Cold Plasma Inactivation of *Bacillus cereus* and *Bacillus anthracis* (Anthrax) Spores

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***Abstract*—*Bacillus* spores represent one of the most resistant organisms to conventional sterilization methods. This paper is focused on the inactivation of the spores of two *Bacillus* species, *Bacillus cereus* and *Bacillus anthracis*, using atmospheric-pressure dielectric-barrier-discharge (DBD) plasma. Spores treated in liquid or air-dried on a solid surface were effectively inactivated within 1 min of DBD plasma treatment at a discharge power of 0.3 W/cm^2. Results of a series of model experiments show that neutral reactive oxygen species and UV radiation play a dominant role in the inactivation of spores. We also show that 45 s of the DBD plasma treatment of air-dried spores placed inside closed plastic or paper envelopes permits up to 7 log reduction of viable spores.**

***Index Terms*—Anthrax, atmospheric-pressure dielectric barrier discharge (DBD), *Bacillus anthracis*, nonequilibrium plasma, nonthermal plasma, spore inactivation, sterilization.**

I. Introduction

NONTHERMAL atmospheric-pressure plasmas are intensively studied for possible use in various biological and medical applications. One of them is the inactivation of microorganisms in water and air and on surfaces [1]–[7], including one of the most attractive applications of plasma—living-tissue sterilization [8]–[13]. *Bacillus* species, which are ubiquitous in the environment, are aerobic or facultative anaerobic gram-positive bacteria [14]–[16]. The genus *Bacillus* is divided into three broad groups, depending, among other characteristics, on the morphology of the spore. *Bacillus cereus*, *Bacillus anthracis* (anthrax), and *Bacillus thuringiensis* belong to the *Bacillus cereus* group [14], [17]. Moreover, morphological and chromosomal similarities between these species have prompted the view that *Bacillus anthracis*, *Bacillus thuringiensis*, and *Bacillus cereus* are all varieties of a single species [14]. Bacilli can produce a dormant cell type called a spore in response to nutrient-poor conditions. Bacterial spores have little or no metabolic activity and can withstand a wide range of environmental assaults including heat, UV, and solvents [18]–[21]. To kill or inactivate *Bacillus* spores, one can apply an 0.88-mol/L hydrogen peroxide at a pH of 5.0 for 3 h to sterilize a spore suspension of 10^6 spores/mL, or 10^6 rad of gamma irradiation to sterilize 10^6 spores/mL [21].

Bacillus anthracis spores, as opposed to vegetative cells, are the infectious form and cause anthrax. The spores of *Bacillus anthracis* represent a noteworthy bioterrorism agent and can be easily distributed in dry form in parcels and letters via postal service (as what occurred in 2001, when anthrax-contaminated letters sent through the U.S. postal service killed 5 people and sickened 23 others [22]), in aerosols, or in contaminated water, for instance. In response to these possibilities, an effective, low-energy, and cost-effective method of spore inactivation or sterilization is required. An attractive method of spore inactivation is plasma treatment. Low-temperature plasma at low pressure, arc discharge plasma, microwave plasma, and other plasmas are effective in the sterilization of spores [23]–[27]. For example, Kuo *et al.* reported that a 3–5 log reduction of *Bacillus cereus* spores in aqueous suspension can be achieved after several seconds of treatment with arc-seed microwave (2.45 GHz and 700 W) plasma torch [24]. Several systems based on different types of discharge have been reported [24]–[27]. In most of these systems, spores were treated either at low pressure or with relatively high power discharges, and 1–5 log reduction of germinated spores was achieved within a few minutes of treatment.

In this paper, we were interested in inactivating *Bacillus* spores both in dry form and suspended in water with the use of atmospheric-pressure dielectric-barrier-discharge (DBD) plasma on surfaces as well as inside closed volumes, e.g., envelopes. We reported previously on the sterilization of bacteria and yeast, including skin flora such as streptococcus and staphylococcus, on agar surfaces with atmospheric-pressure DBD plasma [28]. It took 5–10 s in the case of direct DBD treatment to achieve up to an 8 log reduction of a mixture of staphylococci, streptococci, and Candida yeast species. The results of this paper show that the inactivation of bacteria in spore form both in liquid or air-dried on surface requires higher doses of DBD plasma treatment, and up to 5 log reduction can be achieved within a minute of exposure to plasma. It is also

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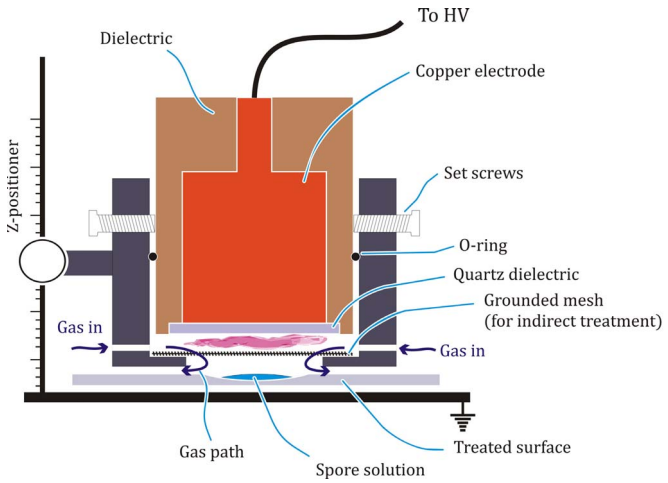


Fig. 1. Principal schematic of the DBD plasma treatment setup with removable mesh, used for indirect treatment. Additional flow of ethanol vapor may be provided as shown by gas flow arrows.

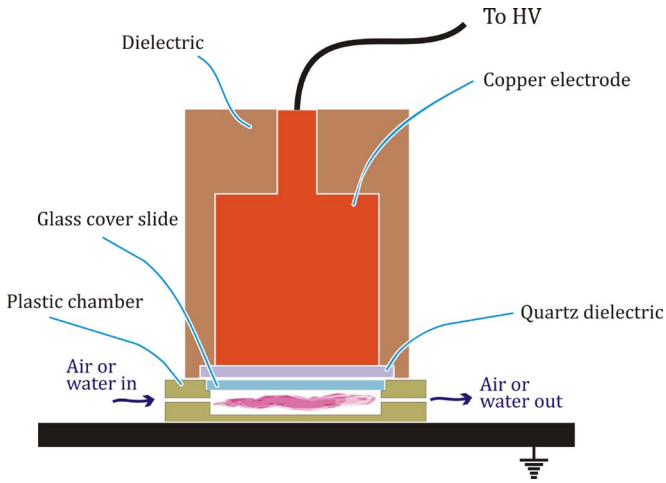


Fig. 2. Schematic of the setup for treatment of air-dried spores with direct DBD plasma.

shown that the mechanisms by which direct plasma inactivates spores and vegetative bacteria may be quite different.

II. Materials and Methods

We used atmospheric-pressure DBD plasma to treat *Bacillus* spores at room temperature in air. The powered electrode was made of a 2.5-cm-diameter solid copper disk covered by a 3.5-cm-diameter 1-mm-thick quartz dielectric. The discharge gap was kept at 1.5 mm. The microsecond-pulsed DBD discharge was ignited by applying an ac-pulsed high voltage of 30-kV magnitude (peak to peak) and 1.3-kHz frequency between the electrodes. Current peak duration was 1.2 μs, and the corresponding plasma surface power density was 0.3 W/cm^2.

The spores were treated in either dry form or in aqueous suspension on glass slides (Fig. 1). The slides were placed on top of grounded metal, and the plasma was ignited directly between the powered electrode and the treated spores. For the treatment of spores in water, in order to avoid the splashing of the solution due to charging during plasma treatment, we used hanging drop slides with well diameters of 15 mm and capacities of 20 μL (Fischer Scientific). To prevent the loss of spores due to disbursement during the treatment of air-dried spores, the spores were dried in a plastic chamber (Fig. 2) placed on a grounded metal surface and covered with a glass cover slide. The chamber was ventilated with air for faster drying of samples and was washed with water to collect spores after treatment.

Another set of experiments was carried out using indirect plasma treatment. In this case, we used a modified plasma system (Fig. 1), where a grounded metal mesh (22 wires/cm, 0.1-mm wire diameter, 0.35-mm openings, 60% open area, and weaved mesh) was used as a second electrode. The gap between the mesh and the second electrode was kept at 1.5 mm, which is the same as that in the direct treatment setup. The distance between the mesh and the treated surface was also kept at 1.5 mm.

In this paper, we used *Bacillus cereus* (ATCC 6464, *Bacillus cereus* Frankland and Frankland) and *Bacillus anthracis* (Sterne strain 7702) spores suspended in distilled water at 10^5–10^8 spores/mL. These experiments were first done using *Bacillus cereus* spores as a surrogate for *Bacillus anthracis* spores and then repeated with *Bacillus anthracis* species. The spores were prepared according to standard protocol [29], [30]. Each experiment was repeated three times. The treated spores were diluted and plated on brain–heart infusion (Fisher Scientific) agar, incubated at 37 °C overnight, and colony-forming units (CFUs) were counted. All work was done in a BSL 2 laboratory, certified by Drexel University and by the CDC.

A. Treatment of Spores in Suspension

Ten microliters of *Bacillus cereus* or *Bacillus anthracis* spores in distilled water at concentrations of 10^7, 10^6, and 10^5 spores/mL were placed in hanging drop glass slide wells and treated for 5–45 s with direct DBD plasma. After treatment, bacteria were appropriately diluted and plated, as aforementioned. Experiments were done in duplicate on three different days.

B. Treatment of Dried Spores in Room Air

Spores were placed inside a plastic chamber (see Fig. 2), covered with a glass cover slide and dried for about 30 min with a constant air flow of about 0.1 L/min (control experiments with only gas flow through the chamber showed no loss of collected spores). Ten microliters of *Bacillus cereus* at 10^8, 10^7, and 10^6 spores/mL in distilled water were used. Dried spores were treated with direct DBD plasma for 5–45 s and then washed out of the chamber with 30 mL of distilled water, appropriately diluted and plated, as aforementioned. Similar experiments were done using *Bacillus anthracis* spores, except that 10 μL of *Bacillus anthracis* spores were placed inside either plastic or paper envelopes, dried in room air for 1 h and treated with DBD plasma. In these experiments, the discharge was ignited in the volume of either the plastic chamber or the paper or plastic envelope [envelopes were slightly inflated to assure that walls are in contact with powered and grounded electrodes (Fig. 3)].

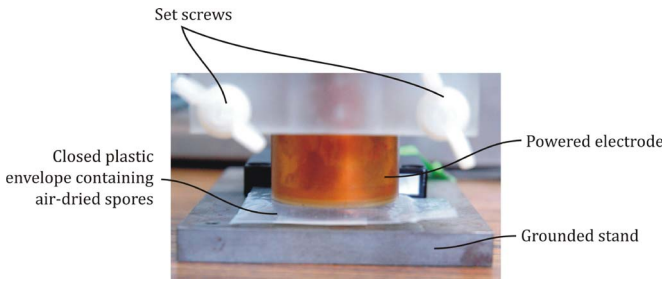


Fig. 3. Setup for DBD plasma treatment of air-dried spores contained inside a closed envelope.

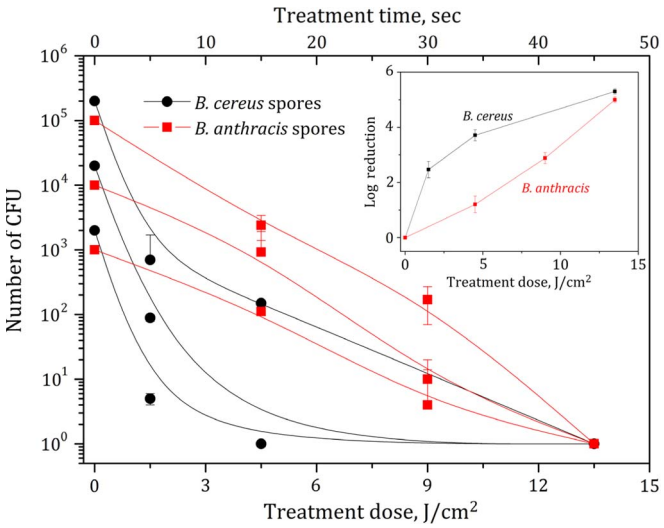


Fig. 4. Inactivation of various concentrations of (filled squares) *Bacillus cereus* and (filled circles) *Bacillus anthracis* spores in water on glass slides using direct DBD plasma treatment.

C. Direct Versus Indirect Treatment of Spores in Room Air

We compared the effects of direct and indirect DBD plasma treatments on *Bacillus cereus* spore viability. Ten microliters of spores in water at 10^5 spores/mL were treated with direct or indirect plasma for 10–60 s and appropriately diluted and plated.

III. Results and Discussion

In the first series of experiments, the spores of two *Bacillus* species, namely, *Bacillus cereus* and *Bacillus anthracis*, were treated in water droplets on the surface of glass slides using atmospheric-pressure DBD plasma in room air. The inactivation kinetics of these *Bacillus* spores of three different concentrations after DBD plasma treatment are shown in Fig. 4. The number of colonies after treatment is plotted on a logarithmic scale as a function of the treatment dose and time, which are directly related. Although the spores of both species were effectively inactivated (up to 5 log reduction in less than a minute of treatment), $\log N$, i.e., killing kinetics, was quite different for *Bacillus cereus* versus *Bacillus anthracis* spores—two-step versus linear, respectively. In addition, anthrax spores appeared to be slightly more resistant to plasma treatment (see inset of Fig. 4). These species have similar morphology, metabolism, and physiology, and a similar behavior of the inactivation kinetics was reported previously, for example, when aqueous

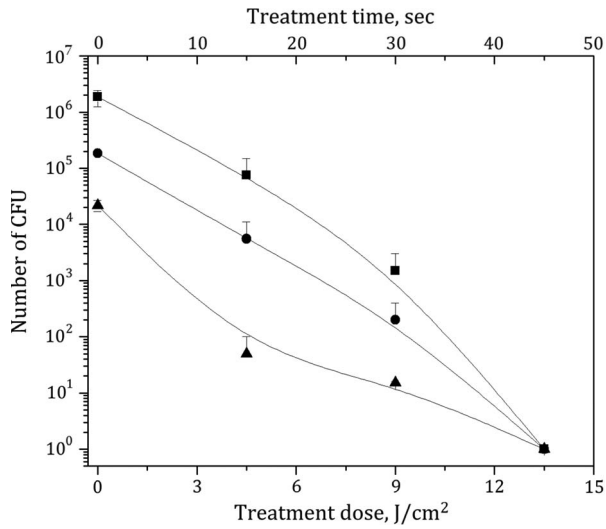


Fig. 5. Inactivation by direct DBD plasma of various concentrations of dry *Bacillus cereus* spores contained inside a closed plastic chamber.

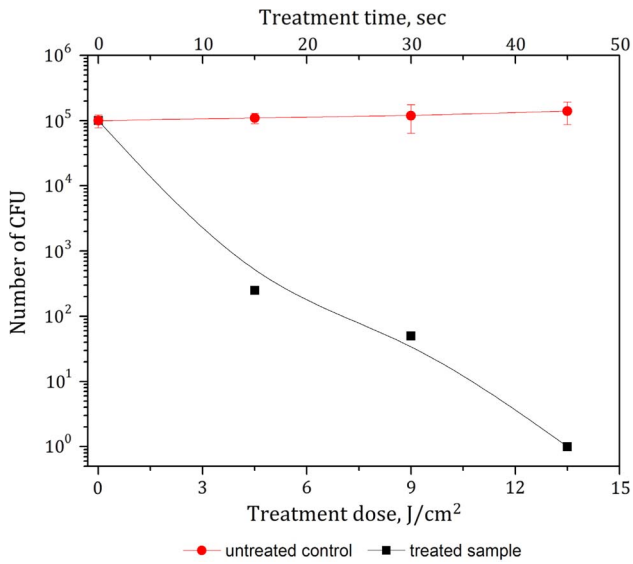


Fig. 6. Inactivation by direct DBD plasma of dry *Bacillus anthracis* spores contained inside a paper envelope.

spore suspensions were treated with UV_{254} irradiation (see [31, Fig. 6]). It was noted by the authors that this difference may be related to virulence plasmids that may influence the UV sensitivity of *Bacillus cereus* and *Bacillus anthracis* spores.

In contrast to the inactivation of spores suspended in distilled water, the inactivations by the plasma treatment of air-dried *Bacillus cereus* and *Bacillus anthracis* spores both appear to be linear. Fig. 5 shows the results of the plasma inactivation of three different concentrations of *Bacillus cereus* spores dried inside a plastic chamber (see Fig. 2). The inactivation of 10^7 per mL anthrax spores inside a closed paper envelope is shown in Fig. 6. Similar results were obtained in the case of the plastic envelope (data not shown). These results are interesting, as they show the high efficiency of DBD-plasma-based systems to sterilize within temperature-sensitive materials. This is important in view of the 2001 bioterrorism attacks when anthrax spores in envelopes were distributed through the U.S. postal service, resulting in illness and death [22].

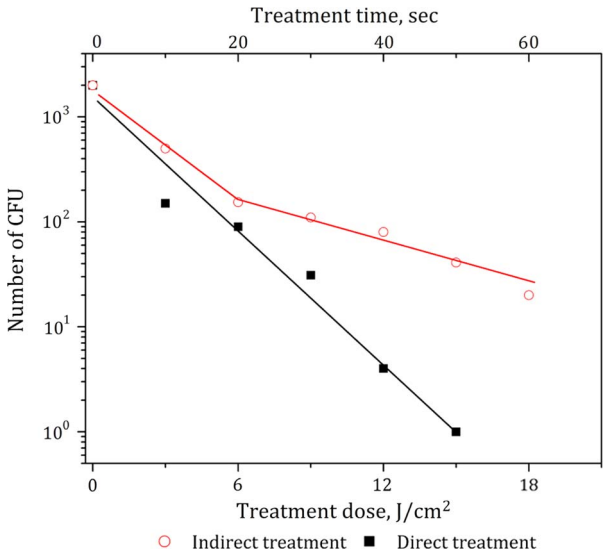


Fig. 7. Inactivation of *Bacillus cereus* spores in water using direct and indirect DBD plasmas.

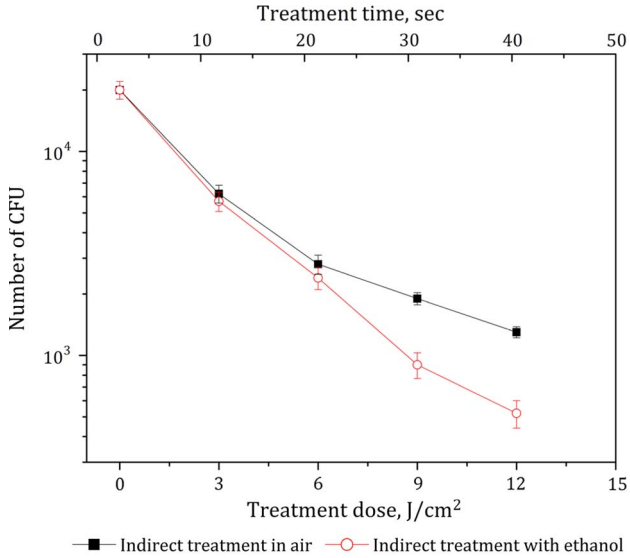


Fig. 8. Effect of ethanol vapor on inactivation of aqueous suspensions of *Bacillus cereus* spores by indirect DBD discharge.

Fridman *et al.* [28] observed a significant difference (a few orders of magnitude) in the killing of bacteria (streptococcus and staphylococcus) and yeast when charged species were excluded from plasma by introducing a grounded metal mesh between the powered electrode and the treated surface. In this case, plasma is generated remotely (indirectly), and active neutral species and radiation are delivered to the object with the plasma afterglow. In our previous experiments [28], we observed that, in the case of the direct plasma treatment of bacteria on agar with an initial concentration of 10^8 CFU/mL in phosphate-buffered saline, complete sterilization was achieved in 5–10 s, and in the case of the indirect plasma treatment, complete sterilization was achieved in more than 15 min. To determine the role of charged species (electrons, ions, and associated electric fields) on the inactivation of spores, we performed similar experiments using *Bacillus cereus* spores in distilled water (Fig. 7). The difference in spore inactivation, about 1.5 log, appears to be not as large as that for vegetative bacteria; however, it is still significant. Moreover, in the case of indirect plasma treatment, the inactivation process clearly consists of two stages, with about the same inactivation rate in the first stage as we observed for direct plasma treatment. The effect of charged species, electrons, and ions may be related to the production of hydrogen peroxide in liquid phase (similar reactions may take place in the gas phase; all experiments were done in room air at about 60% relative humidity)

$$\begin{cases} \mathbf{e}^-_{(\mathbf{H_2O})} + \mathrm{O}_{2(\mathbf{H_2O})} \rightarrow \mathrm{O}^-_{2(\mathbf{H_2O})} \\ \mathbf{2O_2^-} + 2\mathrm{H}^+ \rightarrow \mathbf{H_2O_2} + \mathrm{O}_2 \end{cases} \tag{1}$$

$$\begin{cases} M^+ + \mathrm{H_2O} \rightarrow M + \mathrm{H_2O}^+ \\ \mathrm{H_2O}^+ + \mathrm{H_2O} \rightarrow \mathrm{H_3O}^+ + \mathbf{OH} \\ \mathrm{OH} + \mathrm{OH} \rightarrow \mathbf{H_2O_2} \end{cases} \tag{2}$$

where M^+ is any positively charged ion. Hydrogen peroxide is readily soluble in water and is relatively easily transported through outer spore coats [32]. At appropriate conditions, hydrogen peroxide may be converted inside the spores to hydroxyl radicals through a natural reaction chain termed Fenton mechanism

$$\begin{cases} \mathbf{H_2O_2} + \mathrm{Fe}^{2+} \rightarrow \mathbf{OH} + \mathrm{OH}^- + \mathrm{Fe}^{3+} \\ \mathrm{Fe}^{3+} + \mathrm{O}_2^- \rightarrow \mathrm{Fe}^{2+} + \mathrm{O}_2. \end{cases} \tag{3}$$

To determine the role of neutral OH radicals, which may react directly with spores due to their extremely high reactivity or which may be converted into hydrogen peroxide, we introduced ethanol vapors into indirect DBD plasma discharge and treated *Bacillus cereus* spore suspensions in water (in plasma, ethanol is easily decomposed with the formation of OH radicals and hydrogen peroxide [33], [34]). There was no decrease in spore viability in control experiments where only ethanol vapor flowed through the system (data not shown). The results (Fig. 8) show that additional production of OH radicals allows greater inactivation of spores.

To see if morphological differences occurred, we analyzed the spore morphology before and after DBD plasma treatment. *Bacillus cereus* spores were deposited onto stainless steel coupons and were observed by scanning electron microscopy (SEM). The samples were then exposed to DBD plasma for 1 min (corresponding to a dose of 18 J/cm^2), and SEM photomicrographs of the same spores were taken again. As can be seen when comparing the photomicrographs of spores before and after treatment (Fig. 9), the inactivation mechanism is not the erosion of the spores, since their protective coats appear to be undamaged. Therefore, we believe that the diffusion of chemically active oxygen species (e.g., $\mathrm{H_2O_2}$) into spores followed by the damage of internal macromolecules or molecular systems may be the primary mechanism of spore inactivation.

IV. Conclusion

In summary, the mechanism by which DBD plasma kills *Bacillus* spores appears to be quite different from the

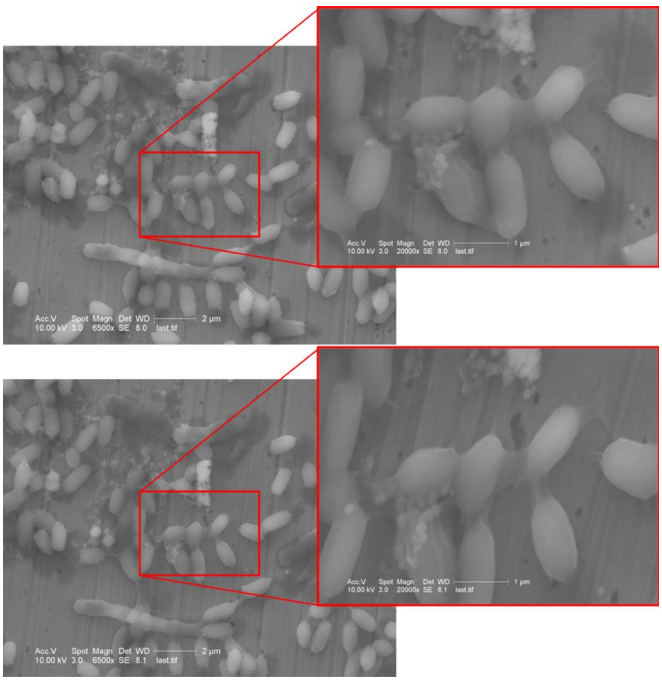


Fig. 9. SEM photomicrographs of *Bacillus cereus* spores on a stainless steel coupon (top) before and (bottom) after exposure to direct DBD plasma (same spot of one sample in both photos).

mechanism by which it kills vegetative bacilli. As indicated previously, a two-stage process occurs in the killing of spores by DBD plasma. The first stage, which is not sensitive to the presence of charged particles, may be dominated by the effect of UV radiation generated by the plasma. This supposition comes from the following observations: 1) The initial rate of inactivation of spores is about the same for direct and indirect plasmas (slightly less for indirect, since the metal mesh allows less radiation to access the sample), and 2) there is a difference in the inactivation rate of *Bacillus cereus* and *Bacillus anthracis* spores, which may be related to different sensitivities due to differences in, for example, plasmid and surface content. The second stage appears related to the production of neutral reactive oxygen species that are transported inside the spore to cause damage to biomolecules critical to the cell's survival or germination. One possible effect may be related to the oxidation of germination proteins located in spore coats or the inactivation of germination receptors which are located on inner spore membranes [35], [36]. Both germination proteins and germination receptors are required for successful germination [35], [36]. Hydrogen peroxide is one possible agent responsible for such effects, as it is known to inactivate spores at low concentrations [18]. It was suggested that lethal action occurs through the inactivation of enzymes, which are mainly located in the spore protoplast, and of the germination apparatus, while no lysis of spores is noticed [18].

We have demonstrated that DBD plasma is able to effectively inactivate *Bacillus* spores both in liquid and in dry form: Up to 5 log reduction of spores was observed after less than a minute of treatment. We also showed that active oxygen radicals play a significant role in the inactivation process.

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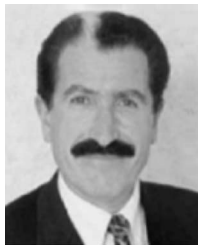
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