Inactivation of *Saccharomyces cerevisiae* and *Candida humilis* in Potassium Phosphate Buffer by Pulsed Electric Fields

by

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Abstract

Pulsed electric field (PEF) is a promising athermal food preservation technology, having the potential to provide a better balance between food safety and food quality. But the high cost of PEF treatment has limited the commercial application of this technology. The aim of this MSc thesis research was to determine the effect of different parameters on the efficacy of PEF. A custom-built PEF system with a gap distance of approximately 14 μm between two parallel plate electrodes was used to provide a homogeneous electric field and temperature distribution. *Saccharomyces cerevisiae* and *Candida humilis* were used as model microorganisms, and potassium phosphate buffer was used as a model fluid. Viable cell enumeration after PEF treatments in the presence or absence of 100 μM of propidium iodide was used to distinguish reversible and irreversible electroporation.

The electrical parameters governing the efficacy of PEF include pulse shape, pulse width, pulse frequency, electric field strength, and specific energy input. Experiments were designed in a systematic way with the variation of field strength from 18 to 71 kV/cm, specific energy input from 8 to 46 kJ/kg, pulse width from 86 ns to 4 μs, pulse frequency of 5 and 10 kHz, and pulse wave shapes of unipolar square pulse and bipolar exponential pulse. Results demonstrated that the specific energy input had the highest correlation (r^2=0.84) with the lethal effect of PEF, followed by field strength (r^2=0.10) and pulse width (r^2=0.003).

In order to avoid electrolysis during PEF treatments, alumina (Al_2O_3) covered gold electrodes were employed. Results indicate that conduction current was not necessary for electroporating and inactivating cells by PEF, but electrolysis had a strong synergy with PEF. The composition of fluid has the ability to greatly influence the efficacy of PEF.

Preface

This thesis is an original work by Qixing Ou.

Chapter 2 has been submitted as Ou, Q. X., Nikonic-Jaric, M., and Gänzle, M. Inactivation of *Saccharomyces cerevisiae* and *Candida humilis* in potassium phosphate buffer by pulsed electric field technology. *Bioelectrochemistry*. Qixing Ou designed and conducted all experiments and drafted the manuscript. Dr. Michael Gänzle and Dr. Marija Nikonic-Jaric helped with experimental design and revised the manuscript.

Experiments in Chapter 3 were designed and conducted by Qixing Ou with the help of Dr. Michael Gänzle.

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1 Introduction

1.1 Pulsed Electric Field (PEF): a promising athermal food processing technology

Pulsed electric field (PEF) is a promising athermal food preservation technology with the potential to provide a better balance between food safety and food quality (Van Loey *et al.* 2001; Barbosa-Cánovas and Altunakar 2006). PEF systems can be either static or continuous. Compared with other athermal food preservation technologies, PEF is more suitable for conductive and pumpable food products, such as milk, juice, beer, wine, liquid egg, and soup (Qin *et al.* 1995). Most foodborne pathogens and spoilage microorganisms, such as *Listeria monocytogenes* and *Salmonella*, can be eliminated by PEF to provide microbiologically safe foods to consumers (Reina *et al.* 1998; Amiali *et al.* 2007). Some other PEF resistant strains, such as *E. coli*, can be inactivated by the combination of PEF with other suitable hurdles, such as mild heating and natural extracts (Amiali *et al.* 2007). The PEF process transfers almost all of the energy to the fluid thereby increasing the fluid temperature (Spilimbergo *et al.* 2003). Generally, the energy consumption of bactericidal PEF treatments ranges from 100 to 1000 kJ/kg (Toepfl *et al.* 2006). Therefore, energy efficiency is considered to be one of the most critical factors for the commercial introduction of PEF processing as an alternative to thermal processing (Heinz *et al.* 2003; Toepfl *et al.* 2006). The heat generated during PEF treatment is typically insufficient to kill microorganisms but may increase the efficacy of PEF. A temperature increase alters the fluidity and physical stability of cell membranes (Stanley and Parkin 1991), and increases the electrical conductivity of the fluid (Toepfl *et al.* 2007).

Besides the inactivation of microorganisms in the food industry, PEF can also be used to improve the drying rate (Lebovka *et al*. 2007), to extract cellular contents (Guderjan *et al.* 2007), such as juice and oil extraction, to increase the total yield, and to soften fruits and vegetables to

improve the life quality of the aged people. PEF can also be used to transfer genes or plasmids into target cells or tissues (Neumann *et al.* 1982; Basu *et al.* 2014) to express target genes in the host cells, and to treat cancers by electrochemotherapy (Matthiessen *et al.* 2012).

1.2 Theoretical mechanism of PEF

In order to promote the application of PEF technology in biotechnology, biology and medicine, and to optimize working conditions for a variety of purposes, it is important to understand the theories behind the technology. However, until now the mechanism of PEF technology has not been fully elucidated. Generally, it is believed that electroporation is the basis of PEF technology. The most commonly accepted model for the mechanism of electroporation is the Electromechanical Model proposed by Zimmermann *et al.* (1973). This model considers the membrane as an elastic body, and the conditions for pore formation are derived from the balance of electrostatic and elastic forces (Zimmermann *et al.* 1973; Dimitrov 1984). The lipid bilayer membrane is used to model the response of cell membranes exposed to an external electric field. When the lipid bilayer membrane is exposed to an external electric field, it begins to increase the charges on both the inner and outer surfaces of the membrane. When the transmembrane potential reaches the critical value, about 0.2 to 1.7 V (Zimmermann *et al.* 1973; Benz *et al.* 1979; Chen *et al.* 2006), the membrane becomes increasingly thin as a result of the attraction of opposite charges, followed by the formation of pores through the lipid bilayer membrane. According to the theoretical models proposed by Weaver in 1993, the principal steps involved in cell membrane electroporation include: (i) the application of an external electric field which is equal to or greater than the critical value of the cell membrane for pore formation by PEF; secondly, (ii) the build up of charges on the inner and outer surfaces of the cell membrane by ion flow; (iii) rapid and localized membrane structure rearrangement and the transient formation of

hydrophobic pores through the membrane; (iv) transient formation of water fingers (or hydrophilic pores) through the cell membrane; (v) increase of membrane permeabilization to charged ions and molecules; (vi) membrane recovery or cell death (Weaver and Chizmadzhev 1996; Weaver 2003; Chen *et al.* 2006). **Fig. 1.1** shows the schematic graph of this process. Electroporation can be reversible (reseal pores after PEF treatment) or irreversible (leading to cell death), depending on many other factors, including the field strength and the total treatment time of pulses applied (Benz *et al.* 1979). Results from modeling cell membranes with a lipid bilayer membrane showed that relatively low induced transmembrane potential (0.15 to 0.4 V) coupled with longer pulse (milliseconds to seconds) is more likely to cause irreversible electroporation, while higher induced transmembrane potential (0.6 to 1 V) coupled with shorter pulses (100 to 400 ns) is more likely to result in reversible electroporation (Benz *et al.* 1979). Reversible and irreversible electroporation can occur at the same time. However, higher applied voltage does not increase the maximum magnitude of transmembrane potential, because a higher density of pores form to compensate the excess stimulus current across the membrane (Benz *et al.* 1979; DeBruin and Krassowska 1999).

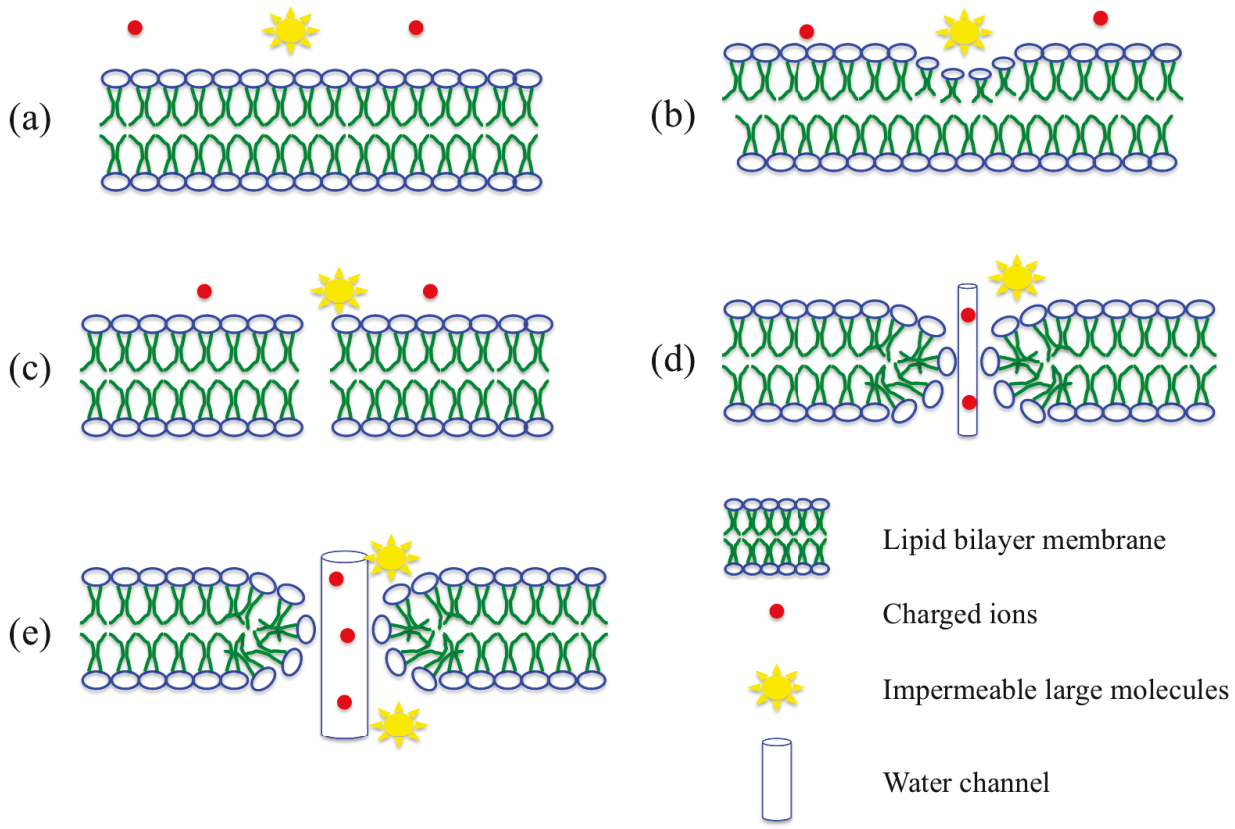

Fig. 1.1 Electromechanical model of electroporation (Zimmermann *et al*. 1973; Weaver 1993). (a) The lipid bilayer membrane is semi-permeable, allowing water and lipid-soluble molecules with low molecular weight to diffuse, while charged ions and large molecules cannot pass through. (b) Application of an external electric field causes the creation of a dimple due to local membrane compression and thinning. (c) The increase of transmembrane potential causes the formation of transient hydrophobic pores through the membranes. (d) Rearrangement of local membrane structure results in the formation of transient hydrophilic pores, which allows small charged ions to pass through the membrane by the small water channel formed. (e) The pore expansion and fusion leads to irreversible electroporation, which allows charged ions and impermeable large molecules to pass through the membrane by the large water channel formed.

The critical field strength of a lipid bilayer membrane is determined by several factors, including pulse width, fluid temperature and ions in the fluid. Firstly, the critical field strength is pulse duration dependent (Dimitrov 1984). Irreversible electroporation usually occurs at longer pulse duration, because pore expansion and fusion, which leads to cell death, is a slow process, on a

time scale of 10~100 μs and higher (Dimitrov 1984). The pores can persist much longer than the pulses, which is likely to be an important mechanism contributing to cell death (Gowrishankar and Weaver 2006). Reversible electroporation is a rather rapid process, on a time scale of 10 μs (Dimitrov 1984). Pore formation itself is on a time scale of 10 ns (Dimitrov 1984; Chen *et al.* 2006), while pore resealing, which leads to cell recovery, is on a time scale of seconds (Chen *et al.* 2006). Secondly, the critical field strength is temperature dependent (Benz *et al*. 1979), decreasing with an increase of the fluid temperature. The critical transmembrane potential of a lipid bilayer membrane dropped from 1.5 to 0.6 V when the fluid temperature increased from 2 to 48 ˚C (Benz *et al*. 1979). The critical transmembrane potential of algae decreased from 1.2 to 0.6 V when the fluid temperature increased from 4 to 30 ˚C (Coster and Zimmermann 1975; Zimmermann *et al.* 1977). Thirdly, certain ions can also influence the critical field strength of the lipid bilayer membrane (Benz *et al*. 1979). Salts with monovalent cations and divalent anions and vice versa, such as $MgCl_2$ and K_2SO_4, and monovalent salts, such as KCl, did not change the critical transmembrane potential of the lipid bilayer membrane for pore formation (Benz *et al*. 1979). However, the addition of divalent salts, such as $MgSO_4$, increased the critical transmembrane potential from 1 to 1.7 V (Benz *et al*. 1979).

1.3 PEF effects on biological cell membranes: do theoretical studies explain the effects?

In order to develop a theory for electroporation, it is helpful to start with a simple model system, such as a lipid bilayer membrane. However, an actual cell membrane is considerably more complex than a planar bilayer membrane with its non-planar shape, a closed topology, a non-zero transmembrane potential induced by active ion pumping, and the presence of membrane proteins (Chen *et al.* 2006). **Fig. 1.2** shows the rest potential across the cell membrane and the cell response to an applied external electric field. Practically, there is no direct proof to show the

existence of pores through the cell membrane after PEF treatment. One study using rapid-freezing electron microscopy showed that there were inverted volcano-shaped membrane openings on the membrane of PEF-treated human red blood cells (Chang and Reese 1990). However, many other facts demonstrate that this theory is plausible, such as the uptake of membrane impermeable dyes and other large molecules (Wouters *et al.* 2001), as well as charged ions (White *et al.* 2004).

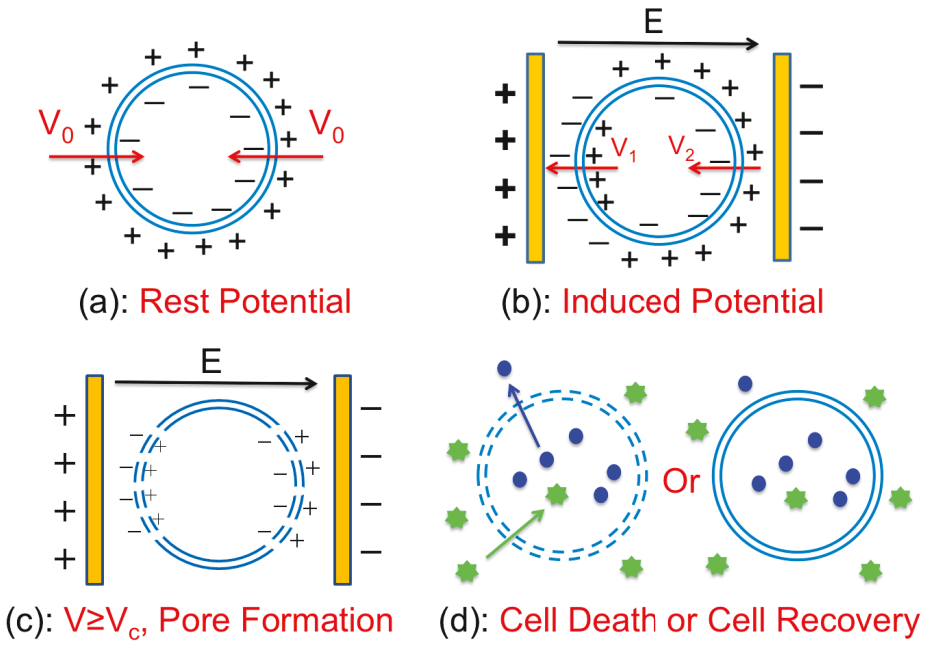

Fig. 1.2 Rest potential of the cell membrane and the response of the cell membrane to an applied external electric field. (a) Rest potential of the cell membrane because of active pumping of ions; the outer and inner surfaces of the cell membrane are positively and negatively charged, respectively. V_0 stands for the rest potential of the cell membrane. (b) and (c) The response of the cell membrane to an applied external electric field. The letter E and the coupled arrow indicate the strength and direction of the applied external electric field between two electrodes, respectively. V_1 and V_2 stand for the transmembrane potentials at each side of the electrodes. Once the transmembrane potential reaches the critical value (V_c) of the cell membrane, electroporation occurs. (d) The electroporation can be reversible and irreversible, leading to cell recovery and cell death, respectively. Once there is pore formation through the cell membrane, interactions between cellular contents (solid circle) and molecules in the fluid (horn star) happen.

Based on the theory of electroporation, electric field strength and pulse duration are the two most important parameters determining the efficacy of PEF (Zimmermann *et al.* 1973; Benz *et al.* 1979; Dimitrov 1984; Weaver 1993; Chen *et al.* 2006). Angersbach *et al*. (2000) studied the effect of PEF on cell membranes in real food systems. They made use of the fact that biological membranes are insulators. Once there were pore formations through the cell membranes induced by PEF, the large conductivity changes in cell membranes and food systems indicated variation in permeability (Angersbach *et al*. 2000). By monitoring the current and voltage simultaneously with a high time resolution, they found that the critical transmembrane potential was about 0.7 to 2.2 V (Angersbach *et al*. 2000), which was greater than the value obtained from artificial membranes (0.2 to 1.7 V). The pore formation was also on the time scale of nanoseconds, which showed consistency with the value detected with a lipid bilayer membrane. The cell membrane recovery was on a time scale of seconds, which was the same as the value obtained with a lipid bilayer membrane. However, the application of a single pulse or repeated pulses with a field strength higher than the critical value was not necessary to cause irreversible electroporation (Angersbach *et al*. 2000), which did not agree with the test done with the model membrane. The dominant process parameter for primary pulse effects (membrane charging process and the initiation of pore formation) was the amplitude of the electric field strength (Angersbach *et al*. 2000). The application of higher electric field strength resulted in earlier occurrence of membrane pore formation. The application of a field strength which was higher than the critical value resulted in a higher transmembrane potential as compared to the application of a field strength which was equal to the critical value (Angersbach *et al*. 2000). These data demonstrated that the dynamics of transmembrane potential build-up vary between the biological cell membrane and the lipid bilayer membrane. The secondary pulse effects, including the opening

time of the pores, the expansion and fusion time of the pores and the recovery time of the pores, were determined by the magnitude and shape of the decaying electric field, the pulse width and the energy intensity (Angersbach *et al*. 2000).

Generally, yeasts are more susceptible to PEF, followed by vegetative bacteria. Spores are highly resistant to PEF treatment. The high sensitivity of yeast to PEF may be because of its large cell size (Grahl and Märkl 1996). The most commonly studied yeast is *Saccharomyces cerevisiae*. **Table 1.1** summarizes the studies of inactivation of *S. cerevisiae* in different treatment media by PEF. However, these data did not show any correlation between the degree of cell count reduction and the treatment parameters, including electric field strength, pulse width, specific energy input, pH and electrical conductivity of the fluid, and the processing temperature. However, the reduction of *S. cerevisiae* was caused by either PEF treatments with high specific energy input, or the combination of PEF treatments with low pH or mild processing temperature. However, the effect of food matrix on the lethal effect of PEF treatment was not determined.

Table 1.1 Parameters of PEF treatments used to inactivate *S. cerevisiae*

Suspension medium	δ (mS/cm)	pH	Log (N_0/N)	Energy density (kJ/L)	T_{outlet}[a] (°C)	E (kV/cm)	Pulse width (μs)	Reference
Apple juice	–	–	3.5	23	25	15	–	Zhang *et al*. 1994a
	2.24	–	6	28	30	50	2.5	Qin *et al*. 1995
	–	–	3.5	33	8	12	60	Zhang *et al*. 1994b
	2.6	3.5	3	50	35	20	2	Timmermans *et al*. 2014
	1.85	–	4	92.4	30	20	2	Cserhalmi *et al*. 2002
	2.1	3.7	5.4	336	57	40	1	Noci *et al*. 2008
Grape juice	0.92	6	6	32.4	41.5	27	3	Huang *et al*. 2014b
	0.98	6	5	50.8	39	24	3	Huang *et al*. 2014a
Orange juice	1.23	3.4	6	144	10	12.5	20	Molinari *et al*. 2004
Watermelon juice	3.0	5.3	1	57	51	20	2	Timmermans *et al*. 2014
Beer	2.2	–	0.2	312	43	45	1.5	Milani *et al*. 2015
	1.38	4.2	3.8	530	31.5	35	1.5	Walkling-Ribeiro *et al*. 2011
Chinese rice wine	1.9	4.3	4.5	151	39	21	3	Huang *et al* 2013
Nutritive treatment medium	4	5	1.8	188	40	25	4	Aronsson and Rönner 2001
	4	5	5	188	40	25	4	Aronsson *et al*. 2001
NaCl buffer	0.8	7	2	71	25	21	20	Sala and Hamilton 1967
	0.5	6.6	0.8	375	30	25	40	Wang *et al*. 2015
Ringer solution	1.25	–	6	80	≤70	16	6.2	Toepfl *et al*. 2007
Trizma buffer	2	7.2	5	60	37	30	3.6	Pataro *et al*. 2011
	2	7.2	4	764	30	30.9	3.11	Donsi *et al*. 2007
Phosphate buffer	4	7	4	153.4	41	20	4	Aronsson *et al*. 2005
Citrate phosphate buffer	2	7	2	120	35	19.5	3.16	Sonolinos *et al*. 2007

[a] T_{outlet}: designates the outlet temperature of fluid after PEF treatment

1.4 Effect of food matrix on PEF effectiveness

PEF treatment done in a real food system is much more complex than that done in a buffer or model food system, because of the presence of proteins, fat, carbohydrates, ions and other compounds. During the process a protein-rich food product by PEF, such as raw milk, a film formed on the surface of the electrodes, especially on the surface of the anode, because of the effect of electrophoretic concentration of charged particles (Bushnell *et al.* 1995). The accumulation of food particles on the surfaces of the electrodes during extended processing periods can cause undesirable side effects, such as electrical breakdown in the treatment chamber, contamination of the PEF system, and the sudden halting of the flow of food products. Because of the presence of ions and other conductive particles, the electrical conductivity of food products is much higher than that of a buffer system or a model food system. The effectiveness of PEF treatment is highly influenced by the electrical conductivity of a fluid (Devlieghere *et al.* 2004). The higher the electrical conductivity, the lower the effectiveness of PEF treatment. When the electrical conductivity of a fluid is high, for a voltage-sourced PEF system, a large portion of the electric field is applied to the fluid instead of the cells, while for a current-sourced PEF system, the strength of the applied electric field decreases, and thus the critical transmembrane potential for cell membrane electroporation is difficult to achieve (Jayaram 2000; Toepfl *et al.* 2007; Huo *et al*. 2010).

1.5 Electrochemistry and high impedance electroporation

Because of the direct contact of conductive electrodes with electrolytes, undesirable side effects from the decomposition of chemical agents or food components and the corrosion of metals happen. The formation and accumulation of gases, such as hydrogen and oxygen, on the surface of electrodes, resulting from the electrolysis of water, can distort or breakdown the electric field

between the two electrodes and even make the fluid flow cease (Sato *et al*. 1996; Saulis *et al*. 2005), and thus PEF treatment homogeneity cannot be achieved. During PEF processing, because of the injection of electrons into the electrolyte and the electrochemical reactions between electrodes and electrolytes, the metallic electrodes may be corroded. In most cases, the electrodes are made of stainless steel, aluminum, iron, carbon or other conductive materials (Roodenburg *et al*. 2005; Saulis *et al*. 2005). Some of the ions are toxic, such as Al^{3+} and Cu^{2+}. At the same time, the durability or lifetime of the electrodes is shortened. In addition, the rough surface of the electrode caused by corrosion cannot generate homogeneous electric fields (Saulis *et al.* 2007). Hydrogen peroxide (H_2O_2) and chlorine gas (Cl_2) are toxic to human cells and animal cells (Sato *et al*. 1996; Saulis *et al*. 2005). Because of the electrophoresis effect of PEF treatment, the fouling or contamination of electrodes and the PEF system may occur as well (Bushnell *et al.* 1995).

Once an electrode is immersed in an electrolyte, electrons transfer between the electrode and the electrolyte, and chemical reactions occur immediately. Reduction and oxidation reactions occur at the same time at the electrode-solution interface. Negatively charged ions are attracted to the surface of the electrode, which causes an electrode-ion double layer to form. The reduction and oxidation reactions can be balanced by the induced electric field at the double layer (Morren *et al*. 2003). However, once a voltage is applied to a pair of electrodes, the thickness of the ionic layers increases and they act as capacitors (Morren *et al*. 2003). If the applied voltage is not high enough to charge the double layer capacitors, electrochemical reactions will not happen. Only when the double layer capacitors are fully charged (over the threshold voltage), will the electrochemical reactions begin (Morren *et al*. 2003). The half-reactions at the two electrodes are independent. The threshold voltage depends on many factors, including the temperature,

electrode material, pH and the chemical composition of the fluid (Morren *et al*. 2003). The half-reaction at one electrode depends on the potential at the electrode-ion double layer. Therefore, the amount of oxidation or reduction reactions correlates to the amount of charges or electrons transferred across the electrode-solution interface. The capacitor charging process depends on the intensity and width of the applied voltage. **Fig. 1.3** shows the structure of the double layer capacitor and the most favourable electrochemical reactions at each electrode.

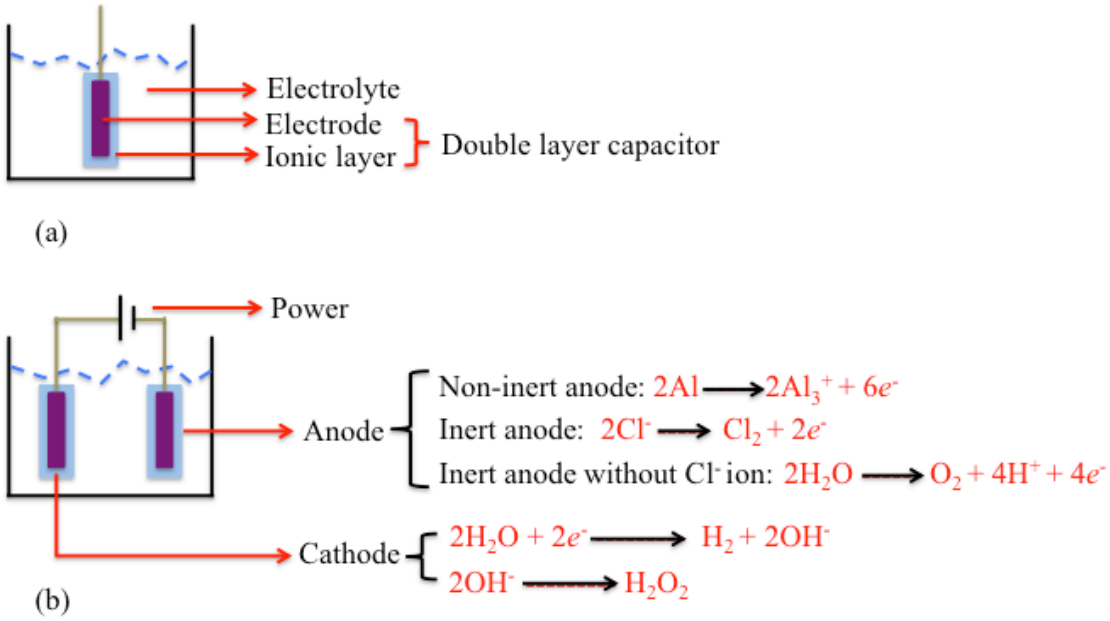

Fig. 1.3 Electrochemical reactions at the electrode-solution interface. (a) The structure of the double layer capacitor formed at the interface between the electrode and the solution (Morren *et al*. 2003). (b) The most favourable electrochemical reactions at the anode and cathode when a voltage is applied to a pair of electrodes (Saulis *et al.* 2005).

Generally, the methods that can discharge or avoid the complete charge of the double layer capacitors have the potential to reduce or minimize the electrochemical reactions during PEF treatments. The most common method used to minimize electrochemical reactions and electrode corrosion is by the application of pulses with high intensity and short duration (Morren *et al.* 2003). Compared with unipolar pulses, bipolar pulses could help minimize electrochemical

reactions and electrode corrosion in a long period of PEF treatment (Morren *et al*. 2003; Roodenburg *et al*. 2005; Saulis *et al*. 2005). Using inert electrodes (Saulis *et al.* 2005), such as gold and platinum, changing the orientation of the anode and cathode, waiting long enough before the application of the second pulse to avoid the cumulative effect of charging (Bushnell *et al.* 1995), and using a strongly buffered solution with low conductivity (Saulis *et al*. 2005) may also work. Another way to reduce electrochemical reactions is by equipping the PEF treatment system with auxiliary circuits to discharge the double layer capacitors after each pulse (Bushnell *et al.* 1995). The application of ion-permeable, membrane-covered electrodes could avoid the direct contact between metallic electrodes and food products, and electron transfers between metallic electrodes and electrolytes could be achieved by ionic electrical connections between them (Dogonadze *et al.* 1977; Dunn and Pearlman 1987).

Covering the bare electrodes with a thin layer of dielectric material can prevent the direct contact of bare electrodes (conductive electrodes) with electrolytes (food products or other conductive fluid), and also avoid the injection of electrons into the fluid. In this case, electrochemical reactions and electrode corrosion could be prevented. However, without the injection of electrons into the electrolyte, how would the covered electrodes, also called high impedance electroporation (HIE) devices (May and Ruben 2012), function during PEF processing? The bare electrodes are conductive, acting as resistors in the electrical circuit, while covered electrodes are capacitive, acting as capacitors in the electrical circuit. The three imperative properties of the dielectric material which can be used to cover bare electrodes include high permittivity, high maximum allowable electric field stress and high volume resistivity, which could substantially forestall conduction current passing through (May and Ruben 2012). The application of a pulsed voltage source to the electrodes could create an electric field across and through the fluid

between the two opposing electrodes. Therefore, the drawbacks of a low impedance electroporation (LIE) device (treatment chamber with bare electrodes), including electrochemical reactions and electrode corrosion, electrophoresis and field shielding by bubble layer formation, could be prevented by the application of a HIE device.

1.6 Effect of electrode configuration on field and temperature distribution homogeneity

In order to achieve consistent results, PEF treatments with accurately defined treatment intensity and homogeneous treatment conditions, including homogeneous electric field strength, flow velocity and temperature distribution, are required (Jaeger *et al*. 2009). The treatment chamber is the key component of a PEF system and its design can greatly influence the effectiveness of PEF treatment (Huang and Wang 2009) because it is the component where an electric field is applied to the fluid. A linear and uniform electric field distribution can be achieved by using parallel plate electrodes (Donsi *et al.* 2007), except for the two edges of the chamber resulting from the roughness of the insulators. However, because of the low electrical resistance of the parallel plate electrodes, there is unwanted high conduction current flow and thus higher energy input is required to maintain the electric field strength across the fluid (Jaeger *et al*. 2009). The conduction current can be avoided by the use of dielectric material-covered electrodes (May and Ruben 2012). In past studies, the typical treatment chamber was a pipe (Yin *et al.* 1997) with co-linear or co-axial electrodes, which was convenient and easy for pumpable food products to pass through. The electric fields generated by these treatment chambers have a less homogeneous field, flow velocity and temperature distribution (Lindgren *et al.* 2002; Jaeger *et al*. 2009). The temperature on the inner surface of electrodes can be 30 to 50 ˚C higher than the average temperature of the fluid (Meneses *et al.* 2011). One of the most important parameters influencing the effectiveness of PEF treatment is the electrical conductivity of the fluid. As described in

section 1.4, high electrical conductivity is counterproductive to the effectiveness of PEF treatment. Moreover, the electrical conductivity is temperature dependent, increasing with the rise of fluid temperature. Therefore, these inhomogeneities substantially confound the assessment of the contribution of an electric field, energy input, and temperature on microbial inactivation (Lindgren *et al.* 2002). However, the homogeneity of flow velocity and temperature distribution can be improved by the insertion of one or more than one grid in the treatment chamber (Cook 1973; Jaeger *et al*. 2009).

1.7 Hypothesis and objectives

Despite the designation of PEF as a "promising emerging technology" in food processing, the number of commercial applications remains limited (Toepfl *et al.* 2007). The reluctant adoption of this technology by the food industry relates to the lack of mechanistic 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 (Sala and Hamilton 1967; Barbosa-Cánovas and Altunakar 2006; Toepfl *et al*. 2007). Membrane electroporation and cell death are determined by additional parameters, including pulse shape, pulse duration and the number of pulses, which corresponds to the total treatment time, the size and shape of the microorganisms, and the properties of the fluid (Sala and Hamilton 1967; Zhang *et al.* 1994b; Toepfl *et al*. 2007). This MSc thesis research aimed to test the hypothesis that the electric field strength and pulse width are not the dominant parameter for the lethal effect of PEF, and that conduction current is not necessary for the efficacy of PEF. The thesis research used a miniaturized and continuous PEF system to determine the contributions of energy input, field strength, and pulse width, shape and frequency on yeast inactivation. *Candida humilis* and

Saccharomyces cerevisiae were used as model microorganisms. This study also investigated the effects of electrolysis and conduction current on the inactivation efficiency of PEF treatment. The treatment chambers used in this study with parallel plate electrodes were able to deliver homogeneous electric fields (Donsi *et al.* 2007). The small scale of the chamber also allowed homogeneous distribution of the temperature during PEF treatment.

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2 Inactivation of *Saccharomyces cerevisiae* and *Candida humilis* in potassium phosphate buffer by pulsed electric fields

2.1 Introduction

Pulsed electric fields (PEF) have the potential to provide minimally processed and microbiologically safe food products (Van Loey *et al*. 2001). PEF can achieve pasteurization temperature, or 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 (Zimmermann *et al.* 1973; Huang *et al*. 2014a). This effect is also termed as electroporation (Saulis 2010). Dielectric permittivity and electrical conductivity of the cell membrane differ from those of cytoplasm and extracellular medium (Agarwal *et al.* 2007), which creates conditions for interfacial polarization. When biological cells are exposed to an external electric field, most of this field concentrates across the membrane (Agarwal *et al*. 2007). When the transmembrane potential reaches a critical value of 0.2~1.7 V, transient pores appear through the membrane (Zimmermann, 1986, Benz *et al*. 1979; Chen *et al.* 2006). The pore formation can be irreversible and lead to cell death if the external electric field is strong enough. Membrane permeabilization and cell death are also determined by pulse shape, duration, and frequency, which corresponds to energy input, the size and shape of microorganisms, and the properties of the treatment medium (Sala and Hamilton 1967; Zhang *et al.* 1994b; Toepfl *et al*. 2007).

PEF treatments transfer energy to the fluid and thus increase its temperature (Spilimbergo *et al.* 2003). The energy consumption of bactericidal PEF treatments ranges from 100 to 1000 kJ/kg (Toepfl *et al.* 2006) and energy efficiency is considered to be a critical factor for commercial use

of PEF processes (Heinz *et al*. 2003; Toepfl *et al.* 2006). Heat generated during PEF treatment is typically insufficient to kill microorganisms but may influence the efficacy of PEF treatment. A temperature increase alters the fluidity and stability of the cell membrane (Stanley and Parkin 1991), and increases the electrical conductivity of the treatment medium (Toepfl *et al*. 2007). An altered conductivity at a constant energy input decreases the field strength (Toepfl *et al.* 2007). In the past studies, the typical treatment chamber was a pipe (Yin *et al*. 1997). These treatment chambers generate non-homogeneous electric fields and temperature distribution; these inhomogeneities substantially confound the assessment of the contribution of the electric field, energy input, and temperature on microbial inactivation (Lindgren *et al.* 2002).

Despite the designation of PEF as "promising emerging technology" in food processing, the number of current commercial applications remains limited (Toepfl *et al*. 2007). 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 (Sala and Hamilton 1967; Barbosa-Cánovas and Altunakar 2006; Toepfl *et al.* 2007). It was therefore the aim of this study to use a miniaturized and continuous 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 (Donsi *et al.* 2007) and to allow a homogeneous distribution of the temperature. *Candida humilis* and *Saccharomyces cerevisiae* were used as model microorganisms.

2.2 Materials and methods

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

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

2.2.2 PEF treatment apparatus

Continuous PEF treatments were performed using a custom-built laboratory scale PEF processing system (**Fig. 2.1**; HIECO Ltd., Calgary, Alberta, Canada). The system has a touchscreen two-syringe pump (Cole-Parmer Canada Company, Montreal, Québec, Canada) to infuse fluid to the treatment chamber through the tubing. One syringe was filled with PB buffer and directed the sheet flow to the two sides of the treatment chamber to avoid exposing cells to inhomogeneous fringe fields (resulting from the roughness of the spacers). The other syringe was

filled with the cell suspension and directed the flow to the central area of the treatment chamber where the laminar fluid flow was undisturbed by the edges of the treatment chamber. The combined flow rate was 0.5 mL/min, except for tests for critical field strength determination where the combined flow rate was set at 0.25 mL/min. The PEF apparatus has a system DC power supply (model 6655A, Hewlett Packard, Palo Alto, California, USA), and the maximum output voltage and current are 120 V and 4 A, respectively. Square pulses were generated by a function/arbitrary waveform generator (model 33220A, 20 MHz, Agilent Technologies, Santa Clara, California, USA). The applied pulse shape and width were monitored by a digital storage oscilloscope (model TDS2014B, 100 MHz, 4 channel, Tektronix, Beaverton, Oregon, USA) and logged into the computer simultaneously. The applied voltage, the output current across the treatment chamber, and the resistance and capacitance were recorded by a 6.5 digit precision multimeter (model 8846A, Fluke Corporation, Everett, Washington, USA) and logged into the computer simultaneously. All PEF treatments done in this study were performed with an initial fluid temperature of 20±2 °C (inlet temperature, T_{in}), and the maximum processing temperature was 32 °C (outlet temperature, T_{out}). The fluid temperatures before (T_{in}) and after (T_{out}) PEF treatment were measured by the thermocouples placed at the entrance and exit of the treatment chamber, monitored by two handheld digital multimeters (model U1233A, Agilent Technologies, Santa Clara, California, USA), and logged in the computer simultaneously. There was a high accuracy digital pressure gauge (Omega, Laval, Québec, Canada) connected between one syringe and the tubing to monitor the pressure of the flow system and to ensure the pressure was stable before collecting samples.

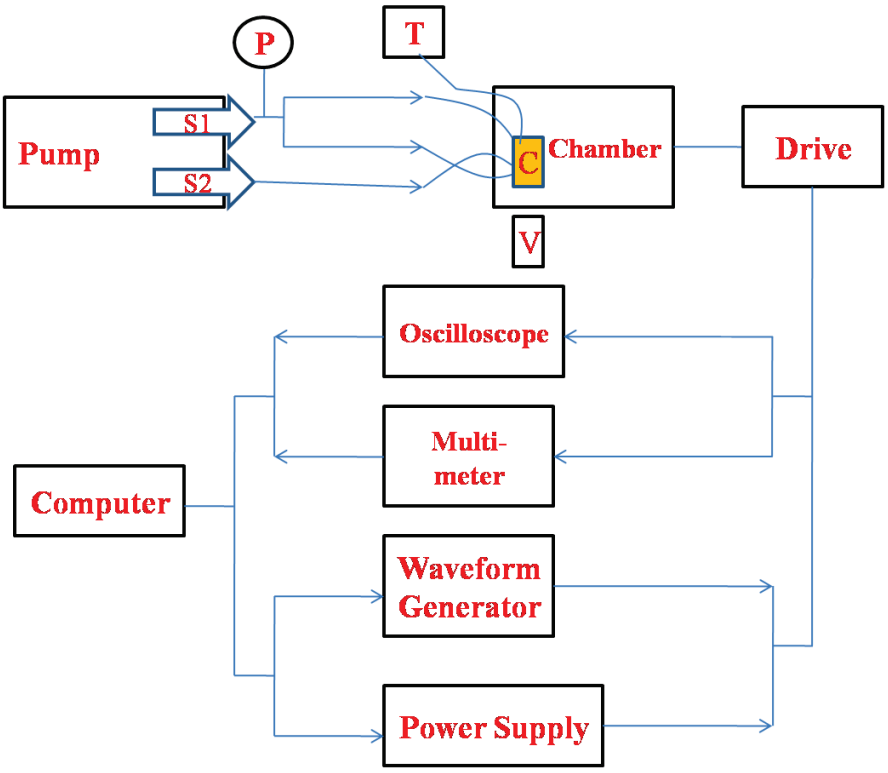

Fig. 2.1 Schematic graph of PEF apparatus. The system has a touchscreen two-syringe pump, a DC power supply, a function/arbitrary waveform generator, a digital storage oscilloscope, a computer, a multimeter, a thermocouple, and a digital pressure gauge. S1 and S2 stand for the two syringes. P and T stand for the pressure gauge and the thermocouple, respectively. C and V stand for the treatment chamber and the Eppendorf tube for sample collecting, respectively.

Unless otherwise specified, bare gold electrodes were used for PEF treatments in this study. These electrodes were predominantly resistive and were employed for low-impedance electroporation (LIE). Alternatively, electrodes covered (**Fig. 2.2**) with a thin layer of dielectric barriers (alumina, Al_2O_3) were employed. These were predominantly capacitive and were employed for high-impedance electroporation (HIE) (May and Ruben 2012). The dielectric barrier of HIE electrodes reduced the flow of conduction current through the fluid while maintaining the electric field. All treatment chambers used in this study were microfluidic chambers with two 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 electrodes (**Fig. 2.3A**), 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 electrodes, dielectric barriers acted as a 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 (5τ). 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 the reverse of 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. 2.3B)**. To obtain comparable bipolar exponential pulses on LIE electrodes, the LIE electrodes were equipped with a capacitor in series. A capacitor was chosen with a specific capacitance to achieve a certain time constant (τ). In this configuration, square pulses applied to LIE electrodes also resulted in bipolar exponential pulses (**Fig. 2.3B)**. **Fig. 2.3C** shows the application of two sequential bipolar exponential pulses.

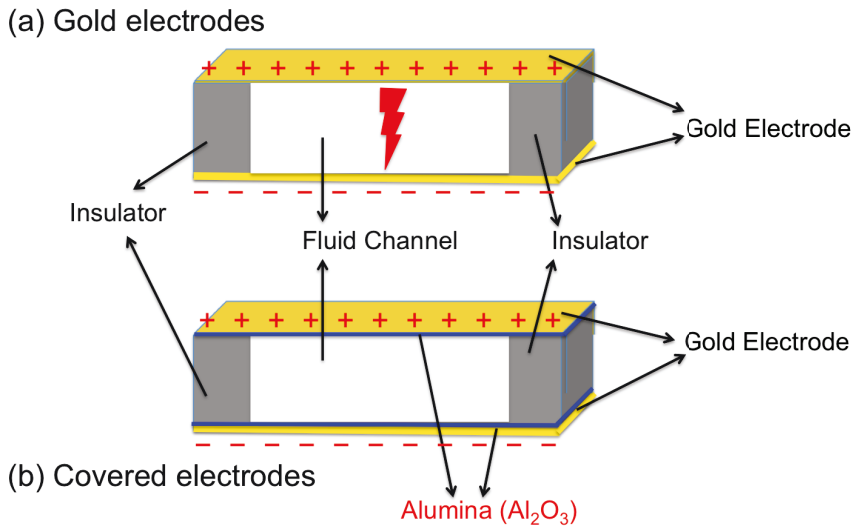

Fig. 2.2 Configuration of gold electrodes and covered electrodes. (a) There is conduction current flowing in the fluid across the gold electrodes. (b) Alumina is one type of dielectric material that can prevent the injection of electrons in the fluid, and thus prevent conduction current flowing in the fluid. (May and Ruben 2012)

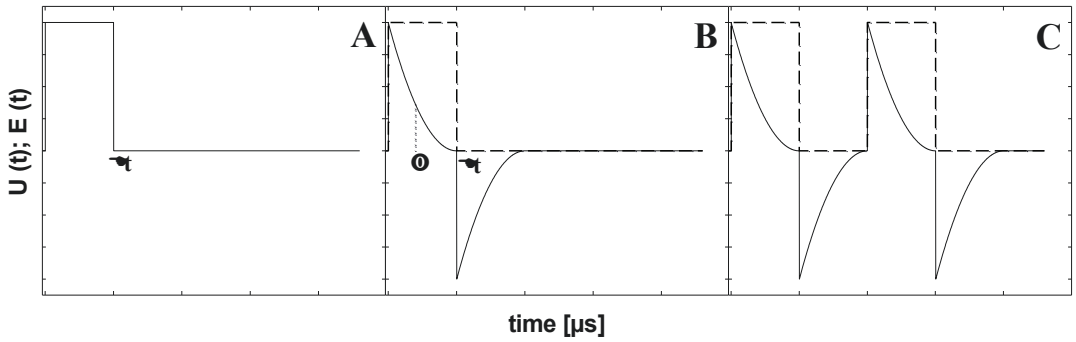

Fig. 2.3 Pulse waveforms used in this study. (A) Square pulse applied to a LIE device resulted in a square pulse across the fluid section. (B) Square pulse (dotted line) applied to a HIE device or a LIE electrodes with a capacitor resulted in a bipolar exponentially decaying pulse (solid line) across the fluid section. The time constant (τ) indicates when the voltage has decayed to $1/e$ of the maximum amplitude. One exponential pulse had a time constant of τ = 86 ns for LIE electrodes with one capacitor in series and τ = 53 ns for high impedance electroporation electrodes. (C) Two sequential bipolar exponential pulses.

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, could be expressed as

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

where $E_s(t)$ was the electric field through the fluid section, and V was the volume of the treatment chamber. For a LIE device powered by a square pulse (**Fig. 2.3A)**, electric field was largely constant over the duration of the pulse Δt, so that $E_s(t) = E_0$ and Eq. (1) simplified to

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

For a bipolar exponentially decaying pulse over the fluid section (**Fig. 2.3B**), total energy dissipation was calculated by replacing $E_s(t)$ in Eq. (1) with $E_0 e^{-t/\tau}$:

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

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

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

When pulses were 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 was equal to

$$N = Vf/\xi. \tag{5}$$

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

$$W = NU_p/\rho V = U_p f/\xi\rho. \tag{6}$$

Finally, the temperature increase of the fluid under treatment could also be calculated as

$$\Delta T = W/c_W. \tag{7}$$

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

2.2.3 PEF treatment

One syringe with 10 mL of PB buffer and the other one with 10 mL of cell suspension with or without PI were fed to the treatment chamber continuously at room temperature. When the pressure of the flow system stabilized, 1 mL of sample was collected as an untreated sample or an initial sample. Then different PEF treatments were applied and 1 mL of each treated sample was collected using 2 mL sterile Eppendorf tubes. The treatment chamber was for one-time use, and the tubing was flushed with 20 mL of sterile water at 15 mL/min both before and after each set of tests. Before each run of experimental tests, the treatment chamber was rinsed with 2 mL of sterile MiliQ water and balanced with 2 mL of sterile PB buffer at flow rate of 0.5 mL/min.

2.2.3.1 Determination of PEF-induced reversible and irreversible permeabilization of yeasts

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

To compare the effect of PEF treatments on the reversible and irreversible permeabilization of the membrane, PI was added to cell suspensions before or after PEF treatments. *C. humilis* and *S. cerevisiae* cell suspensions with and without 10 μM of PI were processed with unipolar square pulses of 0.5 μs and 10 kHz, and with bipolar exponential pulses at a time constant τ of 86 ns and 10 kHz. The samples with PI were subsequently counter-stained with Syto 9 (Life Technologies, Burlington, Ontario, Canada); the final concentrations of PI and Syto 9 were both 5 μM. The ratio of fluorescence intensity of PI (λ_{EX} of 535 nm; λ_{EM} of 617 nm) over that of Syto 9 (λ_{EX} of 485 nm; λ_{EM} of 538 nm) was calculated as a measure of reversible pore formation. Samples

treated by PEF in the absence of PI were subsequently stained with PI and Syto 9 with final concentrations of 10 μM and 5 μM, respectively. The ratio of fluorescence intensity of PI over that of Syto 9 was calculated as a measure of irreversible pore formation. A calibration curve was established for each PEF test by mixing cells treated at 80 °C for 10 min with untreated cells in proportions of 0 %, 50 % and 100 %. The fluorescence intensity was measured with a multiwell plate fluorescence spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

2.2.3.2 Effect of PEF treatment parameters on yeast viability

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

To assess the effect of pulse width, *C. humilis* cell suspensions with and without 100 μM of PI were processed with unipolar square pulses with pulse widths ranging from 125 ns to 2 μs. In order to keep the specific energy input constant across treatments, the increase of pulse width was compensated by the decrease of pulse frequency from 40 to 2.5 kHz. In a second experiment, *C. humilis* cell suspensions with and without 100 μM of PI were processed with unipolar square pulses and bipolar exponential pulses. To keep the specific energy input constant across treatments, the increase of pulse width was compensated by the decrease of pulse frequency. Square pulses of 125 ns and 250 ns were applied at a frequency of 40 kHz and 20 kHz, respectively; exponential pulses with τ = 172 ns and 344 ns were applied at 5 kHz and 2.5 kHz, respectively.

To determine the critical field strength of PEF treatments against *C. humilis*, cell suspensions with and without 100 μM of PI were processed with unipolar square pulses of 2 μs. The flow rate

was set at 0.25 mL/min. To keep the specific energy input constant across treatments with different field strengths ranging from 2 to 18 kV/cm, the decrease of field strength was compensated by the increase of pulse frequency from 1.25 to 80 kHz, from 5 to 320 kHz, and from 10 to 160 kHz for specific energy input at 3, 11 and 21 kJ/kg, respectively.

To determine the effect of energy input, field strength and pulse width, *C. humilis* cell suspensions were processed with unipolar square pulses and bipolar exponential pulses at conditions specified in **Table 2.1**.

Table 2.1 Treatment parameters to determine the effect of specific energy input, electric field 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)				
8	5	–[b]	–	71	50	Exponential[a]
	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	–	

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

[b] –, not done.

To determine the effect of conduction current, *C. humilis* and *S. cerevisiae* cell suspensions with and without 100 μM of PI were processed with LIE (bipolar exponential pulses with τ =86 ns) and HIE (bipolar exponential pulses with τ = 53 ns) devices at 10 kHz (**Fig. 2.3B**). *C. humilis* cell suspensions with and without 100 μM of PI were processed with HIE devices with two

sequential pulses per unit time at 10 kHz **(Fig. 2.3C)**. Treatments were performed with electric field strengths ranging from 18 to 71 kV/cm.

2.2.4 Enumeration of viable cells

The PEF-treated and -untreated samples were serially diluted in PB buffer. Appropriate dilutions were plated on YPD agar plates, and incubated at 30 °C for two days. Results were expressed as $\log_{10}(N/N_0)$, where N_0 is the cell count of untreated sample and N is the cell count of PEF-treated sample. Initial cell counts of *C. humilis* and *S. cerevisiae* were $(6.2 \pm 2.3) \times 10^6$ cfu/mL and $(6.3 \pm 2.4) \times 10^6$ cfu/mL, respectively. The detection limit was 10 cfu/mL. The results are shown as mean ± standard deviation of at least three independent experiments.

2.2.5 Statistical analyses

Results are based on at least three independent replicates done with different cell suspensions prepared on different days. Significant differences between treatments done under different conditions were evaluated with Student's T-test. An error probability of less than 5% ($p<0.05$) was considered significant. Linear regression analyses were conducted using Sigmaplot software (Sigmaplot, Version 12.5, Systat Software Inc., San Jose California, USA).

2.3 Results

2.3.1 Quantification of reversible and irreversible pore formation

This study initially compared the cytotoxicity of PI and bleomycin to electroporated cells (**Fig. 2.4**). Bleomycin is a membrane-impermeable compound which is cytotoxic only to membrane-damaged cells (Sikic 1985). PI is a membrane-impermeable 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 of 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. 2.4** and **Fig. 2.5A)**. The viability of PEF-treated cells in the presence of PI or bleomycin decreased sharply even at a low specific energy input (**Fig. 2.4B**), which indicates that 100 μM of PI has strong cytotoxicity to membrane damaged cells and its cytotoxicity is even higher than that of 1 mg/L of bleomycin.

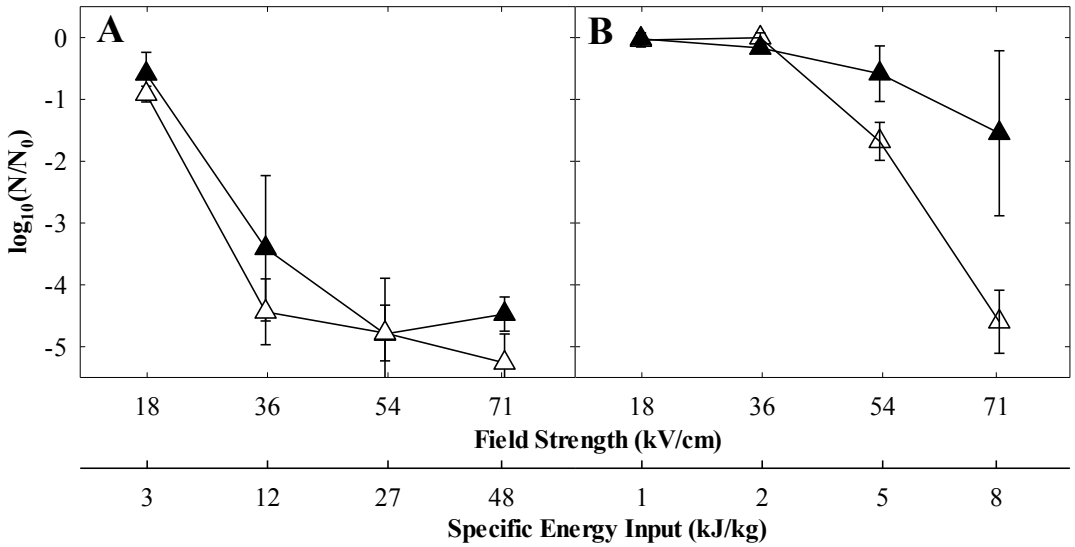

Fig. 2.4 Cytotoxicity of bleomycin (1 mg/L) and propidium iodide (100 μM) to PEF-treated *C. humilis*. Panel A: PEF treatment with square pulses of 0.5 μs and 10 kHz; Panel B: PEF treatment with exponential pulses of 86 ns and 10 kHz. (▲) PEF treatment in the presence of 1 mg/L of bleomycin; (Δ) PEF treatment in the presence of 100 μM of propidium iodide. The results are shown as mean ± standard deviation of three independent experiments. Without PEF treatment, the viability of *C. humilis* in the presence of 1 mg/L of bleomycin or 100 μM of propidium iodide remained unaffected (n=6). N_0 is the cell count of untreated sample and N is the cell count of the PEF-treated sample.

The permeabilization of yeast membrane to PI was also assessed by PEF treatment in the presence of 10 μM of 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 of PI corresponded to the increase of PI fluorescence after PEF treatment in the presence of 10 μM of PI (data not shown). Likewise, the reduction of *C. humilis* or *S. cerevisiae* cell counts in samples exposed to PEF treatment alone corresponded to the increase of PI fluorescence after PEF treatment alone (data not shown). Quantification of PI fluorescence after PEF treatment in the presence of 10 μM of PI and the enumeration of viable cells after PEF treatment in the presence of 100 μM of 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.

2.3.2 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 (Qin *et al.* 1994). 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. 2.5**). 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. 2.5A**). PEF treatments in the presence of PI reduced cell counts of *S. cerevisiae* and *C. humilis* when the specific energy input exceeded 5 kJ/kg and 3 kJ/kg, respectively (**Fig. 2.5B**). The reduction of cell counts was similar when treatments with square pulses and exponential pulses were normalized with respect to specific energy input (**Fig. 2.5B**). These data demonstrate that exponential and square pulses 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 a model organism.

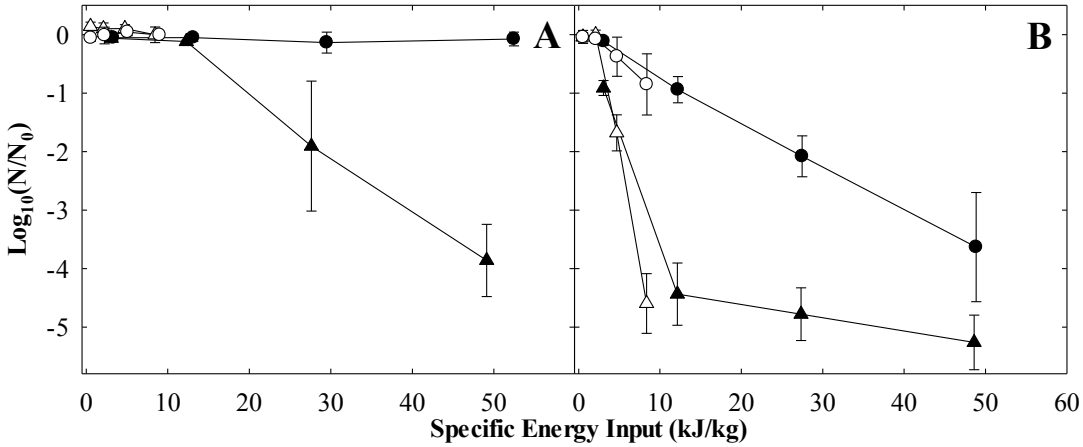

Fig. 2.5 Effect of pulse shape on the inactivation of *C. humilis* and *S. cerevisiae* by PEF. Panel A: PEF treatment alone; Panel B: PEF treatment in the presence of 100 μM of propidium iodide. Symbols indicate treatment combinations as follows: *S. cerevisiae* (○, ●); *C. humilis* (Δ, ▲); square pulses of 0.5 μs and 10 kHz (●, ▲); exponential pulses of 86 ns and 10 kHz (○, Δ). The specific energy input was adjusted by setting the field strength to 18, 36, 54 and 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.

2.3.3 Effect of pulse width on PEF inactivation efficiency

A vast majority of past studies on microbial survival by PEF used pulse widths ranging from 1-10 μs (Pataro *et al.* 2011; Huang *et al.* 2013; Timmermans *et al.* 2014). 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 treatments at 13 kJ/kg and 52 kJ/kg in the absence of PI (**Fig. 2.6)**. Regardless of the pulse width, all treatments at a specific energy input of 52 kJ/kg achieved a cell count reduction of about 4.2 log cycles. 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. 2.6**). A second experiment employed square pulses and

exponential pulses with two different pulse widths. In the second experiment, the pulse width had no effect or inconclusive effect on microbial survival (data not shown). These data indicate that pulse width has a statistically significant effect on the lethality of PEF, but the effect is quite minor, less than 0.5 log cycles.

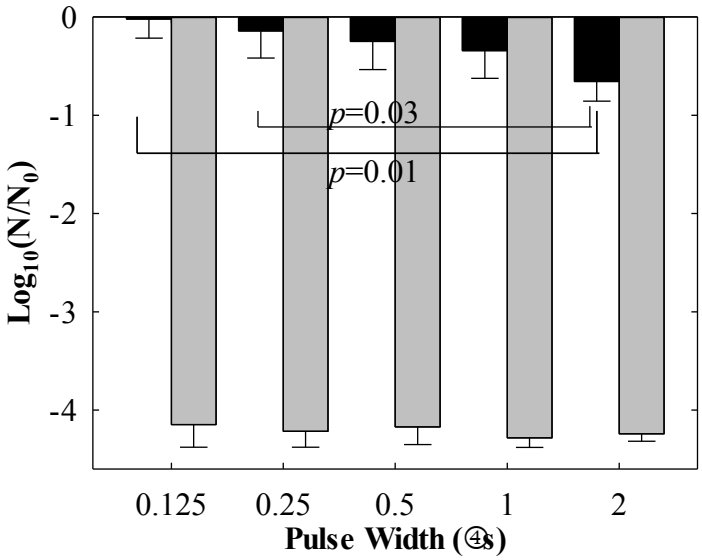

Fig. 2.6 Effect of pulse width on the inactivation of *C. humilis* by PEF with square pulses. Black bars: electric field strength and specific energy input were 36 kV/cm and 13 kJ/kg, respectively; Grey bars: electric field strength and specific energy input were 71 kV/cm and 52 kJ/kg, respectively. Results are shown as means ± 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.

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

Based on the electromechanical model for PEF electroporation mechanism proposed by Zimmermann *et al.* 1973, cell membrane electroporation occurs only when transmembrane potential is equal to or greater than the rupture potential. The critical field strength of PEF treatment against *C. humilis* was determined by treatments with square pulses at a constant

specific energy input (**Fig. 2.7**). PEF treatments in the absence of PI reduced cell counts only when specific energy input exceeded 12 kJ/kg (**Fig. 2.5A**) and field strength exceeded 9 kV/cm (**Fig. 2.7A**), while PEF treatments in the presence of PI reduced cell counts when specific energy input exceeded 3 kJ/kg (**Fig. 2.5B**) and field strength exceeded 2 kV/cm (**Fig. 2.7B**). So the critical field strengths of PEF treatments against *C. humilis* were 2 and 9 kV/cm for reversible and irreversible electroporation, respectively. The cell radius of *C. humilis* in the short axial direction is about 2~3 μm, and 6~10 μm in the long axis. So the minimum transmembrane potential needed to cause reversible and irreversible electroporation against *C. humilis* were 0.4 V and 1.8 V, respectively.

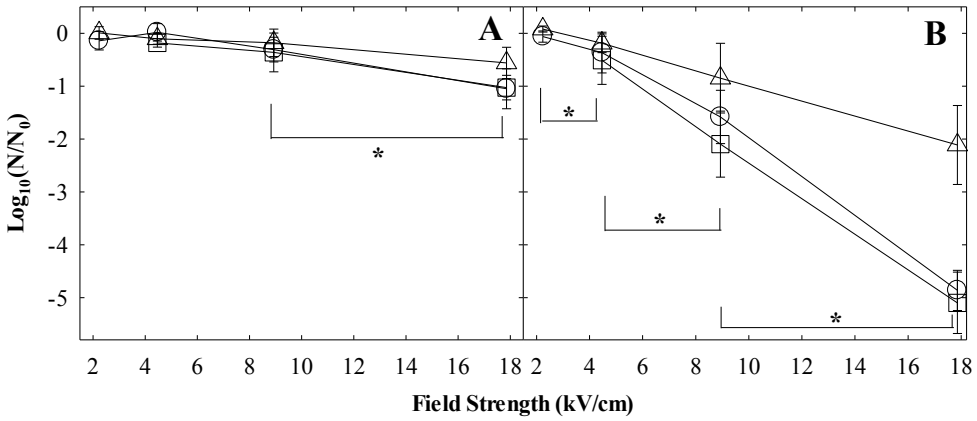

Fig. 2.7 Critical field strength of PEF treatment against *C. humilis*. Panel A: PEF treatment alone; Panel B: PEF treatment in the presence of 100 μM propidium iodide. Symbols stand for PEF treatments with specific energy input at 3 kJ/kg (Δ), 11 kJ/kg (○) and 21 kJ/kg (□). Significant differences (p<0.05) between treatments at the same energy input and pulse width (2 μs) but with different field strength are indicated by aan asterisk. In panel A, only when specific energy input at 11 kJ/kg and 21 kJ/kg showed significant difference (p<0.05) between treatments at 9 kV/cm and 18 kV/cm. In panel B, only when specific energy input at 3 kJ/kg showed significant difference (p<0.05) between treatments at 2 kV/cm and 4 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.

2.3.5 Analysis of the factors describing the lethality of PEF

Past studies evaluated the effect of single parameters on the lethality of PEF (Qin *et al.* 1995; MacGregor *et al.* 2000; Cserhalmi *et al.* 2002); however, the parameters: energy input, electric field strength, pulse shape, pulse frequency and width, are interdependent. Therefore, this study evaluated the lethality of PEF on *C. humilis* with 22 different combinations of field strength, energy input, and pulse width (**Table 2.1**). Cell count reductions were plotted as a function of specific energy input (**Fig. 2.8A**), electric field strength (**Fig. 2.8B**), and pulse width (**Fig. 2.8C**). Field strength or pulse width, when considered on their own, explained only a small portion of the variation in the process lethality (**Fig. 2.8B and 2.8C**). The percentage of variability explained by these parameters was only 10% and 0.3%, respectively. Treatments with a specific energy input of less than 12 kJ/kg reduced the viable cell counts of *C. humilis* by less than 1 log cycle even at high field strength (**Fig. 2.8B**). When only treatments with a specific energy input of greater than 12 kJ/kg were considered, the field strength explained 59% of the variation in the process lethality **(Fig. 2.8B)**. Conversely, specific energy input explained most of the variation in the process lethality (r^2 of 0.84, **Fig. 2.8A**). These data indicate that specific energy input thus represents the most useful parameter of the efficacy of PEF.

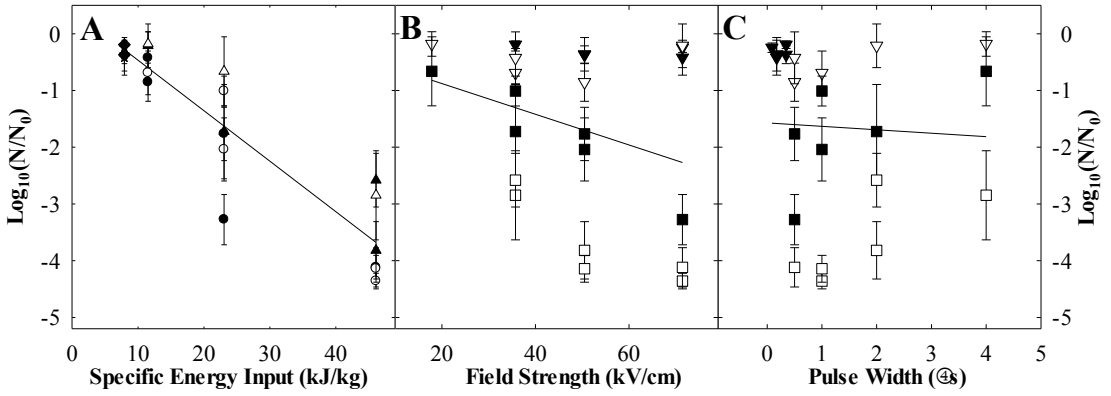

Fig. 2.8 Effect of specific energy input, electric field strength and pulse width on the inactivation of *C. humilis* exposed to PEF treatment alone. Data were plotted as a function of specific energy input (Panel A), field strength (Panel B) and pulse width (Panel C). Panel A: Treatments were carried 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 (Δ). 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 (□). The specific energy input was adjusted by the varying of field strength from 17 kV/cm to 71 kV/cm, or by varying of the effective pulse duration from 86 ns to 4 μs. Pulse frequencies of 5 kHz and 10 kHz were used. Lines show linear regression. 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 an energy input higher than 12 kJ/kg was 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.

2.3.6 Effect of conduction current on PEF inactivation efficiency

The fluid between two electrodes is conductive, allowing ionic current to flow and generate ohmic heating. LIE electrodes act as a resistor in the electric circuit and the conduction current can introduce electrolysis and other chemical reactions in the treatment medium. HIE electrodes are made of alumina-covered gold, acting as a capacitor in the circuit. The alumina layer limits electron transfer to values that can eliminate electrochemical reactions at the electrodes. This experiment was conducted to determine whether conduction current plays a role in the lethality of PEF. PEF treatments in the absence of PI (**Fig. 2.9A**) were not lethal to either *S. cerevisiae* or

C. humilis. PEF treatments in the presence of PI (**Fig. 2.9B**) reduced cell counts of *C. humilis* and *S. cerevisiae.* These data demonstrated that treatments with LIE and HIE electrodes had a comparable ability to electroporate cell membranes when the specific energy input was normalized. The conduction current is thus not essential for membrane electroporation by PEF. The use of a single and two sequential bipolar exponential pulses in a unit of time (**Fig. 2.3B and 2.3C**) also had no influence on the pore formation efficiency **(Fig. 2.9B)**. The results further confirmed, however, that *C. humilis* is more sensitive to PEF, and that a specific energy input of about 3 kJ/kg and 12 kJ/kg are required to achieve reversible and irreversible electroporation, respectively.

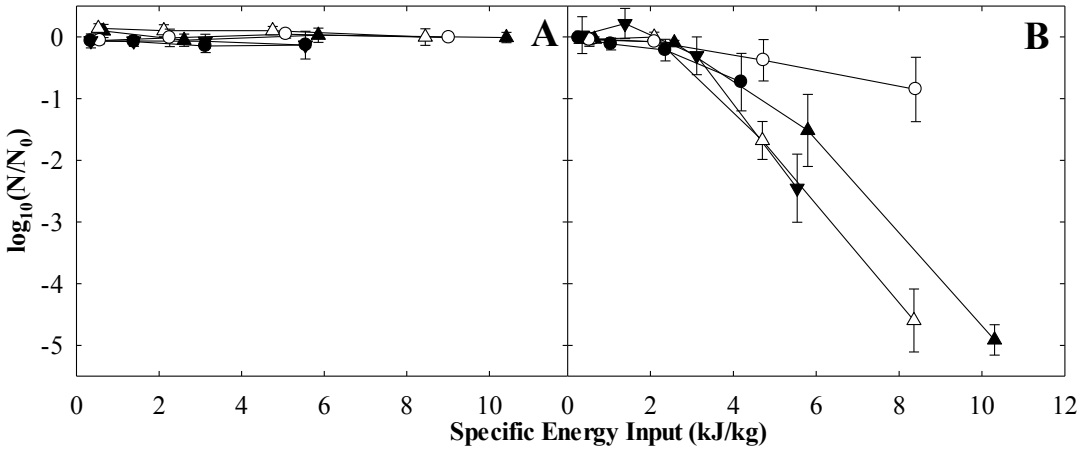

Fig. 2.9 Effect of conduction current on the inactivation of *C. humilis* and *S. cerevisiae* by PEF. Panel A: PEF treatments alone; Panel B: PEF treatments in the presence of 100 μM propidium iodide. Symbols indicate treatment combinations as follows: *S. cerevisiae* (○, ●); *C. humilis* (▲, Δ, ▼); low impedance electroporation device with pulses of 86 ns (○, Δ); high impedance electroporation device with pulses of 53 ns (●, ▼); high impedance electroporation device with pulses of 53 ns and adjusted specific energy input by doubling the number of pulses (▲) (see Fig. 2.2C). 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.

2.4 Discussion

Energy input, electric field strength, pulse width, and the number of pulses or pulse frequency are interdependent. Therefore, simultaneous variation of these parameters allows identification of the factors which best describe the efficacy of PEF. The present study systematically varied specific energy input, electric field strength, and pulse shape and width. This study also compared the effect of square pulses and exponential pulses on the efficacy of PEF. This approach demonstrated that specific energy input best described the efficacy of PEF; field strength was significantly correlated to process lethality against *C. humilis* only when considering treatments with energy input that were higher than a critical energy input of 12 kJ/kg. Accordingly, achieving the same specific energy input by adjustment of electric field strength, pulse width or pulse frequency achieved comparable process lethality. The critical field strength and specific energy input for reversible and irreversible electroporation of *C. humilis* were 2 kV/cm and 3 kJ/kg, and 9 kV/cm and 12 kJ/kg, respectively.

The three main types of PEF treatment chambers, namely co-field (Toepfl *et al*. 2007), coaxial (Zhong *et al*. 2005) and parallel plate (Heinz *et al*. 2003), differ substantially with respect to the homogeneity of treatment conditions. Compared to other treatment chambers, parallel plate electrodes provide more uniform electric fields and temperature distribution (Heinz *et al*. 2003). The small dimensions of the electrode and the application of a sheet flow to keep yeast cells in the center of the treatment chamber where the field is uniform provide additional measures to ensure homogeneous treatment conditions for each microbial cell.

Direct metal-electrolyte contact allows electron injection to the treatment medium (Dogonadze *et al.* 1977). The resulting electrolysis of water causes formation of hydrogen or oxygen bubbles, which may disturb the electric field (Nabil *et al.* 2014). Electrolysis also results in a deposit of

corrosion products on the electrode surface, which may contaminate food products (Jayaram *et al.* 1992). Covering the metallic electrodes with a layer of insulating material, e.g. alumina, prevents electrode corrosion (Escudero *et al.* 1986; May and Rubens 2012). This study indicates that membrane electroporation efficiencies of PEF with bare electrodes and alumina-covered electrodes are comparable. Conduction current is thus not necessary for the electroporation by PEF, and contamination of PEF-treated medium or food products by electrode corrosion products can be prevented.

Propidium iodide (PI), a nucleotide-binding probe excluded by intact cells, is a good indicator of membrane permeabilization introduced by PEF treatments (Wouters *et al.* 2001; Ulmer *et al.* 2002). This study demonstrated that PI shows cytotoxicity to membrane-damaged cells as does bleomycin, a known cytotoxic compound (Kandušer *et al*. 2008). Enumeration of viable cells of PEF-treated samples in the presence and absence of 100 μM of PI correlated to quantification of pore formation by fluorescent detection, and thus provides an alternative method for determination of reversible and irreversible pore formation by PEF treatments.

Past studies plotted the cell count reduction as a function of the number of pulses, electric field strength, or pulse width (Sala and Hamilton 1967; Zhang *et al.* 1994b; Qin *et al.* 1995; MacGregor *et al.* 2000; Cserhalmi *et al.* 2002; Guyot *et al.* 2007). An increase of field strength, pulse width, or the number of pulses also results in an increase in specific energy input. The present study illustrated that an increase of specific energy input increases the lethal effect of the PEF treatment and that this increase in process efficacy is largely independent of whether the increase was achieved by increasing the number of pulses, pulse width, or field strength. To match compare this conclusion with literature data, **Table 1.1** summarizes past studies that investigated PEF inactivation of *S. cerevisiae*. Literature data on the inactivation of *C. humilis* is

scarce. Past studies document a large variability of the process lethality even if the reported process parameters (field strength, pulse frequency or energy input) are comparable. Differences in pH or process temperature, or the inhomogeneity with respect to electric field and temperature distribution, and the flow characteristics (Jaeger *et al.* 2009) only partially account for this large variation in the lethality of PEF reported in past studies. Nevertheless, literature data allow the conclusion that the lethality of PEF is low unless PEF is combined with low pH, a processing temperature higher than 40 °C, or an energy input exceeding 60 kJ/kg (**Table 1.1**). There was no cell death of *S. cerevisiae* after treatment with phosphate buffer at pH 6.5 with the energy input of 50 kJ/kg or less and the electric field strength of 71 kV/cm. These data conform to literature data that was generated at neutral pH and ambient temperature. The use of PI, however, demonstrates that reversible electroporation of *S. cerevisiae* is achieved at a specific energy input ranging from 5~50 kJ/kg. This reversible electroporation may account for the effect of lower pH or higher temperature on the lethality of PEF. Metabolic consequences of electroporation are more severe at a low pH because reversible pore formation may result in dissipation of vital proton gradients before membrane pores are resealed. The cytoplasmic pH of *S. cerevisiae* is closely linked to cellular viability (Imai and Ohno 1995). A temperature of 40 °C is not lethal to *S. cerevisiae*, however, even a modest increase in temperature will increase membrane fluidity which enhances electroporation (Assad-Garcia *et al.* 2008; Kandušer *et al*. 2008). An increase in membrane fluidity also increased the time required for resealing of the membrane after electroporation (Rols *et al.* 1990; Kandušer *et al.* 2006). Taken together, literature data and the present study indicate that inactivation of *S. cerevisiae* by PEF requires a high energy input, or a combination of PEF with enhanced temperature, acidic condition, or cytotoxic compounds.

The critical field strengths tested in this study for *C. humilis* were 0.4 V (2 kV/cm) and 1.8 V (9 kV/cm) for reversible and irreversible electroporation, respectively, which matches the theoretical values (0.2~1.7 V) tested before. Since the thickness of cell plasma membrane is relatively constant from one microorganism to another (about 5~10 nm), the critical field strength depends on the compressibility and permittivity of the membrane, the initial temperature of treatment medium and the valence of the ions (Benz *et al.* 1979; Dimitrov 1984; Barsotti and Cheftel 1999; Heinz *et al.* 2003). There is little information available concerning *C. humilis*, but the cell size and shape of *C. humilis* (2-3 μm×6-10 μm) is quite similar to that of *S. cerivisiae* (3-4 μm×5-9 μm); therefore, it is reasonable to assume that their critical field strengths would be similar. The critical field strength for *S. cerevisiae* is about 4.7 to 8 kV/cm (El Zakhem *et al.* 2006; Pataro *et al.* 2010), which also matches the values tested in this study.

The present study and previous studies relating PEF lethality to the specific energy input employed field strengths that are higher than the critical field strength that is required to induce a transmembrane potential of 0.2~1.7 V (Zimmermann 1986). If this condition is met, modeling of PEF lethality on *S. cerevisiae* and other microorganisms in food is achieved with a high degree of correlation between experimental and predicted values when using specific energy input as a control parameter (Huang *et al.* 2014b; Timmerman *et al.* 2014). It is thus recommended to plot PEF inactivation efficiency as a function of specific energy input. This parameter also provides guidance with respect to the processing cost, which is mainly determined by the energy use.

In conclusion, specific energy input was the parameter that best described reversible electroporation and lethality of PEF treatment of yeasts; the effects of electric field strength, pulse width and frequency, or pulse shape were significant but much less pronounced. Conduction current across the electrode surface was not necessary to achieve reversible

electroporation. In order to increase the cost-effectiveness, it is recommended to combine PEF technology with modest thermal treatment or cytotoxic compounds.

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3 Effect of conduction current and fluid properties on the efficacy of PEF

3.1 Introduction

The results in **Chapter 2** demonstrate that conduction current is not essential for electroporation by PEF. However, whether conduction current is necessary for the lethal effect of PEF is still unknown. When an external electric field is applied to a cell, the charging process and the formation of pores through the cell membrane is the primary effect of the electric field (Angersbach *et al*. 2000), mainly depending on the amplitude of the electric field and the value of specific energy input (section 2.3.4). The fate of these pores formed through the cell membrane is determined by other factors, including the processing temperature (Aronsson and Rönner 2001; Walkling-Ribeiro *et al*. 2011; Huang *et al.* 2013; Timmermans *et al.* 2014), the specific energy input (Huang *et al*. 2014b), the presence of cytotoxic compounds (Somolinos et al. 2007), and the pH of the fluid (Aronsson and Rönner 2001; Somolinos *et al*. 2007; Timmermans *et al.* 2014). Some studies showed that electroporation had a synergistic effect with electrolysis (Sato *et al.* 1996; Saulis *et al*. 2005; Pakhomova *et al*. 2012; Nuccitelli *et al.* 2013; Sandvik *et al.* 2013), but there is no direct proof. Theoretically the addition of cysteine could eliminate the reactive oxygen species (ROS) produced during PEF processing, which is an indirect way to show whether electrolysis has synergistic effect with electroporation. Calcium ions are important for signaling, and electroporation in the presence of high concentrations of calcium ions showed higher lethality of cell lines (Frandsen *et al.* 2014; Pakhomova *et al*. 2014). However, whether or not calcium electroporation could also show high lethality of yeast cells by PEF treatment is unknown yet. Therefore, the objectives of this study were to compare the performance of LIE and HIE devices to show whether electrolysis plays an important role in the lethal effect of PEF, and to figure out the effect of the fluid properties on the efficacy of PEF.

3.2 Materials and methods

3.2.1 Preparation of inoculum and cell suspension

Inoculum was prepared as described in section 2.2.1.

Prior to PEF treatments, *C. humilis* was harvested by centrifugation, washed in potassium phosphate buffer (PB, K_2HPO_4-KH_2PO_4, 4 mM, pH 6.5), and then re-suspended in PB buffer. The optical density (O.D.) of cell suspension in PB buffer at 600 nm was adjusted to 1.00±0.05. Just before PEF treatment, 400 mM of NaCl (Fisher Scientific, Pittsburgh, PA, USA), 200 mM of $CaCl_2$ (Fisher Scientific, Pittsburgh, PA, USA), 1 M of cysteine-HCl conjugate (Sigma-Aldrich, St. Louis, Missouri, USA), or 500 mM of cysteine free base (MP Biomedicals, Santa Ana, California, USA), which was dissolved in MiliQ water and filtered with 0.22 μm Millipore filters separately, was added to PB buffer and cell suspension, as planned, to ensure the final concentrations of NaCl, $CaCl_2$ and cysteine were 4 mM, 2 mM and 4 mM, respectively. There was no precipitation after mixing $CaCl_2$ and PB buffer at room temperature in 24 h. The electrical conductivities of PB buffered solutions are listed in **Table 3.1**. Cysteine is light and air sensitive, therefore the cysteine crystals were stored under nitrogen headspace, and cysteine crystals and solutions were protected from light with aluminum foil. The cysteine solution was kept at 4 ˚C and disposed after 48 h.

Table 3.1 Electrical conductivities of buffered solutions

Solutions	Conductivity (mS/cm)[a]
4 mM PB	0.5/0.6
4 mM PB + 4 mM NaCl	1.0/1.0
4 mM PB + 2 mM $CaCl_2$	0.9/1.0
4 mM PB + 4 mM cysteine free base	0.5/0.6
4 mM PB+ 4 mM NaCl+ 4 mM cysteine free base	1.0/1.0
4 mM PB+ 2 mM $CaCl_2$+ 4 mM cysteine free base	0.9/1.0
4 mM PB + 4 mM cysteine-HCl conjugate	1.2/-[b]
4 mM PB+ 4 mM NaCl+ 4 mM cysteine-HCl conjugate	1.7/-
4 mM PB+ 2 mM $CaCl_2$+ 4 mM cysteine-HCl conjugate	1.6/-

[a]: PB buffer with different electrical conductivities, and the addition of NaCl, $CaCl_2$ or cysteine-HCl conjugate resulted in the increase of conductivity accordingly.

[b]: - indicates there was no mixture of this PB buffer with cysteine-HCl conjugate.

3.2.2 PEF treatment apparatus

PEF treatment apparatus was described in section 2.2.2.

3.2.3 PEF treatment

One syringe with 10 mL of PB buffered solution and the other one with 10 mL of cells suspended in the same solution were fed to the treatment chamber continuously at room temperature. When the pressure of the flow system stabilized, 1 mL of sample was collected as an untreated sample or initial sample. Next, different PEF treatments were applied and 1 mL of each treated sample was collected using 2 mL sterile Eppendorf tubes. The flow rate was set at 0.5 mL/min. The treatment chamber was one time use, and the tubing was flushed with 20 mL of sterile water at 15 mL/min both before and after each set of tests. Before each run of

experimental tests, the treatment chamber was rinsed with 2 mL of sterile MiliQ water and balanced with 2 mL of sterile PB buffer at a flow rate of 0.5 mL/min.

To determine the effect of electrolysis on the inactivation efficiency of PEF, *C. humilis* cell suspensions in PB buffer were processed with LIE devices with unipolar square pulses of 0.5 μs and 10 kHz, or with HIE devices with eight sequential bipolar exponential pulses of 53 ns and 10 kHz. The field strength for PEF treatments with both LIE and HIE devices ranged from 18 to 71 kV/cm, while the specific energy input was from 3 to 43 kJ/kg for PEF treatments with LIE devices and from 3 to 41 kJ/kg with HIE devices.

To determine the effect of calcium ions on the efficacy of PEF, *C. humilis* cell suspensions with 2 mM of $CaCl_2$ or 4 mM of NaCl were processed by PEF with LIE devices with unipolar square pulses of 0.5 μs and 10 kHz, or with HIE devices with six sequential bipolar exponential pulses of 53 ns and 10 kHz. The field strength for PEF treatments with both LIE and HIE devices ranged from 18 to 71 kV/cm, while the specific energy input was from 5 to 46 kJ/kg for PEF treatments with LIE devices and from 3 to 56 kJ/kg with HIE devices.

To determine whether or not cysteine can eliminate the cytotoxic electrochemicals formed from electrolysis during PEF treatments, *C. humilis* cell suspensions with 4 mM of cysteine free base, with or without 2 mM of $CaCl_2$ or 4 mM of NaCl, were processed by PEF with LIE devices with unipolar square pulses of 0.5 μs and 10 kHz, or with HIE devices with bipolar exponential pulses of 53 ns and 10 kHz. In order to normalize the specific energy input, the increase of electrical conductivity after the addition of NaCl or $CaCl_2$ to PB buffer was compensated by the decrease of field strength of PEF treatment with LIE devices and the decrease of the number of sequential pulses from eight to six. The addition of cysteine free base did not change the electrical

conductivity and pH of PB buffered solution. The field strength ranged from 18 to 71 kV/cm and the specific energy input was from 3 to 46 kJ/kg.

To determine the effect of treatment medium with low pH on the efficacy of PEF, *C. humilis* cell suspensions with 4 mM of cysteine-HCl conjugate, with or without 2 mM of $CaCl_2$ or 4 mM of NaCl, were processed by PEF with LIE devices, with unipolar square pulses of 0.5 μs and 10 kHz. In order to normalize the specific energy input, the increase of electrical conductivity after the addition of ions to the PB buffer was compensated by the decrease of field strength. The field strength ranged from 18 to 71 kV/cm and the specific energy input was from 6 to 100 kJ/kg.

3.2.4 Enumeration of viable cells

Enumeration of viable cells was described in section 2.2.4.

3.2.5 Statistical analyses

Results of PEF treatments with LIE devices are based on at least three independent replicates done with different cell suspensions prepared on different days. Treatments with HIE devices had only two independent replicates. Significant differences between treatments at different conditions were evaluated with Student's T-test. An error probability of less than 5% ($p<0.05$) was considered significant.

3.3 Results

3.3.1 Effect of electrolysis and calcium ion on the efficacy of PEF

By covering the conductive electrode with a thin layer of dielectric material, electrolysis can be prevented during PEF treatment (May and Ruben 2012). This experiment was designed to determine the effect of electrolysis on the lethal effect of PEF treatment to *C. humilis* in an approximately direct way. **Fig. 3.1** shows that the inactivation efficiency by PEF treatment with

HIE devices was up to about 2-log cycles lower than that with LIE devices when the specific energy inputs were at 24 kJ/kg and 41 kJ/kg and about 3-log cycles lower at a specific energy input of 26 kJ/kg. These data indicate that conduction current is not necessary for the lethal effect of PEF treatment, but electrolysis plays an important role in the lethal effect of PEF treatment with LIE devices.

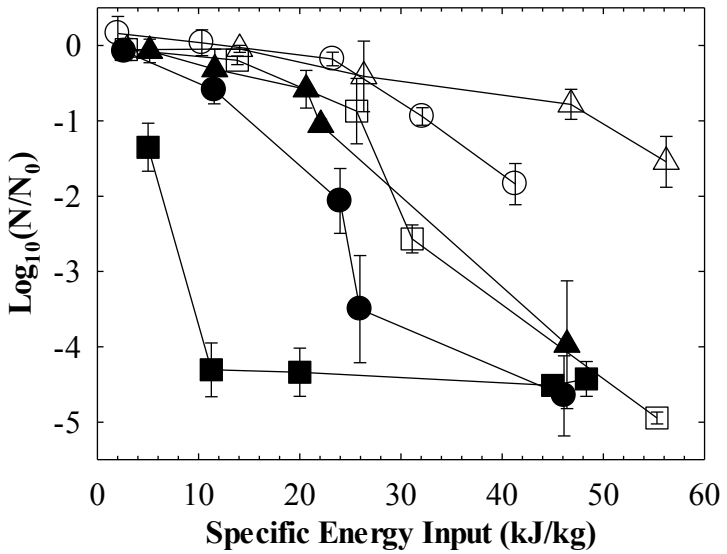

Fig. 3.1 Effect of electrolysis and calcium ion on the inactivation efficiency of PEF. *C. humilis* cell suspensions were treated by PEF with low impedance electroporation (LIE) devices (solid symbols) with unipolar square pulses of 0.5 μs and 10 kHz, or with high impedance electroporation (HIE) devices (open symbols) with bipolar exponential pulses of 0.053 μs and 10 kHz. Different symbols stand for different buffered solutions: (○, ●): PB buffer alone, (▲, Δ): PB buffer with 4 mM of NaCl, and (■, □): PB buffer with 2 mM of $CaCl_2$. The flow rate was 0.5 mL/min. The field strength for PEF treatments with both LIE and HIE devices was ranging from 18 to 71 kV/cm.

Electroporation with calcium, also called calcium electroporation, showed synergistic effects of electroporation and calcium ions on many varieties of mammalian cell lines (Frandsen *et al*. 2014; Pakhomova *et al*. 2014), such as CHO, U937 and BPAE cells, introducing necrotic or apoptotic cell death depending on certain conditions. But the effect of calcium electroporation on

yeast cells has not been elucidated. Therefore, this experiment was designed to determine the effect of calcium electroporation on the lethality of *C. humilis*. The results in **Chapter 2** demonstrate that the specific energy input is the parameter that can best describe the efficacy of PEF treatment. The cell count reduction of *C. humilis* was plotted as a function of specific energy input. **Fig. 3.1** shows that the addition of 2 mM of calcium chloride increased the lethality of *C. humilis* by up to about 4-log cycles when the specific energy input was at about 10 kJ/kg. The increase of lethality may come from calcium ions or chloride ions. In order to distinguish the effect of calcium ions and chlorides ions, 4 mM of sodium chloride was added to the phosphate buffer to make sure the concentration of chloride ions in the two treatment media was the same. **Fig. 3.1** shows that the addition of 4 mM of sodium chloride did not increase the efficacy of PEF instead it decreased the efficacy by up to 2-log cycles when the specific energy input was at 26 kJ/kg. The same tests were also done with HIE devices. **Fig. 3.1** shows that the addition of 2 mM of calcium chloride increased the lethality of *C. humilis* by up to about 2-log cycles when the specific energy input was at about 40 kJ/kg, while the addition of 4 mM of sodium chloride decreased the lethality of *C. humilis* by up to about 1-log cycle. Taken all together, these data indicate that electroporation and electrolysis showed synergistic effect on the lethality of yeast cells; calcium electroporation with both LIE and HIE devices showed higher lethality of yeast cells compared to PEF treatment without calcium ions; the presence of chloride ions decreased the efficacy of PEF treatment; the composition of treatment medium play an important role in the efficacy of PEF treatment.

3.3.2 Effect of cysteine and low pH on the inactivation efficiency of PEF

Electrochemical reactions occur during PEF treatment with LIE devices, producing H_2O_2 or/and HClO depending on the composition of the treatment medium (with or without chloride ions)

(Saulis *et al*. 2005; Pakhomova *et al.* 2012; Sandvik *et al*. 2013). Theoretically, the addition of cysteine could remove these electrochemicals, and thus decrease the lethality of yeast cells. It is an indirect way to show the synergistic effect of electroporation and electrolysis on the efficacy of PEF treatment. The addition of cysteine free base did not change the electrical conductivity and pH of PB buffered solutions. **Fig. 3.2** shows that the addition of 4 mM of cysteine free base did not change the lethality of *C. humilis* by PEF treatment with LIE devices compared with no cysteine control. The addition of cysteine free base also did not change the efficacy of PEF treatment with HIE devices (data not shown).

The addition of cysteine-HCl conjugate increased the electrical conductivity and decreased the pH of PB buffered solutions from 6.5 to about 2.8. The purpose of this experiment was to determine the effect of low pH on the efficacy of PEF treatment. **Fig. 3.2** shows that PEF treatments at a low pH achieved about 1.5 to 2.5-log cycles higher lethality of *C. humilis* compared to that at neutral pH when the specific energy input was equal. These data demonstrate that PEF treatment has a synergistic effect with low pH on the lethality of yeast cells.

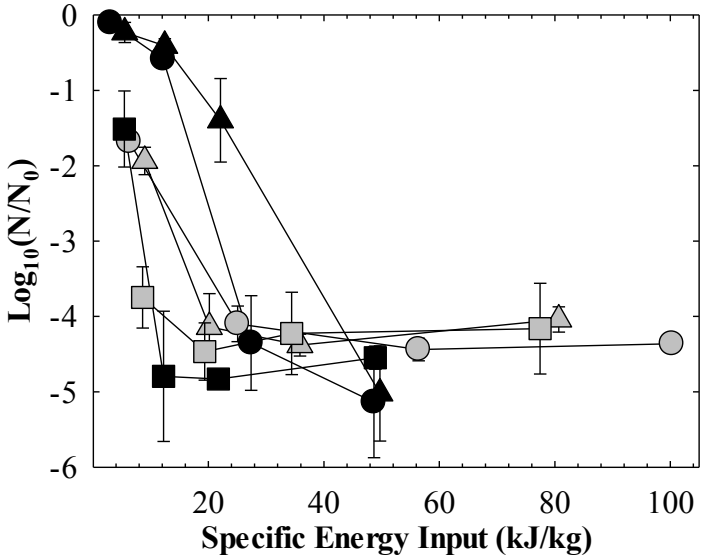

Fig. 3.2 Effect of cysteine and low pH on the efficacy of PEF. *C. humilis* cell suspensions were treated by PEF with low impedance electroporation (LIE) devices with unipolar square pulses of 0.5 μs and 10 kHz. The addition of cysteine free base (black symbols) did not change the pH of PB buffered solutions, while the addition of cysteine-HCl conjugate (grey symbols) changed the pH of PB buffered solutions from 6.5 to 2.8. Different symbols represent PB buffer with different salts: (●, ●): PB buffer without addition of salt, (▲, ▲): PB buffer with 4 mM of NaCl, and (■, ■): PB buffer with 2 mM of $CaCl_2$. The flow rate was 0.5 mL/min. The field strength was from 18 to 71 kV/cm.

3.4 Discussion

This study is the first to compare the lethal effect of PEF treatment with LIE devices and HIE devices, and demonstrated that conduction current was not necessary for the lethal effect of PEF treatment, but that it can enhance the efficacy of PEF greatly. Electroporation and electrolysis had a synergistic effect on the inactivation of *C. humilis*. The addition of calcium ions increased the lethal effect of PEF treatment, while the addition of chloride ions increased the resistance of *C. humilis* to PEF treatment. The addition of cysteine free base did not change the lethal effect of PEF treatment, while the addition of cysteine-HCl conjugate enhanced the efficacy of PEF treatment because it acidified the fluid.

During PEF treatment, because of the direct contact of the conductive electrodes with electrolytes, there are numerous electrochemical reactions taking place at the electrode-liquid interface (**Fig. 1.3**). Depending on the composition of the electrodes and that of the electrolyte, the most favourable electrochemical reactions and the results produced vary (Saulis *et al.* 2005). In this study, the electrodes are made of gold. The most favourable reaction at the inert anode is

$$2H_2O \rightarrow O_2 \uparrow +4H^+ + 4e^- \quad (8)$$

when the treatment medium is potassium phosphate buffer. The presence of H^+ can further produce hydrogen at the anode

$$2H^+ + 2e^- \rightarrow H_2 \uparrow \quad (9)$$

However, once there are chloride ions present in the electrolyte, the most favourable reaction at the inert anode is

$$2Cl^- \rightarrow Cl_2 \uparrow +2e^- \quad (10)$$

The presence of the chlorine gas can further react with water and produce highly oxidative species according to the following reaction

$$Cl_2 \uparrow +H_2O \rightarrow HClO + HCl \quad (11)$$

The most favourable reaction at the cathode is

$$2H_2O + 2e^- \rightarrow H_2 \uparrow +2OH^- \quad (12)$$

The presence of hydroxide ions can further produce hydrogen peroxide according to the reaction

$$2OH^- \rightarrow H_2O_2 + 2e^- \quad (13)$$

The production of gases, including H_2, O_2 and Cl_2, has the potential to distort or break down the electric field in the treatment chamber. If the gases cannot be removed in a timely manner and

sufficiently, they may cease the flow of the fluid through the microfluidic channel of the devices. The production of H_2O_2 and/or HClO can kill microorganisms in the fluid during PEF treatment (Sato *et al*. 1996; Saulis *et al*. 2005; Pakhomova *et al.* 2012; Sandvik *et al*. 2013). The presence of H^+ or OH^- can change the pH of a fluid, which also can contribute to the lethal effect of PEF treatment. Covering the bare electrodes with a thin layer of dielectric material can isolate the conductive electrodes from direct contact with the electrolyte (food products or other conductive fluid), and also greatly limit the injection of electrons into the fluid. In this case, electrochemical reactions and electrode corrosion can be prevented. Results in **Chapter 2** demonstrated that conduction current was not necessary for electroporation by PEF. This study further demonstrated that conduction current was not necessary for the lethal effect of PEF by comparing the performances of LIE and HIE devices, but that the efficacy of PEF could be greatly enhanced by electrolysis. Past studies demonstrated the production of H_2O_2 during PEF processing by the addition of H_2O_2 indicators, like Amplex Red (Pakhomova *et al.* 2012) or catalase (Sato *et al.* 1996) to PEF-treated fluid, and investigated the cytotoxicity of PEF-treated fluid to yeast (Sato *et al.* 1996) and other microorganisms (Sandvik *et al*. 2013). This study and past studies demonstrated the presence of a synergistic effect between electroporation and electrolysis in different ways.

Calcium ions are not essential for the growth of yeast cells, but they play an important role in the synthesis of cell structures, protein-protein interactions, and pH control (Urszula *et al.* 2015). Calcium ions act mainly extracellularly, being actively pumped from cytoplasm to keep the intracellular calcium concentration tight at about 100 nM (Semenov *et al*. 2013). The increase of intracellular concentration of calcium ions can serve as a universal signal for the activation of numerous Ca^{2+}-dependent cascades (Semenov *et al*. 2013), leading to cell death. Calcium

electroporation is a promising method to kill cancer cells, because the presence of high concentrations of calcium (in the range of millimole) during electroporation can greatly increase the lethality of cancer cells by necrosis or apoptosis (Frandsen et al. 2012; Frandsen *et al.* 2014). Compared to bleomycin, calcium is inexpensive and it may be applied without special precaution because it is a cytostatic drug. There are very few studies focusing on the lethal effect of calcium on electroporated microorganisms. In most cases, calcium is used to facilitate DNA binding to cell membranes to increase DNA transformation rates (Benatuil *et al*. 2010). Huo *et al*. (2010) demonstrated that a certain amount of calcium (less than 0.1 mM) could increase the extent of electroporation, because calcium could change the local state of a cell membrane from liquid to solid, and then increase the permeability of cell membrane. The application of an external electric field can also change the status of a transmembrane voltage-dependent Ca^{2+} channel (Saimi *et al.* 1988), and thereby increase the permeability of the cell membrane. Calcium electroporation can also increase the inactivation efficiency of yeast by PEF, which poses a potential for the application of calcium electroporation as a promising food preservation technology.

The addition of chloride ions changed the most favourable reaction at the anode. Theoretically, the presence of chloride ions could generate highly oxidative species, HClO, resulting in higher lethality (Sandvik *et al*. 2013). In this study, however, the presence of chloride ions showed a protective effect on yeast cells instead of a killing effect. The mechanism behind this fact is not clear. One possible reason why the addition of chloride ions did not increase the lethal effect of PEF is that the generation of hypochlorous acid needs extended exposure time and/or higher concentration of chloride ions (Sandvik *et al*. 2013). The reason why the addition of sodium chloride increased the resistance of yeast cells to PEF treatment, as described in section 1.4, is

that the addition of sodium chloride to PB buffer increased the electrical conductivity of the fluid, and thus the lethal effect of PEF decreased. Because the higher the electrical conductivity, the lower the effectiveness of PEF treatment (Jayaram 2000; Toepfl *et al*. 2007; Jaeger *et al.* 2009). This depends on whether the LIE device is voltage sourced or current sourced. If it is voltage sourced, an increase in fluid conductivity causes the current to increase, leaving the resulting electric field at the same intensity (energy consumption increases of course). But the proportion of electric field applied to cells decreased. If it is current sourced, an increase in fluid conductivity will cause a decrease in the applied voltage, thus reducing the intensity of the electric field.

Cysteine is a reducing reagent, having the ability to react with many varieties of reactive oxygen species (ROS), including O_2, H_2O_2 and HClO, because of the presence of free thiol groups (-SH). The formation of ROS in PEF-treated media (extracellular ROS coming from electrolysis) was confirmed by the increased oxidation of fluorescent dye, Amplex Red (Pakhomova *et al*. 2012), and also by the removal of cytotoxicity to the yeast cells after the addition of catalase (Sato *et al.* 1996). The formation of ROS within individual cells exposed to PEF (intracellular oxidative stress induced by nanosecond PEF (nsPEF)) was confirmed by the increased oxidation of various fluorescent dyes, including DHE and carboxy-H_2DCFDA (Pakhomova *et al*. 2012; Nuccitelli *et al*. 2013). The amount of ROS formed during PEF treatment can be reduced by incubating cells with antioxidant Trolox C before exposure to PEF (Nuccitelli *et al*. 2013). Cysteine has been commonly used to determine the presence of ROS induced by high pressure and other treatments (Aertsen *et al.* 2005; Feyaerts *et al*. 2015). In this study, the addition of 4 mM of cysteine free base did not show any effect on the efficacy of PEF. The reason for this might be because the concentration of cysteine (4 mM) is not high enough, or because the incubation time (less than

20 min) with yeast cells before PEF treatment is not long enough. Cysteine-HCl conjugate decreased the pH of fluid from 6.5 to 2.8, and resulted in higher lethality of yeast cells, because cells suspended in acidic fluid were more susceptible to PEF treatment (Timmermans *et al*. 2014).

In conclusion, conduction current was not necessary for the lethal effect of PEF. But electrolysis did have synergistic effect with electroporation. Calcium electroporation had higher efficacy compared to PEF treatment in the absence of calcium ions. PEF treatment with acidic fluid achieved higher inactivation efficiency compared to that obtained with neutral fluid.

3.5 References

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4 General discussion and conclusions

4.1 General discussion

This MSc thesis research determined the effect of electrical parameters, including pulse shape, pulse width, pulse frequency, electric field strength and specific energy input, on the efficacy of PEF in a systematical way, and illustrated that the specific energy input was the parameter that could best explain the efficacy of PEF to inactivate yeast cells. According to membrane modeling tests, the amplitude of electric field strength and the duration of pulses were the two most crucial parameters determining the efficiency of reversible and irreversible electroporation by PEF (Zimmermann *et al*. 1973; Benz *et al*. 1979; Weaver 1993; Chen *et al*. 2006). But this study demonstrated that the most crucial parameters for electroporation were the amplitude of the electric field and the specific energy input. The application of an electric field with a specific energy input higher than the critical values could generate pore formation through the cell membrane, while the pulse shape, pulse width and the number of pulses were not significant. For *C. humilis*, the minimal electric field strength and the specific energy input for electroporation was about 2 kV/cm and 3 kJ/kg, respectively. However, pore formation on the cell membrane was not equal to cell death. The application of higher specific energy input could expand and fuse pores formed on the cell membrane by PEF, leading to cell death. The combination of electroporation with other hurdles, including mild thermal processing and low pH, could help kill microorganisms (Aronsson and Rönner 2001; Huang *et al*. 2013). For the first time this study inactivated yeast cells by PEF treatment with nanosecond pulses (as short as 53 ns), while past studies could only reversibly electroporate cells with nanosecond pulses (Žgalin *et al.* 2012).

PEF treatments with HIE devices could greatly decrease the injection of electrons (nearly zero) from conductive electrodes into electrolyte (May and Ruben 2012). In this case, the production

of gases and other toxic compounds and the corrosion of electrodes could be completely avoided. This study was the first to compare the performances of LIE and HIE devices and demonstrate that conduction current was not necessary for electroporation by PEF and the lethal effect of PEF treatment. However, electrolysis could greatly enhance the lethal effect of PEF, which has also been demonstrated by other researchers in other ways (Sato *et al*. 1996; Sandvik *et al*. 2013).

This study also demonstrated that the combination of electroporation with cytotoxic compounds, calcium ions, could greatly increase the lethality of yeast cells by PEF treatment. Cells suspended in acidic fluid were more susceptible to PEF treatment. Changing the composition of the fluid could change the most favourable electrochemical reaction at anode and the also change the electrical conductivity of fluid, which may also influence the efficacy of PEF treatment.

4.2 Conclusion

In conclusion, specific energy input was the parameter that could best explain the efficacy of PEF. There was a minimal requirement of electric field strength and specific energy input for cell electroporation by PEF. Conduction current was not necessary for electroporation and inactivation of cells by PEF. But electrolysis could greatly enhance the lethal effect of PEF. Calcium ions showed synergistic effect with electroporation by PEF. Cells in acidic fluid were more susceptible to PEF treatment. Changing fluid composition could change the efficacy of PEF.

In food industry, depending on the different purpose, PEF treatments can be employed to form pores through the cell membrane or to inactivate microorganisms. Covered electrodes can be used to form pores through the cell membrane without generating electrolysis; the addition of calcium ions can increase the lethal effect of PEF treatments on foodborne pathogens and food spoilage microorganisms.

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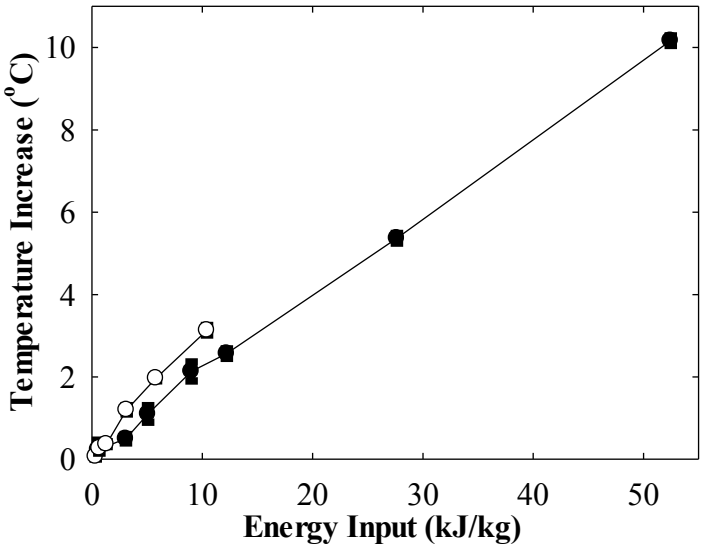
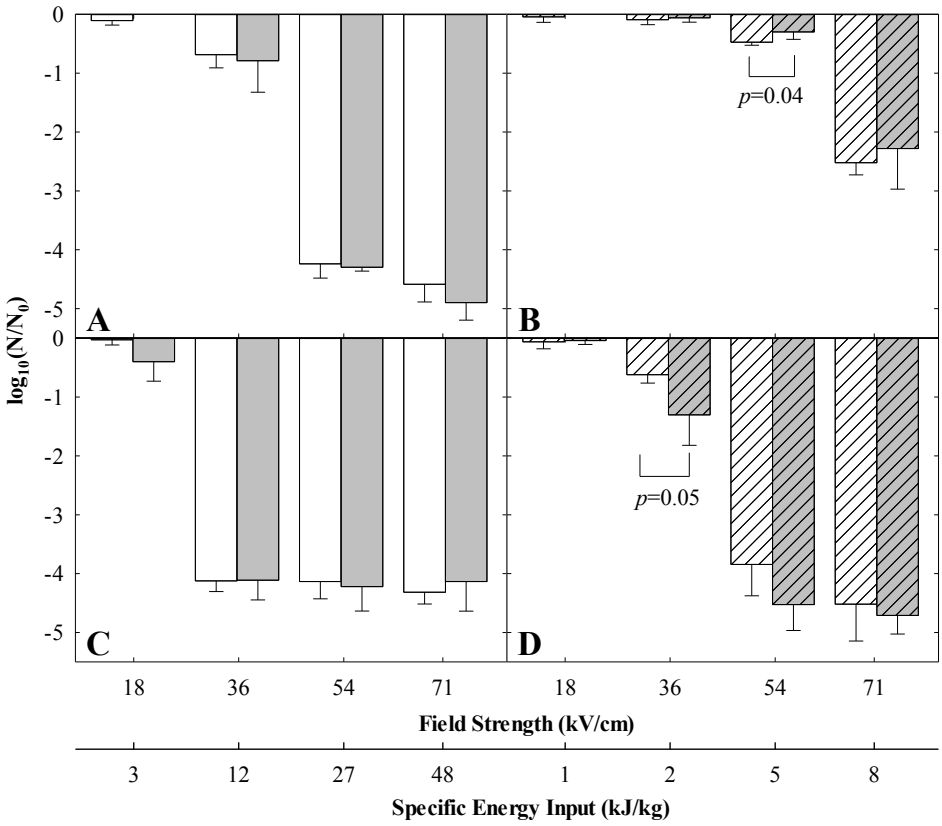
Appendix A: Temperature increase



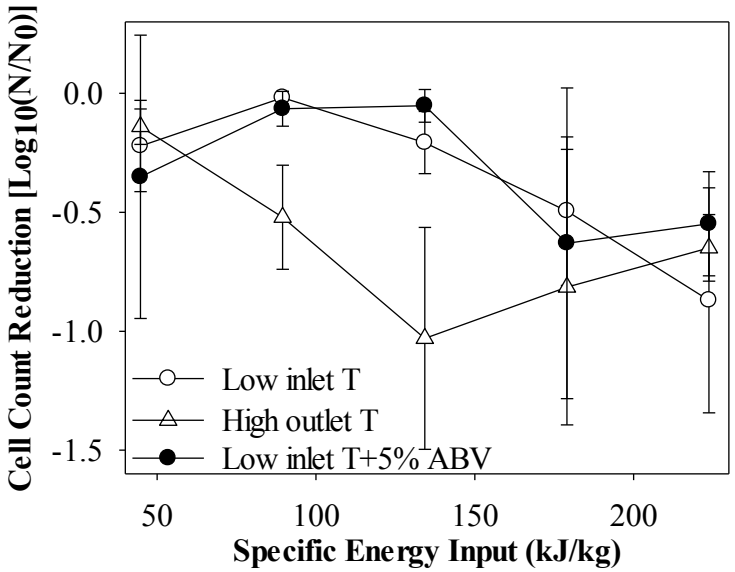
The temperature increase of potassium phosphate buffer during PEF treatment as a function of specific energy input. (○): PEF treatment with high impedance electroporation (HIE) device; (●): PEF treatment with low impedance electroporation (LIE) device.

Appendix B: Effect of pulse width on the efficacy of PEF



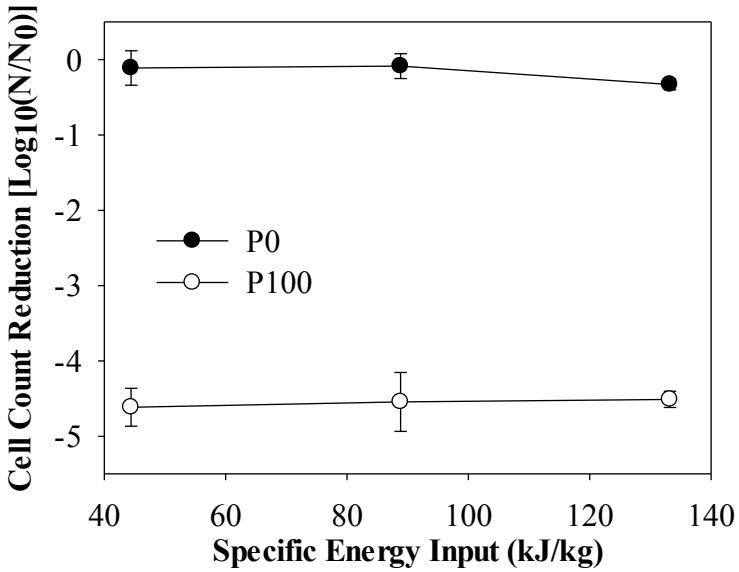
Effect of pulse width on the lethality of *C. humilis* by PEF (gold electrodes). Panel A and B: PEF treatment in the absence of propidium iodide; panel C and D: PEF treatment in the presence of 100 μM of propidium iodide. Panel A and C: PEF treatment with unipolar square pulses; panel B and D: PEF treatment with bipolar exponential pulses. To keep the specific energy input and field strength constant across treatments, the increase of pulse width was compensated by the decrease of pulse frequency. Square pulses of 125 ns (**white bar**) and 250 ns (**grey bar**) were applied at a frequency of 40 kHz and 20 kHz, respectively; exponential pulses with τ = 172 ns (**white hatched bar**) and 344 ns (**grey hatched bar**) were applied at 5 kHz and 2.5 kHz, respectively. The flow rate was 0.5 mL/min. The inlet temperature was about 20 ˚C.

Appendix C: Effect of ethanol on the efficacy of PEF



S. cerevisiae cell suspensions with (●) or without (○) 5 % alcohol by volume (ABV) were chilled on ice to keep inlet temperature at around 6-8 °C. The inlet temperature of another *S. cerevisiae* cell suspension without 5 % ABV (Δ) was adjusted to keep the outlet temperature of samples processed with different specific energy input in the range of 40 to 50 °C. All *S. cerevisiae* cell suspensions with or without 5 % ABV were processed with gold electrodes with unipolar square pulses of 500 ns and 71 kV/cm. Treatments were performed with specific energy input ranging from 45 to 224 kJ/kg, and the flow rate was 0.5 mL/min. The decrease of electrical conductivity of the fluid was neglected in this test.

Appendix D: Effect of processing temperature on the lethality of *S. cerevisiae* by PEF

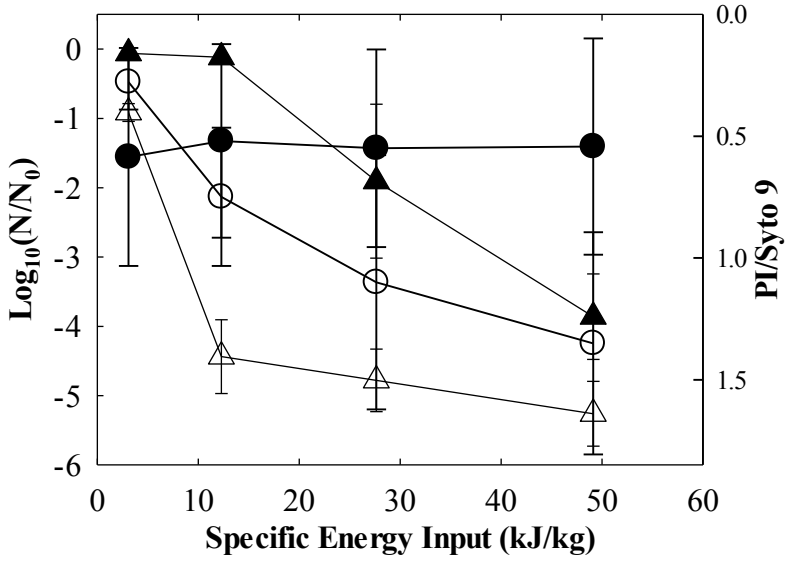


S. cerevisia cell suspensions with (○) and without (●) 100 μM of propidium iodide were chilled on ice before PEF treatments, then processed with gold electrodes with unipolar square pulses of 0.5 μs and 71 kV/cm. Treatments were performed with varying specific energy input from 44 to 133 kJ/kg by increasing pulse frequency from 10 to 30 kHz accordingly. The inlet temperature was kept at 14 °C, and the outlet temperature was below 40 °C. All PEF treatments in the absence of propidium iodide achieved less than 0.5-log cell count reduction, while that in the presence of 100 μM of propidium iodide achieved around 4.5-log cell count reduction. The minimal specific energy input for electroporating *S. cerevisiae* cell membrane was about 5 kJ/kg (see **Fig. 2.7B**). These data indicate that almost all of the cells can be electroporated at a specific energy input of 44 kJ/kg, but they can not be killed even at a high specific energy input, 133 kJ/kg, which means there are some other factors dominating the lethal effect of PEF treatment.

When the inlet temperature of *S. cerevisiae* cell suspension (between 8 and 36 ˚C) was adjusted to ensure the outlet temperature was in the range of 40 to 50 °C, PEF treatments with different specific energy input ranging from 45 to 224 kJ/kg achieved about 0.5 to 1.0-log cell count reduction, except for treatment with a specific energy input of 45 kJ/kg (see **Appendix E**). This may be because of the relatively low outlet temperature (around 40 °C). When the inlet temperature of *S. cerevisiae* cell suspension was kept at about 12 or 20 ˚C, the LIE devices were broken at a specific energy input of 134 and 179 kJ/kg, respectively. The outlet temperature was about 50 ˚C for both of them. However, there was an indication that the

lethality of *S. cerevisiae* by PEF was increased by the combination of thermal processing (higher than 50 ˚C) with PEF treatment. In this experiment, the increase of electrical conductivity of fluid with the increase of temperature was neglected.

Appendix E: Determination of reversible and irreversible electroporation by addition of propidium iodide before or after PEF treatment



C. humilis cell suspensions with (▲, ●) or without (Δ, ○) propidium iodide were processed by PEF treatment with unipolar square pulses of 500 ns and 10 kHz (gold electrodes). The electric field strength ranged from 18 to 71 kV/cm. The flow rate was 0.5 mL/min. The inlet temperature was about 20 ˚C. The Y axis on the left side is the cell count reduction ($Log_{10}(N/N_0)$); The Y axis on the right side is the relative fluorescence intensity (PI/Syto 9). The symbols represent two different methods used to determine the proportion of reversible and irreversible electroporation by PEF: (▲,Δ): enumeration of viable cells by plate count after PEF treatment; (●,○): detection of relative fluorescence intensity after PEF treatment.