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ORIGINAL ARTICLE

Sterilization effect of atmospheric plasma on *Escherichia coli* and *Bacillus subtilis* endospores

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Abstract

Aims: *Escherichia coli* and *Bacillus subtilis* spores were treated with an atmospheric plasma mixture created by the ionization of helium and oxygen to investigate the inactivation efficiency of a low-temperature plasma below 70°C.
Methods and results: An electrical discharge plasma was produced at a radio frequency (RF) of 13·56 MHz, connected to a perforated circular electrode with a discharge spacing of 1–15 mm. The discharge gas was helium with 0–2% oxygen. For the plasma treatment, a dried *E. coli* cell or *B. subtilis* endospore suspension on a cover-glass was exposed to oxygen downstream of the plasma from holes in an RF-powered electrode. The sterilization effect of the RF plasma was highest with 0·2% oxygen, corresponding to the maximum production of oxygen radicals.
Conclusions: Oxygen radicals generated by RF plasma are effective for the destruction of bacterial cells and endospores.
Significance and Impact of the study: Low-temperature atmospheric plasma can be used for the disinfection of diverse objects, especially for the inactivation of bacterial endospores.

Introduction

Recently, atmospheric plasma created by the electrical discharge of a gas has received much attention as a potential physical agent for biological decontamination and sterilization. Low-temperature radio frequency (RF) plasma sources at atmospheric pressure exhibit many characteristics of a low-pressure glow discharge and have been developed for practical applications in industry (Jeong *et al.* 1998; Babayan *et al.* 2001; Kang *et al.* 2003). As an atmospheric plasma source operates at atmospheric pressure, processing and treatments can be implemented continuously, without the need for costly vacuum equipment. This method can be used to destroy bacterial endospores and has attracted much interest for the decontamination of potential biological warfare agents (Schütze *et al.* 1998; Sato *et al.* 2006). The destructive efficiency of various gas plasma sources and temperatures on *Bacillus* spores was compared, and an oxygen-based plasma was found to be more efficient than a pure argon plasma (Hury *et al.* 1998). Purevdorj *et al.* (2003) reported that the highest mortality of *Bacillus pumilis* spores was obtained when air containing water vapour was used as the plasma carrier gas, and suggested that hydroxyl free radicals play a significant role in the inactivation of spores. Recent bacterial inactivation studies using RF atmospheric pressure plasma have demonstrated that plasma can be employed to reduce or sterilize bacterial cells and spores on contaminated surfaces (Rahul *et al.* 2005; Sharma *et al.* 2006).

Here we examined the inactivation efficiency of low-temperature plasma below 70°C, based on helium gas mixed with different concentrations of oxygen. We used an electrical power supply with an operating frequency of 13·56 MHz for the generation of low-temperature plasma at atmospheric pressure. *Escherichia coli* and *Bacillus subtilis* endospores were treated with atmospheric plasma that was electrically discharged between an upper electrode connected to a power supply and a ground electrode. We used transmission electron microscopy and optical emission spectroscopy to examine morphological

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changes in the treated bacteria from the excited oxygen species generated by the plasma.

Materials and methods

Strains

Escherichia coli KCTC 1039 was obtained from the Korean Collection for Type Cultures (Daejon, Korea) and was subcultured twice in tryptic soy (TS) broth for 16 h at 37°C in all experiments. A *B. subtilis* spore suspension was obtained from SGM Biotech, Inc. (Bozeman, MT, USA) and stored in a refrigerator until use to prevent spore germination.

Generation of low-temperature plasma at atmospheric pressure

A diagram of the experimental apparatus is shown in Fig. 1. The electrical discharge was produced in the space between a perforated circular electrode and a ground electrode of the same size as the opposite electrode, and mounted with dielectric material, such as alumina. The electrodes were 45 mm in diameter and had 61 holes, each with a diameter of 0·7 mm, distributed uniformly across the powered electrode through which the gases were fed into the discharge space. The plasma was maintained by an RF of 13·56 MHz connected to the top electrode. The RF power supplier (model number YSR-03 MF) was purchased from Youngsin Engineering Co. (Seoul Korea). The discharge spacing could be varied from 1 to 15 mm without interrupting the stable plasma state, even at atmospheric pressures. The temperature of the plasma was 70°C, measured by using an alcohol thermometer. However, considering the additional dielectric heating of the glass part of the thermometer, the actual temperature was empirically estimated to be slightly below 70°C.

Measurement of oxygen radical intensity

Optical emission spectroscopy was used to observe the excited oxygen species generated by the plasma. The procedure of optical emission measurement has been reported previously (Kang *et al.* 2003). Analysis of the spectra was conducted using an optical emission reference: helium lines at 706·5 and 728·1 nm and atomic oxygen at 777·1 nm.

Plasma treatment of the test organisms

For the plasma treatment of *E. coli*, 0·2 ml of cell suspension at a concentration of 10^5–10^6 cells ml^{-1} was deposited on a thin cover glass of 1 cm^2, and then dried on a clean bench at room temperature overnight. For the *B. subtilis* endospores, 0·2 ml of spore suspension was deposited on a thin cover glass of 1 cm^2 at an initial concentration of 10^5–10^6 spores ml^{-1} and dried on a clean

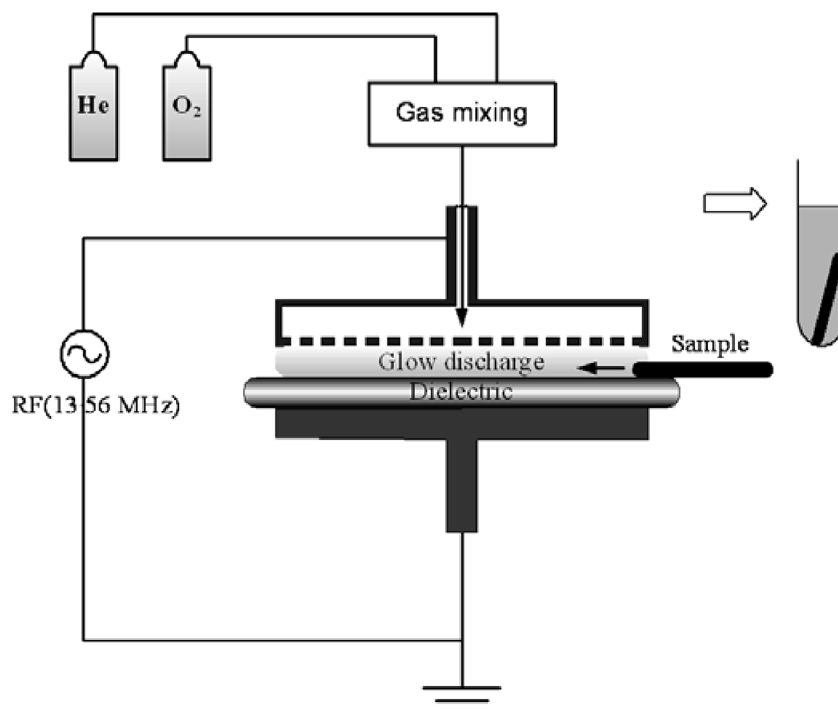

Figure 1 Schematic diagram of the experimental apparatus and process. RF power of 13·56 MHz is supplied to a perforated circular electrode with helium and oxygen gas. The target sample is treated on the ground electrode covered with a dielectric plate.

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bench at room temperature overnight. The dried cells or spores on the cover glass were exposed to the downstream oxygen plasma generated using different concentrations of oxygen from holes in an RF-powered electrode. To examine the inactivation effect of UV and temperature, the cover glass spread with spores was treated under a thin fused quartz plate, which allowed 90% of the UV (190–270 nm) to pass through, and also under a thin glass plate. The treatment time was monitored to determine the plasma exposure duration required for inactivation. After plasma treatment, the cover glass was put into a 50-ml conical tube containing 5 ml of PBS (phosphate buffered saline) and agitated on a shaking incubator at 250 rev min^{-1} for 3 h. Then 1 ml of the suspension was removed for spread plate counting.

Transmission electron microscopy

Escherichia coli were grown in TS broth for 16 h and then centrifuged at 9000 ***g*** for 10 min. The cells were removed to a cover glass and treated with plasma for 2 min, then returned to TS broth and centrifuged for 10 min. Cells were harvested and washed twice with PBS, then mixed with tryptic soy agar. The agar block was fixed with 2·5% glutaraldehyde in 0·1 mol l^{-1} PBS. Samples were postfixed in 1% (w/v) OsO_4 in 0·1 mol l^{-1} PBS for 2 h at room temperature, washed once with the same buffer, dehydrated in a graded ethanol series and embedded in low-viscosity medium. Thin sections of the specimens were double-stained with uranyl acetate and lead citrate. The grids were examined with an H-7000FA transmission electron microscope (Hitachi) at an operating voltage of 75 kV.

Results

The killing effect of RF plasma on *E. coli* as a function of treatment time is shown in Fig. 2. With 1% oxygen in the plasma and an input RF of 75 W, the bacterial tenfold reduction time was 7 s, and sterilization was accomplished within 60 s. With 2% oxygen in the plasma, the 10-fold reduction time was 20 s and the treatment time for sterilization was about 120 s. However, the killing curve showed nonlinear characteristics because the oxygen radicals were not generated uniformly in the plasma jet flame, especially at the initial stage.

The effect of the RF plasma for destruction of *B. subtilis* endospores is shown in Fig. 3. With 0·2% oxygen in plasma at an RF of 75 W, the 10-fold reduction time was 24 s, and sterilization was accomplished within 120 s. However, when the spores were protected from the plasma radicals with a quartz plate, viable counts were only reduced 10-fold after 120 s of treatment. In contrast, when the spores were covered with a thin glass plate to prevent UV and radical exposure, the viability of the spores was not affected. The results show that the UV from the plasma only slightly affected the viability of the spores, while heat-resistant spores were not affected by the low-temperature plasma below 70°C, demonstrating the crucial role of plasma radicals in spore inactivation.

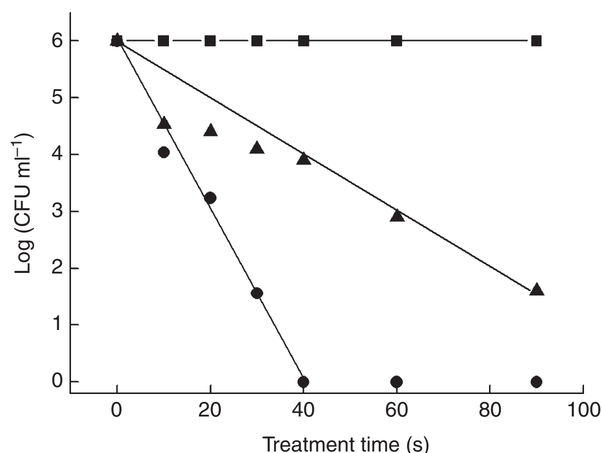

Figure 2 Effect of different gas rates on inactivation of dried cells of *Escherichia coli* with RF of 13·56 MHz at 75 W with helium gas of 4 *lpm* as the main carrier gas: control without plasma treatment (■), helium gas mixed with 2% oxygen (▲), and helium gas mixed with 1% oxygen (●). Values are the average of triplicate.

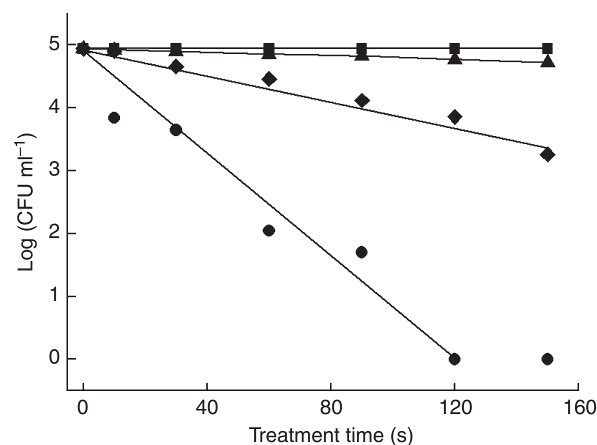

Figure 3 Effect of plasma radicals, UV, and heat from the plasma discharge on the inactivation of dried *Bacillus subtilis* spores. Plasma was produced at a RF of 13·56 MHz at 75 W with gas flow of helium mixed with 0·2% oxygen at 4 *lpm*: control without plasma treatment(■); treated under a thin glass plate to protect from the plasma radicals and UV(▲); treated under a thin quartz plate to protect from the plasma radicals(♦); and exposed to plasma without a glass or quartz plate (●). Values are the average of triplicate.

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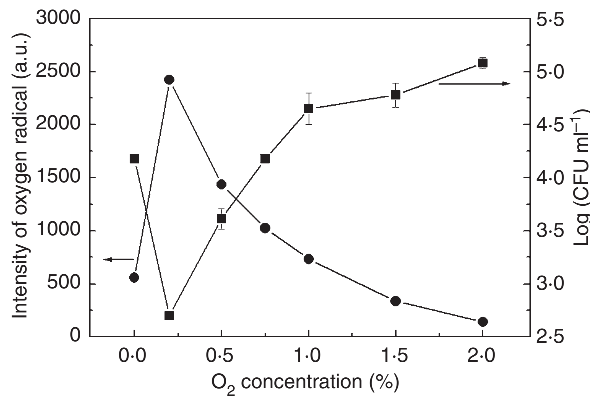

Figure 4 Effect of plasma with different oxygen concentrations on the intensity of oxygen radical (3^5P–3^5S, 777·1 nm) emission (●), and on the inactivation of dried *Bacillus subtilis* spores (■). Plasma was produced with RF at 75 W and a helium gas flow of 4 *lpm* for 60 s. Arbitrary units (a.u.) were used for the intensity of oxygen radicals.

Figure 4 shows survival of plasma-treated *B. subtilis* endospores and the oxygen radical intensity in plasma generated from 0–2% oxygen to determine the most effective oxygen concentration. Plasma with the highest oxygen radical (3^5P–3^5S, 777·1 nm) content was obtained when the oxygen concentration was 0·2%, corresponding to the lowest remaining viable cell number. This indicates that oxygen radicals play a key role in atmospheric plasma for the inactivation of bacterial spores.

We used an electron microscope to observe the effects of the plasma on bacterial cell morphology. Transmission electron micrographs showed morphological changes in *E. coli* cells treated with atmospheric plasma at 75 W for 2 min (Fig. 5). The treated cells had severe cytoplasmic deformations and leakage of bacterial chromosome. The chromosomal DNAs were either attached to the bacterial cells or released freely into the surrounding medium. The results clearly explain the loss of viability of bacterial cells after plasma treatment.

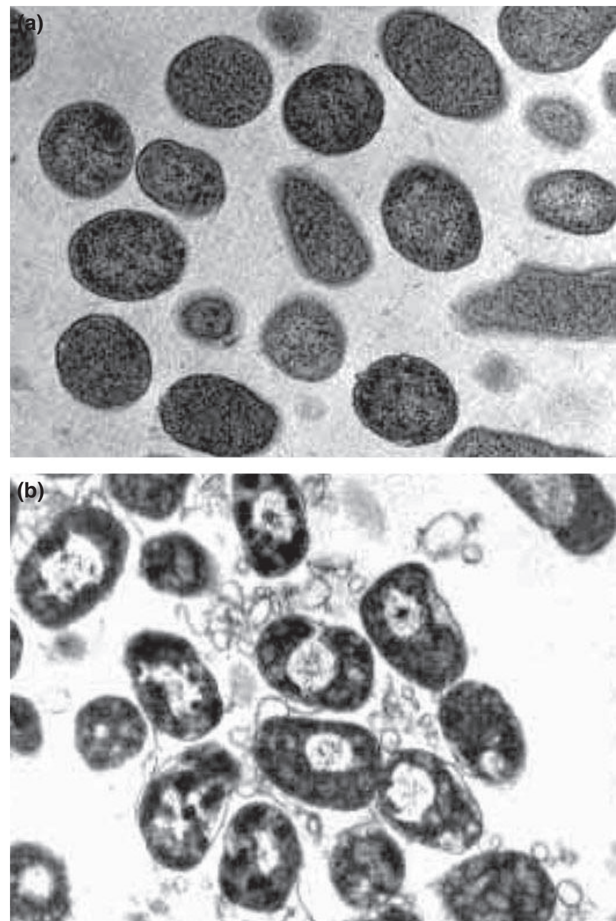

Figure 5 Transmission electron microscopy of *Escherichia coli* (×4400). (a) Control cells. (b) Cells after 2 min of plasma treatment at 75 W.

Discussion

The efficiency of plasma for *E. coli* and *B. subtilis* endospore sterilization matches the oxygen radical intensity to which they were exposed, demonstrating its crucial role in cell destruction. Our results show that an oxygen concentration of 0·2% was optimum for producing the highest oxygen radical intensity in the plasma. Sharma *et al.* (2006) used a mixture of argon and oxygen, and found that the oxygen flow rate had little effect on the inactivation of *E. coli*. They used the device in open air, and the plasma it generated was of the torch type, where there is always some atmospheric oxygen near the surface of the treated object. They found a slightly lower average inactivation rate when the highest amount of oxygen was added to the feed, but explained that the addition of oxygen made the discharge slightly unstable and hence had a net negative influence on the flux of active oxygen species. The present detailed experiment indicates that the highest number of oxygen radicals, measured by optical emission spectroscopy, produces optimum sterilization (Fig. 4). However, the excessively large amount of oxygen in the flow may destabilize the plasma, thereby reducing the number of oxygen radicals and becoming ineffective for sterilization, as mentioned by Sharma *et al.* (2006). Figure 4 clearly demonstrates the effectiveness of the oxygen radicals for sterilization.

Transmission electron microscopy revealed the bactericidal mode of action of atmospheric plasma treatment, showing disrupted cell envelopes and release of cellular components, resulting in loss of cell viability.

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For inactivation of *B. subltilis* endospores, plasma treatment was extremely rapid, considering the high resistance of the bacterial endospores. The aim of this study was to investigate the efficiency of low-temperature plasma treatment for the disinfection of diverse objects that are unsuitable for conventional chemical or physical methods, and, furthermore, for decontamination from bacterial endospores that could be used as biological warfare agents. The results suggest several potential applications for atmospheric cold plasma as a practical method for surface sterilization.

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