Appl Biochem Biotechnol (2010) 160:1978–1984
DOI 10.1007/s12010-009-8817-3

E. coli, *P. aeruginosa*, and *B. cereus* Bacteria Sterilization Using Afterglow of Non-Thermal Plasma at Atmospheric Pressure

Farshad Sohbatzadeh · Abasalt Hosseinzadeh Colagar · Saeed Mirzanejhad · Somayeh Mahmodi

Received: 1 September 2009 / Accepted: 11 October 2009 /
Published online: 1 November 2009

Abstract We developed and employed a new geometrical structure of dielectric barrier discharge in atmospheric pressure for bacterial broad spectrum sterilization. We utilized a plasma source having an AC power supply at 50 HZ and 5,400 V (rms value). We prepared suspensions of the Gram-negative bacteria species (*Escherichia coli*, *Pseudomonas aeruginosa*) and a Gram-positive of *Bacillus cereus* with Luria–Bertani broth media up to $OD_{600\ nm}$=0.25 of McFarland standard. Afterglow of non-thermal atmospheric pressure plasma treated these suspensions. The influence of the atmospheric plasma afterglow on the species was assayed in different time durations 5, 10, and 15 min. The spectroscopic results of this investigation indicated that the survival reduction of the species can reach to 100% for *P. aeruginosa* in an exposure time of 10 min, *E. coli* and *B. cereus* in an exposure time of 15 min.

Keywords Dielectric barrier discharge · Non-thermal atmospheric plasma · Sterilization · *Escherichia coli* · *Pseudomonas aeruginosa* · *Bacillus cereus*

Introduction

Sterilization by means of killing of life is used essentially in the medical-care industry and hospital, as a part of the prevention of infection and sterility assurance system [1, 2]. The inactivation of harmful microorganisms can be accomplished by using chemical or physical different methods, including heat, chemical solutions, or gases and radiation bombardment. However, most of these sterilization methods contain some level of damage to the material or

F. Sohbatzadeh (✉) · A. Hosseinzadeh Colagar · S. Mirzanejhad · S. Mahmodi
Nano and Biotechnology Research Group, University of Mazandaran, Babolsar, Iran
e-mail: f.sohbat@umz.ac.ir

F. Sohbatzadeh · S. Mirzanejhad · S. Mahmodi
Physics Department, Faculty of Basic Science, University of Mazandaran, Babolsar, Iran

A. Hosseinzadeh Colagar
Biology Department, Faculty of Basic Science, University of Mazandaran, Babolsar, Iran

limit the complete sterilization [3, 4]. For example, pressurized steam, autoclave, dry-heat, and other high temperature sterilization methods are not compatible for materials that have low resistance to heat and steam. Furthermore, compatible gaseous compounds such as ethylene oxide may remain on the surface which can be toxic to patients and medical operators. Nowadays, the research on sterilization methods is on the rise. Atmospheric pressure plasmas have been developed for many applications such as air purification and sterilization [1]. Plasma treatment has many advantages in comparison with the other bacterial inactivation methods including: fast, efficient, safe in terms of thermal, chemical, or irradiation damage. Therefore, low temperature plasma sterilization is a green technique and is regarded as one of the most promising sterilization techniques [5]. New methods to sterilize at room temperature at low or atmospheric pressure are actively studied [6]. Among the several plasma types, dielectric barrier discharge (DBD) is most frequently used [1, 2, 7].

In our previous study, we used a comb and a flat electrode structures for *Escherichia coli* sterilization employing alternative current (AC) and direct current (DC) power supplies [8]. In those structures, we showed that the AC power supply could be more efficient in *E. coli* sterilization. In this paper, we introduce a new DBD structure together with a new method for evaluating bactericidal effect of afterglow of non-thermal plasma at atmospheric pressure against *E. coli*, *P. aeruginosa*, and *Bacillus cereus* bacteria.

Materials and Methods

We designed a structure for the plasma source (DBD structure) in such a way to have a nozzle for concentrating afterglow plasma on the species. Oxygen gas with purity 99.99% was employed as the medium for plasma generation. The main structure of DBD consisted of two coaxial right circular copper cones as the electrodes of the system. The spacing between the lateral surfaces of the electrodes was adjustable ranging from 0.1 to 3 mm. We polished the lateral surfaces of the electrodes, too. In order to prevent arcing, a flexible sheet of polyvinyl chloride (PVC) material as the dielectric barrier was shaped to cover the inner electrode. The thickness of the PVC sheet was 0.19 mm. The plasma exit was provided at the apex of the outer cone with diameter of 1 cm which comprised a nozzle. The circular base diameters of the inner and outer copper cones were 50 and 90 mm, respectively. The circular base of the outer electrode was covered by an acrylic glass sheet having 10 mm thickness. A gas inlet and a high voltage cable feed through were installed on the acrylic glass base. In order to prevent arcing and limit the discharge volume, the base of the inner electrode was curved as a conic geometry. This part of the inner electrode had more spacing to the lateral surface of the outer cone. This arrangement made the plasma to be formed at the nearest parts of the gap as indicated in the inset of Fig. 1 by 5.

The electrodes were coupled to an AC voltage up to 5,400 V at the frequency of 50 Hz, and the inner one was grounded. Oxygen gas was fed through acrylic glass base with a controlled flow of 10 l/min. The concentration of ozone was measured by ozone sensor (A-21ZX, Eco Sensors, Inc.) inside the working chamber at the region of 13 in Fig. 1. Ozone concentration was 11.0±0.5 ppm during the experiment.

Two Gram-negative and one Gram-positive lyophilized bacterial strains were obtained from the Pastor Institute of Iran (Table 1). Brain heart infusion (BHI) media solutions (0.5 ml) were added into lyophilized bacterial strain vials and incubated at 37°C overnight. Cultured BHI (0.5 ml) inoculated in to Luria–Bertani (LB) agar (including: 10 g Bacto-tryptone; 5 g Yeast extract; 10 g NaCl; 15 g Agar per liter distilled water). The strains were maintained on standard LB agar slants at 5°C to 6°C.

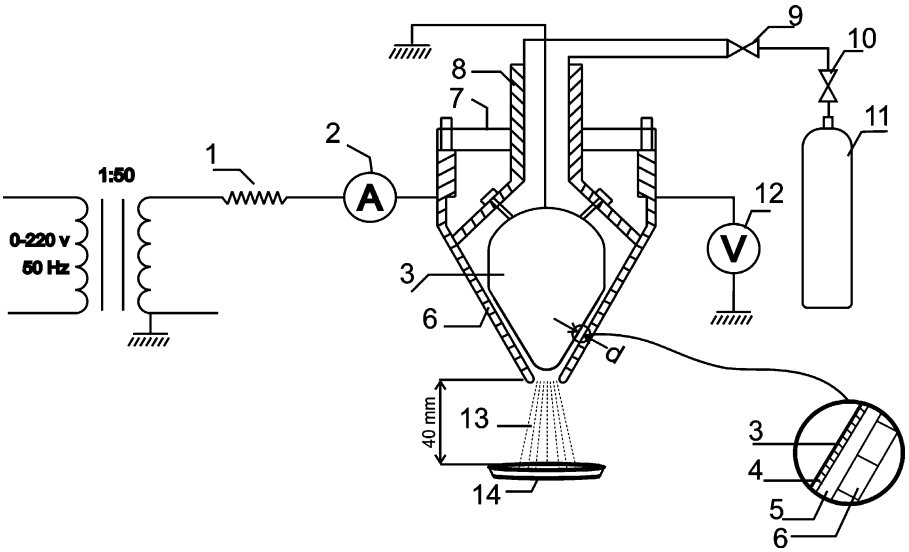

Fig. 1 Schematic picture of the sterilization setup. *1* Ballast resistor R=10 kΩ, *2* Ampere meter, *3* conic copper electrode, *4* PVC dielectric, *5* gap, the plasma formation region, *6* outer copper electrode, *7* acrylic glass, *8* diffuser, *9* flowmeter, *10* valve, *11* oxygen tank, *12* volt meter, *13* flow of afterglow plasma, *14* sample

One loop from either pathogenic Gram-positive/negative bacteria inoculated to 15 ml liquid LB (LB without agar) and incubated at 37°C overnight. One milliliter of bacteria inoculated into 15 ml liquid LB and cultured until 0.5 McFarland standards corresponding to about 3×10^8 CFU/ml for *E. coli*. Then, juvenile cultured bacteria were diluted with liquid LB media up to $OD_{600\ nm}$=0.25. These suspensions contained a concentration of 10^{3-4} CFU/ml for *E. coli*. All bacterial suspensions were performed by this method and used for sterilization by afterglow of non-thermal atmospheric plasma.

Sterilization by Afterglow of Non-Thermal Atmospheric Plasma

Five milliliters of bacterial suspensions with OD_{600}=0.25 was added in glass Petri dish with diameter of 70 mm. We prepared three treatments (5-, 10-, and 15-min treatments by afterglow) and one control Petri dish for each bacterial culture strains. Experiment was conducted in open air with humidity 70% under atmospheric pressure with the room temperature at 25°C. All of glass plates except control one were placed in front of the afterglow plasma nozzle and exposed for 5, 10, and 15 min. The plasma was produced by an AC power supply of 50 HZ and 5,400 V. Oxygen gas flow rate was controlled at 10 l/min

Table 1 Identification and source of bacterial culture strains.

Pathogen	Strain/source	Type/interest in testing
Escherichia coli	ATCC 35218	Gram-negative, clinical significance
Pseudomonas aeruginosa	ATCC 27853	Gram-negative, clinical significance
Bacillus cereus	ATCC 10987	Gram-positive, clinical significance

during the experiment. The distance between the nozzle and the glass plate containing the bacteria was 40 mm. After plasma treatment, 1 ml of treated bacteria was diluted by 15 ml of LB, incubated for 1.5–2 h at 37°C, and then placed on ice powder. Bactericide effects of the atmospheric plasma assayed by turbidimetric measurements within 190–900 nm wavelength range (with UV-1600 UV/VIS Reyleigh) spectrophotometer.

Experimental Results

To produce large volume, uniform, and non-thermal plasma, we designed conical reactor as a plasma nozzle. Atmospheric pressure dielectric barrier discharge in oxygen with purity 99.99% generated for sterilization of the *B. cereus*, *E. coli*, and *P. aeruginosa* strains. Strains were positioned at a constant distance of 40 mm from the nozzle and directly exposed to the afterglow flow when the voltage of 5,400 V was applied. The treatment durations were 5, 10, and 15 min. The influence of the atmospheric afterglow plasma on the bacterial species was studied by using turbidimetric measurement. The spectroscopic measurement curves are shown in Fig. 2.

In Fig. 2, the optical density of the LB is lower than the other treated samples from 500 to 900 nm. The difference between the optical density of the liquid LB and the other treated samples depends on the bacteria type and treatment time. The higher sterilization efficiency, the lower difference between OD of the LB and the treated samples. These results showed that the afterglow plasma of the DBD is very efficient for sterilization. It was seen that the

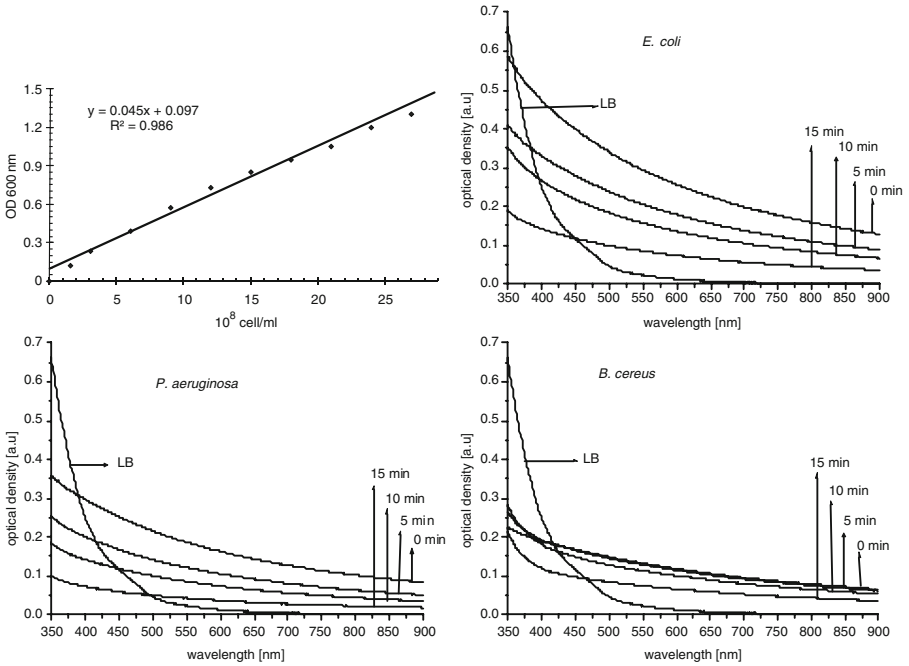

Fig. 2 Sterilization effects of the afterglow of DBD against *B. cereus*, *E. coli*, and *P. aeruginosa* strains in the three time variations of 5, 10, and 15 min. A graph of optical density (OD) versus *E. coli*/ml indicated in the left upper, 0.5 McFarland standard corresponding to about 3×10^8 CFU/ml for *E. coli* with $OD_{600}=0.235$ in this paper

afterglow plasma completely reduced survival of *E. coli* and *B. cereus* after 15 min. On the other hand, the complete survival reduction of *P. aeruginosa* took place after 10 min. A significant difference in survival could be verified by comparing the control and 15-min treated samples with p value<0.05. We used a fast and reliable turbidimetric measurement method. In this measurement method, one can easily recognize handling contamination due to the smooth behavior of the OD curves. Statistical analysis of percentage of survival reduction strains treated with afterglow plasma in the three-time variation summarized in the Table 2. It was seen that 15-min exposure by afterglow of the dielectric barrier discharge is enough to kill *E. coli*, *P. aeruginosa*, and *B. cereus* bacteria, completely. It is also worth noting among the three bacteria, *P. aeruginosa* sterilization was considerable using this structure for exposure time of 5 min.

Discussion and Conclusive Remarks

All of verity sterilization methods including hot steam, dry heat, gas, and irradiation with gamma rays have disadvantages even contain several advantages. For example, residual gaseous agents of the sterilization by ethylene oxide influence patients and medical operators toxically and strong concern to human carcinogenicity is expressed since the early 1990s. They may be structural degradation, harmful to human and environment, or very time-consuming. Non-thermal plasmas are emerging and promising devices for sterilization. They have achieved great progress in recent years. It has been found that many kinds of plasmas can kill vegetative forms, spores, and fungi efficiently [8–12].

In this work, we developed an atmospheric plasma discharge device to produce chemical active agents for sterilization. Employment of oxygen gas as the plasma medium led to the reactive species production such as atomic oxygen and ozone during the treatment. Ozone formation is a two-step reaction, which starts with the dissociation of O_2 molecules by the electron collisions in the plasma reactor [13, 14]

$$O_2 + e \rightarrow O + O + e, \tag{1}$$

The atomic oxygen is an oxidant which leads to the destruction of microorganisms. The second step describes a three-body collision

$$O + O_2 + O_2 \rightarrow O_3 + O_2, \tag{2}$$

$$O + O_2 + O \rightarrow O_3 + O. \tag{3}$$

Ozone is a kind of strong oxidant; it can kill bacteria in different forms: it can decompose enzyme that is desirable in synthesizing glucose in bacteria; react with bacteria

Table 2 The percentage of survival reduction strains treated with plasma in the three time variation.

Strains Time (min.)	*E. coli* reduction percent	*P. aeruginosa* reduction percent	*B. cereus* reduction percent
5	48.6	88	19
10	75.35	100	89.6
15	100	100	100

and virus directly and destroy cell wall, DNA, and RNA of them; permeate the pericellular membrane and invade into the cell, destroy lipoprotein, and lipopolysaccharide. These disinfectant processes are very exhaustive. Ozone is unstable and degrades into molecular and monatomic oxygen. The atomic oxygen will combine into molecular oxygen, so they leave no harmful by-products at the end. Ozone, as a kind of gas, can pervade the whole space and leaves no dead angle.

The structure of the plasma source was designed in such a way to have a nozzle for exiting afterglow plasma. The flow of afterglow in turn led to the production of hydroxyl (OH) as a reactive specie in the humid air. It can be produced by the following collisional pathways due to the air humidity at the exit of the reactor:

$$H_2O + O_3 \rightarrow O_2 + 2OH, \tag{4}$$

$$H_2O + O \rightarrow 2OH, \tag{5}$$

where during the experimnt, the humidity was 70%.

Since heat and UV radiation were expected not to play an important role for non-thermal atmospheric plasmas [13], the destruction of the bacteria was attributed to the chemical reactive species such as ozone, atomic oxygen, and hydroxyl. In order to increase the efficiency of the sterilization, conical geometry structure for electrodes was designed to concentrate the flow on the species. In this way, the workpieces were exposed to the plasma, indirectly. Referring to Fig. 1, this structure reduced the time of flight of the active agents to the workpieces. Since the active radicals have a finite lifetime, the structure caused less reduction of these concentrations. Among the other products of the atmospheric DBD, ozone and atomic oxygen seem to play the major role in killing process. The concentration of ozone was measured by ozone sensor (A-21ZX, Eco Sensors, Inc.) inside the working chamber at the region of 13 which was illustrated in Fig. 1.

UV radiation is another product of the plasmas. The effects of the UV on micro-organisms are well known and include both thymine dimmers and strand breaks. In this work, the effective short-wavelengths UV were absorbed by air within some micrometers and, thus, did not contribute efficiently in sterilization process. In our experiment, ozone, monatomic oxygen, and hydroxyl were the main antimicrobial products of the atmospheric pressure dielectric barrier discharge. We showed that this kind of discharge can be useful not only in killing *E. coli* but also in killing a variety of microorganisms, which is consistent with the other literatures [2, 9, 15].

The results of this investigation showed that the survival reduction percentage of *E. coli* and *B. cereus* by the afterglow of the low-temperature plasma with an exposure time of 15 min and of *P. aeruginosa* with an exposure at least 10 min reached to 100%. On the other hand, the disinfection time strains tested was shorter for *P. aeruginosa* than that of *E. coli* and *B. cereus*. The measurement method which was used in this work seems to be fast and reliable. On the other hand, one can recognize handling contamination easily due to the smooth behavior of the OD curves.

References

1. Trompeter, F. J., Neff, W. J., Franken, O., Heise, M., Neiger, M., Shuhai, L., et al. (2002). *IEEE Transactions on Plasma Science, 30*, 1416–1423.
2. Moreau, M., Orange, N., & Feuilloley, M. G. J. (2008). *Biotechnology Advances, 26*, 610–617.

3. Park, B. J., Takatori, K., Lee, M. H., Han, D. W., Woo, Y. I., Son, H. J., et al. (2007). *Surface and Coatings Technology, 201*, 5738–5741.
4. Ohkawa, H., Akitsu, T., Tsuji, M., Kimura, H., Kogoma, M., & Fukushima, K. (2006). *Surface and Coatings Technology, 200*, 5829–5835.
5. Lerouge, S., Wertheimer, M. R., & Yahia, L. H. (2001). *Plasmas and Polymers, 6*, 175–188.
6. Moisan, M., Barbeau, J., Crevier, M. C., Pelletier, J., Philip, N., & Saoudi, B. (2002). *Pure and Applied Chemistry, 74*, 349–358.
7. Xingmin, S., Yukang, Y., Yanzhou, S., Wang, Y., Fengling, P., & Yuchang, Q. (2006). Plasma. *Science and Technology, 8*, 569–572.
8. Mirzanejhad, S., Sohbatzadeh, F., Hossienzadeh Colagar, A., & Asadian, N. (2008). *Yakhteh Medical Journal, 9*, 248–253. (in Persian).
9. Roth, J. R., Sherman, D. M., Gadri, R. B., Karakaya, F., Chen, Z., Montie, T. C., et al. (2000). *IEEE Transactions on Plasma Science, 28*, 56–63.
10. Philip, N., Saoudi, B., Crevier, M.-C., Moisan, M., Barbeau, J., & Pelletier, J. (2002). *IEEE Transactions on Plasma Science, 30*, 1429–1436.
11. Whittaker, A. G., Graham, E. M., Baxter, R. L., Jones, A. C., Richardson, P. R., Meek, G., et al. (2004). *Journal of Hospital Infection, 56*, 37–41.
12. Laroussi, M., Mendis, D. A., & Rosenberg, M. (2003). *New Journal of Physics, 5*, 41.1–41.10.
13. Laroussi, M., & Leipold, F. (2004). *International Journal of Mass Spectroscopy, 233*, 81–86.
14. Lieberman, M. A., & Lichtenberg, A. J. (2005). *Principles of Plasma Discharges and Material Processing* (2nd ed., p. 274). New Jersey: Wiley.
15. Moreira, A. J., Mansano, R. D., de Jesus Andreoli Pinto, T., Ruas, R., da Silva Zambon, L., da Silva, M. V., et al. (2004). *Applied Surface Science, 235*, 151–155.