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Inactivation of spores and vegetative cells of *Bacillus subtilis* and *Geobacillus stearothermophilus* by pulsed light

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ABSTRACT

The effect of pulsed light (PL) on the inactivation of vegetative cells and spores of *Bacillus subtilis* and *Geobacillus stearothermophilus* at different cell densities was evaluated. The antimicrobial effect of PL decreased when population density increased, both for vegetative cells and spores of *B. subtilis* and *G. stearothermophilus*. For low cell densities, vegetative cells were more sensitive to PL than spores. However, lower reductions in vegetative cell counts were shown for higher cell densities, which could be attributed to the fact that vegetative cell suspensions transmitted less amount of light than spores. Concerning the resistance of both microorganisms, lower reduction in *G. stearothermophilus* than *B. subtilis* counts were found for the same cell density. When cell suspensions with similar light transmittance were compared, vegetative cells of *B. subtilis* were found to be more sensitive than the ones of *G. stearothermophilus*, while the spores of *G. stearothermophilus* were less resistant to PL.

1. Introduction

Pulsed light (PL) consists of a successive repetition of short duration and high power flashes of broadband emission light (190–1000 nm) with approximately 40% of the emitted light corresponding to the ultraviolet (UV) region (Wekhof, 2000). This novel technology appears as a promising alternative to conventional heat preservation processes to ensure the microbial quality and safety of food products. It has been shown to be effective in inactivating a wide range of microorganisms involved in food spoilage and food borne pathogens including bacteria, fungi, viruses and protozoa (Elmnasser, Federighi, Bakhrouf, & Orange, 2010; Gómez-López, Devlieghere, Bonduelle, & Debevere, 2005; Huffman, Slifko, Salisbury, & Rose, 2000). However, one of the main challenges for the application of non-thermal technologies in the food industry is the inactivation of bacterial spores. These microbial forms are extremely resistant to many stresses, including toxic chemicals and biocidal agents, desiccation, high pressure, heat treatment, ionising radiation or ultraviolet (UV) processing (Nicholson, Munakata, Horneck, Melosh, & Setlow, 2000; Setlow, 2006). Their high resistance to UV radiation has been mainly attributed to altered DNA photochemistry caused by the binding of small acid-soluble proteins (SASP), an efficient repair pathway specific for their photoproducts, the accumulation of dipicolinic acid in the dormant spore core or a low core water content and the presence of a thick spore protein coating (Nicholson et al., 2000; Setlow, 2006). Regarding PL, several previous works have reported its use in bacterial spore inactivation (Buschnell, Cooper, Dunn, Leo, & May, 1998; Chaine, Levy, Lacour, Riedel, & Carlin, 2012; Rice & Ewell, 2001). However, only a few studies have pointed out the higher sensitivity to PL of vegetative cells compared to spores (Dunn et al., 1989; Levy, Aubert, Lacour, & Carlin, 2012), so that the impact of the physiological state of the microorganisms on PL effectiveness is not fully elucidated.

PL may have potentiality to decontaminate liquid food products (water, milk, juice …), the surface of solid food products (eggs, meat and fish products …), packaging and processing equipment. The application of PL for liquid food treatment is limited by the penetration capacity of the incident light, which determines the exposure of the microbial cells to the effective wavelengths and therefore, its decontamination effectiveness (Artíguez, Arboleya, & Martínez de Marañón, 2012). Since the microbial population density could determine the degree of light penetration through the liquid, one of the aims of this study was to evaluate its influence on the antimicrobial effectiveness of PL. In addition, the resistance of vegetative cells and bacterial spores was compared to assess the impact of the bacterial physiological state on the effectiveness of PL for microbial inactivation. For these purposes, the antimicrobial effect of PL was evaluated on *Bacillus subtilis* and *Geobacillus stearothermophilus*, bacteria of great concern to the food processing industry due to their ability to form highly resistant spores

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and their spoilage potential (Burgess, Lindsay, & Flint, 2010; From, Pukall, Schumann, Hormazábal, & Granum, 2005).

2. Material and methods

2.1. Bacterial strains and growth conditions

Microbial species, culture media and incubation temperatures used in this study are summarised in Table 1. Bacterial strains were stored at −80 °C in a 20% (w/w) glycerol solution. Thawed stock cultures (100 μL) were transferred to 10 mL of the appropriate broth and precultured at temperatures indicated in Table 1 for 24 h. Each bacterial strain was then inoculated at 10^3 CFU/mL and cultured at the corresponding temperature for 24 h until early stationary growth phase. Cells were harvested by centrifugation (5804R centrifuge, Eppendorf AG, Germany) at 10,000 ×*g* for 15 min at 4 °C, washed twice with Potassium Phosphate Buffered Saline (KPBS; 0.01 M K_2HPO_4, 0.01 M KH_2PO_4, 0.15 M NaCl; pH: 6.7) and finally resuspended in this buffer at a cell density of 10^5–10^7 CFU/mL for *G. stearothermophilus* and 10^7–10^9 for *B. subtilis*.

2.2. Preparation of spore suspensions

For spore preparation, aliquots of 500 μL of bacterial culture prepared as described above were spread on sporulation agar, and then incubated at the appropriate temperatures (Table 1) until >90% of the cells were sporulated. Sporulation was verified by observing the refractile spores using a phase-contrast microscopy. Spore suspensions were not heated to inactivate vegetative cells because thermal treatments could affect the resistance to posterior PL treatments of *B. subtilis* spores (Artíguez & Martínez de Marañón, 2015). Spores were harvested from agar plates with distilled sterile water. Collected spores were centrifuged at 4000 ×*g* for *B. subtilis* and at 8000 ×*g* for *G. stearothermophilus*, for 15 min at 4 °C, washed three times with distilled sterile water and cell density adjusted to 10^5–10^8 spores/mL for *G. stearothermophilus* and 10^7–10^9 for *B. subtilis*.

2.3. Pulsed light device

A desktop SBS-XeMaticA-(L + L) device (SteriBeam Systems GmbH, Germany) was used. The polished stainless steel treatment reactor consists of a vertically mobile quartz shelf located between two xenon lamps (upper and lower), which emit high intensity light pulses of 325 μs duration. The emission spectrum includes wavelengths from 190 to 1000 nm, with about 20% of the emitted light in the UV-C, 8% in the UV-B and 12% in the UV-A region (Wekhof, 2000). A fan cooling system is used to prevent overheating of lamps and samples.

2.4. Pulsed light treatment

Aliquots of 300 μL of each resulting bacterial suspension (prepared as described above) were transferred to sterile UV-transparent Suprasil quartz cuvettes (optical length 1 mm, Hellma, Germany) and immediately subjected to PL treatment at room temperature (23–25 °C). Samples were placed on the centre of the quartz shelf at 6 cm from the upper xenon lamp and treated at 2.2 kV. Fluence per pulse was measured with an energy metre (model QE25-LP-H-MB, Gentec, Canada) used with a Solo2 readout unit and was expressed in joules per square centimetre (Artíguez et al., 2012). Pauses of at least 30 s between measurements were allowed to prevent possible overheating of the detector. All fluence measurements were performed in triplicate. Total fluence or amount of photons striking on the sample per unit area, is the most relevant process factor affecting microbial inactivation by PL (Artíguez & Martínez de Marañón, 2014; Lasagabaster & Martínez de Marañón, 2013). Therefore, total fluence (H) was calculated by multiplying pulse fluence by the number of emitted light pulses (n) ($H = H_0 \times n$). Samples were treated at fluence in the range of 0.14–12 J/cm^2. Each condition treatment was repeated at least three times. Untreated inoculated samples were used as controls.

2.5. Transmittance spectrum of the bacterial suspensions

The spectral transmittance of all samples was determined each 1 nm from 190 to 1000 nm with a Genesys 6 UV/VIS spectrophotometer (Thermo Spectronic, Rochester, USA). All measurements were performed in the same cuvettes used for PL treatments (see above). Distilled water was used as the blank. At least three measurements per sample were performed.

2.6. Microbiological analyses

Bacterial counts in inoculated untreated (control) and PL treated samples were determined immediately after each treatment. Liquid samples were serially diluted in 1% buffered peptone water (Pronadisa, Spain) and 0.1 mL of appropriate dilutions were surface plated onto the corresponding agar media (Table 1). After incubating Petri dishes at the appropriate temperature for 72 h, colonies were enumerated and the results expressed as Log CFU/mL, 1 Log being the detection limit of the plate count method.

2.7. Data analyses

Microbial inactivation was calculated as Log (N_0/N), where N_0 represents initial cell count (untreated samples) and N post-treatment bacterial count (treated samples). Analysis of variance and Tukey's honestly significant difference test were used to determine significant differences among treatments ($p < 0.05$) (SPSS Inc., IL, USA).

2.8. Inactivation modelling

The Geeraerd and Van Impe inactivation model-fitting tool (GInaFiT), a freeware add-in for Microsoft Excel, was used (Geeraerd, Valdramidis, & Van Impe, 2005). The Weibull and the log-linear with shoulder and/or tail model were fitted to experimental data. Results indicated that the log-linear with shoulder and/or tail model, proposed by Geeraerd, Herremans, and Van Impe (2000) best fitted the experimental data (data not shown) and, therefore, it was employed to describe *B. subtilis* and *G. stearothermophilus* inactivation as a function of the total

Table 1
Origin and culture conditions of the tested microorganisms.

Species	Code[a]	Growth media[b]	Sporulation media[b]	Culture media[b]	Incubation temperature (°C)
B. subtilis	DMS 10	TSB	NA (1)	TSA	30
G. stearothermophilus	CECT43	TSB	NA (2)	NA (3)	56

NA (1) Nutrient agar supplemented with 1 mg/L $MnSO_4$ and 0.5 g/L $CaCl_2$ (Prentice, Wolfe, & Clegg, 1972); NA (2) Nutrient agar supplemented with 10 mg/L $MnSO_4{:}H_2O$ (Finley & Fields, 1962); TSA, Tryptic Soy Agar; NA (3), Nutrient agar. All media were provided by Pronadisa (Spain).

[a] DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany; CECT, Spanish Type Culture Collection.

[b] TSB, Tryptic Soy Broth.