The interaction of an atmospheric pressure plasma jet using argon or argon plus hydrogen peroxide vapour addition with *bacillus subtilis**

Deng San-Xi(邓三喜)[a)†], Cheng Cheng(程 诚)[a)], Ni Guo-Hua(倪国华)[a)], Meng Yue-Dong(孟月东)[a)], and Chen Hua(陈 华)[b)]

[a)] *Institute of Plasma Physics, Chinese Academy of Sciences, Hefei* 230031, *China*

[b)] *Key Laboratory of Ion Beam Bioengineering, Chinese Academy of Sciences, Hefei* 230031, *China*

(Received 21 December 2009; revised manuscript received 5 March 2010)

This paper reports that an atmospheric pressure dielectric barrier discharge plasma jet, which uses argon or argon + hydrogen peroxide vapour as the working gas, is designed to sterilize the *bacillus subtilis*. Compared with the pure argon plasma, the bacterial inactivation efficacy has a significant improvement when hydrogen peroxide vapour is added into the plasma jet. In order to determine which factors play the main role in inactivation, several methods are used, such as determination of optical emission spectra, high temperature dry air treatment, protein leakage quantification, and scanning electron microscope. These results indicate that the possible inactivation mechanisms are the synergistic actions of chemically active species and charged species.

Keywords: atmospheric pressure, plasma jet, hydrogen peroxide vapour, inactivation mechanisms, synergistic actions

PACC: 5280, 5275R

1. Introduction

Sterilization is a physical or chemical process to eliminate microorganisms in the environment. Many conventional techniques have been used until now for sterilization involving exposure to toxic compounds such as ethylene oxide, chlorine gas or exposing the specimen to high temperatures and pressures in an autoclave.[1] Most of them are associated with some level of damage to the material or medium supporting the microorganisms, especially, to some non-heat-resistant material.[2−4] In order to reduce the damage on some non-heat-resistant medical devices, a plasma sterilization device using hydrogen peroxide (H_2O_2) as a sterilization agent has been put into practical use in hospitals as an alternative to conventional equipments such as autoclaves and ethylene oxide sterilizer.[5] Unlike ethylene oxide steam sterilization, hydrogen peroxide is not toxic and does not leave residues on the sterilized instruments. As such, operators can use the instruments immediately after reprocessing. However, most medical plasma sterilization devices using hydrogen peroxide, such as the STERRAD^{TM} of Johnson & Johnson Medical Ltd, work at low or medium pressure.[5−7]

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, low-temperature, long operative duration, and economical and simple systems.[2,4,8−11] In this study, a dielectric barrier discharge (DBD) plasma jet using different working gases (argon or argon + H_2O_2 vapour) were operated in the open air for inactivation. In order to test the sterilizing effect of the plasma jet, *bacillus subtilis* (*B. subtilis*) was selected. Furthermore, to examine the possible inactivation mechanisms, a set of physical and chemical methods were used. Firstly, the electronically excited species generated by the plasma jet were determined by optical emission spectrometer, and then scanning electron microscope (SEM) images were obtained after plasma treatment. Finally, the amounts of protein leakage were measured.

*Project supported by the Science Foundation of Hefei Institutes of Physical Science, Chinese Academy of Sciences (Grant No. 085FZ10134).

†Corresponding author. E-mail: dengsanxi@mail.ustc.edu.cn

© 2010 Chinese Physical Society and IOP Publishing Ltd

2. Experimental setup

2.1. Generation of the atmospheric-pressure plasma jet

Figure 1 shows the schematic diagram of self-designed atmospheric pressure DBD plasma jet system. 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. Its geometric details were given elsewhere.[12] In this study, the DBD plasma jet employed either argon (Ar) or Ar + hydrogen peroxide vapour as the working gas. The Ar flow rate was fixed at 21.51 slpm (standard liters per minute), mixed either with or without hydrogen peroxide vapour. The length and gas temperature of plasma jet were about 2–5 cm (changed with flow rate) and 30–80 °C respectively. The distance between the nozzle and the samples was 1 cm, so the samples could be fully covered by the plasma plume.

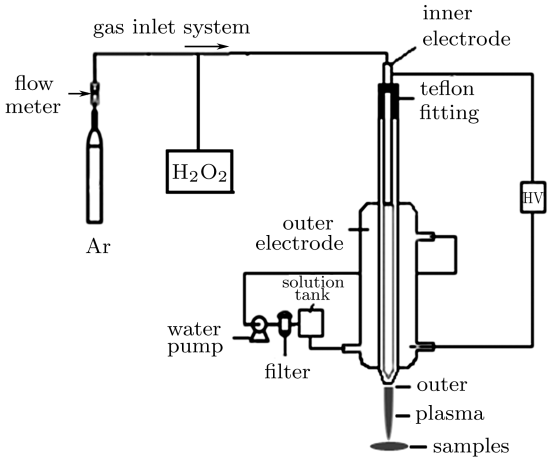


Fig. 1. Schematic diagram of plasma jet discharge system.

2.2. Colony forming unit test

The samples for sterilization were made of 6 mm × 6 mm sheets of polytetrafluoroethylene (PTFE) contaminated 50-μl *B. subtilis* suspension, and then the sheets were dried in a desiccator for 1 h. After that, the samples were exposed to the atmospheric DBD plasma jet with different time. And then, the samples were put into test tubes containing 10-ml germfree distilled 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 study 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) for simulating the inactivation by high temperature. After 5-min treatment by hot air, the follow-up method was the same as the above cfu test.

2.3. Determination of optical emission spectra

In order to examine the optical emission behaviour of plasma jet, an optical emission spectrometer (avaspec-2048 optical fibre emission spectrometer) with a charge-coupled device (CCD) detector was used. The light was transferred through a fibre cable to the spectrometer and the emission was recorded between wavelengths of 197 nm and 720 nm.

2.4. Scanning electron microscope examination

The SEM (XL30 ESEM) was used to detect the effects of plasma treatment on the morphology of the bacteria. 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 morphology changes of controlled and treated samples could be compared by the SEM images.

2.5. Determination of the amount of protein leakage

The *B. subtilis* absorbed on the PTFE sheets were 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 supernatant 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 had an absorption peak at 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.

3. Results and discussion

3.1. Devitalization effect of plasma jet

The inactivation of *B. subtilis* by Ar plasma jet was shown in Fig. 2. About two orders of magnitude of *B. subtilis* cfu reduced after 60-s plasma treatment. Even the *B. subtilis* were treated by plasma at 180 s, less than three *B. subtilis* cfu $\log_{10}$ reduction were achieved, whereas 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.[13] However, when hydrogen peroxide vapour was added into the working gas, the bacterial inactivation efficacy was significantly improved as shown also in Fig. 2. About 4 $\log_{10}$ of *B. subtilis* cfu was reduced after 60-s plasma treatment, which was two orders of magnitude larger than Ar plasma. Finally, the *B. subtilis* were totally inactivated after 120 s.

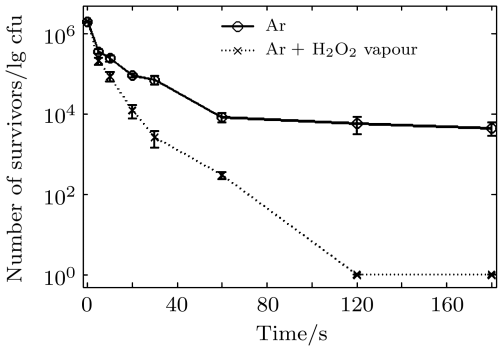


Fig. 2. The survivor curve of *B. subtilis* after Ar and Ar + H_2O_2 vapour plasma exposure (Ar flow rate 21.51 slpm).

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

$$D = t/(\log N_0 - \log N_s), \tag{1}$$

where N_0 is the initial population, and N_s is the surviving population. The acronym D1 indicates the D-value when t is the time to destroy 90% of the initial population (or the time for a one $\log_{10}$ reduction). By the same token, the acronym D2 indicates the D-value when t is the time to destroy 99% (or the time for two $\log_{10}$ reduction).

The D-values of *B. subtilis* after Ar or Ar + H_2O_2 vapour plasma treatment were shown in Fig. 3. The D1 and D2 of Ar plasma were 14 s and 50 s respectively, but they decreased to 5 s and 17 s when H_2O_2 vapour was added in. The significant decrease of D-value suggests that the inactivation of Ar + H_2O_2 vapour plasma is more effective than pure Ar plasma under the same condition.

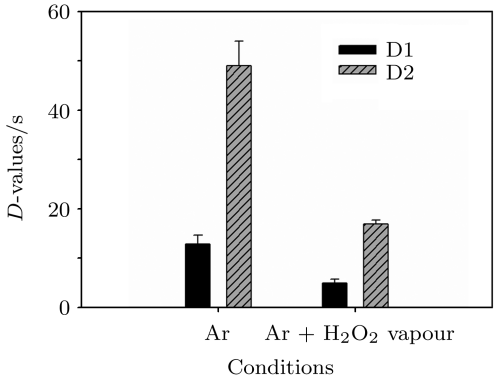


Fig. 3. The D-values of *B. subtilis* after Ar and Ar + H_2O_2 vapour plasma exposure.

From the result of high temperature effect, the number of the survivors decreased with the increase of temperature (from 20 °C to 80 °C). Although the sample was treated at 80 °C hot air for 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 indicates that the temperature is not responsible for *B. subtilis* inactivation in our experiment.

In order to study what kind of role hydrogen peroxide vapour played in inactivation without plasma participation, the *B. subtilis* (initial population of *B. subtilis* cfu was 2.0×10^6) were exposed in H_2O_2 vapour at Ar flow rate 21.51 slpm. The results of the bacteria treated by the plasma on and off were shown in Table 1. Although the flow of hydrogen peroxide vapour caused a decrease of 0.694 $\log_{10}$ in the number of cfu after 60-s treatment, this decrease was much smaller than the one in Ar + H_2O_2 plasma condition (reduced 3.82 orders of magnitude). Therefore, the gas flow of H_2O_2 vapour does not play the main role in cell inactivation but the plasma treatment. The H_2O_2 is decomposed into hydroxyl-radical ($\cdot$OH) in plasma environment. The process of decomposition can be expressed as follows:

$$H_2O_2 \rightarrow 2HO\bullet, \tag{2}$$

where $\cdot$OH is an effective inactivation factor and can inactivate the bacteria by chemically attacking the outer structures of bacterial cells which were mentioned by some researchers.[2,14–16]

Table 1. The results of the bacteria treated by the plasma on and off.

time	plasma on	plasma off
30 s (1)	3790	598400
30 s (2)	1420	453200
30 s (mean)	2605	525800
magnitude of cfu reduction (30 s)	2.89	0.580
60 s (1)	360	463800
60 s (2)	240	345700
60 s (mean)	300	404750
magnitude of cfu reduction (60 s)	3.82	0.694

3.2. Optical emission spectra

There were emission spectra lines of excited $\cdot$OH (306–310 nm), N_2 (336.8 nm), N_2^+ (356.4 nm), and Ar I (696.6, 706.5, and 714.6 nm) radicals[17] in Ar plasma plume (Fig. 4(a)). The $\cdot$OH (generated from water molecules in open air) was considered as an important role in bacterial inactivation. The N_2^+ (as 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.

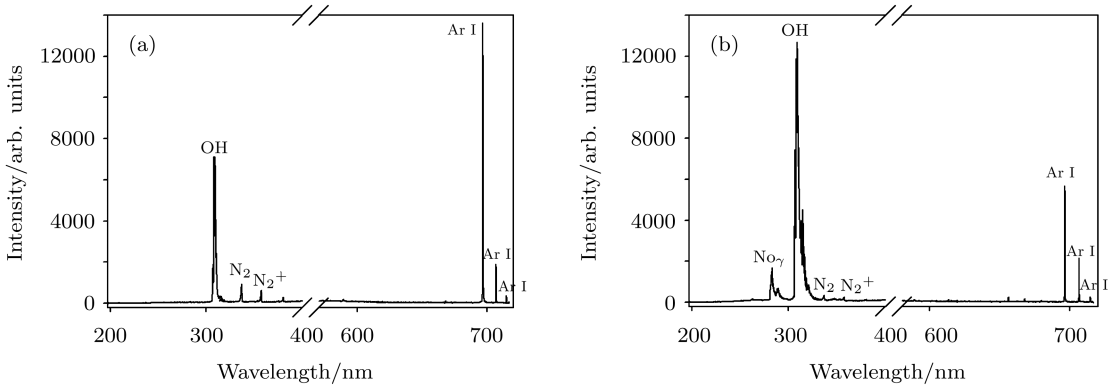


Fig. 4. Optical emission spectrum in the range from 197 nm to 720 nm: (a) Ar plasma; (b) Ar + H_2O_2 vapour plasma.

Besides the above radicals, NOγ (282.9 nm and 289.2 nm)[17] presented in Ar + H_2O_2 vapour plasma plume was shown in Fig. 4(b). We deduce that NOγ radicals are produced because of a set of reactions as Eq. (3) and the following

$$N_2 + 2 \bullet OH \rightarrow N_2O + H_2O, \quad (3)$$

$$N_2O + 2 \bullet OH \rightarrow 2NO + H_2O, \quad (4)$$

$$NO + 2 \bullet OH \rightarrow NO_2 + H_2O. \quad (5)$$

Moreover, compared with Figs. 4(a) and 4(b), the intensity (a.u.) of the $\cdot$OH significantly increased. Obviously, from Eq. (3) the reason of intense increase of the $\cdot$OH can be understood easily. The measured spectral intensity of the $\cdot$OH line (309 nm) normalized by the Ar I line (696.6 nm) in the two different conditions (Ar and Ar + H_2O_2 vapour plasma) was shown in Fig. 5. The intensities of the $\cdot$OH to Ar I in Ar + H_2O_2 vapour plasma jet was nearly five times as many as Ar plasma, which further illustrated that the $\cdot$OH increased after H_2O_2 vapour added in plasma. The inactivation effect of Ar plasma jet or H_2O_2 vapour was much less than Ar + H_2O_2 vapour plasma jet (shown in Fig. 2 and Table 1). It indicates that the $\cdot$OH radicals have an important contribution to inactivation and the deactivation strongly depends on the chemical reactive species.

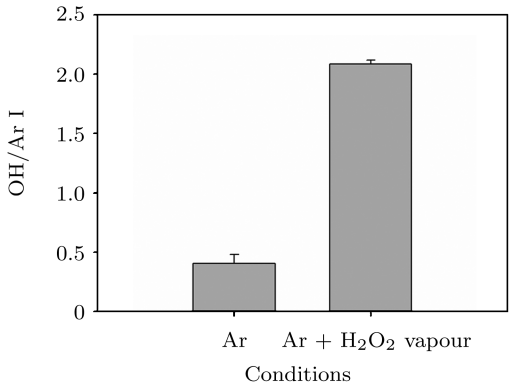


Fig. 5. The intensities of the $\cdot$OH (309 nm) to Ar I (696.5 nm).

3.3. The SEM

Comparison of Figs. 6(a), 6(b) and 6(c) clearly shows that DBD plasma jet caused morphological changes of *B. subtilis*. In Fig. 6(b), a small amount of filamentous substances appeared around the cells obviously, and this phenomenon was even more evidently in Fig. 6(c). The plasma has a direct physical impact on the cells and causes some small cracks on the surface of cell[2,4,16,18] (the cracks are so small that they may be not seen clearly in SEM images), and then the inner substances of cell such as protein leak out from the small cracks. In addition, the chemical active species react with the cells and the leaking substances of cells at the same time.[2,14,15] Finally, the leaking substances become filamentous due to the "erosion" of charged particles and chemically active species.

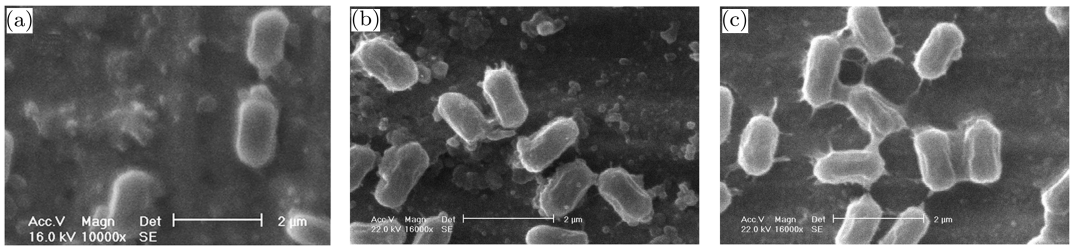


Fig. 6. Scanning electron micrograph of (a) controlled, (b) *B. subtilis* exposed to Ar plasma for 60 s and (c) *B. subtilis* exposed to Ar + H_2O_2 vapour plasma for 60 s.

3.4. Protein leakage

The charged particles in plasma can bombard the bacteria and cause etching action on cell surface. This etching action erode cell materials, as a result, cell membrane rupture and the content such as protein effuse, and cause cell to die finally.[18] From the results of SEM images, there were no obvious ruptures at the surface of cell, but a small amount of filamentous substances appeared around the cells obviously after plasma treatment. The protein leakage was used to prove that the overflow material contained protein and determined the roles of charged particles and chemical reactive species such as $\cdot$OH.

The variational tendency of protein leakage after plasma treatment was shown in Fig. 7. Though there were a small amount of chemical reactive species in pure Ar plasma (mentioned in Subsection 3.2), the charged particles were the main component for cells inactivation. The protein leakage increased with the treatment time in pure Ar plasma (Fig. 7), which indicated that the charged particles played the main role in breaking the cell surface. When Ar + H_2O_2 vapour was used as the working gas, although a large number of the $\cdot$OH radicals was produced in plasma environment, the charged particles also bombarded and etched the bacteria. From the results of SEM images, more filamentous substances appeared around the cells than the one of Ar plasma due to the $\cdot$OH radicals' participation. So it proves that the $\cdot$OH radicals inactivate the bacteria by chemically attacking the outer structures of bacterial cells, but it can react with leaking substances like protein synchronously. Therefore, although the rate of the protein leaking had a significant increase due to the roles of charged particles and the $\cdot$OH radicals, the rate of protein consumption also increased because of the $\cdot$OH radicals' participation. Therefore, when the rate of protein consumption was larger than the one of protein leaking, the measured protein leakage in Ar + H_2O_2 vapour plasma jet was smaller than Ar plasma jet (Fig. 7).

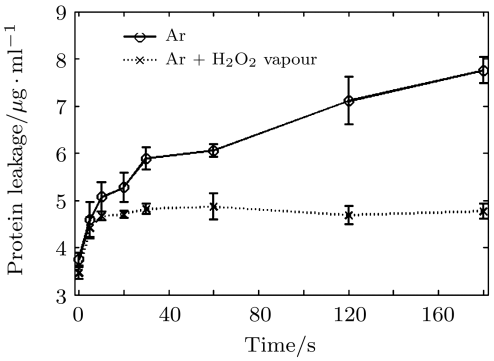


Fig. 7. Amount of protein leakage after plasma treatment at different times.

The test of protein leakage indicates that the cell will be cracked by the synergistic actions of bombard-

ment induced by the charged particles and reaction with the chemical reactive species ($\cdot$OH radicals).

4. Conclusion

In this study, an atmospheric pressure DBD plasma jet using argon (Ar) or argon + hydrogen peroxide vapour as the working gas was designed to sterilize the *bacillus subtilis*. About four orders of magnitude of *B. subtilis* cfu reduced after 60 s Ar + H_2O_2 plasma treatment, which were two orders of magnitude larger than Ar plasma. Moreover, the *B. subtilis* reduced six orders of magnitude cfu after 120 s Ar + H_2O_2 plasma treatment, which were four orders reduction larger than Ar plasma. When plasma was off, the reduction of magnitude was much smaller than that when the plasma was on. It indicates that H_2O_2 can be decomposed into hydroxyl-radical ($\cdot$OH) in a plasma environment, and hydroxyl-radical plays a significant role for inactivation of the bacteria. In the emission spectra, the intensity (a.u.) of the $\cdot$OH radical increased evidently in Ar + H_2O_2 plasma. From the results of SEM images and protein leakage the possible inactivation mechanisms are the synergistic actions of chemically active species ($\cdot$OH radicals) and charged species.

Acknowledgements

The authors thank the help of Key Laboratory of Ion Beam Bioengineering, Chinese Academy of Sciences, Hefei, China.

References

[1] Wintenberg K, Hodge K, Montie A, Deleanu T C, Sherman L, Roth D, Tsai J R and Wadsworth P 1999 *J. Vac. Sci. Technol.* A **17** 1539

[2] Laroussi M 2002 *IEEE Trans. Plasma Sci.* **30** 1409

[3] Cheng C, Liu P, Xu L, Zhang L Y and Zhan R J 2006 *Chin. Phys.* **15** 1544

[4] Ashisharma, Amypruden, Yu Z Q and Collins G J 2005 *Environ. Sci. Technol.* **39** 339

[5] Kyi M S, Holton J and Ridgway G L 1995 *Journal of Hospital Infection* **31** 275

[6] Hofmann J O, Knoll M, Durr M, Schmitt B and Borneff-Lipp M 2005 *Journal of Hospital Infection* **59** 280

[7] Marquez-de-Bar, Bar G, Naumann W and Rusch-G A 2001 *American Journal of Infection Control* **29** 306

[8] Li G, Li H P, Wang L Y, Wang S, Zhao H X, Sun W T, Xing X H and Bao C Y 2008 *Appl. Phys. Lett.* **92** 221504

[9] Bomi Gweon, Kim D B, Moon S Y and Choe W 2009 *Current Applied Physics* **9** 625

[10] Xu L, Zhang R, Liu P, Ding L L and Zhan R J 2004 *Chin. Phys.* **13** 913

[11] Dong L F, Jia P Y, Li L C, Li X C and Liu Z H 2008 *Acta Phys. Sin.* **57** 1001 (in Chinese)

[12] Deng S X, Cheng C, Ni G H, Meng Y D and Chen H 2008 *Jpn. J. Appl. Phys.* **47** 7009

[13] Heise M, Neff W, Franken, Muranyi P and Wunderlich J 2004 *Plasmas and Polymers* **9** 23

[14] Yamamoto M, Nishioka M and Sadakata M 2001 *Proc. 15th Int. Symp. Plasma Chemistry* Orleans, France, August 2–6, 2001 p. 743

[15] Tetsuya Akitsu, Hiroshi Ohkawa, Masao Tsuji, Hideo Kimura and Masuhiro Kogoma 2005 *Surface & Coatings Technology* **193** 29

[16] Joaquin J C, Calvin Kwan, Nina Abramzon, Kurt Vandervoort and Graciela Brelles-Marino 2009 *Microbiology* **155** 724

[17] Pears R W B and Gaydon A G 1976 *The Identification of Molecular Spectra* 4th edn. (London: Chapman & Hall) p. 263

[18] Ferng S F, Castro C E and Afifi A A 1997 *J. Toxicol. Environ. Health* **51** 353