Mechanisms of inactivation of *Candida humilis* and *Saccharomyces cerevisiae* by pulsed electric fields

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Inactivation of yeasts by pulsed electric fields

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Abstract

Aims: This study aimed to determine how electric field strength, pulse width and shape, and specific energy input relate to the effect of pulsed electric fields (PEF) on viability and membrane permeabilization in *Candida humilis* and *Saccharomyces cerevisiae* suspended in potassium phosphate buffer.

Methods and Results: Cells were treated with a micro-scale system with parallel plate electrodes. Propidium iodide was added before or after treatments to differentiate between reversible and irreversible membrane permeabilization. Treatments of *C. humilis* with 71 kV/cm and 48 kJ/kg reduced cell counts by 3.9 ± 0.6 log (cfu/mL). Pulse shape or width had only a small influence on the treatment lethality. Variation of electric field strength (17 - 71 kV/cm), pulse width (0.086 - 4 µs), and specific energy input (8 – 46 kJ/kg) demonstrated that specific energy input correlated to the membrane permeabilization ($r^2 = 0.84$), while other parameters were uncorrelated. A minimum energy input of 3 and 12 kJ/kg was required to achieve reversible membrane permeabilization and a reduction of cell counts, respectively, of *C. humilis*.

Conclusions: Energy input was the parameter that best described the inactivation efficiency of PEF.

Significance and Impact of Study: This study is an important step to identify key process parameters and to facilitate process design for improved cost-effectiveness of commercial PEF treatment.

Key words: pulsed electric fields, *Candida humilis*, *Saccharomyces cerevisiae*, propidium iodide, bleomycin, electric field strength, specific energy input.

1. Introduction

Pulsed electric fields (PEF) have the potential to provide minimally processed and microbiologically safe food products [1]. PEF can be delivered to achieve pasteurization temperature, or to induce only a low temperature increase that avoids heat-induced changes in colour, flavor, taste, and nutrient content of food. PEF induces local structural changes of cell membranes and the breakdown of membrane permeability barrier [2,3]. This effect is also termed as electroporation [4. Dielectric permittivity and electrical conductivity of the cell membrane differ from those of cytoplasm and extracellular medium [5], which creates conditions for interfacial polarization. When biological cells are exposed to an external electric field, most of this field concentrates across the membrane [5]. When the transmembrane potential reaches a critical value of 0.2 – 1.7 V, transient pores appear in the membrane [6,7,8]. The pore formation is irreversible and may lead to cell death. Membrane permeabilization and cell death are also determined by pulse shape, duration, and frequency, which correspond to energy input. Other factors determining the efficacy of PEF include the size and shape of microorganisms, and the properties of treatment medium [9,10,11].

PEF treatments transfer energy to the fluid and thus increase its temperature [12]. The energy consumption of bactericidal PEF treatments ranges from 100 to 1000 kJ/kg [13] and energy efficiency is considered to be a critical factor for commercial use of PEF processes [13, 14]. Heat generated during PEF treatment is typically insufficient to kill microorganisms but may act synergistically with PEF treatment to increase the process lethality. A temperature increase alters the fluidity and stability of the cell membrane [15], and increases the electrical conductivity of treatment medium [11]. An altered conductivity at a constant energy input decreases the field strength [11]. In the past studies, the typical treatment chamber was a pipe [16]. These treatment chambers generate non-homogeneous electric fields and temperature distribution; these inhomogeneities substantially confound the assessment of the contribution of electric field, energy input, and temperature on microbial inactivation [17].

Despite the designation of PEF as "promising emerging technology" in food processing, the number of current commercial applications remains limited [11]. The reluctant adoption of this technology by the food industry relates to the lack of insights on the physical parameters that govern microbial inactivation. The field strength is thought to be the most relevant factor affecting microbial inactivation, but the energy input and the resulting temperature increase may also contribute to microbial inactivation [9,11]. It was therefore the aim of this study to use a miniaturized PEF system to determine the contribution of energy input, field strength, pulse width, shape, and frequency on yeast inactivation. This study used treatment chambers with parallel plate electrodes to deliver homogeneous electric fields [18,19] and to allow a homogeneous distribution of the temperature. *Candida humilis* and *Saccharomyces cerevisiae* were used as model microorganisms.

2. Materials and methods

2.1 Preparation of inoculum and cell suspension

Candida humilis FUA4001 and *Saccharomyces cerevisiae* FUA4011 were grown on Difco Yeast-Peptone-Dextrose agar (YPD, 1 % yeast extract, 2 % peptone, 2 % dextrose, 2 % agar) and

79   incubated at 30 °C for 48 h. Cultures were prepared by inoculation of single colonies in YPD broth,
80   incubation at 30 °C, 250 rpm overnight, followed by sub-culturing in YPD broth with 1 %
81   inoculum. *C. humilis* and *S. cerevisiae* were incubated at 30 °C, 250 rpm for about 24 h and 20 h,
82   respectively, to obtain cultures at late exponential or early stationary phase of growth.

83   Prior to PEF treatments, yeasts were harvested by centrifugation, washed in potassium phosphate
84   buffer (PB, K_2HPO_4-KH_2PO_4, 4 mM, pH 6.5, conductivity: 0.5~0.6 mS/cm), and then resuspended
85   in PB, or PB with 10 µM or 100 µM of propidium iodide (Acros Organics, New Jersey, USA). The
86   optical density (O.D.) of cell suspension in PB buffer at 600 nm was adjusted to 1.00±0.05. The
87   electrical conductivity was measured with an electrical conductivity meter (Hanna Instruments,
88   Laval, Québec, Canada). The cell suspensions were kept at room temperature prior to PEF
89   treatments.

2.2 PEF treatment apparatus

91   Continuous PEF treatments (**Fig. S1** of the online supplementary material) were performed using
92   a custom-built laboratory scale PEF processing system (HIECO Ltd., Calgary, Alberta, Canada).
93   The system has a touchscreen two-syringe pump (Cole-Parmer Canada Company, Montreal,
94   Quebec, Canada) to infuse fluid to the treatment chamber through tubing. One syringe was filled
95   with PB buffer and directed the sheath flow to the two sides of the treatment chamber to avoid
96   exposing cells to the inhomogeneous fringe fields resulting from the roughness of the spacers. The
97   other syringe was filled with cell suspension and directed the flow to the central area of the
98   treatment chamber where the laminar fluid flow was undisturbed by the edges of the treatment
99   chamber. The combined flow rate was 0.5 mL/min, except for tests for critical field strength
100   determination where the combined flow rate was set at 0.25 mL/min. The PEF apparatus has a
101   system DC power supply (model 6655A, Hewlett Packard, Palo Alto, California, USA), and the
102   maximum output voltage and current are 120 V and 4 A, respectively. Square pulses were
103   generated by a function/arbitrary waveform generator (model 33220A, 20 MHz, Agilent
104   Technologies, Santa Clara, California, USA) and delivered by a custom-made source that delivers
105   rectangular pulses with 4 – 6 ns rise/fall times and up to 100 V in amplitude. In keeping with past
106   reports [20,21,22], the pulse shape and width were monitored by a digital storage oscilloscope
107   (model TDS2014B, 100 MHz, 4 channel, Tektronix, Beaverton, Oregon, USA) and logged in a
108   computer simultaneously. The applied voltage, the current across the treatment chamber, and the
109   resistance and capacitance were recorded by a 6.5 digit precision multimeter (model 8846A, Fluke
110   Corporation, Everett, Washington, USA) and logged in the computer simultaneously. All PEF
111   treatments done in this study were with an initial fluid temperature of 20±2 °C (inlet temperature,
112   T_{in}), and the maximum processing temperature was at 32 °C (outlet temperature, T_{out}). The fluid
113   temperatures before (T_{in}) and after (T_{out}) PEF treatment were measured by thermocouples placed
114   at the entrance and exit of the treatment chamber, monitored by digital multimeters (model
115   U1233A, Agilent Technologies), and logged in the computer simultaneously. The fluid
116   temperature increase was lineally correlated to the increase of specific energy input (**Fig. S2**). A
117   high accuracy digital pressure gauge (Omega, Laval, Quebec, Canada) was connected between
118   one syringe and the tubing to monitor the pressure of the flow system and to ensure the pressure
119   was stable before collecting samples.

120   Unless otherwise specified, bare gold electrodes were used for PEF treatments. These electrodes
121   were predominantly resistive and were employed for low-impedance electroporation (LIE).
122   Alternatively, electrodes covered with a thin dielectric barrier (alumina, Al_2O_3) were employed

(**Fig. S3**). These were predominantly capacitive to allow high-impedance electroporation (HIE) [23]. The dielectric barrier of HIE electrodes reduced the flow of conduction current through the fluid to near zero while maintaining the electric field. All treatment chambers used in this study were microfluidic chambers with parallel plate electrodes with an area of 2 mm^2 and a gap distance of 13 – 14 μm. When unipolar square pulses were applied to LIE or resistive electrodes (**Fig. 1A**), the conduction current through the fluid section was constant and in phase with the voltage, and the electric field was in steady state. When the same square pulses were applied to HIE or capacitive electrodes, dielectric barriers acted as capacitor, and continued to charge in an exponential process at the rate related to the characteristic scaling time (referred to as time constant, $\tau = 53$ ns). To fully charge the capacitor, it took approximately five of these intervals. A transient displacement current flows through the capacitor during this time that is predominantly out of phase with the potential across the dielectric barriers. The discharging process was reverse to the charging process, and resulted in a transient electric field across the fluid section in the direction opposite to the original. Overall, the rectangular pulse applied to HIE electrodes resulted in a bipolar exponentially decaying pulse across the fluid section (**Fig. 1C**). To obtain comparable bipolar exponential pulses on LIE electrodes, the LIE electrodes were equipped with a capacitor in series; the time constant (τ) of bipolar exponential pulses on LIE electrodes was adjusted by variation of the capacitance. In this configuration, square pulses applied to the LIE electrodes also resulted in bipolar exponential pulses with a time constant of $\tau = 86$ ns (**Fig. 1B**). Even if the voltage drop over the field section cannot be measured directly for exponential pulses, it can be very accurately estimated using the known parameters electrode area, distance between the electrodes, thickness of the alumina layer, and dielectric permittivity of the alumina and of the aqueous suspension. The calculation accounted for the voltage dissipation that relates to the 100 nm alumina layer in the high impedance electrodes.

PEF processes transferred energy to the fluid and thereby increased the fluid temperature. Energy dissipation U_p as Joule heating from a single pulse of width Δt in a fluid of conductivity σ, can be expressed as

$$U_p = \int_0^{\Delta t} \sigma E_s^2(t)\, V\, dt, \tag{1}$$

where $E_s(t)$ is the electric field over the fluid section, and V is the volume of the treatment chamber. For a LIE device powered by a square pulse (**Fig. 1A**), electric field was largely constant over the duration of the pulse Δt, so that $E_s(t) = E_0$ and Eq. (1) simplifies to

$$U_p^{rect} = E_0^2 \sigma V \Delta t. \tag{2}$$

For bipolar exponentially decaying pulses over the fluid section (**Fig. 1B and 1C**), total energy dissipation was calculated by replacing $E_s(t)$ in Eq. (1) with $E_0 e^{-t/\tau}$:

$$U_p^{exp} = 2 \int_0^{\Delta t} \sigma E_0^2 e^{-2t/\tau}\, V\, dt = E_0^2 \sigma V \tau \left(1 - e^{-2\Delta t/\tau}\right). \tag{3}$$

When $\Delta t \geq 5\,\tau$, term $e^{-2\Delta t/\tau}$ tends to 0, resulting in

$$U_p^{exp} = E_0^2 \sigma V \tau. \tag{4}$$

When pulses are delivered at the frequency f to the fluid pumped through the system at the flow rate ξ, total number of pulses N delivered to the treatment volume V is equal to

| 162 | $$N = Vf / \xi.$$ | (5) |

163 Specific energy input per unit mass of fluid whose density is ρ is then

| 164 | $$W = NU_p/\rho V = U_p f/\xi\rho.$$ | (6) |

165 Finally, the temperature increase of the fluid under treatment was calculated as

| 166 | $$\Delta T = W/c_W,$$ | (7) |

167 where $c_W = 4.18$ kJ/kg.°C is the specific heat capacity of water.

2.3 PEF treatment

169 One syringe with 10 mL of PB buffer and the other one with 10 mL of cell suspension with or
170 without PI were fed to the treatment chamber continuously at room temperature. When the pressure
171 of the flow system stabilized, 1 mL of sample was collected as untreated sample or initial sample.
172 Subsequently, different PEF treatments were applied and 1 mL of each treated samples were
173 collected using 2 mL sterile Eppendorf tubes. The treatment chamber was used only once, and the
174 tubing was flushed with 20 mL of sterile water at 15 mL/min both before and after each set of test.
175 Before each run of experimental test, the treatment chamber was rinsed with 2 mL of sterile MilliQ
176 water and balanced with 2 mL of sterile PB buffer at flow rate of 0.5 mL/min.

177 2.3.1 Determination of PEF-induced reversible and irreversible permeabilization of yeasts

178 To compare the effects of bleomycin and propidium iodide (PI) on the viability of PEF-treated
179 *C. humilis,* cell suspensions in PB with 100 μM PI or 1 mg/L bleomycin (AdooQ BioScience,
180 Irvine, CA, USA) were processed at electric field strengths ranging from 18 to 71 kV/cm with
181 square pulses of 0.5 μs and 10 kHz, and with exponential pulses of 86 ns and 10 kHz.

182 To compare the effect of PEF treatment on reversible and irreversible permeabilization of the
183 membrane, PI was added to cell suspensions before or after PEF treatment. *C. humilis* and *S.*
184 *cerevisiae* cell suspensions with and without 10 μM PI were processed with unipolar square pulses
185 of 0.5 μs and 10 kHz, and with bipolar exponential pulses with a time constant τ of 86 ns and 10
186 kHz. After treatment, samples with PI were counter-stained with Syto9 (Life Technologies,
187 Burlington, Ontario, Canada); the final concentrations of PI and Syto9 were both 5 μM. The ratio
188 of fluorescence intensity of PI (λ_{EX} of 535 nm; λ_{EM} of 617 nm) over that of Syto9 (λ_{EX} of 485 nm;
189 λ_{EM} of 538 nm) was calculated as a measure of reversible pore formation. Samples treated by PEF
190 in the absence of PI were stained with PI and Syto9 with final concentrations of 10 μM and 5 μM,
191 respectively. The ratio of fluorescence intensity of PI over that of Syto9 was calculated as a
192 measure of irreversible pore formation. A calibration curve was established for each experiment
193 day by mixing cells treated at 80 °C for 10 min with untreated cells in proportions of 0 %, 50 %
194 and 100 %. The fluorescence intensity was measured with a multiwell plate fluorescence
195 spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

196 2.3.2 Effect of PEF treatment parameters on yeast viability

197 To determine the effect of pulse shape, *C. humilis* and *S. cerevisiae* cell suspensions with and
198 without 100 μM of PI were processed with unipolar square pulses of 0.5 μs and 10 kHz, or with
199 bipolar exponential pulses with τ = 86 ns and 10 kHz.

200 To assess the effect of pulse width, *C. humilis* cell suspensions with and without 100 μM PI were
201 processed with unipolar square pulses with pulse widths ranging from 125 ns to 2 μs. To keep the

202 specific energy input constant across treatments, the increase of pulse width was compensated by
203 the decrease of pulse frequency from 40 to 2.5 kHz. In a second experiment, *C. humilis* cell
204 suspensions with and without 100 μM PI were processed with unipolar square pulses and bipolar
205 exponential pulses. To keep the specific energy input constant across treatments, the increase of
206 pulse width was compensated by the decrease of pulse frequency. Square pulses of 125 ns and 250
207 ns were applied at a frequency of 40 kHz and 20 kHz, respectively; exponential pulses with τ =
208 172 ns and 344 ns (2 and 4 times the initial exponential pulse width) were applied at 5 kHz and
209 2.5 kHz, respectively.

210 To determine the critical field strength of PEF treatment against *C. humilis*, cell suspensions with
211 and without 100 μM of PI were processed with unipolar square pulses of 2 μs. The flow rate was
212 set at 0.25 mL/min. To keep the specific energy input constant across treatments with different
213 field strengths ranging from 2 to 18 kV/cm, the decrease of field strength was compensated by the
214 increase of pulse frequency from 1.25 to 80 kHz, from 5 to 320 kHz, and from 10 to 160 kHz for
215 specific energy input at 3, 11 and 21 kJ/kg, respectively.

216 To determine the effect of energy input, field strength and pulse width, *C. humilis* cell suspensions
217 were processed with unipolar square pulses and bipolar exponential pulses at conditions specified
218 in **Table 1**.

219 To determine the effect of conduction current, *C. humilis* and *S. cerevisiae* cell suspensions with
220 and without 100 μM PI were processed with LIE (bipolar exponential pulses with τ =86 ns) and
221 HIE (bipolar exponential pulses with τ = 53 ns) electrodes at 10 kHz (**Fig. 1B and 1C**). *C. humilis*
222 cell suspensions with and without 100 μM PI were processed with HIE electrodes with two
223 sequential pulses per unit time at 10 kHz (**Fig. S4C**). Treatments were performed with electric
224 field strengths ranging from 18 to 71 kV/cm.

2.4 Enumeration of viable cells
226 The PEF-treated and -untreated samples were serially diluted in PB buffer. Appropriate dilutions
227 were plated on YPD agar plates, and incubated at 30 °C for two days. Results were expressed as
228 $\log_{10}$ of the survival fraction (N/N_0), where N_0 is the cell count of untreated sample and N is the
229 cell count of PEF-treated sample. Initial cell counts of *C. humilis* and *S. cerevisiae* were (6.2 ± 0.3)
230 $\times 10^6$ cfu/mL and $(6.3 \pm 0.4) \times 10^6$ cfu/mL, respectively. The detection limit was 10 cfu/mL. The
231 results are shown as means ± standard deviation of three independent experiments.

2.5 Statistical analyses
233 Results are based on at least three independent replicates. Significant differences between
234 treatments at different conditions were evaluated with Student's t-test. An error probability of less
235 than 5% (P<0.05) was considered significant. Linear regression analyses were conducted using
236 Sigmaplot software (Sigmaplot, Version 12.5, Systat Software Inc., San Jose California, USA).

3. Results

3.1 Quantification of reversible and irreversible pore formation
239 This study initially compared the cytotoxicity of PI and bleomycin to electroporated cells (**Fig. 2**).
240 Bleomycin is a membrane-impermeant compound which is cytotoxic only to membrane-damaged

cells [24,25]. PI is a membrane-impermeant nucleic acid binding dye. It has been widely used to assess irreversible membrane damage by quantification of PI fluorescence after lethal or sublethal treatments but its cytotoxicity towards yeasts is not documented. The viability of cells in the presence of 10 μM or 100 μM of PI, or 1 mg/L bleomycin remained unaffected (data not shown), confirming that PI and bleomycin are not cytotoxic unless the membrane is permeabilized. PEF treatment at a specific energy input of less than 12 kJ/kg did not reduce cell counts of *C. humilis* and *S. cerevisiae* unless PI or bleomycin was present (**Fig. 2** and **Fig. 3A**). The viability of PEF-treated cells in the presence of PI or bleomycin decreased even at a low specific energy input (**Fig. 2B**), which indicates that the cytotoxicity of 100 μM PI to membrane damaged cells is similar or higher than that of 1 mg/L bleomycin.

The permeabilization of yeast membrane to PI was also assessed by PEF treatment in the presence of 10 μM PI, followed by quantification of PI fluorescence. The reduction of *C. humilis* or *S. cerevisiae* cell counts in samples exposed to PEF treatment in the presence of 100 μM PI corresponded to an increase of PI fluorescence after PEF treatment in presence of 10 μM PI (data not shown). Likewise, the reduction of *C. humilis* or *S. cerevisiae* cell counts in samples exposed to PEF treatment alone corresponded to an increase of PI fluorescence after PEF treatment (data not shown). Quantification of PI fluorescence after PEF treatment in the presence of 10 μM PI, and the enumeration of viable cells after PEF treatment in the presence of 100 μM PI thus represent alternative methods for quantification of reversible pore formation. The determination of viable cells after PEF treatments with and without PI provides a lower limit of detection, and was routinely used to assess the difference between reversible and irreversible electroporation.

3.2 Effect of pulse shape on PEF inactivation efficiency

Square pulses and exponential pulses are two pulse shapes that are often used in PEF inactivation experiments [26]. The effect of pulse shape on PEF inactivation efficiency of yeasts was determined by treatments with square or exponential pulses at a constant energy input (**Fig. 3**). The viability of *S. cerevisiae* was unaffected by PEF treatments in the absence of PI, while the viability of *C. humilis* was reduced if the specific energy input exceeded 12 kJ/kg (**Fig. 3A**). PEF treatments in the presence of PI reduced cell counts of *S. cerevisiae* and *C. humilis* when the specific energy input exceeded 5 and 3 kJ/kg, respectively (**Fig. 3B**). The reduction of cell counts was similar when treatments with square pulses and exponential pulses were normalized with respect to specific energy input (**Fig. 3B**). These data demonstrate that exponential and square pulses of equivalent specific energy input have equivalent effects on membrane pore formation by PEF. *C. humilis* was more sensitive to PEF when compared to *S. cerevisiae*, and was subsequently used as model organism.

3.3 Effect of pulse width on PEF inactivation efficiency

A vast majority of past studies on microbial survival after PEF exposure used pulse widths ranging from 1-10 μs [27,28,29]. A major benefit of short pulses is the reduced energy input per pulse and the associated lower temperature increase. The effect of pulse width on the lethality of PEF was determined by treatment with 13 and 52 kJ/kg in the absence of PI (**Fig. 4).** All treatments at a specific energy input of 52 kJ/kg achieved a cell count reduction of about 4.2 log. Treatments at a specific energy input of 13 kJ/kg had a significantly increased lethality when the pulse width was increased from 0.125 to 2 μs (**Fig. 4**). A second experiment employed square pulses and exponential pulses with two different pulse widths or time constants, respectively. In this second

284 experiment, the pulse width had no effect or inconclusive effects on microbial survival (data not
285 shown). Taken together, results indicate that the pulse width has a significant but relatively minor
286 effect on the lethality of PEF.

3.4 Critical field strength of PEF treatment against *C. humilis*

288 Based on the electromechanical model for PEF electroporation mechanism proposed by
289 Zimmermann *et al.* in 1973 [2], cell membrane electroporation occurs only when the external field
290 imposes a transmembrane potential that is equal to or greater than the rupture potential. The critical
291 field strength of PEF treatment against *C. humilis* was determined by treatments with square pulses
292 at a constant specific energy input (**Fig. 5**). PEF treatments in the absence of PI significantly
293 (P=0.02) reduced cell counts only when specific energy input exceeded 11 kJ/kg and field strength
294 exceeded 9 kV/cm. PEF treatments in the presence of PI significantly (P=0.03) reduced cell counts
295 when specific energy input exceeded 3 kJ/kg and field strength exceeded 4 kV/cm. The critical
296 field strengths of PEF treatments against *C. humilis* were thus 4 and 9 kV/cm for reversible and
297 irreversible electroporation, respectively. The cell radius of *C. humilis* in the short axial direction
298 is about 2-3 μm and 6-10 μm in the long axial direction. The minimal potential needed to cause
299 reversible and irreversible electroporation against *C. humilis* can thus be estimated as 0.8 V and
300 1.8 V, respectively.

3.5 Analysis of the factors describing the lethality of PEF

302 Past studies evaluated the effect of single parameters on the lethality of PEF [30,31,32]; however,
303 the parameters energy input, electric field strength, and pulse shape, frequency, and width are
304 interdependent. Therefore, we evaluated the lethality of PEF on *C. humilis* with 22 different
305 combinations of field strength, energy input, and pulse width (**Table 1**). Data were plotted as a
306 function of specific energy input (**Fig. 5A**), electric field strength (**Fig. 5B**), and pulse width (**Fig.**
307 **5C**). Field strength or pulse width, when considered on their own, explain only a small part of
308 variation in process lethality (**Fig. 5B and 5C**). The percentage of variability explained by these
309 parameters was only 10% and 0.3%, respectively. Treatments with a specific energy input of less
310 than 12 kJ/kg reduced the viable cell counts of *C. humilis* by less than 1 log(cfu/mL) even at high
311 field strength (**Fig. 5B**). When only treatments with a specific energy input of greater than 12 kJ/kg
312 were considered, the field strength explained 59% of the variation in the process lethality **(Fig.**
313 **5B)**. Conversely, specific energy input, the only parameter that includes the information about all
314 other parameters, explains most of the variation in process lethality (r^2 of 0.84, **Fig. 5A**). The
315 specific energy input thus represents the most useful gauge of the lethality of PEF.

3.6 Effect of conduction current on PEF inactivation efficiency

317 The fluid between two electrodes is conductive, allowing ionic current to flow and generate ohmic
318 heating. LIE electrodes allow bare metal (gold) to be in contact with the electrolyte, which
319 facilitates electron injection. The resulting conduction current can introduce electrolysis and other
320 electrochemical reactions at the electrode surface. HIE electrodes are made of alumina-covered
321 gold. The alumina layer limits electron injection to values that all but eliminate electrochemical
322 reactions at the electrodes. This experiment was conducted to determine whether conduction
323 current plays a role in electroporation. PEF treatments in the absence of PI (**Fig. 6A**) were not
324 lethal to either *S. cerevisiae* or *C. humilis*. PEF treatments in the presence of PI (**Fig. 6B**)
325 demonstrated that treatments with LIE and HIE electrodes had a comparable lethality when
326 normalized to the specific energy input. Although voltage across the fluid in HIE electrodes cannot

327   be accessed by direct measurement and was thus estimated numerically, this result suggests that
328   conduction current is not essential for the electroporation of yeasts in phosphate buffer by PEF.
329   The use of single and two sequential bipolar exponential pulses also had no influence on the
330   process lethality **(Fig. 6B)**. The results further confirmed, however, that *C. humilis* is more
331   sensitive to PEF, and that a specific energy input of about 3 and 12 kJ/kg are required to achieve
332   reversible and irreversible electroporation, respectively.

4. Discussion and conclusion

334   Energy input, electric field strength, pulse width, and the number of pulses or pulse frequency are
335   interdependent, therefore, simultaneous variation of these parameters allows identification of the
336   factors which best describe the lethality of PEF treatments. The present study systematically varied
337   the specific energy input, the electric field strength, and pulse shape and width. We also compared
338   the lethal effect square pulses and exponential pulses. Experiments with two different pulse shapes
339   implemented with two different electrode systems provided consistent results, and demonstrated
340   that the specific energy input best described the efficacy of PEF. The critical field strength and
341   specific energy input for reversible and irreversible electroporation of *C. humilis* were 2 kV/cm
342   and 3 kJ/kg, and 9 kV/cm and 12 kJ/kg, respectively. The field strength was significantly correlated
343   to process lethality against *C. humilis* only when considering treatments with energy input that was
344   higher than a critical energy input of 12 kJ/kg. Accordingly, achieving the same specific energy
345   input by adjustment of electric field strength, pulse width or the pulse frequency achieves
346   comparable process lethality.

347   The three main types of PEF treatment chambers, namely co-field [11], coaxial [33] and parallel
348   plate [14], differ substantially with respect to the homogeneity of treatment conditions. Compared
349   to other treatment chambers, parallel plate electrodes provide more uniform electric fields and
350   temperature distribution [14]. The small dimensions of the electrode and the application of a sheath
351   flow to keep yeast cells in the center of the treatment chamber where the field is uniform provide
352   additional measures to ensure homogenous treatment conditions for each microbial cell. The
353   experimental equipment used in this study thus provides an excellent tool to determine parameters
354   governing the lethality of PEF on yeast cells.

355   Direct metal-electrolyte contact allows electron injection to the treatment medium [34]. The
356   resulting electrolysis of water causes formation of hydrogen or oxygen bubbles, which may disturb
357   the electric field [35]. Electrolysis also results in a deposit of corrosion products on the electrode
358   surface which may contaminate food products [36]. Covering the metallic electrodes with a layer
359   of insulating material, e.g. alumina, prevents electrode corrosion [23,37] while movement of ions
360   at the membrane solution interface in response to the electrical field still occurs. Although the
361   voltage across the fluid during treatment with exponential pulses was not measured but estimated
362   numerically, our study indicates that electroporation by PEF with bare electrodes and alumina-
363   covered electrodes is comparable. Conduction current is thus not necessary for electroporation,
364   and contamination of PEF-treated medium or food products by electrode corrosion products can
365   be prevented.

366   Propidium iodide (PI), a nucleotide-binding probe excluded by intact cells, is a good indicator of
367   membrane permeabilization introduced by PEF treatments [22,38]. Our study demonstrated that
368   PI shows similar cytotoxicity to membrane-damaged cells as bleomycin, a known cytotoxic
369   compound [24,25]. Enumeration of viable cells of PEF-treated samples in the presence and

370 absence of 100 μM of PI correlated to quantification of pore formation by fluorescent detection,
371 and thus provides an alternative method for determination of reversible and irreversible pore
372 formation by PEF treatments.

373 Past studies plotted the lethality of PEF as a function of number of pulses, electric field strength,
374 or pulse width [9,10,30,31,32,39]. An increase of field strength, pulse width, or the number of
375 pulses also results in an increase of the specific energy input. In the present study, we report that
376 an increase of specific energy input increases the lethality of the PEF treatment and that this
377 increase of process lethality is largely independent on whether the increase was achieved by
378 increasing the number of pulses, pulse width, or field strength. To compare this conclusion with
379 literature data, **Table 2** summarizes past studies that investigated PEF inactivation of *S. cerevisiae*.
380 Literature data on the inactivation of *C. humilis* is scarce. Past studies document a large variability
381 of the process lethality even if the reported process parameters (field strength, pulse frequency or
382 energy input) are comparable. Differences in pH or process temperature, or the inhomogeneity
383 with respect to electric field and temperature distribution, and the flow characteristics [40] only
384 partially account for this large variation in the lethality of PEF reported in past studies.
385 Nevertheless, literature data allow the conclusion that the lethality of PEF is low unless PEF are
386 combined with low pH, a processing temperature higher than 40 °C, or an energy input exceeding
387 60 kJ/kg (**Table 2**). We observed no cell death of *S. cerevisiae* after treatment with phosphate
388 buffer at pH 6.5 with the energy input of 50 kJ/kg or less and electric field strengths of 71 kV/cm.
389 These data conform to literature data that was generated at neutral pH and ambient temperature.
390 The use of PI, however, demonstrates that reversible electroporation of *S. cerevisiae* is achieved
391 at a specific energy input ranging from 5-50 kJ/kg. This reversible electroporation may account
392 for the effect of lower pH or higher temperature on the lethality of PEF. Metabolic consequences
393 of electroporation are more severe at a low pH because reversible pore formation may result in
394 dissipation of vital proton gradients before membrane pores are resealed. The cytoplasmic pH of
395 *S. cerevisiae* is closely linked to cellular viability [41]. A temperature of 40 °C is not lethal to
396 *S. cerevisiae*, however, even a modest increase in temperature will increase membrane fluidity
397 which enhances electroporation [42,43]. An increase in membrane fluidity also increased the time
398 required for resealing of the membrane after electroporation [44,45,46]. Taken together, literature
399 data and the present study indicate that inactivation of *S. cerevisiae* by PEF requires a high energy
400 input, or a combination of PEF with enhanced temperature, acidic condition, or cytotoxic
401 compounds.

402 The critical field strengths tested in this study for *C. humilis* were 0.8 V and 1.8 V for reversible
403 and irreversible electroporation, respectively, matching theoretical values (0.2-1.7 V) proposed
404 previously [6]. Since the thickness of microbial cytoplasmic membranes is constant (about 5-10
405 nm), the critical field strength depends on the compressibility and permittivity of the membrane,
406 the initial temperature of treatment medium and the valence of the ions [7,14,47]. The size and
407 shape of *C. humilis*, about 2-3 μm × 6-10 μm, is similar to that of *S. cerevisiae,* about 3-4 μm × 5-
408 9 μm, so it is reasonable to assume their critical field strengths are also similar. The critical field
409 strength for *S. cerevisiae* was reported as 4.7 to 8 kV/cm [48,49], matches values obtained in our
410 study.

411 The present study and previous studies relating PEF lethality to the specific energy input employed
412 field strengths that are higher than the critical field strength that is required to induce the critical
413 transmembrane potential of 0.8 – 1.8 V [this study and 6]. If this condition is met, modeling of
414 PEF lethality on *S. cerevisiae* and other microorganisms in food is achieved with a high degree of

415 correlation between experimental and predicted values when using specific energy input as control
416 parameter [29,50]. It is thus recommended to plot PEF inactivation efficiency as a function of
417 specific energy input. This parameter also provides guidance with respect to the processing cost,
418 which are mainly determined by the energy use.

419 In conclusion, specific energy input was the parameter that best described reversible
420 electroporation and lethality of PEF treatment of yeasts; the effects of electric field strength, pulse
421 width and frequency, or pulse shape was significant but much less pronounced. Conduction current
422 across the electrode surface is not necessary to achieve electroporation by PEF. In order to increase
423 cost-effectiveness ratio, it is recommended to combine PEF technology with modest thermal
424 treatment or cytotoxic compounds.

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Conflict of interest

431 The authors declare no conflict of interest.

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Figure legends:

434 **Fig. 1.** Examples for pulse waveforms and current flow used in this study. The voltage setting at
435 the power supply is represented by dotted black lines; the resulting voltage at the electrodes is
436 represented by grey lines; the resulting current in the treatment chamber is represented by a solid
437 black line. (**A**) Square pulse applied to LIE electrodes. (**B**) Square pulse applied to LIE electrodes
438 with a capacitor. (**C**) Square pulse applied to a HIE or capacitive device. Panel **B** and **C** show half
439 of the bipolar exponential pulse. The time constant (τ) indicates when the voltage has decayed to
440 $1/e$ of the maximum amplitude.

441 **Fig. 2.** Cytotoxicity of bleomycin (1 mg/L) and propidium iodide (100 µM) to PEF-treated
442 *C. humilis*. Panel **A**: PEF treatment with square pulses of 0.5 µs and 10 kHz; Panel **B**: PEF
443 treatment with exponential pulses of 86 ns and 10 kHz. (▲) PEF treatment in the presence of
444 1 mg/L of bleomycin; (Δ) PEF treatment in the presence of 100 µM of propidium iodide. The
445 results are shown as means ± standard deviations of three independent experiments. Without PEF
446 treatment, the viability of *C. humilis* in the presence of 1 mg/L of bleomycin or 100 µM of
447 propidium iodide remained unaffected ($n=6$). N_0 is the cell count of untreated sample and N is the
448 cell count of PEF-treated sample. Significant differences (P<0.05) between treatments at the same
449 energy input and field strength but in the presence of different compound are indicated by a star.

450 **Fig. 3.** Effect of pulse shape on the inactivation of *C. humilis* and *S. cerevisiae* by PEF. Panel **A:**
451 PEF treatment alone; Panel **B:** PEF treatment in the presence of 100 µM of propidium iodide.
452 Symbols indicate treatment combinations as follows: *S. cerevisiae* (○, ●); *C. humilis* (Δ, ▲);
453 square pulses of 0.5 µs and 10 kHz (●, ▲); exponential pulses of 86 ns and 10 kHz (○, Δ). The
454 specific energy input was adjusted by setting the field strength to 18, 36, 54 and 71 kV/cm. N_0 is
455 the cell count of untreated sample and N is the cell count of PEF-treated sample. Results are shown
456 as means ± standard deviations of three independent experiments.

457 **Fig. 4**. Effect of pulse width on the inactivation of *C. humilis* by PEF with square pulses. **Black**
458 **bars:** electric field strength and specific energy input were 36 kV/cm and 13 kJ/kg, respectively;
459 **Grey bars:** electric field strength and specific energy input were 71 kV/cm and 52 kJ/kg,
460 respectively. Results are shown as means ± standard deviations of three independent experiments.
461 N_0 is the cell count of untreated sample and N is the cell count of PEF-treated sample. Significant
462 differences between treatments at the same energy input and field strength but with different pulse
463 width are indicated by a bracket and the corresponding P-value.

464 **Fig. 5.** Critical field strength of PEF treatment against *C. humilis*. Panel **A**: PEF treatment alone;
465 Panel **B**: PEF treatment in the presence of 100 µM propidium iodide. Symbols stand for PEF
466 treatments with specific energy input at 3 kJ/kg (Δ), 11 kJ/kg (○) and 21 kJ/kg (□). Significant
467 differences (P<0.05) between treatments at the same energy input and pulse width but with
468 different field strength are indicated by a bracket and a star. N_0 is the cell count of untreated sample
469 and N is the cell count of PEF-treated sample. Results are shown as means ± standard deviations
470 of three independent experiments.

471 **Fig. 6.** Effect of specific energy input, electric field strength and pulse width on the inactivation
472 of *C. humilis* exposed to PEF treatment alone. Data were plotted as a function of specific energy
473 input (Panel **A**), field strength (Panel **B**) and pulse width (Panel **C**). Panel **A:** Treatments were
474 carried out with exponential pulses of 86 ns (■), 172 ns (□) and 344 ns (♦), and with square pulses
475 of 0.5 µs (●), 1 µs (○), 2 µs (▲) and 4 µs (Δ). Panel **B** and **C:** PEF treatments were carried out

476 with exponential pulses and a specific energy input of 8 kJ/kg (▼), and with square pulses and a
477 specific energy input of 11.5 (∇), 23 (■) and 46 kJ/kg (□) **(Table 1)**. Lines show linear regression
478 lines. The regression coefficients for data plotted in Panels **A**, **B** and **C** were 0.84, 0.10 and 0.003,
479 respectively. The regression coefficient for data plotted in Panel **B** was 0.59 when only with an
480 energy input higher than 12 kJ/kg were considered in the regression. N_0 is the cell count of
481 untreated sample and N is the cell count of PEF-treated sample. Results are shown as means ±
482 standard deviations of three independent experiments.

483 **Fig. 7.** Effect of conduction current on the inactivation of *C. humilis* and *S. cerevisiae* by PEF.
484 Panel **A:** PEF treatments alone; Panel **B**: PEF treatments in the presence of 100 μM propidium
485 iodide. Symbols indicate treatment combinations as follows: *S. cerevisiae* (○, ●); *C. humilis* (▲,
486 Δ, ▼); LIE electrodes with pulses of 86 ns (○, Δ); HIE electrodes with pulses of 53 ns (●, ▼);
487 HIE electrodes with pulses of 53 ns and adjusted specific energy input by doubling the number of
488 pulses (▲). Pulse frequency and pulse shape were constant throughout, 10 kHz and exponential
489 decay pulses, respectively. Field strength was adjusted according to specific energy input from 18
490 kV/cm to 71 kV/cm. N_0 is the cell count of untreated sample and N is the cell count of PEF-treated
491 sample. Results are shown as means ± standard deviations of three independent experiments.

492

493 **Table 1** Treatment parameters to determine the effect of specific energy input, electric field
494 strength, and pulse width on the inactivation efficiency of PEF treatments.

Specific Energy Input / kJ/kg	Pulse Frequency / kHz	Exposure time per pulse / μs[a]				Pulse Shape
		0.5	1	2	4	
		Electric Field Strength / kV cm^{-1}[b]				
8	5	–[c]	–	71	50	Exponential[1]
	10	–	71	50	36	
11.5	5	50	36	–	18	Square
	10	36	–	18	–	
23	5	71	50	36	–	Square
	10	50	36	–	18	
46	5	–	71	50	36	Square
	10	71	50	36	–	

495 [a]Time constants of exponential pulses with exposure time of 1, 2 and 4 μs were 86, 172, 344 ns,
496 respectively.

497 [b]The electrical field strength was calculated based on a voltage setting of 100, 75, 50, or 25V at
498 the power generator; the average gap distance across the fluid section was 14 μm. For square
499 pulses, the resulting voltage at the electrode is shown Figure 1A. For exponential pulses, the
500 voltage drop over the field section cannot be measured directly but can be very accurately
501 estimated using the known parameters electrode area, distance between the electrodes, and the
502 dielectric permittivity of the aqueous suspension (Figure 1B).

503 [c] –, not done.

504

505 **Table 2** Parameters of PEF treatments used to inactivate *S. cerevisiae* in past studies

Suspension medium	δ / mS cm^{-1}	pH	Log (N_0/N)	Energy density / kJ L^{-1}	T_{outlet}[1] / °C	E / kV cm^{-1}	Pulse width / μs	Reference
Apple juice	–	–	3.5	23	25	15	–	[51]
	2.24	–	6	28	30	50	2.5	[26]
	–	–	3.5	33	8	12	60	[10]
	2.6	3.5	3	50	35	20	2	[29]
	1.85	–	4	92.4	30	20	2	[32]
	2.1	3.7	5.4	336	57	40	1	[52]
Grape juice	0.92	6	6	32.4	41.5	27	3	[50]
	0.98	6	5	50.8	39	24	3	[3]
Orange juice	1.23	3.4	6	144	10	12.5	20	[21]
Watermelon juice	3.0	5.3	1	57	51	20	2	[29]
Beer	2.2	–	0.2	312	43	45	1.5	[53]
	1.38	4.2	3.8	530	31.5	35	1.5	[54]
Chinese rice wine	1.9	4.3	4.5	151	39	21	3	[28]
Nutritive treatment medium	4	5	1.8	188	40	25	4	[55]
	4	5	5	188	40	25	4	[56]
NaCl buffer	0.8	7	2	71	25	21	20	[9]
	0.5	6.6	0.8	375	30	25	40	[57]
Ringer solution	1.25	–	6	80	≤70	16	6.2	[11]
Trizma buffer	2	7.2	5	60	37	30	3.6	[27]
	2	7.2	4	764	30	30.9	3.11	[19]
Phosphate buffer	4	7	4	153.4	41	20	4	[58]
Citrate phosphate buffer	2	7	2	120	35	19.5	3.16	[59]

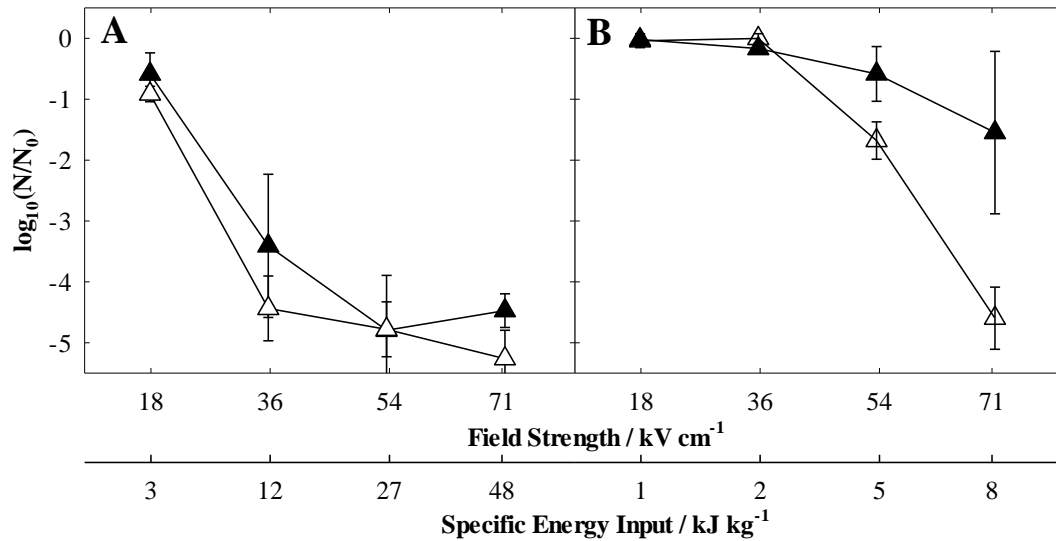506 [1] T_{outlet}: designates the outlet temperature of fluid after PEF treatment
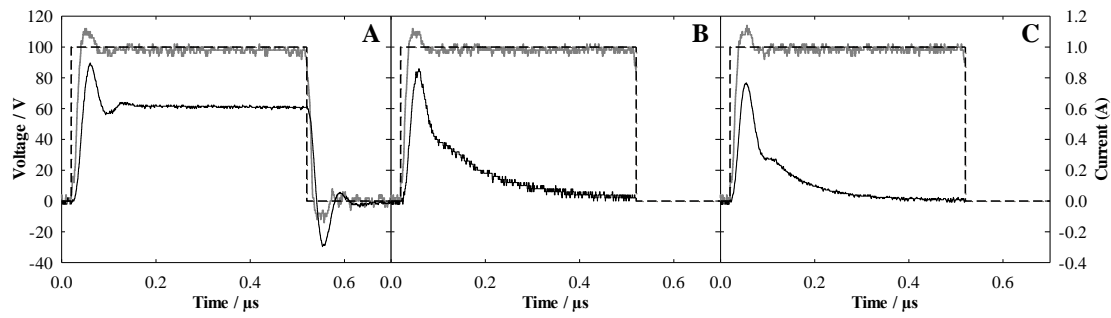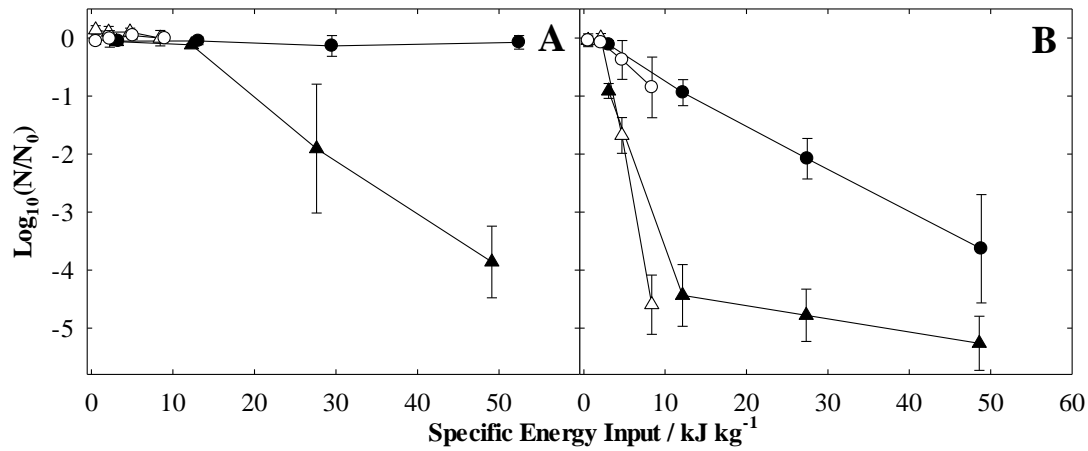
507

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528

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536

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Fig. 4. Effect of pulse width on the inactivation of *C. humilis* by PEF with square pulses. **Black bars:** electric field strength and specific energy input were 36 kV/cm and 13 kJ/kg, respectively; **Grey bars:** electric field strength and specific energy input were 71 kV/cm and 52 kJ/kg, respectively. Results are shown as means $\pm$ standard deviations of three independent experiments. N_0 is the cell count of untreated sample and N is the cell count of PEF-treated sample. Significant differences between treatments at the same energy input and field strength but with different pulse width are indicated by a bracket and the corresponding P-value.
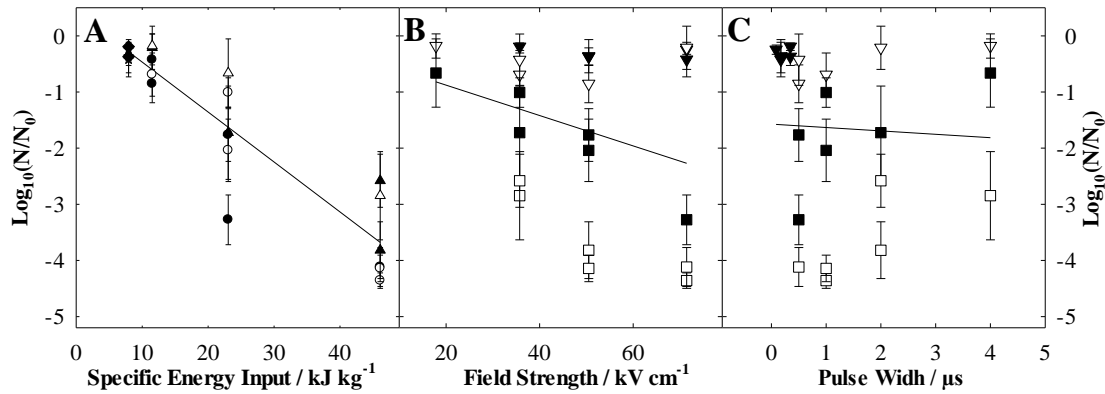
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Fig. 5. Critical field strength of PEF treatment against *C. humilis*. Panel **A**: PEF treatment alone; Panel **B**: PEF treatment in the presence of 100 μM propidium iodide. Symbols stand for PEF treatments with specific energy input at 3 kJ/kg (Δ), 11 kJ/kg (○) and 21 kJ/kg (□). Significant differences ($P < 0.05$) between treatments at the same energy input and pulse width but with different field strength are indicated by a bracket and a star. N_0 is the cell count of untreated sample and N is the cell count of PEF-treated sample. Results are shown as means ± standard deviations of three independent experiments.
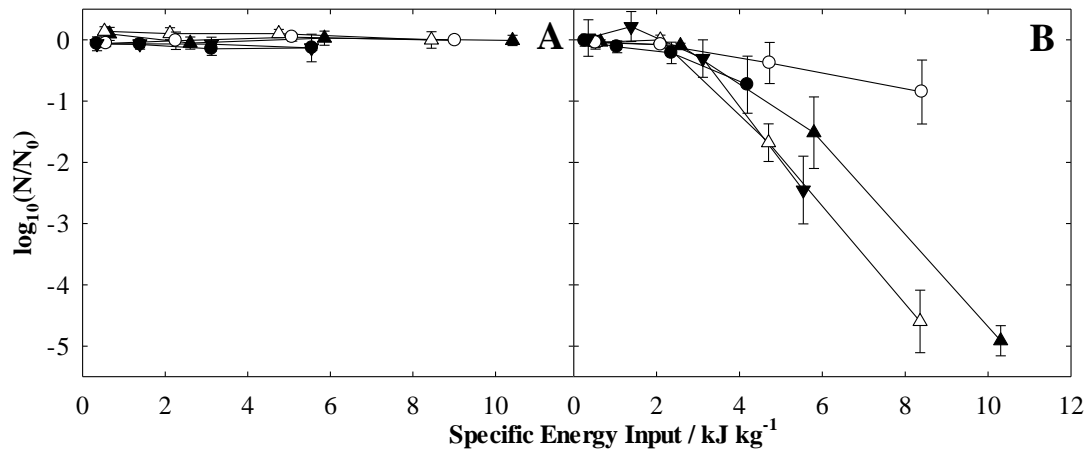
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Fig. 6. Effect of specific energy input, electric field strength and pulse width on the inactivation of *C. humilis* exposed to PEF treatment alone. Data were plotted as a function of specific energy input (Panel **A**), field strength (Panel **B**) and pulse width (Panel **C**). Panel **A:** Treatments were carried out with exponential pulses of 86 ns (■), 172 ns (□) and 344 ns (♦), and with square pulses of 0.5 μs (●), 1 μs (○), 2 μs (▲) and 4 μs (△). Panel **B** and **C:** PEF treatments were carried out with exponential pulses and a specific energy input of 8 kJ/kg (▼), and with square pulses and a specific energy input of 11.5 (▽), 23 (■) and 46 kJ/kg (□) **(Table 1)**. Lines show linear regression lines. The regression coefficients for data plotted in Panels **A**, **B** and **C** were 0.84, 0.10 and 0.003, respectively. The regression coefficient for data plotted in Panel **B** was 0.59 when only with an energy input higher than 12 kJ/kg were considered in the regression. N_0 is the cell count of untreated sample and N is the cell count of PEF-treated sample. Results are shown as means ± standard deviations of three independent experiments.

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Fig. 7. Effect of conduction current on the inactivation of *C. humilis* and *S. cerevisiae* by PEF. Panel **A:** PEF treatments alone; Panel **B**: PEF treatments in the presence of 100 µM propidium iodide. Symbols indicate treatment combinations as follows: *S. cerevisiae* (○, ●); *C. humilis* (▲, Δ, ▼); LIE electrodes with pulses of 86 ns (○, Δ); HIE electrodes with pulses of 53 ns (●, ▼); HIE electrodes with pulses of 53 ns and adjusted specific energy input by doubling the number of pulses (▲). Pulse frequency and pulse shape were constant throughout, 10 kHz and exponential decay pulses, respectively. Field strength was adjusted according to specific energy input from 18 kV/cm to 71 kV/cm. N_0 is the cell count of untreated sample and N is the cell count of PEF-treated sample. Results are shown as means ± standard deviations of three independent experiments.

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