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Bacillus subtilis devitalization mechanism of atmosphere pressure plasma jet

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ABSTRACT

Bio-applications of atmospheric pressure plasma have been widely studied in recent years. However, the devitalization mechanisms of micro-organisms by atmosphere pressure plasma have not been clearly explained. This paper was to find the possible sterilization mechanisms and define the major sterilization factors with the atmospheric pressure plasma jet. For the sterilization target, the *Bacillus subtilis* was selected. In this paper, a set of physical or chemical methods were used to characterize the following sterilization factors: heat, ultraviolet radiation, charged species and chemical reactive species generated by the plasma. It was found that the bacterial inactivation efficacy had a significant improvement when oxygen was introduced into the plasma jet system. Comparing with the result of sterilization, the inactivation process was dominantly controlled by synergistic actions of chemically active species and charged species, rather than heat, or ultraviolet radiation.

1. Introduction

Eradication of harmful micro-organisms in the environment has been an important goal since they were first discovered and understood. Many reliable conventional techniques were used until now for sterilization involving exposure to toxic compounds such as ethylene oxide or chlorine gas or exposing the specimen to high temperatures and pressures in an autoclave [1]. Yet, most of them involved some level of damage to the medium sustaining micro-organisms and showed relatively low efficiencies. Especially, some chemicals such as ethylene oxide used for sterilization induced serious damage to the user or the environment. As a novel sterilization technique, non-equilibrium atmospheric pressure plasma has been paid more attention in recent years due to their many advantages including operation at atmospheric pressure, no presence of toxic substances, low-temperature, long operative duration, and economical and simple systems [2–6].

In order to make the atmospheric pressure plasma effective in sterilization, the study of sterilization mechanisms was very significative [6,7]. As important factors of the sterilization, heat, charged particles, ultraviolet (UV) radiation, chemically active species, or the synergistic actions of preceding factors were recommended [2–5,8]. However, because the main sterilizing factors were dependent on the plasma source type or the plasma characteristics strongly, the different sterilization results would be obtained due to the different plasma parameter [9]. For example, at low pressure, the UV radiation in the plasma played a crucial role because of their little absorption [10,11], thus, at the atmospheric pressure the UV radiation effect was still under debate [2,3,5,6,9].

Wintenberg et al. [12] and Uhm et al. [13,14] found that oxygen-containing atmospheric pressure plasmas have a profound antimicrobial influence on bacterial spores. In this paper, a dielectric barrier discharge (DBD) plasma jet using different working gas (Argon or Argon + O_2) was operated in the open air for inactivation. In order to test the sterilizing effect of the plasma jet, the *Bacillus subtilis* (*B. subtilis*) (on the surface of medical polytetrafluoroethylene sheet) was selected. Furtherly, the influence of the plasma on the sterilization efficiency was discussed. In order to analyze the sterilization mechanisms clearly, a set of physical or chemical methods were used to characterize the sterilization factors such as heat, charged particles, ultraviolet (UV) radiation, chemically active species, or the synergistic actions respectively. Firstly, the effect of the heat or UV radiation produced by plasma jet were measured by the colony forming unit test. Secondly, the electronically excited species generated by the plasma jet were determined by optical emission spectrometer, and then the amount of protein leakage and the production of lipid peroxide, whose marker was the malondialdehyde (MDA) as being one of the end products of the lipid peroxidation, were measured and SEM images were obtained after plasma treatment.

2. Experimental set-up

2.1. Plasma generator

The schematic diagram of self-designed atmosphere pressure dielectric barrier discharge plasma jet system was showed in

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Fig. 1, and the discharge picture was showed in Ref. [15]. In the reactor, a quartz condenser tube was with dimensions of 12 mm (inside diameter), 40 mm (outside diameter), 180 mm (length) and wall thickness of 1 mm was used as the dielectric layer. It had a quartz condenser tube surrounded by the sodium chloride (NaCl) solution as the ground electrode. The powered electrode was a copper rod and connected with an AC power supply which operated between 10 and 40 kHz. The operating gas blew through two electrodes, and the plasma jet was formed at the nozzle. The length of Ar plasma jet, changed with flow rate, was about 2–5 cm, and the gas temperature of plasma jet was about 30–50 °C. A digital real-time oscilloscope (TDS200-Series) was used to record the waveform of the discharge peak-to-peak voltage and peak-to-peak current, the typical voltage and current waveforms of the Ar plasma jet were showed in Fig. 2. The discharge peak-to-peak voltage was 9.9 kV and the discharge peak-to-peak current was 162.0 mA.

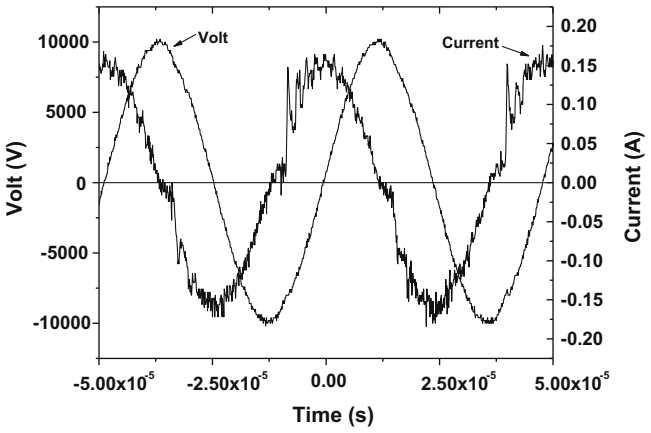

Fig. 2. Characteristic wave forms of the discharge voltage and current corresponding to the stable stage.

2.2. Colony forming unit test

The samples for sterilization were made of 6 mm × 6 mm sheets of PTFE contaminated by 50 μl *B. subtilis* suspension, and then the sheets were dried in a desiccator for 1 h. After that, the samples were exposed in the atmospheric DBD plasma jet with different time. And then, the samples would be put into test tubes containing 10 ml germfree water and mixed fully for 30 min by a vortex mixer. The bacterial strains were serially diluted and dispersed into the agar in Petri dishes, and the number of the colony forming unit (cfu) was counted after incubation at 30 °C for 24 h. In order to reduce error, two independent experiments were completed at the same condition, and every colony counting result was average of the two independent experiments.

In order to understand the effect of high temperature produced by plasma jet on bacteria, the samples were put into the desiccator which could maintain different temperature (20–100 °C) environment for simulating the inactivation of high temperature. After 5 min treatment by hot air, the follow-up method was the same as the above colony forming unit test. Since the quartz glass could be penetrated by UV radiation from the plasma jet while the radicals and charged particles could not, the samples covered by the quartz glass could simulate the sterilization of only UV radiation from the plasma plume.

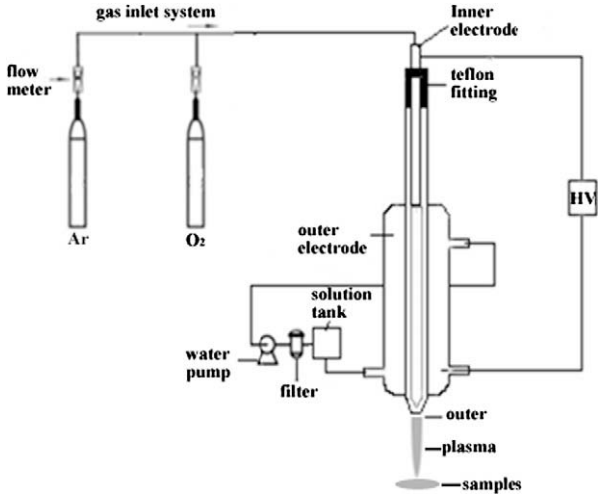

Fig. 1. Schematic diagram of plasma jet discharge system (Ar flow rate 21.51 slpm).

2.3. Determination of optical emission spectra

The emission spectra from the plasma source were acquired through the equipped optical fiber in the range of 197–720 nm. The optical emission spectrometer (avaspec-2048 optical fiber emission spectrometer) with a charge-coupled device (CCD) detector, was used to analyzed the spectra emitted and the electronically excited species generated by the plasma jet.

2.4. Determination the amount of protein leakage

B. subtilis absorbed on the PTFE sheets was treated by Argon (Ar) plasma, and then eluted in the phosphate buffered saline (PBS) to form suspensions of *B. subtilis*. These suspensions were centrifuged, and then put the upper pellucid solution to mix with the coomassie brilliant blue dye and placed for 3 min at room temperature. Coomassie brilliant blue dye and protein could form a complex which was determined at the wavelength 595 nm by colorimetry. The amount of protein leakage of cell after plasma treatment could be determined by putting the determined absorbance value in the regression equation of standard curve of bovine serum albumin.

2.5. Determination the production of MDA

The absorbency of the supernatant solution prepared in the way described in Section 2.4 was determined at the wavelength 532 nm by colorimetry. The measuring reagents of MDA were obtained from Nanjing Jiancheng biological engineering institute. The MDA concentration was calculated by the following equation according to the manual of the reagents:

$$\mathrm{MDA} = \frac{\text{determination tube absorbency} - \text{determination blank tube absorbency}}{\text{standard tube absorbency} - \text{standard blank tube absorbency}} \times 10\,\mathrm{nmol/ml} \times \text{sample diluted multiple} \quad (1)$$

2.6. SEM examination

The effects of plasma treatment on the morphology of the bacteria were examined using a SEM (XL30 ESEM). The controlled and the plasma-treated samples were placed in the fixation and then coated with a thin layer of plasma sputtered aurum. So the changes of the cells morphology of controlled and treated samples could be compared by the SEM images.

3. Results and discussion

3.1. Devitalization by plasma jet

Fig. 3 shows the cell survival curve of the *B. subtilis* upon Ar plasma exposure. About two orders of magnitude of *B. subtilis* cfu reduced after 60 s pure Ar plasma treatment. Even the *B. subtilis* treated by plasma at 180 s, less than three orders of magnitude of *B. subtilis* cfu reduced, but they were not totally inactivated. Heise et al. named this phenomenon as "shadowing effects", and they mainly attributed this phenomenon to the protection of the underlying layers [16]. However, when 3.59% oxygen was added into the working gas, the bacterial inactivation efficacy improved significantly, and it was showed in Fig. 3. About four orders of magnitude of *B. subtilis* cfu reduced after 60 s plasma treatment, which were two orders of magnitude larger than Ar plasma, and they were totally inactivated at 180 s.

In the devitalization experiment, decimal reduction time values (D-value) was a very important parameter. It could be expressed as follows:

$$D = t/(\log N_0 - \log N_s) \tag{2}$$

where N_0 was the initial population, and N_s was the surviving population. The D-value indicated when t was the time to destroy 90% of the initial population (or the time for one log 10 reduction). As to Ar plasma, the D-value was about 14 s. However, the D-value was only 5 s to Ar + O_2 (3.59%) plasma, and the D-value was significant decrease than Ar plasma. This result suggested that the sterilization of Ar + O_2 plasma were more effective than pure Ar plasma under the same conditions.

The number of the survivors decreased with the temperature increasing (from 20 to 80 °C). Although the sample was treated at 80 °C hot air 5 min (much longer than plasma treatment time, 1 min) in the desiccator and caused a decrease about 30% in the number of cfu, this decrease was much smaller than the one in plasma jet. Therefore, this result clearly indicated the temperature was not responsible for *B. subtilis* inactivation in our experiment, and which was consistent with Refs. [13,14]. In addition, about ten percent of the bacilli reduced after 3 min UV radiation generated by plasma jet. Though the number of survivors reduced with the exposing time increase, this decrease was much smaller than direct plasma jet treatment. So the UV radiation generated by the plasma jet played a little role to bacterial inactivation, and which was consistent with Ref. [14].

3.2. Determination of optical emission spectra

The optical emission spectrum of the Ar and Ar + O_2 atmospheric pressure plasmas were showed in Fig. 4. There were emission spectra lines of excited ·OH (306–310 nm), N_2 (336.8 nm), N_2^+ (356.4 nm), O IV (589.2 nm) and Ar I (696.6, 706.5, and 714.6 nm) radicals [17] in the Ar plasma plume (Fig. 4a). ·OH (as a result of the dissociation of water molecules, present as moisture in air or plasma feed gas) considered in the bacterial inactivation playing an important role. O IV (as a result of the dissociation of oxygen molecules, present in the open air) as a kind of reactive oxygen species, also had an important effect on the bacterial inactivation [12–14]. N_2^+ (as a result of the Penning ionization process of N_2 molecules with the electron in plasma plume) and Ar I (as a result of exciting Ar) also could bombard and react with the bacteria.

Besides the above radicals, Ozone (O_3) (313.7 nm) [17] presented in Ar + O_2 plasma plume was showed in Fig. 4b. O_3 was produced because of the reactions as the follows:

$$3O_2 \rightarrow 2O_3 \tag{3}$$

$$O + O_2 \rightarrow O_3 \tag{4}$$

Ozone, which interfered with cellular respiration, had been known to have a strong bactericidal effect to bacteria. Moreover, compared with Fig. 4a and b, the intensity (a.u.) of O IV was significant increase. The measured spectral intensity of the O IV line (589.2 nm) was normalized by the Ar I line (696.6 nm) at the two different conditions (Ar and Ar + O_2 plasma) was showed in Fig. 5. The intensities of O IV and Ar I in Ar + O_2 (3.59%) plasma jet was nearly two times as many as pure Ar plasma. This was further illustrated that the O IV did increase after O_2 added. Comparing Fig. 3 with Fig. 4, it could be drew the conclusion that the O_3 and O IV radicals had an important contributor to inactivation and the sterilization strongly depended on the chemical reactive species. This was the reason of Ar + O_2 plasma had a better inactivation effect than pure Ar.

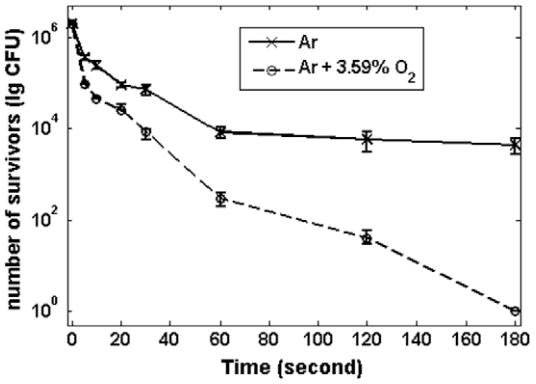

Fig. 3. The survivor curve of *B. subtilis* after Ar and Ar + 3.59% O_2 plasma exposure.

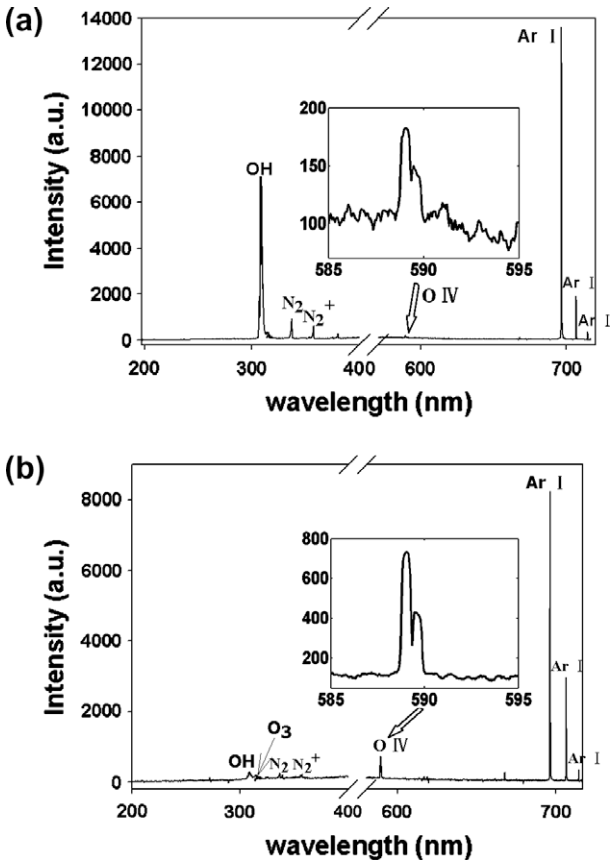

Fig. 4. Optical emission spectrum in the range from 197 to 720 nm: (a) Ar plasma and (b) Ar + 3.59% O_2 plasma.

3.3. Protein leakage

The charged particles in plasma bombarded the bacteria and caused etching action on cell surface. This etching action eroded cell materials, as a result, cell membrane ruptured and the content effused, and caused cell to die finally [18]. In addition, the chemical reactive species also reacted with the cell materials at the same time.

The protein leakage vs. plasma treatment time was showed in Fig. 6. There were similar variational trends of protein leakage after exposure in plasma jet which used different working gases (one was pure Ar, and the other was Ar + O_2). The protein leakage increased with plasma treatment time, which indicated that more damage presented in cell surface with treatment time increase. The charged particles were the main component of pure Ar plasma jet to bombard the bacteria and cause etching action on bacteria surface, though there were small amount of chemical reactive species (mentioned in Section 3.2), then the cell ruptured and the content such as protein effused. When Ar + O_2 was used as the working gas, although a large number of reactive oxygen species (such as O IV and O_3) were produced in plasma environment, the charged particles also bombarded and etched the bacteria, and these reactive oxygen species were far more than pure Ar plasma jet. Though the plasma jet used the different working gases, the amount of protein leakage did not change obviously at the same treatment time (shown in Fig. 6). This phenomenon showed that the oxygen-containing active species generated by Ar + O_2 plasma jet did not play main role in breaking cell surface but the charged particles. In addition, the reactive oxygen species could react with protein and consume a small part of protein, thus the protein leakage quantity in Ar + O_2 was less than pure Ar.

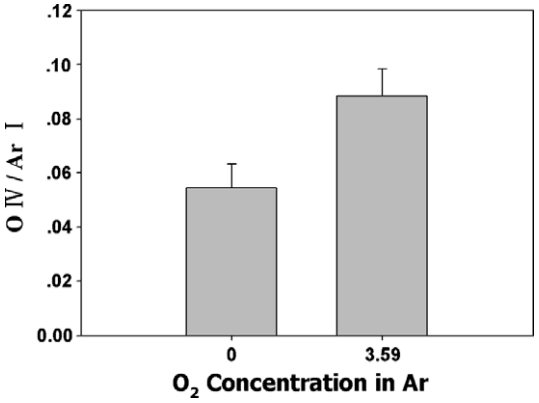

Fig. 5. The intensities of O IV (589.2 nm) to Ar I (696.5 nm).

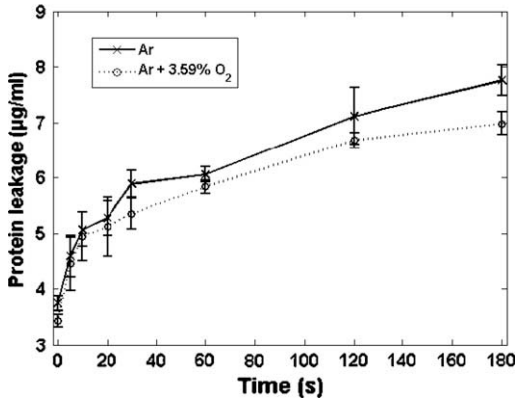

Fig. 6. Amount of protein leakage after plasma treatment at different time.

The charged particles played the main role in breaking bacteria surface and led the bacteria content such as protein to effuse, in addition, the chemical reactive species also played a certain synergistic actions.

3.4. MDA production

When the working gas was Ar, the reactive oxygen species were still produced because the electric charges would react with oxygen in the open air. Moreover, with Ar + O_2 as the working gas, the reactive oxygen species were produced more because the reactive probability of electric charges and oxygen would increase. In addition, the reactive oxygen species could react with polyunsaturated fatty acids (PUFA) in cell membrane, and then produced the MDA, which was the marker as one of the end products of the lipid peroxidation. So the changes of MDA concentration in suspensions of bacilli after plasma treatment could reflect the actions between reactive oxygen species and cells of bacteria [19].

Fig. 7 showed the variational tendency of MDA production in the suspensions of bacilli after sterilization in the different treatment time and working gas. When Ar was the working gas, the MDA production increased with the treatment time. However, when Ar + O_2 was the working gas, a large amount of reactive oxygen species (such as O IV) was produced in plasma environment. So the probability of reactive oxygen species reacting with the bacteria was larger than pure Ar, which led the MDA production much larger than Ar plasma.

The test of MDA production proved the reactive oxygen species reacted with bacteria assuredly and destroyed the intrinsic material of cell membranes. Consequently, these destroyed bacteria lost their activity.

3.5. SEM

Comparison of Fig. 8a (controlled), Fig. 8b (after 60 s Ar plasma treatment) and Fig. 8c (after 60 s Ar + 3.59% O_2 plasma treatment) clearly showed that the DBD plasma jet caused morphological changes of *B. subtilis*. In Fig. 8b, a small amount of filamentous substances and rupture (indicated by arrow) appeared around the cells obviously, and this phenomenon was even more evidently in Fig. 8c. The plasma had a direct physical impact on the cells and made them "burst", and then the inner substances of cell leaked out. In addition, the chemical active species reacted with the cells and the leaking substances from cells at the same time. Finally, the leaking substances became filamentous due to the "erosion" of charged particles and chemically active species. Hong et al. [14] reported the plasma-treated *Escherichia coli* cells had severe cytoplasmic deformations and leakage of bacterial chromosome. Yet, the filamentous substances in our SEM were significantly different

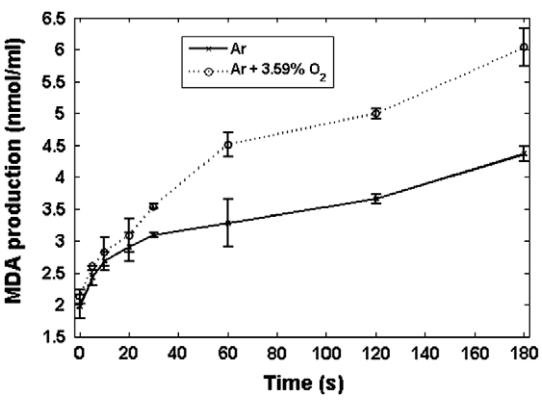

Fig. 7. Amount of MDA after plasma treatment at different time.

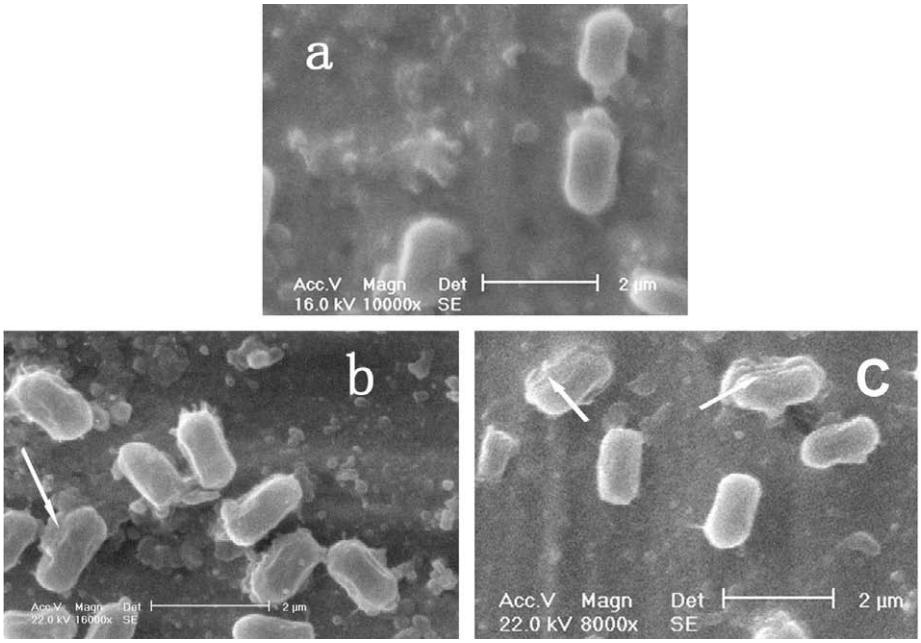

Fig. 8. Scanning electron micrograph of: (a) untreated, and (b) exposed to plasma for 60 s, (c) exposed to Ar + 3.59% O_2 plasma for 60 s.

from leakage of bacterial chromosome showed in literature [14]. Because the cell in our experiment did not damage seriously, the filamentous substances were mainly the leaking substances like protein.

4. Conclusions

In this study, an atmospheric pressure dielectric barrier discharge plasma jet using Ar or Ar + O_2 as the working gas was designed to sterilize the *Bacillus subtilis*. About four orders of magnitude of *B. subtilis* cfu reduced after 60 s plasma treatment, which were two orders of magnitude larger than Ar plasma, and they were totally inactivated at 180 s. It was found that the bacterial inactivation efficacy had a significant improvement when oxygen was introduced into the plasma jet. The emission spectra result indicated that O_2 in plasma environment could be decomposed reactive oxygen species and produced O_3, and these chemically active species played a significant role in for inactivation the bacteria. Moreover, the intensity (a.u.) of O IV radical increased evidently in Ar + O_2 plasma. From the results of heat, UV, protein leakage, MDA production and SEM images, these were showed that heat or UV radiation was not significant in the deactivation. Instead, synergistic actions of charged particles and chemically active species played the dominant role.

Acknowledgements

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