Thermal Inactivation of *Eupenicillium Javanicum* Ascospores in Pineapple Juice: Effect of Temperature, Soluble Solids and Spore Age

Evelyn¹, Chairul¹, S R Muria¹, L Adella¹, R Ramadhani¹

¹Department of Chemical Engineering, Faculty of Engineering
Universitas Riau, Pekanbaru 28293, Riau, Indonesia
evelyn@eng.unri.ac.id

Abstract. Food spoilage leading to food waste and substantial economic losses is a major issue for the food industry. The spoilage is often due to bacterial and fungal contamination in foods. Spores from microorganisms pose a great concern since they can survive pasteurization and grow in specific foods. The objectives of this work were to investigate the effect of temperature (T: 80-90°C), soluble solid concentration (11-20°Brix), and spore age (30-60 days) on the log reductions of *Eupenicillium javanicum* ascospores in pineapple juice. It was also carried out to estimate the first-order kinetic parameters (D and z-values) from the log survivor curves. Increasing the temperature from 80 to 90°C for 10 min increased the spore inactivation in 11°Brix pineapple juice by 6.4 log. For 5-log inactivation, increasing the percentage weight (% wt.) of sucrose from 11°Brix to 20°Brix at 90°C, increased the time needed to inactivate the spores by 2.6 min. Likewise, increasing the age of ascopores from 30 days to 60 days, also increased the time required by 16.7 min. The estimated D-values for 30-days old spores in 11°Brix juices were 19.84 min at 80°C, 4.99 min at 85°C and 1.45 min at 90°C, with z-values of 8.6-8.8°C. The results obtained provide useful data to design and predict pasteurization process targeting *E. javanicum* ascospores.

1. Introduction

*Eupenicillium* species is widely distributed in soil and have been known from time to time as heat-resistant survivors of fruit juices [1]. Among 37 recognized species of *Eupenicillium*, four important species in these foods are *Eupenicillium brefeldianum*, *Eupenicillium cinnamopurpureum*, *Eupenicillium hirayamae*, and *Eupenicillium javanicum* [2]. *Eupenicillium javanicum* (anamorph *Penicillium indonesiae* Pitt) is a fast-growing filamentous fungus or mould that produces bright colors in exudate and reverse colonies [1]. Despite their benefits in enzyme production [3], detrimental effect or spoilage due to the presence of this mould in fruit juices has been known [2]. Previous studies have shown that the decimal reduction (D) values of *E. javanicum* ascospores in strawberry pulp (15°Brix) were 15 min, 3.7 min, and 0.8 min at 80°C, 85°C, and 90°C, respectively [4]. It has also been reported to produce mycotoxins xanthomenigin, a little palitantin, and patulin [5, 6].

Fruit and vegetable juices are still the most important consumed beverages next to tea and water. Pineapple is one of the most popular tropical fruit processed into juices due to its attractive flavor and richness in vitamins. Brazil, Thailand, Philippines and China are the biggest producers of pineapple in the world, contributing to 52% of the total outputs [7]. This fruit develops in contact with soil thus is more prone to filamentous mould contamination. A proper preservation method should be carried out to avoid the major concern i.e. spoilage or economic loss and wastage of fruit. Furthermore, processing aims to achieve food safety and maintain food quality. Currently there is very little...
information available in literature on the effect of thermal processing on the ascospores of this species. It is well known that the effect of thermal treatments on microorganisms varied with the type of microorganism (e.g. species, strain) as well as processing conditions and suspending medium.

Thermal treatment is still the most widely applied technology to preserve foods in industries. A minimum 5-log reduction of contaminant microorganism i.e. vegetative forms in a fruit juice by pasteurization at 90–95°C for 4–10 seconds has been recommended by FDA [8]. Nowadays, the definition of pasteurization has deviated to include the inactivation of spores of public health risk [9]. Therefore, microbial and fungal spores are now also the pasteurization targets, in which 5-6 log reductions in the spores are required [10]. The effect of the temperature, soluble solid and ascospore age has been reported during microbial inactivation with thermal processes [11]. Due to little information and the importance of *E. javanicum* in fruit products, therefore, the objectives of this study were: (i) to investigate the effect of temperature on the log reductions of *E. javanicum* spores after thermal inactivation, (ii) to investigate the effect of soluble solids (SS) on the log reductions of *E. javanicum* spores after thermal inactivation; (ii) to investigate the effect of ascospore age on the log reductions of *E. javanicum* spores after thermal inactivation, and; (iv) to model the inactivation of these spores after thermal treatments. It is believed that such a study will provide information on the resistance of this mould under thermal treatment, which could be useful for designing fruit juice pasteurization.

2. Materials and Methods

2.1 Mould

*Eupenicillium javanicum* InaCC F154 isolated from soil was obtained from Indonesian Culture Collection (InaCC), Research Center for Biology, Indonesian Institute of Sciences or LIPI. The strain was revived according to the suppliers’ instruction.

2.2. Ascospore production

Ascospores of *E. javanicum* were obtained after cultivation of the stock cultures after periods of 30, 45, and 60 days at 25°C on malt extract agar. This period of cultivation was considered as the age of the ascospores. The spores were collected by flooding the surface of the culture plates with 5 mL sterile distilled water (SDW) and gently rubbing from the agar surface with a sterile bent glass rod. The spore suspension was subsequently filtered through layers of gauze to remove any remaining hyphal fragments. Spore pellets were obtained after centrifugation in sterile SDW at 4,000×g, 15 min, 4°C, and the procedure was repeated three times. The spores 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

The pineapple juice used in this study (pH 4.2±11°Brix) was purchased from a local supermarket and used as the treatment medium for the *E. javanicum* spore inactivation. The juice contained added flavour, citric acid, and preservatives (sodium metabisulfite). Depending on thermal experiments, the juices were adjusted to 15 or 20°Brix with sucrose. A small portion (ca. 1.0 mL) of spore solution was inoculated into 3.0 mL of pineapple juice to yield an initial spore concentration of roughly 10⁹ cfu/mL of juice.

2.4. Spore enumeration

The mould ascospore concentration in pineapple juice before and after processing was determined by spread plating onto potato dextrose agar [11]. 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 plated twice. 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. Thermal processing
Thermal resistance of *E. javanicum* ascospores was carried out according to previous method applied for heat-resistant ascospores [11]. Briefly, a thermostatic water bath was initially heated until the treatment temperature i.e. 80, 85 or 90°C was reached (±1°C). Then, thermal death tubes containing the inoculated pineapple juice were submerged into the thermostatic water bath and heated for various times. Treated samples were taken out and kept in an ice water bath until microbial enumeration.

2.6. Data model fitting
First-order kinetic equation is usually used to describe microbial survivors after thermal processes due to its simplicity, thus is also used in this study (Eq 1 and 2) [12]:

\[
\log \frac{N}{N_0} = -\frac{t}{D_t}
\]  \(\text{(1)}\)

\[
\log \frac{D_T}{D_{T_{ref}}} = \frac{T_{ref}-T}{z_T}
\]  \(\text{(2)}\)

Initially, the average data of \(\log N/No\) versus time were plotted in a chart, in which \(No\) represents untreated ascospore population (cfu/mL) and \(N\) represents the number of ascospores after being exposed to a lethal (heat) treatment for a specific time \(t\). Then, decimal reduction times \(D_T\)-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. \(D_{T_{ref}}\) is \(D\)-value at the reference temperature \(T_{ref}\) (can be any reference temperature, °C), \(T\) is the temperature of the isothermal treatment (°C). Inactivation of spores in terms of log reductions and \(D_T\)-values for different processing conditions (temperature/soluble solid/age) was investigated using a T-test (with significance assigned at \(p<0.05\)). All experiments were carried out in triplicate.

3. Results and Discussion

3.1. Effect of temperature on the log reductions of *E. javanicum* ascospores
The log survivors as a function of time of a month-old *E. javanicum* InaCC F154 ascospores in pineapple juice (11°Brix) processed by 80-90°C thermal is illustrated in Figure 1. The important role of thermal processing temperature has been known. As can be seen from Figure 1, higher temperatures produced greater log reductions. For example, for a 10 min process, 6.9 log was obtained at 90°C compared to 2.0 log at 85°C and 0.5 log at 80°C \((p<0.05)\). Thus, only the 90°C thermal process was able to achieve the recommended 5-log reduction in a short time. The mechanism of spore killing by moist heat has been suggested as follows: release of dipicolinic acid leading to damage of one or more key proteins; followed by significant loss of DPA and protein unfolding or denaturation by subsequent heat [13]. Aragão [2] obtained 11.8 log increase for thermal inactivation of a month-old *E. javanicum* ascospores in 15°Brix strawberry pulp after increasing the temperature from 80°C to 90°C for 10 min. Likewise, Vander Spuy [14] reported an increase (9.6 log) of *Penicillium brefeldianum* spores in apple juice after 10°C increase of similar processing temperature-time. Salomão et al. [15] used lower temperatures (50-60°C) for the thermal inactivation of 10-days old *Penicillium expansum* spores in which 6D reduction can be obtained at much lower temperature (56°C) after 6.84 min, suggesting lower resistance of younger spores. To date, very few literatures available on the thermal inactivation of *Penicillium sp.* in fruit beverages and products. Therefore, this study was carried out in order to confirm their resistance and concern in these high-acid food products.
3.2. Effect of soluble solid on the log reductions of *E. javanicum* ascospores

The effect of increasing soluble solid from 11°Brix to 20°Brix in a thermal process at 90°C on the time needed to inactivate 5-log ascospores of *E. javanicum* InaCC F154 in pineapple juice is illustrated in Figure 2. As can be seen from the figure, the time required to result in a 5-log increased steadily at all soluble solids. For example, increasing the percentage (%) weight of sucrose from 11°Brix to 20°Brix at 90°C, increased the time needed by 2.6 min. The same increase in time required to inactivate 5-log ascospores were also observed at lower temperatures (6.5 min for 85°C and 46.2 min for 80°C) (p<0.05). These results suggest combination of lower temperature and higher soluble solid will result in a more difficult technique to achieve the required pasteurization. Several past studies have also shown the protective effect of soluble solid on the resistance of bacterial (e.g. *Alicyclobacillus acidoterrestris*) and mould (*Talaromyces flavus*, *Byssoclamys nivea* and *Neosartorya fischeri*) spores in fruit juices and other non-liquid food medium [11, 16-18].

![Figure 2](image2.png)

*Figure 2.* Effect of soluble solid content (11, 15 and 20°Brix) on the 90°C thermal inactivation of a month-old *Eupenicillium javanicum* ascospores in pineapple juice.
3.3. Effect of ascospore age on the log reductions of E. javanicum ascospores

Figure 3 shows the effect of ascospore age from 30 to 60 days on the time required to inactivate 5-log E. javanicum InaCC F154 ascospores at 90°C. It can be seen that the age of ascospores affected the time (in min) values. Generally, an increase of cultivation days of mould on the plates resulted in an increase of inactivation times. It is known that the older the spores, the more protected the ascospores of heat-resistant fungi to thermal or non-thermal methods [16, 19-24], which has substantial impacts in the pasteurization design of beverages. The times needed to inactivate 5-log E. javanicum ascospores by 90°C thermal processes were 7.3 min for 30 day-old-spores, 13.75 min for 45 day-old-spores and 23.95 min for 60 day-old-spores (p<0.05). Similarly, the times needed for 5D at 85°C thermal treatments increased, when the age of ascospores were increased from 30 to 60 days (25.0 min to 106.5 min). Several authors suggested the causes in higher resistance of older ascospores were due to changes in the spore ultrastructure observed through formation of multilayers in the ascospore’s wall during cultivation times [25, 26]. Higher ratio of asci free ascospores in older spores might also become another cause responsible for the heat resistance of older spores [23].

![Figure 3](image_url)

**Figure 3.** Effect of ascospore age (30, 45 and 60 days) on the 90°C thermal inactivation of Eupenicillium javanicum ascospores in pineapple juice (11°Brix).

3.4. Modelling the thermal inactivation of Eupenicillium javanicum ascospores in pineapple juice

E. javanicum spore inactivation after thermal processing at 80, 85 and 90°C showed a linear pattern (Fig. 1), and was therefore consistent with first-order kinetics. The kinetic parameters were estimated (Table 1) to compare with past results. In general, the first-order kinetic models are supported by the D-values temperature dependence (R²=0.90-0.99). The 90°C thermal process was successful in inactivating ≈5 log of a month-old E. javanicum ascospores in 11-20°Brix pineapple juice after 7.3-9.9 min (D₉₀°C-values of 1.45, 1.76, and 1.97 min for 11, 15, and 20°Brix, respectively). This makes the commercial pasteurization conditions suggested for fruit juice preservation such as 90°C for 10 s inadequate for pasteurization processes aimed at E. javanicum mould ascospores. Further increase in the age of the ascospores, resulting in the increase for the time needed for a 5D reduction: D₉₀°C-value of 2.75 min for 45-day old spores and D₉₀°C-value of 4.79 min for 60-day old spores (p<0.05). Aragão [4] reported slightly lower D-values (D₉₀°C=0.8 min, D₈₅°C=3.7 min and D₉₀°C=15 min) for E. javanicum ascospores (isolated from strawberry pulp) in 15 °Brix strawberry pulp as opposed to our study. Vander Spuy [14] worked with P. brefeldianum ascospores in apple juice and obtained D-values of 25, 4.9 and 1.0 min after 80, 85 and 90°C. Salomão et al. [15] obtained a total inactivation of 10-days old P. expansum spores at 80°C, indicating lower resistance for younger spores. The ε-values estimated for the 30-day old spores in this study were 8.6-8.8°C (Table 1), which are within the expected values for most mould ascospores. These results showed that E. javanicum ascospores pose a
concern in fruit juice pasteurization, similar to the ascospores of heat resistant moulds *B. nivea* and *N. fischeri* [27, 28].

Table 1. *D* and *z*-values of a month-old *Eupenicillium* spp. ascospores in pineapple juice

| Species       | Temperature (°C) | Fruit products       | *D*-value (min) | *z*-value (°C) ±SE | Reference               |
|---------------|------------------|----------------------|-----------------|-------------------|-------------------------|
| *E. javanicum*| 80               | Pineapple juice      | 19.84           | 8.8±0.1           | This study              |
| InaCCF154     | 85 (30 days)     | 1.45                 |                 |                   |                         |
|               | 80               | Pineapple juice      | 25.41           | 8.6±0.2           |                         |
|               | 85               | (11°Brix)            | 5.89            |                   |                         |
|               | 90               | 1.76                 |                 |                   |                         |
|               | 80               | Pineapple juice      | 29.07           | 8.6±0.2           |                         |
|               | 85               | (15°Brix)            | 6.29            |                   |                         |
|               | 90               | 1.97                 |                 |                   |                         |
| (45 days)     | 80               | Pineapple juice      | 22.2            | 11.0±0.4          |                         |
|               | 85               | (11°Brix)            | 11.2            |                   |                         |
|               | 90               | 2.75                 |                 |                   |                         |
| (60 days)     | 80               | Pineapple juice      | 31.92           | 12.1±0.3          |                         |
|               | 85               | (11°Brix)            | 21.30           |                   |                         |
|               | 90               | 4.79                 |                 |                   |                         |

*D*-value and *z*-values are the first-order kinetic parameters (Eq. 1 and 2); *R*²=0.96-0.99.

4. Conclusion

The heat resistance of *E. javanicum* ascospores was greatly affected by temperature (80-90°C) and ascospore age (30-60 days). Concentration of soluble solid (11-20°Brix) was also influenced the inactivation. A linear relationship in the log survivors vs. time was observed, thus was well described by the first-order kinetics. *E. javanicum* survives pasteurization process of 90°C for 10 s, in which the *D*-values for 5D in 11-20°Brix pineapple juices were 7.3-9.9 min. Thus, designing sufficient processing conditions and/or combination with preservatives is needed to prevent spoilage by this heat resistant mould.

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