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Influence of treatment temperature on the inactivation of *Listeria innocua* by pulsed electric fields

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Abstract

The effect of treatment temperature on the bactericidal effectiveness of pulsed electric fields (PEF) applied on *Listeria innocua* suspended in McIllvaine buffer was investigated. Electric field intensity and number of applied pulses were applied in the ranges of 31–40 kV/cm and 5–35 pulses, respectively. Studied treatment temperatures were sustained for 10 s, and ranged between 19°C and 59°C depending on the amount of energy delivered by the PEF treatment. The application of PEF at higher temperatures proved to be more effective than either PEF at low temperatures or the applied thermal treatments by themselves. A maximum bacterial inactivation of 6-log cycles was obtained by applying either: 20 pulses of 40 kV/cm at 65°C, 25 pulses of 36 kV/cm at 61°C, or 31 pulses of 31 kV/cm at 56°C. On the other hand, a thermal treatment of 66°C sustained for 30 s reduced the bacterial population on its own by only 5-log cycles, and the application of 60 pulses of 31 kV/cm at 30°C caused only 3-log cycles of bacterial inactivation. The findings in this study suggest that PEF technology may be effectively used as an enhanced mild thermal preservation method.

Keywords: Pulsed electric fields; PEF; *Listeria innocua*; Bacterial inactivation

1. Introduction

Pulsed electric fields (PEF) technology has been extensively studied as a nonthermal preservation method used to inactivate undesirable microorganisms in liquid food products without the need for high temperatures that may modify their nutritional and sensory characteristics (Barbosa-Cánovas, Góngora-Nieto, Pothakamury, & Swanson, 1999). This preservation method is considered to be a nonthermal method because it has been demonstrated that microbial inactivation is mainly caused by the presence of electric fields, apart from the influence that other factors such as temperature may have on the effectiveness of the process (Sale & Hamilton, 1967). Application of PEF is attained by delivering high-voltage pulses to a product placed between two conductive electrodes within a treatment chamber, and it is accompanied by a temperature increase caused by electric current flowing through the treated product. The intensity of the electric current, and thus of the temperature increase, varies depending on the electrical characteristics of the treated product and equipment (Heinz, Alvarez, Angersbach, & Knorr, 2002). High voltage pulses produced by capacitor discharge contain a finite amount of energy (Q_{pulse}) that reaches the treatment chamber, as defined by Eq. (1)

$$Q_{\text{pulse}} = \left(\frac{R_{\text{Ch}}}{R_{\text{T}}}\right)\frac{CV^2}{2}, \tag{1}$$

where C is the capacitance of the discharging capacitor, V is the charging voltage, R_{Ch} is the electrical resistance of the treatment chamber and R_{T} is the total electrical resistance of the system through which the capacitor is being discharged (a series system including R_{Ch}). Repetitive application of high voltage pulses causes heating of the treated product (ΔT) as energy is released into the treated product as defined by Eq. (2)

$$\Delta T = \frac{fQ_{\text{pulse}}}{F\rho C_p}, \tag{2}$$

where f is the pulsing frequency, F is the flow rate of the liquid product pumped through the treatment chamber,

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ρ is the density of the treated product, and C_p its specific heat.

From the nonthermal food processing perspective, treatment temperature has to be kept low in order to avoid heat damage to the treated product, hence indirect cooling through heat exchangers located either in the treatment chamber itself, or between treatment chambers in the case of systems with more than one treatment chamber is usually employed (Zhang, Barbosa-Cánovas, & Swanson, 1995; Qin, Pothakamury, Barbosa-Cánovas, & Swanson, 1996). Removal of heat produced during PEF treatment may be achieved by the use of heat exchangers in the treatment chamber; however, as heat is generated volumetrically throughout the treated liquid, product heating can only be avoided either by limiting the number of applied pulses or by using heat exchangers between treatment chambers or treatment steps.

Application of PEF at mild temperatures has been suggested as a way to enhance the effectiveness of PEF as a preservation method (Hülsheger, Pottel, & Niemann, 1981; Dunn & Pearlman, 1987). Although application of treatments at high temperatures is not desirable, use of mild sub-lethal thermal conditions in combination with PEF has the potential to facilitate the application of effective treatments without the need for multiple chambers or processing steps. The exact reasons behind the enhancement of bacterial inactivation by combining PEF and mild temperatures have not been determined yet, however several hypotheses have been proposed. Examples of such hypotheses are the reduction of the charging time of bacterial membranes due to increased electrical conductivity of the media caused by higher temperatures (Schwan, 1957), changes in the phase state of cell membranes (Jayaram, Castle, & Margaritis, 1992), and reduced trans-membrane breakdown potentials (Kinosita & Tsong, 1977; Zimmermann, 1986).

The objective of this research was to determine the effectiveness of PEF treatments under mild thermal conditions on the inactivation of *Listeria innocua* suspended in a buffer solution. *L. innocua* was selected in view of its known particular resistance to PEF treatments (Heinz et al., 2002), and because it is a nonpathogenic surrogate of *L. monocytogenes* (Kamat & Nair, 1996), which is a pathogenic microorganism of relevance in food preservation processes (Calderon-Miranda, Barbosa-Cánovas, & Swanson, 1999).

2. Materials and methods

The effect of the combination of PEF and mild thermal treatments was studied at several electric field intensities and treatment times to determine the effect of these processing parameters on bacterial inactivation, and was compared with both purely thermal treatments and PEF treatments at low treatment temperatures. In a first stage, *L. innocua* suspended in McIllvaine buffer was subjected to PEF treatments (31 kV/cm) under controlled temperature conditions (step processing, limited treatment time) such as to maintain treatment temperature in the range 33–34°C. PEF treatments with the same electric field intensity were then applied on *L. innocua* suspended in buffer without limiting the treatment temperature, reaching temperatures up to 65°C. In a second stage, PEF treatments with higher electric field intensities (36 and 40 kV/cm) were applied also in combination with their self-generated electric heating and their effectiveness was assessed.

2.1. Preparation of inoculum and mcIllvaine buffer

L. innocua (ATCC 51742, Rockville, MD) was purchased freeze-dried and rehydrated in 10 ml of tryptic soy broth (Difco, Kansas MO) enriched with 0.6% yeast extract (TSBYE). After 30 min, the cell suspension was inoculated into 150 ml of TSBYE and incubated for 18 h at 37°C with continuous agitation at 225 rpm in an orbital shaker (MSB-3322A-I, GS Blue Electric, Blue Island, IL). The culture was then dispensed into sterile vials containing 1 ml of 20% sterile glycerol as a cryo-protective agent and stored at −70°C until used. Prior to every experimental run, one vial containing frozen culture was thawed and 1 ml of the media was inoculated into 150 ml of TSBYE and incubated at 37°C with continuous agitation at 225 rpm for 5 h until the early stationary phase was reached. Once the desired bacterial concentration was reached ($\sim 10^9$ cfu/ml), 6 ml of the inoculum were transferred into 6 l of McIllvaine buffer to obtain an initial bacterial concentration of approximately 1×10^6 cfu/ml. McIllvaine buffer pH 7 consists of a solution of 0.1 M citric acid and 0.2 M sodium phosphate, diluted with distilled water in a 1:6 ratio in order to adjust its electric conductivity to 0.45 S/m at 25°C. Bacteria were subjected to the desired PEF treatments as soon as the inoculum was homogeneously suspended in the buffer previously set at 4°C.

2.2. PEFs treatments

A pilot plant PEF system manufactured by Physics International (San Leandro, CA), and a continuous cylindrical concentric-electrodes treatment chamber (Qin, Zhang, Barbosa-Cánovas, Swanson, & Pedrow, 1995) were used to apply the desired treatments to the prepared samples. A total of four different treatments were investigated in this study.

Treatment A: The first condition under study was the application of 15, 30, 45, and 60 exponentially decaying pulses with maximum electric field intensity of

31 ± 0.6 kV/cm and 2.1 ± 0.1 μs pulse width (FWHM; full-width at half-maximum: pulse width measured at half the peak voltage) by discharging a 0.5 μF capacitor. In order to maintain treatment temperature in the range 33–34°C, the PEF treatment was applied by steps, 15 pulses at a time with cooling through heat exchangers between steps in order to return the product's temperature to 4°C (initial temperature) before applying the next set of pulses.

Treatment B: The second applied treatment consisted of the application of 5, 10, 15, 20, 25, and 30 exponentially decaying pulses with maximum electric field intensity of 31 ± 0.5 kV/cm and 2.0 ± 0.5 μs pulse width (FWHM) by discharging a 0.5 μF capacitor. In this treatment all pulses were applied at once (no cooling or step treatment), hence allowing for higher treatment temperatures, sustained for less than 10 s. Pulse width varied within the mentioned range from a minimum at the highest treatment temperature to a maximum at the lowest treatment temperatures.

Treatment C: The third treatment consisted of the application of 5, 10, 15, 20, and 25 exponentially decaying pulses with maximum electric field intensity of 36 ± 0.7 kV/cm and 1.9 ± 0.5 μs pulse width (FWHM), applied all at once, allowing for temperature increase, sustained for less than 10 s. Pulse width varied within the mentioned range from a minimum at the highest treatment temperature to a maximum at the lowest treatment temperatures.

Treatment D: Finally, the fourth treatment consisted of the application of 5, 10, 15, and 20 exponentially decaying pulses with maximum electric field intensity of 40 ± 0.5 kV/cm and 1.7 ± 0.5 μs pulse width (FWHM), again applying all pulses without intermediate cooling steps to allow heating of the product sustained for less than 10 s. Pulse width varied within the mentioned range from a minimum at the highest treatment temperature to a maximum at the lowest treatment temperatures.

The intensity of the electric fields, pulse width and shape were directly measured by a digital oscilloscope (Hewlett-Packard 54530A, Colorado Springs, CO), connected to the treatment chamber through high-voltage probes. Temperatures before and after the treatment chamber were monitored with digital thermometers (Cole-Palmer, Vernon Hills, IL).

2.3. Assessment of the effectiveness of PEF-inactivation treatments

Each study was conducted in triplicate using independently prepared batches of buffer and fresh bacteria for every replicate. Samples of treated and untreated media were taken after each treatment and pour plated into Tryptic Soy Agar (Difco) enriched with 0.6% yeast extract (TSAYE) after serial dilutions with sterile 0.1% peptone water. All samples were plated in duplicate and incubated at 27 ± 0.5°C for 48 h in a controlled temperature chamber. After incubation, colonies were quantified following standard procedures (AOAC, 1995). Inactivation effectiveness was quantified by determining the logarithmic difference between the number of bacteria before and after treatment, expressing it as "logarithmic cycles reduction".

Table 1
Treatment temperatures achieved by the application of selected number of exponentially decaying pulses of approximately 2 μs at different electric field intensities

Treatment	Data set	Electric field intensity (kV/cm)	Number of pulses	Treatment temperature (°C)
A	1	31	15	33
A	2	31	30	34
A	3	31	45	33
A	4	31	60	34
B	5	31	5	19
B	6	31	10	28
B	7	31	15	38
B	8	31	20	44
B	9	31	25	51
B	10	31	30	56
C	11	36	5	21
C	12	36	10	31
C	13	36	15	45
C	14	36	20	56
C	15	36	25	61
D	16	40	5	24
D	17	40	10	39
D	18	40	15	55
D	19	40	20	65

The data obtained from the four described PEF experiments was further subdivided into 19-independent sets of data defined by different combinations of electric field intensity, number of applied pulses and treatment temperature (see Table 1). A one-way ANOVA was conducted and a LSD multiple comparison test with $\alpha = 0.05$ was used to determine whether the application of different experimental conditions caused different degrees of bacterial inactivation or not.

3. Results and discussion

Inactivation plots showing the bacterial inactivation obtained from the applied treatments are presented in Fig. 1. Treatment time (number of pulses times the pulse width) is presented in this plot instead of the number of applied pulses, as pulse width slightly varied (around ± 0.5 μs) depending on the treatment temperature. The statistical analysis of the data divided the area between null inactivation and maximum inactivation (inactivation values cannot go beyond that of the initial population level, which is around 1×10^6), into 10

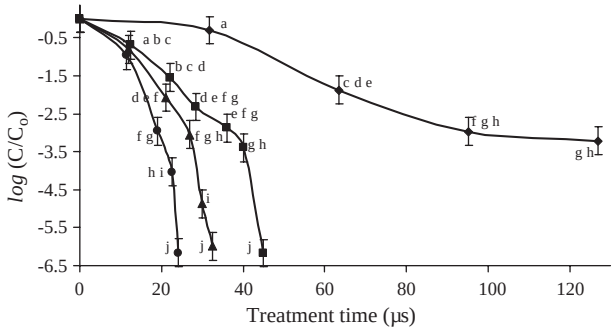

Fig. 1. Inactivation plot showing the bacterial inactivation obtained by applying electric field pulses of approximately 2 μs pulse width, and peak electric field intensity of: (♦)31 kV/cm at temperatures below 35°C, (■)31 kV/cm at temperatures up to 56°C, (▲)36 kV/cm at temperatures up to 61°C, and (●)40 kV/cm at temperatures up to 65°C. Plotted values and error bars correspond to the average of three replicates and its standard error, respectively. Mean values with the same letter are not significantly different.

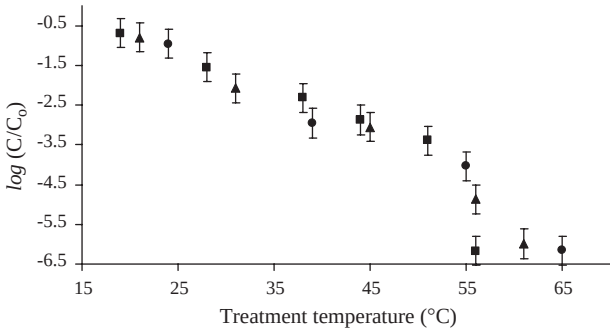

Fig. 2. Combined PEF/thermal bacterial inactivation data plotted against treatment temperature. (■)5, 10, 15, 25, and 30 pulses at 31 kV/cm, (▲)5, 10, 15, and 25 pulses at 36 kV/cm, and (●)5, 10, 15, and 20 pulses at 40 kV/cm. Treatment temperature increases with the number of applied pulses for each studied treatment starting at 5 pulses (coldest). Indicated temperatures were sustained for no more than 10 s in all cases. Plotted values and error bars correspond to the average of three replicates and its standard error, respectively.

different inactivation zones, identified by letters *a* to *j*. PEF treatments branding same superscript produced statistically equivalent bacterial inactivation. Table 1 complements the information of Fig. 1, presenting the corresponding treatment temperatures reached after the application of PEF on each one of the nineteen studied experimental conditions. Use of higher treatment temperatures during application of PEF treatments resulted in enhanced inactivation effectiveness, as found in previous studies (Zhang et al., 1995; Pothakamury, Vega, Zhang, Barbosa-Cánovas, & Swanson, 1996; Reina, Jin, Zhang, & Yousef, 1998; Wouters, Dutreux, Smelt, & Lelieveld, 1999; Aronsson, Lindgren, Johansson, & Ronner, 2001). Fifteen pulses of 31 kV/cm applied at 38°C caused equivalent inactivation as 45 pulses of the same field intensity applied at 33°C, reducing the required treatment time by about two-thirds, while 20 pulses applied at 44°C, caused equivalent inactivation as 60 pulses applied at 33°C, again reducing the required treatment time to around one-third of the time required at lower temperature. This enhancement of the inactivation effectiveness increased markedly when 30 pulses of 31 kV/cm were applied at 56°C. Although no inactivation data are available at this time for a treatment of 90 pulses at 33°C (which from the relationship established by the previous points in the curves would be the equivalent treatment at 33°C to 30 pulses at 56°C), the curve's observed trend makes doubtful that such inactivation levels could be reached by this lower temperature treatment. Furthermore, it is possible to notice that an abrupt jump in the inactivation effectiveness occurs between 25 and 30 pulses in the "31 kV/cm up to 56°C" curve, suggesting that some sort of relevant event involving a reduction of the resistance of *L. innocua* to PEF occurred between these two points.

Increasing the intensity of the applied electric field also increased the inactivation effectiveness of PEF treatments applied in combination with mild thermal treatments. Curves "36 kV/cm up to 61°C" and "40 kV/cm up to 65°C" required 25 and 20 pulses, respectively, instead of 30 as in the case of "31 kV/cm up to 56°C" to reach the maximum inactivation level, representing an increase in the inactivation efficiency of about one-sixth and one-third, respectively. Analysis of the inactivation data plotted against treatment temperature (Fig. 2), resulted in a surprising observation. The effect of the electric field intensity appears not to be as relevant defining bacterial inactivation as the treatment temperature, since similar results are obtained by the application of different combinations of electric field and number of pulses at treatment temperatures below 55°C where inactivation abruptly increases. Interestingly enough, application of PEF at different intensities and treatment times at 55°C coincides with the experimental condition where inactivation effectiveness markedly increased in the plot of inactivation versus treatment time, hence suggesting that the observed enhancing effect may have a thermal origin. Phospholipid molecules in cell membranes undergo temperature-related phase transitions, changing from a packed gel-like structure at low temperatures to a looser liquid–crystalline phase at higher temperatures, reducing the thickness and mechanical resistance of cell membranes (Stanley, 1991). The marked increase of PEF inactivation effectiveness at 55°C may be indicative of the occurrence of such phase transition on the cell membrane of *L. innocua* at this temperature, since it is possible to argue that a thinning of the bacterial membrane would render bacterial cells more susceptible to disruption by electric fields (Jayaram et al., 1992), as indicated by the dielectric breakdown theory (Zimmermann, Pilwat, & Riemann,

1974). Similar studies conducted on *Lactobacillus brevis* in which PEF were applied at high temperatures reported a considerable increase on the inactivation effectiveness of PEF at 60°C (Jayaram, Castle, & Margaritis, 1991). The discrepancy on the temperatures at which enhancement of PEF effectiveness was observed in these two studies may respond to differences on the lipid composition of the cellular membranes of *L. innocua* and *L. brevis*, assuming that the bactericidal enhancement is effectively related to a phase transition phenomenon. Based on these findings, it may be hypothesized that the phase transition temperature of bacterial cell membranes is a relevant processing parameter that must be taken into account when defining a PEF treatment applied in combination with mild thermal treatments, but further work confirming the relevance of thermally induced phase transitions on the effectiveness of PEF treatments is needed.

In order to determine the effect that temperature, by itself, may have on the inactivation process, besides the simple effect or interaction caused by the application of PEF, some thermal inactivation data obtained from literature were used to characterize the applied treatments as if they were purely thermal (Doyle, Mazzotta, Wang, Wiseman, & Scott, 2001). The selected data correspond to the thermal inactivation of *L. monocytogenes*, not *L. innocua,* which was used in this study; nevertheless, it has been found that the thermal resistance of *L. monocytogenes* is very similar to that of *L. innocua*, and that when different, lower than the thermal resistance of *L. innocua*, providing an extra safety factor to ensure that the thermal effect is not underestimated (Fairchild & Foegeding, 1993). The bacterial strain with the lowest resistance to thermal treatments was selected for comparison based on the same reasons. Temperatures reached during the application of PEF treatments were closely monitored; however, the thermal treatment time can only be analytically estimated since it not only depends on the flow rate, but also on the nature of both the flow regime and the heat exchange process. Given the characteristics of the employed PEF system and processing conditions it was determined that the residence time of the treated media in the "hot"area was about 10 s before temperature was lowered to at least 30°C. However, to be on the safe side, treatment times of 30, 60 and 120 s were chosen for comparison purposes. Fig. 3 shows the effect of the thermal treatments applied in combination with PEF, compared to the effect of thermal treatments applied on *L. monocytogenes* under conditions similar to those employed in the present study. Given the low temperatures and short times employed, purely thermal treatments had almost null inactivating action. From this comparison it is well established that although increasing the treatment temperature enhances the bactericidal effect of PEF, this in no way must be interpreted as the result of thermal inactivation, since clearly the applied combinations of treatment time and temperature constitute sub-lethal conditions for *L. innocua* under the studied circumstances.

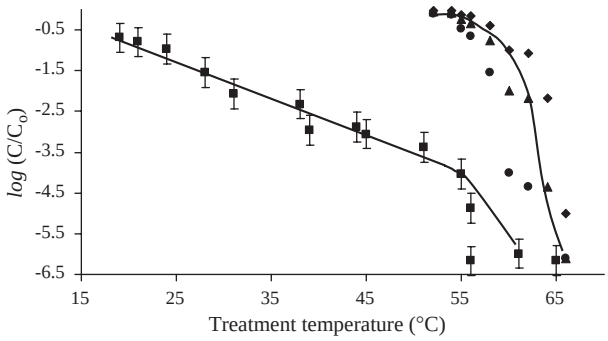

Fig. 3. Inactivation plot comparing thermal treatments and PEF/thermal combined treatments. (■) PEF treatments of 31, 35 and 40 kV/cm combined with mild thermal treatments for less than 10 s, (♦) Thermal treatments sustained for 30 s, (▲) Thermal treatment sustained for 60 s, and (●) Thermal treatments sustained for 120 s.

4. Conclusions

Application of PEF in combination with mild thermal treatments were able to significantly reduce the studied bacterial populations of *L. innocua* under circumstances in which neither the applied PEF at low temperatures nor the thermal treatments by themselves were able to cause significant bacterial inactivation. Hence, it was demonstrated that treatment temperature is a very important parameter defining microbial inactivation by PEF; moreover, it was determined that the application of PEF on *L. innocua* at temperatures over 55°C presents unusually high effectiveness, which was attributed to temperature-related phase transition of phospholipids in the cell membrane. It was also suggested that the phase transition temperature of bacterial membranes could be used as a processing parameter to define PEF treatments applied in combination with mild thermal treatments. This study provides evidence of the potential for the successful use of PEF on food preservation processes at mild temperatures, reaching a compromise between effective bacterial inactivation and the preservation of quality and nutritional attributes as well.

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