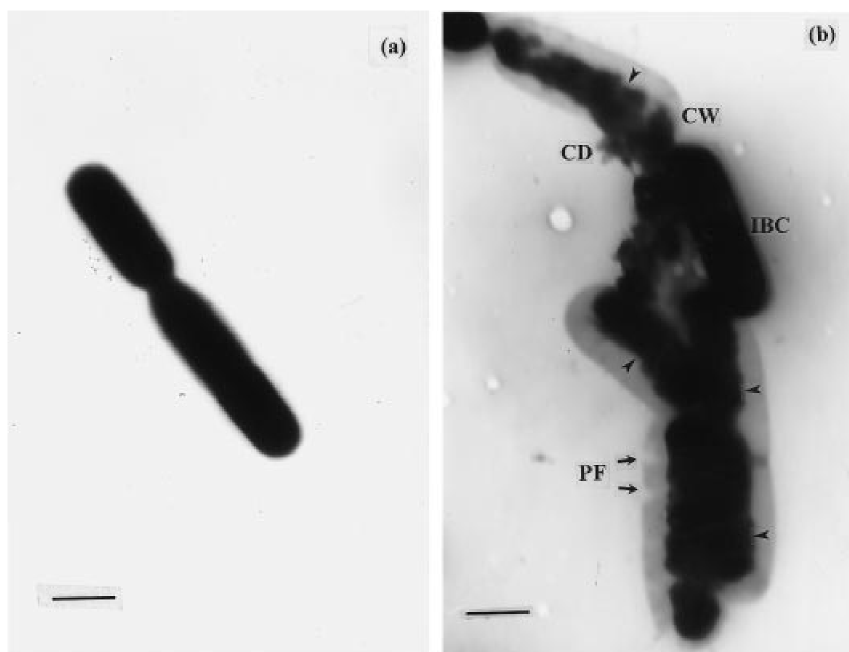

Fig. 3 TEM of untreated (a) and PEF-treated (b) cells of *Bacillus cereus*. Bar, 1·5 μm. CW, cell wall; CD, cellular debris; PF, PEF-induced pore formation; IBC, intact *B. cereus*. Arrowheads (→) indicate shrinkage pattern

the survival data for PEF-treated *B. cereus* shown in Table 1.

The TEM results did reveal some relatively minor shrinkage of the cytoplasmic membrane away from the outer membrane in the treated bacterial cells. There was no visual evidence of any partial or complete disintegration of the cell envelope in these PEF treated organisms. Cells of *Escherichia coli* (ATCC) and *Staphylococcus aureus* (ATCC 6538) suspended in simulated milk ultrafiltrate (SMUF), were observed with scanning (SEM) and transmission (TEM) electron microscopy following electric field treatment at 60 kV cm^{-1}. However, the SEM technique did not indicate any significant differences between PEF-treated and untreated *E. coli* and *Staph. aureus* cells (cited in Barbosa-Cánovas 1997). The TEM results presented here with *B. cereus* are in agreement with the findings of Harrison (1997), who showed that PEF inactivation of the food spoilage yeast *Saccharomyces cerevisiae* (ATCC 16664) occurs by cell envelope rupture and loss of intracellular contents.

In conclusion, studies have been carried out with a simple buffered system that have permitted an assessment to be made on the influence of PEF on the integrity of bacterial cells by TEM. PEF inactivation of diarrhoeagenic *B. cereus* occurs through irreversible electroporation, and causes cell death. The TEM results showed that PEF-induced pore formation occurred randomly in these rod-shaped bacterial cells, resulting in a large-scale leakage of cytoplasmic and nuclear material. This study has provided a clear understanding of the mechanism of PEF-induced lethality in micro-organisms, information that is required in order to allow optimization of this electrotechnology and to help satisfy the regulatory requirements associated with the introduction of such a novel food-processing technology.

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