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Effects of microbial loading and sporulation temperature on atmospheric plasma inactivation of *Bacillus subtilis* spores

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Current inactivation studies of *Bacillus subtilis* spores using atmospheric-pressure glow discharges (APGD) do not consider two important factors, namely microbial loading at the surface of a substrate and sporulation temperature. Yet these are known to affect significantly microbial resistance to heat and hydrogen peroxide. This letter investigates effects of microbial loading and sporulation temperature on spore resistance to APGD. It is shown that microbial loading can lead to a stacking structure as a protective shield against APGD treatment and that high sporulation temperature increases spore resistance by altering core water content and cross-linked muramic acid content of *B. subtilis* spores. © 2005 American Institute of Physics. [DOI: 10.1063/1.2103394]

Conventional decontamination strategies including autoclaving, ethylene oxide, and hypochlorite treatments can have undesirable effects when applied to certain foods and polymer-based medical devices, such as catheters and endoscopes.¹⁻³ Atmospheric-pressure glow discharges (APGD) offer the prospect of low-temperature and nontoxic inactivation of micro-organisms in these and other applications. So far the extent of their biocidal efficacy has been established typically using bacterial spores including those of *Bacillus subtilis*.⁴⁻⁸ However, comparison of available efficacy data poses difficulties as surface microbial loadings, the concentration of bacteria applied per unit surface area, differ widely between different studies. For low-pressure vacuum plasmas, microbial loading is known to affect their inactivation efficiency⁹ and so should be considered also in APGD inactivation studies. Meaningful comparison of inactivation kinetics is further compounded by the fact that spores used in previous APGD studies have often been generated under different environmental conditions. In particular sporulation temperature has been shown to influence spore resistance to many decontamination treatments such as wet heat and hydrogen peroxide.^{10,11} In this letter, we present a study of the effects of microbial loading and sporulation temperature on APGD inactivation of *B. subtilis*.

The strain of *B. subtilis* used here was ATCC 6633/NCIMB 8054, and spores were produced using the method of Harnulv and Snygg.¹² Specifically, after an incubation period of 8, 6, and 4 days, respectively, at sporulation temperatures of 22, 30, and 47 °C, most *B. subtilis* cells had sporulated. The resulting spores were harvested by centrifugation at $7367 \times g$ at 5 °C for 20 min. They were then washed four times in order to remove all unwanted constituents. To ensure that the spore samples were free from vegetative cells, a

heat shock was applied by leaving the suspension in a 70 °C water bath for 30 min. The spore suspensions were refrigerated until required.

For convenience and ease of reproducibility, spores were deposited onto the surface of Whatman polycarbonate membranes. To prepare samples for plasma treatment the spore stock solutions were pipetted at the required concentration (100 μ l) onto the membrane filters. The spore-laden membranes were left to dry in a laminar flow cabinet for 45 min before plasma treatment. Ringer solution (Oxoid) was used to recover plasma-treated spores from the membranes, after which they were plated onto Tryptone Soya Agar (Oxoid) and incubated for 30 °C overnight. Counts are expressed in terms of colony forming units per filter (100 μ l). The APGD system used for this work was based on a coaxial configuration consisting of a powered copper electrode with ceramic coating and a concentric glass tube enclosing the powered electrode. Helium (99.9995% purity) was fed through the space between the powered electrode and the glass tube for ionization. The ionized gas was flushed out of the electrode unit and into ambient air towards a point downstream where the membrane filter was placed. The helium flow was fixed at 3 slm.

Figure 1 shows survival curves of *B. subtilis* spores against time, with different initial spore densities at a fixed sporulation temperature of 30 °C. Although not shown in Fig. 1, control experiments were performed using a helium flow of 3 slm and with the plasma switched off. No inactivation occurred under these conditions. Figure 1 shows that the resistance of *B. subtilis* spores is dependant on the initial spore density. For an initial spore density of up to 10^6 per filter, a 3-log reduction was achieved in less than 200 s, whereas an initial spore density of 10^9 per filter required a longer plasma treatment of about 360 s. This represents an increase of more than 80%. The greater resistance at higher spore densities may result from stacking where the spores

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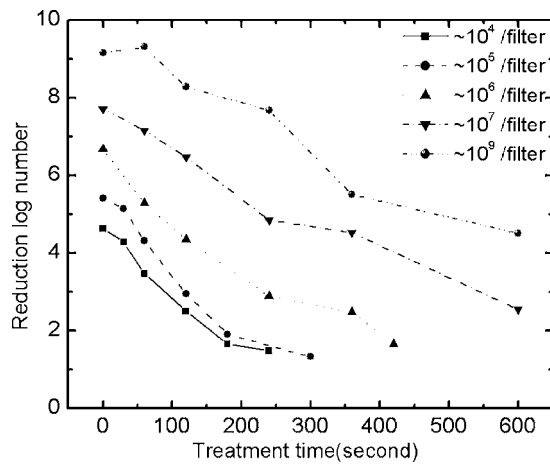


FIG. 1. Plasma inactivation curves of *Bacillus subtilis* spores at different initial densities.

form a multilayered structure on the membrane filter. In such circumstances, the top layers of spores, even if inactivated, could form a physical barrier to shield those beneath them from plasma penetration and hence contribute to increased survival. A simple estimate can be made to confirm the possible existence of the earlier-mentioned stacking structure in the range of microbial loadings of Fig. 1. *B. subtilis* spores are rod shaped with a length of 1.5–1.8 μm and a diameter of 0.8 μm . Hence, when lying flat on a surface, a single *B. subtilis* spore takes up a surface area of approximately $1.65 \times 0.8 = 1.3 \mu\text{m}^2$. In our experiments, the spores were confined to a region on the filter having a surface area of 0.78 cm^2 . Therefore, the maximum density occupied by a single monolayer of *B. subtilis* spores is about $0.78 \text{ cm}^2 / 1.3 \mu\text{m}^2$ or 6.0×10^7 per filter. In reality, it is highly unlikely that the spores would neatly stack against one another in a single monolayer and multilayers would start to form at spore densities below the figure quoted above. Indeed, examination of scanning electron microscopy images confirms that spore distribution was not uniform on the membrane filter and that considerable stacking may form at initial bacterial densities above 10^6 per filter. In other words, an initial microbial loading above 10^6 per filter is likely to produce a bacterial stacking structure and lead to a marked increase in spore resistance to APGD treatment. This correlates very well with the longer treatment time observed in Fig. 1 for a 3-log reduction at initial spore densities above 10^6 /filter.

To see this more clearly, Fig. 2 shows spore density dependence of D value, the time required to achieve 90% reduction of viable bacteria.^{12,13} The spore density dependence of the D value appears to have three phases, a low-density phase below 10^6 /filter in which the D value remains roughly unchanged at around 70 s, a mid-density phase of 10^6 – 10^8 /filter in which the D value changes rapidly, and a high-density phase above 10^8 /filter in which the D value again stays roughly unchanged. The D value starts to change rapidly at spore densities above 10^6 /filter. This correlates well with the estimate made above at which stacking structures begin to form, suggesting that the physical barrier of these structures appears to be a dominant cause for the increased spore resistance at microbial loadings above 10^6 /filter. It is worth mentioning that bacterial stacking has also been considered important in microbial inactivation us-

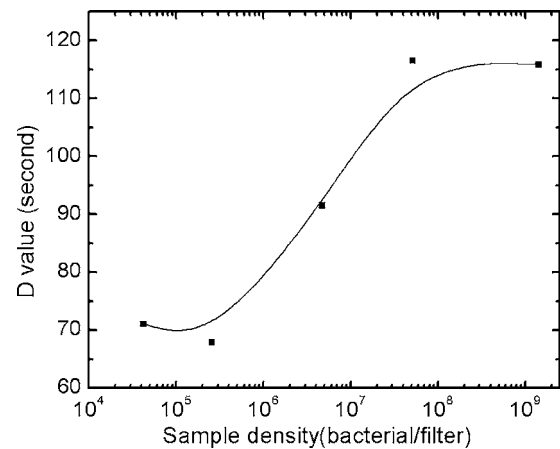


FIG. 2. Dependence of the D value on initial bacterial densities of *Bacillus subtilis* spores.

ing low-pressure vacuum plasmas.⁹ The range of D values in Fig. 2 is comparable with those obtained in other APGD inactivation studies^{1,5–8} but significantly lower than what has typically been achieved with low-pressure vacuum plasmas.⁹

Sporulation temperature has recently been shown to affect the resistance of *B. subtilis* spores against many inactivation agents including wet heat, hydrogen peroxide, Beta-dine, formaldehyde, glutaraldehyde, superoxidized water, and UV radiation.¹⁴ It seems highly likely that it may also affect spore resistance to gas plasmas. To this end, *B. subtilis* spores were prepared at three different sporulation temperatures, namely 22, 30, and 47 $^\circ\text{C}$, with all other procedures kept unchanged. Figure 3 shows the normalized log reduction against APGD treatment time for *B. subtilis* sporulated at different temperatures. It is clear that the spore survival declines as the sporulation temperature increases. APGD treatment for 10 min leads to approximately a 6-log reduction for *B. subtilis* spores at a sporulation temperature of 22 $^\circ\text{C}$ and only 3.5-log reduction at 47 $^\circ\text{C}$. Initial spore densities for these two cases were 5×10^7 and 6×10^7 per filter, respectively, and such a difference in microbial loadings should, according to Fig. 2, make little difference in their resistance to plasma treatment. Therefore the considerable change in spore resistance to the plasma in Fig. 3 is likely to be due to sporulation temperature alone.

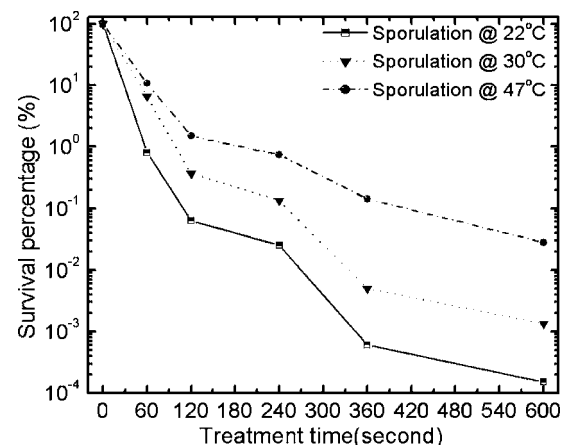


FIG. 3. Survival curves of *Bacillus subtilis* spores at different sporulation temperatures and similar initial spore densities of around 5×10^7 per filter.

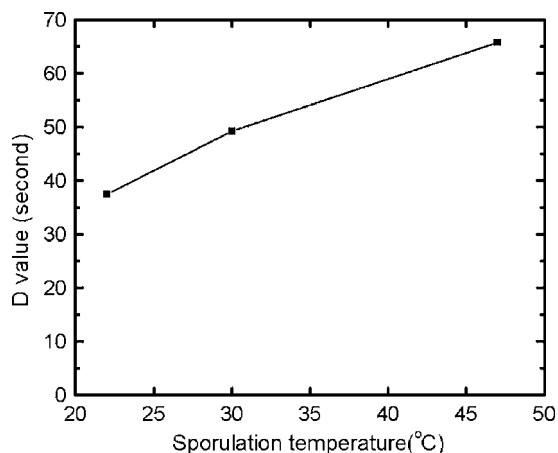


FIG. 4. Dependence of the D value on the sporulation temperature.

D values obtained from Fig. 3 are plotted against sporulation temperature in Fig. 4, showing an almost linear dependence with a 76% increase in sporulation temperature from 22 to 47 °C. The survival curve of Fig. 3 suggests that resistance of *B. subtilis* spores to APGD is very similar to their resistance to wet heat and hydrogen peroxide.¹⁴ It is known that the wet heat resistance of *Bacillus subtilis* spores depends on several factors including (a) the spore core water content and (b) cross-linked muramic acid content in the peptidoglycan of the spore cortex.¹⁴ Measurement of these two spore properties for *B. subtilis* strain PS3302 suggests that they also change in an approximately linear fashion as the sporulation temperature increases from 22 to 47 °C. Specifically the core water content decreases by 12% whereas cross-linked muramic acid content increases by 21%.¹⁴ While these data were obtained for a different *B. subtilis* strain from that used in this study (ATCC 6633), the effects of sporulation temperature are almost certainly to be much greater than effects arising from using different spore strains.¹⁵ Therefore the linear sporulation temperature dependence of the D value observed in our APGD study can be correlated to that of the core water content and cross-linked muramic acids. This suggests that the biological targets of

our APGD are likely to be related to (a) core water content and (b) cross-linked muramic acid.

This letter shows the significant effects of initial microbial loadings and sporulation temperature on the resistance of *B. subtilis* spores against atmospheric-pressure glow discharges. At microbial loadings above 10^6 per filter, a stacking structure is likely to develop and thereby restrict plasma penetration to access deep-embedded spores. Also *B. subtilis* spores prepared at a higher sporulation temperature are shown to be more resistant to APGD, and the increased resistance is likely to relate to (a) reduced core water content and (b) increased muramic acids with crosslink of *B. subtilis* spores.

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