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Highly effective fungal inactivation in $He+O_2$ atmospheric-pressure nonequilibrium plasmas

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Highly effective (more than 99.9%) inactivation of a pathogenic fungus *Candida albicans* commonly found in oral, respiratory, digestive, and reproduction systems of a human body using atmospheric-pressure plasma jets sustained in $He+O_2$ gas mixtures is reported. The inactivation is demonstrated in two fungal culture configurations with open (Petri dish without a cover) and restricted access to the atmosphere (Petri dish with a cover) under specific experimental conditions. It is shown that the fungal inactivation is remarkably more effective in the second configuration. This observation is supported by the scanning and transmission electron microscopy of the fungi before and after the plasma treatment. The inactivation mechanism explains the experimental observations under different experimental conditions and is consistent with the reports by other authors. The results are promising for the development of advanced health care applications.
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I. INTRODUCTION

Successful applications of thermally nonequilibrium plasmas for inactivation of a large variety of microorganisms (e.g., pathogens) have enormously expanded in the past few years as more and more advantages of such plasmas are found.[1–8] Atmospheric-pressure plasma jet (APPJ) devices can generate numerous reactive atomic/radical species (O, OH, etc.), as well as ions and electrons which interact with the surfaces of the microorganisms during the treatment process. The roles and specific effects of different plasma species produced in the APPJ have not yet been understood completely.[9–12] Significant advance has been made in the treatment of the simplest prokaryotic cells, which have no organelles other than ribosomes, and no membrane-enclosed nucleus.[13–15] Disruption of the living system of a prokaryotic cell is much simpler compared to multicellular fungal organisms, whose cells contain a membrane-enclosed nucleolus, multiple organelles, and hypha.[16–19] Low-pressure cold plasmas of a toxic sulfur hexafluoride (SF_6) gas in air have been used to inactivate *Aspergillus parasiticus* on a nut surface.[20] However, the treatment efficacy typically remains quite low, i.e., only about 50% of *Aspergillus parasiticus* has been inactivated even after 20 min treatment.[20] Cultures of pathogenic *Candida albicans* fungi have been treated using low-pressure dielectric barrier discharge plasmas.[21] However, low-pressure treatment is not practical from the point of view of clinical applications.

Here we report that cold nonequilibrium atmospheric-pressure plasmas generated using the APPJ show much better efficacy in inactivation of *Candida albicans*. This plasma is cold and does not produce any thermal or electric shocks in contact with unprotected human skin. The *Candida albicans* is one of the most common fungal pathogens present in the oral cavity, the upper respiratory tract, as well as in some parts of gastrointestinal, blood, and genital tracts of the human body. In certain conditions, this fungus can cause many types of diseases, for example, dermatocandidiasis, and may also lead to morbidity and mortality of immunocompromised (e.g., via HIV infection) patients. Furthermore, the profound presence of *Candida albicans* in the human body leads to biofilm formation on implantable biomedical devices. Traditional therapeutic procedures to cure diseases caused by *Candida albicans* are lengthy and in most cases need to be repeated due to high rates of recurrent fungal infestation. This is why complete cure is rarely achieved and *Candida albicans* remains a significant health care issue despite years of research.

In this paper, we demonstrate an unusually high inactivation efficacy of the atmospheric-pressure plasma jet sustained in a $He+O_2$ gas flow. It is shown that it only takes a few minutes to inactivate more than 99% of *Candida albicans* under conditions of restricted access to atmosphere. The scanning electron microscopy (SEM) and transmission electron microscopy (TEM) have been used to examine the effects of fungal treatment by the atmospheric-pressure nonthermal plasma. The SEM results show clear signs of major changes to the external cellular structure, whereas the TEM analysis also confirms substantial changes to the interior of the cells. Our experimental results clearly demonstrate that even a short plasma exposure leads to significant damages to the structure of the cell wall and the cell membrane. As the

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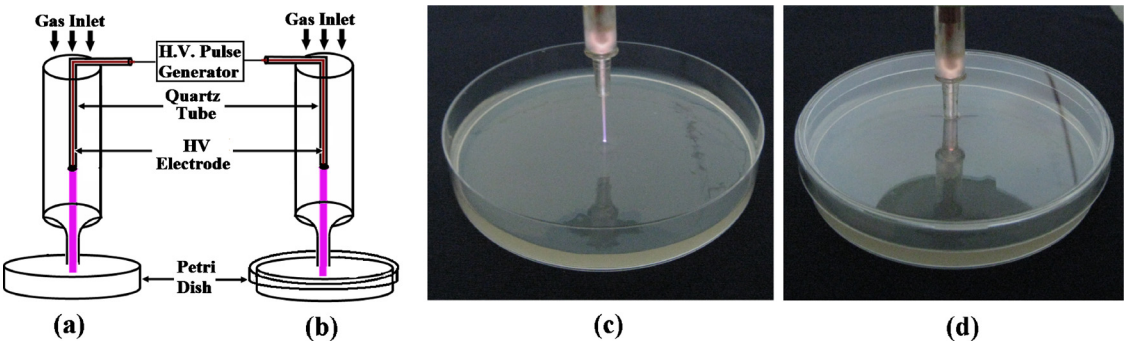

FIG. 1. (Color online) Experimental setup and photographs of the plasma plume. (a) and (b) are the schematics of the two treatment configurations used with (a) and without (b) contact of the cellular culture with open air. (c) and (d) are the photographs corresponding to sketches in (a) and (b), respectively.

treatment time becomes longer, the interior structure of the fungus also undergoes notable changes.

The article is organized as follows. In Sec. II, the details of the experimental setup, cell culture, and electron microscopy are presented. Section III contains the systematic results on fungal inactivation using the APPJ device in two configurations with and without access of the treated cultures to the atmosphere. The inactivation mechanism, which is also discussed in this section, is based on the analysis of the reactive species production and residence time in the plasma jet and involves the reaction kinetics calculations and optical emission spectroscopy from the plasma discharge. The results of the electron microscopy analysis of the control and plasma-treated cellular samples are also presented. The paper concludes in Sec. IV by a brief summary of the results and outlook for future research.

II. EXPERIMENTAL SETUP AND PROCEDURES

Figures 1(a) and 1(b) show the schematic of the experimental setup for the two main treatment configurations, with and without contact of the cellular culture with atmosphere, respectively. In the first case, the Petri dish (of a diameter 5.5 cm) is not covered and is directly exposed to the plasma; in the second case, the dish is covered, as shown in Fig. 1(b). In the second configuration sketched in Fig. 1(b), a hole with the same diameter of the syringe nozzle (about 5 mm) is made in the center of the cover to insert the nozzle of the hollow syringe, so that the plasma plume could reach the fungi. The distance between the nozzle exit and the sample surface was approximately 8 mm. Figures 1(c) and 1(d) show the photographs of the plasma plume in the two configurations, respectively. From Fig. 1(d), one can notice that the intensity of optical emission from the plasma plume significantly decreases when the air access to the Petri dish is restricted.

The main component of the APPJ device is a high-voltage (HV) electrode made of a copper wire with a diameter of about 2 mm; the HV electrode is inserted into a 5-cm-long quartz tube; one of the ends of the tube is sealed. The quartz tube has the inner and outer diameters of 2 and 4 mm, respectively. This quartz tube is mounted coaxially inside a hollow glass syringe. The inner diameter of the hollow syringe is 6 mm. More details about the plasma jet device can be found elsewhere.[22] The plasma discharge is sustained by pulsed dc voltage. The voltage amplitude, the pulse frequency, and the pulse width have been fixed at 8 kV, 8 kHz, and 1.6 μs, respectively. For all the experimental results reported in this paper, the working $He+O_2$ gas mixture was let into the APPJ device with the flow rate of 2 l/min and contained 3% of oxygen diluted in helium.

To prepare the cellular culture, freeze-dry *Candida albicans* was inoculated using the Sabouraud's medium (40 g glucose, 10 g peptone, 0.5 g chloramphenicol, 20 g agar, and 1000 ml distilled water) and incubated for 24 h at 37 °C. The most viable cellular colonies selected for the treatment were diluted with sterile physiological saline to produce an $\sim 10^7$ colony-forming units (CFUs)/ml suspension, and then stored in a refrigerator at 4 °C for use in the experiments.

The surface morphology of the *Candida albicans* with and without the plasma exposure was analyzed using a Hitachi S-570 scanning electron microscope using the 5–15 kV electron energy range. The transmission electron microscopy analysis was carried out through the use of a FEI Tecnai G212 transmission electron microscope equipped with a 200 kV field emission gun.

The optical emission spectroscopy of the atmospheric-pressure plasma jet discharge was implemented using an Acton SpectraHub 2500i (Princeton Instruments) spectroscope and photomultiplier tubes. The resolution of the spectroscope was 0.05 nm.

III. RESULTS AND DISCUSSION

A. Sterilization of *Candida albicans*: Efficacy and possible mechanisms

All of the samples have been divided into two groups, i.e., with and without access of the cellular culture to the atmosphere during the treatment. Both of the two groups have been divided into five smaller groups, each containing five samples. Four of the five groups have been treated by the plasma plume for 2, 3.5, 5, and 8 min, while the last group represented control samples which were treated by a neutral gas flow (no plasma discharge). After the treatment, the samples were incubated for 72 h at 37 °C. Thereafter, digital photographs of the fungal samples were taken and the number of viable colonies was estimated. The photographs of *Candida albicans* samples treated in the two configura-

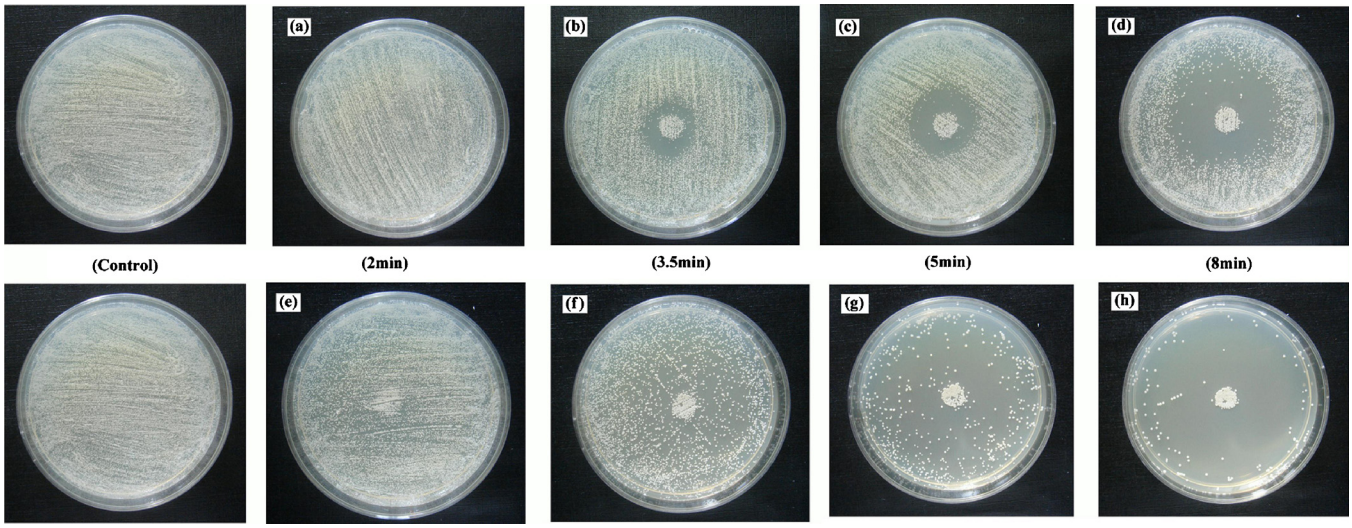

FIG. 2. (Color online) Photographs of *Candida albicans* samples in Petri dishes. $He+O_2$ (3%) working gas mixture with flow rate of 2 l/min is used. Panels (a)–(d) show the *Candida albicans* samples treated in the open-air configuration for 2, 3.5, 5, and 8 min, respectively. Panels (e)–(h) correspond to the samples in covered Petri dishes treated for 2, 3.5, 5, and 8 min, respectively.

tions are shown in Fig. 2. The treatment times corresponding to images (a)–(d) and (e)–(h) are 2, 3.5, 5, and 8 min, respectively. Images (a)–(d) are for the samples treated without covering the Petri dish, while images (e)–(h) are for the limited air exposure configuration.

The cell viability (survival CFU counts) plots are shown in Fig. 3. One can see a striking difference between the two treatment configurations. Only a small fraction of fungal cells is effectively inactivated in the open-air configuration even after a long treatment time of 8 min. On the other hand, more than 99.9% of *Candida albicans* has been inactivated after only 3.5 min treatment when the Petri dish was covered. As the treatment continues, the treatment efficacy becomes even better. In fact, the number of viable cells per colony in the latter case is more than four orders of magnitude less than in the open-air exposure case. In addition, Fig. 2 suggests that when no cover is used, there is a clear edge between the plasma-affected and nonaffected areas. The diameter of the affected area increases with treatment time. On the other hand, when a cover is used, the whole surface of the fungal culture is fairly equally affected by the plasma treatment. This experimental observation indicates that the presence of the boundary (Petri dish cover) improves the confinement of reactive species and the uniformity of their distribution over the surface.

In the following, we will estimate the lifetime of the reactive species. At the given gas flow rate of 2 l/min and the inner diameter of the nozzle of 1.2 mm, the gas flow rate can be estimated to be ~30 m/s. Under such conditions, the minimum time for the reactive species to reach the edge of the Petri dish is ~1 ms. In the gas diffusion approximation, this time is expected to appear one order of magnitude longer. Furthermore, if one assumes that oxygen atoms play the key role in the inactivation process, according to chemical reaction kinetics, the main destruction mechanisms of O-atoms at atmospheric pressure are through heavy-particle reactions,[2,23,24]

$$O + O_2 + M \rightarrow O_3 + M, \quad (1)$$

$$2O + M \rightarrow O_2 + M, \quad (2)$$

and

$$O + O_3 \rightarrow 2O_2, \quad (3)$$

where M is another neutral molecular species in the discharge, such as He, N_2, or O_2. The reaction rates for reactions (1)–(3) at room temperature can be found elsewhere.[23,24] The O-atom formation mechanism in a rf-driven atmospheric-pressure microplasma jet was studied using optical diagnostics and numerical simulations of the plasma chemical kinetics.[23] It was observed that the absolute densities of O-atoms had steep gradients at the interface between the plasma core and the effluent region; it was

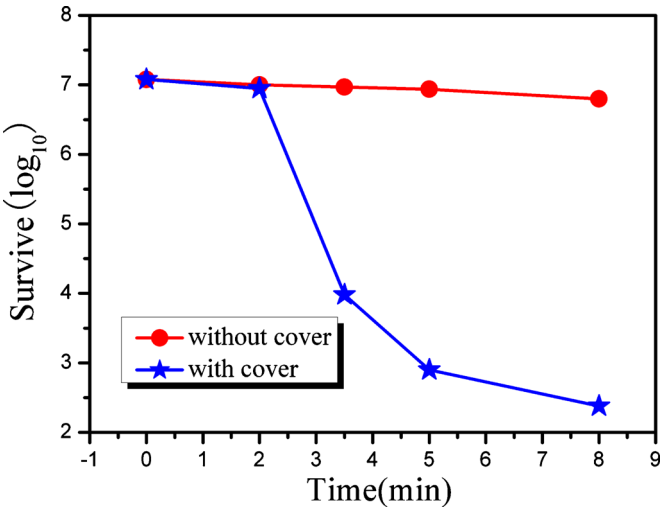

FIG. 3. (Color online) Cell viability (survival) plots for the two treatment configurations. Filled circles and stars correspond to the open-air and closed-dish cases, respectively.

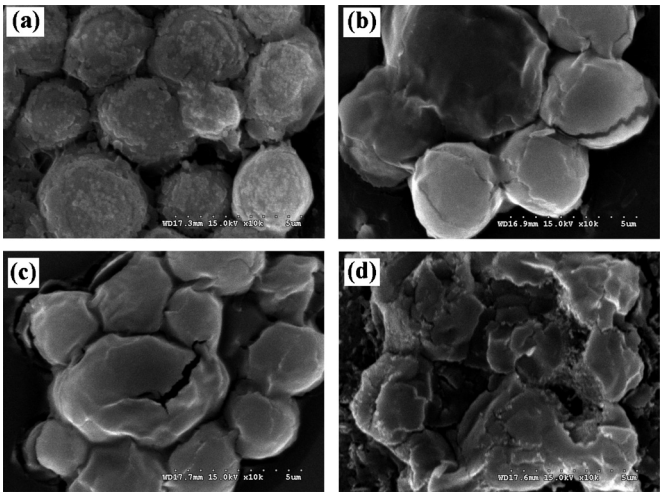

FIG. 4. The SEM micrographs of the *Candida albicans*. Panel (a) corresponds to the untreated (control) sample while panels (b)–(d) correspond to the samples treated for 2, 4, and 8 min, respectively.

$\sim 10^{15}$ cm^{-3} in the plasma core and steeply decreased along the direction of effluence.[23] Park *et al.*[24] calculated the densities of oxygen atoms and ozone (O_3) molecules using a global model of $He+O_2$ atmospheric-pressure plasmas. The results showed that the densities of these species were strongly affected by the partial pressure of O_2 in the gas mixture and reached their maximum values ($\sim 10^{16}$ cm^{-3}) when only 0.5% oxygen admixture was used. The densities of reactive O and O_3 species decreased when the oxygen partial pressure was further increased. Therefore, based on reactions (1)–(3) and their rates, the lifetime of oxygen atoms in the open-air configuration can be estimated to be of the order of 0.1 ms, which is less than the time required to reach the edge of the Petri dish. This is consistent with the observed better treatment efficacy in the areas around the center of the Petri dish [Figs. 2(b)–2(d)]. There is a possibility that other long-lived reactive radicals (e.g., reactive oxygen species) also play important roles in the inactivation process. Further studies are needed to clarify this issue.

An interesting observation from Fig. 2 is that fungal colonies appear to be relatively unaffected in the area with the diameter of a few millimeters when oxygen gas flow is present; this has not been observed in pure He discharges. There is a possibility that the flow of heavy neutral species (O_2, etc.) aligned with the center of the dish effectively pushes the cells inside the agar which then protects the cells from the plasma plume.

B. Electron microscopy analysis

SEM/TEM has been used to investigate the effect of the plasma plume on the cells. The fungal colonies were collected by repeated washing the surface of the culture medium with 0.9% normal saline. After that, the collected colonies were further prepared as appropriate for the SEM and TEM cell analysis.[25]

Figure 4 is the SEM images of the control and plasma-treated *Candida albicans* samples; labels (a)–(d) correspond to the treatment times of 0, 2, 4, and 8 min, respectively. It is clear that the structure of untreated *Candida albicans* is viable, while the treated cells have clear slits/ruptures on the surface, which evidences damage to the structure of the cell wall and membrane. As the treatment time increases, the damage to the structure becomes more and more pronounced. One can conclude that after 8 min of treatment, the integrity of the cellular structure is completely broken.

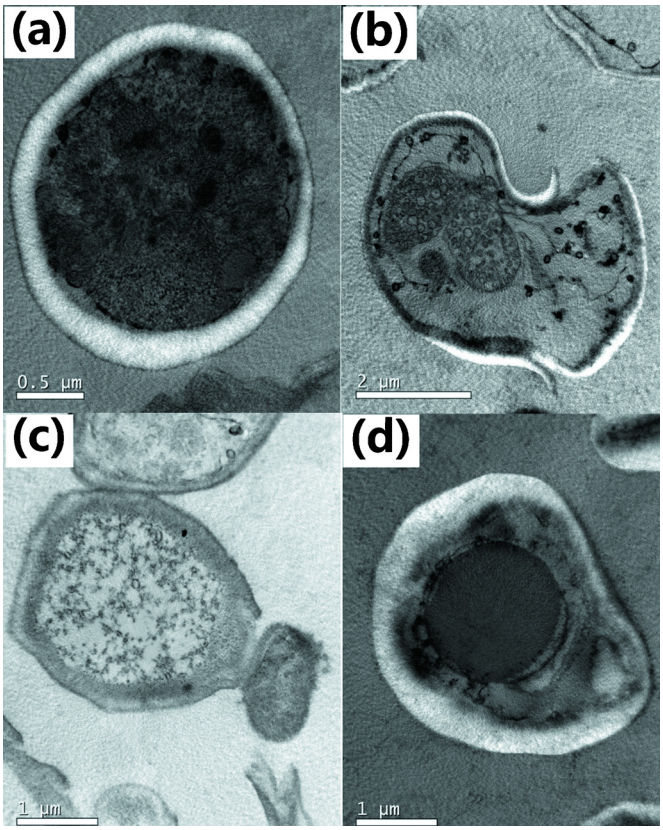

FIG. 5. (Color online) The TEM images of the *Candida albicans* revealing three typical types of damage caused by the APPJ treatment: (a) control sample; [(b)–(d)] samples treated for 8 min.

Figure 5 shows three typical types of plasma-induced damage to the cellular structures imaged by the TEM. Figure 5(a) shows the TEM image of a typical *Candida albicans* without the plasma treatment. It could be seen that the cell wall is uniform, the cell membrane is complete, and the cytoplast inside the cell is homogeneous. Images (b)–(d) are the three typical patterns of the observed damage to the cellular structures after an 8-min-long plasma treatment. From image (b), one can clearly see that a hole appears on the cell wall and the membrane. The intensity of the transmitted electron beam is not uniform and is quite low throughout the cellular interior; a possible explanation of this observation is the cytoplasm partial leakage out of the cell through the hole. In addition, no clear contrast between denser and rarefied areas in the cell interior indicates on the possibility of the plasma-induced damage to the cell nucleus; the specific mechanism of the observed disappearance (e.g., dissolution) of the cell nucleus is unclear and further studies are warranted. A different damage pattern is observed in Fig. 5(c); most of the cytoplasts split out of the cell and we could not see any complete structure of any organelle inside the cell.

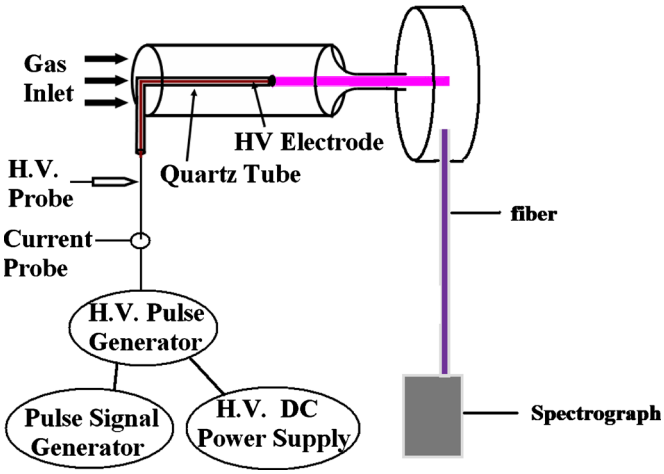

FIG. 6. (Color online) Schematic of the optical emission spectra measurements in the configuration with the covered Petri dish.

The interesting point is that the image shows exactly the case of the cytoplasts of the cell splitting out. Figure 5(d) shows a typical example of karyopyknosis—a cytologic condition characterized by a major shrinkage of the cell nucleus; in addition, the cell wall and the membrane become much thicker compared to the untreated cells. In this case, no clear rupture of the cell surface can be observed.

C. Optical emission spectroscopy

Optical emission spectroscopy was used to measure the optical emission of the plasma plume inside the covered Petri dish. Figure 6 is the schematic of the experimental setup to measure the optical emission spectra. An optical fiber was inserted through a small hole (with a diameter of 5 mm) drilled in the side wall of the Petri dish. The distance of the fiber end to the plasma plume was ~1.5 cm in order to avoid any effect on the discharge. Figure 7 shows the typical emission spectra (300–800 nm) produced by the plasma plume. One can clearly see that excited O, OH, N_2, N_2^+, and He are present in the plasma plume. It is worth pointing out

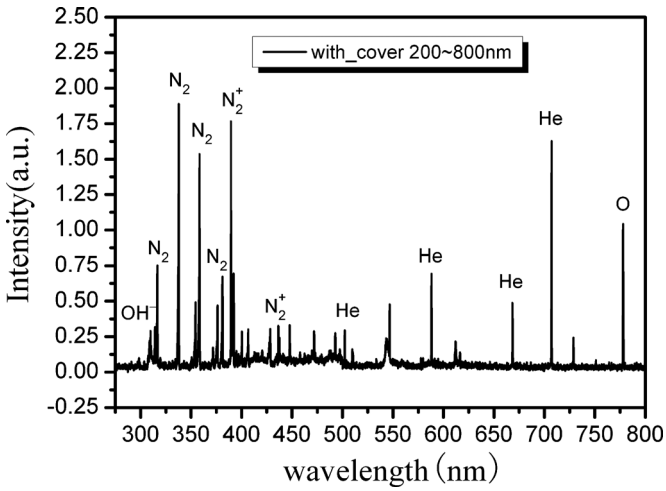

FIG. 7. Optical emission spectra of the APPJ in the same configuration as in Fig. 6.

that the relative emission intensity of O species (emission wavelength of 777 nm) is about two orders of magnitude larger than in the open air.[22] Since O-atoms play an important role in biomedical applications and physiological responses,[26] this observation suggests that the restricted exposure of the treated cellular samples to atmosphere can lead to substantial improvements of the treatment efficacy; this is consistent with the results presented in Secs. III A and III B.

It should also be noted that the length of the APPJ plume depends on the pulse duration. It reaches a maximum length of about 3 cm when the pulse duration is increased to 800 ns. It does not change any more with further increase of the pulse duration.[27] This important feature can also be exploited in a variety of different uses including plasma-based nanotechnology and related biomedical applications.[28–30]

IV. CONCLUSION

The inactivation of *Candida albicans* by using an atmospheric-pressure plasma jet (APPJ) device was investigated. When treated with a cover on the Petri dish, the inactivation effect is greatly enhanced (the numbers of survived cells decrease by several orders of magnitude). This effect may be attributed to the difference in transport and chemical kinetics of reactive species in the limited space between the surface of the cell culture and the cover of the Petri dish. The scanning and transmission electron microscopy imaging have revealed typical patterns of damage to the fungal cells caused by the atmospheric-pressure, low-temperature nonthermal plasma treatment. The results of the optical emission spectroscopy studies have shown that in the configuration with the covered Petri dish, the intensity of the optical emission which originated from oxygen atoms is about two orders higher compared to the open-air treatment. This is an additional manifestation of the possibility to significantly increase the inactivation efficacy by restricting the contact of the cellular cultures with open air. Similar conclusions may apply to other cellular types of importance to medicine, food hygiene, and preventative health care applications. Research in this direction should continue to improve the understanding of the mechanisms of interactions of reactive plasma species with cellular structures and to apply this generic approach to different cellular types.

ACKNOWLEDGMENTS

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