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Influence of electrical process parameters to inactivate microorganisms in orange juice by PEF technology

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Abstract
Pulsed electric field (PEF) is a non-thermal/chemical food processing method for microbial inactivation in liquid food. This method finds its application to prolong the shelf-life of the seasonal fruit juices which helps to maintain the supply chain throughout the year. So, in the present study, the most commonly found microorganisms of *Escherichia coli* (*E. coli*), *Staphylococcus aureus* (*S.aureus*) and *Saccharomyces cerevisiae* (*S.cerevisiae*) inoculated in orange juice are considered. PEF impact on microbial inactivation based on the experimental results are analyzed with respect to pulse frequency, electric field intensity and treatment time. The PEF treatment conditions set for the investigation are as follows. Electric field intensity: - 5 kV/cm and 10 kV/cm; Pulse frequency: - 1, 10, 20 and 50 kHz; Pulse width: - 1.2 µs; Treatment time: - 10 s, 20 s, 30 s, 40s and 50s. A maximum inactivation of log10 reduction 2.62 ± 0.122 is observed when *E. coli* was subjected to 10 kV/cm at 50 kHz. Meanwhile *S.cerevisiae* and *S.aureus* are inactivated at the level of log10 reduction 2.4 + 0.16 and 2.1 + 0.9 respectively at the same treatment conditions when the treatment time is 50 s. It is also observed that the electric field intensity along the higher frequency enhances the inactivation level of microorganisms and the variations in the log reduction might be due to the different biological structure of the microorganisms.

Keywords: Electric field intensity, *E. coli*, *S. aureus*, *S. Cerevisiae*, pulse repetition frequency, treatment time, process time

Introduction
The fundamental mechanism of electroporation for microbial inactivation is associated with the charge also plays an important role in determining the efficiency of the PEF treatment [5] So, the direct influence on the efficiency of electroporation must be studied based on biological factors along with the PEF system parameters. The relationship between the cell membrane voltage and electric field intensity has been investigated in several research papers [6-8]. Most of the investigations in PEF processing stated that electric field intensity and the number of pulses applied can be the control parameters and only very few studies concentrate on pulse frequency in inactivation process [9-11]. The electrical energy delivered to the food product depends on electric field intensity, pulse frequency, pulse width and treatment time and these variables are correlated to each other to define the amount of electrical energy. Geveke [12] and applied electric field strength of 26 kV/cm over a frequency range of 15 to 70 kHz to inactivate *E. coli* k12 in apple juice and found 1.8 log10 reductions. He again reported in another investigation that electric field strength of 0.5 kV/cm at 18 MHz can be used to inactivate microorganisms in liquids [10]. As the electric field intensity majorly influences the cost of the PEF generator, the electric field can be decreased by either increasing the pulse frequency of treatment time to maintain the required amount of electrical energy. From the literature, it is observed that the impact of the individual factor on the survival reduction cannot be parsed out in PEF technology for food processing [13-15]. Prokaryotic type (Gram-negative –*E. coli*, Gram-positive –*S. aureus*) and Eukaryotic type (*S. Cerevisiae*) are considered for the study. A laboratory-type PEF generator which can generate 1 kV square pulses of pulse width 1.2 µs with variable pulse frequency of 1 kHz, 10 kHz, 20 kHz and 50 kHz is used in the investigation. This paper explores the impact of pulse frequency along with electric field intensity and treatment time.

Materials and Methods

Culture preparation

The microorganisms, *E. coli* (ATCC 25922), *S.aureus* (ATCC 6538) and *S.cerevisiae* (ATCC 13007) were obtained from American Type Culture Collection, Manassas, USA. Cell cultures were maintained on Muller Hinton Agar (MHA) (Hi-media, India) plates at 4°C with a subculture period of one week. Each microorganism culture was prepared by transferring one colony from MHA plate to 4ml of Nutrient Broth in a test tube and incubated at 37 °C for 24h. Then the cell culture was transferred to 50 ml Nutrient Broth and again incubated at 37 °C in order to obtain cells in stationary phase. The concentration of the inoculum was maintained at ~109 colony forming units (CFU/ml) by comparing with a 0.5 McFarland standard solution. A separate suspension was used for each experiment, and it is also very important that all cell suspensions were prepared from the same culture. The prepared cell suspensions were refrigerated throughout the experiment. Before each treatment, the samples were allowed to reach the temperature of 31 °C. Fresh squeezed orange juice was prepared by using oranges purchased from the local market. The cell culture was mixed with prepared orange juice and incubated for 30 min and then treated immediately.

PEF experiment

A laboratory-type high voltage pulse generator that can generate 1 kV unipolar square pulses of pulse width 1.2 µs with adjustable frequency in the range of 1 kHz to 50 kHz shown in the figure 1 is used for the investigation. The prototype PEF system consists of power MOSFETs and power diodes.

The detailed experimental protocols, instruments and software etc. used in the study should be described here with their proper references. The details of the study area should also be provided.

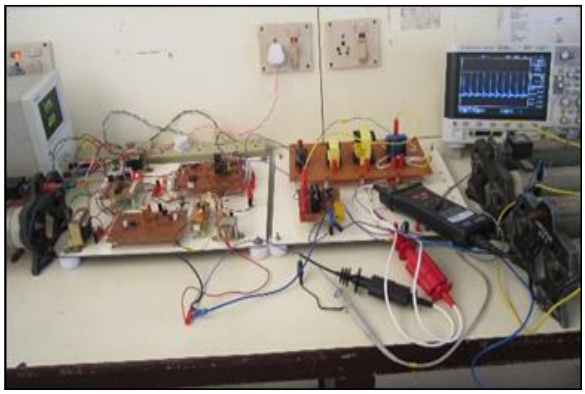

Fig 1: Prototype PEF generator

The high voltage pulses are generated by interrupting the current through the inductor, connected in series with DC power supply. The interruption is done by switching ON and OFF the power MOSFETs at regular intervals. The advantage of this prototype PEF system is its compactness and able to generate high voltage unipolar pulses at flexible frequency range. The PEF generator output terminals are designed suitably to connect CU500 cuvette chamber (Nepa Gene Co., Ltd., Japan) and two different types of standard Nepa Electroporation cuvettes. The disposable Nepa Electroporation cuvettes consist of aluminum electrodes with 1 mm and 2 mm gap. The 1 mm cuvette can hold maximum of 80 µl cell suspensions as whereas 2 mm cuvette can hold 200 µl. The 1 mm and 2mm cuvette can develop electric field intensities of 10 kV/cm and 5 kV/cm respectively when used along with the implemented PEF generator and so the experiments were conducted at these electric field intensities of 5 and 10 kV/cm.

The focus of the study is to investigate the effect of pulse repetition frequency and treatment time on the different types of microorganisms. So, the samples were taken in the cuvettes and high voltage pulses were applied across the electrodes at different PRF of 1 kHz, 10 kHz, 20 kHz and 50 kHz and the process time was set at 10 s, 20 s, 30s, 40s and 50s. It was observed that the temperature was reached maximum when the electric field intensity of 10 kV/cm was applied at the maximum frequency of 50 kHz for the time period of 50 s while conducting the experiments and so the 50 s time period was set as maximum for the other experiments conducted at PRF of 1, 10 and 20 kHz.

Microorganisms count

Samples of 100µl were taken using 1000 µl micropipette from treated suspensions and serially diluted using sterile Nutrient broth. After appropriate dilution, the sample of 4µl had taken from treated suspension for streaking on Nutrient Agar plate. The plates were incubated at 37°C for 24h, after which colonies were counted. Each count was determined by the mean values of three plates.

Statistical Analysis

Experiments were repeated in triplicate and the results were expressed as the measurement of the mean ± standard deviation. ANOVA test was performed for all the experiments to determine the significant differences.

Results

Although the electric field intensity is the major factor involved in the PEF treatment to inactivate the different microorganisms, the present study focuses the effect of PRF to inactivate the different microorganisms *E. coli*, *S. aureus*, and *S. Cerevisiae*. The results shown in Figure 2 compare the effect of PRF on the inactivation of microorganisms of *E. coli*, *S. aureus*, and *S. Cerevisiae*. Values are expressed in terms of mean ± standard error. Though the treatment conditions and the medium (orange juice) were the same for all the microorganisms considered for the present study, a significant difference clearly exists in their inactivation level, and it might be due to the nature of the microorganisms.

Pulse repetition frequency

The effect of PRF on inactivation of microorganisms was determined. The observed results show that the PRF has a significant impact on inactivation process irrespective of the nature of the microorganisms. The experiments were conducted at 1, 10, 20 and 50 kHz and all the four frequencies were investigated. A maximum of 2.63 ± 0.122 log10 reductions was achieved when *E. coli* was undergone to PEF of 50 kHz, 10 kV/cm, while 2.4 ± 0.16 log10 reductions was achieved for *S. Cerevisiae*. It was found that *S. aureus* was also inactivated but at a lesser level than *E. coli* and *S. aureus*. It could be seen from figure 2 that there was a noticeable difference in the level of inactivation when the PRF was raised. Error bars are not included in figure 1 if

it is less than 0.05. But there was no noticeable difference in the inactivation process of *S. Cerevisiae* at the frequency of 1, 10 and 20 kHz. The Gram-positive bacterium, *S. aureus* was less inactivated than the Gram-negative bacterium, *E. coli* at all frequencies.

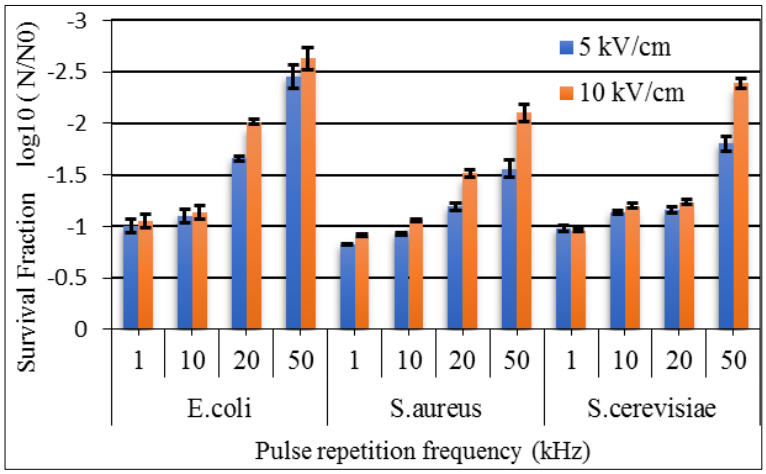

Fig 2: Inactivation of microbes in orange juice at 10 kV/cm and 5 kV/cm

From the results, it can be stated that the PRF play important role in the PEF inactivation process and the inactivation of all the three microorganisms can be increased at higher frequencies. There was inactivation significantly different ($P < 0.02$) in the inactivation level at 50 kHz among all other frequencies considered in the present study.

Electric field intensity

The experiments were repeated at two different electric field intensities of 5 and 10 kV/cm. The effect of electric field intensity was studied at different frequencies. The population of *E. coli* was reduced by 2.45 ± 0.01 log10 reduction after treated by 5 kV/cm and increased to 2.63 ± 0.122 log10 reductions at 10 kV/cm. Similarly, the other two microorganisms were inactivated significantly ($p < 0.05$) and found that the population was reduced better when the electric field intensity was increased to 10 kV/cm at higher frequencies. But, there was no significant difference ($p > 0.05$) found on the *S. Cerevisiae* in orange juice at both electric field intensities when the frequency was set at 1, 10, 20 kHz. It was also observed that there was no significant death rate when *E. coli* and *S. aureus* were subjected to 5 and 10 kV/cm at the PRF of 1 and 10 kHz. When the frequency was increased to 20 kHz, the *E. coli* and *S. aureus* population were reduced better at 10 kV/cm than 5 kV/cm.

Treatment Time: The experiments were performed to study the effect of treatment time. The microorganisms in orange juice were subjected to 1 kV, 1.2 µs by varying the process time from 10 to 50s in steps of 10s for different PRFs of 1 kHz, 10 kHz, 20 kHz and 50 kHz. But the treatment time (actual time during which the electrical energy is transferred to the food) can be calculated by multiplying the pulse width and number of pulses applied (number of pulses applied = pulse frequency * process time). The effect of treatment time on inactivation of different microorganisms, *E. coli*, *S. aureus*, S. scerevisiae are shown in Figures 3-5 at 10 kV/cm and 5 kV/cm respectively. The *E. coli* population reduction was more than *S. aureus* and *S. Cerevisiae* for all the frequencies and also the inactivation level had been increased during the treatment time. A maximum log10 reduction of *E. coli* was observed as 2.62 ± 0.122 at the treatment time of 3s. Meanwhile, *S. aureus* population was reduced by 2 ± 0.05 log10 reduction and *S. Cerevisiae* by 2.4 ± 0.07 at the same treatment time. Table 1 shows the treatment time for different PRF used in the present study.

The effect of treatment time on inactivation of different microorganisms, *E. coli*, *S. aureus*, *S. Cerevisiae* are shown in Figures 3-5 at 10 kV/cm and 5 kV/cm respectively. The *E. coli* population reduction was more than *S. aureus* and *S. Cerevisiae* for all the frequencies and also the inactivation level had been increased during the treatment time. A maximum log10 reduction of *E. coli* was observed as 2.62 ± 0.122 at the treatment time of 3s.

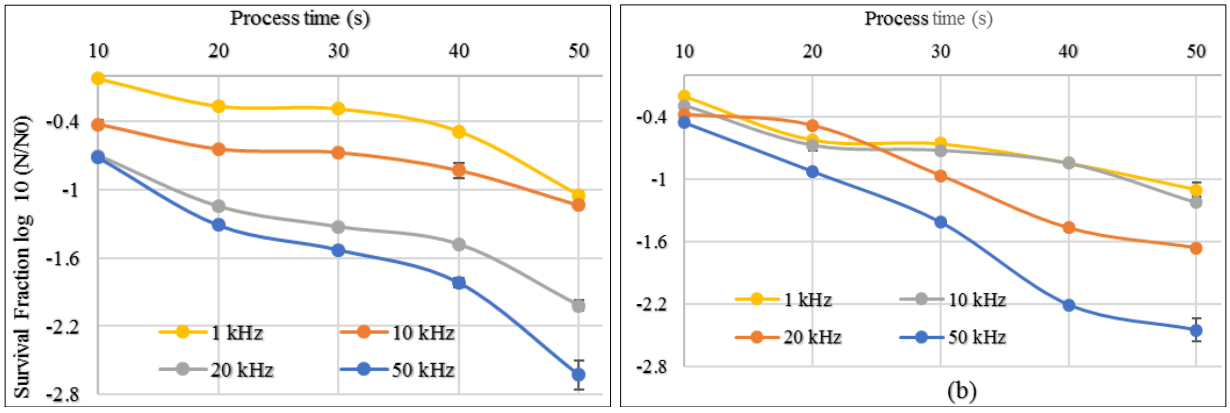

Fig 3: Inactivation of *E. coli* at PRF of 1 kHz, 10 kHz, 20 kHz and 50 kHz at (a) 10 kV/cm (b) 5 kV/cm

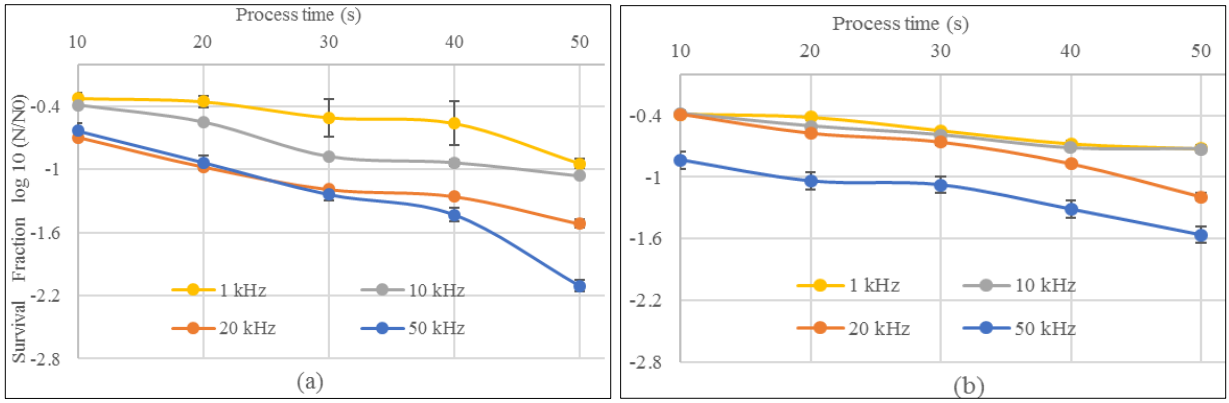

Fig 4: Inactivation of *S. aureus* at PRF of 1 kHz, 10 kHz, 20 kHz and 50 kHz at (a) 10 kV/cm (b) 5 kV/cm

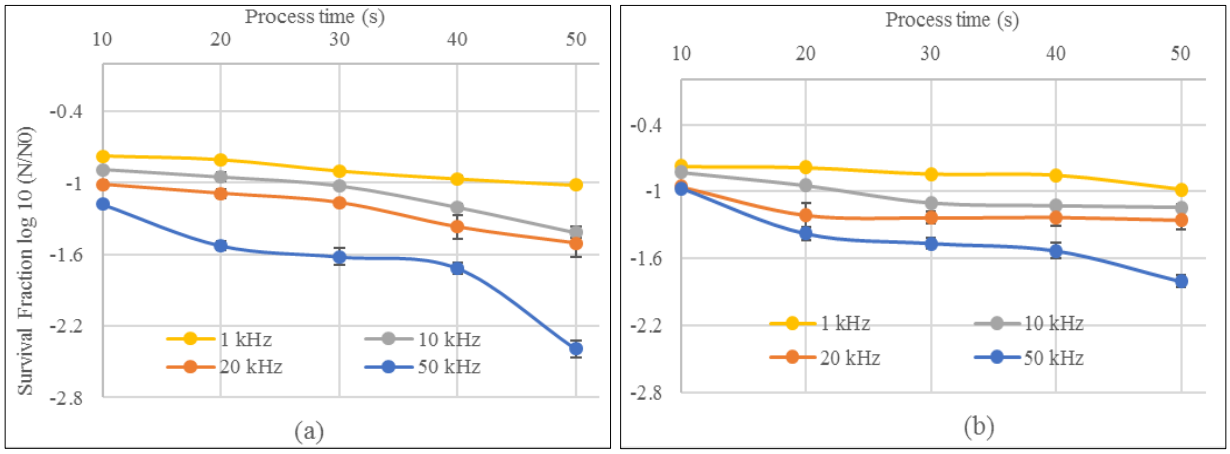

Fig 5: Inactivation of *S. Cerevisiae* at PRF of 1 kHz, 10 kHz, 20 kHz and 50 kHz at (a) 10 kV/cm (b) 5 kV/cm

Meanwhile, *S. aureus* population was reduced by 2 ± 0.05 $\log_{10}$ reduction and *S. Cerevisiae* by 2.4 ± 0.07 at the same treatment time. The *E. coli* $\log_{10}$ reduction of 0.717 ± 0.01 and 1.13 ± 0.018 was achieved for the same treatment time of 0.6s but the corresponding PRFs were 50 kHz and 10 kHz respectively. The difference in $\log_{10}$ reduction at the same treatment time was extended throughout the experiments conducted on *S. aureus* and *S. Cerevisiae*. So, the treatment time and PRF had their own control over the inactivation process on the microorganism.

Temperature

The temperature variation was determined from the experiments conducted from the initial temperature of 31°C which was set before starting the PEF treatment. The temperature variations while inactivating the microorganisms *E. coli*, *S.aureus*, and *S.cerevisiae* found during the experiments are shown in figures 6 to 8 respectively at both electric field intensities of 5 and 10 kV/cm.

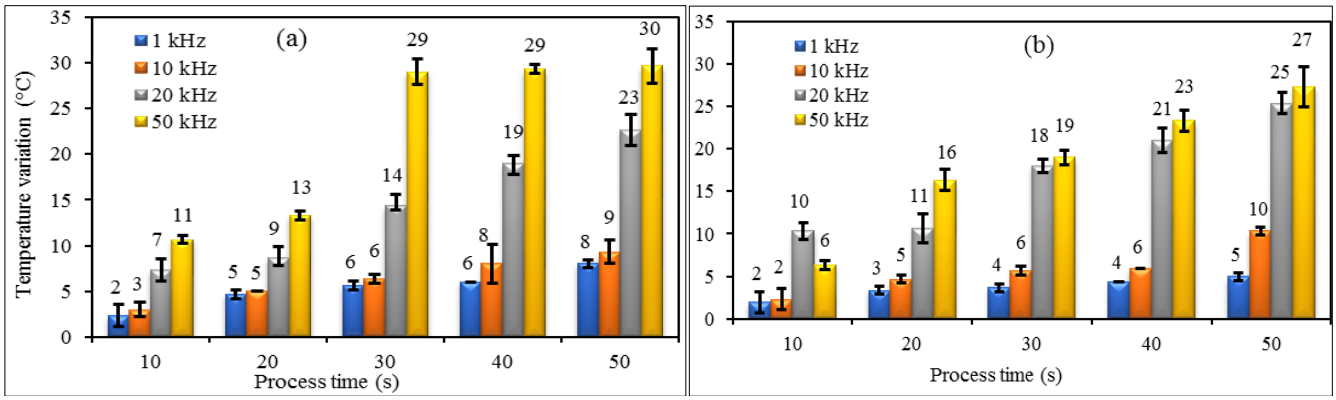

Fig 6: Temperature variation in inactivation process of *E. coli* at PRF of 1 50 kHz at (a) 10 kV/cm (b) 5 kV/cm

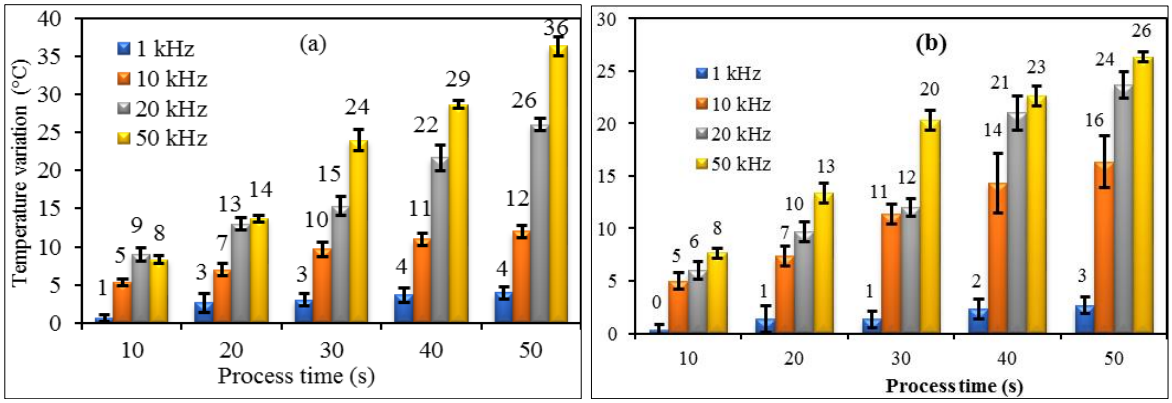

Fig 7: Temperature variation in inactivation process of *S. aureus* at PRF of 1-50 kHz at (a) 10 kV/cm (b) 5 kV/cm.

Higher PRF and longer treatment time increase the process temperature. The maximum temperature variation of 36°C was found for *S.aureus* with the log10 reductions of 2.1 ± 0.05 at the process time of the 50s at 10 kV/cm which was lesser than the E.coli population reduction at the same treatment conditions.

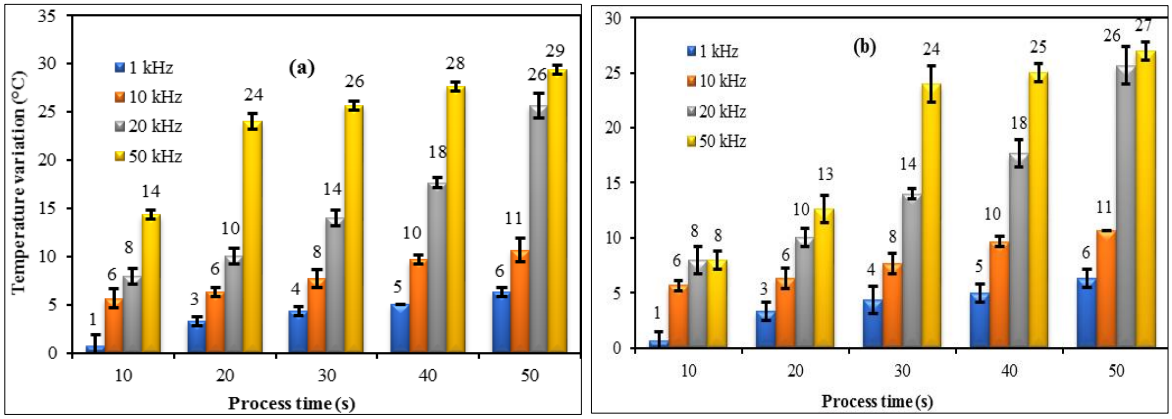

Fig 8: Temperature variation in inactivation process of *S. Cerevisiae* at PRF of 1-50 kHz at (a) 10 kV/cm (b) 5 kV/cm.

The temperature variation of 30°C was observed for *E. coli* with the maximum of 2.62 ± 0.122 log10 reductions, while 29°C was observed for *S. Cerevisiae* with 2.4 ± 0.07 log10 reductions. So, this re-ascertains that there was no thermal inactivation involved in the conducted PEF process.

Discussion

The obtained results can be compared with the work done by Geveke [12] in which 1.4 ± 0.1 log10 reductions of *E. coli* in apple juice has been achieved when subjected to 24 kV/cm at 20 kHz. In this present study, *E. coli* population has been reduced by 2.01 ± 0.045 log10 reductions with the application of 10 kV/cm at 20 kHz. In another investigation, Geveke [11] reported a log10 reduction of 3 of S. cerevsiae in water at 20 kV/cm and 20 kHz at the outlet temperature of 55°C. But the same log10 reduction was achieved at 10 kV/cm itself but the temperature was found as 57° in the present study. Moreover, there was no significant difference (p< 0.05) for all the microorganisms in the frequency range of 1 kHz to 10 kHz whereas the inactivation level of microorganisms has been elevated in the range of PRF of 20 kHz to 50 kHz. The major problem associated with the comparison of cell inactivation is the experimental conditions adopted and the different PEF systems used by investigators are not comparable directly. However, the PRF can be considered as one of the key parameters on PEF process in the food industry. Although it is very difficult to predict the relationship between electric field intensity and PRF, the voltage can be significantly reduced to achieve maximum level by increasing PRF. In the present study, the *E. coli* population was reduced by 2 ± 0.045 log10 reductions at 10 kV/cm when PRF was 20 kHz and at the same time, 2.45 ± 0.11 log10 reduction was achieved at 5 kV/cm but PRF was 50 kHz. The temperature variations in the present study are in agreement with the results reported by Geveke [16]. There were significant differences in temperature (p < 0.05) for *E. coli*, *S. aureus*, and *S. Cerevisiae* at same PRF when the process time varied from 10s to 30s.

In order to quantify the effect of the PRF in the present experimental work, it is worthwhile to compare the results with the previous results published in the PEF food processing technology. Hojo *et al.* [17] found that as the cell size of the *S. Cerevisiae* increased, the cell electro-permeabilization is decreased in both logarithmic and the stationary growth phases. The authors contributed such a behavior to the change in the membrane conductivity due to electroporation. In the present study, the inactivation level of *S. Cerevisiae* was lesser than the *E. coli* bacteria which are very tiny compared to *S. Cerevisiae* under same PEF conditions. Qin *et al.* [18] studied the effect of PEF on three microorganisms of *E. coli*, *S. aureus*, and *S. Cerevisiae* and it has been found that *S. Cerevisiae* is easier to inactivate than other two microorganisms. The authors attributed the higher inactivation rate of *S. Cerevisiae* to its larger cell size compared to *E. coli* and *S. aureus* microorganisms. The effect of PEF treatment on spores, bacteria, and yeast was

studied by MacGregor *et al*. [19] and no significant difference in the inactivation rate was found between the different microorganisms under test conditions. On the contrary, Narsetti *et al*. [20] observed that *E. coli* is easily inactivated than B. subtilis under the same test condition. So, such differences in the inactivation rate can be attributed to the fact that *E. coli* is more susceptible to the PEF treatment.

Although it is very difficult to predict the relationship between electric field intensity and PRF, the voltage can be significantly reduced to achieve a maximum death rate by increasing PRF. In the present study, the *E. coli* population was reduced by 2 ± 0.045 log10 reductions at 10 kV/cm when PRF was 20 kHz and at the same time, a 2.45 ± 0.11 log10 reduction was achieved at 5 kV/cm when PRF was increased 50 kHz. The temperature variations agree with the results reported by Geveke *et al*. [16]. There were significant differences in temperature ($p< 0.05$) for *E. coli*, *S. aureus*, and *S. Cerevisiae* at same PRF when the process time varied from 10 to 50s.

Table 1: Treatment time

Process time (s)	1 kHz	10 kHz	20 kHz	50 kHz
	Treatment time (s)			
10	0.012	0.12	0.24	0.6
20	0.024	0.24	0.48	1.2
30	0.036	0.36	0.72	1.8
40	0.048	0.48	0.96	2.4
50	0.06	0.6	1.2	3

Conclusion

In summary, high-frequency PEF process is capable of inactivating *E. coli*, *S. aureus* and *S. Cerevisiae* inoculated in orange juice at non-thermal conditions. The study has shown that the level of inactivation depends on the electric field intensity, treatment time and PRF might be enhanced along with the electric field intensity. In general, the results showed that the reduction of microorganisms was higher when the PRF of PEF treatment is increased and so it can be concluded that high frequency enhances the level of inactivation at lesser electric field intensity. Moreover, the results show that the inactivation of Gram-negative microorganism is higher than the other two types of microorganisms considered in this study. However, the mechanisms of PEF with the effect of individual parameters on the inactivation process are not fully understood and the developments of PEF technology in the food industry must be still addressed. The work can be extended by studying the impact of each individual parameters and select a proper PEF process parameter with the help of simulation and experimental work.

Acknowledgment

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