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by Evelyn Evelyn

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Modeling the inactivation of *Neosartorya fischeri* ascospores in apple juice by high pressure, power ultrasound and thermal processing

Evelyn [a, b], H.J. Kim [a], F.V.M. Silva [a, *]

[a] Department of Chemical and Materials Engineering, University of Auckland, Private Bag 92019, Auckland 1142, New Zealand
[b] Department of Chemical Engineering, University of Riau, Pekanbaru 28293, Indonesia

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ABSTRACT

Neosartorya fischeri is a mould that spoils acid foods and can produce mycotoxins. In this work, the efficacy of high pressure processing (HPP, 600 MPa) and power ultrasound (24 kHz, 0.33 W/mL) in combination with 75 °C for the inactivation of four week old *N. fischeri* ascospores in apple juice was investigated and compared with 75 °C thermal processing alone. The HPP-75 °C process was the most effective technique for inactivating *N. fischeri* spores, resulting in 3.3 log reductions after 10 min vs. no inactivation for thermosonication (TS) and thermal processing. Unexpectedly, activation shoulders were observed during the TS process. Then, the effect of different temperatures on the ascospore inactivation in apple juice by HPP-thermal, TS and thermal processing was investigated, and the log survivors vs. time were modeled. Faster inactivation was achieved at higher temperatures for all the technologies tested, indicating the significant role of temperature for the spore inactivation, alone or combined with other processes. The Weibull model described the spore inactivation better by 600 MPa HPP-thermal (50, 60, 75 °C) and thermal (85, 90 °C), whereas Lorentzian was more appropriate for the TS treatment (65, 70, 75 °C). In conclusion, HPP is the best food preservation technology due to higher spore inactivation in apple juice at the same temperature.

1. Introduction

Extremely heat resistant ascospores from moulds *Byssochlamys*, *Neosartorya*, and *Talaromyces* have been found (Hocking & Pitt, 1984; Silva & Gibbs, 2004, 2009; Silva, Gibbs, Nunez, Almonacid, & Simpson, 2014). These are often associated with the spoilage of pasteurized fruit products such as juices, purees, jellies, jams, and canned fruits (Beuchat, 1998; Pitt & Hocking, 1997; Silva et al., 2014). *Neosartorya fischeri* (anamorph *Aspergillus fischerianus*) is also a public health concern because of its capacity to produce mycotoxins terrein, fumitremorgins A and B, and verruculogen (Frisvad & Samson, 1991; Misawa, Nara, Nakayama, & Kinoshita, 1962; Nielsen, Beuchat, & Frisvad, 1989; Tournas, 1994). This species is widely distributed in soil (Pitt & Hocking, 1997) and was first isolated from canned strawberries in 1963 (Kavanagh, Larchet, & Stuart, 1963). *N. fischeri* can grow at temperatures between 10 and 52 °C (the optimal temperature is around 26–45 °C), in oxygen levels as low as 0.1% at 25 °C (Nielsen et al., 1989), and a broad range of pH (3–8) as most fungi (Pitt & Hocking, 1997). The extremely heat resistant ascospores formed by the teleomorphs or sexual reproductive stage survive 85 °C for 10 min (Houbraken, Dijksterhuis, & Samson, 2012) and drought (<0.5% relative humidity) (Wyatt, 2014). Pitt and Hocking (1997) reported that the degree of heat resistance of ascospores of *N. fischeri* is comparable with that of many bacterial spores, and is higher than that of *Byssochlamys fulva* ascospores, the most heat resistant mould ascospores known. The heat resistance of *N. fischeri* also increased with the ascospores age (Slongo, Miorelli, & Aragão, 2009; Tournas & Traxler, 1994), with 25 day old ascospores exhibiting changes in their ultrastructure and chemical composition when compared with 11 day old ascospores (Conner, Beuchat, & Chang, 1987). Based on the ascospore ornamentation, three varieties of *N. fischeri* (*var. fischeri*, *var. glabra*, and *var. spinosa*) have been identified (Samson, Nielsen, & Frisvad, 1990).

Temperatures between 85 and 95 °C are commonly used to prolong the shelf life of fruit juices (Sant'Ana, Rosenthal, & Massaguer, 2010). However, it has been recognized that the thermal process may activate the dormant ascospores of moulds which

* Corresponding author.
E-mail addresses: filipa.silva@auckland.ac.nz, filipavinagresilva@gmail.com (F.V.M. Silva).

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subsequently cause deterioration, hence resulting in economic loss (Katan, 1985; Slongo & Aragão, 2006; Splittstoesser, Nielsen, & Churey, 1993). Increasing the intensity (temperature or processing time) of the heat treatment is not desirable, due to quality reasons and consumer demands for 'fresh-like' fruits. Food preservation by non-thermal methods such as high pressure processing (HPP) and power ultrasound in combination with mild heat have been investigated due to reduced treatment temperatures and processing times (Evelyn & Silva, 2015a, 2015b, 2015c). HPP is an established commercial food processing technology and can be combined with temperature for the inactivation of resistant microbial spores (Evelyn & Silva, 2015c; Sarker, Akhtar, Torres, & Paredes-Sabja, 2015; Wilson, Dabrowski, Stringer, Moezelaar, & Brocklehurst, 2008) and enzymes (Sulaiman, Soo, Yoon, Farid, & Silva, 2015). With respect to the heat resistant mould ascospores such as *Byssochlamys fulva, Byssochlamys nivea, N. fischeri, Neosartorya spinosa, Talaromyces avellaneus, Talaromyces macrosporus*, the efficacy of 600–900 MPa of HPP pressure (cycle, oscillatory or continuous) in conjunction with heat (25–90 °C) using 3–15 week old spores was up to 5.7 log reductions (Butz, Funtenberger, Haberditzl, & Tauscher, 1996; Chapman et al., 2007; Ferreira, Rosenthal, Calado, Saraiva, & Mendo, 2009; Hocking, Begum, & Stewart, 2004; Maggi, Gola, Spotti, Rovere, & Mutti, 1994; Palou et al., 1998; Reyns, Veraverbeke, & Michiels, 2003; Voldřich, Dobiáš, Tichá, Čeřovský, & Krátká, 2004). Among the research studies, only Voldřich et al. (2004) modeled the inactivation kinetic for *Talaromyces* spores, reporting a first order kinetics and a decrease in the decimal reduction time (*D-value*) at 600 MPa as the temperature increased from 17 to 60 °C.

Power ultrasound (frequency ranging from 20 to 100 kHz) is a promising non-thermal technology for food preservation. This technology relies on the application of pressure waves called cavitation to the food/beverage, causing microbial cell death (Feng & Yang, 2011; Piyasena, Mohareb, & McKellar, 2004). Power ultrasound has been combined with mild heat (thermosonication, TS) to inactivate bacterial and fungal vegetative cells and spores, and a synergistic effect was observed (Earnshaw, Appleyard, & Hurst, 1995; Evelyn & Silva, 2015a; Garcia, Burgos, Sanz, & Ordonez, 1989; López-Malo, Palou, Jiménez-Fernández, Alzamora, & Guerrero, 2005; Ordoñez, Aguilera, Garcia, & Sanz, 1987; Zenker, Heinz, & Knorr, 2003). With respect to mould ascospores, Jimenez-Munguia, Arce-Garcia, Argaiz, Palou, and López-Malo (2001) reported that the inactivation of *Penicillium digitatum* and *Aspergillus flavus* ascospores by TS (20 kHz, 40–45 °C) in sabouraud broth increased with the treatment time and amplitude. The addition of boiling chips and air bubbles to the broth medium reduced the *D*-values. López-Malo et al. (2005) found lower *D*-values for TS (20 kHz, 40–60 °C) inactivation of *P. digitatum* and *A. flavus* ascospores in sabouraud broth compared to the thermal treatment alone. The authors also concluded an increase in the ultrasound amplitude and decrease in pH resulted in lower *D*-values. Coronel, Jiménez, López-Malo, and Palou (2011) proposed the Weibull model for the inactivation of *A. flavus* ascospores in broth by TS combined with vanillin. No studies have been carried out on the TS inactivation and kinetics modeling of heat resistant mould ascospores relevant to the fruit industry, such as *N. fischeri*. In particular, no work using fruit products has been reported, being broth inoculated with microorganisms the medium processed.

Due to the importance of *N. fischeri* spores in high acid fruit products, more research is needed to provide predictive models for the HPP-thermal and TS inactivation and design appropriate processes. Therefore, in this research the inactivation of *N. fischeri* ascospores in apple juice by HPP-thermal and TS processes were carried out, and the main objectives were as follows: (i) to compare the HPP-thermal, TS and thermal inactivation of ascospores at 75 °C; (ii) to model the 600 MPa HPP-thermal inactivation of ascospores; (ii) to model the TS inactivation of ascospores; and (iv) to model the thermal inactivation of ascospores.

2. Material and methods

2.1. Microbiology

2.1.1. Mould

N. fischeri var fischeri JCM 1740 was obtained from the Japan Collection of Microorganism (= ATCC 1020, DSM 3700, CBS 101.12, IAM 13864). This strain was isolated from canned apples in the USA.

2.1.2. Ascospore production

Ascospores of *N. fischeri* were obtained after growth for four weeks at 30 °C on malt extract agar (MEA). The spores were collected by flooding the surface of the culture plates with 5 mL sterile distilled water (SDW), and gently rubbing 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 4000 × g, 15 min, 4 °C and the procedure was repeated three times. The final spore suspension was then stored at 2 °C in SDW containing glass beads until use.

2.1.3. Apple juice inoculation and preparation

Apple juice (pH 3.7, 10.6 ± 0.1°Brix) was obtained from a local supermarket and used as the treatment medium to suspend *N. fischeri* ascospores. For HPP-thermal and thermal experiments, aliquots (ca. 0.5 mL) of *N. fischeri* spore solution were inoculated into 3.0 mL of apple juice to yield an initial juice spore concentration of approximately 10^6 cfu/mL of juice. The inoculated juice was packed in 8 × 8 cm food grade retort pouches (Cas-Pak, New Zealand) composed of polyester coated with silicon oxide, and laminated to nylon and cast polypropylene (PET-SIOX(12)//ON(15)//RCPP(70)). The pouches can withstand temperatures of up to 130 °C which are suitable for thermal processing and high pressure applications. Regarding the TS experiments, *N. fischeri* spore solution was inoculated aseptically by adding a small volume of inoculum to the apple juice contained in a round-bottom flask (5 mL of spore solution into 95 mL of apple juice) before the TS thermal pretreatment. The initial spore concentration after the thermal pretreatment and before TS was approximately 10^5 cfu/mL of juice.

2.1.4. Spore enumeration

The mould ascospore concentration in apple juice before and after processing (thermal, HPP and TS) was determined by spread plating onto MEA. A heat shock (75 °C, 5 min) of raw unprocessed apple juice was required to obtain the initial ascospore count (1.3 × 10^7 cfu/mL) in the untreated juice for HPP and thermal processes (Katan, 1985; Splittstoesser et al., 1993). N_0 for TS process was thermally pretreated apple juice at 80 °C for 30 min. Prior to plating, spore samples were decimal diluted using 9 mL 0.1% (w/v) sterile buffered peptone water (BPW; Difco, Becton Dickinson, USA). 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 3–5 days until visible colonies were formed. Plates with 20–100 colonies were used for enumeration. Ascospore concentration was expressed in cfu per milliliter (cfu/mL) of juice sample.

2.2. Processing

2.2.1. Experimental design

In the first experiment, the effectiveness of mould ascospores inactivation in apple juice at 75 °C by 600 MPa HPP-thermal and TS vs. sole thermal processing for treatment times up to 40 min were compared. The long processing time was chosen for HPP-thermal and TS so that changes in microbial numbers are observed, especially at the lower temperatures, and to be able to model the kinetics of spore inactivation. At least two independent experiments were carried out for each HPP-thermal, thermal or TS condition, and duplicate samples were processed for each treatment time. A *t*-test was used to compare the ascospore numbers (log N/N_0) by different methods at the same processing time and to check if the processing time for each processing method (TS, HPP-thermal and thermal) resulted in significantly different survivors (Statistica 8, Statsoft Inc., USA).

In the other experiments, HPP-thermal, TS and thermal treatments of apple juice were carried out at different temperatures and treatment times, as described in the following sections. The logarithmic number of survivors (log N/N_0) versus time was plotted for each survival experiment to model and to estimate the kinetic parameters. Two samples were processed for each time and three survival experiments were carried out for each treatment temperature. Detailed procedures for apple juice HPP-thermal, TS and thermal treatments are explained as follows.

2.2.2. High pressure combined thermal (HPP-thermal) processing

A QFP 2L-700 high pressure food processing system from Avure Technologies-USA distilled water as the working fluid was used for the HPP-thermal treatments. The maximum operation pressure and temperature were 690 MPa and 90 °C, respectively. High pressure at 600 MPa combined with temperatures of 50, 60 and 75 °C were used with processing times up to 40 min. 600 MPa high pressure treatment at room temperature (38 °C) was also carried out. No prior heat treatment of the inoculated apple juice was carried out before the HPP experiments, since 500–600 MPa pressures have been recognized as the most effective method for activation of *T. macrosporus* ascospores (Dijksterhuis & Teunissen, 2004; Reyns et al., 2003). Propylene glycol was the external heating medium for the water contained in the HPP chamber. Two internal thermocouples were used to monitor the temperature in the distilled water contained in the pressure chamber, and another internal thermocouple was used to register the glycol bath temperature during the process time. The pressure come up times were ≤1.5 min for 600 MPa and the depressurization took less than 30 s. Only the constant pressure phase was accounted for HPP processing time. The inoculated apple juice contained in the plastic pouches was submitted to different high pressure processing conditions. The HPP treated samples were submerged into an ice water bath prior to spore enumeration.

2.2.3. Thermosonication

Ultrasonic processor Hielscher UP200S (Hielscher-Ultrasonic Gmbh, Germany) with a sonotrode tip of 3 mm was used for all the thermosonication (TS) experiments. The processor has a high frequency (24 kHz) and was operated at 100% amplitude (210 μm, 460 W/cm^2, 0.33 W/mL apple juice). Prior to the TS experiments, the round bottom-flask containing 100 mL of apple juice inoculated with the mould was thermally processed at 80 °C for 30 min in a water bath, inside a laminar flow hood, to avoid aerial contamination. This heat shock process can break the dormant states of mould spores and increase the number of spores able to germinate (Sussman, 1976), leading to a loss of stability during the transition to the germinating stage (Eicher & Ludwig, 2002). This could possibly increase sensitivity of the moulds to the TS treatments. Preliminary experiments revealed that this procedure allowed a reduction of 15 min in the TS treatment time for the same spore inactivation in apple juice. Then, the TS treatments were carried out in the thermostatic water bath inside the laminar flow hood. The temperature of the juice sample during processing was monitored and the thermostatic water bath was used to keep it at the desired value during the process. At each temperature (65, 70, and 75 °C), the flask containing the pre-heated apple juice sample was placed in the water bath and the pre-sterilized sonotrode was readily submerged in the juice more or less 1 cm from the flask bottom. Ultrasonic treatments were carried out for up to 70 min depending on the TS treatment temperatures. Juice samples (0.5 mL) were taken from the flask at pre-specified intervals, cooled in an ice water bath for subsequent spore survivor counts, which were immediately carried out.

2.2.4. Thermal processing

Thermal resistance of *N. fischeri* ascopores was carried out at three temperatures (75, 85 and 90 °C). Initially, the thermostatic water bath was heated until the treatment temperature was reached. The inoculated apple juice samples contained in the plastic pouches 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.

2.3. Modeling the N. fischeri ascospore inactivation in apple juice

Linear and non-linear models were used to analyze *N. fischeri* ascospores inactivation by HPP-thermal, TS and thermal process (TableCurve 2D, version 5.01, SYSTAT Software Inc., Chicago, USA). Table 1 shows the mathematical models used in this study: first order kinetics (Equations 1a and 1b), Weibull (Equation 2), three parameters log-logistic (Equation 3), four parameters logistic (Equation 4) and Lorentzian (Equation 5). The logarithmic microbial survival ratio was evaluated with log N/N_0 and expressed as mean ± standard deviations (SD). N was the ascospore concentration (cfu/mL) in the juice after processing for a specific time t (min), and N_0 was the initial spore concentration of raw juice (HPP, thermal) and preheated juice (before TS).

2.3.1. First order kinetics

First order kinetic was used to model the thermal inactivation results in order to compare with literature results. In this model, decimal reduction time (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 1a (Bigelow, 1921). 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 (Eq. 1b). 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).

2.3.2. Weibull model

The Weibull equation (Eq. 2), written in the decimal logarithmic form, was used to model the log survivors by HPP and thermal (Peleg & Cole, 1998; Weibull, 1951), where b (the scale factor) is a rate parameter which is related to the velocity of inactivation of the microorganism. $n < i$ s the survival curve shape factor: $n < 1$ and $n > 1$ correspond to survival curves with concave-upwards (tailings) and concave-downwards (shoulders), respectively. When $n = 1$, t he Weibull model becomes the simple first-order kinetics.

Table 1
Mathematical models used in this study[a].

No.	Model name	Equation	Parameters	References
1a.	First order	$\log \frac{N}{N_0} = -\frac{t}{D_T}$	D_T	(Bigelow, 1921)
1b.	First order	$\log\left(\frac{D}{D_{ref}}\right) = \frac{T_{ref}-T}{z_T}$	z_T	(Bigelow, 1921)
2.	Weibull	$\log \frac{N}{N_0} = -bt^n$	b, n	(Peleg & Cole, 1998; Weibull, 1951)
3.	Log-logistic	$\log \frac{N}{N_0} = \frac{A}{1+e^{4\sigma(\tau-\log t)/A}} - \frac{A}{1+e^{4\sigma(\tau+\log t)/A}}$	A, σ, τ	(Chen & Hoover, 2003; Cole et al., 1993)
4.	Logistic	$\log \frac{N}{N_0} = a + 4b + \frac{e^{\left[-\left(\frac{t-c}{d}\right)\right]}}{\left[1+e^{\left[-\left(\frac{t-c}{d}\right)\right]}\right]^2}$	a, b, c, d	(Hubbert, 1956; Systat, 2002)
5.	Lorentzian	$\log \frac{N}{N_0} = a + \frac{b}{1+\left(\frac{t-c}{d}\right)^2}$	a, b, c, d	(Lorentz, 1875; Peng & Lu, 2006; Systat, 2002)

[a] N was the ascospore concentration (cfu/mL) in the juice after processing for a specific time t (min), and N_0 was the initial spore concentration in the raw apple juice.

2.3.3. Log-logistic

The three parameters of the log-logistic (A, σ, τ) model are shown in Eq. 3 (Chen & Hoover, 2003; Cole, Davies, Munro, Holyoak, & Kilsby, 1993) and used to compare the Weibull model for modeling the HPP inactivation results. $A = \omega - \alpha$ = lower asymptote − upper asymptote (log cfu/mL), σ is the maximum inactivation rate (log (cfu/mL)/log min), τ is the log time to achieve the maximum inactivation rate (log min) and commonly decreases as the lethal effect increases. A small value of t ($t \sim 10^{-6}$ min) was used to approximate t = 0 due to undefined log t = 0.

2.3.4. Logistic and Lorentzian

The four parameter logistic (Eq. 4) and Lorentzian (Eq. 5) curve peak functions (a, b, c, d) were used to model the TS log survivors (Hubbert, 1956; Lorentz, 1875; Peng & Lu, 2006; Systat., 2002), where a illustrates the log N/N_0 intercept; b acts as an amplitude of curve that is the height at the center of the distribution in log N/N_0 units; c is the center, which is the time (t) value at the center of the distribution, and d is the width, a measure of the width of the distribution in the same units as t.

2.3.5. Model evaluation

Random residuals, mean square error (MSE), coefficient of determination (R^2) and accuracy factor (A_f) were used to compare the goodness of fit of the models. A relatively small MSE, and R^2 and A_f values close to 1 indicate the adequacy of the model to describe the survival data. Additionally, the temperature dependence of the parameters estimated was checked.

3. Results and discussion

3.1. Ascospore activation and activation shoulders

Figs. 1–4 show the effects of different food preservation technologies on the log number of *N. fischeri* ascospores. With respect to thermal processing, a slight activation (increase in the number of spores with the processing rather than reduction) in the *N. fischeri* ascospores was registered at 75 °C (0.6 log-15 min, Fig. 1), but not at 85 and 90 °C. With fungal spores, activation is a mechanism caused by the application of heat, a chemical or other factor under certain conditions, which causes breaking of the spore dormancy for germination, leading to an increase in the viable counts by several logs (Dijksterhuis, 2007; Sussman, 1976; Tournas, 1994). Beuchat (1986) also observed *N. fischeri* FRR 1833, FRR 2334, and FRR 110483 ascospore activation up to 4.5 logs in buffer by 75 and 80 °C thermal processes during the first 15–45 min. Other investigators reported lower spore activation (0.25–0.8 log) between 10 and 50 min with *N. fischeri* (E7, C3, and isolated from spoiled papaya fruit) spores in buffer at the same temperatures which is similar to our results (Amaeze & Ugwuanyi, 2011; Rajashekhara, Suresh, & Ethiraj, 1996). Slongo and Aragão (2006) found that temperature of 85 °C between 10 and 20 min was required for maximum activation of *N. fischeri* ascospores in pineapple and papaya nectar, however the level of activated spores was not mentioned. Activation of spores has also been reported with thermal processed (105–120 °C, 5–30 min) bacterial spores such as *Bacillus stearothermophilus* spores (Corradini, Normand, Eisenberg, & Peleg, 2010; Finley & Fields, 1962).

Regarding thermosonication treatments, we have registered activation shoulders of at least +2.4 log after TS (65, 70 and 75 °C) during the first 10–30 min of processing (Figs. 1 and 3), demonstrating the mould spores are more sensitive to the ultrasound + heat than heat alone. Sonication is recognized as a tool to help separate fungal spore clusters and to produce suspensions of free ascospores (Amaeze, 2012; Beuchat, 1986; Michener & King, 1974). This can also lead to higher activation and viable counts in the TS compared to thermal alone. In sum, ultrasound played a significant role in the spore activation.

No activation was observed in the 600 MPa HPP-thermal survival lines (Figs. 1 and 2). This observation is in agreement with previous inactivation works with *B. nivea* ascospores employing

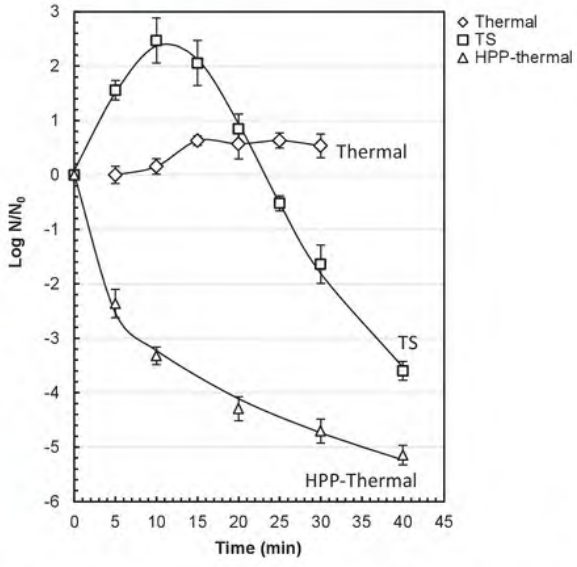

Fig. 1. Thermal, 600 MPa HPP-thermal and thermosonication (24 kHz, 0.33 W/ml) inactivation of four week old *Neosartorya fischeri* ascospores in apple juice at 75 °C.

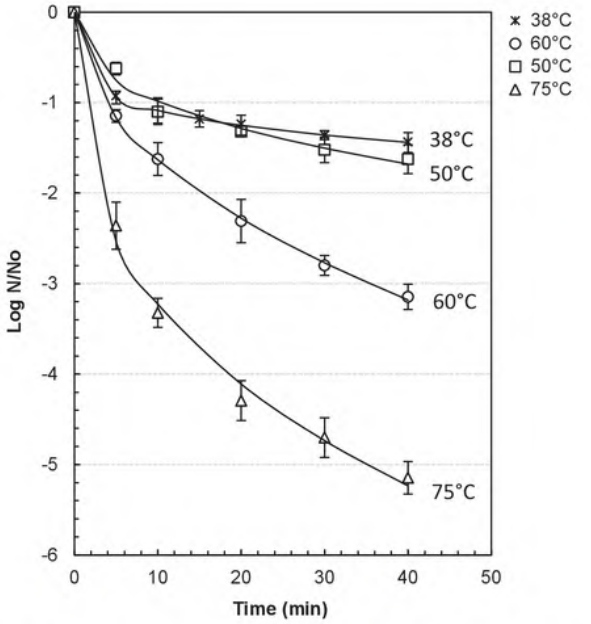

Fig. 2. Weibull curve fitting for 600 MPa HPP-thermal inactivation of four week old *Neosartorya fischeri* ascospores in apple juice.

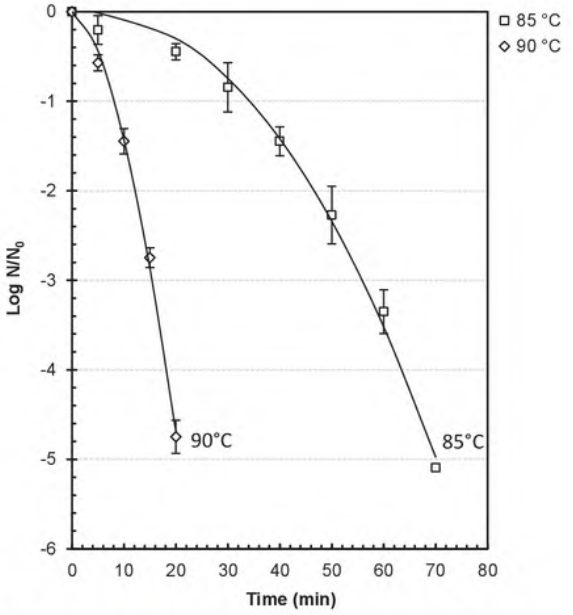

Fig. 4. Thermal inactivation kinetics and Weibull curve fitting (85 °C, 90 °C) of four week old *Neosartorya fischeri* ascospores in apple juice.

sustained pressure treatment of 600 MPa HPP and 40–70 °C thermal (Butz et al., 1996; Ferreira et al., 2009) although almost no spore reduction observed at 40 °C in Ferreira et al. (2009). Some authors reported that the combination of 600–900 MPa with temperatures ≤21 °C for treatment time ≤25 min activated up to 2.4 log of not only four weeks but also older spores of *Byssochlamys* spp., *Neosartorya* spp., and *T. macrosporus* (Chapman et al., 2007; Ferreira et al., 2009; Maggi et al., 1994; Palou et al., 1998; Reyns et al., 2003), which was attributed to the spores release from the ascus. However, this behavior was not observed in our experiments, probably due to the combination of HPP with heat (≥21 °C).

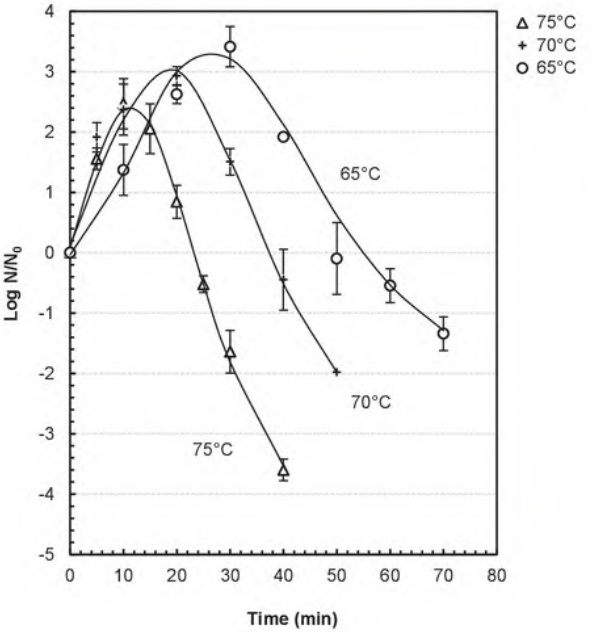

Fig. 3. Lorentzian curve fitting for thermosonication (24 kHz, 0.33 W/ml) inactivation of four week old *Neosartorya fischeri* ascospores in apple juice.

3.2. HPP-thermal, TS and thermal inactivation of N. fischeri ascospores in apple juice at 75 °C

The effects of 600 MPa high pressure combined with thermal (HPP-thermal), thermosonication (TS) with prior thermal treatment, and thermal processing at 75 °C on *N. fischeri* ascospores for up to 40 min are illustrated in Fig. 1. The HPP-thermal was the best method for the ascospore inactivation, since the spores reduced steadily with the processing time reaching nearly 4.3 log after 20 min, and declining more slowly after 20 min. Regarding the 75 °C TS, the spore numbers increased (activation), reaching a maximum (+2.4 log) at 10 min, which was followed by a steady linear inactivation. For thermal, a significant spore activation was observed after 15 min ($p < 0.05$), and then remained constant until 30 min. Overall, for a 30 min process at 75 °C, 4.7 log reductions in *N. fischeri* spores for HPP-thermal vs. 1.6 log reductions for TS vs. 0.5 log increase for thermal were obtained. It has been known that short time treatments at very high pressures (400–800 MPa) can induce maximal spore activation and quicker spore germination, followed by inactivation by mild heat or pressure (Black et al., 2007; Dijksterhuis & Teunissen, 2004). This could be the cause of the quicker inactivation by HPP-thermal treatment than TS or thermal processing. However, for 600 MPa–75 °C long treatment times (>40 min) are still needed to achieve 5 log inactivation, as recommended by USFDA (2001) for fruit juice pasteurization.

With respect to TS at 75 °C, the spore activation shoulder registered during the first 10 min, makes the TS not feasible for a commercial application, which requires shorter times for better

Table 2
Weibull model parameters estimation for the survival of four week old *Neosartorya fischeri* ascospores in apple juice after 600 MPa HPP-thermal processing[a].

Temperature (°C)	*b*	*n*	R^2	MSE	A_f
75	1.44 ± 0.12	0.35 ± 0.03	0.996	0.021	1.03
60	0.54 ± 0.02	0.48 ± 0.01	0.999	0.001	1.01
50	0.40 ± 0.07	0.39 ± 0.05	0.982	0.009	1.07
38	0.67 ± 0.02	0.20 ± 0.01	0.999	0.0003	1.01

[a] *b* and n are the Weibull scale and shape factors, respectively; low mean square errors (MSE), and R^2 and A_f close to 1 are indication of good fit, with all the temperatures tested showed random residuals.

industrial productivity. However, ≥25 min TS process showed higher inactivation results (0.5 log) than thermal (no inactivation). The results indicate higher susceptibility of the spores after the TS activation. Higher spore inactivation by 75 °C TS as opposed to activation by 75 °C thermal alone could be explained by the cell membrane damage caused by the cavitation bubbles generated by the ultrasonic waves, which was enhanced by the heat, therefore resulting in spore killing (Earnshaw, 1998; Evelyn & Silva, 2015a; Garcia et al., 1989; Nayak, 2014). López-Malo et al. (2005) also observed the effectiveness of TS with *Aspergillus flavus* ascospores in sabouraud broth. Higher spore inactivation by TS vs. thermal was also found at around 70 °C by Garcia et al. (1989) with *Bacillus subtilis* spores in milk and Evelyn and Silva (2015a) with psychrotrophic *Bacillus cereus* spores in skim milk and beef slurry. The benefit of ultrasound pretreatment to enhance the thermal inactivation of heat resistant bacterial spores such as *Clostridium perfringens* has also been reported (Evelyn & Silva, 2015b). Although overall the 75 °C was the best temperature for the TS inactivation, long spore activation (around 10 min) makes the TS process not feasible for a commercial application, as opposed to HPP technology.

3.3. Modeling the 600 MPa HPP-thermal inactivation of N. fischeri ascospores in apple juice

The log survivors of *N. fischeri* ascospores by 600 MPa HPP-thermal for up to 40 min is illustrated in Fig. 2. The number of *N. fischeri* spores reduced with the processing time for each treatment temperature (38, 50, 60, 75 °C), with the fastest reduction occurring at 75 °C. For example, 3.3 log after 10 min at 75 °C compared to 1.0–1.6 log at 38–60 °C. These results demonstrate the significant effect of high pressure combined with thermal on the *N. fischeri* ascospores. As the processing temperature increased from 38 to 75 °C, the spore inactivation after 40 min also increased from 1.4 log to 5.2 log, also indicating the significant role of temperature on the spore inactivation. These results confirm the benefit of the 600 MPa HPP-thermal as a successful approach for a commercial application aiming the inactivation of the heat resistant mould *N. fischeri* ascospores in apple juice.

Based on the non-linearity observed in the survival lines, Weibull (Equation 2, Table 1) and three parameter log logistic (Equation 3, Table 1) were attempted to model the spore survival data. Both worked well, the Weibull model performance and the parameters estimated (*b* and *n*) are presented in Table 2. The Weibull model showed MSE ≤0.021, $R^2 \geq 0.982$, and A_f values of 1.01–1.07. The Weibull *b* values (scale factors) increased from 0.40 at 50 °C to 1.44 at 75 °C, demonstrating this parameter is temperature dependent within this range of temperatures ($R^2 = 0.92$). The Weibull *n* values (shape factors) were between 0.20 and 0.48 (≤1), indicating an upward concavity. These results are in agreement with past reports with other microorganisms. When using HPP technology, non-linear inactivation of spores is observed and Weibull can often predict the inactivation results well (van Boekel, 2009; Buzrul, Alpas, & Bozoglu, 2005; Evelyn & Silva, 2015c; Serment-Moreno, Barbosa-Cánovas, Torres, & Welti-Chanes, 2014; Wang, Li, Zeng, Huang, Ruan, Zhu, et al., 2009). The inactivation of *T. avellaneus* mould ascospores by 500–600 MPa HPP at 17–60 °C, the only kinetic modeling reported with the mould ascospores, also seemed to follow a non-linear pattern. However, fitting was only carried out with the conventional first order kinetics (Voldřich et al., 2004).

3.4. Modeling the TS inactivation of N. fischeri ascospores in apple juice

The log survivors of thermal pretreated (80 °C, 30 min) apple juice containing the *N. fischeri* ascospores by TS are shown in Fig. 3. As mentioned, the maximum temperature supported by the ultrasound equipment (TS at 75 °C), was the best temperature. Activation shoulders were observed for all TS temperatures tested (75, 70, and 65 °C) with a maximum at 10 min for 75 °C, 20 min for 70 °C and 30 min for 65 °C, followed by approximately linear spore inactivation until 40 min. Longer and higher spore activation were obtained as the TS temperature was reduced from 75 to 65 °C. The better spore reduction by 75 °C TS compared to 65 °C TS and 70 °C TS shows the role of temperature in this process. Overall, while 30 min at 75 °C achieved ≃ 2 log reductions, 50 min at 70 °C and >80 min at 65 °C were required to obtain the same spore inactivation.

Due to the activation shoulders as well as tails observed in all the TS spore survival curves, the first order kinetics was not appropriate. Initially, four non-linear models (double Weibullian, Peleg, log-logistic and Lorentzian) were attempted to describe the ascospore inactivation in apple juice. However, the double Weibullian and Peleg's models suggested by Corradini et al. (2010) for heat activated *Bacillus* spores were inappropriate presenting high A_f and standard errors for the estimated parameters (results not shown). On the contrary, logistic (four parameters, Equation 4, Table 1) and Lorentzian (Equation 5, Table 1) worked well. The Lorentzian distribution was a better model, presenting 0.02–0.09 MSE, 0.971–0.997 R^2, and 1.04–1.26 A_f (Table 3). The Lorentzian *b* parameters increased from 6.3 to 8.5 as the temperature was increased from 65 to 75 °C, whereas the Lorentzian *a*, *c*, and *d* parameters decreased with the processing temperatures, exhibiting temperature dependence ($R^2 \geq 0.90$). There has been little to no research carried out on kinetic modeling of heat resistant mould ascospores such as *N.* spp. by TS, therefore there are no other results to compare. López-Malo et al. (2005) and Coronel et al. (2011) attempted TS without prior thermal pretreatment and

Table 3
Lorentzian model parameters estimation for the survival of four week old *Neosartorya fischeri* ascospores in apple juice after thermosonication (24 kHz, 0.33 W/ml)[a].

Temperature (°C)	*a*	*b*	*c*	*d*	R^2	MSE	A_f
75	−6.1 ± 0.40	8.5 ± 0.37	11.5 ± 0.22	18.6 ± 1.06	0.997	0.018	1.04
70	−4.9 ± 0.80	7.9 ± 0.75	18.3 ± 0.45	23.9 ± 2.70	0.997	0.029	1.05
65	−3.1 ± 0.63	6.3 ± 0.57	27.6 ± 0.67	26.4 ± 3.38	0.971	0.091	1.26

[a] *a*, *b*, *c* and *d* are the Lorentzian temperature dependent parameters, *a* illustrates the log N/N_0 intercept; *b* is the amplitude of the curve, which is the height at the center of the distribution in log N/N_0 units; *c* is the time value at the center of the distribution, and *d* is the width of the distribution in time units; low mean square errors (MSE), and R^2 and A_f close to 1 are indication of good fit; all the temperatures tested showed random residuals.

Table 4
Weibull model parameters estimation for the survival of four week old *Neosartorya fischeri* ascospores in apple juice after thermal processing[a].

Temperature (°C)	*b*	*n*	R^2	MSE	A_f
90	$3 \times 10^{-2} \pm 7 \times 10^{-3}$	1.71 ± 0.09	0.997	0.012	1.07
85	$4 \times 10^{-4} \pm 2 \times 10^{-4}$	2.24 ± 0.12	0.995	0.019	1.52

[a] *b* and n are the Weibull scale and shape factors, respectively; low mean square errors (MSE), and R^2 and A_f close to 1 are indication of good fit.

demonstrated the first order kinetics for *P. digitatum* and *A. flavus* ascospore inactivation as opposed to the non-linear Lorentzian model used in our study as a result of the activation shoulders and tails registered in the *N. fischeri* spore survival curves (Fig. 3). This suggests those mould spores are less resistant and dormant than the *N. fischeri* spores.

3.5. Modeling the thermal inactivation of *N. fischeri* ascospores in apple juice

The log survivors of *N. fischeri* ascospores after thermal processing at 85 and 90 °C were plotted in Fig. 4. The 90 °C thermal process was able to inactivate 4.8 log of *N. fischeri* ascospores in apple juice after 20 min. This result indicates the commercial pasteurization conditions of 77–88 °C for 25–30 s, suggested for apple juice preservation (Moyer & Aitken, 1980), are far from the minimum pasteurization required for pasteurizing apple juice prone to contamination by *N. fischeri*. Thus, this mould requires 90 °C and ≥20 min or equivalent for apple juice pasteurization. This process negatively affects the fruit product sensory quality, and may result in the loss of raw fruit nutrients such as antioxidants (Silva, Sims, Balaban, Silva, & O'Keefe, 2000).

The non-linear Weibull model was a good model to describe the survivors (0.998–0.999 R^2, 0.001–0.005 MSE, 1.05–1.36 A_f), and the parameters were estimated (Table 4). The Weibull *n* values (shape factor) were more than 1, indicating downward concavity. The Weibull model has been used by Sant'Ana et al. (2010) for the thermal inactivation of *Byssochlamys fulva* ascospores in apple juice. The majority of previous thermal studies found non-linearities for *N. fischeri* log survivors (Beuchat, 1986; Kotzekidou, 1997; Rajashekhara et al., 1996; Salomão, Slongo, & Aragão, 2007), but used first order or linearized first order model proposed by Alderton & Snell (1970) to estimate the kinetic parameters. Although the error is higher and fitting not so good (0.924 R^2, 0.365 MSE, 1.27 A_f), the first order $D_{90°C}$ of 4.9 min was also determined to be able to compare with past results. This value was lower than the values (between 9.9 and 23.4 min) reported earlier for *N. fischeri* ascospores in tomato and pineapple juice (Amaeze, 2012; Kotzekidou, 1997), confirming the influence of the suspending medium and the strain on the heat resistance of *N. fischeri* ascospores.

4. Conclusion

600 MPa HPP-75 °C was a better method than 75 °C thermosonication (TS) and sole 75 °C thermal for the inactivation *N. fischeri* ascospores in apple juice, confirming the benefit of HPP technology. Approximately 3.3 log reduction was obtained for HPP-75 °C after 10 min in contrast to no inactivation for 75 °C TS and 75 °C thermal processes. Long treatment time is still needed to achieve a 5 log inactivation under the 600 MPa HPP-75 °C. Thus, future studies should focus on the optimization of processing parameters to inactivate the *N. fischeri* spores or combination with other method (hurdles) such as addition of preservatives to ensure the juice stability. A TS process at 75 °C for 10 min induced *N. fischeri* activation followed by approximately 2 log reduction per 10 min processing time. The TS process might be applicable at higher temperatures. However, further research must be undertaken to design an ultrasound probe that can withstand higher temperatures. With respect to the exclusively thermal process, temperatures ≥90 °C are still required to achieve the inactivation of spores. Weibull modeled the inactivation of *N. fischeri* ascospore by the 600 MPa HPP-thermal processes, while Lorentzian was more suitable for the TS modeling. The results from this study show that 600 MPa HPP-thermal is a better option for the preservation of fruit juices prone to *N. fischeri* ascospores contamination.

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