Synergistic Effect of Pulsed Electric Fields and Temperature on The Inactivation of Microorganismsill

Zeyao Yan
Fudan University
Li Yin
Fudan University
Chunjing Hao
Fudan University
Kefu Liu (kfliu@fudan.edu.cn)
Department of Light Sources and Illuminating Engineering, Fudan University, Shanghai 200433, China
Jian Qiu
Fudan University

Original article

Keywords: Pulsed electric fields, initial temperature, critical electric field intensity, nucleic acid content, protein content

Posted Date: January 15th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-144619/v1

Version of Record: A version of this preprint was published on March 23rd, 2021. See the published version at https://doi.org/10.1186/s13568-021-01206-8.

Synergistic effect of pulsed electric fields and temperature on the inactivation of microorganisms

Zeyao Yan, Li Yin, Chunjing Hao, Kefu Liu*, Jian Qiu

(Department of Light Sources and Illuminating Engineering, Fudan University, Shanghai 200433, China)

Abstract

Pulsed electric fields (PEF) as a new pasteurization technology played an important role in the process of inactivating microorganisms. At the same time, temperature could promote the process of electroporation, and achieve better inactivation effect. This article studied the inactivation effect of PEF on *Saccharomyces cerevisiae*, *Escherichia coli*, and *Bacillus velezensis* under different initial temperatures (room temperature-24 ℃, 30 ℃, 40 ℃, 50 ℃). From the inactivation results, it found temperature could reduce the critical electric field intensity for microbial inactivation. After the irreversible electroporation of microorganisms occurred, the nucleic acid content and protein content in the suspension increased with the inactivation rate because the cell membrane integrity was destroyed. We had proved that the electric field and temperature could promote molecular transport through the finite element simulation. Under the same initial temperature and electrical parameters (electric field intensity, pulse width, pulse number), the lethal effect on different microorganisms was *Saccharomyces cerevisiae* > *Escherichia coli* > *Bacillus velezensis.*

* Corresponding author at: No.220 Handan road, Yangpu district, Shanghai, China.
E-mail address: kfliu@fudan.edu.cn (K. Liu). Tel: +86 21 55665184 Fax :+86 21 55665350
Zeyao Yan E-mail address: 17110720008@fudan.edu.cn Li Yin E-mail address: 19210720016@fudan.edu.cn
Chunjing Hao E-mail address: chunjinghao@126.com Kefu Liu E-mail address: kfliu@fudan.edu.cn
Jian Qiu E-mail address: jqiu@fudan.edu.cn

Keywords Pulsed electric fields; initial temperature; critical electric field intensity; nucleic acid content; protein content

Introduction

As we know, food safety and quality, which are humans pursuing health and quality of life concerns, are the dominating purpose of food testing and analysis (Weng&Neethirajan,2017). More and more consumers want the food they eat to be green, pollution-free, nutritious, as fresh as possible, and free from microbial contamination (Bisconsin-Junior, Alvarenga, Rosenthal&Monteiro,2015). The traditional thermal pasteurization technology has a good effect in controlling the quantity of microorganisms. However, thermal treatment damages the heat-sensitive substances and nutrients of food, motivating the development of new pasteurization technologies. The new pasteurization technology is expected to be energy-saving, efficient, and causes less damage to nutrients with minimal impact on food quality (Pasha, Saeed, Sultan, Khan&Rohi,2014, Wang, Wang, Bekhit, Yang, Hou, Wang, Dai&Zeng,2018, Yogesh,2016). PEF processing is one of these non-thermal techniques that has been investigated during the last decades as an alternative for food pasteurization without damage to food (Timmermans, NieropGroot, Nederhof, van Boekel, Matser&Mastwijk,2014). The PEF pasteurization technology refers to applying a high pulsed voltage between two electrodes to form a uniform or non-uniform electric field region having a lethal effect on the microbes (Toepfl, Heinzb&Knorr,2007). When the liquid solution containing microbes flows through the intense electric fields region, the integrity of the cell membrane is damaged and the

membrane permeability increases significantly (Huo, Li, Yu, Feng, Lu, Wu, Yu&Hu,2018). The genetic material inside the cells flows out to achieve the purpose of completely killing microbes (El Zakhem, Lanoiselle, Lebovka, Nonus&Vorobiev,2007). Many studies have scientifically reported the effective application of PEF in food pasteurization and it promises to be a new method of pasteurization (Mahnic-Kalamiza, Vorobiev&Miklavcic,2014).

In recent years, most reports have examined the effects of single electrical or environmental conditions on microbial inactivation (González-Arenzana, Portu, López, Santamaría, Gutiérrez, López&López-Alfaro,2019, van Wyk, Silva&Farid,2019). Factors affecting the results of microbial inactivation involve various aspects, including PEF parameters (pulse amplitude, duration, shape, number, rising edge, polarity), cell parameters (size, shape, orientation), and medium parameters (temperature, composition, conductivity, ionic strength, pH) (Bermúdez-Aguirre, Dunne&Barbosa-Cánovas,2012, Grahl&Markl,1996, Saulis,2010). PEF has proven to be capable of inactivating microorganisms, and increasing the electric field intensity is the most effective way to increase the inactivation rate of microorganisms.

However, excessive electric field intensity will cause bubbles in the liquid food to be processed and abnormal breakdown, which is very unfavorable and undesirable in the food processing process (Wei, Li, Wang&Zhu,2017). It is hoped to be combined with other methods that may synergistically inactivate microorganisms to achieve more sufficient pasteurization (Garner,2019, Vadlamani, Detwiler, Dhanabal&Garner,2018). Applying combined PEF-thermal treatments can induce membrane pore formation and

damage, including lysis (El Zakhem, Lanoiselle, Lebovka, Nonus&Vorobiev,2006). Choosing appropriate temperature and electrical parameters can effectively inactivate microorganisms, ensure food quality, extend the shelf life, and avoid breakdown of liquid food caused by excessive electric field intensity.

Some articles have reported the method of inactivating microorganisms that combine PEF with temperature in quasi-isothermal or non-isothermal conditions (Amiali, Ngadi, Smith&Raghavan,2007, Saldaña, Puértolas, Álvarez, Meneses, Knorr b&Raso,2010). However, these reports do not provide details on the inactivation effect of pure temperature, the inactivation effect of PEF at room temperature, and the inactivation effect of combined temperature-PEF. Moreover, the microbial species treated are relatively single, and the research is not universal, so it is not possible to explain systematically the inactivation effect of combined thermal-PEF treatments.

In this study, we have investigated the inactivation of *Saccharomyces cerevisiae*, *Escherichia coli* and *Bacillus velezensis* under pure thermal treatment and pure PEF treatment (room temperature-24 ℃ as a standard temperature) and combined thermal-PEF treatments (30 ℃, 40 ℃, 50 ℃). These three microorganisms include fungi, bacteria, prokaryotes, eukaryotes, gram-positive bacteria, gram-negative bacteria, bacteria with spore structure and bacteria without spore structure, demonstrating the universality of combined temperature-PEF on microbial inactivation. At the same time, through the linear fitting, it can be obtained the temperature can reduce the critical electric field intensity, which can prove that temperature promotes the occurrence of irreversible electroporation. The content of nucleic acid and protein that spilled from

the cells into the suspension is related to the inactivation rate of microorganisms.

Materials and methods

Cultivation of microorganisms

Saccharomyces cerevisiae ATCC 201238 was inoculated into the prepared sterile yeast extract peptone dextrose medium (YPD) composed from 20 g/L of peptone, 20 g/L of glucose, 10 g/L of yeast extract powder in a sterile environment, and cultured at 30 ℃ for approximately 12 h. The medium and culture conditions for culturing *Escherichia coli* CICC 10899 and *Bacillus velezensis* CICC 24434 were the same. The medium used was a nutrient broth medium composed of 5 g/L sodium chloride, 10 g/L peptone, and 3 g/L beef extract powder, and the culture condition was 12 h at 37 ℃. We set the initial concentration of microorganisms at 10^8 to 10^9 CFU/ml. The electric conductivity and pH of the sample were measured by conductivity meter and pH meter, respectively, and they were 3.7mS/cm and 6.2. The specific flow chart of microbial culture and the system diagram of the whole experiment are shown in Fig. 1.

Experiment system

The high voltage pulse generator was independently developed by Fudan University high power electronics research group (Shanghai, China). The high voltage pulse generator could output a high voltage ranging from 0-30 kV, the pulse width ranging from 0.5-2.5 μs, and the frequency ranging from 0.1-10 Hz. Typical voltage and current waveforms used in this experiment are shown in Fig.2. Since we chose exponential waves in our experiments, it was necessary to explain the definition of the pulse width. We defined the full width at half maximum of the pulse waveform as the

pulse width, which was adjusted by changing the capacitance in the circuit.

In the experiment, the treatment chamber adopted a flat plate structure, the distance between the two plate electrodes was 4 mm, the width of a single plate was 10 mm, the height was 20 mm, the cross-sectional area was 200 mm^2, and the total treatment volume was 0.8 mL. PEF pasteurization was suitable for high-resistance loads. Long electrode spacing and small facing areas were conducive to forming high-resistance loads. However, long electrode distances caused a reduction in the electric field intensity, which was bad for pasteurization. Considering all factors, the structural parameters of the above-mentioned treatment chamber were determined. The treatment chamber with the flat structure could form a uniform electric field, so the pasteurization effect was more uniform than other structures.

The experiment system is shown in Fig.3. The voltage characteristics across the load and the current characteristics through the load were measured respectively by a four-channel oscilloscope DSO7104B (Agilent Technologies) with an external high voltage probe (1000:1) and a current loop (100:1). The thermostatic oil field played two purposes in this system. One was to control the initial temperature of the sample to be treated by changing the temperature of the oil field. The other was to make the Joule heat generated during the action of the PEF dissipate as quickly as possible. Thermocouple was used to detect the initial temperature of the sample and the final temperature after PEF treatment.

Thermal treatment, PEF treatment, and PEF-thermal treatments

In order to study the influence of the temperature on the inactivation of

131 microorganisms by PEF, we conducted three sets of experiments. 1) pure thermal
132 inactivation, 2) pure PEF inactivation (at room temperature) and 3) PEF-thermal
133 inactivation.

134 The effect of microbial inactivation under specific treatments was measured by the
135 amount of viable microbial by the plate counting before and after treatments. The plate
136 counting was the best way to define the effect of inactivation because it prevented pore
137 healing, which could lead to suspended animation of microorganisms
138 (Rols&Teissie,1990). Defined survival rate $S = Log(N/N_0)$ as a criterion for judging
139 inactivation effect, N (CFU/mL) is the number of viable microbial after processing, and
140 N_0 (CFU/mL) is the number of viable microbial before processing. Each experiment
141 was repeated at least three times, and the mean value of the three experimental results
142 were calculated to determine the final experimental results, including error bars being
143 by standard error. Statistical analysis was performed using one-way analysis of variance
144 (ANOVA; $P < 0.05$).

145 **Data fitting analysis**

146 Based on the least squares fit method, the data were fitted and analyzed by OirginPro
147 software (Version 2017). R^2 was the statistical parameter to show the goodness of the
148 fits.

149 # Results

150 **Electric field distribution in the treatment chamber**

151 Different treatment chambers would produce different load characteristics,
152 resulting in different electric field distributions, which could have different effects on

inactivation effects. The uniform electric field generated by the parallel plate electrodes could avoid the problem of under-treatment or over-treatment, which was crucial for food pasteurization compared with the non-uniform electric field generated by coaxial electrodes (Huang&Wang,2009). The electric field distribution in the treatment chamber was obtained through the numerical simulation in Fig.4.a. Simulations were performed using finite element simulation software COMSOL Multiphysics v5.3a. The simulated electric field was uniformly distributed throughout the chamber except for distortion along the corners and edges where the electrodes were in contact with the insulated medium to produce a strong electric field intensity at the tip. The distorted electric field region occupied a quite small part of the whole electric field region, and its influence could be ignored. The distorted electric field region was enlarged and could be seen in the upper right corner of Fig.4.a. From Fig.4.b, the homogeneous electric field (E = 20 kV/cm) was obtained within the active part of the chamber between the two electrodes, while the electric field outside the active region is zero (the blue line). There were obvious electric field distortions at the corner of the chamber, resulting in a significant increase in the electric field (the red line).

Analysis of microbial inactivation

Pure thermal inactivation. High temperatures had a lethal effect on microorganisms because the high temperature could denature proteins structure of microorganisms, thereby killing microorganisms. In this paper, we chose a temperature range of 30 to 50 ℃ in increments of 5 ℃, because the temperature in this range was mild and would not have much impact on substances other than microorganisms. What

needs to be emphasized was that the processing time for microbial inactivation by pure temperature was 1 min, which ensured the same time as the pure PEF treatment and PEF-thermal treatment. As shown in Fig.5, we got the curve of the survival rate of three microorganisms (*Saccharomyces cerevisiae, Escherichia coli*, and *Bacillus velezensis*) with temperature. The survival rate of *Saccharomyces cerevisiae* after pure thermal treatment decreased by 0.02, 0.11 and 0.32 logs at the processing temperature of 30, 40 and 50 ℃. The survival rate of *Escherichia Coli* decreased by 0.01, 0.09, and 0.17 logs at the processing temperature of 30, 40, and 50 ℃. Because of *Bacillus velezensis* with spore structure, which made it heat-resistant, there was no change in the survival rate of *Bacillus velezensis* within this temperature range.

Pure PEF inactivation. We investigated the effect of the three electrical parameters (electric field intensity, pulse width, pulse number) on microbial inactivation. The method used to study any of three electrical parameters was the controlled variable method. In this experiment, the electric field intensity adopted 5, 10, 15, 20, and 25 kV/cm. The pulse widths were 0.5, 1.2, 1.7, and 2.1 μs. The number of pulses were 50, 100, 150, 200, 150, 300, 600, 900, and 1200. In all experiments, the frequency of PEF was chosen to be 10 Hz.

The effect of electric field intensity on microbial survival rate. The experimental conditions to study the effect of electric field intensity on microbial inactivation as follows: the electric field intensity varied from 5 to 25 kV/cm, increasing at intervals of 5 kV/cm, under the condition of pulse width of 0.5 μs, pulse number of 600 and the pulse frequency of 10 Hz. In Fig.6.a, the curve drawn in red square described the

change of *Saccharomyces cerevisiae* survival rate with the electric field intensity. The electric filed intensity changed from 5 to 25 kV/cm , and the survival rate correspondingly changed from -0.11 to -3.02 causing approximately 2.9 logs change. The survival rate decreased significantly with the increase of electric field intensity ($P<0.05$). In Fig.6.b, we could found that the survival rate of *Escherichia Coli* decreased significantly with the increase of the electric field intensity from the red square curve ($P<0.05$). At 25 kV/cm, the survival rate of *Escherichia Coli* was -2.52, which was more than that of *Saccharomyces cerevisiae.* The red square curve in Fig.6.c described the influence of electric field intensity on the survival rate of *Bacillus velezensis*. The survival rate of *Bacillus velezensis* decreased significantly with the increase of the electric field intensity ($P<0.05$). Compared with *Saccharomyces cerevisiae* and *Escherichia coli*, the PEF had the worst inactivation effect on *Bacillus velezensis*, and the survival rate corresponding to the electric field intensity of 25 kV/cm was -2.06.

The effect of pulse width on microbial survival rate. Under the condition of electric field intensity of 20 kV/cm, pulse number of 600, pulse frequency of 10 Hz, we studied the microbial survival rate as a function of pulse width from 0.5 to 2.1 μs. From Fig.7, the red square curve described that the survival rates of *Saccharomyces cerevisiae*, *Escherichia coli* and *Bacillus velezensis* decreased with the increase of the pulse width ($P>0.05$). The pulse width changed from 0.5 to 2.1 μs , the survival rate of *Saccharomyces cerevisiae* was changed from -2.74 to -2.90, the survival rate of *Escherichia coli* was changed from -1.99 to -2.17, and the survival rate of *Bacillus velezensis* changed from -1.53 to -1.70. The increase in pulse width could reduce the

survival rate of microorganisms, but the degree of change in survival rate is not significant ($P>0.05$).

The effect of pulse number on microbial survival rate. Consistent with the method of studying electric field intensity and pulse width, we investigated the effect of pulse number on microbial inactivation. The experimental electrical parameters were as follows, electric field intensity of 20 kV/cm, pulse width of 0.5 μs, and pulse frequency of 10 Hz. We could found that increasing pulse number could effectively reduce the survival rate of *Saccharomyces cerevisiae*, *Escherichia coli* and *Bacillus velezensis* from the red square curve in Fig.8. After pulse number increased to 600, the survival rate changed not significantly ($P>0.05$). Therefore, when we studied the effect of electric field intensity and pulse width on the survival rate, we chose to set pulse number to 600.

PEF-thermal inactivation. We first raised the samples to different initial temperatures (30 ℃, 40 ℃, 50 ℃) through the constant temperature oil domain, and then conducted PEF pasteurization at different initial temperatures. The selection of electrical parameters was consistent with that of pure PEF treatment. It took about 46 s for the untreated sample to grow from room temperature to the highest initial temperature 50 ℃. Therefore, before the PEF treatment, we waited for 1 min for the sample to reach the required initial temperature, and then performed PEF pasteurization. Similarly, the samples processed by pure thermal treatment and pure PEF treatment were placed in the treatment chamber for 1 min to reach the required temperature.

The effect of initial temperature and electric field intensity on microbial survival rate.

In Fig.6.a, the increase in temperature and electric field intensity caused survival rate of *Saccharomyces cerevisiae* to decrease. At 25 kV/cm and 50 ℃, the survival rate decreased by 5.04 logs, which was much greater than 0.32 logs obtained by pure thermal treatment at 50 ℃ and 3.02 logs obtained by pure PEF treatment at 25 kV/cm. At 25 kV/cm, by increasing the initial temperature of the sample, the survival rate of *Saccharomyces cerevisiae* could be effectively reduced, and the survival rate changed from -3.02(room temperature-24 ℃) to -5.04 (50 ℃). In Fig.6.b and Fig.6.c, we could find that the inactivation effect of temperature and electric field intensity on *Escherichia coli* and *Bacillus velezensis* had the same trend as *Saccharomyces cerevisiae.* When *Escherichia coli* and *Bacillus velezensis* were exposed to higher electric field intensity and temperature, the corresponding survival rate became lower. At 25 kV/cm and 50 ℃, the survival rate of *Escherichia coli* decreased by 4.56 logs, which was much greater than 0.17 logs treated by pure thermal treatment (50 ℃) and 2.52 logs obtained by pure PEF treatment at 25 kV/cm. At 25 kV/cm and 50 ℃, the survival rate of *Bacillus velezensis* decreased by 4.05 logs. Inversely, the survival rate of *Bacillus velezensis* decreased by 2.06 logs obtained by pure PEF treatment at 25 kV/cm. It was worth mentioning that pure thermal treatment did not have the inactivation effect on *Bacillus velezensis*, but a higher initial temperature promoted the inactivation effect on *Bacillus velezensis* by the PEF.

The effect of initial temperature and pulse width on microbial survival rate. In Fig.7.a, the survival rate of *Saccharomyces cerevisiae* changed significantly from -4.11 to -5.21 when the pulse width changed from 0.5 to 2.1 μs at 50 ℃ ($P<0.05$). This change in

survival rate was obvious compared with that in pure PEF inactivation. The same pulse width change only caused the survival rate to change from -2.74 to -2.9 under pure PEF treatment, which was not significant ($P>0.05$). From Fig.7.b, and Fig.7.c, we found that increasing the pulse width and initial temperature could decrease the survival rate of *Escherichia coli*, and *Bacillus velezensis*. At 50℃, the pulse width changed from 0.5 to 2.1 μs, the survival rate of *Escherichia coli* was changed from -4.01 to -4.76, and the survival rate of *Bacillus velezensis* changed from -3.28 to -3.61.

The effect of initial temperature and pulse number on microbial survival rate. Fig. 8 a, b, c respectively described the survival rate curve of *Saccharomyces cerevisiae*, *Escherichia coli*, and *Bacillus velezensis* under different initial temperature and pulse number. The initial temperature and pulse number reduced the survival rate of microorganisms, which was consistent with the influence of electric field intensity and pulse width on microbial inactivation. When the pulse number was less than 600, the microbial survival rate decreased significantly with the increase of pulse number ($P<0.05$). After the pulse number reached 600, the decreasing trend of the microbial survival rate became slower with the increase of the pulse number.

We studied the inactivation of *Saccharomyces cerevisiae*, *Escherichia coli*, and *Bacillus velezensis* by three different electrical parameters (electric field intensity, pulse width, pulse number) under different initial temperatures. Under the same initial temperature and electrical parameters, *Saccharomyces cerevisiae* had the lowest survival rate, followed by *Escherichia coli*, and *Bacillus velezensis* had the highest survival rate. This was because *Saccharomyces cerevisiae* was the largest and had the

strongest sensitivity to electric fields (DeBruin&Krassowska,1999, Krassowska&Filev,2007). *Escherichia coli* was a gram-negative bacterium, and it was moderately sensitive to electric fields (Evrendilek, Z.T, Ruhlman, Qiu, Zhang&Richter,2000). *Bacillus velezensis* had the least sensitive to electric fields because it was a gram-positive bacterium (Heinz, Alvarez, Angersbach&Knorr,2001, Heinz, Phillips, Zenker&Knorr,2009). What is more, *Bacillus velezensis* had spore structure which was resistant to electric field.

The synergistic effect of PEF-thermal treatment

PEF-thermal treatments induced greater inactivation than either PEF or thermal treatments individually. Here we discussed a more important issue. We compared the inactivation effect of combined PEF-thermal treatments with the sum of the inactivation effect of their two separate treatments. Based on equation (1), the sum survival rate S_{T+PEF} of pure temperature and pure PEF treatments could be obtained.

$$S_{T+PEF} = \mathrm{Log}\left(\left(\frac{N}{N_0}\right)_T \times \left(\frac{N}{N_0}\right)_{PEF}\right)$$

$$= \mathrm{Log}(N/N_0)_T + \mathrm{Log}(\mathrm{N}/N_0)_{PEF} = S_T + S_{PEF} \quad (1)$$

Where S_T is the survival rate under pure thermal treatments, and S_{PEF} is the survival rate under pure PEF. All the results were obtained under the experimental conditions of pulse width of 0.5 μs, pulse number of 600 and frequency of 10 Hz. We defined the synergy coefficient *k* to characterize the synergistic effect of PEF-thermal treatments on microorganism inactivation.

$$k = \frac{S}{S_{T+PEF}} = \frac{Log(N/N_0)}{Log(N/N_0)_T + Log(N/N_0)_{PEF}} \quad (2)$$

306 S is the survival rate of combined PEF-thermal treatments.

307 As shown in Fig.9, we could find that k was bigger than 1, which approved the
308 synergistic effect between temperature and PEF. The inactivation effect of combined
309 thermal-PEF treatment was better than the sum inactivation effect of pure thermal
310 treatment and pure PEF treatment. For *Saccharomyces cerevisiae*, *Escherichia coli*, and
311 *Bacillus velezensis*, the electric parameter and initial temperature of the maximum
312 synergy coefficient were 10 kV/cm and 50 ℃. Especially for *Bacillus velezensis*,
313 pure thermal treatment of 50 ℃ had no lethal effect on *Bacillus velezensis*. However,
314 we control the initial temperature to 50 ℃, combined with PEF treatment, and the
315 survival rate was significantly decreased compared with pure PEF treatment.

316 **Temperature rise caused by Joule heating**

317 When the PEF was used to inactivate microorganisms, the current passing through
318 the suspension generated Joule heating, thereby increasing the temperature of the
319 suspension. The temperature change of the suspension had a very important relationship
320 with the quality of the liquid food, so it was necessary to detect the temperature of the
321 sample after the PEF treatment. Here we studied the changes in temperature after pure
322 PEF treatment and combined PEF-thermal treatment when the electric field intensity
323 was a variable. The temperature rise under different conditions is shown in Table 1.

324 **Table 1.** The temperature rise under different initial temperatures and electric field
325 intensities.

Initial temperature (℃)	Electric field intensity (kV/cm)	Temperature rise ΔT(℃)

Room temperature (24)	5	0.9
	10	4.7
	15	12.7
	20	20.3
	25	25.2
30	5	0.5
	10	4.0
	15	10.8
	20	17.2
	25	23.1
40	5	0.3
	10	1.4
	15	7.2
	20	14.0
	25	19.6
50	5	0.2
	10	1.1
	15	6.8
	20	12.6
	25	18.3

Detection of nucleic acid content and protein content in suspension

Microorganisms underwent electroporation under the action of PEF. Nucleic acids

and proteins in the cells overflowed into the suspension, thereby killing microorganisms completely. The bases that made up the nucleic acid molecule had the characteristic of ultraviolet absorption due to the aromatic ring structure. The absorption value was between 250-270 nm and the maximum absorption wavelength was 260nm (Huo, Luo, Xie, Feng, Jiang, Wang&Hu,2017). Therefore, the content of nucleic acid could be calculated by measuring the light absorption value of nucleic acid at 260nm. The protein contained conjugated double bonds tyrosine and tryptophan, and the absorbance at 280 nm was used to estimate the protein content. As shown in Fig.10, we used a micro-spectrophotometer to measure the content of nucleic acid and protein in the suspension under different initial temperatures and electric field intensities. The content of nucleic acid and protein in the suspension increased significantly with the increase of the electric field intensity and the initial temperature ($P<0.05$). The lower the survival rate of microorganisms, the higher the nucleic acid and protein content in the suspension. The nucleic acid content and protein content in the suspension were negatively correlated with the survival rate of microorganisms. After microorganisms were treated by the PEF, the permeability of the cell membrane increased, and nucleic acids and proteins overflowed to the outside of the cell, destroying the homeostasis of the cell and the normal physiological process of the cell, and resulting in inactivation of microorganisms.

The effect of temperature and electric field on molecular transport

The material inside and outside the cell was exchanged through the cell membrane. When electroporation occurred, the outflow of genetic material in the cell destroyed the

stability of the cell and achieved the goal of completely inactivating microorganisms. Molecular transport in porous media under an electric field maintained the Nernst-Planck equation.

$$\frac{\partial c}{\partial t} = \nabla \cdot \left(D\nabla c + \frac{DzF}{RT} cE \right) \tag{3}$$

$$D = \mu_P k_B T \tag{4}$$

Where c is the concentration of molecules, D is the diffusion coefficient, μ_P is molecules velocity, k_B is Boltzmann constant, z is the charge number, F is Faraday's constant, R is the ideal gas constant, T is absolute temperature and E is electric field intensity.

We had carried out simulation research of transport of molecules pass through pores in the cell membrane on the conditions of 1). 30 ℃, 5 kV/cm, 2). 40 ℃, 5 kV/cm, 3). 50 ℃, 5 kV/cm, 4). 30 ℃, 15 kV/cm, 5). 40 ℃, 15 kV/cm, 6). 50 ℃, 15 kV/cm, 7). 30 ℃, 25 kV/cm, 8). 40 ℃, 25 kV/cm, and 9). 50 ℃, 25 kV/cm. We assumed that the initial concentration of molecules inside cell was 2×10^{-4} mol/m^3, the initial concentration of molecules outside cell was 0 and the pore radius was 10 nm. All parameter values and boundary conditions could be obtained from this reference (Jayasooriya&Nawarathna,2017).

From Fig.11, we presented the different diffusion results under nine different conditions at 2 s. In Fig.11, we calculated the area where the concentration of the extracellular molecule was greater than 2×10^{-5} mol/m^3. We could find that increasing the electric field intensity and temperature promoted the transport of intracellular materials, and the concentration of extracellular molecules was higher.

Both temperature and electric field accelerated the transport of molecules, destroyed the integrity of cells, increased the degree of irreversible electroporation, and increased the inactivation rate of microorganisms. According to equation (3), we could find that the diffusion of molecules was proportional to temperature and electric field intensity, which was consistent with simulation results. At the same time, the simulation results further proved that in our previous part of the experiment, increasing the temperature and electric field intensity could increase the nucleic acid content and protein content in the suspension.

Microbial inactivation kinetics of combined PEF-thermal treatment

The initial model of survival rate as a function of electric field intensity was proposed by Huelsheger (Hulsheger, Potel&Niemann,1981). The survival rate S was related to the electric field intensity by

$$S = -b_e(E - E_C) \tag{5}$$

Where b_e is a regression coefficient and E_c is the electric field intensity at the extrapolated survival fraction of 100%. We fitted the experiment data of microbial inactivation under different initial temperatures and electric field intensities according to equation (5), and the results obtained by fitting were shown in Fig.12. The value of parameters b_e and E_c are shown in Table 2. The increasing temperature caused E_c to decrease and b_e to increase. For *Saccharomyces cerevisiae*, E_c decreased from 5.632 to 1.859 and b_e increased from 0.1676 to 0.2254. The increasing temperature reduced the critical electric field intensity of *Saccharomyces cerevisiae* inactivation and increased the slope of *Saccharomyces cerevisiae* inactivation, which represented a

394 better and faster inactivation effect. Similarly, for *Escherichia coli* and *Bacillus*
395 *velezensis*, the higher initial temperature led to a decrease in critical electric field
396 intensity and an increase in slope of microbial inactivation. E_c for *Escherichia coli*
397 decreased from 6.091 to 1.422, and b_e increased from 0.1338 to 0.2068. E_c for
398 *Bacillus velezensis* decreased from 6.266 to 1.960, and b_e increased from 0.1090 to
399 0.1816. The higher temperature reduced the value of the critical electric field intensity,
400 irreversible electroporation was more likely to occur, and the corresponding survival
401 rate was lower. Under the same initial temperature and electrical parameters, we could
402 get that the critical electric field intensity of *Bacillus velezensis* was the largest, and that
403 of *Saccharomyces cerevisiae* was the smallest. This also proved that PEF had the
404 strongest inactivation effect on *Saccharomyces cerevisiae* and the worst effect on
405 *Bacillus velezensis.*

406 **Table 2.** Parameters from regression analysis for experiments.

407 a. *Saccharomyces cerevisiae*

Experiment conditions	b_e	$E_c(\mathrm{kV/cm})$	R^2
Pure PEF	0.1676	5.632	0.9255
PEF+30℃	0.1868	5.043	0.9339
PEF+40℃	0.1974	3.732	0.9597
PEF+50℃	0.2254	1.859	0.9875

408 b. *Escherichia coli*

Experiment conditions	b_e	$E_c(\mathrm{kV/cm})$	R^2

Pure PEF	0.1338	6.091	0.9523
PEF+30℃	0.1396	4.857	0.9616
PEF+40℃	0.1892	4.112	0.9736
PEF+50℃	0.2068	1.422	0.9683

c. *Bacillus velezensis*

Experiment conditions	b_e	$E_c(\mathrm{kV/cm})$	R^2
Pure PEF	0.1090	6.266	0.9530
PEF+30℃	0.1236	5.502	0.9640
PEF+40℃	0.1756	4.351	0.9831
PEF+50℃	0.1816	1.960	0.9837

Scanning electron microscope (SEM) characteristics

We took *Saccharomyces cerevisiae* as an example to observe the changes in surface morphology after thermal treatment, PEF treatment, and combined thermal-PEF treatment. For Fig.13.a, the sample was smooth, integrated and imporous which was used as a control without any treatments. The second sample was treated by pure thermal treatments at 50 ℃ for 1 min. The second sample was not obviously different from the first sample on the surface, and it was also smooth, integrated and imporous from Fig.13.b. The third sample treated by pure PEF had a rough, incomplete and porous surface, which formed a sharp contrast with the first two samples from Fig.13.c. The surface of the sample treated with PEF+50 ℃ was rougher, more incomplete and more porous from Fig.13.d. It could be seen that the damage degree of cell membrane was greater and the electroporation degree was higher. The surface morphology of the

samples treated in different ways was obviously different, which showed that different methods had different degrees of damage to *Saccharomyces cerevisiae*. Increasing the initial temperature, PEF increased the degree of damage to the microbial morphology, and the corresponding microbial survival rate was lower.

Discussions

This article studied the effects of PEF inactivation on *Saccharomyces cerevisiae*, *Escherichia coli*, and *Bacillus velezensis* at different initial temperatures. Increasing the initial temperature could effectively decrease microbial survival rate by PEF, and avoid dielectric breakdown caused by excessive electric field intensity. At the same time, the article studied the temperature rise of the liquid under the action of different electric field intensities and initial temperatures. The higher the electric field intensity, the greater the temperature rise, which was not good for liquid food. Therefore, it was necessary to weigh in the actual application process to match different initial temperatures and electric field intensities to meet specific requirements. The result of linear fitting proved that temperature could reduce the critical electric field intensity for microorganism inactivation. Therefore, increasing the initial temperature could effectively reduce the survival rate of microorganisms. What is more, the synergy coefficient k was bigger than 1, which proved the synergistic effect between temperature and PEF.

The content of nucleic acid and protein in the suspension was negatively correlated with the survival rate of microorganisms. This was because the more inactivated microorganisms, the more substances overflow from the cells. We had verified that

444 temperature and electric field could promote the diffusion of molecules and destroy the
445 steady state of cells through the simulation of molecular transport. This article
446 systematically studied the effects of different initial temperatures (room temperature,
447 30 ℃, 40 ℃, 50 ℃) and different electrical parameters (electric field intensity, pulse
448 number, and pulse number) on different microorganisms (*Saccharomyces cerevisiae*,
449 *Escherichia coli*, *Bacillus velezensis*). These microorganisms include fungi, bacteria,
450 prokaryotic, eukaryotic, gram-positive bacteria, gram-negative bacteria, bacteria with
451 spore structures, and bacteria without spore structures, proved the universality that
452 temperature could promote inactivation of microorganisms by PEF.

453 In this paper, we had studied the influence of temperature on microbial inactivation
454 by PEF from the perspective of microbial inactivation rate, critical electric field
455 intensity for microbial inactivation, synergy coefficient, nucleic acid content and
456 protein content in suspension, and molecular transport. We could see that there were
457 many articles explaining the promoting effect of temperature on the inactivation of
458 microorganisms by PEF, including phase change (Liu&Conboy,2004), cell membrane
459 fluidity (Kanduser, Sentjurc&Miklavcic,2008), cell membrane permeability (Blicher,
460 Wodzinska, Fidorra, Winterhalter&Heimburg,2009). Of course, the mechanism of
461 temperature promoting the inactivation of microorganisms by PEF was complicated
462 and unclear, and it needed further research and exploration.

463 # Declarations

464 ## List of abbreviations

465 PEF: pulsed electric fields

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no conflict of interest.

Funding

We thank the Jihua Laboratory Project of Guangdong Province (No.18002U100), National Natural Science Foundation of China (No.51877406) and Fudan University-CIOMP Joint Fund (No.FC2017-002).

Authors' contributions

Z.Y, C.H, and K.L designed research; Z.Y, L.Y performed research; Z.Y and J.Q designed the high voltage pulse generator; Z.Y analyzed data; Z.Y wrote the paper; and all authors read and approved the manuscript.

Acknowledgements

We thank Professor Yao Yu for his help in microbial culture.

Author details

Department of Light Sources and Illuminating Engineering, Fudan University, Shanghai 200433, China

References

Amiali M., Ngadi M. O., Smith J. P.&Raghavan G. S. V.(2007). Synergistic effect of temperature and pulsed electric field on inactivation of *Escherichia coli* o157 h7 and *Salmonella enteritidis* in liquid egg yolk. J FOOD ENG. 79:689–694

Bermúdez-Aguirre D., Dunne C. P.&Barbosa-Cánovas G. V.(2012). Effect of processing parameters on inactivation of *Bacillus cereus* spores in milk using pulsed electric fields. INT DAIRY J. 24:13-21

Bisconsin-Junior A., Alvarenga J. F. R., Rosenthal A.&Monteiro M.(2015). Effect of high hydrostatic pressure on ascorbic acid, phenolic compounds and antioxidant activity of pera rio orange juice. J Food Process Technol. 06:416-422

Blicher A., Wodzinska K., Fidorra M., Winterhalter M.&Heimburg T.(2009). The temperature dependence of lipid membrane permeability, its quantized nature, and the influence of anesthetics. BIOPHYS J. 96:4581-4591

DeBruin K. A.&Krassowska W.(1999). Modeling electroporation in a single cell. I. Effects of field strength and rest potential. BIOPHYS J. 77:1213–1224

El Zakhem H., Lanoiselle J. L., Lebovka N. I., Nonus M.&Vorobiev E.(2006). Behavior of yeast cells in aqueous suspension affected by pulsed electric field. J COLLOID INTERF SCI. 300:553-563

El Zakhem H., Lanoiselle J. L., Lebovka N. I., Nonus M.&Vorobiev E.(2007). Influence of temperature and surfactant on *Escherichia coli* inactivation in aqueous suspensions treated by moderate pulsed electric fields. INT J FOOD MICROBIOL.120:259-265

Evrendilek G. A., Z.T J., Ruhlman K. T., Qiu J., Zhang Q. H.&Richter E. R.(2000). Microbial safety and shelf-life of apple juice and cider processed by bench and pilot scale PEF

systems. INNOV FOOD SCI EMERG. 1:77-86

Garner A. L.(2019). Pulsed electric field inactivation of microorganisms: From fundamental biophysics to synergistic treatments. APPL MICROBIOL BIOT. 103:7917-7929

González-Arenzana L., Portu J., López N., Santamaría P., Gutiérrez A. R., López R.&López-Alfaro I.(2019). Pulsed electric field treatment after malolactic fermentation of tempranillo rioja wines: Influence on microbial, physicochemical and sensorial quality. INNOV FOOD SCI EMERG. 51:57–63

Grahl T.&Markl H.(1996). Killing of microorganisms by pulsed elctrcic fields. APPL MICROBIOL BIOT. 45:148-157

Heinz V., Alvarez I., Angersbach A.&Knorr D.(2001). Preservation of liquid foods by high intensity pulsed electric fields - basic concepts for process design. TRENDS FOOD SCI TECH. 12:103-111

Heinz V., Phillips S. T., Zenker M.&Knorr D.(2009). Inactivation of *Bacillus subtilis* by high intensity pulsed electric fields under close to isothermal conditions. FOOD BIOTECHNOL. 13:155-168

Huang K.&Wang J.(2009). Designs of pulsed electric fields treatment chambers for liquid foods pasteurization process: A review. J FOOD ENG. 95:227-239

Hulsheger H., Potel J.&Niemann E.-G.(1981). Killing of bacteria with electric pulses of high field strength. RADIAT ENVIRON BIOPH. 20:53-65

Huo Z.-Y., Luo Y., Xie X., Feng C., Jiang K., Wang J.&Hu H.-Y.(2017). Carbon-nanotube sponges enabling highly efficient and reliable cell inactivation by low-voltage electroporation. ENVIRON SCI-NANO. 4:2010-2017

Huo Z. Y., Li G. Q., Yu T., Feng C., Lu Y., Wu Y. H., Yu C. X., X.&Hu H. Y.(2018). Cell transport prompts the performance of low-voltage electroporation for cell inactivation. SCI REP. 8:15832

Jayasooriya V.&Nawarathna D.(2017). Simulation of molecular transport through an electroporated cell using comsol multiphysics. 13th COMSOL conference, Boston.

Kanduser M., Sentjurc M.&Miklavcic D.(2008). The temperature effect during pulse application on cell membrane fluidity and permeabilization. BIOELECTROCHEMISTRY. 74:52-57

Krassowska W.&Filev P. D.(2007). Modeling electroporation in a single cell. BIOPHYS J. 92:404–417

Liu J.&Conboy J. C.(2004). Phase transition of a single lipid bilayer measured by sum-frequency vibrational spectroscopy. J AM CHEM SOC.126:8894-8895

Mahnic-Kalamiza S., Vorobiev E.&Miklavcic D.(2014). Electroporation in food processing and biorefinery. J MEMBRANE BIOL.247:1279-1304

Pasha I., Saeed F., Sultan M. T., Khan M. R.&Rohi M.(2014). Recent developments in minimal processing: A tool to retain nutritional quality of food. CRIT REV FOOD SCI. 54:340-351

Rols M.-P.&Teissie J.(1990) .Electropermeabilization of mammalian cells. Quantitative analysis of the phenomenon. BIOPHYS J.58:1089-1098

Saldaña G., Puértolas E., Álvarez I., Meneses N., Knorr b D.&Raso J.(2010). Evaluation of a static treatment chamber to investigate kinetics of microbial inactivation by pulsed electric fields at different temperatures at quasi-isothermal conditions. J FOOD ENG. 100:349–

356

Saulis G.(2010). Electroporation of cell membranes: The fundamental effects of pulsed electric fields in food processing. FOOD ENG REV. 2:52–73

Timmermans R. A. H., NieropGroot M. N., Nederhof A. L., van Boekel M. A. J. S., Matser A. M.&Mastwijk H. C.(2014). Pulsed electric field processing of different fruit juices impact oh ph and temperature on inactivation of spoilage and pathogenic micro organisms. INT J FOOD MICROBIOL.173:105–111

Toepfl S., Heinzb V.&Knorr D.(2007). High intensity pulsed electric fields applied for food preservation. CHEM ENG PROCESS.46:537–546

Vadlamani A., Detwiler D. A., Dhanabal A.&Garner A. L.(2018). Synergistic bacterial inactivation by combining antibiotics with nanosecond electric pulses. APPL MICROBIOL BIOT. 102:7589-7596

van Wyk S., Silva F. V. M.&Farid M. M.(2019). Pulsed electric field treatment of red wine: Inactivation of brettanomyces and potential hazard caused by metal ion dissolution. INNOV FOOD SCI EMERG. 52:57-65

Wang M. S., Wang L. H., Bekhit A. E. A., Yang J., Hou Z. P., Wang Y. Z., Dai Q. Z.&Zeng X. A.(2018). A review of sublethal effects of pulsed electric field on cells in food processing. J FOOD ENG. 223:32-41

Wei X., Li J., Wang H.&Zhu B.(2017). Study on the breakdown characteristics of liquid food under pulsed electrci voltage. CHN SCI. 12:1262-1268

Weng X.&Neethirajan S.(2017). Ensuring food safety: Quality monitoring using microfluidics. TRENDS FOOD SCI TECH. 65:10-22

576 Yogesh K.(2016). Pulsed electric field processing of egg products: A review. J FOOD SCI
577 TECH MYS.53:934-945

578 **Figure titles**

579 **Fig.1.** Experimental flow chart of the process including microbial culture, PEF and
580 thermal treatments, plate counting, SEM detection, and spectrophotometer detection.

581 **Fig.2.** Typical voltage and current waveforms.

582 **Fig.3.** PEF and thermal treatment system.

583 **Fig.4.** The distribution of the electric field (a. in XY cross section plane, b. along X axis.)

584 **Fig.5.** The survival rate of *Saccharomyces cerevisiae, Escherichia coli,* and *Bacillus*
585 *velezensis* changes with temperature.

586 **Fig.6.** The effect of electric field intensity on the survival rate at different temperatures
587 (a. *Saccharomyces cerevisiae,* b. *Escherichia coli,* c. *Bacillus velezensis*).

588 **Fig.7.** The effect of pulse width on the survival rate at different temperatures (a.
589 *Saccharomyces cerevisiae,* b. *Escherichia coli,* c. *Bacillus velezensis*).

590 **Fig.8.** The effect of pulse number on the survival rate at different temperatures (a.
591 *Saccharomyces cerevisiae,* b. *Escherichia coli,* c. *Bacillus velezensis*).

592 **Fig.9.** Synergy coefficient under different electric field intensities and initial
593 temperatures (a. *Saccharomyces cerevisiae,* b. *Escherichia coli,* c. *Bacillus velezensis*).

594 **Fig.10.** Nucleic acid content and protein content in suspension under different electric
595 field intensities and initial temperatures (a. *Saccharomyces cerevisiae*, nucleic acid, b.
596 *Saccharomyces cerevisiae*, protein, c. *Escherichia coli*, nucleic acid, d. *Escherichia coli*,
597 protein, e. *Bacillus velezensis*, nucleic acid, and f. *Bacillus velezensis*, protein).

598 **Fig.11.** The effect of temperature and electric field on molecular transport

599 **Fig.12.** Critical electric field intensity fitting analysis at different initial temperatures

600 (a. *Saccharomyces cerevisiae,* b. *Escherichia coli,* c. *Bacillus velezensis*).

601 **Fig.13.** SEM characteristics (a. *Saccharomyces cerevisiae* without any treatment

602 (control); b. *Saccharomyces cerevisiae* with thermal treatments (50 ℃); c.

603 *Saccharomyces cerevisiae* with PEF treatments (25 kV/cm); d. *Saccharomyces*

604 *cerevisiae* with PEF and thermal treatments (25 kV/cm +50 ℃)).

605

Figures

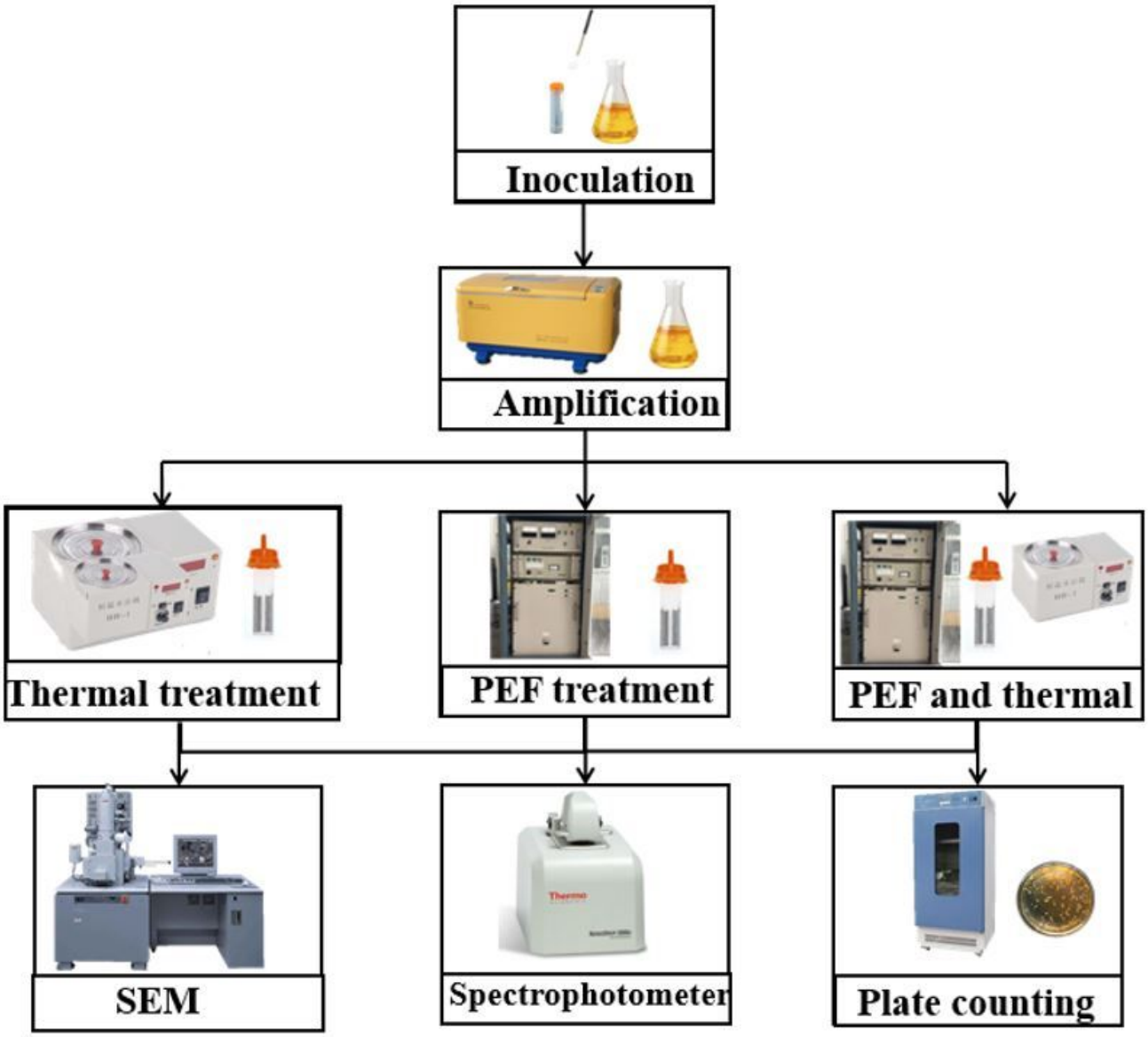

Figure 1

Experimental flow chart of the process including microbial culture, PEF and thermal treatments, plate counting, SEM detection, and spectrophotometer detection.

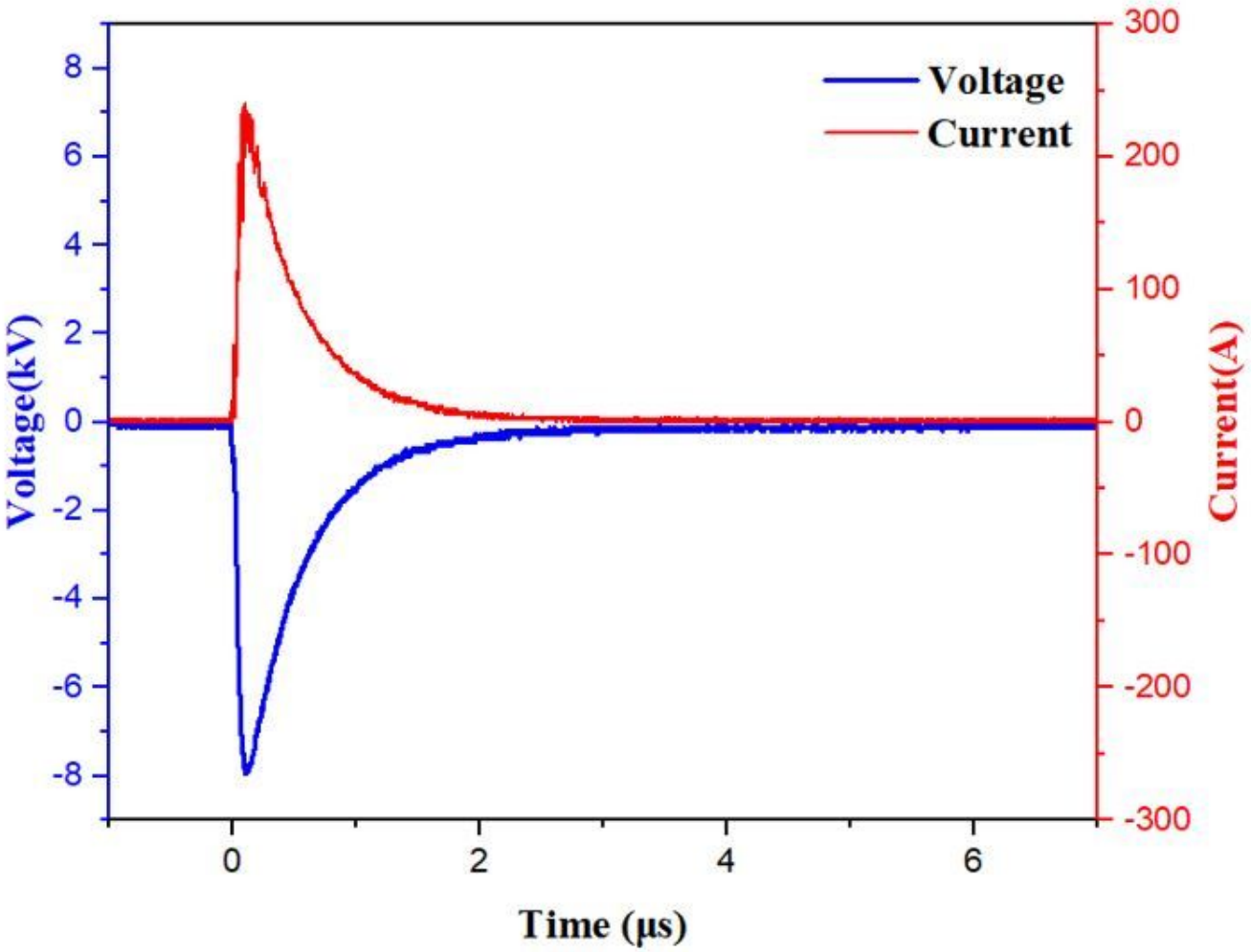

Figure 2

Typical voltage and current waveforms.

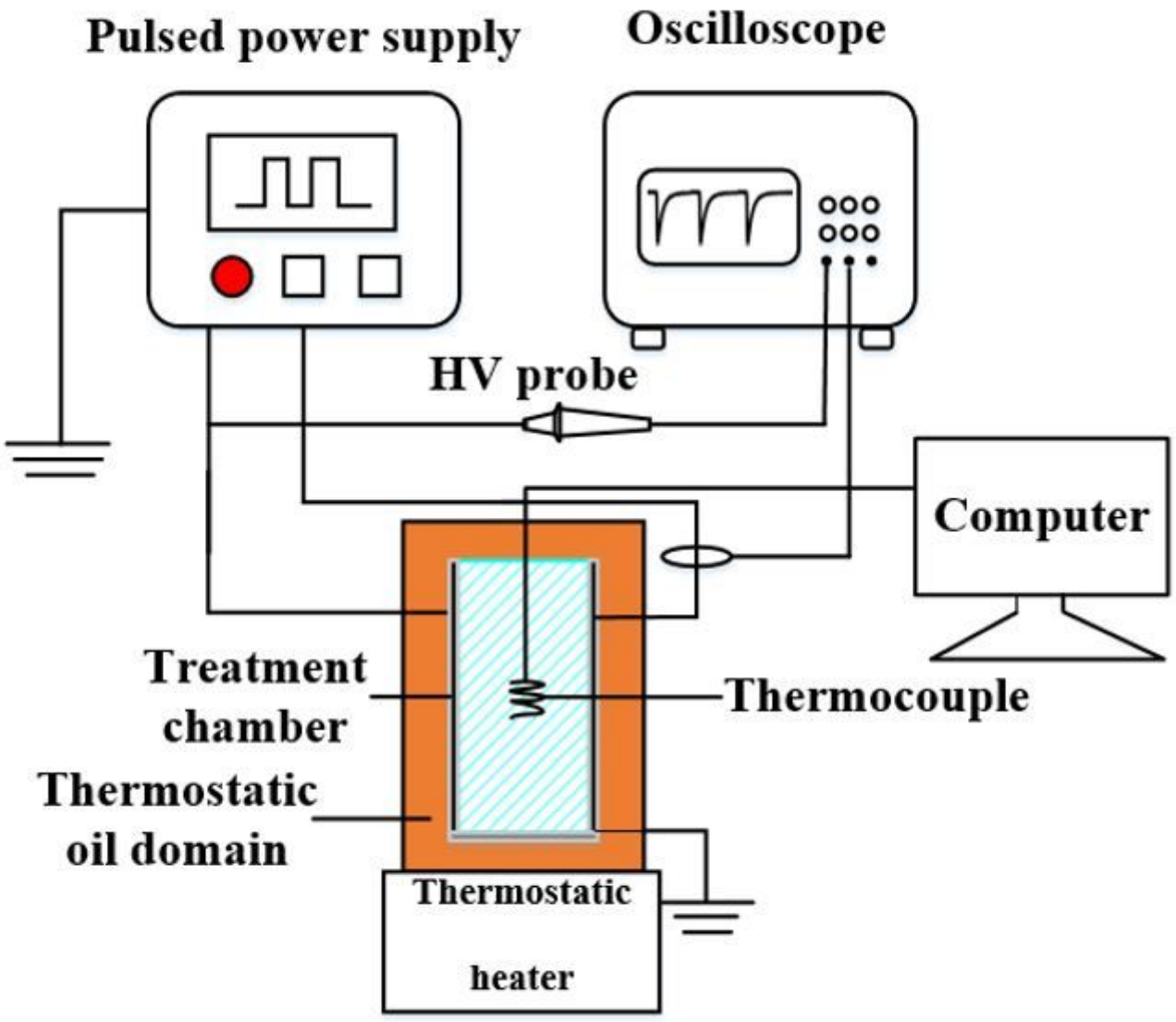

Figure 3

PEF and thermal treatment system.

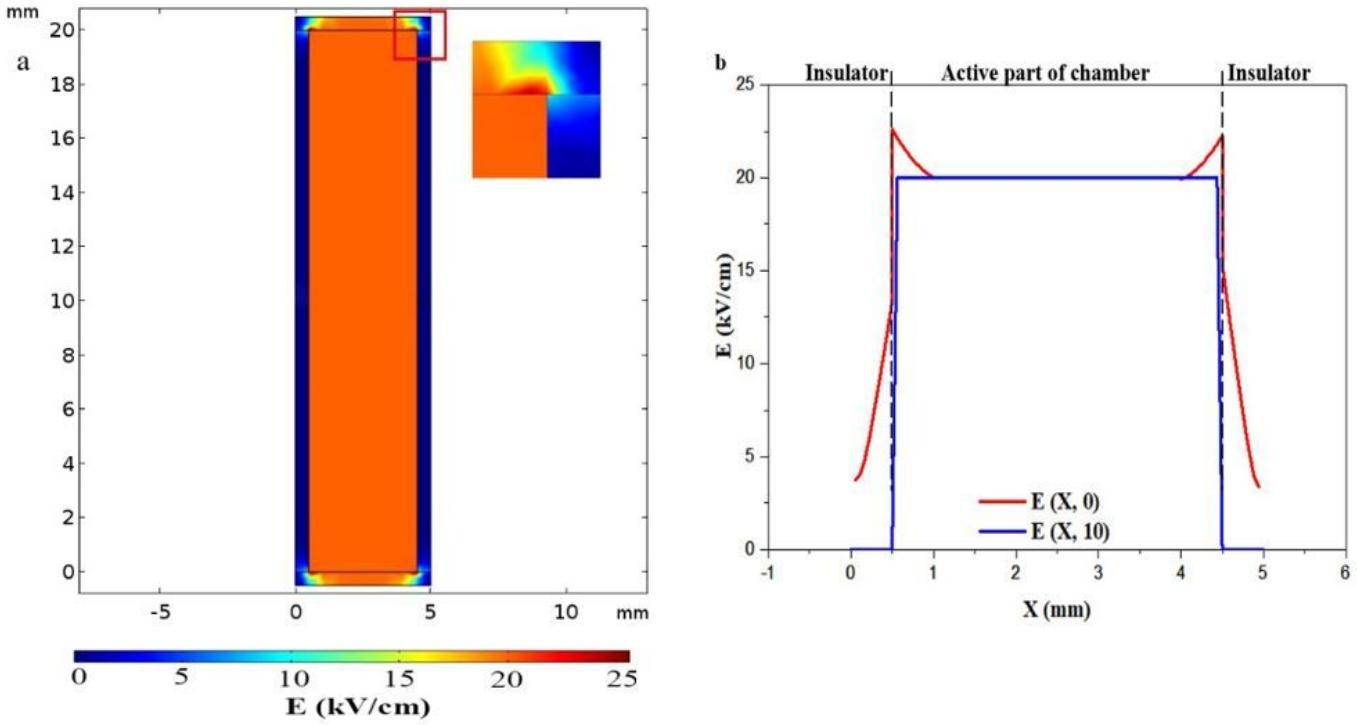

Figure 4

The distribution of the electric field (a. in XY cross section plane, b. along X axis.)

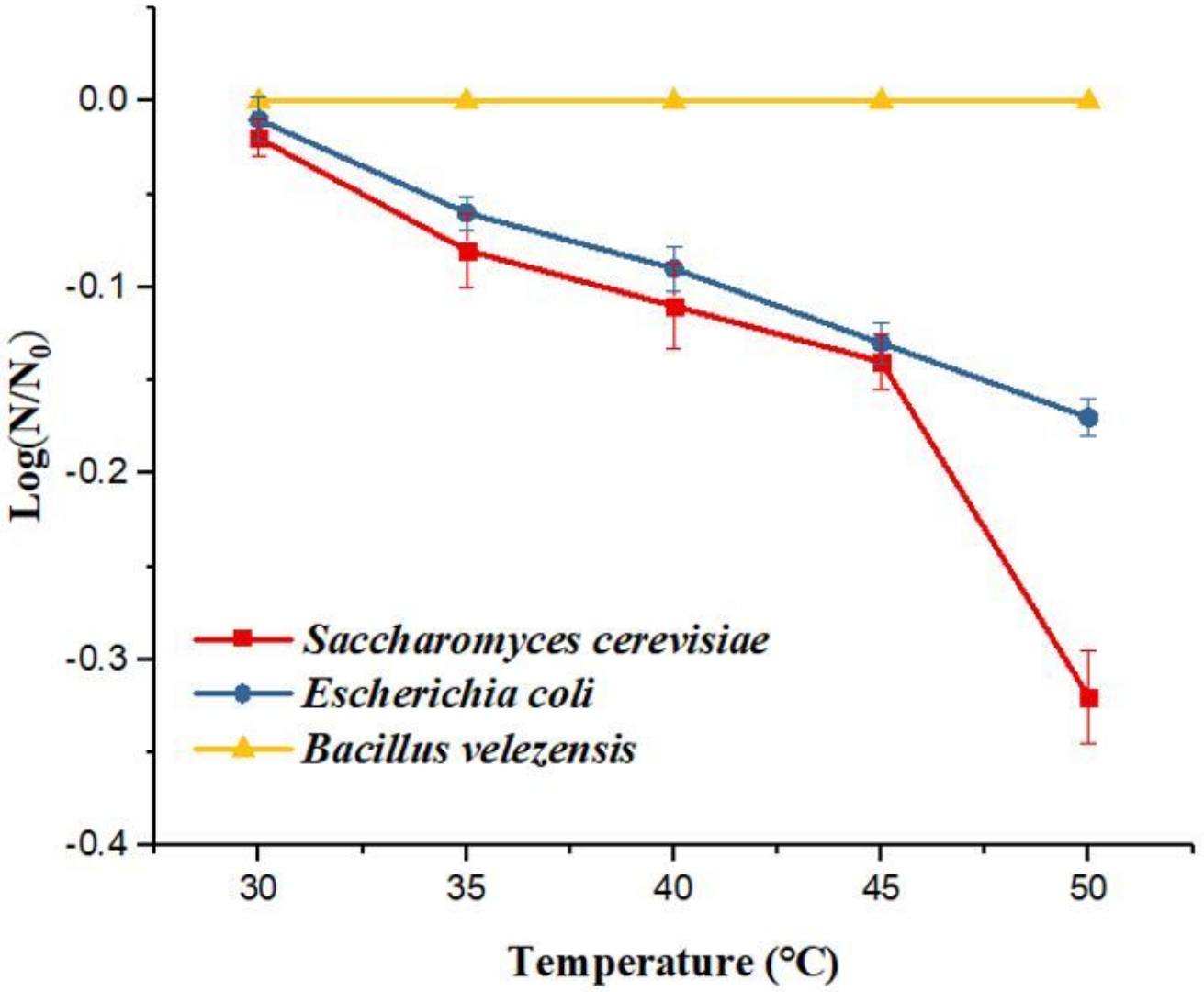

Figure 5

The survival rate of Saccharomyces cerevisiae, Escherichia coli, and Bacillus velezensis changes with temperature.

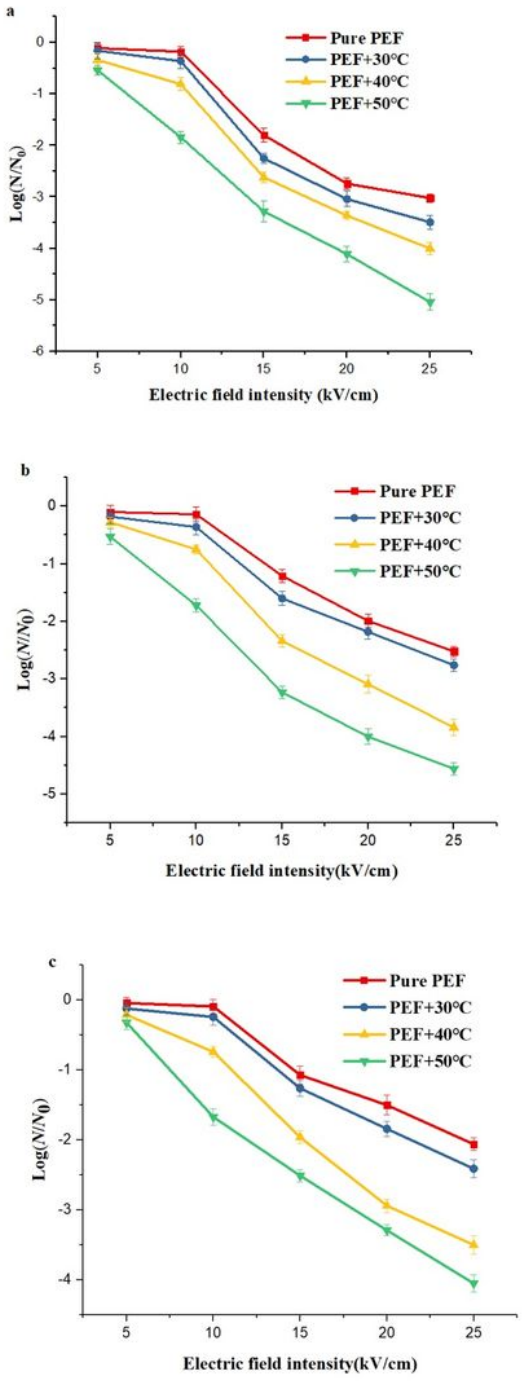

Figure 6

The effect of electric field intensity on the survival rate at different temperatures (a. Saccharomyces cerevisiae, b. Escherichia coli, c. Bacillus velezensis).

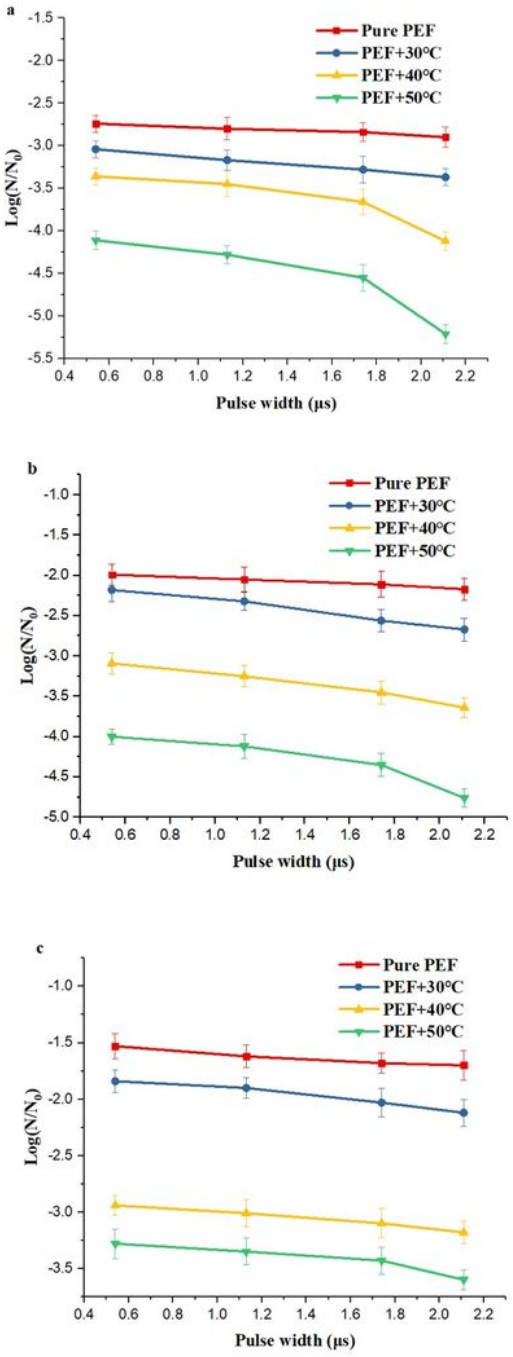

Figure 7

The effect of pulse width on the survival rate at different temperatures (a. Saccharomyces cerevisiae, b. Escherichia coli, c. Bacillus velezensis).

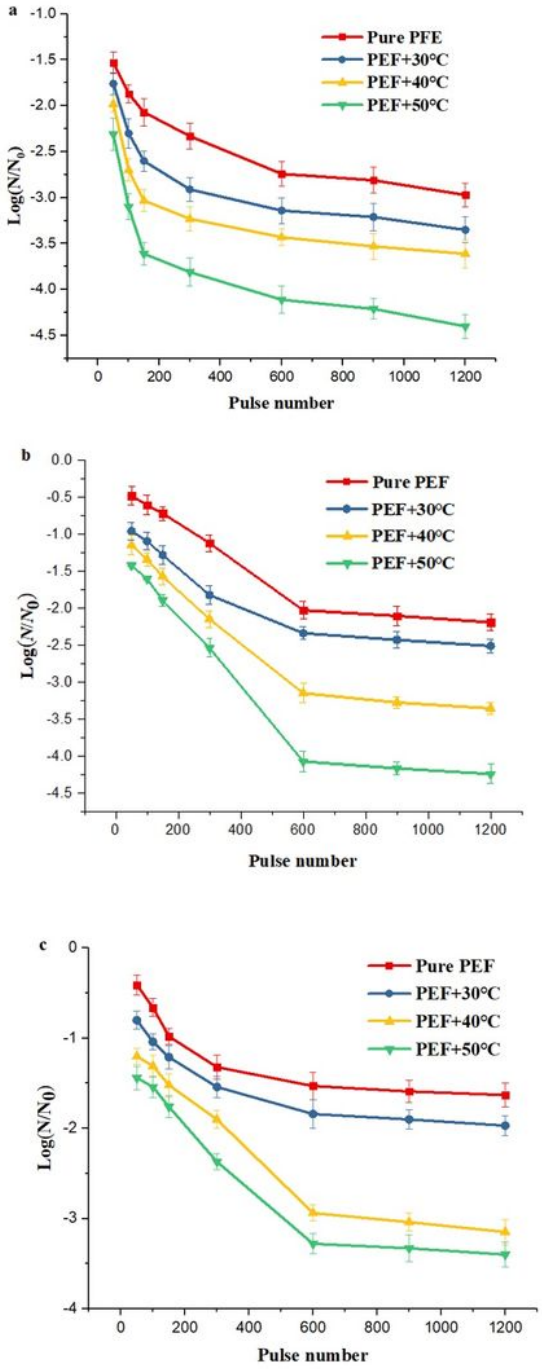

Figure 8

The effect of pulse number on the survival rate at different temperatures (a. Saccharomyces cerevisiae, b. Escherichia coli, c. Bacillus velezensis).

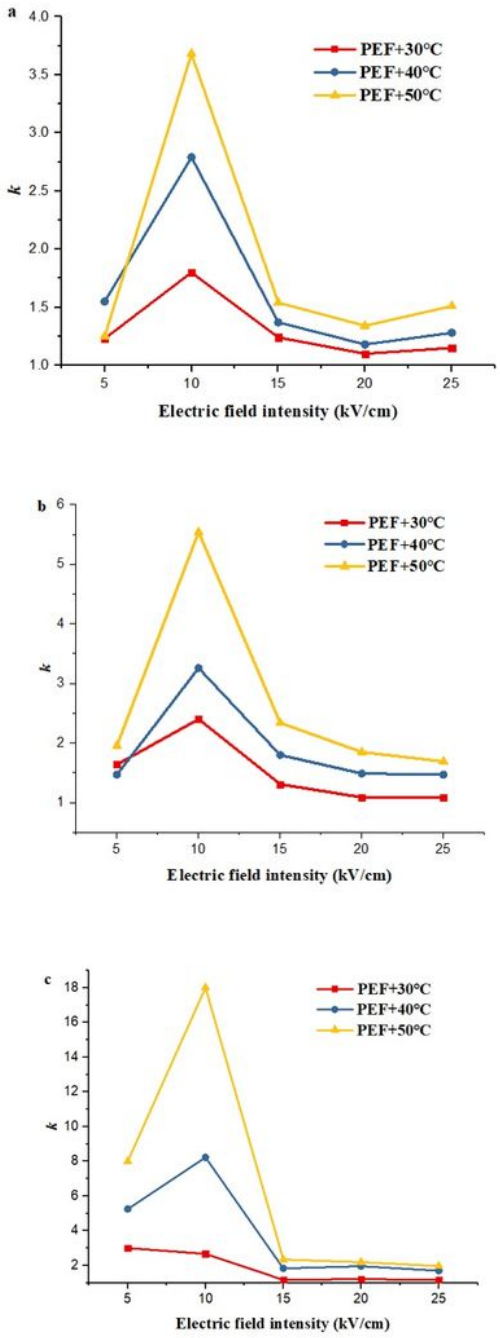

Figure 9

Synergy coefficient under different electric field intensities and initial temperatures (a. Saccharomyces cerevisiae, b. Escherichia coli, c. Bacillus velezensis).

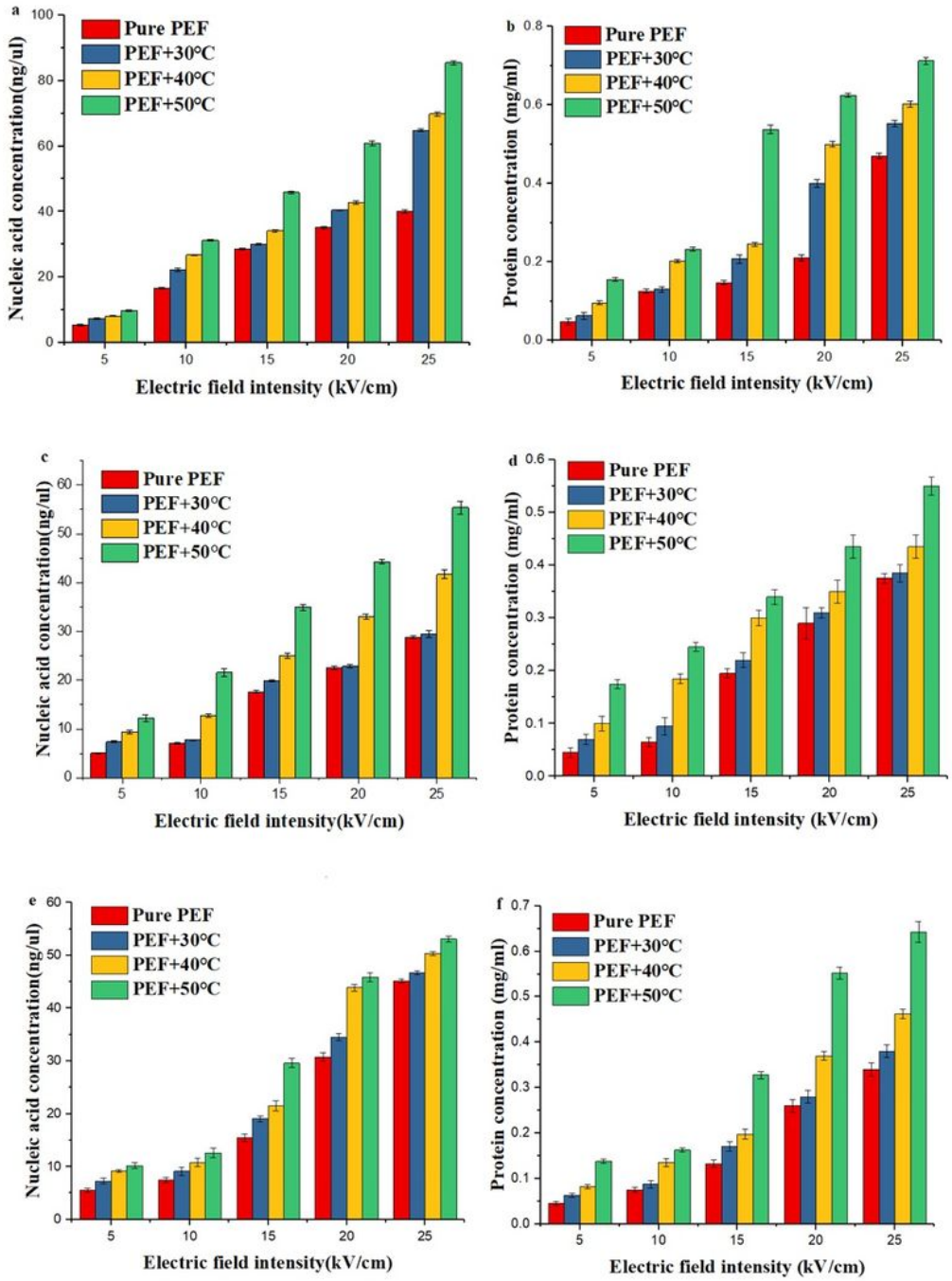

Figure 10

Nucleic acid content and protein content in suspension under different electric field intensities and initial temperatures (a. Saccharomyces cerevisiae, nucleic acid, b. Saccharomyces cerevisiae, protein, c. Escherichia coli, nucleic acid, d. Escherichia coli, protein, e. Bacillus velezensis, nucleic acid, and f. Bacillus velezensis, protein).

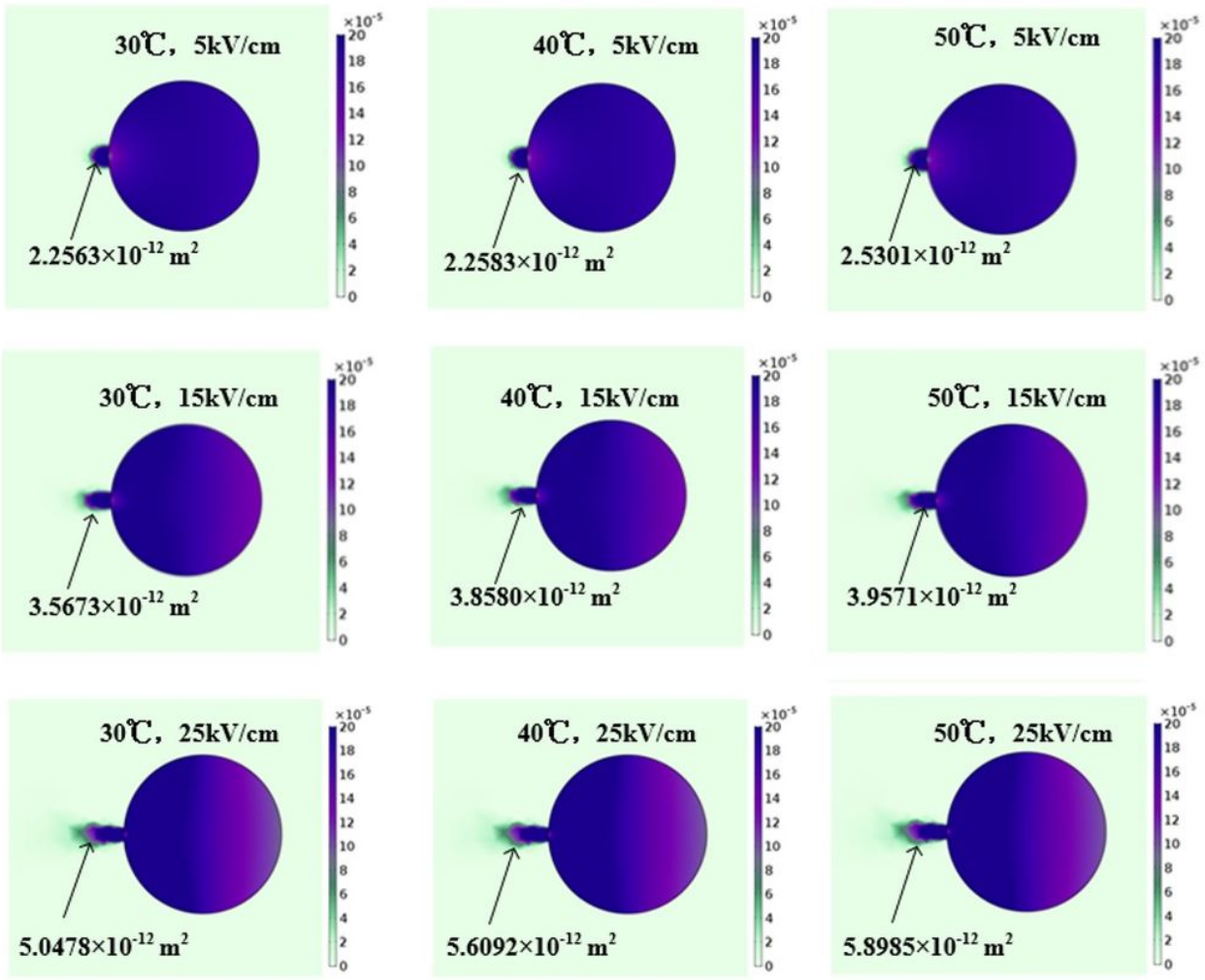

Figure 11

The effect of temperature and electric field on molecular transport

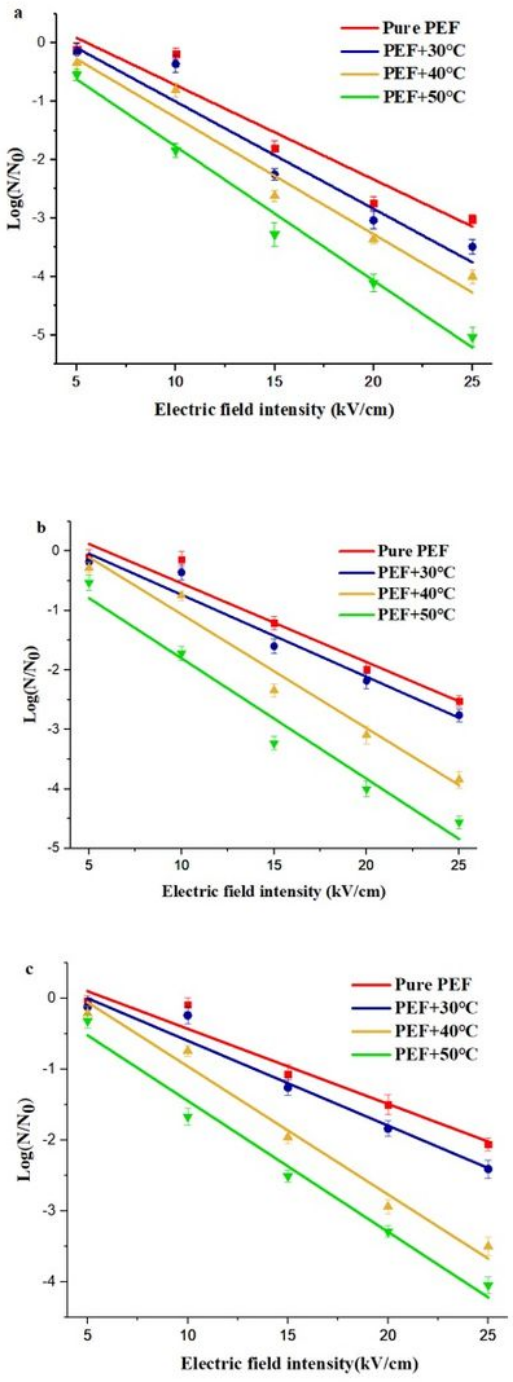

Figure 12

Critical electric field intensity fitting analysis at different initial temperatures (a. Saccharomyces cerevisiae, b. Escherichia coli, c. Bacillus velezensis).

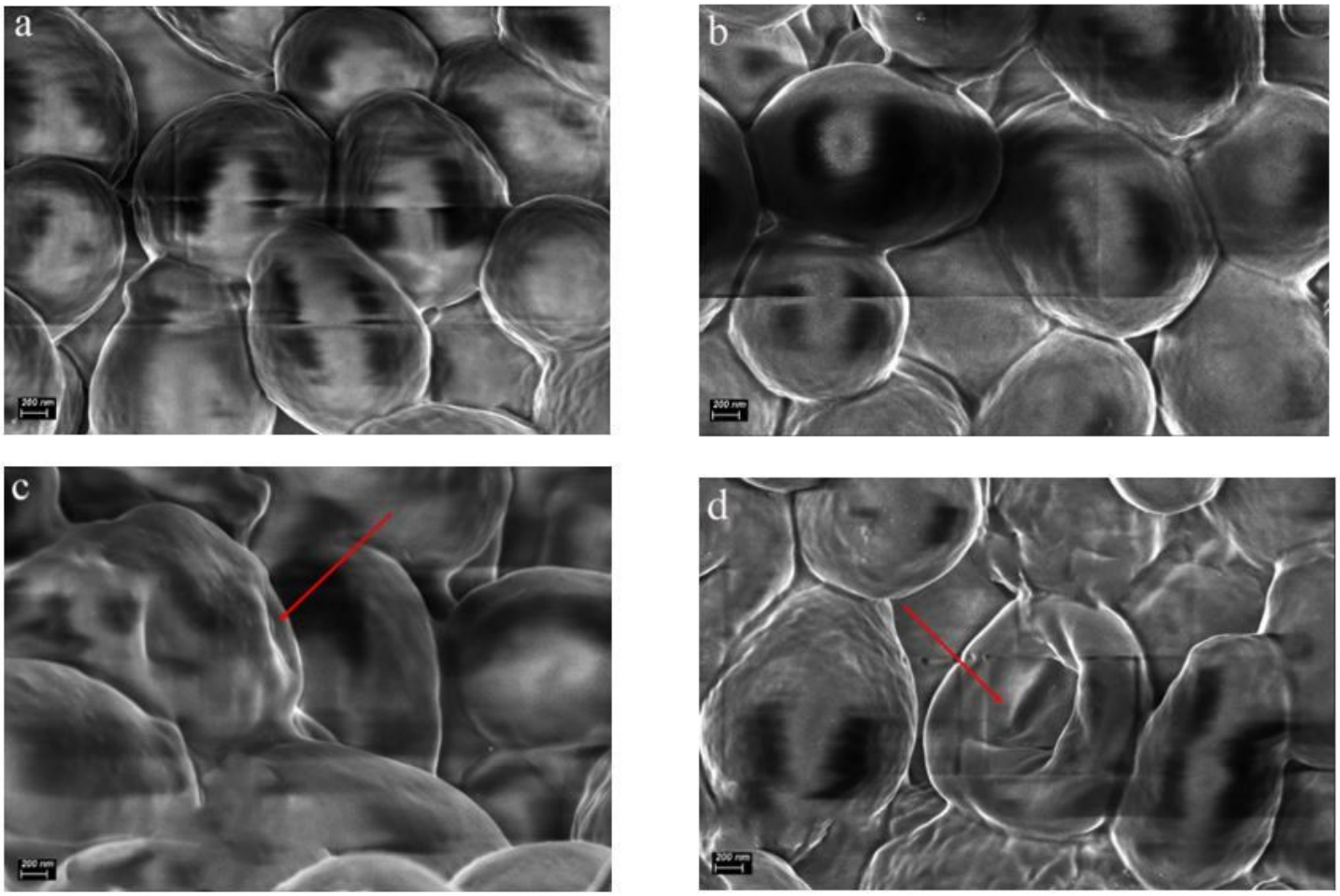

Figure 13

SEM characteristics (a. Saccharomyces cerevisiae without any treatment (control); b. Saccharomyces cerevisiae with thermal treatments (50 □); c. Saccharomyces cerevisiae with PEF treatments (25 kV/cm); d. Saccharomyces cerevisiae with PEF and thermal treatments (25 kV/cm+50 □)).