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Effect of pH, ethanol addition and high hydrostatic pressure on the inactivation of *Bacillus subtilis* by pulsed electric fields

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Abstract

In this study the inactivation of vegetative *Bacillus subtilis* ATCC 9372 cells in response to high intensity pulsed electric fields (PEF) is presented. The effect of medium pH in a range between 5.5 and 7 as well as the addition of 5% (v/v) of ethanol was studied. Furthermore, it was investigated how a simultaneous compression of the microbial suspension can impact its susceptibility to PEF. The logarithmic inactivation of *B. subtilis* was independent of medium pH and progressively increased with rising field strengths. The extent of this enhancement strongly depends on the specific energy of the pulses which are released into the suspension. Applying 100 pulses at 2.1 kJ/kg a 4 log-cycle inactivation was obtained. By the addition of 5% (v/v) ethanol a further drastic increase in lethality was observed but only when the pH was reduced to 5.5. At pH 7 the presence of ethanol produced a higher resistance against PEF. A synergistic effect of high pressure was only observed when the compression time prior to PEF was extended to 10 min. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Pulsed electric fields; High pressure; pH; Ethanol; Inactivation; *Bacillus subtilis*

Industrial relevance: This paper is the first in depth report on a real combination process of high pressure and electric field pulses and the prototype equipment developed. It also demonstrates the dramatic impact medium composition (i.e. 5% ETOH) can have on the field strength dependent inactivation of vegetative microorganisms.

1. Introduction

It is often observed that traditional preservation methods such as heat treatment fail to produce microbiologically stable materials at the desired quality level. It has already been demonstrated that high intensity pulsed electric fields (PEF) processing has the potential of providing safe and shelf-stable products such as fruit juices or milk with fresh-like character and high nutritional value (Grahl & Märkl, 1996). Prior to the commercial exploitation of PEF as an alternative to traditional preservation techniques process safety, cost-effectiveness, and consumer benefits of PEF have to be confirmed. From experimental data (Wouters, Dutreux, Smelt & Lelieveld, 1999; Simpson, Whittington, Earnshaw & Russel, 1999) it is evident that sufficient microbial reduction can be achieved. However, the degree of inactivation strongly depends on the intensity of the pulses in terms of field strength, energy and number of pulses applied on the microbial strain and on the properties of the food matrix under investigation. In the presence of membrane affecting agents such as nisin, an increased inactivation in response to PEF was observed (Pol, Mastwijk, Bartels & Smid, 2000). Hence, for the optimization of process design it is necessary to consider the mechanisms of action of PEF on the level of microbial cells.

It is a well-known fact that an increase of the 10 mV resting potential across the cell membrane by the exposure to an external electrical field up to potentials higher than approximately 1 V leads to rapid electrical breakdown and local conformational changes of bilayer structures (Neumann & Rosenheck, 1972) and cell membranes (Zimmermann, Schulz & Pilwat, 1973). A drastic increase in permeability re-establishes the equi-

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librium of the electrochemical and electric potential differences of the cell plasma and the extracellular medium forming a Donnan-equilibrium (Glaser, 1996). Simultaneously, the neutralization of the transmembrane gradient across the membrane irreversibly impairs vital physiological control systems of the cell like osmoregulation and consequently cell death occurs.

The factors affecting the efficiency of PEF are many and diverse. Besides field strength (Heinz, Phillips, Zenker & Knorr, 1999), pulse duration (Muraji, Taniguchi, Tatebe & Berg, 1999) or energy input (Gaskova, Sigler, Janderova & Plasek, 1996) the ionic composition of the extracellular medium is crucial (Muraji, Tatebe & Berg, 1998). Also, side effects like precipitation of biological macromolecules by solubilized metal ions of the electrode (Stapulionis, 1999) or the fragmentation of nucleic acids (Hofmann, Ohnimus, Scheller, Strupp, Zimmermann & Jassoy, 1999) might play a role.

In spite of the substantial knowledge of the impact of PEF on microorganisms (Wouters & Smelt, 1997) or eucaryotic cells (Zimmermann, 1996) until now there is no clear evidence on the basic action of membrane permeabilization on a molecular level. As breakdown of the membrane occurs in the range of nanoseconds and experimental techniques still fail to yield sufficient information at the required time resolution, several models that have been put forward during the last three decades are still a matter of discussion.

An early approach (Crowley, 1973; Zimmermann, Pilwat & Riemann, 1974) suggested that the cell membrane when exposed to PEF acts like a spheric shell filled with an elastic dielectric material. Induced charges on both sides with different polarity impose compressive forces on the membrane which can be equilibrated by elastic deformation and generated strains. An increase in the external electrical field reduces the thickness of the membrane. Electrical breakdown occurs when a critical value is reached which depends upon the compressibility, the permittivity, and the initial thickness of the membrane. Using macroscopic compressibility data of lipids the model predicts a spatially restricted reduction of the membrane thickness by 30% which is not very likely with regard to its molecular conformation and without experimental evidence (Winterhalter, Klotz & Benz, 1996).

A refined version of this model tried to include the duration of the pulse to the parameters that determine the electrical breakdown (Dimitrov, 1984). The membrane is regarded as a thin viscoelastic film of dielectric material and it is assumed that permanent thickness fluctuations occur. The main improvement to the previous model is that the compressive forces are counterbalanced not only by local elastic deformation but also by long-range intermolecular forces which can increase the total area of the membrane. Another aspect of the concept of membrane thinning prior to permeabilization is that possibly the energy barrier for ions crossing the low dielectric membrane decreases (Parsegian, 1969).

Another approach (Powell, Derrick & Weaver, 1986) did not use the concept of membrane thinning and postulated that a population of statistically fluctuating membrane defects, which are naturally present in the absence of PEF, determine the membrane resistance and the critical transmembrane voltage. More recently it was reported (Neumann, Toensing, Kakorin, Budde & Frey, 1998) that a model using a minimum number of three different (hypothetical) pore states can accurately predict the permeabilization and the resealing behaviour during and shortly after the exposure of cells to electric fields.

In spite of the experimental evidence of the latter concepts it is also undoubted that deformation forces caused by Maxwell stress are imposed on the cell during the time course of a pulse (Sukhorukov, Mussauer & Zimmermann, 1998). Attempts were made to enhance the stress by using hydrostatic pressure to precompress the membrane prior to PEF (reviewed in Zimmermann, 1982). These works yielded that an increase in turgor pressure of 0.3 MPa reduced the breakdown voltage of algal cells by approximately 5% (Coster, Steudle & Zimmermann, 1977). The corresponding effective compressive modulus was estimated to be 6.9 MPa (Zimmermann, Beckers & Coster, 1977).

By the exposure of eucaryotic cells to hydrostatic pressure it could be demonstrated that the potassium efflux is significantly increased when the pressure exceeds 60 MPa (Zimmermann, Pilwat, Pequeux & Gilles, 1980). It was concluded that a critical hydrostatic pressure exists at which reduction of membrane thickness is sufficient to induce mechanical breakdown. Although these findings might be interpreted in analogy to the electromechanical model of bilayer breakdown, it has to be considered that pressure induced phase transitions of membrane components can yield similar macroscopic effects (Macdonald, 1984; Tauc, Mateo & Brochon, 1998). The effects of hydrostatic pressure on the integrity and functionality of biological membranes and bilayers is diverse (Kato & Hayashi, 1999). Some membrane anchored functional proteins and peptides are reported to change their conductance state in response to an external transmembrane pressure by molecular rearrangements (Marsh, 1996). On the other hand, it was found that increased hydrostatic pressure has an ordering effect upon the lipid acyl chains which should stabilize the membrane (Bernsdorff, Wolf, Winter & Gratton, 1997).

External chemical stress like an increased proton concentration should induce changes in membrane functionality which affect the charging properties during PEF. However, a reduction in pH down to pH 5

was without effect on the breakdown voltage which was assumed to be due to the protonation of the anionic groups within the tonoplast and plasmalemma (Zimmermann, Büchner & Benz, 1982).

2. Materials and methods

In this study the effect of variations in medium composition as well as the simultaneous application of high hydrostatic pressure on the inactivation of vegetative cells of *Bacillus subtilis* by PEF were investigated. This organism was used because its response to high pressure has been investigated previously (Heinz & Knorr, 1996). Medium pH and ethanol content were chosen as environmental factors that are supposed to affect the functionality and the composition of the microbial membrane (Sajbidor, 1997). Increasing proton concentration of the medium can have an impact on the activity of enzymes and force the cell to stabilize its internal pH by energy consuming homeostatic action like proton translocation by membrane-bound ATPase.

Chemical stress is imposed by the addition of ethanol to the suspending liquid as it is toxic in high concentrations even for ethanol producing microorganisms. Alterations in plasma membrane proteins as well as in fatty acid composition can produce an increased permeability. Additionally, a reduction of the level of H^+-ATPase in the membrane reduces the homeostatic potential (Piper, 1995).

High pressure in the range of several hundreds of MPa can produce a considerable shift of dissociation equilibria in the external medium as well as in the cell plasma. The rather narrow range of tolerated internal pH is stabilized by an energy demanding proton transport across the membrane mediated by the H^+-ATPase. As proteins are sensitive to high hydrostatic pressure and can be unfolded, the total H^+-ATPase activity can be reduced by the treatment which can cause net protonization of the cell plasma (Wouters, Glaasker & Smelt, 1998). Additionally, as mentioned earlier, high pressure can induce phase transitions of membrane lipids from the crystalline to the liquid–crystal state which can possibly affect the susceptibility to PEF, too.

To avoid interference between the above specified variables and to ensure stable boundary conditions all experiments were carried out in batch systems. Compared to continuous systems it is further advantageous that the diversity in exposure time due to the flow pattern has not to be considered.

Taking into account that the maximum field strength of a pulse and the time course of the transient electrical field from which the total energy input is derived cannot be treated separately (Gaskova et al., 1996) the basic experimental design was to either keep the specific energy input constant to allow variations in field strength or vice versa. Additionally, the temperature which strongly affects the breakdown voltage (Coster & Zimmermann, 1975) should remain under almost constant conditions. Furthermore, inactivation data obtained from batch experiments with constant boundary conditions and homogeneous electric field can be used to predict the lethal effect of PEF under transient conditions in continuous flow.

Hence, it was decided to design an experimental setup which would provide an accurate adjustment of field strength and pulse energy, an extremely good heat removal from the treatment chamber, and a homogeneous electric field (Heinz et al., 1999). To meet these requirements, a high voltage switching system with variable on-time was used together with a small dimensioned batch treatment chamber with parallel circular electrodes. The setup can be operated at hydrostatic pressures up to 200 MPa.

2.1. Discharge circuit

To obtain variable field strength and square wave pulses with adjustable on-time a suitable experimental setup was developed and has been described in detail previously (Heinz et al., 1999). A semi-conductor switch is used together with a 2-μF storage capacitor and a 15 Ω protective resistor is included to limit the current in the case of arching in the treatment chamber. A scheme of the used circuit and the specifications of all components is presented in Fig. 1. Depending on the electrode system used, the electrical load varied between 55 and 400 Ω. Voltage and current are measured directly at the treatment chamber and detected by a digital storage oscilloscope. From these signals the peak field strength and the specific pulse energy is calculated.

The switch is rated to a maximum voltage level of 10 kV and a maximum repetition rate of 6 kHz. The current capability is limited to 800 A. Typical pulse characteristics were a turn-on rise time of approximately 40 ns and a variable on-time from 0.4 μs to infinity.

The standard treatment chamber consisted of two polished stainless steel cylinders (length: 2 cm) which were fitted into the sample containing PVC tube made from a syringe shell (length: 2 cm). The electrode area was 2.01 cm^2. The gap was adjusted to 1.2 mm. With an electrical conductivity of the sample of 0.15 S/m at 20°C the ohmic resistance of the treatment chamber can be approximated to 40 Ω.

2.2. High pressure unit

An apparatus was designed at the Department of

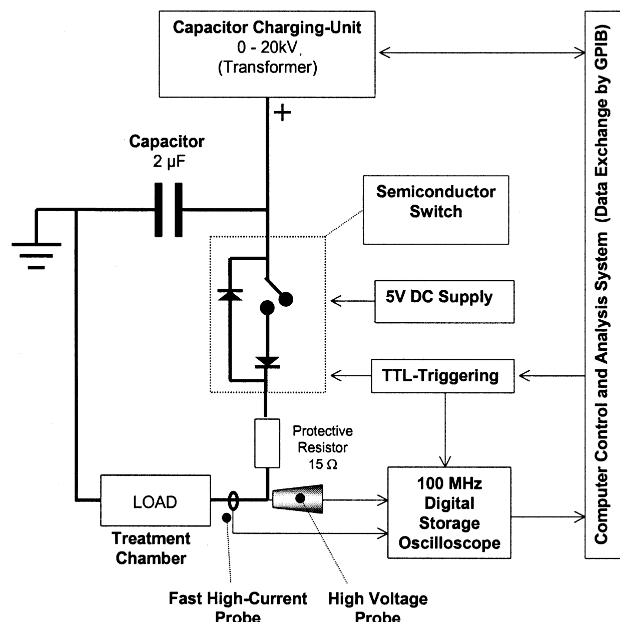

Fig. 1. Capacitator discharge circuit. The discharge circuit is operated by a central PC-based control unit. The storage capacitor is charged by a stabilized high voltage charging unit with maximum 20 kV and 50 mA output. A semi-conductor on/off switch protected by two diodes and a series resistor is used to partially discharge the capacitor across the electrical load to ground. The switching system is limited to 10 kV and 800 A. Triggering of high voltage discharges is done by standard TTL-signals delivered from a function generator which is programmed and controlled by the PC. A 100-MHz digital storage oscilloscope is used for acquisition of voltage and current at the treatment chamber. Signal conditioning is performed by a 75-MHz high voltage and a 100-MHz current probe connected to an amplifier system.

Food Biotechnology and Food Process Engineering, Berlin University of Technology in cooperation with Unipress (High Pressure Research Center, Warzaw) that could be used for the simultaneous application of high pressure (UHP) and pulsed electric fields (PEF) in conductive liquid samples. A schematic view of the unit and all its relevant components is presented in Fig. 2. The main purpose of the development of the PEF/UHP apparatus was to provide a system that can be used as electrical load of the previously described discharge circuit. For that reason the sample container as the basic component of the unit is similar to the standard treatment chamber. The electrode area is 0.072 cm^2 and the gap is adjusted to approximately 2.3 mm. A sample conductivity of 0.15 S/m yields an ohmic resistance of the treatment chamber of approximately 390 Ω. The stainless steel electrodes are part of the sealing system which further consists of the PTFE tube and the high pressure vessel. All these components are manufactured with high accuracy to optimize the sample container's fit into the pressure vessel which is made of ceramics customized for high compressive strength, and externally supported by a stainless steel tube. When the vessel is placed between the supporting metal plates of the yoke press the two electrical connectors (which are coupled to the capacitor discharge circuit) can be brought in contact with the electrodes. The upper connector which is driven by an external oil pump moves downward and puts a compressive force on the sample container. The intensifying ratio derived from the force transmitting areas of the electrical connectors is 11. Because of the small tolerances the contact surfaces between the electrode and the sealing effect of the PTFE tube is sufficient to reach the rated maximum pressure of the system of 200 MPa. The pressure inside the sample container can be derived from the measured oil pressure multiplied by the intensifying ratio.

2.3. Sample preparation

Bacillus subtilis ATCC 9372 was obtained from the German Culture Collection (DSM, Braunschweig, Germany). A stock culture was inoculated into Standard I nutrient broth (MERCK, No. 7882, Darmstadt, Germany) and incubated at 30°C for 24 h. A subset of this culture was stored on slanted Standard I nutrient agar (MERCK, No. 7881, Darmstadt, Germany) at 4°C. A stock culture was inoculated into Standard I nutrient broth shaken and incubated with 140 RPM at 30°C for 24 h. The suspension (20 ml) was centrifugated for 2 min at $4000 \times g$ and resuspended in 6 ml 1:10 diluted

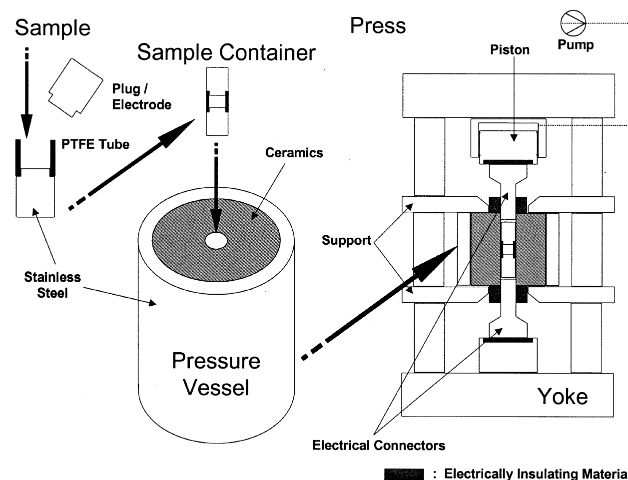

Fig. 2. Experimental setup for the combination of pulsed electric field and high pressure treatment. The PEF/UHP apparatus consists of three components: the sample container, the high pressure vessel, and the yoke press. To run the system a liquid sample is filled into the opened sample container. The sample container is then prepared to be inserted into the central bore of the high pressure vessel by pressing the upper electrode into the PTFE tube and squeezing out the excess liquid. The completed high pressure vessel is placed at the yoke press between the electrical connectors and the supporting metal plates. Finally, the system is pressurized by a moving piston driven by an external oil pump.

Ringer solution (MERCK, No. 15525, Darmstadt, Germany) and mixed with 54 ml McIlvaine buffer (Rauscher, Voigt, Wilke & Wilke, 1968) of pH 7.0, 6.0, or 5.5. The conductivity was adjusted to 0.15 S/m (if not stated otherwise) by adding either deionized water or Ringer's solution. In those experiments where the effect of alcohol was tested, a previously determined volume of concentrated ethanol (96% v/v) which yields a final concentration of 5% EtOH v/v was added to the buffered microbial suspension immediately before the sample was exposed to PEF. In less than 1 min after the treatment the ethanol concentration was reduced to approximately 0.5% (v/v) by dilution with sterile Ringer solution.

2.4. Microbiological analysis

Immediately after treatments, the dilution series were made using Ringer's solution (MERCK, No. 15525, Darmstadt, Germany). Viable counts of vegetative cells were determined using the drop plating method (Baumgart, 1986). The agar plates consisted of 5.0 g/l peptone (MERCK, No. 7214, Darmstadt, Germany), 3.0 g/l meat extract (MERCK, No. 3979, Darmstadt, Germany) and 12 g/l bacteriological agar (OXOID, Code L11, Basingstoke, UK). The plates were then incubated at 30°C for 48 h.

2.5. Experimental designs

The effect of field strength, pulse energy, hydrostatic pressure, pH, and ethanol on the inactivation of *Bacillus subtilis* was investigated. Variation of the electric field strength was performed in a range between approximately 3 and 53 kV/cm. To discriminate the effect of field strength and energy input, it was necessary to compensate the increase in specific pulse energy with rising field strength by reducing the on-time of the discharge pulse from 80 to 0.5 μs. In Fig. 3 examples of voltage signals which produce identical specific energy dissipation at a 40-Ω load resistor in response to partial 2 μF capacitor discharges are presented.

In this way inactivation experiments were carried out in the standard treatment chamber on four different specific pulse energy levels (0.5 ± 0.061; 0.97 ± 0.094; 1.48 ± 0.156, 2.06 ± 0.289 kJ/kg) with constant pulse count (100 pulses) and repetition rate (5 pulses/s) at pH 7. The indicated pulse energy values were averaged from all trials of the experimental design carried out at the same energy level. The pulsing system allowed to keep the setting of this independent variable within a range of $\pm 15\%$ of the scheduled specific pulse energy.

In a second approach the effect of medium composition was investigated by repeating the previous experiment on reduced pH levels (pH 6 and pH 5.5) and by the addition of alcohol to yield an ethanol concentration of 5% v/v. Also the impact of liquid compression was tested on the same specific pulse energy levels (except at 2.06 kJ/kg) with the PEF/UHP apparatus. High pressure of 200 MPa was applied for less than 1 min and the PEF treatment was done simultaneously during the isobaric holding time.

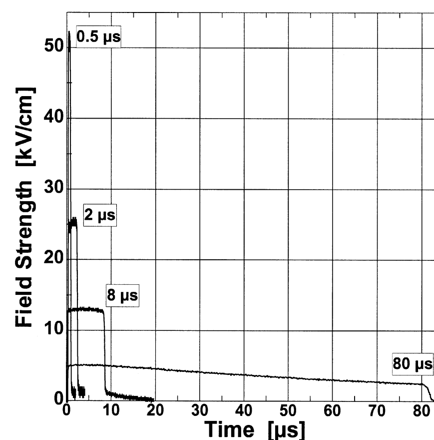

Fig. 3. Discharge pulses with variable on-time. Examples of voltage signals at a 40Ω load in response to partial 2 μF capacitor discharges. The presented pulses were adjusted by varying on-time (0.5, 2, 8 or 80 μs) and maximum field strength (53, 25, 13 or 5 kV/cm) to release the same amount of energy (1.48 ± 0.156 kJ/kg) to the sample between the electrodes.

Furthermore, it was investigated how the duration of a high pressure treatment at 200 MPa can affect the inactivation by pulsed electric fields. At a constant field strength level (24.7 ± 0.61 kV/cm) 200 pulses were applied (rate: 20 pulses/s) immediately before pressure release. At a pH of 7 the conductivity was adjusted to 0.3 S/m. Three different specific pulse energies were tested (1.10 ± 0.064; 1.66 ± 0.071; 2.78 ± 0.193 kJ/kg) which were obtained by adjusting the on-time of the pulse to 1, 1.5, and 2.5 μs, respectively. Experiments were carried out with the PEF/UHP apparatus.

Inactivation data presented in the figures below show the average of the logarithmic survivor portion of at least two independent experiments.

3. Results and discussion

The effect of field strength in a range between 2.5 and 52.8 kV/cm on the inactivation of *Bacillus subtilis* ATCC 9372 suspended in buffer (pH 7; conductivity 0.15 S/m) is demonstrated in Fig. 4. Due to the experimental conditions which allowed the compensation of the increasing pulse energy with increasing field strength, the inactivation has been investigated on four fixed energy levels: *A* 0.5 kJ/kg, *B* 0.97 kJ/kg, *C* 1.48 kJ/kg and *D* 2.06 kJ/kg. The number of pulses applied and the repetition rate was kept constant at 100 pulses and 5 pulses/s. From previous work (Heinz et

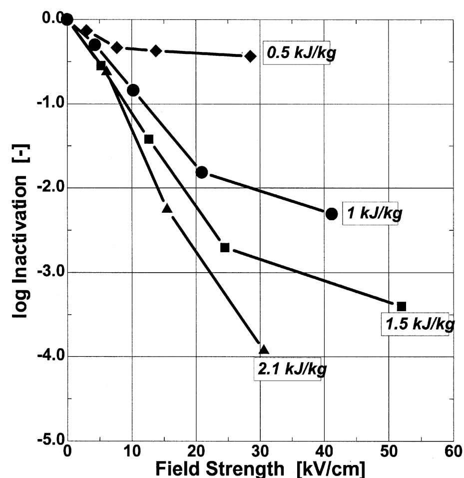

Fig. 4. Effect of the electrical field at constant specific pulse energy. Experiments were carried out on four different energy levels (*A*: ◆0.5 ± 0.061 kJ/kg; *B*: ●0.97 ± 0.094 kJ/kg; *C*: ■1.48 ± 0.156 kJ/kg, D: ▲2.06 ± 0.289 kJ/kg) with constant pulse count (100 pulses) and with constant repetition rate (5 pulses/s). Constant energy levels were obtained by adjusting the on-time between 0.5 and 80 μs (see example in Fig. 3). The average initial temperature was 22°C and the heating up by PEF was less than 8°C.

al., 1999) it is known that under these conditions the temperature of the sample does not exceed 30°C.

Although a significant reduction in survivor count with increasing field strength was observed, it is evident that the level of the specific pulse energy input determines the lethal effect of the treatment. The progressive inactivation as a function of the maximum field strength is strongly reduced if specific energy input levels below 1 kJ/kg per pulse are used. Within 2.5 and 52.8 kV/cm the occurrence of a critical field strength was not observed. Although it is possible that such a threshold level exists below 2.5 kV/cm the identification by survival counts after repeated pulses would be rather vague because of the diversity of the population.

At a given field strength the four different energy levels tested can be interpreted as variations of the on-time of the square wave pulses. As the turn-on rise time of the pulses is identical the improved inactivation with increasing energy input must be related to a prolonged current flow across the membrane and through the cell. Because of the used experimental setup which immediately equilibrates the pulse energy dissipation in the suspending medium, an overlapping thermal effect can be excluded.

Similar to the previous experiment 100 pulses on four different levels of specific pulse energy were applied to *Bacillus subtilis* cells in modified suspensions and during high pressure treatment. In all experiments the conductivity of the medium was adjusted to 0.15 S/m. In Fig. 5 the logarithmic survivor fraction is plotted vs. field strength.

Variations in medium pH are regarded to be of minor influence because the inactivation curves obtained at pH 5.5, 6, and 7 are not significantly different. Taking into account the previous assumption that at a constant field strength level more cells are inactivated when the time of current flow through the cell is increased, the independence of pH might be due to the action of homeostatic regulation mechanisms which maintains the proton concentration and prevent changes of the ionic composition in the protoplasma. Hence, the ohmic resistance of the cell interior is not affected by variations in medium pH within the investigated range.

The addition of 5% (v/v) ethanol to the medium at pH 7 resulted in a clearly increased resistance of *Bacillus subtilis* against PEF at a specific pulse energy of 1 kJ/kg and at 1.5 kJ/kg. However, a drastically enhanced effect of PEF on cell viability was obtained at all energy levels tested when the same ethanol concentration was used at pH 5.5. In both cases an influx of ethanol and a partial damage of the membrane bound ATPase is likely to occur. Additionally, the susceptibility of the cells to PEF is largely determined by the proton concentration of the medium. Hence, in consequence of limited homeostatic proton translocation mediated by the ATPase the ionic content of the cell protoplasma will raise. Furthermore, it is interesting to note that an almost similar field strength dependence of the inactivation was observed at 0.5 kJ/kg in the presence of ethanol and at 2.1 kJ/kg without ethanol. The finding that short pulses with less specific energy are similar efficient in the presence of ethanol at pH 5.5 than the control suggests that a higher portion of the energy from capacitor discharge is dissipated at the microbial cells. It can be speculated that the ionic composition of the protoplasma determines the fraction of energy released into the cell rather than an increased permeabilization of the membrane. The stabilizing effect of ethanol at pH 7 might be due to the reduced proton concentration and an increased portion of undissociated ethanol molecules compared to the control is in line with this assumption.

Further investigations are required to explain the high sensitivity of *Bacillus subtilis* towards PEF treatment at the comparably low specific pulse energy level of 1 kJ/kg at pH 5.5 and in the presence of 5% ethanol.

Simultaneous application of high hydrostatic pressure at a level of 200 MPa for less than 1 min and PEF did not result in an improved inactivation compared to the PEF treatment at atmospheric pressure. At a specific pulse energy of 1 kJ/kg and at 1.5 kJ/kg even a stabilizing effect was observed. This result is in contradiction to mechanistic models of membrane breakdown which assume a thinning of the membrane in response to contractive forces. There is experimental evidence that hydrostatic pressure of 200 produces a percentage

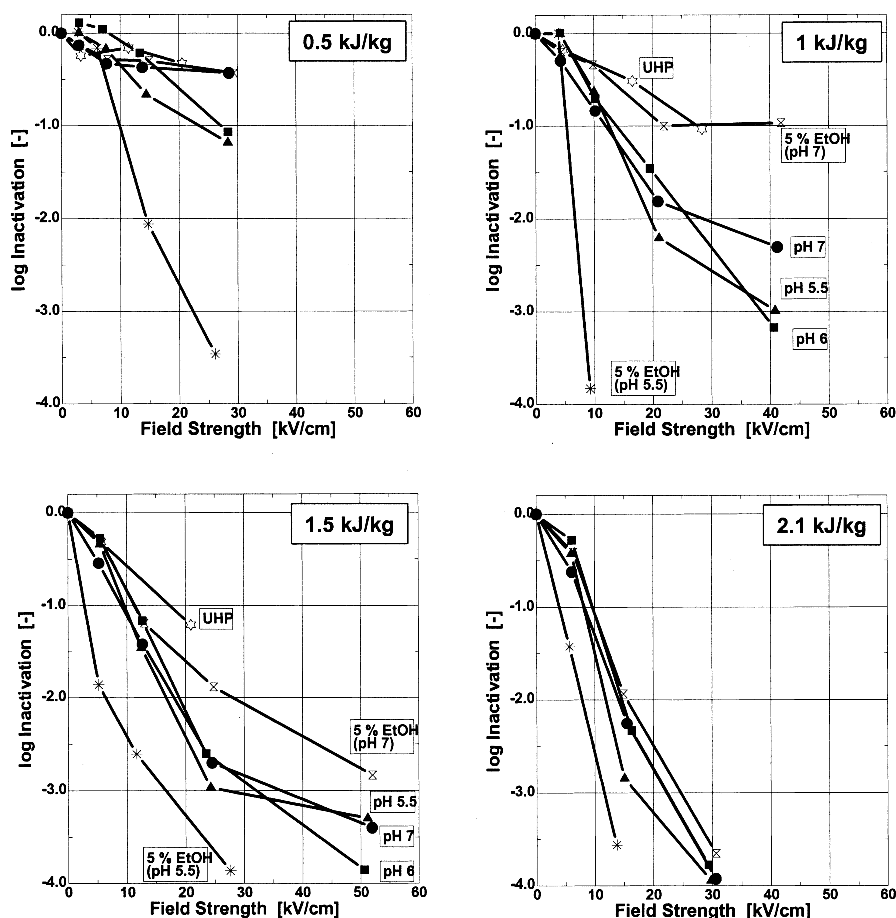

Fig. 5. Effect of environmental factors. Experiments were carried out on four different energy levels (top left: 0.5 ± 0.061 kJ/kg; top right: 0.97 ± 0.094 kJ/kg; bottom left: 1.48 ± 0.156 kJ/kg, bottom right: 2.06 ± 0.289 kJ/kg) with constant pulse count (100 pulses) and with constant repetition rate (5 pulses/s). The average initial temperature was 22°C. The following variations in media composition were tested: ● pH 7; ■ pH 6; ▲ pH 5.5, ⧗ 5% (v/v) ethanol at pH 7, *5% (v/v) ethanol at pH 5.5. Additionally the impact of pressurization was investigated: ☆ simultaneous PEF and 200 MPa high pressure treatment (duration: less than 1 min) at pH 7.

decrease in chain spacing of the bilayer in the order of 5% (Braganza & Worcester, 1986). That this precompression did not produce any synergistic effect on the lethality of the PEF treatment might be explained by an counterbalancing ordering effect of the pressure upon the lipid acyl chains (Bernsdorff et al., 1997).

In Fig. 6 the logarithmic survivor fractions after 200 pulses at a peak field strength of 24.7 kV/cm are presented. Except for the control samples which were exposed to PEF at atmospheric pressure all other samples were treated during a simultaneous pressurization of 200 MPa at the indicated specific pulse energy. Prior to PEF the samples were incubated at 200 MPa and at 22°C for 5 s, 5 min, and 10 min, respectively. Increasing specific energy input yielded no significant difference in inactivation between the control and the 5-s or 5-min pressure treatment. A synergistic lethal effect was only observed when the pressure treatment time prior to the pulsed electric field treatment was extended to 10 min. This suggests that alterations in the membrane structure imposed by compressive forces do not immediately increase the susceptibility to pulsed electric fields but that time dependent reactions in response to sublethal pressure stress can produce a destabilization. If no pulses were applied at the end of the treatment a 30-min pressure application at 200 MPa did not reduce the number of viable cells (data not shown) indicating that repair mechanisms are active during a subsequent incubation under optimum growth conditions.

4. Conclusion

Microbial survival in response to PEF strongly depends on the field strength applied and the specific energy input into the microbial suspension. By increasing the field strength from approximately 5 to 50 kV/cm at the same level of specific pulse energy more than three log cycles difference in survivor counts of *Bacillus subtilis* were obtained. This finding might become especially crucial for the design of energy efficient commercial PEF units. The medium pH which was

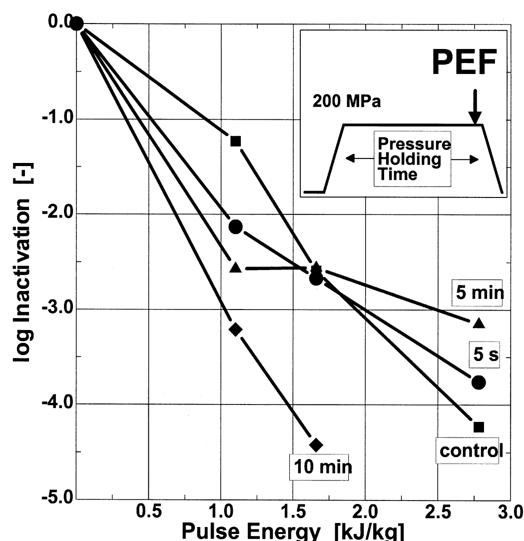

Fig. 6. Effects of pressure holding time. Experiments were carried out at constant field strength level (24.7 ± 0.61 kV/cm) releasing 200 pulses at a repetition rate of 20 pulses/s. The average initial temperature was 22°C and the pH was adjusted to pH 7. The logarithmic surviving portion of *B. subtilis* is plotted vs. the averaged energy levels investigated: 1.10 ± 0.064; 1.66 ± 0.071; 2.78 ± 0.193 kJ/kg) which were obtained by adjusting the on-time of the pulse to 1, 1.5, and 2.5 μs, respectively. Except for the control ■ all samples were exposed to high pressure treatments with different holding times prior to the PEF treatment: ● 5 s; ▲ 5 min and ◆ 10 min. The pulses were applied immediately before the system was decompressed (see pressure profile top right).

tested in a range from pH 5.5 to 7 seems to be of minor impact on the lethality of the treatment. This observation is consistent with earlier results (Zimmermann et al., 1982) that state that down to pH 5 the breakdown voltage is not affected by the external proton concentration. However, in this study we could demonstrate that at pH 5.5, in combination with 5% (v/v) of ethanol, the efficiency of PEF can be enhanced drastically. It is assumed that ethanol molecules that entered the protoplast damage the membrane-bound ATPase which is the key enzyme in internal pH regulation by proton translocation. The resulting proton accumulation increases the ionic content and produces a higher dissipation of the pulse energy at the protoplasma.

The simultaneous application of PEF and a sub-lethal high pressure treatment at 200 MPa produced a stabilizing effect compared to PEF at atmospheric pressure. The expected synergistic effect of the precompression, e.g. by reducing the critical breakdown voltage (Coster et al., 1977) was not observed. However, when the cells were exposed to 200 MPa for 10 min significantly improved inactivation was observed with increasing pulse energy. This indicates that pressure induced kinetic changes of membrane components like phase transitions of lipids or proteins can increase the susceptibility against PEF.

Future work will focus on the effect of temperature of the combined PEF/pressure treatment and the pressure range will be extended up to 1000 MPa.

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