Effect of Short HV Pulses on Bacteria and Fungi

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ABSTRACT

The survival of three kinds of microorganisms under strong-pulse electric field conditions was investigated with a possible application of the electric pulse method for sterilization of consumable liquids. The results of the investigations of survival ratio of Gram-negative (Escherichia coli, Yersinia enterocolitica) and Gram-positive (Staphylococcus aureus, Listeria monocytogenes) bacteria and yeastlike fungi (Candida albicans) are presented. The HV pulses with peak voltage $U = 0$ to 100 kV and rise time $t_n = 0.5$ to 1.2 μs were applied. The microorganisms were suspended in an NaCl solution with $\gamma = 6$ to 13 mS/cm conductivity and pH = 7.2. The experimental setup and the dependency of the microorganism survival ratio on the rise time, peak voltage and on the number of pulses applied, are presented. It has been found that the lethal effect on microorganisms caused by HV pulses depends on the pulse parameters as well as on the kind of microorganism being treated.

1. INTRODUCTION

DURING the action of a strong electric field pulse on biological cells the occurrence of phenomena which lead to electroporation of the cell membrane is observed [1, 2]. Depending on the peak electric field intensity and its rise time, reversible electroporation (after the HV pulse the membrane recovers its shape and properties) or irreversible electroporation (the membrane is permanently disrupted) can be induced [1]. The technique of reversible electroporation is being used in genetic engineering [1, 2], and recently the phenomenon of irreversible electroporation has been investigated in order to use it for sterilization of consumable liquids [3–10].

The results of investigations concerning the technique of irreversible electroporation indicate that it is possible to reduce the number of living cells per 1 cm^3 of suspension by 1 to 9 orders of magnitude [3–6]. This level of reduction depends on the temperature of samples and on the conductivity of the suspending media [3, 4, 8]. At the same time, the energy input for inactivating microorganisms by the application of the HV pulse method is several times lower than for the thermal method of sterilization, which is widely used in the food industry [5, 8]. Because of energy efficiency and of the fact that the thermal method affects the nutritive and taste value of products [4], the interest in sterilization with the HV method has increased.

The mechanism of electroporation is described in several publications [1, 2]. However, it has not been examined sufficiently, and the models presented (*e.g.* for lipid bilayers [2] or the electromechanical model [1]) have not fully explained the physical relations in this phenomenon.

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It is most frequently assumed that the lethality of biological cells results from the mechanical disruption of the cell membrane, which is caused by the pressure arising from the interaction between the surface charges accumulated on both sides of the membrane while the HV pulse is acting [1, 2]. The potential difference over the cell membrane wall (an insulator which displays low conductivity, relative permeability within the range of $\varepsilon = 2$ to 10 and which has a thickness of $\sim$ 10 nm [1]), which is enough for electroporation to take place, is within the range of 0.8 to 3 V [1, 2, 10]. This difference in the normal cell is $\sim$ 80 to 120 mV [11]. Generally, for a separated spherical cell, under the assumption that the rise time of the voltage pulse is high enough for completely charging the cell membrane, the spherical capacitor model is considered. Its electrodes are the internal and external media of the cell and the cell membrane is between them [1, 2, 12, 13]. Such an estimation makes it possible to determine the charging time constant of the cell membrane, which is also the shortest rise time of the HV pulse for inducing electroporation. The time constant τ (s) can be calculated by

$$\tau = \left[\frac{1}{2}\rho_e + \rho_i\right] C_m a \qquad (1)$$

where $\rho_i = 10$ to 10^2 Ωcm is the resistivity of the internal fluid of the cell, $\rho_e = 10^2$ to 10^4 Ωcm the resistivity of the external medium, $C_m = 1$ μF/cm^2 the capacity of the cell membrane for the surface unit and $a = 10^{-4}$ is the radius of the cell [1, 2, 7]. Using the values in brackets to calculate the time constant, one obtains 500 ns, *i.e.* the rise time of the pulse has to be higher than that value.

In addition, there are more limitations than the one mentioned above. The HV pulses used for sterilization of consumable liquids should not affect them, but at the same time the effectiveness of the inactivation of microorganisms must be high. The upper level of the rise time of the pulse is estimated by taking into account the heat generated in the liquid being treated (for $E = 100$ kV/cm its duration should be < 10 μs [7]) as well as its electrical strength (prebreakdown and breakdown states should be avoided). Taking into consideration the mechanisms of the electrical breakdown in conducting liquids, where the time to form the breakdown channel depends on the electric field intensity, the conductivity of the liquid and on the polarization of the electrodes, the treatment duration should be 2 to 5 μs [14, 15]. The time for the point-plate electrode system which indicates the real conditions for broad area electrodes, is 2 and 10 μs for positive and negative points, respectively, for an electric field $E = 200$ kV/cm and conductivity $\gamma = 1$ mS/cm [15]. The most suitable value of the pulse rise time in respect of the effectiveness of the lethal effect and of the influence of HV pulses on the medium being treated, should be > 0.5 and < 2.5 μs at a field $E \approx 300$ kV/cm.

2. EXPERIMENTAL METHODS

2.1. PREPARATION OF THE SAMPLES AND ESTIMATING THE SURVIVAL RATIO

The investigations have been carried out on two types of bacteria: Gram-negative Escherichia coli, Yersinia enterocolitica, and Gram-positive Staphylococcus aureus, Listeria monocytogenes, and also for the yeastlike fungus Candida albicans. The above microbes occur widely in food [16]. S. aureus causes food decomposition and produces toxins which lead to food poisoning [16]. Y. enterocolitica and L. monocytogenes can develop and multiply at a temperature of 4°C at which food is stored. E. coli also occurs in the digestive tracts of people and animals, and the amount of this kind of bacteria per 1 cm^3 is the measure of biological purity of food products [16].

The standard bacterial strains of Escherichia coli PCM 2057 and Staphylococcus aureus PCM 520 (209 P) were used in the experiments. Their origin was the collection of microorganisms at the Institute of Immunology and Experimental Therapy of the Polish Academy of Sciences in Wroclaw. Use was also made of Listeria monocytogenes (serological type 4 b), Yersinia enterocolitica (4-MYO serological type 3) and Candida albicans (ATCC 10231) strains from the collection of the Chair of Veterinary Medicine of Wroclaw Agricultural Academy. E. coli and S. aureus were grown on a Tryptic Soy Agar (Difco) base at a temperature of 37°C for 24 h. On the same base L. monocytogenes and Y. enterocolitica were grown at a temperature of 22°C for 48 h. The Candida albicans fungus strains were bred on the Sabourada base and incubated at a temperature of 37°C for 72 h. Those microbe cultures were dispersed in NaCl solution which had a pH within the range of 7.2 to 7.4 and conductivity of 6 to 10 mS/cm. The influence of HV pulses on the microorganisms has been measured as a survival ratio ($s = N/N_o$, where N and N_o are a number of active microbes per 1 cm^3 after and before the voltage treatment, respectively) by estimating the number of active cells with the use of the serial dilution method. The suspended microorganisms were sown in a Petrie plate with a tryptic-soy agar substrate. The plates with the inoculation were held in an incubator at a temperature of 37°C for 24 h. 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 unit volume.

2.2. ELECTRICAL METHODS

The survival ratio of E. coli, Y. enterocolitica, S. aureus, L. monocytogenes and C. albicans was measured as

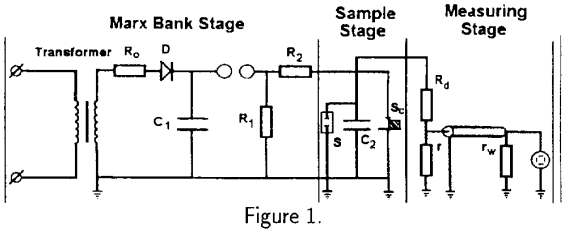

Figure 1.

Diagram of the electrical setup of the modified Marx generator used in investigations. S_c cutting system, C_2 water capacitor, R_d/r voltage divider, S sample, S_c cutting electrode system, R_1, R_2 shaping resistors, C_1 shaping capacitor, r_w = 50 Ω fitting resistor.

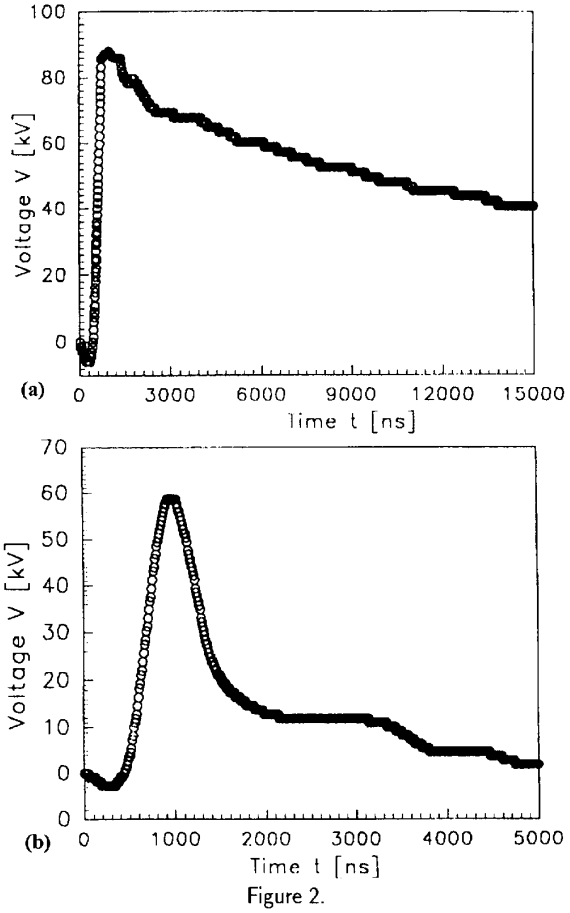

Figure 2.

Impulse waveforms. (a) generated by one stage generator only, (b) waveform obtained with the sample stage connected.

a function of peak voltages, rise times and the number of pulses applied. HV pulses were generated by means of the modified one-stage Marx generator (Figure 1). The impulse wave obtained (1/22 μs) was cut by the discharge on the surface of the ceramic insulating disc in the point-plate electrodes system (S_c) in order to achieve the required rise time of the pulse and to avoid overcurrent through the sample being tested (S). The water capacitor (C_2) at the output of the generator was used for shaping the voltage wave as well as for inducing a fast energy transfer to the sample. The pulses were applied with the frequency f = 1 Hz. The runs of the pulses were measured with the use of an impulse wave voltage divider (R_d/r) with the attenuation ratio 10 kΩ/5.7 Ω connected to a Kikusui digital oscilloscope COM7101 E. Figure 2 shows the voltage waveforms recorded, one without cutting and connecting the sample (Figure 2(a)), and the other one with the complete sample stage connected (Figure 2(b)).

The measurements were carried out by using two kinds of electrode systems without any flow of the suspension in the electrode gap. The effect of various peak voltages and of the number of pulses applied on the survival of microbes being treated was measured with the use of the bowl electrodes shown in Figure 3(a). The distance between the electrodes was h = 5 mm, and the volume of the suspension placed in the gap was v = 30 cm. Figures 3(b) and (c) show the cylindrical electrode system used in the measurements of the survival ratio of the microbes tested, depending on the rise time. The peak voltage was 60 kV and 50 pulses were applied in these investigations. The rise time was adjusted both by the value of the shaping resistor R_2 in the Marx stage and by the thickness of the insulating disc between the cutting electrodes in the sample stage. The range of the rise time 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. HV pulses were applied to the internal electrode. The electric field intensity in the vicinity of the external cylindrical electrode for ε = 80 and U = 60 kV was E = 46 kV/cm and the number of pulses applied was n = 25.

3. RESULTS

3.1. SURVIVAL RATIO OF GRAM-NEGATIVE AND GRAM-POSITIVE BACTERIA

The results of the survival ratio $s = N/N_o$ of Gram-negative (Y. enterocolitica, E. coli) and Gram-positive (S. aureus, L. monocytogenes) bacteria depending on the number of pulses applied are shown in Figure 4. The initial amount of microorganisms N_o was within the range of 10^7 to 10^8 cells/cm^3. The survival ratio of E. coli for U = 45 kV declines 6 orders of magnitude for n = 15 pulses. When only two pulses are applied, a decrease of 2 orders of magnitude is observed. A strong dependence of the survival ratio of the rodshape E. coli

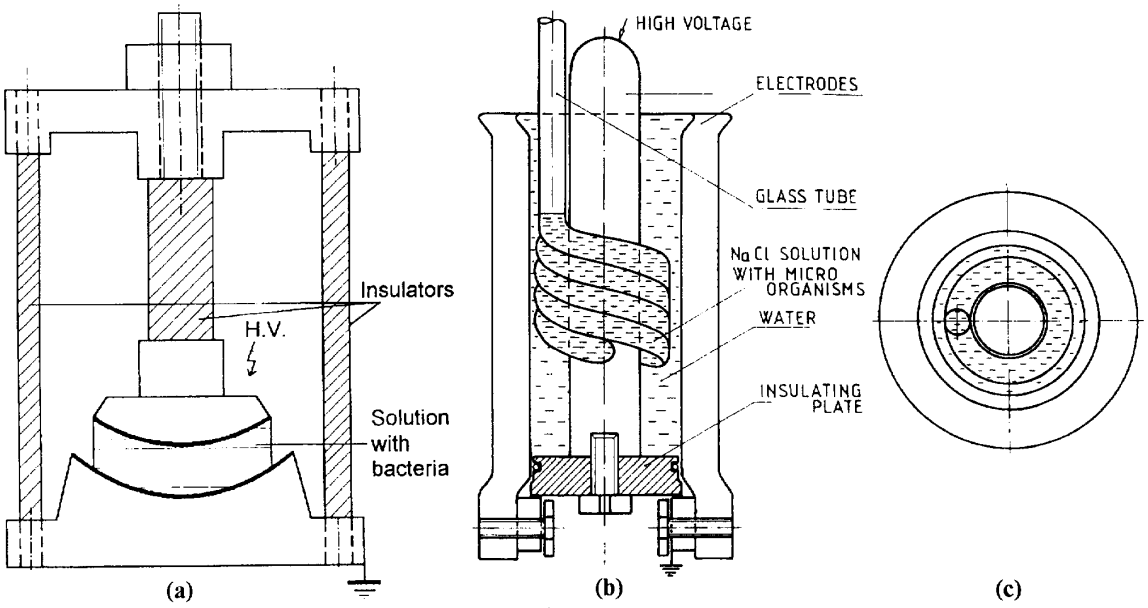

Figure 3.

Electrode systems used in measurements. (a) bowl electrodes, (b) cylindrical electrodes with a glass tube immersed, (c) cross section.

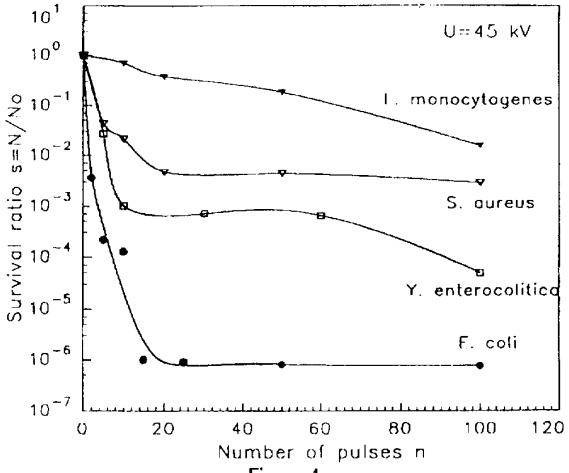

Figure 4.

The survival ratio $s = N/N_o$ of the bacteria tested *vs.* the number of pulses for the rise time $t_n = 0.8$ s.

on the number of pulses applied is observed within the range of $n = 1$ to 15 pulses. The number of the living cells per 1 cm^3 of Y. enterocolitica bacteria decreases by 4 orders of magnitude for $U = 45$ kV and $n = 100$ pulses. The survival ratio of this spherically shaped bacterium is also strongly dependent on the number of pulses applied within the range of $n = 25$ to 100 pulses. Contrary to Gram-negative bacteria, the decrease in the number of microorganisms per 1 cm^3 is not as significant as for Gram-positive ones. The survival ratio of S. aureus decreases by *ca.* 2 orders of magnitude for the peak voltage to $U = 45$ kV and $n = 20$ pulses. However, the dependence of this kind of spherical bacterium on the number of pulses applied is smaller than for Gram-negative ones within the range of $n = 1$ to 20. The effect of pulses on the survival ratio of the rod-shaped L. monocytogenes bacteria is insignificant. The decrease in its survival ratio is barely 2 orders of magnitude for $n = 100$ pulses. It probably results from its very small size compared to the other bacteria tested.

In Figure 5 the survival ratio of the bacteria is shown as function of the peak voltage. The number of pulses applied was $n = 50$, for which value the survival ratio depends insignificantly on the number of pulses. Total inactivation of E. coli has been obtained for $U = 40$ kV. The survival ratio of these bacteria decreases by 6 orders of magnitude even for $U = 10$ kV. For Y. enterocolitica a decrease of 4 orders takes place for $U = 46$ kV. It is evident that the number of living cells per 1 cm^3 of this type of Gram-negative bacterium decreases by 6 orders of magnitude within the range of voltage $U = 10$ to 40 kV. Total inactivation of Gram-positive bacteria has not been found within the range of applied voltages. The survival ratio of S. aureus decreases by 4 orders of magnitude for $U > 60$ kV, and barely by 2 orders of magnitude for $U > 70$ kV in the case of L. monocytogenes bacteria.

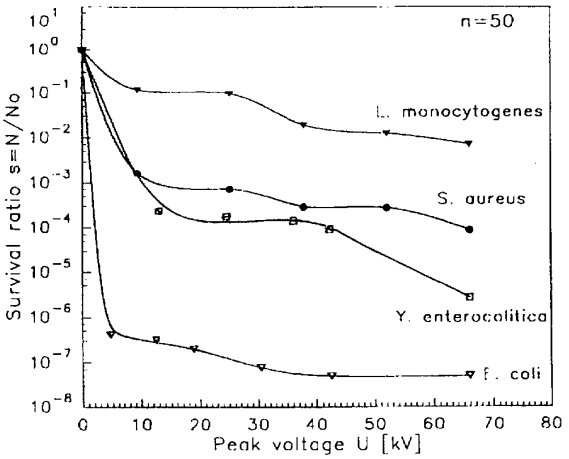

Figure 5.

The survival ratio $s = N/N_o$ of the bacteria tested *vs.* the peak voltage value of pulses for the rise time $t_n = 0.8$ s.

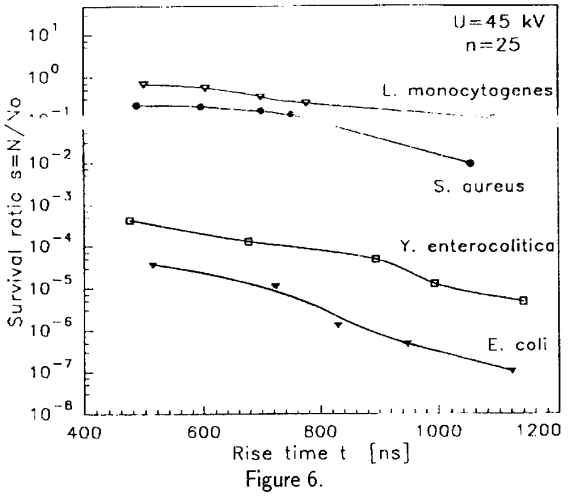

Figure 6.

The survival ratio $s = N/N_o$ of the bacteria tested *vs.* the rise time of pulses.

Thorough investigations have proved that the inactivation of microorganisms carried out by means of the HV pulse method with the use of the electrode system shown in Figure 3(b) is possible, on condition that the interelectrode space is filled with an insulating liquid with a permittivity equal to or larger than the sample treated. That is why distilled water with the conductivity of 10 μS/cm was used for that purpose. The characteristics of the survival ratio as function of the rise time of the pulses (Figure 6) show that there is no significant effect of the rise time within the range of $t_n = 500$ to 1100 ns on the lethality of the bacteria tested. It is similar to the results described above which have proved that the decrease in the survival ratio is much larger for Gram-negative bacteria. In the case of E. coli there is a change of $s = N/N_o$ from *ca.* 10^{-5} to 10^{-7} for $t_n = 500$ and 1100 ns, respectively. If the rise time increases from 500 to 1100 ns, the survival ratio decreases from 10^{-3} to 10^{-5} in the case of Y. enterocolitica, from 10^{-1} to 10^{-2} for S. aureus and from 10^{0} to 10^{-1} for L. monocytogenes.

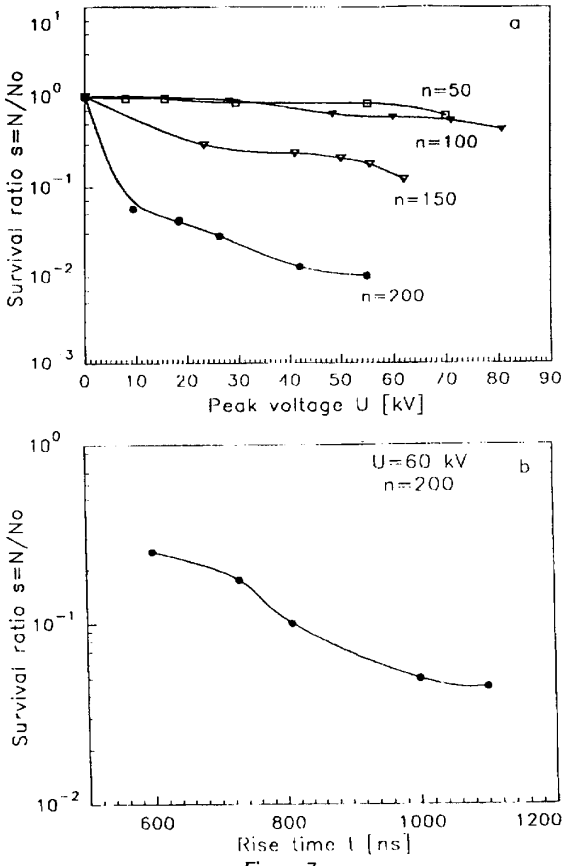

Figure 7.

The survival ratio $s = N/N_o$ of yeastlike fungi C. albicans *vs.* (a) peak voltage value for various number of pulses and rise time $t_n = 0.8$ s, (b) rise time of pulses applied.

3.2. SURVIVAL OF YEASTLIKE FUNGI CANDIDA ALBICANS

The dependency of the survival ratio of C. albicans fungi (N_o was within the range of 10^7 to 10^8 cells/cm^3) on the peak voltage value for various numbers of pulses applied and on the rise time of the pulse (Figure 7) indicate that the decrease in the number of cells per unit volume is similar to the results obtained for L. monocytogenes bacteria. For $n = 200$ pulses and $U = 55$ kV the

survival ratio decreases by 2 orders of magnitude. Based on Figure 7(a) it is evident that the $s = N/N_o$ ratio of this kind of microbe is strongly dependent upon the number of pulses, whereas the influence of the peak voltage on $s = N/N_o$ has less of an effect than for the bacteria tested. There is a decrease in the survival ratio value by 2 orders of magnitude within the range of $n = 15$ to 200. When the rise time of the voltage pulse increases within the range of $t_n = 500$ to 1300 ns, the survival ratio of C. albicans yeastlike fungi decreases by < 1 order of magnitude (Figure 7(b)).

4. DISCUSSION

In general, the results obtained confirm the decrease in the survival ratio of microorganisms being treated by HV pulses with an increase of the peak voltage and the total time of voltage treatment (the number of pulses × duration of the pulse). According to the data included in [3–10], irreversible electroporation is caused by the electric field intensity within the range of 5 to 40 kV/cm, dependent on the size and shape of microorganism for the pulse rise time of 1 to 100 μs. In the investigations described, use was made of the pulses within the range of the rise time $t_n = 0.5$ to 1.3 μs, and a stronger inhomogeneous electric field was applied (*ca.* 200 kV/cm). Similar conditions ($E = 100$ kV/cm and $t_n = 1$ μs) were successfully applied to induce a lethal effect of S. typhirium and P. fragi bacteria [7]. However, the inhomogeneity of the electric field (our investigations were carried out with the use of bowl and cylindrical electrode systems) and a relatively high volume of the suspension tested as well as the short rise time of the pulses applied have brought about small differences between our results and those presented in the earlier papers published [3–10]. The pulses applied in the investigations described above have caused a total inactivation of E. coli bacteria and a relatively high decrease in the survival ratio of Y. enterocolitica and S. aureus bacteria. The differences between the effects of HV pulses on Gram-negative and Gram-positive bacteria are probably due to differences in the morphology of the cell membranes of these two species. The differences of the decrease in survival ratio within the same type of the bacteria being treated indicate the dependence of the lethal effect of HV pulses on the shape and size of the microbes. According to the investigations made by means of electron transmission microscopy (the samples were prepared by using the ultrafine scrap method), the largest bacteria have been the rod-shaped E. coli and spherical S. aureus, and the smallest ones have been the rod-shaped L. monocytogenes strains. The relatively low level of the inactivation of C. albicans has already been noted [10] and explained in connection with the large size of this strain. It is surprising that the level of the inactivation of L. monocytogenes bacteria is not as high as it might be expected in respect of the small size of this strain. It does not correspond with the potential theory [17–19] which states that a lower electric field is needed to bring about the membrane breakdown in cells with smaller sizes. It has not been found on the basis of the investigations that, contrary to the paper [9], the initial amount of microbes per volume unit N_o affects the dependences of the survival ratio $s = N/N_o$ on the various parameters of the pulses being applied for each kind of the microorganisms tested.

Taking into consideration a possible application of the HV pulse method for sterilization of consumable liquids, it is interesting to present the dependence of the survival ratio of microorganisms on the energy input per 1 cm^3 of the suspension treated. This energy (J/cm^3) can be calculated with

$$\frac{U_{max}^2 C_p n}{2V} \tag{2}$$

where $C_p = 45$ nF is the capacity of the sample, U_{max} the peak voltage of the pulse, n the number of pulses applied, and $V = 30$ cm^3 the volume of the sample.

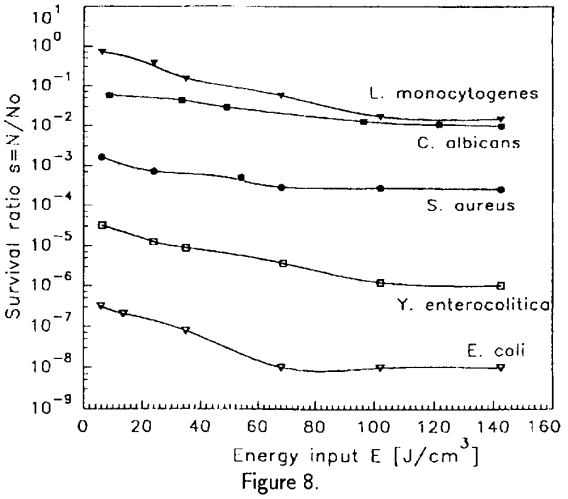

Figure 8.

The survival ratio $s = N/N_o$ of the microbes treated *vs.* the energy input (J/cm^3) of suspension.

Figure 8 illustrates the dependence of the survival ratio of microorganisms tested on the energy input per volume unit of the suspension. It is evident that an energy of 147 J/cm causes total inactivation of E. coli, the decrease in the s ratio of Y. enterocolitica bacteria by 6 orders of magnitude, 4 orders of magnitude in the case of S. aureus and 2 orders of magnitude for L. monocytogenes bacteria as well as in the case of C. albicans fungi. If we take into consideration that the number

of microorganisms in unpasteurized consumable liquids is much smaller than that in the suspensions tested (*e.g.* in unpasteurized beer E. coli < 50 cm^{-3} and yeast Saccharomyces cerevisae < 50 cm^{-3}), and if the level of a decrease in the survival ratio were the same, then the total inactivation of microbes could be expected for energy input to be within the range of 126 to 168 J/cm^3, which is lower than that needed for thermal sterilization methods. The reduction of bacteria in the conductive liquid (physiological fluid obtained on NaCl base) displays the possibility of using the HV impulse method for sterilizing consumption liquids. The above method would require a muchs lower energy than the thermal method used in industry. Its suitability for food processing will be determined by physico-chemical and microbiological investigations and, in some cases, sensory studies of consumption liquids sterilized by means of the above method.

5. CONCLUSIONS

THE destruction of Gram-negative and Gram-positive bacteria and yeastlike fungus cells has been carried out by the application of HV pulses with a rise time of 0.5 to 1.3 μs. The electrode systems of the samples tested and the peak voltages applied make it possible to achieve electric field intensities of $\sim$ 200 kV/cm. The survival ratio of each species being treated declined at least by 2 orders of magnitude for 147 J/cm^3 energy input to the suspension treated. The decrease in the survival ratio of microbes depends upon the kind of microorganism especially upon its size, shape and morphology of the cell membrane. The Gram-negative bacteria are more susceptible to being killed by HV pulses than Grampositive and yeastlike fungi C. albicans. The survival ratio of all the microbes being tested decreases with an increase in the peak voltage and in the time of voltage treatment.

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