COMBINING MODERATE PULSED ELECTRIC FIELDS WITH TEMPERATURE AND WITH ORGANIC ACIDS TO INACTIVATE *ESCHERICHIA COLI* SUSPENSIONS

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ABSTRACT **:** The aim of this work was to study the efficiency of inactivation of *Escherichia coli* cells in aqueous suspensions using combined moderate pulsed electric field (PEF) and thermal treatments. The inactivation kinetics of *E. coli* cells in aqueous suspensions (1 wt%) was monitored using conductometric technique. The electric field strength E was within 5-7.5 kV/cm, the effective PEF treatment time t_{PEF} was within 0-0.75 s, the pulse duration t_i was within 0.3-1 ms, the medium temperature was 30-50°C, and the time of thermal treatment t_T was within 0-7000 s. The organic acid concentration was within 0-0.5 g/L.The damage of *E. coli* was accompanied by release of intracellular components. The synergy between the PEF and thermal treatments in *E. coli* inactivation was clearly demonstrated. The damage efficiency was noticeably improved by addition of organic acids, especially lactic acid.

KEY WORDS*: Escherichia coli, Pulsed electric fields, Thermal treatment, Electrical conductivity, Organic acids.*

1. INTRODUCTION

Pulsed electric field is a new technology recently studied and designated for inactivation of micro-organisms. Promising results were obtained using high electric fields within the intensity of 20-80 kV/cm. The pulsed electric field (PEF) application to an aqueous suspension of colloidal bio-organisms can result in noticeable changes in the structure of cells and in the state of their aggregation. Electric pulses of high intensity and of small duration (typically 1–100 μsec) cause temporary loss of the cell membrane

permeability (electroporation) and ion leakage [1] without significant temperature increase and undesirable effects on cell components. Numerous studies have investigated the effect of PEF applications for microbial inactivation [2], electrofusion of cells [3], and transport of nanoparticles across the cell wall [4]. Electropulsation treatment of some microbial species (*E. coli, S. cerevisiae*) is expected to be a promising method for recovery of the homogeneous and heterogeneous intracellular proteins having wide biotechnological applications [5,6].

2. MATERIALS AND METHODS

2.1 Preparation of *E. coli* suspensions

In this research cells of gram negative bacteria *Escherichia coli* (ATCC strain #25255) were used. *E. coli* was cultured in Nutrient Broth (Biokar diagnostics, Beauvais, France). Overnight cultures were inoculated in 100 mL of an adapted medium composed from 16 g/L of Tryptone, 10 g/L of yeast extract, 5 g/L of lactose, 1.5 g/L of fresh bile, 5 g/L of NaCl, 9.4 g/L of K_2HPO_4, and 2.2 g/L of KH_2PO_4. All chemical products were from Fisher scientific (Leicestershire, UK). Then cultures were grown in a shaker at 200 rpm (INFORS AG, Bottmingen, Switzerland) at 37°C for 24 hours.

Cells were harvested by centrifugation using the Sigma 4K10 centrifuge (Bioblock Scientific, Osterode, Germany) at 3000 g and at 4°C for 3 min. Then cells were washed by saline water, centrifuged again under the same conditions, and the supernatant solution was removed. The saline water used in this study had conductivity of 300 µS/cm at 25°C, and the background electrolyte (NaCl) was used for the suspension conductivity adjustment. The above procedure was repeated at least 3 times in order to remove residual substances from the original samples of cells. The suspensions were prepared by one minute vortexing (rotation speed 100 rpm and amplitude 4.5 mm) of centrigugated cells with the saline water (Top Mix Bioblock Scientific, Germany). The agitation and centrifugation conditions were rather gentle, in order to prevent any additional damage of the cells. The viability of cells at the beginning was 98%. Finally, the exact concentrations were calculated on the basis of the dry matter content after drying of suspensions at 105°C for 24 h.

In PEF thermal treatment experiments, the aqueous *E. coli* suspensions had dry matter concentration (after heating in the oven at 105ºC for 24h) about 1 wt%, which corresponds to the cellular density of approximately 3.5×10^{10} cells/mL.

In experiments with organic acids additives, the initial cellular density of *E. coli* cells was approximately 14×10^8 cells/mL.

2.2 Analysis instruments

K-type thermocouple, connected to the data logger thermometer Center 305/306 (JDC Electronic SA, Yverdon-les-Bains, Switzerland) was used to control the temperature.

Following PEF-thermal exposure, 2 mL of each replicate was centrifuged at 7903 g for 5 min, and the supernatant was used for UV absorption measurements by Anthelie Advanced spectrophotometer (Secomam, Domont, France). The wavelength range was within 190 - 340 nm (with the precision of ±1 nm). The path length of the quartz cell (Starna, Optiglass Ltd, Essex, UK) was 5 mm and the scan speed was 1800 nm/min.

In experiments with organic acids additives the treated and untreated suspensions were diluted by physiological water and plated (Nutrient agar, Biokar diagnostics, Beauvais, France) in three parallels. The plates were incubated at 37°C for 24 h and cells reduction was expressed by calculating $\mathrm{Log}(N_o/N)$.

Each experiment was repeated, at least, in triplicate to calculate the mean value of the experimental data and the standard deviations.

2.3 PEF treatment

In PEF-thermal treatment experiments, suspensions were treated by pulsed electric field at the field strength E = 5 kV/cm, and simultaneously heated within the temperature range T = 30-50°C, while in organic acid additives experiments the pulsed electric field strength E was 7.5 kV/cm and samples were treated at ambient temperature.

The PEF generator, 1500 V–20 A (Service Electronique UTC, Compiègne, France) provided monopolar pulses of near-rectangular shape. The trains of pulses were used for the PEF treatment. An individual train consisted of n pulses with the pulse duration t_i and pulse repetition time Δt. There was a pause Δt_t after each train, and then polarity of the next train was changed to the opposite.

Suspensions were pumped in the continuous flow treatment chamber (OSU-4B, Ohio State University, U.S.A.) through six co-field flow, tubular stainless steel electrode gaps, connected in series. The suspension temperature was regulated after each pair of gaps using a water thermostat maintained at the required temperature T.

PEF-thermal treatment experiments were carried out with the electric field strength E = 5 kV/cm, number of pulses by train n = 1000, pulse duration $t_i = 10^{-3}$ s, pulse repetition time $\Delta t = 5 \times 10^{-2}$ s, number of trains N = 100-150, and inter-train time Δt_t = 1 s. All the output data (resistance, current, voltage, and temperature) were recorded using a data logger and software developed by Service Electronique UTC, Compiègne, France. The PEF treatment time during one train was nt_i. The effective PEF treatment time t_{PEF} was calculated as [7]

$$t_{PEF} = nt_i NW_g / W \tag{1}$$

where $W_g = N_g Sh$ is the treatment volume, $S = \pi r^2$, r = 0.115 cm is the radius of a cylindrical electrode, h = 0.29 cm is the distance between the electrodes, N_g = 6 is the number of treatment chambers, W = 50 cm^3 is the total volume of suspension. The effective PEF treatment time t_{PEF} corresponds to the thermal treatment time $t_T = N(n\Delta t+\Delta t_t)$. At such protocol of the PEF treatment, $n\Delta t >> \Delta t_t$ and t_T can be approximated as

$$t_T \approx t_{PEF} (\Delta t/t_i)(W/W_g) \approx 3.46 \times 10^4 t_{PEF} \tag{2}$$

The electrical conductivity of suspension σ was measured with a conductivity meter InoLab pH/cond Level 1 (WTW, Weilheim, Germany) at the frequency of 50 Hz. The electrical conductivity was compared under different modes of PEF-thermal treatments.

The conductivity disintegration index Z was estimated as [8]

$$Z = (\sigma - \sigma_o)/(\sigma_{max} - \sigma_o) \quad (3)$$

Where σ_o is the initial electrical conductivity of the suspension and σ_{max} is the saturation electrical conductivity of the suspension with maximally disintegrated cells.

The value of σ_{max} was estimated by measuring the electrical conductivity of a suspension initially treated by PEF at 50°C during $t_{PEF} = 0.12$ s and then heated at 70°C during 1 hour. After such electrical and thermal treatment, the disintegration index was $Z \approx 1$ and the surviving ratio $S \approx 1\text{-}Z_d$, i.e., the ratio of the number of undamaged cells to the total number of cells (values obtained by direct colony counting in the agar plates), was $S \approx 1.1 \times 10^5$ CFU/mL /3.5×10^{10} CFU/mL $\approx 3.14 \times 10^{-6}$.

In organic acid additive experiment series, batch treatment chamber was used. Experiments were carried out with electric field strength $E = 7.5$ kV/cm, number of pulses by train $n = 50$, pulse duration $t_i = 0.3 \times 10^{-3}$ s, pulse repetition time $\Delta t = 10 \times 10^{-2}$ s, number of trains $N = 50$, and inter-train time $\Delta t_t = 40$ s. The pulsed electric field effective treatment time $t_{PEF} = n\, t_i\, N = 0.75$ s.

3. RESULTS AND DISCUSSION

The electrical conductivity measurements of *E. coli* suspensions in the course of treatment time reveal the conductivity disintegration index Z kinetics shown in Fig. 1. Theses results were also confirmed by the direct counts on Petri dishes. The conductivity disintegration index Z grows with the PEF treatment time (t_{PEF}), the thermal treatment time (t_T) and the temperature T increase. To achieve a high *E. coli* disintegration degree at only thermal treatment, a long treatment time is required, while, by combining PEF with thermal treatment noticeable increase of the conductivity disintegration index Z was observed. For example for $t = 3600$ s, the disintegration index of combined PEF thermal treatment was approximately 1, 4 and 1.3 times greater than the disintegration index obtained with sole thermal treatment at T = 30°C, 40°C and 50°C respectively. Furthermore, disintegration index Z reached approximately 1 after t_T = 4000 s with combined PEF-thermal treatment. Therefore, synergism between the simultaneously applied electric and thermal treatments is evidently present.

The absorbance spectra of the supernatant solutions for untreated and PEF-thermally treated 1.0 % (wt) aqueous suspensions of *E. coli* were shown in Fig. 2. The effective PEF treatment time was $t_{PEF} \approx 0.116$ s, which corresponds to the thermal treatment time $t_T \approx$ 4000 s (Eq.(2)). The supernatant solutions were analysed after centrifugation of the bacterial suspensions. The absorbance analysis evidently reveals release of the intracellular components because absorbance values of PEF thermally treated suspensions are greater than absorbance values of untreated *E. coli* suspension. Increase of the treatment temperature T leads to the peak intensity increase at the wavelength $\lambda_{max} \approx 260$ nm ($A \approx 0.4$, 0.75 and 1.06 for T = 30, 40 and 50ºC respectively). Peak at $\lambda_{max} \approx 260$ nm corresponds to the released nucleic acids [9], and the absorbance analysis evidence the leakage of nucleic acids owing to the *E. coli* cells damage induced by PEF-thermal treatments.

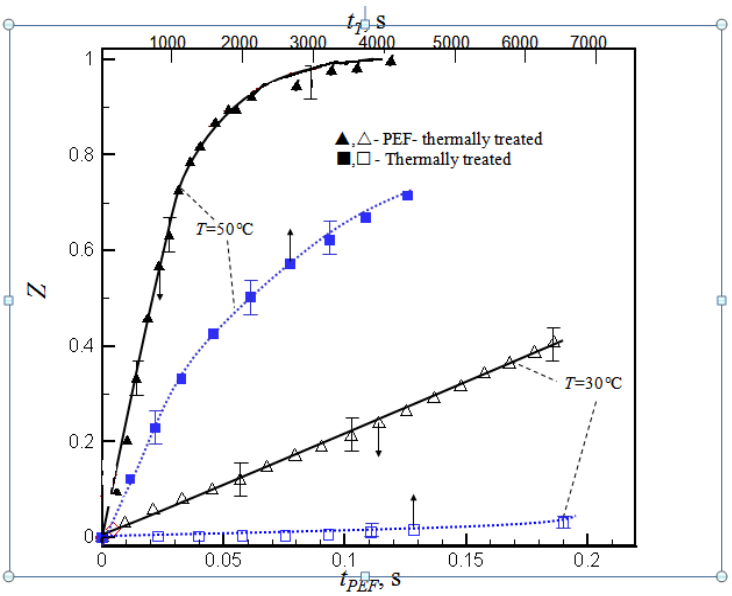

Fig. 1: The electrical conductivity disintegration index Z versus effective PEF treatment time (t_{PEF}) and thermal treatment time (t_T) at different temperatures T. Error bars are the standard deviations.

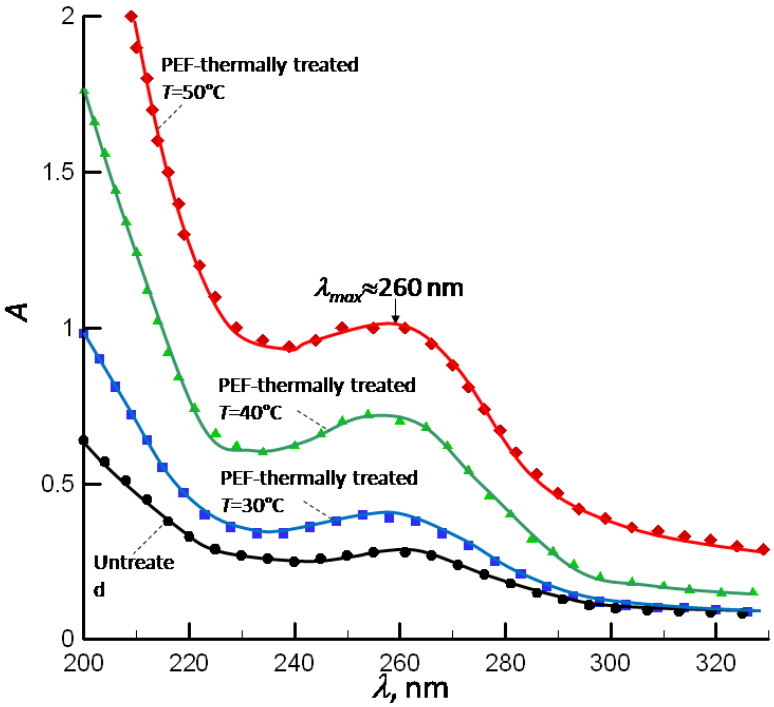

Fig. 2: Absorbance spectra of the supernatant solutions for the PEF-thermally treated 1.0 % (w/w) aqueous suspensions of *E. coli* at different temperatures

The effects of addition of the organic acids, such as citric, malic and lactic acids, on the PEF treatment efficiency was studied. The advantage of theses acids is that they can be used as food additives, or even occur naturally in some fruit juices. The lactic acid seems to be the most efficient with 8 log cycles of destruction (Fig. 3). Lactic acid has greater effect than other organic acids because lactic acid, in addition to its antimicrobial property due to the lowering of the pH, also functions as a permeabilizer of the gram-negative bacterial outer membrane [10] and may act as a potentiator of the effects of pulsed electric fields.

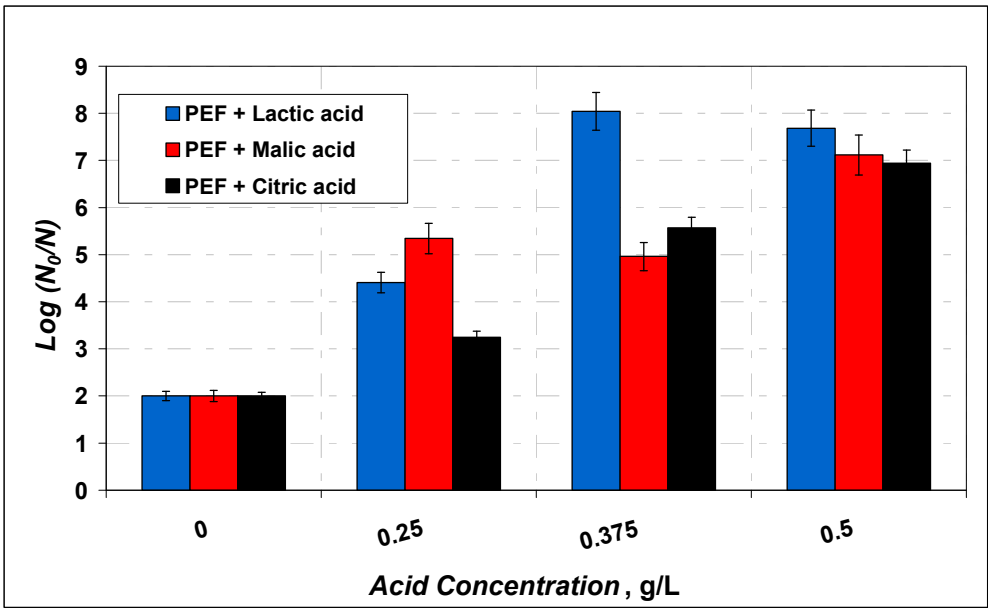

Fig. 3: Histogram of the effects of combining organic acids with PEF treatments. Error bars are the standard deviations.

We can suppose certain stagnation of the effect of lactic acid after 0.375 g/L concentration as well as continuous increasing of the efficiency with the increase of citric acid concentration.

4. CONCLUSIONS

We have demonstrated that measurement of electrical conductivity during PEF treatment of *E. coli* suspensions correlated with the Petri dishes plating and could be used for monitoring of the extent of cell damage. The synergy between the PEF and thermal treatments in *E. coli* inactivation was clearly shown. The release of nucleic acids increased with temperature increase in the PEF-thermal treatments. The damage efficiency was noticeably improved by addition of organic acids in small concentrations ($C_{organic\ acid} \leq 0.5$ g/L) and 8 log cycles reduction was reached by using 0.375 g/L of lactic acid.

5. ACKNOWLEDGEMENT

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7. NOMENCLATURE

E	Pulsed electric field strength	kV/cm
h	Distance between electrodes	cm
n	Number of pulses by train	-
N	Number of trains	-
N_g	Number of treatment chambers	-
r	Radius of a cylindrical electrode	cm
S	Surviving ratio	-
t_i	Pulse duration	s
t_T	Thermal treatment time	s
t_{PEF}	Effective PEF treatment time	s
T	Suspension temperature	°C

W_g	Treatment volume	cm^3
W	Total volume of suspension	cm^3
Z	Conductivity disintegration	-

Greek letters

σ_o	Initial electrical conductivity of the suspension	S/cm
σ_{max}	Suspension saturation electrical conductivity	S/cm
Δt	Pulse repetition time	s
Δt_t	Inter-train time pause	s