Innovative Food Science and Emerging Technologies 26 (2014) 116–123

Contents lists available at ScienceDirect

Innovative Food Science and Emerging Technologies

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

Bacterial spore inactivation by ultra-high pressure homogenization

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ARTICLE INFO

Article history:
Received 9 June 2014
Accepted 13 August 2014
Available online 22 August 2014

Editor Proof Receive Date 4 September 2014

Keywords:
Ultra high pressure homogenization UHPH
Bacterial spore
Sterilization
Continuous processing
Bacillus subtilis
Geobacillus stearothermophilus

ABSTRACT

A new generation of high pressure homogenizers reaching up to 400 MPa offers opportunities for spore inactivation and high pressure sterilization of foods. A Stansted Fluid Power ultra-high pressure homogenization (UHPH) unit was tested to inactivate bacterial spores in a model buffer system. *Bacillus subtilis* PS832 and *Geobacillus stearothermophilus* ATCC7953 spores' thermal resistances were assessed (D, z-value and E_a). The pressure and valve temperature were monitored during UHPH. Residence times under pressure and high temperature were estimated and enabled comparison with thermal inactivation, indicating the estimated thermal contribution to inactivation. Spore germination was also assessed but no germination was observed. Up to five $\log_{10}$ *B. subtilis* and two $\log_{10}$ *G. stearothermophilus* spores were inactivated by the harshest treatments (>300 MPa –T_{valve} >145 °C for ~0.24 s). The inactivation profiles were similar to the predicted thermal inactivation suggesting that the valve temperature might be a dominant parameter leading to bacterial spore inactivation.

Industrial relevance: This work showed the UHPH potential for spore inactivation and the need of highly thermoresistant surrogates for process validation, for instance, *G. stearothermophilus* ATCC7953 spores. Both these findings are highly relevant for industrial application as, to date, no surrogate for this process was suggested while the first patents for the technology transfer to industry are being issued.

1. Introduction

Mechanical homogenization has been defined as the ability to generate a distribution of particles of homogeneous size, in a liquid, by forcing the liquid under high pressure through a disruption valve (Donsì, Ferrari, & Maresca, 2009). High pressure homogenization (HPH), also known as dynamic high pressure homogenization, utilizes pressures 10 to 15 times higher than those of conventional homogenizers. It covers the full range of 100 to 400 MPa and the range 300–400 MPa is generally referred to as ultra-high pressure homogenization (UHPH). HPH has confirmed its potential for low temperature pasteurization of food matrices (Diels & Michiels, 2006; Dumay, Chevalier-Lucia, Picart-Palmade, Benzaria, Gràcia-Julià, & Blayo, 2013; Popper & Knorr, 1990) and the disruption of vegetative microorganisms was suggested to result from a combination of temperature, cavitation, shear, turbulence, impingement and high pressure (Dumay, et al., 2013; Kleinig & Middelberg, 1998). However, previous work showed that bacterial spores resist treatments at low homogenization pressure and/or low valve temperature (Bevilacqua, Cibelli, Corbo, & Sinigaglia, 2007; Feijoo, Hayes, Watson, & Martin, 1997), thus limiting applications to the domain of pasteurization.

UHPH extension of the homogenization pressure range offers a new area to investigate bacterial spore inactivation and to attempt to achieve commercial sterility through a single, continuous step combining dynamic high pressure and other stress factors, such as temperature, shear and cavitation. Bacterial spores are resistant to most conventional treatments and are the main reason for the use of high thermal intensity treatments to sterilize food (Georget, Reineke, Heinz, Knorr, Ananta, & Mathys, 2013). Detailed investigations were conducted on the impact of high hydrostatic pressure on bacterial spores and it was demonstrated that pressure and temperature have a synergetic impact on bacterial spore inactivation (Heinz, 1997; Mathys, 2008; Reineke, 2012; Reineke, Mathys, Heinz, & Knorr, 2013). Fewer investigations have looked at the use of UHPH for spore inactivation partially due to the previous unavailability of the equipment. Also, some of the studies looking at spore inactivation by UHPH focused on the control of the homogenization pressure but did not consider the temperature at the valve, thus making it hard to assess the causes for the absence of inactivation (Chen, Harte, Davidson, & Golden, 2013). Existing studies showing successful inactivation of bacterial spores through UHPH were reviewed (Georget, Miller, Callanan, Heinz, & Mathys, 2014), and it was concluded that the best inactivation could be achieved by combining high homogenization pressures and high inlet and, consequently, valve temperatures

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http://dx.doi.org/10.1016/j.ifset.2014.08.004
1466-8564/

(Amador-Espejo, Suàrez-Berencia, Juan, Bárcenas, & Trujillo, 2014; Cruz, 2008; Cruz, Capellas, Hernandez, Trujillo, Guamis, & Ferragut, 2007; Poliseli-Scopel, 2012; Valencia-Flores, Hernandez-Herrero, Guamis, & Ferragut, 2013). In most of the studies inactivation of bacterial spores was hypothesized to be the result of a synergetic effect of pressure, shear, cavitation, temperature and turbulence but this hypothesis was not demonstrated. Although a direct association could be made with the processing temperature, no studies attempted to quantify the expected thermal inactivation through the decimal reduction time $D_{\theta,}$ z value and energy of activation E_a of the strains combined with the residence time. This approach could provide a rapid estimation of the effective contribution of temperature to spore inactivation by UHPH. The absence of a clear understanding of the factors leading to bacterial spore inactivation has also prevented the scientific community from reaching an agreement on the most relevant surrogate to use for future work and process parameters validation. Most studies in food matrices focused on *Bacillus cereus* spores or endogenous mesophilic spore strains and claimed sterility based on the full inactivation of these strains. This was without clear establishment of the contribution of different process parameters which is necessary to select the appropriate surrogate, and without high level of inoculation necessary to prove the robustness and reliability of the process (Amador-Espejo, Suàrez-Berencia, et al., 2014; Poliseli-Scopel, Hernández-Herrero, Guamis, & Ferragut, 2012; Valencia-Flores, et al., 2013). A study conducted with *Geobacillus stearothermophilus* spores, a particularly thermostable strain, showed that high pressure of 300 MPa and 84 °C at the UHPH valve were not sufficient to inactivate this strain, but unfortunately did not assess higher valve temperatures (Pinho, Franchi, Tribst, & Cristianinia, 2011). Recently published work initiated exploration of UHPH thermophilic spore inactivation with inoculation of whole UHT milk and showed that with an inlet temperature of 85 °C, *G. stearothermophilus* (CECT 47) spores could be inactivated (Amador-Espejo, Hernández-Herrero, Juan, & Trujillo, 2014). Further studies with this indicator and higher inlet and valve temperatures could be useful to validate the UHPH sterilization over a broader range of resistant spore formers and improve our understanding of the role of different process parameters.

In this work, the authors investigated the inactivation of spores of two bacterial strains, *Bacillus subtilis* PS832 and *G. stearothermophilus* ATCC7953. Both strains were inoculated at high concentration in a model buffer system and UHPH was applied with different combinations of homogenization pressure (350 MPa ≥ P ≥ 200 MPa) and inlet temperature in order to assess the role of both parameters in the inactivation. In particular, the thermal inactivation kinetics of both strains were also assessed in order to model the impact of temperature during UHPH spore inactivation. For clarity, the pressure range investigated in this work is referred to throughout the manuscript as UHPH.

2. Material and methods

2.1. Bacterial strains and sporulation

The strains used in this work were *G. stearothermophilus* ATCC7953 obtained from the DSMZ-German Collection of Microorganisms and Cell Cultures, and *B. subtilis* PS832, courtesy of Professor Peter Setlow, University of Connecticut Health Center, USA.

G. stearothermophilus spores were obtained following a sporulation method enabling >95 % spores, as established in previous work (Georget, Kapoor, Winter, Reineke, Song, Callanan, Ananta, Heinz & Mathys, 2014). Sporulation was induced at 55 °C on solid Difco medium agar plates without antibiotics. *B. subtilis* spores were obtained using a method described elsewhere (Nicholson & Setlow, 1990; Reineke, Mathys, & Knorr, 2011a). Sporulation was induced at 37 °C on solid 2xSG medium agar plates without antibiotics. In both cases, the spore suspensions were cleaned by repeated centrifugation until the supernatant was clear (minimum 3-fold at 4800 g), washed with cold distilled water (4 °C), and were treated twice with sonication for 1 min (35 kHz - 160 W_{eff}) (Bandelin Sonorex RK 510H, Berlin, Germany). The cleaned spore suspensions contained >95 % phase bright spores without agglomerates as was verified by phase contrast microscopy and were stored in the dark at 4 °C until use.

2.2. UHPH unit and treatment conditions

For the high pressure homogenization treatment, a Stansted UHPH unit, model FPG11300 (Stansted Fluid Power Ltd, Harlow, UK) was used as depicted in Fig. 1 (numbers under brackets [1], [1'] and [2]-[6] in the text hereafter refer to Fig. 1). This unit consisted of two pistons generating the homogenization pressure – the first homogenization valve [3] – a cooling step [4] – the second homogenization valve [5] and the product outlet for sampling [6]. The Stansted first valve [3] design was such that the fluid streamed axially under high pressure along the mobile part of the valve (valve spindle) and then flowed at high velocity through the radial narrow gap formed between the valve seat and the spindle, before leaving the valve seat at the low pressure maintained by the second homogenization valve (~10 MPa). Bacterial spores were re-suspended in PBS buffer 0.01 M (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4) at pH 7.0, at initial concentrations of 10^6 CFU/mL for *B. subtilis* spores and 10^4 CFU/mL for *G. stearothermophilus* spores [1]. PBS buffer was selected for its high stability at the high temperatures which were targeted in this experimental work and avoided potential pKa variation at high temperatures as was reported in previous work (Reineke, Mathys, & Knorr, 2011b).

The parameters controlled were the inlet temperature and the operating pressure before the first valve. The target inlet temperatures were 37 and 80 °C for *B. subtilis* spores and 55 and 80 °C for *G. stearothermophilus* spores. The low temperature corresponds to the optimal growth temperature of the respective indicators and was selected to assess a potential germination under pressure during the high pressure stage of the process. For 37 and 55 °C, the inlet temperature was directly adjusted by means of a water thermostat and plate heat exchanger (DIL e.V., Quakenbrueck, Germany) connected ahead of the UHPH unit [2]. For the trials at high inlet temperature, the spore suspension was first preheated by recirculation through a separate plate heat exchanger (DIL e.V., Quakenbrueck, Germany) and then processed through the heat exchanger and UHPH set-up as described above, allowing for an inlet temperature of ~80 °C [1']. The cooler consisted of a tubular heat exchanger localized at the outlet of the first valve and connected to a cooling unit using glycol water at −10 °C as the cooling agent [4]. The cooling step allowed for a rapid cooling after the first valve, bringing the process medium from the valve temperature down to temperatures below 50 °C within < 1 s.

For each temperature, three homogenization pressures were tested: 200, 300, and 350 MPa. The second valve was present on the UHPH unit in order to maintain a minimal pressure (~10 MPa) after the decompression at the first valve, thus minimizing the formation of steam at the first valve and cavitation phenomena. The valve temperature was recorded during the trial. This parameter directly resulted from the homogenization pressure and the inlet temperature selected and enabled comparison to thermal inactivation kinetics (via $D_{\theta,}$ z value and E_a see Section 3).

Each trial was done at least in triplicate using a single batch for both indicators and the bars in the graphs correspond to the standard deviation. The same spore batches were used for the determination of the thermal inactivation kinetics as described hereafter.

2.3. Microbiological analyses

Following the UHPH trials, plate counts were performed before and after a thermal treatment at 80 °C during 20 min to inactivate the potentially heat-sensitized spores and establish the extent of germination. Samples were plated at least in triplicate on nutrient agar and incubated at