Highly Effective Large-Area Inactivation of *B. subtilis* Spore Using a Planar Dielectric Barrier Discharge Jet With CF_4/Air Mixture

Yi-Wei Yang, Jane-Yii Wu, Ming-Hung Chiang, and Jong-Shinn Wu

***Abstract*—In this paper, we demonstrate that, by adding a small amount of CF_4 (2%) into a compressed-air planar dielectric barrier discharge (DBD), one can effectively inactivate a *B. subtilis* spore up to 50 mm in width in the postdischarge jet region after ten passes of exposure of plasma plume (equivalent plasma exposure time of 1 s) for treatment distance of up to 14 mm for the spore concentration in the range of 10^5-10^7 CFU/mL. A possible mechanism is proposed, i.e., the chemically active F atoms and CF_n $(n = 1 - 3)$ are generated through the help of abundant excited nitrogen molecules in the postdischarge region.**

***Index Terms*—Air and *B. subtilis* spore, atmospheric-pressure plasma (APP), CF_4, dielectric barrier discharge (DBD), inactivation, sterilization.**

I. Introduction

CONVENTIONAL sterilization technologies include, for example, autoclaves, ovens, chemicals (e.g., ethylene oxide), and radiation (gamma rays) [1], [2]. However, these methods have several disadvantages, such as high energy consumption, high temperature for medical product (e.g., polymer) and foodstuff [3], or harmful to human beings (e.g., radiation). Recently, plasma has been considered as one of the promising technologies, which could possibly replace these techniques for sterilization.

There are many studies focusing on the use of low-pressure plasmas as alternatives for sterilization (e.g., [4]–[6]). However, handling the low-pressure test pieces imposes several disadvantages, such as expensive vacuum equipment and inconvenience of vacuum break. Recently, the use of nonthermal atmospheric-pressure plasma (APP) has demonstrated a promising alternative to the low-pressure one and attracted tremendous attention (e.g., [7] and [8]). Moreover, by treating the test species in the postdischarge region, it can render the APP jet (APPJ) source a stand-alone module and also protect substrates from thermal damage and ion bombardment.

Manuscript received February 21, 2012; revised June 7, 2012; accepted August 11, 2012. Date of publication September 14, 2012; date of current version November 6, 2012.

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Digital Object Identifier 10.1109/TPS.2012.2214238

Many studies [9]–[12] have shown that air atmospheric-pressure dielectric barrier discharge (DBD) can sterilize a *B. subtilis* spore, which is a well-known tough target bacterium. However, treatment time usually required a few minutes for total sterilization, which may not be efficient enough for practical applications. In addition, most of them sterilized the spore directly in the discharge region that is often very narrow in APP, which might be inconvenient in practical applications.

In this paper, we demonstrate that a planar cold DBD-APPJ with air mixing with a small quantity of CF_4 (2%) can be used to sterilize large-area of *B. subtilis* spore efficiently after 10 passes of exposure (equivalent plasma exposure time: 1.0 s) for treatment distance of up to 14 mm.

II. Experimental Methods

Fig. 1 shows the experimental setup of the DBD-APPJ for the sterilization. The discharge is formed by two-parallel electrode plates (5 × 5 cm) with a ceramic material of 2 mm in thickness at the inside and a gap distance of 1 mm. The plasma was a typical Townsend-like homogeneous discharge that was maintained by distorted sinusoidal voltages at a frequency of 30 kHz and 300 W (EN Technologies Inc. model Genius 2). Pure air and air with 2% of CF_4 was controlled to flow through the gap, respectively, at the flow rate of 5 slm. A single-axis moving plate with a *B. subtilis* spore on a Petri dish was placed under the DBD that can provide different exposure times of treatment by simply controlling the number of passes and the plate moving velocity. Throughout this paper, the treatment distance and stage moving speed were kept as 14 mm and 1 cm/s, respectively. The *B. subtilis* spore on the Petri dish was controlled at three different concentrations (10^5, 10^6, and 10^7 CFU/mL).

The optical emission spectra (OES) intensities of the APPJ were measured using a monochromator (PI Acton SP 2500) with a photomultiplier tube (Hamamatsu R928), which was mounted on a mobile 3-D table. The spectral range is 180–900 nm with 1200-g/mm grating (holographic, 300-nm blaze, and 500-nm blaze). Linear dispersion is 1.489 nm/mm at a central wavelength of 400 nm. The gas temperatures in the postdischarge jet region were measured using a K-type thermocouple. Surface chemical composition of the *B. subtilis* spore before and after application of the APPJ were measured using an X-ray photoelectron spectrometer (ESCA PHI 1600, Mg anode at 250 W and 15 KV, 1253.6 eV, an electron

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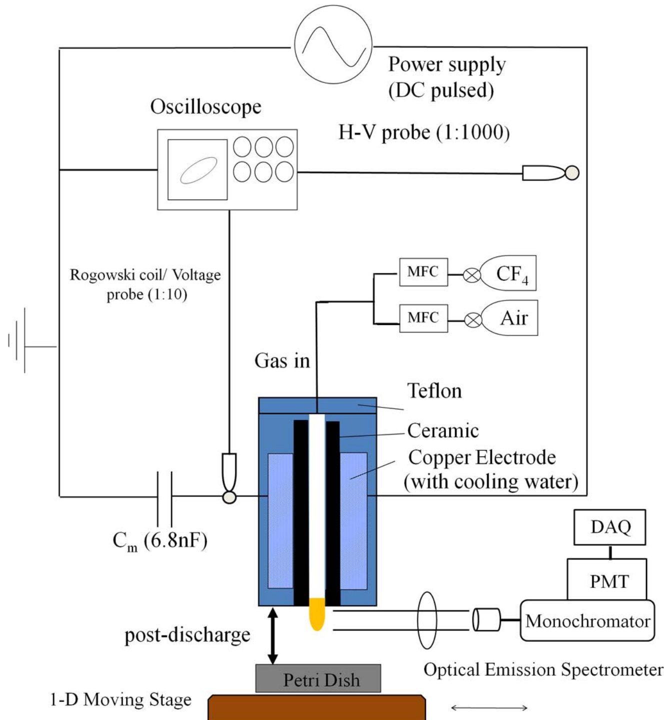

Fig. 1. Schematic of a planar DBD-APPJ.

take-off angle respect to surface at 45°, and chamber pressure below $2 \times 10 - 8$ torr).

In the experiments, the *B. subtilis* spore was first maintained for 18 h at 37 °C. Then, add 0.1-mL culture suspension to sterile Petri dishes containing 20 mL of nutrient agar (beef extract, 3 g/L; peptone, 5 g/L; and agar, 15 g/L, with the pH adjusted to 6.8 ± 0.2). After being incubated at 37 °C for two weeks, the sporulated cells were collected by suspending in approximately 5 mL of water per plate. The spores were washed five times by centrifugation, suspended in sterile water, and finally were stored at 4 °C in water at concentrations of about 1.24×109 spores/mL, diluted according to the required thickness of the experiment separately. Then, add 0.1-mL diluted solutions to sterile Petri dishes (diameter 80 mm) containing 20 mL of nutrient agar, and spread it on the central area of the Petri dishes (4 cm × 4 cm) to make sure that all bacteria can be treated by the plasma jet with 5 cm in width. After the plasma jet treatment, the Petri dishes were incubated at 37 °C for 24 h, prior to determine the resulting number of colony-forming units (CFU/mL) by NIH ImageJ [13]. This software can easily calculate the area of grown bacteria based on pixel counting, which results in the survival rate of the bacteria in the Petri dish.

For spore morphological observation, the spore of the controls and the plasma-treated samples were rinsed with distilled water and fixed with 2.5% glutaraldehyde solution overnight. The fixed spore cells were dehydrated in a graded series of acetone concentration, dried in a CO_2 atmosphere (HCP-2, Hitachi, Japan) under critical conditions, and then coated with a thin layer of gold. The effects of plasma treatment on the structures of the bacteria were examined using a scanning electron microscopy (SEM) (Hitachi S-470 type II, electron voltage: 5 kV; amplification factor: 30 k).

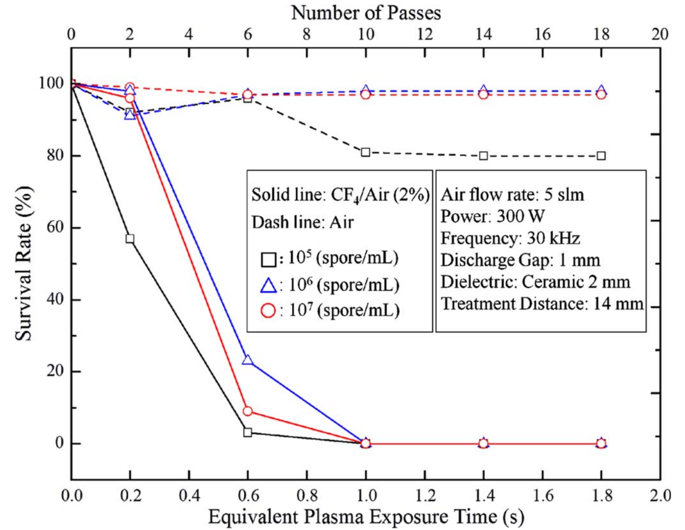

Fig. 2. Survival rate of the *B. subtilis* spore on the Petri dish after incubation with different exposure times of plasma jet treatment.

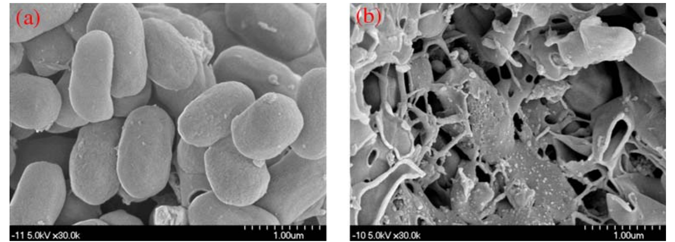

Fig. 3. SEM images of *B. subtilis* spores with a parallel-plate DBD APPJ using CF_4/air (2%): (a) untreated and (b) plasma-treated with 1 s of equivalent plasma exposure time.

III. Results and Discussion

Fig. 2 shows that the CF_4/air (2%) APPJ treatment can result in total inactivation of *B. subtilis* spore after only ten passes of exposure (equivalent plasma exposure time: 1 s). The gas temperature at the treatment position is measured to be 37 °C. In contrast, pure compressed-air APPJ treatment is found to be ineffective even after 50 passes, although it has been demonstrated that it can efficiently sterilize both E. coli and *B. subtilis* [9].

The SEM images of the *B. subtilis* spore with and without the treatment of CF_4/air (2%) APPJ are shown in Fig. 3. Obviously, APPJ treatment with CF_4/air (2%) causes *B. subtilis* spore serious membrane erosion, as compared with the untreated case. The phenomenon of ruptured cell surfaces after CF_4/air (2%) plasma treatment is similar to the appearance of etching in polymer materials. Note that peptidoglycan exists in periplasmic space between cytoplasmic membrane and outer membrane. The broken peptidoglycan could affect the proper function of solute transportation function for carrying protein in cytoplasmic membrane, which can result in effective cell inactivation [9]. By using X-ray photoelectron spectroscopy (XPS) analysis, C1s concentrations are found to be 71.3% and 66.2%, respectively, before and after plasma jet treatment. Through the observations of SEM and XPS analysis, we confirm that etching process by the plasma-generated reactive species is

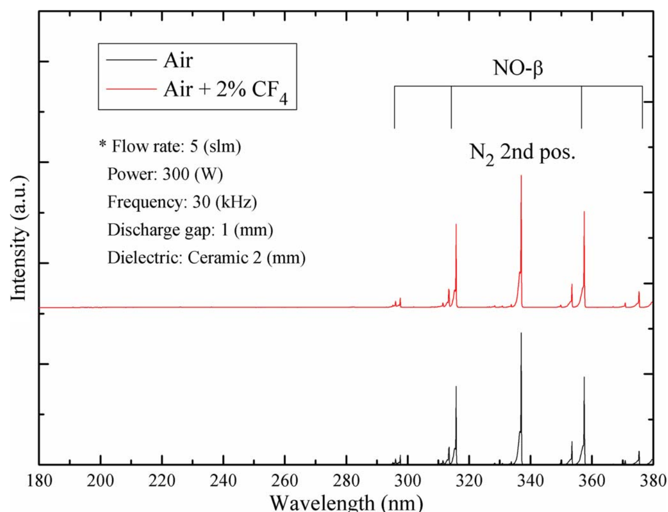

Fig. 4. OES of air and CF_4/air (2%) plasma for the postdischarge plasma at 14 mm and 180–380 nm.

mainly responsible for the inactivation of the *B. subtilis* spore in this paper, which is explained in the following.

Fig. 4 shows that OES data in the UV regime (180–380 nm) measured in the postdischarge jet regions (distance $= 14$ mm) of air and CF_4/air (2%) DBDs. Results show that the N_2 second positive (N_2-SPS) (337.1 nm) and NO-β emissions can be observed. Furthermore, with addition of 2% of CF_4, OES data does not change much in the UV range. It shows that the intensities of N_2-SPS (337.1 nm) and NO-β emissions remain almost the same.

The measured N_2-SPS emission indicates the existence of long-lived metastable nitrogen molecule $N_2(A^3\sum_u^+)$ [14] due to $N_2(C^3\prod_u)$. $N_2(C^3\prod_u)$ is mainly responsible for N_2-SPS emission due to the reaction $N_2(C^3\Pi_u) \rightarrow N_2(B^3\Pi_g) + h\nu(337.1 \text{ nm})$ [15], which is mainly generated by long-lived $N_2(A^3\sum_u^+)$ in the postdischarge region through "pooling reaction" $2N_2(A^3\Sigma_u^+) \rightarrow N_2(C^3\Pi_u) + N_2$ [16]. In addition, the energy level of $N_2(A^3\sum_u^+)$ is 6.2 eV.

NO-β emissions is observed through the reaction $NO(B^2\Pi) \rightarrow NO(X^2\Sigma) + h\nu_\beta$ [16]. For $NO(B^2\Pi)$, it is mainly generated by a three-body collision reaction between O, N, and N_2, i.e., $N + O + N_2 \rightarrow NO(B^2\Pi) + N_2$ [16]. Thus, the existence of the O atom in the postdischarge region is certainly guaranteed through the presence of NO-β emissions [14] based on our OES measurements.

The O atom can lead to very effective inactivation of both sterilized E. coli and *B. subtilis* in our earlier study [17]. However, without the addition of 2% CF_4, the present results have shown that the generated oxygen atoms in the postdischarge region of pure air DBD are not enough to etch the thick cell membrane of the spore within the tested period of exposure time. With the addition of 2% CF_4, etching enhancement could be attributed to the generation of F atoms, which is more reactive than O atom. Unfortunately, the excited F atom is very difficult to measure using OES since very high excitation energy is required. We thus hypothesize the effective inactivation of *B. subtilis* spore next.

These excited and long-lived metastable nitrogen molecule, $N_2(A^3\sum_u^+)$, which reach the treatment position (14 mm) based on the OES measurement, can easily break CF_3–F bond with bond strength of 5.6 eV to generate more F atoms in the postdischarge region. Moreover, the O atom can further react with CF_3 to generate the F atom through $CF_3 + O \rightarrow COF_2 + F$ [18]. With these two contributions, abundant F atoms can be generated. In addition, for example, while CF_3 lands on spore surface, it can possibly increase the number of active sites on the cell surface via the reaction of microorganisms with CF_n (e.g., $C_xO_yH_z + CF_n \rightarrow HF + active\ sites; n = 1-3$) to further enhance the etching effectiveness [18]. In brief, not only the generation of more aggressive etchant F atom but also the active sites created by CF_n molecules contribute to the serious membrane erosion of the *B. subtilis* spore.

A little amount of highly toxic byproducts, HF and COF_2 [19], [20] might be generated during the inactivation procedure with addition of 2% of CF_4. For safety consideration, their quantity should be seriously evaluated and proved to be lower than the safety standard before future practical applications. How to eliminate the toxic byproducts to make this CF_4/air plasma treatment more practical and safer deserves further investigation.

IV. Concluding Remarks

In brief summary, we have demonstrated that, by adding a small amount of CF_4 (2%) into an air planar DBD, one can effectively inactivate *B. subtilis* spore in the postdischarge region within 1 s of equivalent plasma exposure time for the spore concentration in the range of 10^5-10^7 CFU/mL. Possible mechanisms should be attributed to the F atoms generated through the help of abundant excited nitrogen molecules in the postdischarge region.

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