# INACTIVATION OF *Yersinia enterocolitica* GRAM-NEGATIVE BACTERIA USING HIGH VOLTAGE PULSE TECHNIQUE

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*Abstract* - **High voltage pulses of peak voltages U=5-75 kV and rise times $t_p$=500-1300 ns were applied with repetition frequency f=1Hz in order to cause the irreversible electroporation of Gram negative bacteria *Yersinia enterocolitica*. The bacteria were suspended in NaCl solution of pH=7.2 and conductivity $\gamma$ =0.8-1.3 S/m. The suspension was placed in glass tube immersed in the cylindrical electrode system gap filled with distilled water. Such an electrode system will protect the bacteria suspension from the chemical processes at the electrode-liquid interface due to conduction and prebreakdown phenomena. The current chopping electrode system was connected in parallel to the sample in order to avoid heat generation from direct discharge of the pulse through the suspension. The dependence of the survival ratio s=N/$N_0$ (the number of bacteria per $cm^3$ after pulse treatment, N, divided by the number of bacteria per $cm^3$ before treatment, $N_0$) of *Y. enterocolitica* on peak voltage of the pulse, number of pulses applied and on various rise times of pulses have been measured. The reduction by 6 orders of magnitude of *Y. enterocolitica* living cells per $cm^3$ was achieved. The results show that considerable inactivation of microbes can be achieved by the application of short ($t_p$<1000 ns) high voltage pulses for bacteria suspension without directly exposing the bacteria suspension to the electrodes. It is therefore possible to use the electrode system proposed as a means for sterilization of liquid foods.**

## 1. INTRODUCTION

### *A. Phenomenon of electroporation*

The disruption of cell membrane has been recognized as the cause of the lethal effect of pulsed high electric field on bacteria [1-3]. This phenomenon, known recently as electroporation, occurs in living cells (in bacteria, yeasts, tissue cells, protoplasts) when the transmembrane potential (TMP - potential difference between inner and outer surfaces of cell membrane) is within a range of 0.5-1.2 V dependent on kind and size of a cell [4,5]. It has been noted that three possible events take place in the cell membrane when the duration of voltage pulse is t<1 μs [6]: 1. increase in membrane conductivity - membrane conductance increases while the pulse is acting and slowly (few microseconds) returns to its original value; 2. reversible electric breakdown - membrane conductance increases by up to eight orders of magnitude and causes a fast discharge of the membrane in time t<<1 μs; 3. irreversible electrical breakdown (rupture) - membrane conductance increases rapidly after a time of a few microseconds following the voltage pulse and it never recovers its original value (membrane is irretrievably disrupted). The fate of the membrane is determined by its

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properties depending on the kind and size of cell, and by duration and magnitude of voltage pulse [4].

In order to cause electroporation of a cell membrane, the voltage magnitude must be high enough to induce suitable value of TMP for breakdown of the membrane, and at the same time, duration of the voltage pulse must be at least higher than the relaxation time of a bacteria suspension. Using the Maxwell-Wagner mixture polarization equations for spherical particles, one can derive the formula for relaxation time $\tau$ [7]:

$$\tau = \frac{2\varepsilon_3+\varepsilon_1+\varepsilon_2\frac{2d}{R}}{4\pi\left(2\gamma_3+\gamma_1+\gamma_2\frac{2d}{R}\right)}, \quad (1)$$

where: $\varepsilon_1$, $\varepsilon_2$, $\varepsilon_3$ - complex permittivities of the medium, shell and core of the particle, respectively; R - radius of the core which is approximately equal to radius of the core with shell; d - thickness of the shell. The time constant can be calculated by substituting the typical values for bacteria and NaCl solution: $\varepsilon_1$ = 80, $\varepsilon_2$ = 2-10 [5,7,8], $\varepsilon_3$ = 45-60 [5,7,8,9], $\gamma_1 = 10^{-3}$-$10^{-2}$ S/cm, $\gamma_2 = 10^{-3}$ S/cm$^2$ [5,8,9],$\gamma_3 = 10^{-2}$ S/cm, d=$10^{-8}$ m, R=$10^{-6}$ m. For those values, time constant $\tau$~2-5 ns. This is the time needed to build up the TMP, which is the minimum time of the voltage pulse duration for electroporation to occur.

For the rise times of the pulses much greater than $\tau$, i.e. cell membrane is completely charged, one can use the potential theory for describing the potential difference between surfaces of the membrane in the direction of electric field intensity vector E (on the assumptions that the pulse shape is rectangular, cell membrane is non-conducting and cell is spherical) [5,8]:

$$V_m = -\left(0.5ER + \Delta\phi_m / \cos\Theta\right)\left|\cos\Theta\right|, \quad (2)$$

where: E - electric field intensity, $\Theta$ - the angle between directions of E and R, $\Delta\phi_m$ - the TMP which exists in normally functioning cells, $\Delta\phi_m$ =50-120 mV [4]. Formula (2) has been experimentally verified for a low number of pulses applied. In this case the TMP depends on the size of the cell.

*B. High voltage pulse sterilization*

Recently, reversible electroporation has been used in biotechnology and genetic engineering for introducing new genetic materials into living cells and for cell fusion [8,10]. The goals in this field are a stable and long-lasting state of increased permeability of the cell membrane as well as avoiding an occurrence of irreversible electroporation, both of which can be reached by suitable manipulating with repetition frequency (0.1-few Hz for pulses and 2500 Hz for use of rotating electric field), magnitude (up to 20 kV/cm [4]) and width (from few to several hundreds microseconds [4]) of applied pulses [11,12].

At the same time the phenomenon of irreversible electroporation has been investigated in order to put it to use for sterilization of consumable liquids [13-21]. The non-thermal pulsed high electric field method for food preservation is proved to be efficient, energy saving and environment friendly. Electric field strength and duration of the pulses used in this method vary within a range of 10-35 kV/cm (and 100 kV/cm in experiment described in [16]), and from 500 ns to even 15 ms [13-21]. The results of investigations concerning the technique of irreversible electroporation indicate that it is possible to reduce the number of living cells suspended in various electrolytes such as NaCl solution, phosphate buffer and in consumable liquids e.g. milk, yogurt and orange juice within the range of 1-9 orders of magnitude, and the level of reduction strongly depends on the peak value and shape of the voltage pulse and on the number of pulses applied [13-21]. It has been reported that the temperature and the conductivity of suspending media during high voltage pulse treatment affect the survival rate of the microorganisms [13,14].

Several types of microorganisms have been tested by using pulse electric field sterilization method and it has been concluded that the level of inactivation depends on the kind of microorganisms and on the stage of their development. Gram-negative bacteria can be killed easily, but electroporation of Gram-positive ones is difficult because of the barrier posed by the Gram-positive cell wall [22]. This barrier has been overcome in genetic engineering by enzymatic treatment [22]. A higher electric field must be used for electroporation of yeast cells than that for inactivation of bacteria [23].

### C. Drawbacks of high voltage pulse treatment

Using the pulsed electric field for food sterilization, special attention should be paid for preserving the safety of the medium being treated. The phenomena which can take place in consumable liquids exposed for high voltage pulses application are: 1. electrolysis; 2. overheating of the medium; 3. conduction phenomena occurring in the bulk of liquid treated; 4. prebreakdown phenomena occurring on the electrode-liquid interface. The occurrence of first two phenomena of above mentioned can be efficiently minimized by suitable adjustment of the pulse width and its repetition frequency. However, the conduction and prebreakdown phenomena are especially important when voltages with relatively high magnitude are used.

In the pulsed high electric field sterilization method, pulses are discharged by the volume of liquid being treated. In that case, typical conduction processes take place in the sterilized food products. Six different mechanisms are suspected to contribute to charge carrier generation, and in the conductivity of polar and nonpolar liquids [24,25]: 1. generation of charge carriers in the electrochemical processes at the electrode surface, 2. enhanced electronic emission from the electrodes arisen from small value of work function (comparing to the interface metal-vacuum, the work function for the interface metal-liquid is inversely proportional to the permittivity of a liquid) [24]; 3. field-enhanced dissociation of liquids, 4. field-enhanced ionization of liquids, 5. collision ionization and avalanche formation in high electric field ~$10^9$ V/m [24]; 6. field-enhanced ionic mobility - EHD motion [24]. The first two mechanisms are interfacial in nature, surface mechanisms, while the last four occur in the bulk of the medium. In order to limit the effect of the above mentioned mechanisms on the quality of food sterilized by the pulsed electric field method, it is reasonable to apply very short pulses and/or to connect a system for chopping the pulses to avoid the full discharge through the product being treated.

It is known that the value of the local field in some micro sites in the vicinity of the broad area electrode surface exceeds the value of the applied field by 100-500 times [24]. Therefore, for high electric fields used for sterilization of the consumable liquids, the prebreakdown and breakdown phenomena must be taken into consideration as those ones which can affect the quality and structure of products. It is obvious in the case of pulsed high voltage sterilization that the initiation step of the breakdown may occur during the process. The existing models of the breakdown mechanisms in liquids assume that the interfacial processes at the electrode-liquid interface are responsible for the initiation of the breakdown [24]. High prebreakdown currents can be generated by field-enhanced thermionic emission (Schottky), by Fowler-Nordheim tunneling, by creation of impurity particles during spark erosion of the electrodes, or by the occurrence of both the Schottky and Fowler-Nordheim emission because of sufficient decrease of the energetic barrier caused by the electrochemical processes taking place in the vicinity of electrode surface [24,26]. Those currents have an energy high enough to either vaporize a liquid or cause the electrochemical reactions (reduction and oxidation) in the vicinity of the electrode surface. As a result of the prebreakdown processes, the formation of chemical compounds (such as free radicals $H^{\cdot}$ and $OH^{\cdot}$, $H_2O_2$) and the introduction of electrode material into the liquid placed between the electrodes can take place [24,26,27].

Optimal electrical properties of the medium and suitable parameters of high voltage pulses allow one to minimize the risk of an initiation of prebreakdown processes. However, it is apparent that the most efficient way to avoid the influence of the prebreakdown phenomena on the product treated by high voltage pulses is the separation of the electrode surface and volume of the liquid to be sterilized. The electrode and the chopping system proposed provide a pulsing technique with significant reduction of microbes yet minimizing the influence of prebreakdown and conduction phenomena on consumable liquid exposed to high voltage pulses.

## 2. EXPERIMENTAL METHODS

### A. Preparation of samples and estimation of the survival

The investigations have been carried out for one type of Gram-negative bacteria *Yersinia enterocolitica*. This kind of bacteria can develop and multiply even at a temperature of 4 $^{o}$C at which food is stored. The standard bacterial strains of *Yersinia enterocolitica* (4-MYO serological type 3) were obtained from the collection of the Chair of Veterinary Medicine of Wroclaw Agricultural Academy. Bacteria were grown on a Tryptic Soy Agar (Difco) base at a temperature of 22 $^{o}$C for 48 hrs. The microbe culture was

dispersed in NaCl solution which had a pH within the range of 7.2-7.3 and a conductivity of 0.8-1.3 S/m. The influence of high voltage pulses on the microorganisms has been measured as a survival ratio ($s=N/N_0$, where N and $N_0$ are a number of active microbes per $cm^3$ before and after the voltage treatment, respectively) by estimating the number of active cells with the use of the serial dilution method. The suspended bacteria were sown in Petrie dishes with a tryptic-soy agar substrate. The dishes with the inoculation were held in an incubator at a temperature of 37 $^oC$ for 24 hrs. Then the colonies grown were counted and their number was multiplied by the dilution of the suspension in order to obtain the number of active cells per volume unit.

### *B. Preparation of the bacteria for transmission electron microscopy (TEM)*

The bacteria were prepared by means of ultra fine scraps method. The initial amount of bacteria was much higher than that used for previously described investigations $N_0 = 10^{16}/cm^3$. After voltage treatment (U=30 kV, n=10 pulses, $t_p$=800 ns), this amount was reduced to $N = 10^{12}/cm^3$. That high number of bacteria was necessary for centrifugal separation of the cells in the first step of preparation for TEM investigations. After this separation, bacteria were treated by 1% of $OsO_4$ soluted in $(NaCH_3)_2Sao_2x3H_2O$ buffer. The samples were lyophilized in ethanol and propylene oxide. After that treatment, the bacteria samples were placed in resin EPON 812. The ultra fine pieces were achieved by using Reichert ultramicrotome. The photographs were made by means of Philips transmission electron microscope with accelerating voltage being equal to 80 kV and 100 kV.

### *C. Electrical experimental setup*

The survival ratio of *Y. enterocolitica* was measured as a function of peak voltages, rise times and the number of pulses applied. High voltage pulses were generated by means of the one-stage Marx generator. The generated impulse wave (2/20 μs) was chopped by the discharge on the surface of the ceramic insulating disc in a point-plate electrodes system. The chopping electrode system was connected in parallel to the bacteria suspension in order to limit the influence of the conduction effects on the medium by avoiding the full discharge of the pulse through the sample. A breakdown on the surface of ceramic spacer was used for that purpose, because of the high velocity of development of the surface flashover which is several times higher than that for the similar effect appearing in gases and liquids [26]. The water-capacitor was placed at the output of the generator. It was used for shaping the voltage wave as well as for inducing a fast energy transfer to the sample. The detailed description of the electrical setup used can be found in [15]. The pulses were applied with the repetition frequency f=1 Hz. The runs of the pulses were measured with the use of an copper sulfate impulse wave voltage divider connected with KIKUSUI Digital Oscilloscope COM7101E. Fig. 1 shows the voltage waveform generated by setup described above.

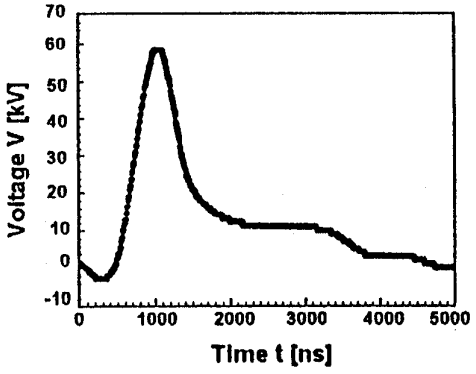



Fig. 1. Voltage waveform used for bacteria treatment.

The measurements were carried out under stationary conditions, i.e. without any flow of the suspension. Fig. 2 shows the cylindrical electrode system used in the measurements of the survival ratio of the microbes tested. The rise time of pulses was adjusted both in the Marx bank and in chopping electrode system. The range of the rise time $t_p$ value obtained was from 0.5 to 1.3 μs. The samples (volume v=20 $cm^3$) were placed inside the glass tube immersed in distilled water filling the electrode gap. Such a configuration allows the separation of electrodes and liquid treated. Before this electrode system was used, the measurements of the survival ratio of *Y. enterocolitica* depending on the kind of liquid filling the electrode gap had been made. The results have shown that a significant decrease in survival ratio could be achieved for the liquids with dielectric constant similar to that of the bacteria

suspension. There was no destruction effect when the electrode gap was filled with the transformer or silicon oil. Distilled water with a conductivity of $10^{-3}$ S/m was used. High voltage pulses were applied to the internal electrode. The electric field intensity in the vicinity of the external cylindrical electrode (minimum field area) for $\varepsilon$=80 and U=60 kV was E=46 kV/cm.

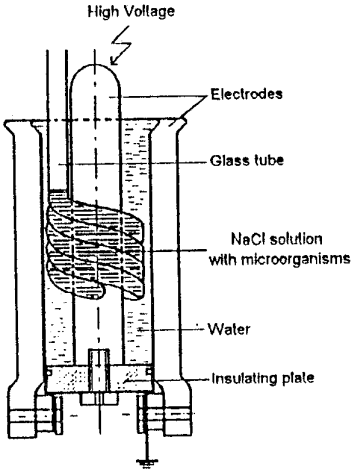



Fig. 2. The way of placing the bacteria solution - cylindrical electrode system.

## 3. RESULTS

### *A. Survival ratio of Yersinia enterocolitica*

The results of the survival ratio $s=N/N_0$ of Gram-negative *Y. enterocolitica* bacteria depending on the number of pulses applied for three values of peak voltage are shown in Fig. 3. The initial amount of microorganisms was $N_0 = 2x10^8$ cells/cm$^3$. The number of the living cells per cm$^3$ decreases by ~6 orders of magnitude for U=75 kV and n=250 pulses. The survival ratio of this spherical-shape bacterium is strongly affected by the number of pulses applied within the range of n=1-50 pulses. It is apparent that the survival ratio of the bacteria treated depends on the value of peak voltage applied. The increase in the voltage magnitude from 45 kV to 75 kV brings about the decrease of the survival ratio by ~3 orders of magnitude.

The characteristics of the survival ratio depending on the rise time of the high voltage pulses applied (Fig. 4) have shown that there is no remarkable effect of the increasing rise time within the range of $t_n$=500-1300 ns on the lethality of the bacteria tested. The results show that the survival ratio is considerably affected by the value of voltage. The difference in survival ratio between U=5 kV and U=45 kV is 4 orders of magnitude. If the rise time increases from 500 to 1300 ns, the survival ratio decreases only by 2 orders of magnitude in the case of U=45 kV applied, and by 1 order of magnitude in the case of U=5 kV applied.

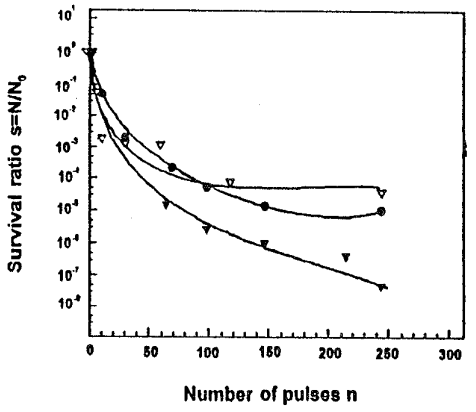



Fig. 3. The dependence of the survival ratio of the *Y. enterocolitica* bacteria on the number of high voltage pulses applied for the three values of the peak voltage:● - 45 kV, ∇ - 60 kV, ▼ - 75 kV.

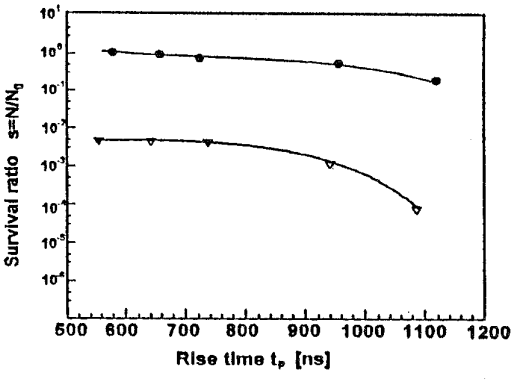


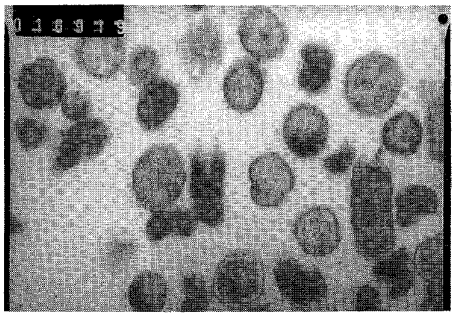

Fig. 4. The dependence of the survival ratio of the *Y. enterocolitica* on the rise time of the pulse for two values of the peak voltage ● - 5 kV, ∇ - 45 kV.

### *B. The results of the TEM investigations*

The TEM photographs are shown in Fig. 5. The photographs have been made for bacteria before and after treatment. TEM images were taken for magnitude of the pictures being equal to 29000x. The photographs show the destructive effect of high voltage pulses applied. The shapes of bacteria after treatment are not

the same as the regular ones shown in the photographs made before high voltage application. It is apparent that the changes in shape have been caused by the disruption of the cell membranes. The difference in the number of living cells before and after high voltage treatment was in this case equal to 4 log cycles.

a.)



b.)

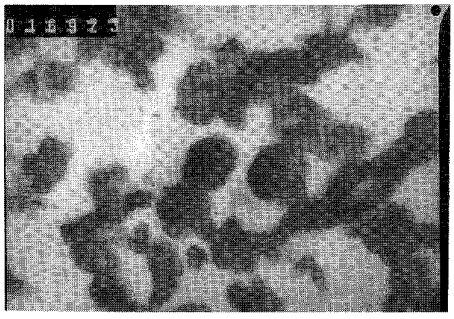


Fig 5. The transmission electron microscopy photographs of the *Y. enterocolitica* bacteria made: a.) before and b.) after high voltage pulse treatment (28000x).

## 4. CONCLUSIONS

The results obtained confirm the decrease in the survival ratio of microorganisms being treated by high voltage pulses with an increase of the peak voltage and the total time of voltage treatment. The pulses applied in the investigations have caused a relatively high decrease by 6 log cycles in the survival ratio of *Y. enterocolitica*. The survival ratio of this bacteria is strongly dependent on the magnitude of voltage and on the number of pulses applied. The dependence of the survival ratio on the rise time of the pulse within a range of $t_p$=500-1300 ns was insignificant. Electron transmission microscopy results have shown that the lethal effect was directly caused by the rupture of the cell membrane.

The energy calculated on the basis of capacitance of the electrode system (45 nF) has shown that the energy input being equal to 122 J/cm$^3$ (energy input in thermal sterilization method is equal ca. 270 J/cm$^3$) causes the decrease in the survival ratio of *Y. enterocolitica* bacteria by 6 orders of magnitude. If we take into consideration that the number of microorganisms in unpasteurized consumable liquids is much smaller than that in the suspensions tested, and if the level of a decrease in the survival ratio were the same, then the total inactivation of this kind of microbe presented in food could be expected.

The proposed high voltage pulse treatment could cause a significant reduction of microorganisms despite no direct contact between the electrodes and the liquid treated as the pulse is not fully discharged through the sample. The use of the proposed electrode system could therefore minimize the influence of the conduction and prebreakdown effects on the quality of liquid being treated.

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