Use of Pulsed Electric Field for the Inactivation of *Eupenicillium Javanicum* Ascospores in Pineapple Juice

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Abstract. Heat resistant molds are principle spoilage agents in foods and beverages with low acidity. The main objective of this work was to investigate the effect of 65 kV/cm PEF on the log reductions of *Eupenicillium javanicum* ascospores in 10–30°Brix pineapple juice as well as the modelling. Then, the first-order and Weibull parameters of the 65 kV/cm PEF inactivation of *E. javanicum* ascospores was estimated and compared. Further, the effect of PEF in combination with ultraviolet (UV) light treatment on the log reductions of *E. javanicum* ascospores was studied. Decreasing the soluble solid content of the juice from 30 to 10°Brix for 11.3 pulses increased the spore inactivation in pineapple juice by 2.7 log. A pulse number of 16 would be required by the 65 kV/cm PEF to achieve a 5-log reduction in juice. The Weibull model described spore inactivation by pulsed electric field. The estimated *b*-values for the 65 kV/cm PEF were 0.673 at 10°Brix, 0.041 at 20°Brix and 0.010 at 30°Brix, with *n*-values between 0.73 and 2.08. Although the combination of the PEF and UV light resulted in a slightly greater microbial inactivation, however two hurdles were not suggested. The results of this study confirmed the advantage of PEF technology for the inactivation of *E. javanicum* ascospores in pineapple juice.

1. Introduction

It has been known that heat resistant moulds and specific bacteria such as *Alicyclobacillus acidoterrestris* are undesirable microorganisms in fruit juices, pulps, and concentrates, which could lead to a huge economic loss in fruit juice industry. Among the listed moulds are *Neosartorya fischeri*, *Byssochlamys nivea*, *Byssochlamys fulva*, *Talaromyces* sp. and *Eupenicillium* sp. [1, 2]. The resistance of these ascomycetes moulds are claimed due to their ability to form ascospores, usually in groups of eight which formed inside asci [3]. Mycotoxins such as patulin, xanthomengin and palitantin have been registered during growth in fruit products from some of these heat-resistant molds which are public health concern [1, 4, 5]. The genus *Eupenicillium* such as *Eupenicillium brefelidanum*, *Eupenicillium hirayamae*, *Eupenicillium digitatum*, *Eupenicillium expansum* and *Eupenicillium javanicum* from time to time has been reported as studied microorganisms in thermal or non-thermal food processing [6-12]. Example heat resistance or decimal reduction (*D*) values for ascospores of *E. javanicum* in fruit pulp or juice were 15-19.8 min, 3.7-5 min, and 0.8-1.5 min at 80°C, 85°C, and 90°C, respectively [6, 12].

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Non-thermal food preservation methods have emerged and some have been employed in commercial scale in the industry. These technologies have been claimed to have more advantages over conventional methods in terms of microbial food safety and product quality [13]. Among the technologies are high-pressure processing (HPP), carbon dioxide processing, pulsed electric fields (PEFs), ultrasound, ozonation, UV light, etc. The main goal of the non-thermal pasteurization of foods through the inactivation of pathogenic and spoilage vegetative microorganisms usually by 5 or 6 D followed by cold chain distribution [14]. Pulsed electric field (PEF) application involves the generation of electric field ranging from generally 10 to 80 kV/cm for only a few micro to milliseconds [15]. Process parameters governing microbial inactivation by PEF are the treatment parameters (i.e., strength of electric field, pulse width and shape, number of pulse and temperature), the product nature (pH, water activity and electrical conductivity), and the characteristics of microbes (type of microorganisms, species and strains) [15, 16]. PEF is considered as a suitable pasteurization technology for pumpable foods such as fruit juices, and its combination with other methods are also possible. Microbial inactivation utilizing light emitting diodes (LEDs) has also received attention in past decades. It is a semiconductor that emits light equivalent to a certain wavelength when an electric current flows through it. Thus, LED are classified into several types namely ultraviolet light-emitting diode (UVLED), visible light-emitting diode (Vis-LED), and deep ultraviolet light-emitting diode (DUVLED) [17]. Several factors affecting photodynamic inactivation by LEDs in foods include wavelength used, temperature, nature of foods (acidity, surface properties, water activity), and engineering factors (irradiance, dose and flux distribution) [18].

Currently, there is limited information available in literature on the effect of PEF to ascospores of heat resistant moulds as well as the modelling. Due to the concern and the importance of Eupenicillium sp. in fruit products, therefore the objectives of this study were: (i) to investigate the log reductions and modelling of 65 kV/cm PEF resistance of Eupenicillium javanicum ascospores in pineapple juice, (ii) to estimate the first-order and Weibull parameters of the 65 kV/cm PEF inactivation of E. javanicum ascospores in pineapple juice with different soluble solid content; and (iii) to investigate the effect of PEF in combination with ultraviolet (UV) light treatment on the log reductions of E. javanicum ascospores. It is believed that this study will provide more information on the resistance and kinetic modelling of mould ascospores after PEF, which could be useful for designing fruit juice pasteurization.

2. Materials and Methods

2.1 Mould

The mould Eupenicillium javanicum InaCC F154 was used for the experiments and purchased from Indonesian Culture Collection (InaCC), Research Center for Biology, Indonesian Institute of Sciences or LIPI. The strain was revived according to the suppliers’ instruction. A culture was obtained after 5 day-incubation at 25°C on potato dextrose agar.

2.2. Ascospore production

The 5-day culture was used to prepare a-month-old culture (27°C on malt extract agar) and to obtain the ascospores of E. javanicum. The procedures described by Evelyn et al. were followed [12]. Briefly, 5 mL sterile distilled water (SDW) was poured onto the agar surfaces followed by gently rubbing with a sterile bent glass rod. The spore suspension was obtained after removing any remaining hyphal fragments using few layers of gauze. Then, centrifugation (4,000g and 15 min) of the spore suspension followed by three time-washing in sterile SDW were carried out to obtain the spore pellets. The presence of ascospores were confirmed using an optical microscope. The final spore suspension was then stored at 2°C in SDW before used.
2.3. Pineapple juice preparation and inoculation
Pasteurized pineapple juices (pH 4.2±0.1°Brix) for suspending *E. javanicum* were obtain from a local supermarket. The juices were adjusted to 20 and 30°Brix with sucrose addition depending on the required experiments. A spore volume of 20 mL was inoculated into the juice to make a total PEF working volume of 700 mL and an initial spore concentration of 10^6 cfu/mL of juice. The PEF treated suspension were further taken to UV or BlueLED chamber when PEF+UVLED or PEF+BlueLED light experiments were needed.

2.4. Spore enumeration
The *E. javanicum* ascospore concentration in pineapple juice before and after processing was determined by spread plating onto potato dextrose agar [12]. Appropriate decimal dilutions were performed in 0.9% saline prior to plating. Each tube dilution was mixed repeatedly using a high-speed vortex mixer to yield a uniform spore suspension and surface plated. The average colonies were enumerated after an incubation at 25°C for 4 to 5 days. Ascospore concentration was expressed in cfu/mL of juice sample.

2.5. Pulsed electric field (PEF) and PEF+UVLED processing
Batch experiments of PEF were carried out in a local-made and lab-scale PEF system (Fig. 1). The equipment contains two parts i.e. the high voltage pulse generator and the treatment chamber with a total working volume of 700 mL. The pulse generator can produce a maximum electrical field intensity of 65 kV/cm (distance between electrode = 1cm) and 250 Hz frequency. Prior to each experiment, the PEF chamber was thoroughly cleaned and sterilized and this procedure was repeated after the treatment. Then, the inoculated juice was transferred inside the chamber and treated for up to maximal equivalent to 11.3 pulses depending on the treatment time. As mentioned for PEF+UVLED experiments, these treated samples were further processes to compare with the PEF only results. All final treated suspensions were kept in an ice water bath until microbial enumeration. Two survival experiments were carried out for PEF and PEF+UVLED or PEF+BlueLED.

![Figure 1. PEF treatment chamber](image)

2.6. Data fitting and kinetic modelling
The log survivors (log N/No) versus number of pulses after PEF treatments were drawn and two kinetic models were selected for modelling. First-order kinetics with one parameter i.e. *D*-value is usually used to describe microbial survivors in food preservation due to its simplicity, thus is also used in this study (Eq 1 and 2) [12]:

\[
\log N(t) = \log N_0 - D \times t
\]

\[
\log N(t) = \log N_0 - D \times t + \log A
\]
\[ \log \frac{N}{N_0} = -\frac{t}{D_T} \]  

\[ \log \frac{D_T}{D_{T \text{ref}}} = \frac{T_{\text{ref}} - T}{z_T} \]

in which \( N_0 \) represents untreated ascospore population (cfu/mL) and \( N \) represents the number of ascospores after being exposed to usually a heat treatment for a specific time \( (t) \) (in this case the lethal treatment was 65 kV/cm PEF). \( D_T \)-values, the time in min at a certain temperature necessary to reduce microbial population by 90% were calculated from the reciprocal of the slope in Equation 1. Next, the temperature coefficients (\( z_T \)-value in °C, the temperature increase that results in a 10-fold decrease in the \( D_T \)-value) were estimated from the negative reciprocal of the slope as in Equation 2. \( D_{T \text{ref}} \) is \( D \)-value at the reference temperature \( T_{\text{ref}} \) (can be a reference temperature, °C), \( T \) is the temperature of the isothermal treatment (°C). The \( T \)-value in Equation 1 and 2 can also be replaced with \( SS \) to describe the effect soluble solid on the inactivation [19].

Weibull equation written in the decimal logarithmic form with two parameters was also used to compare the first-order kinetic model for the log survivors of \( E. javanicum \) ascospores after PEF processing [14, 20-22]:

\[ \log \frac{N}{N_0} = -bt^n \]  

where \( b \) (the scale factor) is a rate parameter which is related to the velocity of inactivation of the microorganism. \( n \) is the survival curve shape factor: \( n<1 \) and \( n>1 \) correspond to survival curves which are concave-upwards (tailings) and concave-downwards (shoulders) respectively. When \( n = 1 \), the Weibull model becomes a simple first-order kinetic model. \( t \) is the treatment time in min.

3. Results and Discussion

3.1. Log reductions and modelling of 65 kV/cm pulsed electric field resistance of \( E. javanicum \) InaCC F154 ascospores in pineapple juice

Figure 1 and 2 show the log survivors of \( E. javanicum \) InaCC F154 ascospores in pineapple juice with different soluble solid content (10, 20, and 30°Brix) after a 65 kV/cm pulsed electric field (PEF) process of up to 11.3 pulses. These survival curves were modelled using the first-order kinetics and Weibull, and the derived parameters will be discussed in the next section. As can be seen from these figures, there was a steady decrease in the number of spores at all soluble solids after the PEF treatments. For example, for a 11.3 pulse-process, around 4.0 log was obtained at 10°Brix compared to 3.1 log at 20°Brix and 1.3 log at 30°Brix. Pulsed electric field between 33 and 100 kV/cm, frequencies between 2 Hz and 466 Hz, alone or in combination with temperatures (56-123°C) were reported to inactivate bacterial and fungal spores by 2.5-5.9 log [23-28]. There are several mechanism suggested for microbial inactivation by PEF. According to previous investigators, electroporation caused by PEF could result in a significant increase in the membrane rupture and irreversible electro permeabilization, giving rise to cell death [15, 29, 30]. Meanwhile with respect to spores, partial destruction of coat protein nanostructures which associated with internal alterations of cortex, core and inner membrane or peptidoglycan degradation after pulsed electric field application were observed [28, 31]. Severe destruction of this spore organizational structure might lead to the death of cell. Many past studies have also shown the protective effect of soluble solid on the resistance of spores of bacteria such as \( Alicyclobacillus acidoterrestris \) and mould such as \( Talaromyces flavus \), \( B. nivea \) and \( N. fischeri \) in fruit juices and other non-liquid food medium under thermal or non-thermal tretments [32-35]. Regarding fungal spores, Raso et al. obtained slightly lower spore inactivation (3.4 log) of \( Z. balii \) ascospores after 33 kV/cm-2.2μs pulse width versus around 4 log in this study with \( E. javanicum \) ascospores after 65
kV/cm-11.3 pulses, both in unadjusted pineapple juice [23]. In another studies, these authors achieved almost 5 log inactivation of Byssochlamys fulva conidiospores after 30 kV-11 pulses and 0.6 cm electrode gap [24]. These results suggest that the ascospores of E. javanicum might be more resistant to pulsed electric field than the ascospores of Z. balii, and the conidiospores have lower resistance than the ascospores. With respect to bacterial spores, other investigators attempted B. subtilis and B. cereus spore inactivation using a synergistic effect of PEF (up to 100 kV/cm) combined with heat treatments (50-123°C), and obtained 2.5 to 4.5 log reductions of these spores [26-29]. This indicates that PEF inactivation is species dependent and bacterial spores present higher resistance than fungal spores.

![Figure 1](image1.jpg)

**Figure 1.** First-order model fitted to the inactivation of *Eupenicillium javanicum* InaCC F154 ascospores in 10, 20, and 30°Brix pineapple juice by pulsed electric field (data points are average from two experiments).

![Figure 2](image2.jpg)

**Figure 2.** Weibull model fitted to the inactivation of *Eupenicillium javanicum* InaCC F154 ascospores in 10, 20, and 30°Brix pineapple juice by pulsed electric field (data points are average from two experiments).
3.2. First-order and Weibull parameters of the 65 kV/cm pulsed electric field inactivation of \( E. javanicum \) InaCC F154 ascospores in different soluble solid pineapple juice

The first-order and Weibull models were attempted to describe the survival curves of \( E. javanicum \) strain (Fig. 1 and Fig. 2) after pulsed electric field (PEF) treatment and the parameters were estimated in Table 1. The \( D_{35} \)-values obtained in the first-order model for InaCC F154 spores were 7.1 ± 0.20 min at 10ºBrix, 11.0 ± 0.04 at 20ºBrix and 27.0 ± 0.30 at 30ºC, with a \( z_{55} \)-value of 34.3ºBrix. Higher \( D \)-values at higher °Brix values confirming more difficult inactivation. The \( z_{55} \)-value indicates that a reduction of 34.3ºBrix in the soluble solid is needed in order to decrease the \( D_{35} \)-values by a factor of 10. Although the first-order model is a simpler model with one parameter, however the Weibull model with two parameters resulted in better performance indices (0.034 ≤ MSE ≤ 0.107, 0.911 ≤ \( R^2 \) ≤ 0.976) and was suggested for describing the data. In the past, thermal and non-thermal processing have been largely adopted this model to describe the spores and vegetative cells inactivation in foods.

In Weibull distribution, the \( b \) and \( n \) parameters are the scale and shape factor that are related to the spore inactivation rate (\( b \)) and the deviation from linearity (\( n \)). As is shown from Table 1 and Figure 2, the higher the soluble solid, the lower the \( b \) value. For the investigated fungal spores, the \( b \) value decreased from 0.673±0.13 to 0.010±0.005 as the soluble solid or % weight of sucrose concentration increased from 10ºBrix to 30ºBrix, suggesting lower inactivation rate at higher °Brix. Although there were several previous researches on fungal spore inactivation by PEF and showed concavity trend in the survival curves obtained, however none seems have studied their kinetic models. Nonetheless as mentioned, many investigators have used the Weibull model for describing the log inactivation of microbial spores after the thermal and non-thermal food processes.

**Table 1.** First order and Weibull model parameters for 65 kV/cm pulsed electric field inactivation of \( Eupenicillium javanicum \) InaCC F154 ascospores in pineapple juice*

| SS (ºBrix) | First order | Weibull |
|-----------|-------------|---------|
|           | \( D_{35} \)-value ± SE (min) | \( z_{55} \)-value ± SE (ºBrix) | \( b \) ± SE | \( n \) ± SE |
| 10        | 7.1 ± 0.20  | 34.4±0.08 | 0.673 ± 0.13 | 0.73 ± 0.09 |
| 20        | 11.0 ± 0.04 | R\(^2\) = 0.96 | 0.041 ± 0.03 | 1.80 ± 0.36 |
| 30        | 27.0 ± 0.30 | 0.010 ± 0.005 | 2.08 ± 0.53 |

*\( D_{35} \) is the decimal reduction time at certain soluble solid and \( z_{55} \)-value is the soluble solid required that results in a 10-fold decrease in the \( D_{35} \)-value (Eq. 1 and 2). \( D_{35} \)-values are means ± standard error (SE) and obtained from two experiments; \( b \) and \( n \) are the Weibull scale and shape factors, respectively (Eq. 3); Weibull model worked better than the first-order model presenting low MSE values (0.034−0.107) and high \( R^2 \) (0.911−0.976).

The \( n \) values vary from 0.73±0.09 to 2.08±0.53 (Table 1), indicating both concave-upward (\( n \) less than 1) and concave-downward (\( n \) more than 1) in the spore survivor curves for pulsed electric field processes (Fig. 2). A concave-upward type of concavity suggests that there is a mixed resistance of the spore population to the lethal treatment [20, 21], in which the most sensitive spore population is inactivated at a faster rate, followed by the slower and steady decline of a more resistant population. Meanwhile a concave-downward type of concavity could suggest spore resistance to the sublethal treatment (due to lower number of pulses) shown by a slight activation shoulder followed by an increase in the destruction rate with the pulsed electric field exposure pulse or time (due to accumulated damage). From Table 1, the \( n \) values obtained were 0.73±0.09 at 10ºBrix, 1.80 ± 0.36 for 20ºBrix and 2.08 ± 0.53 for 30ºBrix in pineapple juice, also seem to show soluble solid dependence for
this strains with $R^2$ of 0.90 (not shown). Similarly, the log $b$-values was also a linear function of the soluble solid content of juice with $R^2 = 0.97$ for InaCC F154 strain. The dependence of $b$ and $n$ on the lethal parameters has been observed with other non-thermal methods such as HPP temperature and pressure [36-39]. Based on the results, increasing the pulse number of pulsed electric field to 15 or 16 in 10 and 20ºBrix juice would be able to reduce the ascospores by 5 log.

3.3. Effect of pulsed electric field in combination with ultraviolet light treatment on the log reductions of E. javanicum InaCC F154 ascospores

Figure 3 shows a comparison of log inactivation of E. javanicum ascospores in pineapple juice (10, 20, and 30 ºBrix) after 65 kV/cm-11.3 pulses pulsed electric field (PEF) and 65 kV/cm-11.3 pulsed electric field followed by ultraviolet LED (PEF+UVLED) treatment. The combination of PEF with UV was possible according to Noci et al. [2008]. As can be seen from the figure, addition of other lethal treatment i.e. UV light to the PEF treated spores increased the log reductions at all soluble solids and more prone at lower concentration (≤20ºBrix). The obtained log reductions values for PEF vs. PEF+UVLED were higher by 0.7 log at 10ºBrix and 0.5 log at 20ºBrix, and 0.4 log at 30ºBrix for PEF+UVLED. The results for PEF+BlueLED resulted in smaller increases and not significant for the spore inactivation (thus was not shown). The combination of PEF and UV could result in almost 5D, FDA recommended for fruit juice pasteurization [14]. However, these added lethal effects were small compared to total inactivation therefore the hurdle method was not suggested. There were limited reports on the combination of PEF and UV. However, these results are in agreement with Noci et al. that showed a slightly greater microbial inactivation (6.2 log) after PEF+UVLED compared to PEF alone (5.4 log) [40]. The results of PEF+UVLED also suggest the protective effect of soluble solid or SS on E. javanicum spore inactivation in the pineapple juices. This phenomenon has been observed in other treatment methods [33, 34, 41].

![Figure 3](image_url)

**Figure 3.** Effect of pulsed electric field (PEF) and pulsed electric field followed by ultraviolet light (PEF++UV) treatment on the inactivation Eupenicillium javanicum ascospores in 10, 20, and 30ºBrix pineapple juice.

4. Conclusion

In this study, we demonstrated the use of 65 kV/cm and 11.3 pulses pulsed electric field (PEF) to inactivate Eupenicillium ascospores in unadjusted pineapple juice by around 4.0 log. Increasing the soluble solid content of the juice to 20 and 30º Brix resulted in less spore inactivation. Inactivation
kinetics showed a better fit of the Weibull model than the first-order kinetic model for describing the spore survival curves after the PEF application. A hurdle method of PEF followed by ultraviolet treatment did not seem to have a significant added lethal effect on spore reductions. To conclude, these results would indicate a potential role of 65 kV/cm PEF processing for pineapple juice contaminated by *E. Javanicum* ascospores.

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