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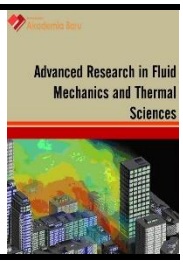

Thermal Inactivation of *Talaromyces flavus* Ascospores in Pineapple Juice as Influenced by Temperature, Soluble Solids, and Spore Age

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ABSTRACT

The ascospores of *Talaromyces* species can survive pasteurization and grow in high acid fruit juices. In this study, the thermal inactivation of *Talaromyces flavus* ascospores in pineapple juice was carried out and the log reductions were counted. First, the effect of temperature (T: 80-90°C), soluble solid content (SS: 10–30°Brix), and spore age (30–60 days) on the viability of ascospores were investigated. Then, the first-order kinetic parameters (D and z-values) were determined from the log survivor curves. Results of the thermal processes showed that higher log reductions for higher temperatures (5.2 log for 90°C vs. 0.89 log for 85°C and 0.28 log for 80°C) were obtained at 10°Brix after 15 min heat treatments. Lower spore reductions were observed at higher SS (1.5 log for 30°Brix vs. 2.4 log for 20°Brix and 3.0 log for 12°Brix after 9 min at 90°C). Longer time needed to inactivate the older spores (53.8 min for 30 day-old-spores vs. 67.1 min for 60 day-old-spores). The log survivors are better described by the first-order kinetics at 80-90°C. The estimated D-values in 12°Brix juice were 63.8 min, 16.9 min and 2.89 min for 80, 85 and 90°C, respectively, with z-value of 7.9°C. This result emphasizes the importance of temperature, SS, and ascospore age on the heat resistance of *T. flavus* ascospores in high acid fruit juice.

Keywords:
Thermal processing; mold spores; survival; kinetic; modelling

1. Introduction

Talaromyces is a mold that is widely distributed in soil. For example, *Talaromyces flavus* has been isolated in 16 countries around the world [1]. *Talaromyces* species is a great concern in fruit products since the ascospores can be very resistant to heat. In favorable conditions (storage temperature and food characteristics such as pH, water activity, food constituents) after pasteurization, the ascospores can germinate and grow in fruit products to attain high numbers e.g. 10^5-10^6/g or mL and cause spoilage or food-borne diseases. Decimal reduction values (D-values) at 91°C between 0.9 min in strawberry pulp and 2.9 min in grape juice have been reported for this species [2, 3]. Few

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Talaromyces sp. such as *Talaromyces macrosporus* and *Talaromyces wortmannii* are known as mycotoxin producers [4].

Indonesia is rich in the production of tropical fruits, placing the country in the top twenty for world fruits producers. According to Indonesian Central Bureau of Statistics in 2015 [5], Indonesia produced 18.4 million tons of fruits with the major commodities are banana, mango, orange, pineapple and durian. Pineapple is in the fourth place with 1.7 million tons, which some of the products are coming from Riau Province. Brazil, Thailand, Philippines, China are also main pineapple producers in the world, contributing to 52% of the total outputs [6]. The abundant production of these fruits should be followed with a proper preservation method to avoid significant economic losses and to maintain food safety as well as sensorial and nutritional quality. Contamination and isolation of *T. flavus* in pineapple juice have been reported by King and Halbrook [7].

Despite development of many non-thermal pathogen and spoilage inactivation technologies in recent decades [8-10] and high energy consumption drawback of thermal processing [11], heat treatment is still the most widely applied technology in the food industries. Traditional heat treatment using hot-filling process applies 92 and 105°C for 15-30s to process the foods [12]. However, these conditions were not specifically reported for pineapple juice. Due to the importance of *T. flavus* in pineapple juice, therefore the objectives of this study were: (i) to investigate the effect of temperature on the log reductions of *T. flavus* spores after thermal inactivation, (ii) to investigate the effect of soluble solids (SS) on the log reductions of T. *flavus* spores after thermal inactivation; (ii) to investigate the effect of ascospore age on the log reductions of *T. flavus* spores after thermal inactivation, and; (iv) to model the inactivation of these spores after thermal treatments.

2. Methodology

2.1 Mold

Talaromyces sp. InaCC F155 was obtained from Indonesian Culture Collection (InaCC), Research Center for Biology, Indonesian Institute of Sciences or LIPI. The species was isolated from soil at Mt. Bromo, Ngadisari, Pasuruan Regency, Indonesia and identified as *Talaromyces flavus*.

2.2 Ascospore Production

Ascospores of *T. flavus* were obtained after growth for four weeks at 30°C on potato dextrose agar (PDA) [13]. 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 final spore suspension was then stored at 2°C in SDW.

2.3 Pineapple Juice Preparation and Inoculation

Pineapples from local market in Riau Province were cut into small pieces and blended in a sterile laboratory scale blender. The juice (12°Brix, pH 4.3) was used as the medium to suspend the *T. flavus* ascospores and adjusted to 20 or 30°Brix with sucrose, depending on thermal experiments. Aliquots (ca. 1.0 mL) of *T. flavus* spore solution were inoculated into 2.0 mL of pineapple juice to yield an initial spore concentration of approximately 10^6 cfu/mL of juice.

2.4 Spore Enumeration

The mold ascospore concentration in pineapple juice before and after processing was determined by spread plating onto PDA [14]. For the initial spore concentration, the number was determined after a heat shock (75°C, 5 min) of spores in pineapple juice in a thermostatic water bath [15]. Prior to plating, 1 mL spore samples were decimal diluted using 9 mL saline solution (0.85%). Each tube dilution was mixed repeatedly using a high-speed vortex mixer to yield a uniform spore suspension and plated twice. The plates were then incubated at 30°C for 3 to 5 days until visible colonies were formed. Plates with 20 to 100 colonies were used for enumeration, and average colony counts were calculated. Ascospore concentration was expressed in cfu/mL of juice sample.

2.5 Thermal Processing

Thermal resistance of *T. flavus* ascopores was carried out at three temperatures: 80, 85 and 90°C, according to previous method applied for heat-resistant ascopores [16]. Initially, thermostatic water bath was heated until the treatment temperature was reached (±1°C). The inoculated pineapple juice samples contained in thermal death tubes were then submerged into the preheated thermostatic water bath, and heated for various times. Treated samples were taken out at different time intervals and kept in an ice water bath until microbial enumeration.

2.6 Data Model Fitting and Statistical Data Analysis

For each spore age or temperature, two survival experiments were carried out for thermal treatments. For each survival experiment, duplicate samples were processed each time. Then, the average data of log N/No versus time were plotted in a chart, in which a log-linear relation was observed. No is the initial or untreated ascospore population (cfu/mL), N is the number of ascospores after being exposed to a lethal (heat) treatment for a specific time (t). Thus, first-order kinetics was used to model the thermal inactivation results in order to compare with literature results. In this model, 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 Eq. (1) [17]. The temperature coefficient, z_T-value (°C) is the temperature increase that results in a 10-fold decrease in the D_T-value and was estimated from the negative reciprocal of the slope as in Eq. (2). D_{Tref} is D-value at the reference temperature T_{ref} (can be any reference temperature, °C), T is the temperature of the isothermal treatment (°C). A t-test (Statistica 8.0, Statsoft Inc., USA) was used to compare the log reductions of mold spores at different temperature/soluble solids/spore age, after a specific processing time.

$$log \frac{N}{N_0} = -\frac{t}{D_t} \quad (1)$$

$$log \frac{D_T}{D_{Tref}} = \frac{T_{ref}-T}{z_T} \quad (2)$$

3. Results

3.1 Effect of Temperature on The Log Reductions of T. Flavus Ascospores

Figure 1 shows the log survivors of *T. flavus* ascospores in pineapple juice (12°Brix) after thermal processes at 80-90°C. As expected, the higher the temperature the higher the log reductions obtained, indicating the important role of temperature for inactivating the ascospores. For example, increasing the temperature from 80°C to 90°C at 12°Brix for 15 min increased the *T. flavus* spore inactivation by almost 5 log ($p<0.05$). Quintavalla and Spotti [3] obtained 4.6 log increase for thermal inactivation of *T. flavus* CBS 317.63 in grape juice after increasing the temperature from 88°C to 90°C for 15 min (estimated from the D_T-values). Likewise, Scott and Bernard [18] obtained 4.8 log increase for thermal inactivation of *T. flavus* 83-41 in apple juice after increasing the temperature from 87.8°C to 90.6°C for 15 min. Aragão [2] achieved much higher log reduction (12 log) of *T. flavus* also in apple juice when the temperature was changed from 85°C to 90°C. Decreasing of the heat resistance of several strains *Talaromyces sp.* with increasing temperature were also observed in other medium i.e. 16°Brix glucose-tartrate heating medium [8]. It has been known that mechanism inactivation of spores by heat is due to dipicolinic acid (DPA) release leading to spore core hydration, followed by protein denaturation of the more hydrated spore core [19]. Variations in *T. flavus* ascospore reductions after thermal treatments in the literature might be due to many factors, for example different spore preparations [8], type and temperature of growth medium [20], as well as age and sugar content in the inactivation medium [21].

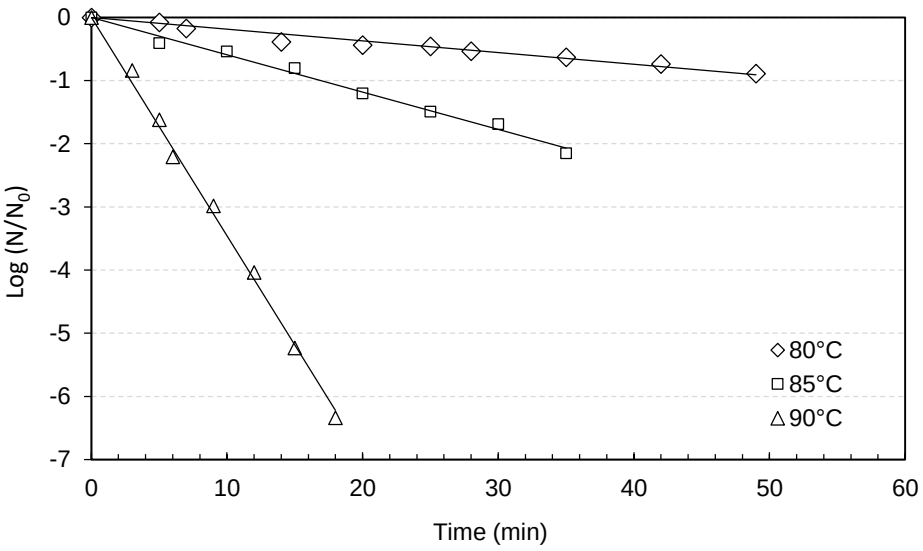

Fig. 1. Effect of temperature on the thermal inactivation *Talaromyces flavus* ascospores in pineapple juice (12°Brix)

3.2 Effect of Soluble Solid Content on The Log Reductions

The effect of soluble solid contents or SS (10-30°C) on the log survivors of *T. flavus* ascospores in pineapple juice after thermal processes at 90°C is illustrated in Figure 2. A decrease in the number of inactivated ascospores was observed with increasing percentage (%) weight of sucrose. As can be seen from the figure, a 3-log reduction was obtained for 12°Brix compared to 2.4 log for 20°Brix and 1.5 log for 30°Brix pineapple juices after 9 min ($p<0.05$). The typical level of microbial contamination in juice is around 10^3 cfu/mL [22], indicating the difficulty in reducing the spores to that amount with higher SS (20 and 30°Brix) content. These results are in agreement with Beuchat [20] in which lower

log reductions were observed with higher sucrose concentration (60% as opposed to 15%), reducing the water activity (a_W) from 0.99 to 0.96. Silva *et al.,* [23] also reported the protective effect of SS (5-60°Brix) on the heat resistance of *Alicyclobacillus acidoterrestris* spores suspended in malt extract broth, whereas other authors demonstrated generally an increase in the heat resistance of *Byssochlamys nivea* and *Neosartorya fischeri* mold ascospores suspended in pineapple and papaya juices after increasing the SS from 13 to 27°Brix [24]. The same phenomenon i.e. a significant increase in the D-values observed upon increasing the sucrose concentration, was also reported by other authors [25].

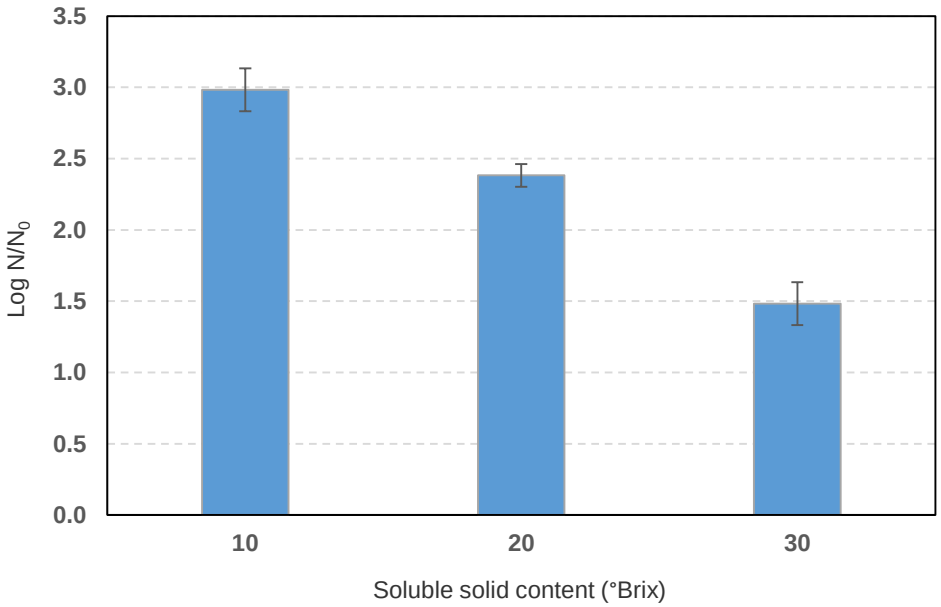

Fig. 2. Effect of soluble solid content (12, 20 and 30°Brix) on the 90°C-thermal inactivation *Talaromyces flavus* ascospores in pineapple juice

3.3 Effect of Ascospore Age on The Log Reductions

Figure 3 shows the effect of ascospore age from 30 to 60 days on the time required to inactivate 1-log T. flavus ascospores at 80, 85, and 90°C. Generally, the older the spores, the longer the time needed for the inactivation at all temperatures. For 80°C thermal treatment, the time needed to inactivate 1-log of the ascospores were 53.8 min for 30 day-old-spores, 59.88 min for 45 day-old-spores and 67.11 min for 60 day-old-spores ($p<0.05$) (Figure 3(a)). Similarly, only 2.89 min was needed to inactivate 1-log of 30 day-old-spores as opposed to 4.80 min 60 day-old-spores at 90°C ($p<0.05$) (Figure 3(c)). Changes in the spore ultrastructure or nanostructure through formation of multilayers in the ascospore's wall can contribute to the higher resistance of older ascospores [26, 27]. Higher ratio of asci free ascospores in older spores might also become another cause for the heat resistance of older spores [28]. Higher resistance of older ascospores than younger ascospores were also observed in other studies under thermal and non-thermal inactivation methods [28, 29, 30].

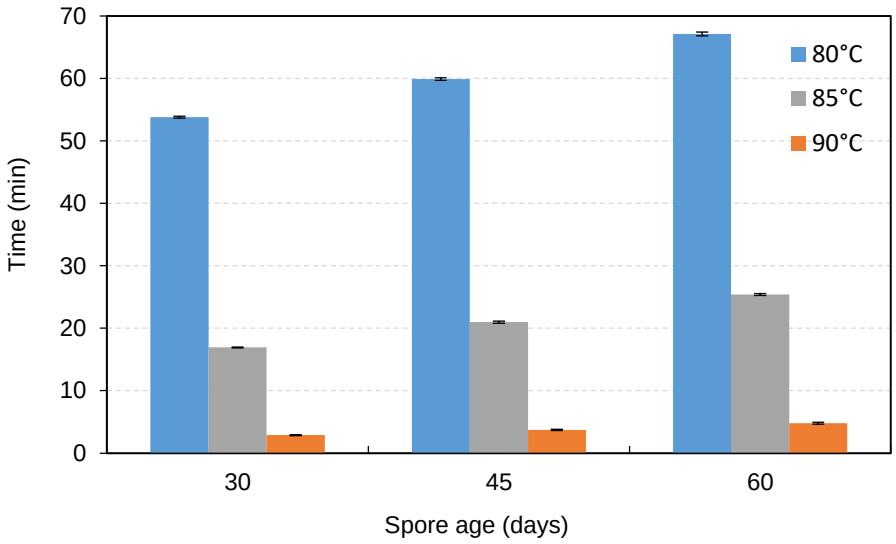

Fig. 3. Effect of ascospore age (30, 45 and 60 days) on the 80, 85, and 90°C-thermal inactivation of *Talaromyces flavus* ascospores in pineapple juice (12°Brix)

3.4 Modelling the Thermal Inactivation of Talaromyces Flavus Ascospores In Pineapple Juice

Based on the general appearance of the thermal survival lines in Figure 1, a linear equation was attempted to model the spore survivors, and the kinetic parameters were estimated (Table 1). In general, the first-order kinetic models are supported by the D-values temperature dependence (R^2=0.96-0.99). Exposure to higher temperatures resulted in higher lethality of heat treatments, thus lowering the minutes required to inactivate the ascospores. At 90°C, the D-value obtained was 2.89 min as opposed to 15.9 min at 85°C and 53.8 min at 80°C. DPA release leading to damage of one or more key proteins has been a common phenomenon for spore killing by moist heat [19]. The theory suggests that more heat resulted in significant loss of DPA and protein unfolding or denaturation. Scott and Bernard [18] and Aragão [2] reported almost similar $D_{90°C}$-values of *T. flavus* and *T. macrospores* ascospores in apple/grape juices as reported in this study (2.77-2.89 min) despite the large $D_{80°C}$-values reported (>265.5 min), whereas Beuchat [31] reported a wide range of $D_{90°C}$-values *T. flavus* NFPA-2 strain in bluberry/peach/strawberry puree (1.13-7.5 min). To conclude, variation in the D-values of *Talaromyces sp*. ascospores might be attributed to interaction of various influencing factors, namely composition of fruit products and strain. The z-values estimated in this study were 7.9°C (Table 1), which are within the expected values for most mold ascospores.

Table 1
D and *z*-values of *Talaromyces flavus* ascopores in fruit products

Temperature (°C)	Fruit products	D_T-value (min)	z-value (°C)[a]	Reference
80	Pineapple juice	53.8	7.9±0.09	This study
85	(12°Brix)	16.9	R^2=0.96-0.99	
90		2.89		
80	Blueberry puree	648.6	5.4	[24]
85		69.9		
90		7.5		
80	Cherry puree	224.0	7.7	
85		25.38		
90		1.13		
80	Strawberry puree	449.8	5.3	
85		51.9		
90		5.99		
80	Apple juice	265.5	5.2	[18]
85		27.7		
90		2.89		
80	Grape juice	384.6	4.8	[2]
85		32.6		
90		2.77		

[a] The *z*-values of the past studies were obtained from calculation of the reported *D* and z-values

4. Conclusions

The current study demonstrates that 10°C increase in the temperature resulted in 5 more log reductions of *Talaromyces flavus* ascopores in pineapple juice after 15 min. Soluble solid (SS) content caused a protective effect towards temperature inactivation of spores, with lower reductions occurring at higher SS. Additionally, longer inactivation was needed for older spores (2.89 min for 30 day-old-spores as opposed to 4.80 min 60 day-old-spores at 90°C). The first-order kinetics described well the thermal inactivation of *T. flavus* ascopores. The heat resistance analysis of *T. flavus* ascopores might provide further information for pasteurization processes required for pineapple juice.

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