Food Microbiology 82 (2019) 409–415

Contents lists available at ScienceDirect

Food Microbiology

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

Inactivation of *Bacillus subtilis* spores at various germination and outgrowth stages using intense pulsed light

Hye-Lim Jo[1], Hee-Jeong Hwang[1], Myong-Soo Chung*

Department of Food Science and Engineering, Ewha Womans University, Seoul, 120-750, South Korea

ARTICLE INFO

Keywords:
Intense pulsed light (IPL)
Bacillus subtilis
Germination
Dipicolinic acid (DPA)

ABSTRACT

It is important to inactivate spore-forming bacteria in foods because their spores are highly resistant to various stresses. Although thermal treatment is an effective inactivation method, the associated high temperatures can cause changes in food quality. Intense pulsed light (IPL) is a nonthermal technique that can effectively improve food safety. This study evaluated the inactivation effects of IPL at various fluences on *Bacillus subtilis* spores. IPL treatment at a total fluence of 7.40 J/cm^2 resulted in a 7 log reduction, indicating the potential of IPL to effectively inactivate bacterial spores. The sensitivity of *B. subtilis* spores to IPL during germination and outgrowth was also measured. The resistance to the IPL increased temporarily until 1 h after the start of incubation, and then gradually decreased for longer incubation periods. This temporary increase in resistance at the early stage of incubation was attributed to the leakage of dipicolinic acid from the spores. The results also showed that the inactivation efficiency increases after 1 h pre-incubation because the numbers of vegetative cells increased with the incubation time.

1. Introduction

Spore-forming bacteria are widespread in the environment, and contamination can easily occur from *Bacillus* spp. in the agricultural environment (Carlin, 2011; te Giffel et al., 1995). It is known that while the resistance of these bacterial spores depends on their specific strain and the environmental conditions during the sporulation, all of those tested have been found to be more resistant than vegetative cells to various stresses such as radiation, chemicals, heat, and UV light (Atrih and Foster, 2002; Setlow, 1994, 2006). It has been reported that the fluence, the total amount of IPL energy which is expressed J/cm^2, required to reduce spores by 5 log CFU/ml is up to 18-fold higher than for vegetative cells (Levy et al., 2012). Many studies have found that several factors underlie this difference in resistance, such as the spore coat (which is known to protect against chemicals), α/β-type small acid-soluble proteins (SASP), and repair mechanisms (Nicholson et al., 2000; Setlow, 2006). In particular, the UV-resistance-related factors include changes in the UV photochemistry of DNA, DNA repair mechanisms, and dipicolinic acid (DPA) (Setlow, 2006). Vegetative cells exposed to UV irradiation produce photoproducts such as cyclobutene pyrimidine dimers (CPDs) and pyrimidine-(6-4)-pyrimidone photoproducts (6-4 PPs), which are formed between adjacent pyrimidines in the same DNA strands. In contrast, these CPDs and 6-4 PPs are produced at only very low levels when spores are irradiated with UV light, with instead thyminyl-thymine adduct (termed the spore photoproduct) mainly forming. This photoproduct is repaired during the early stages of spore growth via three recovery mechanisms (Setlow, 2006). Since dormant spores do not cause food spoilage or poisoning, they themselves do not represent hazards in foodstuffs (Artíguez and de Marañón, 2015a). However, if spores are provided with an appropriate environment for germination and outgrowth, vegetative cells that germinate from the spores can cause food spoilage and food-borne diseases (Paidhungat et al., 2000; Setlow et al., 2003). Therefore, ensuring the effective inactivation of bacterial spores and the prevention of germination is very important in the food industry. Thermal sterilization is very effective at inactivating dormant spores, but the required high temperatures can cause deviations of the sensory characteristics and nutritional properties of food. This aspect indicates that importance of developing nonthermal techniques that can ensure stability without causing significant changes in food quality.

Intense pulsed light (IPL) is a nonthermal technique which involves the application of broad-spectrum irradiation in the form of intense short pulses. IPL covers a wavelength range from 200 to 1100 nm, and the light is typically 20,000 times more intense than sunlight at the Earth's surface (Dunn et al., 1995). Recent studies of IPL inactivation effects on spores have mainly involved either spores or vegetative cells,

* Corresponding author.
E-mail address: mschung@ewha.ac.kr (M.-S. Chung).
[1] These authors contributed equally to the work.

https://doi.org/10.1016/j.fm.2019.03.013
Received 22 November 2017; Received in revised form 21 August 2018
Available online 15 March 2019

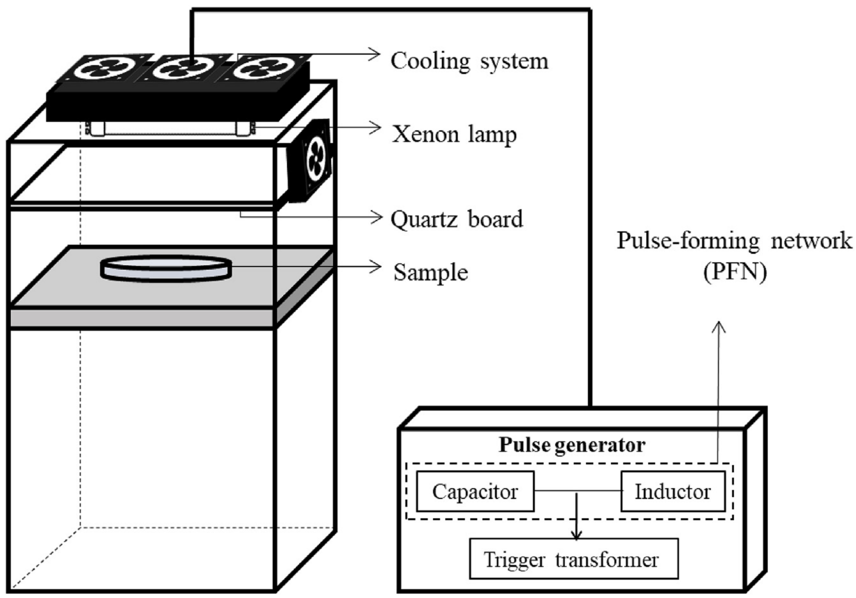

Fig. 1. Schematic of the laboratory-scale intense pulsed light (IPL) system.

or investigated the inactivation mechanism of IPL using mutant strains (Aguirre et al., 2015; Artíguez and Marañón, 2015a; Esbelin et al., 2016; Levy et al., 2011, 2012). These studies have shown that the SASP, DNA repair system, and spore coats contribute to IPL resistance in experiments using a mutant strain to reveal the spore-killing mechanism of IPL. Artíguez and de Marañón (2015a) studied the effects of IPL treatment on the germination and outgrowth of spores. However, it was not clear exactly what germination stage and growth stage the spores were based on the incubation time. Since the germination of spores have a complex processes in dependency of the environmental conditions, it is very important to monitor the sensitivity to IPL throughout this process.

The present study investigated the inactivation effects of IPL on *Bacillus subtilis* spores and how the sensitivity against IPL changes as spores become vegetative cells during the germination and outgrowth stages, while identifying these stages as a function of incubation time. In addition, changes in heat sensitivity were investigated for various incubation times in order to identify whether or not the change in sensitivity to IPL differs from the change in sensitivity to the heat treatments applied for general spore sterilization.

2. Materials and methods

2.1. Preparation of microbial suspensions

2.1.1. Spore preparation

B. subtilis KCCM 11315 acquired from the Korean Culture Center of Microorganisms (Seoul, Korea) was cultured on Brain Heart Infusion (Difco™, Sparks, MD, USA) broth at 30 °C for 24 h. The culture medium was inoculated with 50 μl of each sample on sporulating agar (AK Agar #2, Difco™) at 30 °C for 5 days. Sterile 0.85% NaCl solution (5 ml) was then added to the plate medium on which the spores had formed. Collected spores were centrifuged (406G, Gyrozen, Incheon, Korea) at 8000 g for 10 min, the supernatant was removed, and then the obtained pellet was washed three times with sterile distilled water. The washed pellet was then suspended in sterile 0.85% NaCl solution. Heat treatment was carried out at 80 °C for 10 min to kill any remaining vegetative cells. The suspension was centrifuged and then purified by washing three times as described above. The spore preparations were examined using modified spore (Wirtz-Conklin) staining under an optical microscope (Olympus CX21LED, Olympus Corporation, Tokyo, Japan), which confirmed that more than 99% of spores had formed (Hamouda et al., 2002). These spore were resuspended in 0.85% NaCl solution. These were contained in plastic cryopreservation tubes (MCT-150-C, AXYGEN, CA, USA) and stored at −70 °C until further analysis (Cho et al., 2007).

2.1.2. Germination and outgrowth condition

The formed spores were treated by heat at 70 °C in a 0.85% NaCl solution for 30 min based on our preliminary experiments. This heat treatment activated the spores and enhanced the initiation of germination, thereby making the degree of spore germination more homogeneous and killing any remaining cells (Pandey et al., 2013). The heat-activated spores were then cultured at 30 °C in shaking incubators (HB-201SF, Hanbaek Scientific Technology, Bucheon City, Korea) in tryptic soy broth (TSB; Difco™) supplemented with 10 m ML-alanine (Avocado Research Chemicals, Heysham, UK). The addition of L-alanine increased the homogeneity of spore germination.

Spores that had been cultured for 0.5, 1, 2, 3, 4, 5, and 6 h were analyzed to identify changes in IPL sensitivity according to incubation time. The degree of germination and outgrowth of spores were determined using optical microscopy after staining with modified spore (Wirtz-Conklin) stain according to the same method as described above (Hamouda et al., 2002).

2.2. IPL treatments

2.2.1. IPL system

The laboratory-scale IPL system used in this study was self-designed in the laboratory of Department of Food Science and Engineering, Ewha Womans University in Seoul, Korea. Fig. 1A shows a schematic of the system, which consists of a pulse generator, a treatment chamber, and a xenon lamp within this chamber (Hwang et al., 2015). When the pulse generator is turned on, electrical energy is stored in the capacitor and this is then transferred to the xenon lamp (type NL4006, XAP series, Heraeus Noblelight, Cambridge, UK) to produce the IPL. Cooling fans are installed in the upper and both sides of the chamber to prevent the temperature inside the chamber from increasing by more than 10 °C during IPL sterilization treatment. As shown in Fig. 1, cooling fans are installed in the upper and both sides of the chamber. These fans can not only prevent the overheating inside the chamber, but also circulate the air continuously. So ozone can be released out of the chamber by these fans (Jo, 2017).

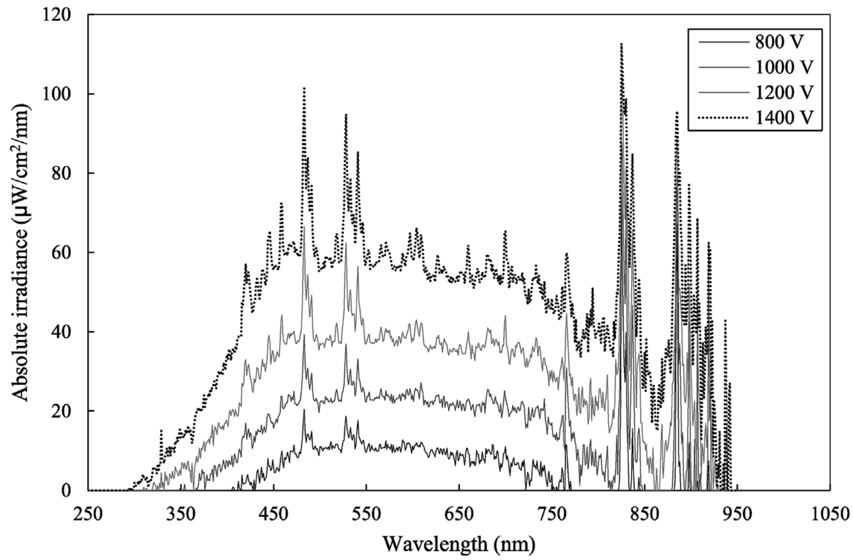

Fig. 2. Typical instantaneous emission spectrum of the xenon lamp used for IPL irradiation.

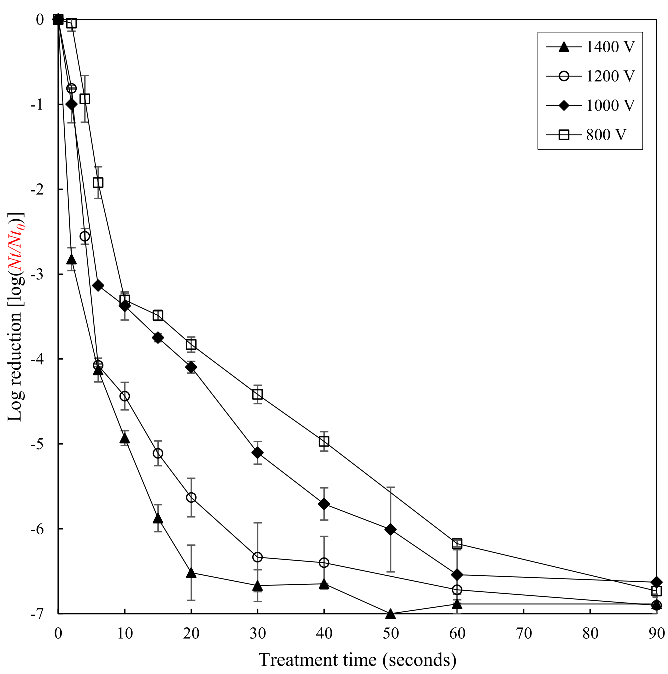

Fig. 3. Inactivation level of *B. subtilis* spores exposed to IPL as a function of the lamp voltage and treatment time. Data are mean and SD values.

2.2.2. Measurement of total IPL fluence

The wavelength distribution and intensity of the radiation emitted by the lamp were measured using a spectroradiometer (ILT-900, International Light Technologies, Peabody, MA, USA), as shown in Fig. 2. The total IPL fluence was calculated by integrating the area under the instantaneous light spectrum.

2.2.3. Treatment conditions

Spores and vegetative cells obtained for different incubation times were centrifuged at 8000 g for 10 min, the supernatant was removed, and then the obtained pellet was suspended in sterile 0.85% NaCl solution to adjust the initial spore count to 10^7 CFU/ml. The solution was then serially diluted in sterile 0.85% NaCl solution to produce 100-μl volumes that were spread on plate count agar (PCA; Difco™) plates with a diameter of 90 mm. Uncovered plates were treated with IPL at a lamp voltages of 800–1400 V, treatment times of 0–90 s, a frequency of 5 Hz, 0–450 flashes, and a pulse width of 100 m s. The distance between the lamp and the sample was 3.5 cm and applied IPL fluences to the samples at this point was 3.54–29.60 mJ/cm^2/pulse. All samples with different germination stages were treated IPL with the same range of the fluences. Untreated samples were control. In order to prevent the germination and outgrowth of spores caused by nutrients in PCA, the plates were treated by IPL within 1–2 min, in which it is enough time for the spore to be attached to the surface and be dried, after the inoculation of spores (Esbelin et al., 2016; Levy et al., 2012). All experiments were conducted in triplicate.

2.3. Thermal treatments

The heat treatment was performed using an autoclave (HB-506-4, Hanbaek Scientific Technology). The *B. subtilis* suspension (15 ml) with an initial microbial number of 10^7 spores/ml was sterilized at 118 °C for 60 s. There were a come-up time required for the temperature to rise from 100 °C to 118 °C (5 min) and cooling time to drop from 118 °C to 100 °C (8 min), so actual treatment time is much longer than 60 s. The bacterial suspension was then removed from the autoclave and immediately cooled in 20 °C of cold water for 10 min.

2.4. Microbial analysis

2.4.1. Estimation of viability

The PCA plates treated with IPL were incubated at 30 °C for 48 h. The reduction of the initial microbial load was expressed on a logarithmic scale, and calculated as log (Nt/Nt_0), where Nt_0 is the initial number of spores and Nt is the number of colonies that survived after IPL or heat treatment. Data are presented as mean values ± standard deviation obtained from three individual samples (n = 3).

2.4.2. Monitoring of optical density at 600 nm

We monitored the optical density at 600 nm (OD_{600}) in order to investigate the stages of germination and outgrowth of spores during incubation in TSB (Hitchins et al., 1963). OD_{600} was measured with a spectrophotometer (HNBT-3000 UV/VIS, Hanbit Nano Biotechnology, Seoul, Korea) during incubation periods of up to 6 h.

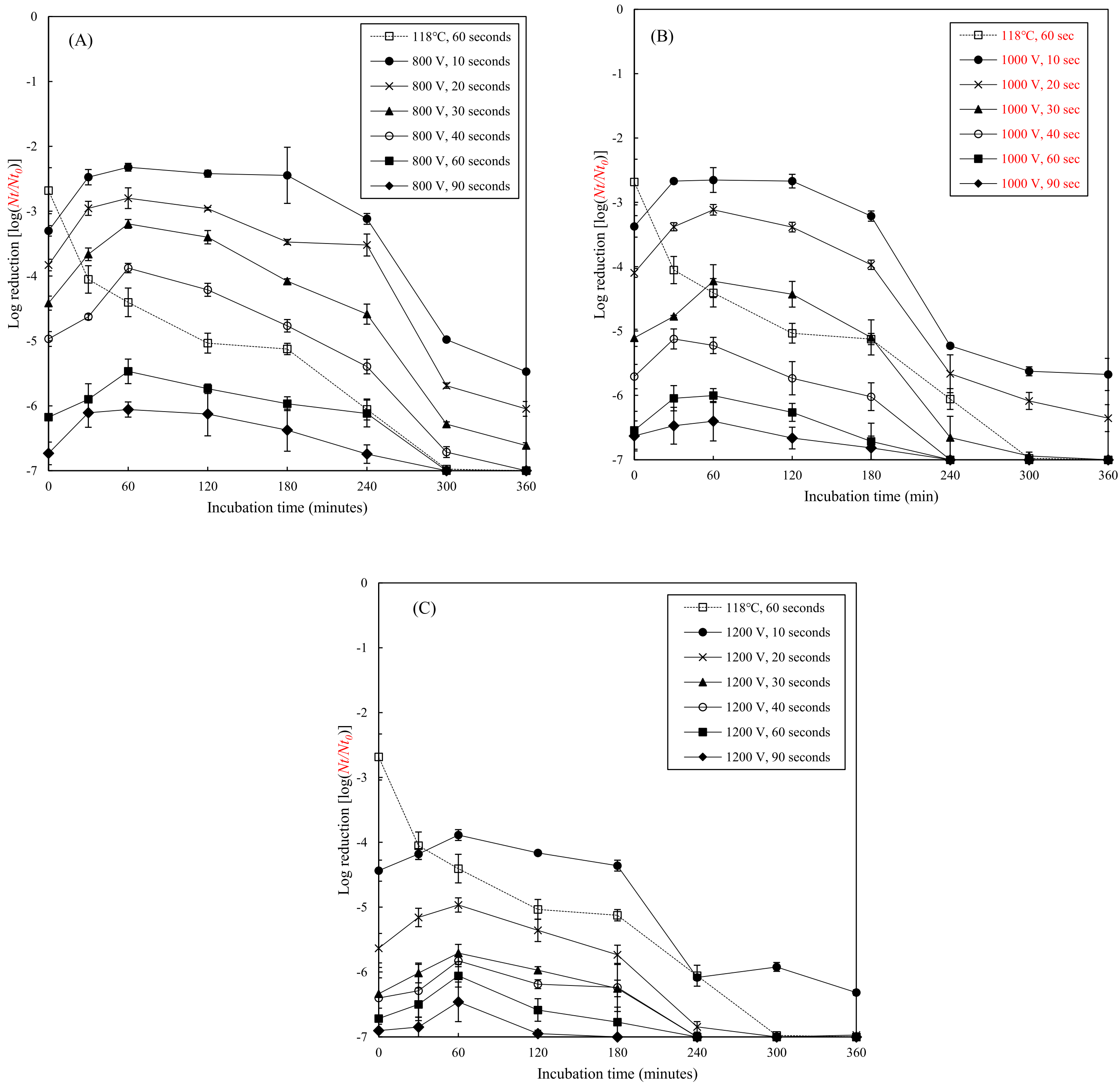

Fig. 4. Inactivation level of *B. subtilis* spores exposed to IPL as a function of the lamp voltage and treatment time for different incubation times (each dashed line is the inactivation level of heat treatment for comparison). Data are mean and SD values.

3. Results and discussion

3.1. IPL inactivation effects on B. subtilis spores

The inactivation effects of IPL on *B. subtilis* spores were investigated for different lamp voltages (800–1400 V), treatment time (0–90 s), and frequency (5 Hz, so total flashes of 0–450), which resulted in fluences of 3.54–29.60 mJ/cm^2/pulse. The results confirmed that the inactivation effects on spores increased with the lamp voltage and treatment time. As shown in Fig. 3, there were decreases of 5.7, 6.1, and 7.0 log CFU/ml after treatments at 800, 1200, and 1400 V, respectively for 50 s, corresponding to total fluences of 0.88, 4.56, and 7.40 J/cm^2. Levy et al. (2012) also treated *B. subtilis* spores on agar medium using pulsed light, and they obtained 3 and 5 log reductions at total fluences of 0.5 and 1.25 J/cm^2, respectively; similar log reductions were obtained at total fluences of 0.29 and 1.48 J/cm^2, respectively, in the present study. These findings confirm that *B. subtilis* spores were effectively inactivated by the IPL treatment.

3.2. Monitoring of IPL and heat sensitivity during incubation

We examined the changes in the IPL sensitivity of *B.subtilis* spores during incubation in TSB for up to 6 h to monitor changes in the sensitivity of incubating spores to IPL. As shown in Fig. 4, the sensitivity of the spores to IPL varied with the incubation time. The resistance to IPL gradually increased for up to 1 h of incubation under lamp voltages of 800, 1000, and 1200 V. Dormant spores that had not been incubated were reduced more (by a maximum of 1.16 log CFU/ml) than spores

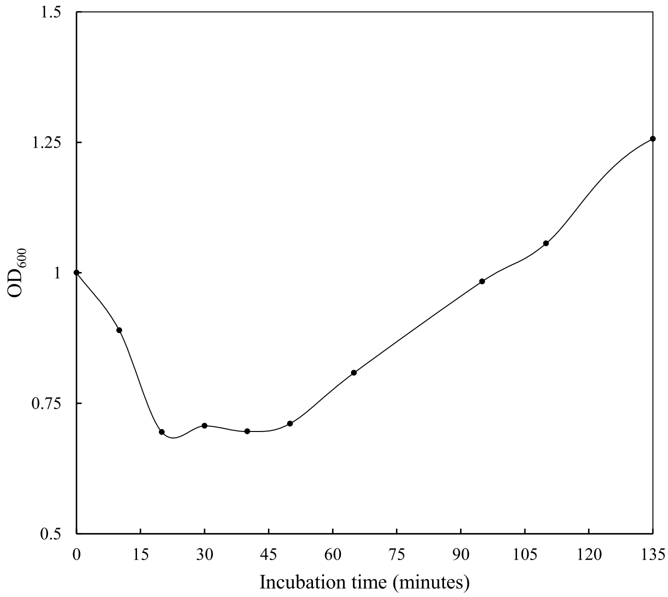

Fig. 5. Changes in OD_{600} during the germination and outgrowth of *B. subtilis* spores.

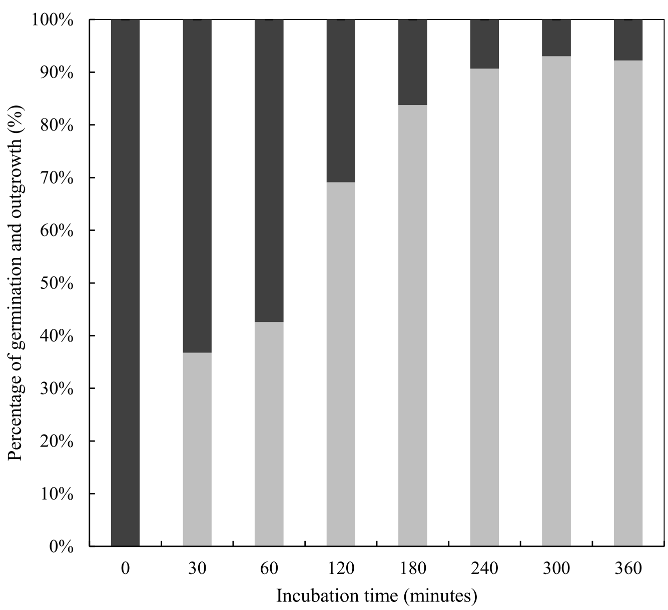

Fig. 6. Ratio of dormant spores to germinated spores plus vegetative cells during incubation (■ dormant spores, ■ germinated spores and vegetative cells).

incubated for 1 h. After 1 h, the resistance to IPL gradually decreased as the incubation time lengthened. This tendency is consistent with the findings of several other studies, such as Irie et al. (1968) and Munakata (1974) showing that germinated *B. subtilis* spores exhibited temporarily higher resistance to UV light during the early germination stage. Cho et al. (2012) found that the resistance of *B. subtilis* spores to UV light was slightly greater for those germinated for 1 h than for dormant spores. In addition, Artíguez and de Marañón (2015a) reported that spores cultured for 1 h were the most resistant to the pulsed light.

To compare the sensitivities of *B. subtilis* spores to IPL and heat treatment, spores incubated for different times were heated at 118 °C for 60 s, which revealed that their sensitivity to heat decreased gradually as the incubation time lengthened. Dormant spores were reduced by approximately 2.6 log CFU/ml, while the heat treatment of spores that had been incubated for 30 min were reduced by about 4 log CFU/ml. After 5 h of incubation the inactivation was about 7 log CFU/ml. When comparing the results of heat treatment and IPL treatment for 60 s (the actual time was much longer than 60 s because of come-up and cooling times), the inactivation effect was higher for IPL treatment regardless of the spore incubation time. Artíguez and de Marañón (2015b) also observed that individual exposure to heat treatment at 90 °C for 10 min or to total fluences of 0.5 or 1 J/cm^2 of IPL reduced *B. subtilis* spore counts by 0.6, 1.1, and 2.7 log, respectively. It is therefore clear that IPL is a nonthermal technique that can effectively control spores. An especially notable observation was that the sensitivities of spores to heat and IPL treatments different significantly for incubation times of 0–60 min—it is important to identify the associated changes in the spores. Although this study was conducted by inoculating on agar surface and the effects of the food structure and other disturbance factors were avoided, this result identifies the high possibility for the IPL to be applied to the food industry.

3.3. Identifying germination and growth stages of spores during incubation

The germination and growth stages of spores were identified at each time during incubation for 6 h by performing three measurements: (1) OD_{600}, (2) the degree of germination using the plate count method, and (3) the degree of spore staining using an optical microscope.

Firstly, OD_{600} was measured in dependency the incubation time. As shown in Fig. 5, the tendencies of OD_{600} differed between before and after 60 min, decreasing up to 20 min and with the decreased value then being maintained for a certain period of time, while OD_{600} gradually increased after 60 min of incubation. Previous studies have shown that spore germination can be monitored by observing changes in OD_{600} as the spores germinate (Hitchinset al., 1963; Keijser et al., 2007; Rousseau et al., 1972). Those authors reported that during germination, DPA, calcium, and spore mucopeptides rapidly flow out from inside the spores. Ghosh and Setlow (2009) have also reported that water uptake and the swelling of the core occur during spore germination. The loss of these materials finally leads to a decrease in optical density (Hitchins et al., 1963). In our study, it was therefore expected that spore germination occurred up to 60 min, with emergence and elongation occurring after 60 min.

Secondly, heat treatment was carried out at 80 °C for 15 min for different incubation times in order to quantitatively determine the extent of spore germination. Heat treatment at 80 °C for 15 min can kill germinated spores and vegetative cells, but does not affect dormant spores (Wuytack et al., 2000). As shown in Fig. 6, the numbers of germinated spores and vegetative cells increased with the incubation time. After the first 30 and 60 min of incubation, proportions of germinated spores and vegetative cells were 38% and 42%, respectively. The percentage of dormant spores after 1 h was still over 50%. Therefore, ungerminated spores were still remained at that time, so initiation of germination was still occurred after 1 h. When incubated for over 240 min, however, almost 90% of all the cells had germinated into vegetative cells.

Lastly, to confirm the degree of germination and outgrowth of spores, spore staining was performed and then observed with the aid of optical microscopy. Fig. 7 shows that more than 99% of the cells appeared greenish-blue at 0 h of incubation time, and the proportion of red vegetative cells gradually increased with the incubation time. After cells had been cultured for 6 h, the ratio of red stained vegetative cells was remarkably high. Overall it was considered that the spores germinated to become vegetative cells after about 60 min of incubation time, and some changes in the spore induced during this time affected their sensitivity against IPL.

3.4. Assumption of germination steps of spores and their relationship with IPL sensitivity

The process of germination and outgrowth of spores into vegetative

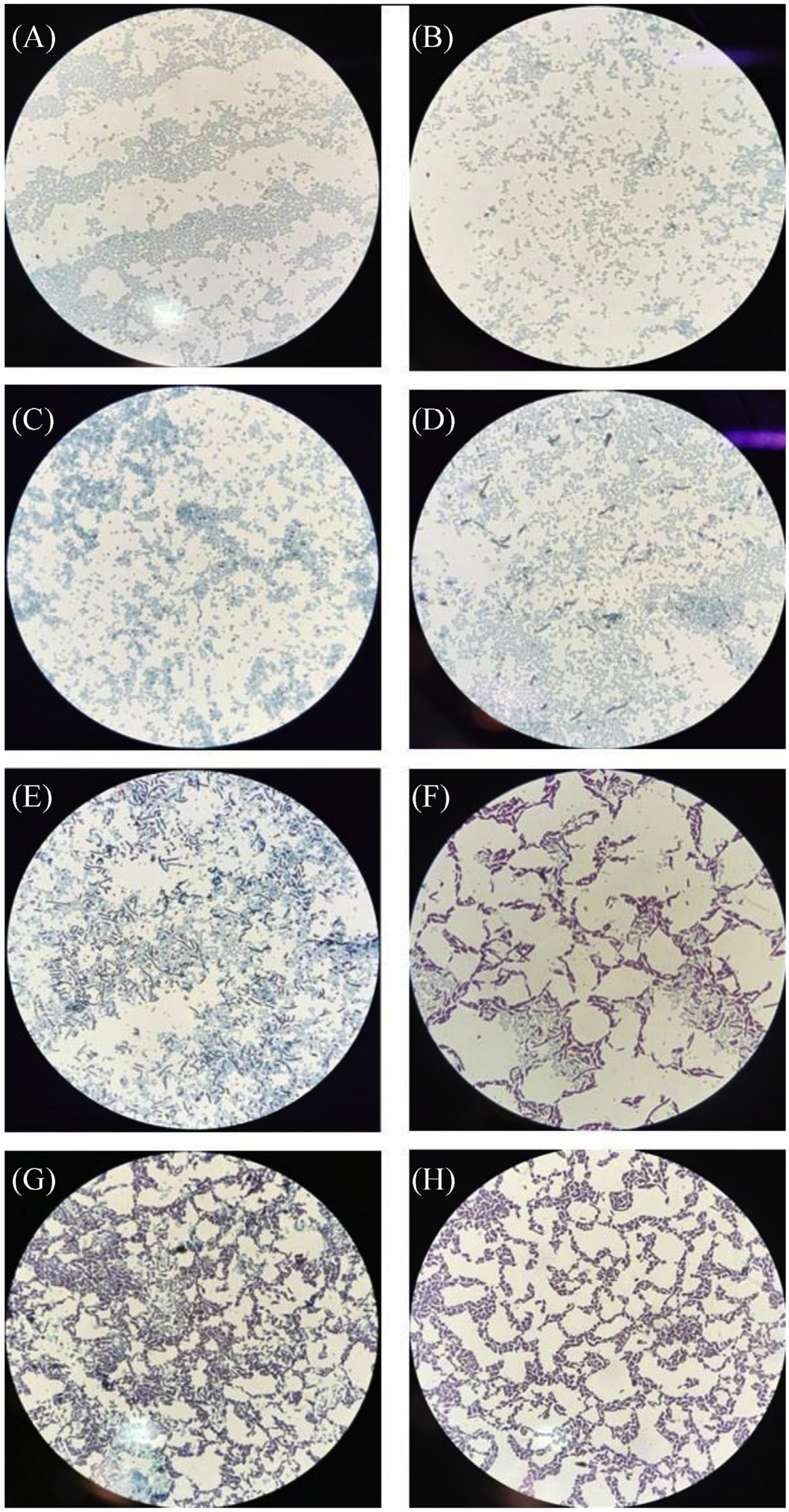

Fig. 7. Micrographs of *B. subtilis* spores stained with a modified Wirtz-Conklin stain for the following incubation times (in hours): (A) 0 (dormant spores), (B) 0.5, (C) 1, (D) 2, (E) 3, (F) 4, (G) 5, and (H) 6.

cells is a complex process in dependency of the environmental conditions. Germination is divided into stages I and II, and thereafter spores undergo an outgrowth process to finally become vegetative cells (Black et al., 2007; Setlow, 2003). In stage I of germination, cations and DPA are released and the spore core is partially hydrated. After passing through this process, in stage II of germination cortex hydrolysis and protoplast swelling occur, and finally the spores lose their dormancy (Black et al., 2007; Santo and Doi, 1974; Setlow, 2003). In the present study, a culturing period of up to 60 min was considered as stage I of germination (the early germination stage). Although not all spores began to germinate homogeneously, most spores were predicted to be in stage I of germination up to 60 min. We found that OD_{600} actually decreased for an incubation time of 60 min, demonstrating that internal materials of the spores had been released. Most importantly, it can be inferred that the leakage of DPA in the spores had a significant impact on the inactivation efficiency of the investigated pulsed light treatment.

DPA is known to affect the sensitivity to UV light (Germaine and Murrell, 1973). Spores of *B. subtilis* mutants with a low DPA content were found to be more resistant than wild-type spores to UV light because DPA acts as a photosensitizer (Paidhungat et al., 2000; Setlow et al., 2001). Also, DPA is implicated as being responsible for the heat resistance of spores (Setlow, 2006; Slieman and Nicholson, 2001), and so consideration of DPA can also explain why the inactivation level decreased continuously with the incubation time when the spores were heat treated in the present study. Although the DPA leakage of spores during IPL treatment has not been confirmed yet, it is inferred that IPL would exert similar effects on spores because IPL radiation includes the UV part of the spectrum.

4. Conclusion

This study has confirmed that *B. subtilis* spores can be reduced by IPL, which is one type of nonthermal sterilization technology. Since spores germinate rapidly to grow into vegetative cells when they are in an appropriate growth environment, it is very important to monitor how the sensitivity against the IPL changes during germination and outgrowth process as well as in dormant spores. This study found that the resistance to IPL temporarily increased during the initial germination period of spores, and then decreased thereafter, which contrasts with the resistance gradually decreasing during incubation for heat sterilization. The increase in IPL resistance during the early incubation period was attributed to intracellular changes during spore germination. DPA in the spore—which is the substance associated with the sensitivity to IPL—is released during the early stage, and hence this could explain the results of this study.

Before applying IPL treatment to food products, it will be necessary to confirm its inactivation effects by inoculating spores into such foods. Furthermore, it will be necessary to confirm how the IPL sensitivity changes during spore germination and outgrowth after the inoculation of food products.

Acknowledgement

This work was supported by Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry (IPET) through High Value-added Food Technology Development Program, funded by Ministry of Agriculture, Food and Rural Affair (MAFRA) (317030-3)..

References

Aguirre, J.S., de Fernando, G.G., Hierro, E., Hospital, X.F., Ordóñez, J.A., Fernández, M., 2015. Estimation of the growth kinetic parameters of *Bacillus cereus* spores as affected by pulsed light treatment. Int. J. Food Eng. 202, 20–26.

Artíguez, M.L., de Marañón, I.M., 2015a. Effect of pulsed light treatment on the germination of *Bacillus subtilis* spores. Food Bioprocess Technol. 8 (2), 478–485.

Artíguez, M.L., de Marañón, I.M., 2015b. Inactivation of *Bacillus subtilis* spores by combined pulsed light and thermal treatments. Int. J. Food Microbiol. 214, 31–37.

Atrih, A., Foster, S.J., 2002. Bacterial endospores the ultimate survivors. Int. Dairy J. 12 (2–3), 217–223.

Black, E.P., Setlow, P., Hocking, A.D., Stewart, C.M., Kelly, A.L., Hoover, D.G., 2007. Response of spores to high-pressure processing. Compr. Rev. Food Sci. Food Saf. 6 (4), 103–119.

Carlin, F., 2011. Origin of bacterial spores contaminating foods. Food Microbiol. 28 (2), 177–182.

Cho, W.I., Cheigh, C.I., Chung, M.S., Park, K.H., Chang, P.S., Chung, M.S., 2012. The combined effect of UV irradiation and ethanol extract from *Torilis japonica* fruit on inactivation of *Bacillus subtilis* spores. J. Food Saf. 32 (4), 474–480.

Cho, W.I., Choi, J.B., Lee, K., Cho, S.C., Park, E.J., Chung, M.S., Pyun, Y.R., 2007. Antimicrobial activity of medicinal plants against *Bacillus subtilis* spore. Food Sci. Biotechnol. 16 (6), 1072–1077.

Dunn, J., Ott, T., Clark, W., 1995. Pulsed-light treatment of food and packaging. J. Food Technol. 49 (9), 95–98.

Esbelin, J., Malléa, S., Clair, G., Carlin, F., 2016. Inactivation by pulsed light of *Bacillus subtilis* spores with impaired protection factors. Photochem. Photobiol. 92 (2), 301–307.

Germaine, G.R., Murrell, W.G., 1973. Effect of dipicolinic acid on the ultraviolet radiation resistance of *Bacillus cereus* spores. Photochem. Photobiol. 17 (3), 145–153.

Ghosh, S., Setlow, P., 2009. Isolation and characterization of superdormant spores of *Bacillus* species. J. Bacteriol. 191 (6), 1787–1797.

Hamouda, T., Shih, A.Y., Baker Jr., J.R., 2002. A rapid staining technique for the detection of the initiation of germination of bacterial spores. Lett. Appl. Microbiol. 34 (2), 86–90.

Hitchins, A.D., Gould, G.W., Hurst, A., 1963. The swelling of bacterial spores during germination and outgrowth. J. Genet. Micobiol. 30 (3), 445–453.

Hwang, H.J., Cheigh, C.I., Chung, M.S., 2015. Relationship between optical properties of beverages and microbial inactivation by intense pulsed light. Innov. Food Sci. Emerg. Technol. 31, 91–96.

Irie, R., Yano, N., Kembo, H., 1968. Kinetic analysis of the UV-resistant transient stage in spore germination of *Bacillus subtilis*. J. Gen. Appl. Microbiol. 14 (3), 279–293.

Jo, H.R., 2017. Inactivation of *Bacillus Subtilis* Spores Using Intense Pulsed Light (IPL) According to Incubation Times (Master's thesis). Retrieved form. http://dcollection.ewha.ac.kr/common/orgView/000000142429.

Keijser, B.J., TerBeek, A., Rauwerda, H., Schuren, F., Montijn, R., van der Spek, H., Brul, S., 2007. Analysis of temporal gene expression during *Bacillus subtilis* spore germination and outgrowth. J. Bacteriol. 189 (9), 3624–3634.

Levy, C., Bornard, I., Carlin, F., 2011. Deposition of *Bacillus subtilis* spores using an airbrush-spray or spots to study surface decontamination by pulsed light. J. Microbiol. Methods 84 (2), 223–227.

Levy, C., Aubert, X., Lacour, B., Carlin, F., 2012. Relevant factors affecting microbial surface decontamination by pulsed light. Int. J. Food Microbiol. 152 (3), 168–174.

Munakata, N., 1974. Ultraviolet sensitivity of *Bacillus subtilis* spores upon germination and outgrowth. J. Bacteriol. 120 (1), 59–65.

Nicholson, W.L., Munakata, N., Horneck, G., Melosh, H.J., Setlow, P., 2000. Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. Microbiol. Mol. Biol. Rev. 64 (3), 548–572.

Paidhungat, M., Setlow, B., Driks, A., Setlow, P., 2000. Characterization of spores of *Bacillus subtilis* which lack dipicolinic acid. J. Bacteriol. 182 (19), 5505–5512.

Pandey, R., TerBeek, A., Vischer, N.O.E., Smelt, J.P.P.M., Brul, S., Manders, E.M.M., 2013. Live cell imaging of germination and outgrowth of individual *Bacillus subtilis* spores; the effect of heat stress quantitatively analyzed with SporeTracker. PLoS One 8 (3), e58972.

Rousseau, P., Halvorson, H.O., Bulla Jr., L.A., Julian, G.S., 1972. Germination and outgrowth of single spores of *Saccharomyces cerevisiae* viewed by scanning electron and phase-contrast microscopy. J. Bacteriol. 109 (3), 1232–1238.

Santo, L.Y., Doi, R.H., 1974. Ultrastructural analysis during germination and outgrowth of *Bacillus subtilis* spores. J. Bacteriol. 120 (1), 475–481.

Setlow, P., 1994. Mechanisms which contribute to the long-term survival of spores of *Bacillus* species. J. Appl. Bacteriol. 76 (S23), 49S–60S.

Setlow, P., 2003. Spore germination. Curr. Opin. Microbiol. 6 (6), 550–556.

Setlow, P., 2006. Spores of *Bacillus subtilis*: their resistance to and killing by radiation, heat and chemicals. J. Appl. Microbiol. 101 (3), 514–525.

Setlow, B., Melly, E., Setlow, P., 2001. Properties of spores of *Bacillus subtilis* blocked at an intermediate stage in spore germination. J. Bacteriol. 183 (16), 4894–4899.

Setlow, B., Cowan, A.E., Setlow, P., 2003. Germination of spores of *Bacillus subtilis* with dodecylamine. J. Appl. Microbiol. 95 (3), 637–648.

Slieman, T.A., Nicholson, W.L., 2001. Role of dipicolinic acid in survival of *Bacillus subtilis*spores exposed to artificial and solar UV radiation. Appl. Environ. Microbiol. 67 (3), 1274–1279.

teGiffel, M.C., Beumer, R.P., Slaghuis, B.A., Rombouts, F.M., 1995. Occurrence and characterization of (psychrotrophic) *Bacillus cereus* on farms in The Netherlands. Neth. Milk Dairy J. 49, 125–138.

Wuytack, E.Y., Soons, J., Poschet, F., Michiels, C.W., 2000. Comparative study of pressure-and nutrient-induced germination of *Bacillus subtilis* spores. Appl. Environ. Microbiol. 66 (1), 257–261.