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by Evelyn Evelyn

Submission date: 19-Feb-2019 06:58PM (UTC+0700)
Submission ID: 1080203700
File name: Evelyn_2015d.pdf (594.53K)
Word count: 8774
Character count: 44511

International Journal of Food Microbiology 214 (2015) 129–136

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Inactivation of *Byssochlamys nivea* ascospores in strawberry puree by high pressure, power ultrasound and thermal processing

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ARTICLE INFO

Article history:
Received 26 April 2015
Received in revised form 8 July 2015
Accepted 30 July 2015
Available online 1 August 2015

Keywords:
High hydrostatic pressure
Thermosonication
Heat
Mold
Fungi
Modeling

ABSTRACT

Byssochlamys nivea is a mold that can spoil processed fruit products and produce mycotoxins. In this work, high pressure processing (HPP, 600 MPa) and power ultrasound (24 kHz, 0.33 W/mL; TS) in combination with 75 °C for the inactivation of four week old *B. nivea* ascospores in strawberry puree for up to 30 min was investigated and compared with 75 °C thermal processing alone. TS and thermal processing can activate the mold ascospores, but HPP–75 °C resulted in 2.0 log reductions after a 20 min process. For a 10 min process, HPP–75 °C was better than 85 °C alone in reducing *B. nivea* spores (1.4 vs. 0.2 log reduction), demonstrating that a lower temperature in combination with HPP is more effective for spore inactivation than heat alone at a higher temperature. The ascospore inactivation by HPP–thermal, TS and thermal processing was studied at different temperatures and modeled. Faster inactivation was achieved at higher temperatures for all the technologies tested, indicating the significant role of temperature in spore inactivation, alone or combined with other physical processes. The Weibull model described the spore inactivation by 600 MPa HPP–thermal (38, 50, 60, 75 °C) and thermal (85, 90 °C) processing, whereas the Lorentzian model was more appropriate for TS treatment (65, 70, 75 °C). The models obtained provide a useful tool to design and predict pasteurization processes targeting *B. nivea* ascospores.

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1. Introduction

Byssochlamys species are abundant in soil and recognized as important spoilage molds in fruit and fruit products (Beuchat, 1998; Pitt and Hocking, 1997; Silva et al., 2014). *Byssochlamys* can produce 8 spores, called ascospores, inside an ascus. The ascospores of *Byssochlamys fulva*, *Byssochlamys nivea* and *Byssochlamys spectabilis* are very heat resistant, and may require an inactivation temperature above 90 °C (Houbraken et al., 2008; Silva and Gibbs, 2004; Silva and Gibbs, 2009; Silva et al., 2014; Tournas, 1994). Bayne and Michener (1979) reported that seven out of 25 strains of *Byssochlamys* were able to survive heating at 90 °C for 25 min or longer, when initial numbers were frequently near 10^6/mL. The ascospores also show great resistance to acids and chemicals such as chlorine and alcohol (Tournas, 1994). A combination of heat with nonthermal processes for spore inactivation and food pasteurization can better retain the food's sensory properties and quality (Alexandre et al., 2011; Krebbers et al., 2003; Vervoort et al., 2012).

B. nivea (anamorph *Paecilomyces niveus*) is able to grow at temperatures between 11 and 43 °C (the optimal temperature is around 30 °C), water activity between 0.892 and 0.992 (Panagou et al., 2010), over a wide range of pH (3–8) (Pitt and Hocking, 1997), and under reduced oxygen conditions inside food packs and in carbonated beverages (Taniwaki et al., 2009). Month old ascospores of *B. nivea* survived in thermally treated pineapple nectar at 103 °C for 7 min (Ferreira et al., 2011). The most resistant strain of month old ascospores of *B. nivea* (strain 162) also survived 90 °C for 20 min in tomato juice (Kotzekidou, 1997). Temperature time combinations of 87.5 °C–10 min and 90 °C–2 min did not kill *B. nivea* in processed canned fruits (Luthi and Hochstrasser, 1952; Put and Kruiswijk, 1964). Other investigators also reported the isolation of *B. nivea* from pasteurized fruit concentrates (Palou et al., 1998; Salomão et al., 2014). Milk and milk products (Engel and Teuber, 1991), cucumber brine (Yates and Ferguson, 1963), cream cheese (Pitt and Hocking, 1997) and palm wine (Eziashi et al., 2010) have also been contaminated by this species. In addition to the high thermal resistance, *B. nivea* is also a concern to human and animal health since it can produce the mycotoxins patulin (Roland and Beuchat, 1984; Sant'Ana et al., 2010; Taniwaki et al., 2009), byssochlamic acid (Escoula, 1974), and byssotoxin A (Beuchat and Rice, 1979). These toxins may act upon the central neural system causing sustained tremors and convulsions (Tournas, 1994).

Preservation of foods by nonthermal technologies such as high pressure processing (HPP) and power ultrasound are attractive alternatives because they have little or minimal effects on the nutrients and taste of

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http://dx.doi.org/10.1016/j.ijfoodmicro.2015.07.031
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food (Cullen et al., 2012; Farkas and Hoover, 2000). HPP technology, generally with pressures between 400 and 600 MPa at ambient or chilled temperatures, and processing times under 10 min, have been used commercially for the preservation of acidic fruit juices and beverages (Cheftel, 1995). In general bacterial spores, mold ascospores and enzymes in food are difficult to inactivate by HPP alone (Evelyn and Silva, 2015a; Larson et al., 1918; Patterson, 2005; Sulaiman and Silva, 2013; Sulaiman et al., 2015a; Timson and Short, 1965). However, most of the heat resistant bacterial spores, including pathogenic species, do not germinate and grow in the acidic environment (pH < 4.6) of the fruit juices (Silva and Gibbs, 2004). The combination of HPP with a mild heat treatment (HPP–thermal) is usually required to inactivate bacterial spores (Evelyn and Silva, 2015a; Sarker et al., 2015; Wilson et al., 2008). Unlike bacterial spores, only a few reports about the inactivation of ascospores of *B. nivea* mold by HPP–thermal are available in the literature and none modeled the kinetics. High pressures of up to 900 MPa (oscillatory or continuous) in conjunction with temperatures of 20–90 °C have been used to inactivate *B. nivea* ascospores in buffer, juice or concentrate, and the degree of inactivation was dependent on the strain, ascospore age, and °Brix or water activity of the suspending medium (Butz et al., 1996; Chapman et al., 2007; Ferreira et al., 2009; Maggi et al., 1994; Palou et al., 1998). HPP prior to or followed by thermal treatment has also been attempted, with prior thermal treatment being less effective than thermal treatment after the HPP processes (Butz et al., 1996; Maggi et al., 1994).

With respect to power ultrasound, cavitation (20–100 kHz) combined with heating causes bacterial spore (Evelyn and Silva, 2015b; Feng and Yang, 2011) and enzyme inactivation (Sulaiman et al., 2015b). Similar to HPP technology, power ultrasound alone is also ineffective for inactivation of these spores (Butz and Tauscher, 2002; Evelyn and Silva, 2015b). Thus a combination with thermal treatment, often referred to as thermosonication (TS), is needed. The inactivation of the conidia of *Penicillium digitatum* and *Aspergillus flavus* by TS (20 kHz, 40–60 °C) has been reported (Coronel et al., 2011; Jimenez-Munguia et al., 2001; López-Malo et al., 2005), being higher when using heat. The TS inactivation and kinetic modeling of *B. nivea* ascospores have not been reported.

In this research, the HPP–thermal, TS and thermal methods of inactivation of *B. nivea* ascospores in strawberry puree were compared for the first time and the inactivation was modeled. The main objectives were: (i) to compare the HPP–thermal, TS and thermal methods of inactivation of ascospores at 75 °C for up to 20 min processing; (ii) to study the 600 MPa HPP–thermal inactivation of ascospores at different temperatures; (iii) to study the TS inactivation of ascospores at different temperatures; and (iv) to study the thermal inactivation of ascospores at different temperatures.

2. Material and methods

2.1. Microbiology

2.1.1. Mold

B. nivea JCM 12806 (=CBS 696.95) was obtained from the Japan Collection of Microorganisms. This strain was isolated from pasteurized strawberry in the Netherlands.

2.1.2. Ascospore production

Ascospores of *B. nivea* were obtained after a growth period of four weeks at 30 °C on potato dextrose agar (PDA). The spores were collected by flooding the surface of the culture plates with 5 mL sterile distilled water (SDW) and gently rubbing biomass 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 SDW at 4000 ×*g* for 15 min at 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. Microscopic observation revealed that a mixture of asci and free ascospores were present. The survivor experiments were carried out without any attempt to free ascospores from their asci. Under natural conditions, the ascospores in fruits would be expected to be present as a mixture of asci and free ascospores so carrying out the inactivation experiments in this way should reflect the reality of fruit juice processing.

2.1.3. Strawberry puree inoculation and packaging

New Zealand strawberries obtained locally were pureed (pH 3.4, 8.1 ± 0.1°Brix) in a sterile laboratory scale blender and used as the medium to suspend and process the *B. nivea* ascospores. For HPP–thermal and thermal experiments, aliquots (ca. 0.1 mL) of *B. nivea* spore solution were inoculated into 3.4 mL of strawberry puree to yield an initial spore concentration of ≈ 10^5–10^6 cfu/mL of puree. The inoculated puree was packed into 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, so were suitable for thermal processing and high pressure applications. Regarding TS experiments, the *B. nivea* spore solution was inoculated aseptically by adding a small volume of inoculum to the strawberry puree contained in a 200 mL round-bottom flask (3 mL of spore solution into 97 mL of strawberry puree) before the pretreatment and TS treatment. The initial spore concentration was approximately 10^5–10^6 cfu/mL of puree.

2.1.4. Spore enumeration

The mold ascospore concentration in strawberry puree before and after processing (thermal, HPP and TS) was determined by spread plating onto PDA. A heat shock (75 °C, 5 min) of raw unprocessed strawberry was required to obtain the initial count (N_0) in the untreated strawberry for HPP and thermal processes (Katan, 1985; Splittstoesser et al., 1993). This procedure allows the growth of colonies on the plate and ascospore enumeration in the untreated raw puree (N_0). With respect to TS, N_0 was determined in already thermally pretreated strawberry (see Section 2.2.3), so there was no need for additional heat shock for enumeration in this case. Prior to plating, the whole content of strawberry contained in the pouches (3.5 mL, HPP and thermal treatments) and in the flask (100 mL, ultrasound processing) was placed in 110 × 230 mm sterilized stomacher bags (Interscience, France) and diluted 2-fold with 0.1% (w/v) buffered peptone water. Samples from HPP and thermal treatments were then homogenized in a stomacher bag for 1 min, whereas larger volume samples from ultrasound processing were manually mixed. Homogenized half diluted strawberry samples (1 mL) were then decimal diluted using 9 mL of 0.1% (w/v) sterile buffered peptone water (Difco, Becton Dickinson, USA). Each tube dilution was mixed repeatedly using a high speed vortex mixer to yield a uniform spore suspension, and 0.1 mL from each tube dilution was plated on two PDA plates. 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. Ascospore concentration was expressed in cfu per milliliter (cfu/mL) of strawberry puree.

2.2. Processing

A heat pretreatment of 80 °C for 15 min was applied to the inoculated strawberry puree before the TS survival experiments, since preliminary experiments revealed that this procedure allowed a reduction of 15 min in the TS treatment time for the same spore inactivation in strawberry puree. Butz et al. (1996) have demonstrated no effect of prior heat treatment (80 °C, 30 min) on the 700 MPa-HPP inactivation of *B. nivea* spores. Thus we did not heat pretreated the inoculated puree before HPP–thermal and exclusively thermal experiments.

2.2.1. Experimental design

In the first experiment, the *B. nivea* ascospore inactivation in strawberry puree by 600 MPa HPP–thermal, TS and sole thermal processing at 75 °C for treatment times of up to 30 min was investigated. At least two independent survival experiments were carried out for each technology and duplicate samples were processed for each treatment time. Tukey's test was used to compare the log ascospore numbers ($\log N/N_0$) by different methods at the same processing time to check if different methods (TS, HPP–thermal and thermal) resulted in significantly different survivors (Statistica 8, Statsoft Inc., USA). N was the microbial count after processing.

In the other survival experiments, HPP–thermal, TS and thermal treatments of strawberry puree were carried out at different temperatures, as described in detail in the following sections. The logarithmic number of survivors ($\log N/N_0$) versus time was plotted for each temperature to model and estimate the inactivation parameters. Two samples were processed for each time and three survival experiments were carried out for each treatment temperature.

2.2.2. HPP–thermal processing

A QFP 2 L-700 high pressure food processing system from Avure Technologies (USA) with 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 50, 60 and 75 °C was used with processing times of up to 40 min. 600 MPa high pressure treatment without heating (38 °C) was also carried out. This pressure treatment (600 MPa) was selected for the inactivation experiments because the literature shows that high pressure ≥600 MPa was more effective for the inactivation of heat resistant ascospores (Butz et al., 1996; Chapman et al., 2007; Palou et al., 1998), and 600 MPa was the maximum working pressure of the Avure HPP unit. Propylene glycol was the medium used to heat the water contained in the HPP chamber without direct contact. Two internal thermocouples were used to monitor the temperature in the distilled water contained in the pressure chamber and another 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 processes and the depressurization took less than 30 s. During the pressurization phase adiabatic heating occurs and an increase of 3 °C per 100 MPa in the temperature of the food sample is expected (Balasubramanian and Balasubramaniam, 2003). Only the constant pressure phase was counted for the HPP processing time. The average temperature during the constant pressure phase was considered as the HPP temperature. The inoculated strawberry puree contained in the plastic pouches was submitted to different high pressure processing thermal conditions. The HPP treated samples were submerged in an ice water bath for subsequent survivor enumeration.

2.2.3. Thermosonication

A Hielscher UP200S ultrasonic processor (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, 33 W, 0.33 W/mL of strawberry puree) and continuous energy supply. A 200 mL round bottom-flask with a narrow neck containing 100 mL of puree was used for TS to minimize the water evaporation from the puree sample. Prior to TS experiments, the flask containing the inoculated puree was thermally processed at 80 °C for 15 min in a water bath inside a biosafety cabinet (class II, type A2, AC2-4E1, Esco Micro Pte. Ltd., Singapore) to avoid aerial contamination. Preliminary experiments revealed that this procedure allowed a reduction of 15 min in the TS treatment time (the best method) for the same spore inactivation in strawberry puree. Then, the TS treatments were carried out in a thermostatic water bath inside the biosafety cabinet under aseptic conditions. The temperature of the puree during processing was monitored and the thermostatically controlled water bath was used to keep it at the desired value. At each temperature (65, 70, and 75 °C), the round bottom flask containing the preheated strawberry puree sample with mold spores was placed in the water bath and the disinfected sonotrode was submerged into the puree more or less 1 cm from the flask bottom. Ultrasonic treatments were carried out for up to 60 min depending on the TS treatment temperatures. For each pre-specified processing time, the whole sample of strawberry puree was taken from the water bath, cooled in ice water, and survivors were enumerated.

2.2.4. Thermal processing

The experiments for determining the thermal resistance of *B. nivea* ascospores were carried out at three temperatures (75, 85 and 90 °C). Initially, a thermostatically controlled water bath was heated until the treatment temperature was reached. The inoculated strawberry puree samples contained in the plastic pouches were then submerged into the preheated water bath and heated for various times. The large surface area of the bags (8 × 8 cm) compared to the small volume of the puree (3.5 mL) packed resulted in a small depth of puree and allowed a very quick heat transfer to the puree center. Treated samples were taken out at different time intervals and kept in an ice water bath until microbial enumeration.

2.3. Modeling the B. nivea ascospore inactivation in strawberry puree

A non-linear Weibull model was used to describe *B. nivea* ascospore inactivation by HPP–thermal and thermal processes, while the Lorentzian model was used for the TS (TableCurve 2D, version 5.01, SYSTAT Software Inc., Chicago, USA). The Weibull model (Eq. (1)) written in the decimal logarithmic form (Peleg and Cole, 1998; Weibull, 1951) is shown as follows:

$$\log\frac{N}{N_0} = -bt^n \tag{1}$$

where b (the scale factor) is a rate parameter which is related to the velocity of microbial inactivation. n is the survival curve shape factor: $n < 1$ and $n > 1$ correspond to survival curves with concave-upwards (tailings) and concave-downwards (shoulders) respectively. When $n = 1$, the Weibull model becomes a simple first-order kinetics.

The four parameter Lorentzian (Eq. (2)) curve peak function (a, b, c, d) was used to model the TS log survivors (Lorentz, 1875; Systat, 2002):

$$\log\frac{N}{N_0} = a + \frac{b}{1 + \left(\frac{t-c}{d}\right)^2} \tag{2}$$

where a illustrates the $\log N/N_0$ intercept; b acts as the amplitude of the curve that is the height at the center of the distribution in $\log N/N_0$ units; c is the center that 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.

Random residuals, mean square error (MSE) and coefficient of determination (R^2) were used to compare the performance of the different models. A relatively small MSE and R^2 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. Activation shoulders and increase in ascospore numbers

Figs. 1, 2, 3 and 4 show the effects of different food preservation technologies on the log number of *B. nivea* ascospores. With fungal

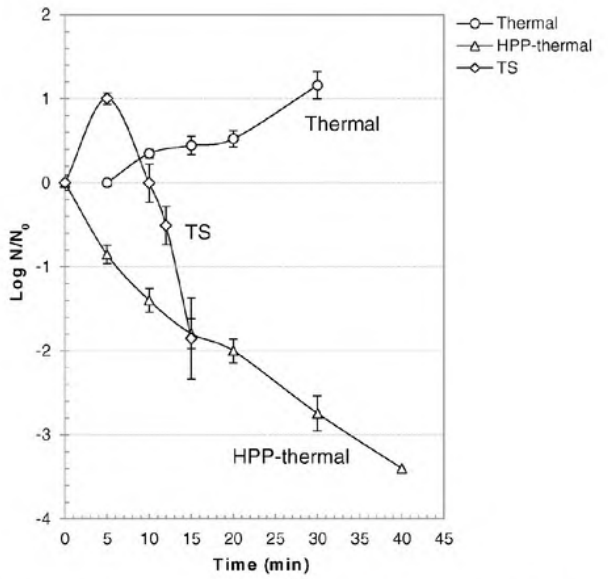

Fig. 1. Thermal, 600 MPa HPP–thermal and thermosonication (24 kHz, 0.33 W/mL) inactivation of four week old *Byssochlamys nivea* ascospores in strawberry puree at 75 °C.

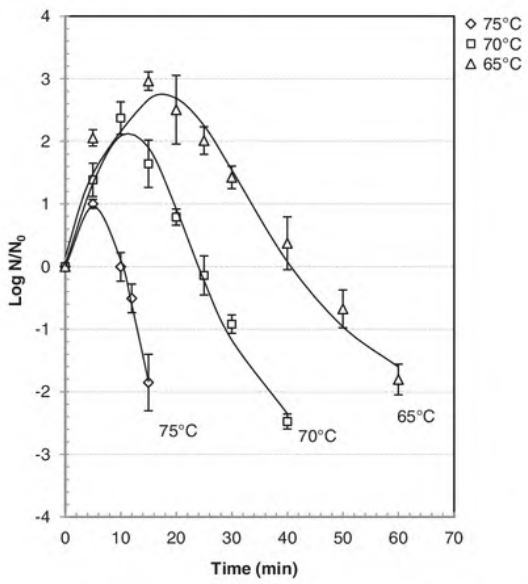

Fig. 3. Lorentzian curve fitting for the thermosonication (24 kHz, 0.33 W/mL) inactivation of four week old *Byssochlamys nivea* ascospores in strawberry puree submitted to a heat shock pretreatment.

ascospores, the increase in their numbers is a mechanism caused by the application of heat, a chemical or another factor under certain conditions, which causes the breaking of the spore dormancy for germination, leading to an increase in the viable counts by several logs (Dijksterhuis, 2007; Sussman, 1976; Tournas, 1994).

Activation shoulders were observed during thermosonication treatments (Figs. 1 and 3). The shoulders of 1 log after 5 min TS at 75 °C and 3 log after 15 min TS at 65 °C demonstrated that the mold ascospores are more sensitive to the ultrasound plus heat than heat alone. Sonication is recognized as a tool to release the fungal spores from the asci and to produce suspensions of free ascospores (Amaeze, 2012; Beuchat, 1986; Michener and King, 1974). The combination of ultrasound and heat (TS) can further facilitate the increase in the free spore numbers.

With respect to thermal processing, a steady increase of *B. nivea* ascospores was registered with processing time at 75 °C, reaching 1.2 log after 30 min (Fig. 1). On the contrary, only inactivation was observed at 85 and 90 °C thermal processing for up to 60 min. Ferreira et al. (2011) reported a 0.5 log increase of *B. nivea* ascospores in fruit nectars after treatment at 85 °C for 10 min. Increases in ascospore numbers have also been observed with other thermally processed heat resistant fungal ascospores such as *Neosartorya* and *Talaromyces* spp. (70–85 °C, 7–30 min) (Beuchat, 1986; Dijksterhuis and Teunissen, 2004; Katan, 1985), and thermally processed bacterial spores such as *Bacillus stearothermophilus* (105–120 °C, 5–30 min) (Corradini et al., 2010).

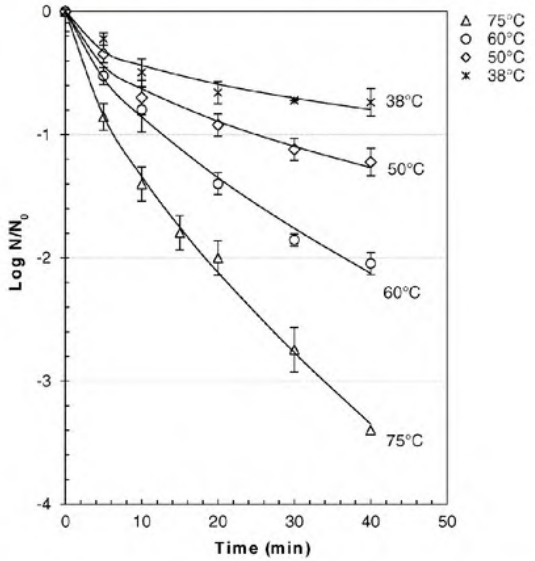

Fig. 2. Weibull curve fitting for 600 MPa HPP–thermal inactivation of four week old *Byssochlamys nivea* ascospores in strawberry puree.

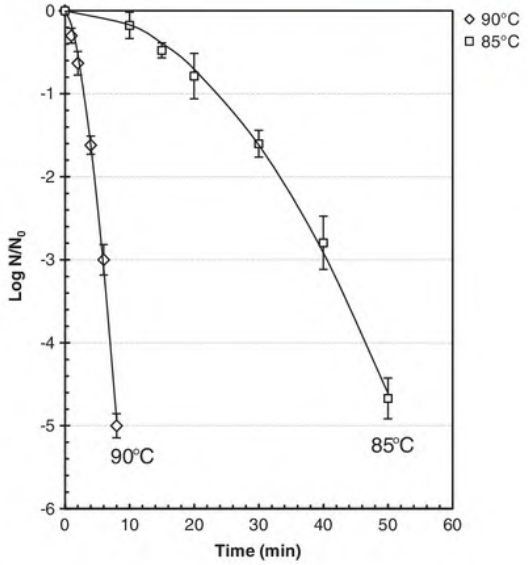

Fig. 4. Thermal inactivation kinetics and Weibull curve fitting of four week old *Byssochlamys nivea* ascospores in strawberry puree.

No increase in ascospores was observed after non-thermal 600 MPa HPP at 38 °C and thermal 600 MPa HPP processes at temperatures ranging between 50 and 75 °C (Fig. 2). This observation is in agreement with previous work with *B. nivea* ascospores employing 600 MPa HPP combined with a 40–70 °C temperature (Butz et al., 1996; Ferreira et al., 2009). Other authors reported that 600–900 MPa without heat (≤21 °C) for ≤25 min treatment times increased ascospore numbers up to 2.4 log in four week old *B. nivea*, *Neosartorya*, and *Talaromyces macrosporus* ascospores (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 release of spores from asci.

3.2. HPP–thermal, TS and thermal inactivation of B. nivea ascospores in strawberry puree at 75 °C

The effects of a 600 MPa high pressure process combined with thermal (HPP–thermal), thermosonication (TS), and thermal processing at 75 °C on *B. nivea* ascospores for up to 30 min are illustrated in Fig. 1. For a 75 °C and 10 min process, 1.4 log reductions in *B. nivea* ascospores was obtained for HPP–thermal vs. no reductions for TS and thermal. As mentioned in the previous section the spore numbers increased with TS, reaching a maximum (+1.0 log) at 5 min, which was followed by a steady linear inactivation. For thermal processing, an increase in spore numbers was also observed, with a 0.5 log spore increase after 20 min processing ($p < 0.05$) and continued to increase to 1.2 log after 30 min processing ($p < 0.05$). As opposed to TS and thermal treatments, the HPP–thermal process reduced the spores steadily, reaching nearly 2.0 log reduction after 15 min and 3.4 log at 40 min. It is known from the literature that HPP can activate the dormant spores to germinate, rendering them more susceptible to inactivation treatments. For example, short time treatments at pressures between 400 and 800 MPa induced *T. macrosporus* ascospore activation and quicker germination (transformation from a dormant state with low metabolic activity to one of high activity in which spores lose much of their extreme resistance), followed by inactivation of the germinated form by pressure or mild heat (Black et al., 2007; Dijksterhuis and Teunissen, 2004; Reyns et al., 2003). The results obtained in our research showed that long treatment times (>40 min) are needed to achieve 5 log reduction recommended by the US Food and Administration (USFDA, 2001) for fruit juice pasteurization with a 600 MPa HPP–75 °C process, which is not feasible for commercial application. This process could be sufficient if there are lower initial loads of microbial contamination in the strawberry puree. Better results could be achieved at higher HPP temperatures, but currently commercial HPP machines cannot reach very high temperatures.

With respect to TS at 75 °C, the ascospore activation shoulder registered during the first 10 min makes the TS unfeasible for commercial applications, which require shorter times for better industrial productivity. However, ≥15 min TS processes showed a comparable or higher inactivation than the HPP–thermal (1.8 log, 15 min). The results indicate higher susceptibility of the spores after the TS treatment, demonstrated by a higher inactivation rate with a sharp decrease in TS log survivors after the maximum activation shoulder peak was reached. The spore inactivation by 75 °C TS as opposed to spore viable count increase by 75 °C thermal treatment 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 destruction (Earnshaw, 1998; Evelyn and Silva, 2015b; Garcia et al., 1989; Nayak, 2014). López-Malo et al. (2005) also found the benefit of TS such as lower *D*-values for *Aspergillus flavus* and *P. digitatum* conidia inactivation in Saboraud broth between 45 and 52.5 °C. 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 (2015b) 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 and Silva, 2015c).

3.3. Modeling the 600 MPa HPP–thermal, thermosonication and thermal inactivation kinetics of B. nivea spores in strawberry puree

3.3.1. Modeling the 600 MPa HPP–thermal inactivation

The log survivors of *B. nivea* ascospores after 600 MPa HPP–thermal processing for up to 40 min are illustrated in Fig. 2. The number of *B. nivea* spores reduced with processing time for all the temperatures (38, 50, 60, 75 °C), with the fastest reduction occurring at 75 °C. For example, after 10 min, reductions of 1.4 log, 0.8 log, 0.7 log, and 0.5 log at 75, 60, 50 and 38 °C respectively were achieved. These results demonstrate the significant effect of high pressure temperature on the *B. nivea* ascospore inactivation. At 40 min the effect of temperature is more evident: while 0.7 log was registered at 38 °C, a value of 3.4 log was observed for HPP at 75 °C. These results confirm the benefit of the 600 MPa HPP–thermal method as a successful approach aiming at the inactivation of the heat resistant mold *B. nivea* ascospores in strawberry puree. HPP combined with ≥75 °C heat for a short processing time will be needed to achieve 5-log inactivation, suggesting a better quality of strawberry puree than exclusively thermally treated puree.

Based on the non-linearity observed in the log survivors, Weibull (Eq. (1)) and three parameter log logistic (Chen & Hoover, 2003) were initially attempted to model the log spore survival data. The log logistic model showed large standard errors in the estimated parameters at some temperatures (results not shown), thus the Weibull model was selected and the model performance and parameters estimated (b and n) are presented in Table 1. The Weibull model showed an MSE ≤ 0.013 and $0.940 \le R^2 \le 0.997$. The Weibull b values (scale factors) increased from 0.15 at 38 °C to 0.29 at 75 °C, demonstrating that this parameter is temperature dependent ($R^2 = 0.90$). The Weibull n values (shape factors) were between 0.46 and 0.66 (≤1), indicating an upward concavity. These results are in agreement with past reports with other microorganisms, also obtaining non-linear inactivation and showing the Weibull model capable of predicting the inactivation results (Evelyn and Silva, 2015a; Serment-Moreno et al., 2014; van Boekel, 2009; Wang et al., 2009). The inactivation of *Talaromyces avellaneus* mold ascospores by 500–600 MPa HPP at 17–60 °C, also seemed to follow a non-linear pattern. However, fitting was only carried out with the conventional first order kinetics (Voldřich et al., 2004).

3.3.2. Modeling the thermosonication inactivation

The log survivors of thermal pretreated (80 °C, 15 min) strawberry puree by TS are shown in Fig. 3. TS at 75 °C, the maximum temperature supported by the ultrasound equipment, was the best. Short treatments did not inactivate the spores, since activation shoulders were observed for all TS temperatures tested (75, 70, and 65 °C) with a maximum of 5 min at 75 °C, 10 min at 70 °C and 15 min at 65 °C. The peaks in the log counts were followed by approximately linear spore inactivation. A higher spore increase for longer periods was obtained when lowering the TS temperature. Overall, while 15 min at 75 °C achieved ≈2 log

Table 1
Weibull model parameters estimation for the survival of four week old *Byssochlamys nivea* ascospores in strawberry puree after 600 MPa HPP–thermal processing.[a]

Temperature (°C)	b	n	R^2	MSE
75	0.29 ± 0.02	0.66 ± 0.03	0.997	0.006
60	0.19 ± 0.03	0.65 ± 0.05	0.993	0.005
50	0.16 ± 0.01	0.57 ± 0.01	0.959	0.013
38	0.15 ± 0.05	0.46 ± 0.11	0.940	0.007

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

reductions, 35 min at 70 °C and ~60 min at 65 °C were required to obtain the same spore inactivation.

Due to the activation shoulders observed in all the TS spore survival curves, the first order kinetics was not appropriate and modeling was a challenge. Initially, four non-linear models (double Weibullian, Peleg, logistic and Lorentzian) were attempted. However, the double Weibullian and Peleg models suggested by Corradini et al. (2010) for heat activated *Bacillus* spores were inappropriate, presenting high standard errors for the estimated parameters (results not shown). On the contrary, the four parameter logistic (TableCurve 2D, version 5.01, SYSTAT Software Inc., Chicago, USA) and Lorentzian (Eq. (2)) models worked well. The Lorentzian distribution was a better model (0.025–0.248 MSE and 0.940–0.994 R^2), although the performance slightly decreased at 65 °C (Table 2). The Lorentzian *b* parameters increased from 5.7 to 10.1 as the temperature was increased from 65 to 75 °C, whereas the Lorentzian *a*, *c*, and *d* parameters decreased with the TS temperature, exhibiting temperature dependence ($R^2 \geq 0.81$). There has been little to no research carried out on the kinetic modeling of heat resistant mold ascospores such as *Byssochlamys* spp. by TS. López-Malo et al. (2005) and Coronel et al. (2011) attempted TS without prior thermal pretreatment and obtained first order kinetics for *P. digitatum* and *A. flavus* conidia inactivation as opposed to the non-linear Lorentzian model used in our study (Fig. 3). This confirms that conidia are less resistant than sexually produced ascospores (Pitt and Hocking, 1997).

3.3.3. Modeling the thermal inactivation

The log survivors of *B. nivea* ascospores after thermal processing at 85 and 90 °C were plotted in Fig. 4. The 90 °C thermal process was successful in inactivating ≈5 log of *B. nivea* ascospores in strawberry puree after 8 min. This makes the commercial pasteurization conditions suggested for fruit juice preservation such as 85 °C for 20 min inadequate for pasteurization processes aimed at *B. nivea* mold ascospores. Thus *B. nivea* requires 90–95 °C for fruit pasteurization, which negatively affects the fruit product sensory quality (Silva et al., 2000) and may result in the loss of raw fruit nutrients such as antioxidants.

The first-order kinetic parameters (Bigelow, 1921) were first determined as able to compare with past results: $D_{90°C} = 1.8$ min, $D_{85°C} = 13.7$ min, and z-value = 5.7 °C. Aragão (1989) reported higher $D_{90°C}$-values (6.4 min) for *B. nivea* ascospores (isolated from strawberry pulp) in 15 °Brix strawberry pulp as opposed to the 8 °Brix puree used in our study. Kotzekidou (1997) found higher $D_{90°C}$-values (3.5 min) with *B. nivea* spore strains 102 and 162 in 16 °Brix tomato juice. Engel and Teuber (1991) found much lower $D_{90°C}$-values (0.05–0.07 min) for *B. nivea* spores 6607 and 6611 in cream. These results indicate the influence of the strain and the food on the thermal resistance of *B. nivea* ascospores. The *z*-value of 5.7 °C obtained in our study was in the range of previously published *z*-values between 4 and 7 °C (Aragão, 1989; Engel and Teuber, 1991; Kotzekidou, 1997).

Table 2
Lorentzian model parameters estimation for the survival of four week old *Byssochlamys nivea* ascospores in strawberry puree after thermosonication (24 kHz, 0.33 W/mL)[a].

Temperature (°C)	*a*	*b*	*c*	*d*	R^2	MSE
75	−9.1 ± 6.1	10.1 ± 6.0	5.1 ± 0.3	15.9 ± 6.5	0.994	0.025
70	−3.9 ± 0.7	6.1 ± 0.6	11.6 ± 0.6	16.7 ± 2.5	0.983	0.072
65	−2.7 ± 1.2	5.7 ± 1.1	17.7 ± 1.5	24.0 ± 6.5	0.940	0.248

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 close to 1 are indication of good fit; all the temperatures tested showed random residuals.
[a] The spores were submitted to heat shock pretreatment (80 °C, 15 min).

Table 3
Weibull model parameters estimation for the survival of four week old *Byssochlamys nivea* ascospores in strawberry puree after thermal processing[a].

Temperature (°C)	*b*	*n*	R^2	MSE
90	$1.8 \times 10^{-1} \pm 3 \times 10^{-2}$	1.6 ± 0.01	0.998	0.010
85	$2 \times 10^{-3} \pm 4 \times 10^{-4}$	2.1 ± 0.07	0.998	0.007

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

Given the high MSE (≥0.21) given by the first order kinetic model, the non-linear Weibull model was a better choice for modeling, and the Weibull parameters were also estimated (Table 3). The Weibull *n* values (shape factor) were more than 1, indicating downward concavity. Weibull distribution function was also reported by Sant'Ana et al. (2009) to model *B. fulva* ascospore inactivation in clarified apple juice by thermal process.

4. Conclusion

600 MPa HPP–75 °C for a short time period (≤10 min) was a better method than 75 °C thermosonication (TS) and 75 °C thermal for the inactivation of *B. nivea* ascospores in strawberry puree, confirming the benefit of HPP technology. A reduction of approximately 1.4 log was obtained for HPP–75 °C after 10 min in contrast to no inactivation for 75 °C TS and 75 °C thermal processes. A longer treatment time is still needed to achieve a 5 log inactivation at 600 MPa HPP–75 °C processing conditions, thus a temperature of ≥75 °C should be used. However, the HPP–thermal process could be sufficient for typical loads (below 10^5 cfu/mL) of ascospore contamination in strawberry puree. The TS process might be applicable at longer treatment times (≥15 min) and higher temperatures (~75 °C). However, further research must be conducted to reduce the activation shoulders and design an ultrasound probe that can withstand higher temperatures. With respect to exclusively thermal processes, temperatures ≥90 °C are still required to achieve the efficient inactivation of spores. The Weibull model described the inactivation of *B. nivea* ascospores by the 600 MPa HPP–thermal and thermal processes, while the Lorentzian model was more suitable for the TS. The results from this study show that the 600 MPa HPP–thermal process might be a better option for the preservation of fruit products prone to *B. nivea* ascospore contamination.

Acknowledgments

The PhD grant no. 246/D4.4/PK from the Directorate General of Higher Education, Ministry of Education and Culture of Indonesia is acknowledged. Project No. 3701175 "Non-thermal pasteurization of foods", funded by the Faculty of Engineering Research Development Fund, University of Auckland is also acknowledged. The support from laboratory and administrative staff from the Chemical and Materials Engineering Department, University of Auckland, is also appreciated.

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