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Electric Field Effects on Bacteria and Yeast Cells

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Summary. Comparative studies were carried out describing the lethal effects of electric pulses on GRAM-negative bacteria, GRAM-positive bacteria, and yeast cells. Microorganisms are killed by the pulse treatment without visible morphological destruction. The observed survival rates are figured as functions of the field strength E and the treatment time t (pulse number × time constant) revealing three explicit parameters as sufficient to explain the kinetics of the results. These parameters are determined by the species of microorganism used and moreover depend on the physiological properties of the microbial population. GRAM-positive bacteria and yeasts were found to be less sensitive to electric pulse treatment than GRAM-negative bacteria, when low pulse numbers are applied. Treatment with high pulse numbers reveals survival rates below 1% for all microorganisms examined. Cells from the logarithmic growth phase are killed in markedly higher percentage than cells harvested from the stationary growth phase. The obtained results as well as further studies confirm the hypothesis of an electric induced selective damage of inner cell membranes.

Introduction

Electric fields of high strength (kV) applied as short time pulses (μs) to aqueous suspensions of living cells have remarkable effects on the cell membranes or even kill the organisms. Among the biological systems examined in many studies are blood cells [1–4], algal cells [3, 5], bacteria [6–12], and yeasts [7, 13]. Electric fields can be applied to cell suspensions by use of capacitor discharges as a part of a high voltage circuit [7, 10]. The published results, particular about erythrocytes, widely agree that the observed effects can be explained as a selective response of the cell membrane to the external field leading to an induced trans-membrane potential [2, 3, 8]. At critical potential values of about 1 V the membrane looses intrinsic properties, such as electrical resistance, membrane potential, and barrier function [1, 3]. The molecular transformations

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underlying the 'dielectric breakdown' may be reversible or irreversible, mainly depending on the maximal field strength as well as on the field pulse duration. The mechanism of the induced membrane labilisation was also explained by the formation of aqueous pores leading to drastic conductivity shifts of the cell membrane [1].

For practical application, it is possible to employ electric field treatment for loading of blood cells with extraneous molecules or pharmacological substances [14–16]. Further on, high voltage fields are an useful tool for cell fusion experiments which have been established with protoplasts of plant cells as well as blood cells in order to produce hybrids of different cell types [17, 18].

Studies concerning the electric field treatment of microorganisms, such as bacteria and yeasts, described a lethal action of high voltage pulses with killing rates of more than 99.99% [7, 10, 11, 13]. Field peak values up to 20 kV/cm and several ms duration of the pulsed electric fields were employed in these studies. Yeast cells were described to be more sensitive than bacteria due to larger cell diameters [7]. The lethal effect was found to be mainly governed by the field strength value and the treatment time (pulse number × decay time constant) but also influenced by electrolytic production of toxic substances, once chloride is present in the suspension [10, 11]. In recent studies we demonstrated that the kinetics of the obtained survival rates with *E. coli* can be described in a mathematical relation:

$$\hat{s} = (t/t_c)^{-(E-E_c)/k} \tag{1}$$

where $\hat{s}$ = relative survival rate (0–1), E_c = threshold value of field strength, t_c = threshold value of treatment time, k = additional factor [11]. The definition reveals a logarithmic dependency between $\hat{s}$ and E and a double logarithmic relation between $\hat{s}$ and t, provided that the physiological conditions of the cell population are kept constant.

Presence of bivalent cations as Ca^{2+} and Mg^{2+} in the treated suspensions slightly shifts the survival rates which is assumed to be due to a specific reaction of these ions with the cell membrane [11]. It has previously been shown that nucleotides such as ADP, AMP, NAD, and NADH are released from bacteria and yeast cells after pulse treatment [12]. A release of enzymes could not be detected but is found after subjection of the respective cell populations to ultrasonic stress. Thus, the increase of the membrane permeability is not sufficient to allow the passage of macromolecules, as obtained with electric field treatment of red blood cells [16].

In contrast to other methods employed for microbial cell inactivation, electric pulse treatment does not lead to visible cell damage, which is also proved by electron microscopy [7]. This might distinguish high voltage pulses to represent an useful method when morphological alterations of variable cells are to be avoided.

In this study different GRAM-positive, GRAM-negative bacteria and the yeast *C. albicans* were examined. The inactivation kinetics are reported as functions of the field peak values E and the pulse number, i.e., the treatment time. For each species used, the characteristic model parameters E_c, t_c, and k are calculated and comparatively interpretated.

Material and Methods

The electric pulse treatment technique was performed as described before [10]. The high voltage generator was equipped with a capacitor of 1 μF which was discharged across the treatment chamber of 4 ml volume, via a triggered spark gap. The pulse repetition rate was adjusted to 0.5 Hz and varying pulse numbers in one experiment were used. Peak values of 20 kV/cm field strength are obtained in the suspensions with the time course of the field following a decaying exponential function [10].

The following microorganisms were examined: *Escherichia coli* K12, *Klebsiella pneumoniae* ATCC 27736, *Pseudomonas aeruginosa* lab. nr. PA-103, *Staphylococcus aureus* ATCC 25923, *Listeria monocytogenes* I (Serovar 1/2a) lab. nr. 85 H (smooth, S-form), *Listeria monocytogenes* II (Serovar 1/2a) lab. nr. 46 J (rough, R-form), *Candida albicans* lab. nr. 2468. To be able to compare similar strains of different cell sizes, two different strains of *L. monocytogenes* were employed. Strain I grows regular rod shaped whereas strain II appears in a rough form growing in threads with up to 100 μm length [19]. GRAM-negative bacteria were cultivated in nutrient broth (8 g/ml, DIFCO) and GRAM-positive bacteria and *C. albicans* in tryptone soya broth (30 g/l, OXOID). As a rule, the cell populations were harvested in the stationary growth phase (30 h incubation), except *L. monocytogenes* which was incubated for 16 h because of decreasing viable count for longer incubation time. In the series of experiments, described in the beginning, *E. coli* cultures with only 4 h incubation time were also used.

The conditions of inoculation, incubation and harvesting of cell populations were carefully kept constant in order to obtain identical cell properties [11].

Electric pulse treatment was performed in phosphat buffer solutions (Na_2HPO_4/KH_2PO_4, pH 7.0), the concentration of which was adjusted to get an electrical resistivity of 600 Ωcm at 20° C. 10^8 bacteria/ml and 10^7 yeast cells/ml were employed in the suspensions. Due to an electrode area of 8.3 cm^2 with a distance of 0.5 cm, the pulse time constant (decay) was 36 μs at room temperature. After pulse treatment and suitable dilution, the viable count was determined by evaluating colony forming cells on agar plates, incubated for 24 h. In the experiments, the field strength was varied in steps of 2 kV/cm with pulse numbers of 2–30 for each tested strain. The related treatment time t is given as a product of the time constant (36 μs) and the pulse number used. The relative survival rate s is defined as the proportion of surviving cells with values between 0 and 1. One obtained point is calculated as the mean from at least two experiments.

Results

Experiments with E. coli Bacteria from Different Growth Phases

In former studies we employed *E. coli* cell cultures harvested after 16 h incubation time, i.e., in the beginning of the stationary growth phase [10, 11]. To

examine the influence of a different cell age on the survival rate, *E. coli* cultures incubated for 4 h and for 30 h were employed here. The cell density in these cultures was measured to 2×10^7 and 2×10^9 cells/ml, respectively.

The survival rates obtained after high voltage pulse treatment are drawn in Figs. 1 and 2 with the corresponding statistical parameters listed in Table 1. E_c and t_c are defined as ordinate intersections of the straight survival curves [11]. The parameter k is given by (1) and can be statistically calculated by fitting the obtained results to a correlation line, defined as:

$$\ln s = a_c - b_c \ln \hat{s} \tag{2}$$

where s = measured rate; $\hat{s}$ = calculated rate by means of (1); a_c and b_c = parameters of correlation line indicating the confirmity between the measured rates and the mathematical model. Optimal values are $a_c = 0$ and $b_c = 1$ (see also [11]).

When comparing the results for cells from the logarithmic phase and for those harvested from the stationary phase, a distinct difference of the E_c values is observed whereas t_c remains almost unchanged. Moreover the parameter k is higher for the younger cell population caused by small differences between the survival curve slopes (Fig. 1), which also leads to higher scattering of the confidence limits. The respective parameter values for cultures incubated 16 h are $E_c = 4.9$ kV/cm, $t_c = 12$ µs, and $k = 6.3$, as obtained before [11].

In conclusion, the alterations of physiological properties, a microbial cell population undergoes during continous growth in batch cultures, fairly influence the sensitivity to electric field action. A particular change is obtained for the threshold value E_c which gets below 1 kV/cm when the tested *E. coli* population is harvested during the optimal proliferation rate.

Further examinations, performed under otherwise identical conditions, revealed a continously increasing survival rate in dependence of the growth age. The sensitivity reaches a minimum at about 30 h incubation time. The variation of the killing effect does not correlate to the change of mean cell sizes which already achieve a minimum at about 14 h incubation, i.e., at a cell density of approximately 1×10^9 cells/ml. Thus, the E_c increase can not only be explained by a decrease of the average cell sizes (see below). A similar tendency was found by other authors concerning dielectric breakdown measurements with *E. coli* [2].

Additionally, the survival rates are influenced by the chosen concentration of nutrient in the cultured medium, as shown in Fig. 3. In these experiments the cells were harvested after 16 h incubation and treated with five pulses of 12 kV/cm each. Low medium concentration causes a shift in the number of viable bacteria up to 10 times as much as obtained with cells from nutrient enriched cultures. This effect is partly due to the altering time courses of the growth behaviour. The yield of cells is lower in diluted cultures and thus the stationary growth phase reached earlier. Additional effects are probably to be expected with differing medium composition as well as with varying suspension preparation techniques.

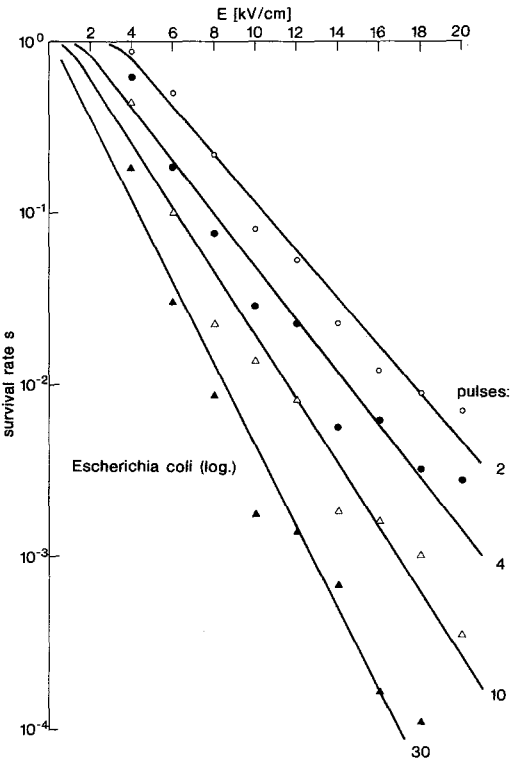

Fig. 1. Survival rates for *E. coli* from the logarithmic growth phase (4 h culture)

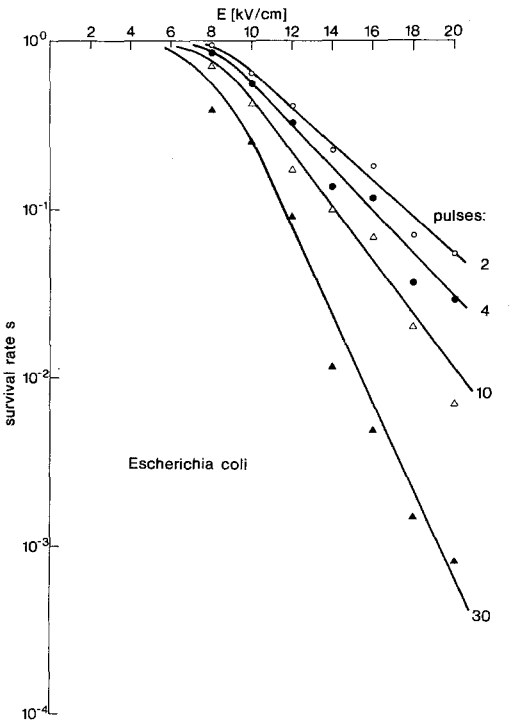

Fig. 2. Survival rates for *E. coli* from the stationary growth phase (30 h culture)

Table 1. Values and confidence limits of calculated model parameters (Figs. 1, 2, and 4–9): E_c = threshold value of electric field strength, t_c = threshold value of treatment time, k = additional model parameter [see text, (1)], a_c and b_c = parameters of correlation line [see text, (2)], r = correlation coefficient of correlation line. Confidence limits are calculated for 5% probability of error

	E (kV/cm)	t (ms)	E_c (kV/cm)	t_c (μs)	k	a_c	b_c	r (%)
E. coli (4 h)	4–20	0.07–1.1	0.7 ± 3.1	11 ± 9.6	8.1 ± 1.8	0.07 ± 0.19	1.00 ± 0.08	97.7
E. coli (30 h)	10–20	0.07–1.1	8.3 ± 0.3	18 ± 5.7	6.3 ± 1.0	− 0.12 ± 0.25	1.03 ± 0.10	97.6
K. pneumoniae	8–20	0.07–1.1	7.2 ± 2.0	29 ± 16	6.6 ± 1.4	0.04 ± 0.35	0.99 ± 0.12	95.7
P. aeruginosa	8–20	0.07–1.1	6.0 ± 0.4	35 ± 6.1	6.3 ± 1.1	0.01 ± 0.20	0.99 ± 0.07	98.4
S. aureus	14–20	0.07–1.1	13 ± 0.9	58 ± 17	2.6 ± 0.7	0.03 ± 0.37	1.01 ± 0.12	97.7
L. monocytogenes I	12–20	0.07–1.1	10 ± 2.6	63 ± 12	6.5 ± 2.5	0.02 ± 0.40	0.96 ± 0.11	97.2
L. monocytogenes II	10–20	0.07–1.1	8.7 ± 1.1	36 ± 19	6.4 ± 1.8	0.00 ± 0.23	1.02 ± 0.08	98.5
C. albicans	10–20	0.14–1.1	8.4 ± 7.5	110 ± 33	2.2 ± 0.9	− 0.03 ± 0.45	1.05 ± 0.14	96.6

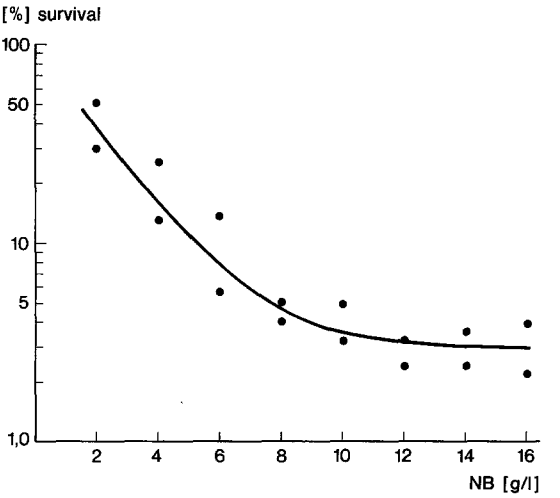

Fig. 3. Survival rates for *E. coli* in dependence on nutritive substance concentration (NB = nutrient broth) in the culture; treatment with five pulses of 12 kV/cm each

Lethal Effect on Different Microorganisms

Since the results obtained had shown that the sensitivity of cells to electric treatment reaches a minimum in the stationary growth phase, the following experiments were performed with microbial populations harvested after 30 h incubation time. *L. monocytogenes* was harvested after 16 h (see above). Besides *E. coli,* the GRAM-negative rod shaped species *K. pneumoniae* and *P. aeruginosa* were chosen. From the group of GRAM-positive bacteria *S. aureus* and *L. monocytogenes* were tested, the latter used in two types, a S-form (strain I) and a R-form (strain II). Representative for yeasts, the species *C. albicans* was examined.

The results obtained with the tested microorganisms can be seen in Figs. 4–9. The related parameters are quoted in Table 1. As shown in the diagrams, the kinetics of the survival rates in dependence on the field strength and the pulse number show the expected qualitative course. Similar graphs would be obtained when drawing the rates as double logarithmic functions of the treatment time.

An interesting deviation of the survival kinetics is obtained for *K. pneumoniae* (Fig. 4). The decline of these curves is bended at a field strength of about 14 kV/cm for all pulse numbers adjusted. This effect also alters the relation between the survival rates and the variable *t*. Accordingly, the application of the model function (1) leads to higher scattering of the parameter values. Nevertheless, for reasons of comparison the model parameters were calculated in the same way as usual. In contrast to other species, *K. pneumoniae* is a capsula forming microorganism. It seems reasonable to assume that the observed specific effect is due to this particular attribute. Cells surrounded by a capsula might dispose of an additional protecting mechanism leading to a lower decline of the survival curves until a certain field strength is exceeded. However, we are not able to explain the observation in terms of a specific cell related process.

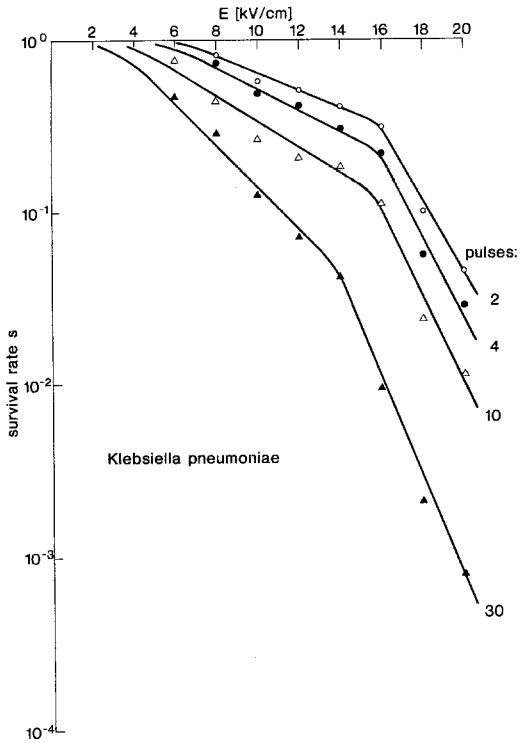

Fig. 4. Survival rates for *K. pneumoniae* from the stationary growth phase (30 h culture)

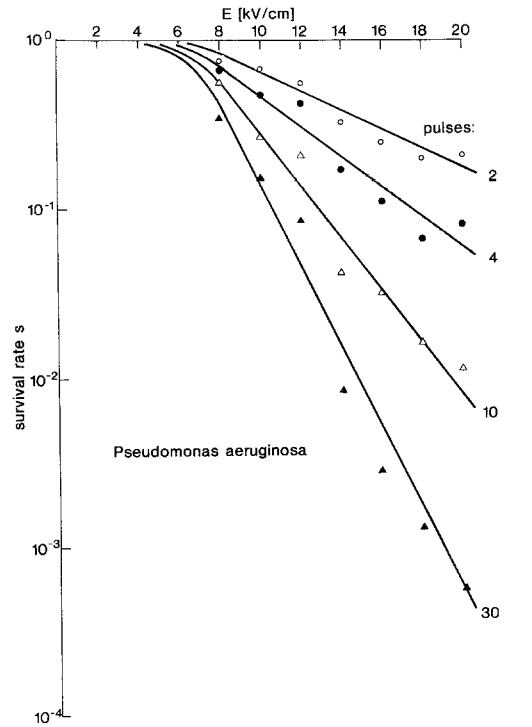

Fig. 5. Survival rates for *P. aeruginosa* from the stationary growth phase (30 h culture)

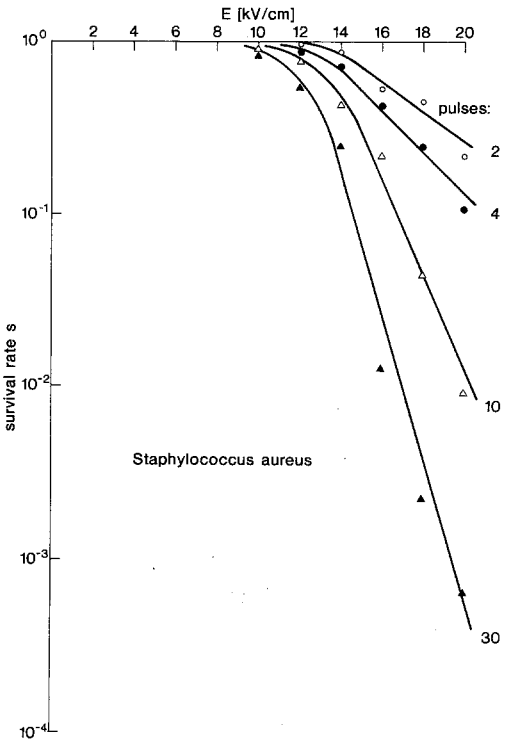

Fig. 6. Survival rates for *S. aureus* from the stationary growth phase (30 h culture)

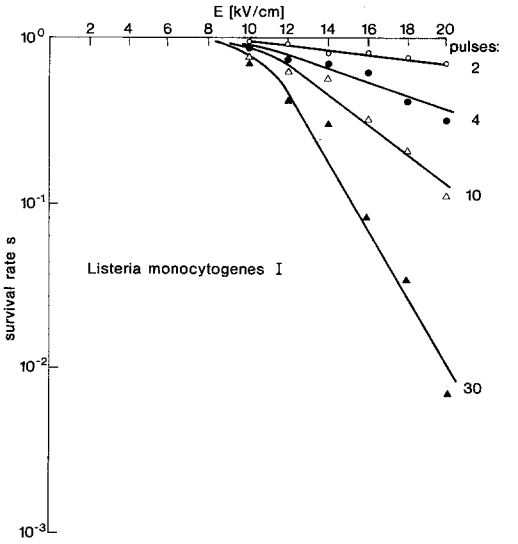

Fig. 7. Survival rates for *L. monocytogenes* I (smooth form) from the stationary growth phase (16 h culture)

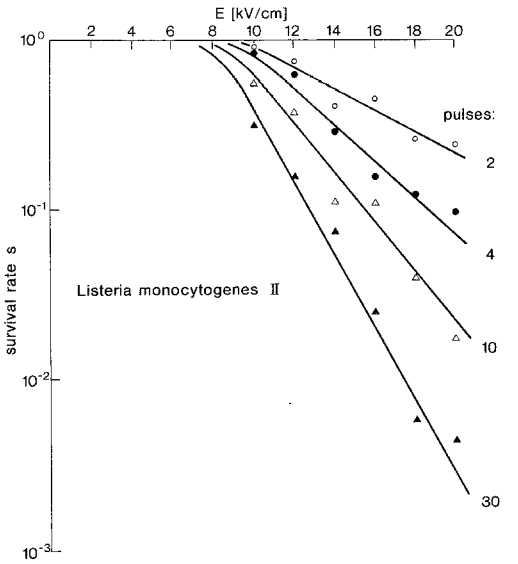

Fig. 8. Survival rates for *L. monocytogenes* II (rough form) from the stationary growth phase (16 h culture)

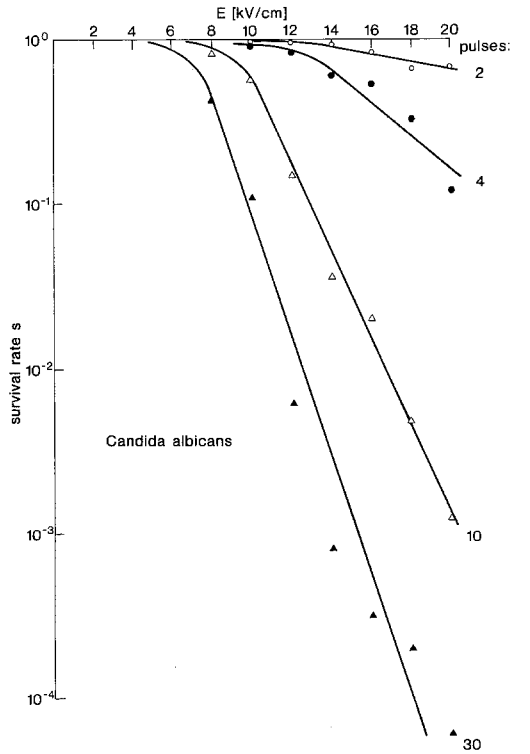

Fig. 9. Survival rates for *C. albicans* from the stationary growth phase (30 h culture)

A comparison between the mean threshold values obtained for GRAM-negative and GRAM-positive bacteria reveals a significant difference of E_c (7.2 kV/cm and 11.5 kV/cm, respectively) except *L. monocytogenes* II. Just so, the mean value of t_c is higher for GRAM-positive cells (61 μs) than for GRAM-negative bacteria (27 μs). These differences for E_c and t_c may be due to the varying morphological and biochemical properties of the respective cell types, particularly the cell membranes. In contrast to this, the parameter k has a distinctly lower value for the spherical cells of the species *S. aureus* only, as indicated by the degree of variation in the curve slopes (Fig. 6).

For the R-strain of *L. monocytogenes* II, lower values for E_c as well as for t_c are obtained when compared to the other GRAM-positive bacteria. Due to a physiological defect in the membrane assembly, the R-strain does not form regular rods in the culture but appears in elongated threads. The differing results may be caused by the enlarged cell size together with specific membrane defects leading to a labilization of the stressed cells. However, it could be demonstrated by these results that the characterization of a GRAM-positive reacting cell type alone is not sufficient to explain the relation between electric field treatment and obtained survival rates.

The pulse treatment of the yeast *C. albicans* leads to a high killing effect only when high pulse numbers are applied (Fig. 9). Pulse numbers below 10 additionally shift the threshold value E_c. As stated in previous studies [11], the applicability of the mathematical model provides that E and t values, used in the experiments, remarkably exceed the threshold values E_c and t_c, obtained for the respective cell species. If not so, the evaluation of these parameters leads to deviating results. We have therefore not considered the results of experiments with two pulses applied in the statistical evaluation of *C. albicans* (Table 1). The threshold value t_c calculated for *C. albicans* (110 μs) is much higher than the mean value obtained for bacteria (about 40 μs). On the other hand, the field strength E_c does not distinctly differ from the results for bacteria. For the parameter k, a relatively low value (2.2) similar to the corresponding value of *S. aureus* (2.6) is calculated. As a result, the killing effect of *C. albicans* exceeds the rates obtained for bacteria only if high pulse numbers are employed, i.e., the treatment time has to be comparatively long to get a low survival rate of yeast cells.

Relation Between Cell Size and Critical Field Strength

The theory about electric field effects on cell membranes of viable cells is derived from potential theory [20]. It implies that induced trans-membrane potentials depend on the cell size. For the assumption of spherical cells surrounded by non-conducting membranes, the induced potential is given by the equation:

$$V_m = f\ a\ E_c \tag{3}$$

where V_m = membrane potential induced by an external field of the strength E_c, a = cell radius, f = form factor for spherical shape (= 1.5). Zimmermann et al. have derived a mathematical relation in order to calculate the membrane potential V_m for non-spherical cells [2]. It is based on the assumption that the cell shape consists of a cylinder with two hemispheres on each end. The shape factor f for such particles is given by:

$$f = l/(l - 0.33\ d) \tag{4}$$

Table 2. Cell size and induced membrane potential of examined microorganisms: d = mean diameter, l = mean length, V = mean volume, f = shape factor [see text, (4)], V_m = membrane potential induced by an external electric field E_c under the assumption of parallel long particle axis and field vector

	d (μm)	l (μm)	V (μm³)	f	V_m (V)
E. coli (4 h)	1.15	6.9	7.2	1.06	0.26
E. coli (30 h)	0.88	2.2	1.4	1.15	1.05
K. pneumoniae	0.83	3.2	1.7	1.09	1.26
P. aeruginosa	0.73	3.9	1.6	1.07	1.25
S. aureus	1.03	–	0.6	1.50	1.00
L. monocytogenes I	0.76	1.7	0.8	1.17	0.99
C. albicans	4.18	–	38	1.50	2.63

with l = length of particle, d = diameter. This formula, substituted in (3), allows an approximate calculation of the induced potential for rod shaped bacteria.

By use of microphotographs of viable cells fixed on glass slides covered with agar, the sizes of the microorganisms employed in this study were measured. The measurements are calculated as average values of about 300 individual cells for each species used. Table 2 shows the results and the values V_m calculated by means of (3). The calculation is based on the assumption that the long axis of the rod shaped bacteria is parallel to the field vector. By consideration of the respective cell sizes, a critical membrane potential V_m of about 1.1 V for all bacteria from the stationary growth phase is revealed. This corresponds well with results obtained in former publications [2, 8]. Despite of that, the potential of young cells is markedly lower, as quoted for *E. coli* from the early logarithmic growth phase, but affected by larger confidence ranges. Calculations for *E. coli* cells from 16-h cultures lead to a membrane potential of 0.67 V (E_c = 4.9 kV/cm), as was recently found out [11]. A relatively high potential of more than 2 V is obtained for *C. albicans* with the large mean cell diameter of about 4 μm.

Apart from these results, it has to be considered that the stated potentials are no exact calculations, particularly for non-spherical cells. The assumption of the long particle axis to be parallel to the field vector probably leads to higher calculated V_m values than actually obtained, due to the unknown distribution of cell alignment during pulse treatment.

Nevertheless, it can be concluded that bacteria and yeasts do not possess identical membrane characteristics when comparing the inactivation by electric fields. Provided that field induced membrane processes are responsible for cell death, yeast cell membranes are of higher stability than bacterial membranes. Additionally, the sensitivity of the latter strongly depends on the culture conditions, i.e., the physiological age of the examined population.

Discussion

Bacteria and yeast cells are killed when subjected to high voltage field pulses of sufficient strength and time of treatment. For all microorganisms examined, threshold values of the field strength E_c and the treatment time t_c (pulse number

× time constant) could be calculated. Together with a third parameter k this allows the evaluation of the killing rates which are to be expected. The obtained results show that the sensitivity of microbial cells depends more on the physiological status of the tested cell population than on the type of microorganism chosen. Young *E. coli* cells (4 h incubation time) lead to significantly lower values of E_c as well as V_m with no distinct decrease of t_c. This demonstrates that the alterations in cell populations, occurring during proliferation reduction in growth cultures, primarily influence the critical value of the induced membrane potential. The threshold value of the treatment time, however, is almost not affected.

GRAM-postive bacteria reveal higher E_c values as well as higher t_c values, when the R-strain *L. monocytogenes* II is not considered. The GRAM-negative species *K. pneumoniae* shows an additional effect indicated in particularly bended survival curves, otherwise appearing as straight lines in logarithmic dependence on the field strength. The yeast *C. albicans* shows a markedly higher t_c value but no significant changing of the E_c value, when compared to bacteria.

In recent studies it could be shown that electric pulse treatment causes a release of nucleotides, such as AMP, ADP, NAD, and NADH [12]. These substances were also released after ultrasonic treatment of the respective cell suspensions. In contrast, a release of intracellular enzymes could solely be demonstrated after mechanical cell destruction by ultrasound but not after electric field treatment. Additionally, ultrasonic stress was found to be less destructive to GRAM-positive bacteria than to GRAM-negative bacteria. When the release of intracellular substances was observed, yeast cells were even left unaffected. This indicates the mechanical stability of yeast cells as well as of GRAM-positive bacteria to be higher than that of GRAM-negative bacteria. It is inclined to ascribe this effect to the mechanical solidity of the respective cell walls.

Nevertheless, in all cases electric pulse treatment leads to very low survival rates ($< 1\%$) when the microbial suspensions are subjected to 30 pulses of 20 kV/cm. In comparison, the quantity of nucleotides released after pulse treatment was found to be similar in all cases. Thus, the mechanism by which viable cells are killed from electric fields differs from that of ultrasonic stress in terms of a different stress target. In contrast to ultrasound, electric fields can be assumed to affect the molecular structure of the inner cell membrane of microbial cells leading to a permeability increase for small molecules.

It seems reasonable to assume that this cell damage finally causes the death of the viable cells, also considering further chemical factors presumably involved. On the other hand, the degree of membrane damage is not excessive enough to allow the passage of macromolecules, such as enzymes [12]. This might be due to a remaining stabilization in connection with cell wall structures, the latter being practically unaffected by electric field stress.

In conclusion, the kinetics of the inactivation process caused by electric pulse treatment could be demonstrated to be similar in quality for different microorganisms. The differences in quantity revealed for *E. coli* cell populations from varying growth phases prove that specific alterations in the membrane composition or structure are to be observed while the cells undergo progressive growth phases. The same has to be considered when different species are compared, particularly bacteria and yeasts. The killing effect is additionally influenced by the relevant cell shape and size. When this is considered in the evaluation of a critical membrane potential, an average value of $V_m = 1.1$ V can be calculated for bacteria from

stationary growth phases. The membrane of *C. albicans*, however, reveals a value of about 2.6 V for critical membrane potential. Following this interpretation, it has to be considered that the membrane potential values are not a result of direct measurements so that they are bound to be inexact within a certain range.

Nevertheless, it can be concluded that the lethal action of electric fields is primarily governed by the intrinsic properties of the microorganism, under which the inner cell membrane may play the most important role. The process involved strongly depends on specific membrane properties which are remarkably changing during growth in cultures.

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