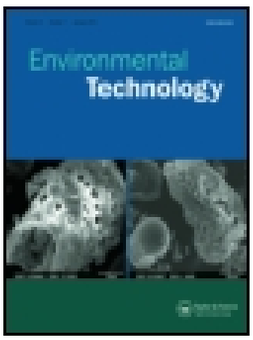

Environmental Technology

ISSN: 0959-3330 (Print) 1479-487X (Online) Journal homepage: https://www.tandfonline.com/loi/tent20

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To cite this article: Yu-Jung Chen, Yung-Chih Chen, Wei-Chieh Chung, Bao-Shun Tong & Moo Been Chang (2019): Evaluation of the Effectiveness of Nonthermal Plasma Disinfection, Environmental Technology, DOI: 10.1080/09593330.2019.1583289

To link to this article: https://doi.org/10.1080/09593330.2019.1583289

Accepted author version posted online: 13 Feb 2019.

Publisher: Taylor & Francis & Informa UK Limited, trading as Taylor & Francis Group
Journal: *Environmental Technology*
DOI: 10.1080/09593330.2019.1583289

Evaluation of the Effectiveness of Nonthermal Plasma Disinfection

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Abstract

As concern has increased regarding the interaction between efficiency and safety of disinfection, plasma becomes a viable alternative for disinfection in comparison with traditional methods. Dielectric barrier discharge (DBD) is applied to deactivate *B. subtilis* and *E. coli*, respectively, and disinfection efficiency is experimentally evaluated in this study. Tests are conducted with different working gases to investigate their effects on disinfection. Results show that Ar plasma diluted with 25% O_2 enhances the germicidal effects to 5.9 and 6.9 (log reduction) for *B. subtilis* and *E. coli*, respectively, and OES results show that active Ar and O species play important roles to weaken cell wall of microbes and further disinfect *E. coli* and *B. subtilis*. Analysis of protein and total sugar release indicates that active species such as ozone, NO and Ar^* produced by plasma result in hydrolysis of cell. In the meantime, charged particles produced by plasma would affect the amount of sugar released, resulting in different germicidal effects. Overall, plasma can disinfect microorganisms mainly via the generation of oxidizing agents including ozone, NO and UV with a comparatively short treatment period, which is typically less than 5 minutes.

Keywords: Plasma **disinfection**, DBD, Germicidal effect, Ozone **disinfection**, Damage of cell wall

1. Introduction

Disinfection is a cleansing technique that destroys or prevents the growth of microorganisms such as virus, bacteria and yeast via either physical or chemical processes. Conventional disinfection technologies including thermal disinfection, [1],[2]

ultraviolet (UV) irradiation [3],[4] and chemical disinfection (e.g., ethylene oxide and hydrogen peroxide) [5],[6] may not be suitable for disinfecting biomedical apparatus because these techniques rely on irreversible metabolic inactivation or breakdown of the structural components of microorganisms, resulting in damaging the heat-sensitive material. Additionally, chemical residues of disinfectant adsorbed on materials or devices may cause side effects [7]-[10].

Thus, new tools have been developed for disinfection to avoid the drawbacks associated. Plasma technology has found wide applications in many processes, including surface treatment (e.g., etching, coating and surface cleaning) and waste gas treatment such as electrostatic precipitator (EP) and fast selective catalytic reduction (fast SCR). During the last decade, utilizing non-thermal plasma (cold plasma or non-equilibrium plasma) to disinfect bacteria has attracted much attention. Especially, applying non-thermal plasma to remove microorganism has become an emerging disinfection technology. Non-thermal plasma generates free electrons, ions, UV radiation and reactive species which directly contact with the bacterial cell. The disinfection mechanism of reduced pressure plasma has become clear during the past two decades [11],[12]. Several gas-plasma disinfection studies conclude that UV radiation plays the key role in reduced-pressure plasma disinfection [9]. Nevertheless, UV irradiation may be absorbed by gas molecules in atmospheric plasmas since the density of gas molecules is much higher than that of reduced-pressure plasmas, resulting in lower UV irradiation and inferior disinfection efficiency. Moreover, the dosage of UV radiation must be strong enough to induce lethal effects, such as the rupture of cell wall and the breakage of DNA segment. Reduced-pressure plasma generates uniform glow discharge and the discharge volume is bigger than that of atmospheric-pressure plasma, therefore, it can easily treat thicker material. However, the reduced-pressure plasma must be operated under near vacuum condition and

requires a vacuum pump, which is expensive and may limit its application.

Previous studies have identified the active species as a main factor leading to disinfection [13]-[23]. So far the disinfection mechanism of plasma at atmospheric pressure is still not well understood. Chiang et al. (2010) applied atmospheric-pressure plasma jet (APPJ) as non-thermal plasma source, because its post-discharge jet region is effective in inactivating typical bacterial cells with good mobility, and the APPJ reactor is relatively compact [24]. Since APPJ results in good mixing with atmospheric air, it is difficult to distinguish the lethal active species between working gases and atmospheric air. Moreover, inactivation of aquatic microorganisms by plasma has been proposed and studied by Chen et al. [28],[29]. Inactivation of microorganism in water by plasma is applicable for water purification and treatment. However, it may not be good for the disinfection of precise and sensitive medical devices.

In this study, an AC pulse power supply is applied to generate dielectric barrier discharge (DBD) plasma at atmospheric pressure for disinfection due to the effectiveness in producing radicals and ozone [20]. Various working gases including air, nitrogen, argon, and oxygen are used to compare disinfection efficiencies. Previous studies indicate that addition of oxygen into working gases increases the disinfection efficiency [18],[19]. One of Gram-positive bacteria (*Bacillus subtilis 1A757, B. subtilis*) and one of Gram-negative bacteria (*Escherichia coli BL21(DE3), E. coli*) are selected as target strains. Besides, optical microscopy is applied to detect bacterial cell morphology and the mechanism of disinfection via atmospheric-pressure plasma is elucidated.

2. MATERIALS AND METHOD

2.1. Sample preparation

Before culturing, 8 g of nutrient broth (Merck) were added into 1 L of deionized water as broth and 40 g of Trypticase soy agar (Merck) were added into 1 L of deionized water as agar. The bacterial strains used for disinfection tests include B. subtilis and E. coli which were cultured in 10 mL nutrient broth. B. subtilis and E. coli were then selected and cultured in agar, respectively. Small amount of bacteria were inoculated firstly into 10 mL of nutrient broth and further cultured at 37^{o}C for 48 h. Next 0.1 mL of nutrient broth is drawn to the nutrient agar and cultured at 37°C for 24h and repeated for three times. The 3rd generation of bacteria was applied for the sample preparation because this generation has stable growing and good adaptation ability. Firstly, 1 mL of bacterial cell-containing liquid was added into 9 mL of PBS (phosphate buffered saline) solution. Then, the solution was diluted continuously for five times to obtain appropriate bacteria concentration for germicidal tests. All samples were prepared to have number of colonies ranging from 10^5 to 10^7 CFU/mL to compare the germicidal effects. All diluted bacterial solution was spread on nutrient agar. When the agar surface was dried, the agar-containing petri dish was placed in the center of plasma area for disinfection test. After plasma treatment, sample was placed in an incubator (LM570R) at 37°C for 24-hour culturing for further analysis. The image of treated petri dish was recorded by a camera (Moticam 2300) and further analyzed via the software (Image J, National Institute of Health) to calculate the total number of colonies.

2.2. Disinfection system

A DBD reactor with fixed parallel-plate electrodes was applied and the agar-containing petri dish was placed in the center of top electrode for disinfection

test. Fig. 1 (A) is the schematic diagram of the experimental system. It consists of two separate gas inlets: one inlet for humid gas consisting of air and water vapor and the other inlet for the working gas consisting of pure gas and O_2. The relative humidity of gas stream is controlled at 30%, 60% and 80%, respectively, to evaluate its influence on germicidal effect. The working gases include N_2 (99.99%), O_2 (99.99%), Ar (99.99%) and air (21% O_2 and 79% N_2). Both electrodes are made of stainless steel. Top electrode is installed in the ceramic tube and a quartz plate with a thickness of 2 mm is placed between electrode and ceramic tube, while a ceramic plate is placed on the bottom electrode. Both electrodes are immersed in a cooling water bath to prevent the arcing or overheat. The discharge gap between two electrodes is 5 mm, and the thickness of agar-containing petri dish is 4 mm. The DBD plasma is generated by an AC pulse power supply with adjustable frequency of 6 – 12 kHz and voltage of 5 – 50 kV, the power supply can provide current and power up to 100 mA and 5 kW, respectively. In this study, all plasma tests are conducted with fixed applied voltage (20 kV) and frequency (6 kHz) and total flow rate of working gas is controlled at 5 lpm. An acrylic barrier is installed between the gas inlet and outlet holes to facilitate the flowing of gas through the discharge area to ensure good contact between agar and reactive species generated as presented in Fig 1 (B) and (C).

To measure power consumption during discharge, oscilloscope equipped with two voltage probes is applied to measure the power. One voltage probe is connected with two electrodes in parallel. One of electrodes is connected with one conductor with electrical capacitance of 1 μF, and one of voltage probe is connected with two sides of conductor to draw the Lissajous figure in oscilloscope for measuring the power.

To elucidate the roles of ozone and UV in disinfection, different ozone concentrations are generated and two wavelengths of UV irradiations (254 nm and

356 nm) are tested, respectively. Various ozone concentrations are generated by adjusting the feeding flow rate to evaluate influence on germicidal effect, and the operation times of ozone disinfection are fixed at 60 and 240 seconds, respectively. Ozone concentrations are recorded via an ozone analyzer (Model 450 Ozone monitor serial No. 162, Advanced Pollution Instrumentation). For UV disinfection, UV lamps (Techintro) with two different wavelengths, i.e., 254 nm and 356 nm, are applied to disinfect both *B. subtilis* and *E. coli* and the irradiances of UV lamps are measured by a photometer (Digilux 9500, Konica Minolta Group) and fixed at 0.4 mW/cm^2. Disinfection tests are conducted for three times for each operating parameter to confirm the reproducibility of experimental results. The germicidal effect of both UV lamps and the comparison between UV and plasma disinfection will be discussed later.

Optical emission spectroscopy (OES, Mikropack, PlasCalc–2000–UV/VIS/NIR equipped with PLASUS SpecLine) is also applied to understand the species including ions, radicals and molecules produced during discharge. The range of emission detected is between 200 and 900 nm, and the resolution is 1 nm.

Figure 1

2.3. Germicidal Effect

The survival curve represents the decrease rate of bacterial colonies achieved with disinfection. Survival curves indicate not only disinfection efficiency but also decimal time which is also known as D-value. However, the survival curve does not give us the baseline information on original colony number for comparison. Therefore, the disinfection efficiency is expressed by germicidal effect in this study. The germicidal effect (GE) is defined as Equation (1):

GE (log reduction) = $\log_{10}N_0 - \log_{10}N$ (1)

where N_o and N represent colony forming units (CFU/mL) of controlled and disinfected set, respectively.

2.4. Total sugar and protein analysis

Protein and total sugar amounts before and after disinfection process were determined with a spectrophotometer to check whether cell wall the bacteria was ruptured. For total sugar analysis, agar was scraped from the petri dish and put into a 50-mL microtube, and 10 mL PBS solution was applied to the sample 10-20 times to wash away the bacteria remained on sample. The solution from 50 mL microtube was transferred into a 15 mL microtube and centrifuged at 5,000 rpm for 10 minutes. 0.5 mL supernatant of centrifuged solution was taken and 0.5 mL (5%) phenol was added, and 2.5 mL of sulfuric acid was added thereafter. Last, the solution was placed in a spectrophotometer for analysis with OD 490 nm. Regarding protein analysis, the sample was put into a 50-mL microtube, and 10 mL PBS solution was applied to the sample 10-20 times to wash away the bacteria remained on sample. Then, the solution from 50 mL microtube was transferred into a 15 mL microtube and centrifuged at 3000 rpm for 10 minutes. Thereafter, 0.8 mL supernatant of centrifuged solution was taken and 0.2 mL of Coomassie blue staining solution was added thereafter. The solution was placed in a spectrophotometer for analysis with OD 595 nm and bovine serum was used as reference.

3. RESULTS AND DISCUSSION

3.1. Influence of humidity on disinfection efficiency

Air with various relative humidity (0%, 30%, 60% and 80%, respectively) is

applied as working gas with the flow rate of 5 lpm, to evaluate its effect on disinfection. During discharge, operating parameters are fixed with the voltage of 20 kV, current of 6 mA, frequency of 6 kHz, feeding flow rate of 5 lpm and operating time of 60 seconds. Germicidal effect as a function of relative humidity is presented in Fig. 2. It shows that higher humidity results in higher germicidal effect. Germicidal effect of *B. subtilis* increases from 2.6 to 2.9 (log reduction) as the relative humidity is increased from 0% to 80% while germicidal effect of *E. coli* increases from 2.9 to 3.1 (log reduction).

During discharge, water vapor can be dissociated into H and OH via electron impact dissociation, then H and OH can collide with working gas (air) to produce reactive species, e.g., H_2O_2 and NO_x. These species can be adsorbed on cell membrane to oxidize or destroy the membrane, resulting in higher germicidal effect. [30]-[31]

Figure 2

3.2. Influences of ozone concentration and UV irradiation on disinfection efficiency

Ozone is an effective disinfectant since it has strong oxidizing capacity. In this study, ozone concentration with N_2/O_2 as carrier gas are controlled as 5, 25, 50, 75 and 100 ppm, respectively, by adjusting the total feeding flow rate and N_2/O_2 ratio from 5 to 50 lpm and 79:21 to 95:5, respectively, and operation time of 60 and 240 seconds to elucidate the influence of ozone concentration on disinfection efficiency and the results are presented in Fig. 3. Fig. 3 (A) shows the germicidal effects of *B. subtilis* and *E. coli* increase from 2.1 and 2.3 to 3.1 and 3.3 (log reduction) after 60 seconds of operation, respectively, as the ozone concentration is increased from 5 to 100 ppm, indicating that high ozone concentration is favorable for disinfection.

Moreover, no significant difference is observed between germicidal effects of *B. subtilis* and *E. coli*, indicating that ozone can disinfect *B. subtilis* and *E. coli* with comparable efficiency. After 240 seconds of operation, the germicidal effects of *B. subtilis* and *E. coli* increase from 3.1 and 3.3 to 3.4 and 3.7 (log reduction), respectively, with ozone concentration of 100 ppm as shown in Fig. 3 (B). When operation time is increased from 60 to 240 seconds, germicidal effects of *B. subtilis* and *E. coli* increased by 10% with ozone concentrations lower than 100 ppm, indicating that germicidal depends on operation time and ozone concentration.

Influences of UV irradiation including 254 nm and 356 nm on germicidal effect are tested and the results are presented in Fig. 4. Since the irradiances of both 254 nm and 356 nm are kept the same, the amounts of radiation energy are equal. However, wavelength close to the size of cell wall is easier to be absorbed by cell wall to kill microorganism [32]. For 356 nm UV irradiation, there is minor difference in germicidal effects between *B. subtilis* and *E. coli*, while the difference is rather significant for 254 nm UV irradiation. Germicidal effects of *B. subtilis* and *E. coli* increase from 4.0 and 5.5 to 4.5 and 6.0 (log reduction), respectively, as the operating time is increased from 5 to 60 minutes with 254 nm UV irradiation, indicating that *E. coli* is UV-sensitive especially to 254 nm UV.

Figure 3

Figure 4

3.3. Influence of working gases on disinfection efficiency

Argon, nitrogen, oxygen and air are individually applied to generate plasma to evaluate the effectiveness in disinfecting microorganism and reactive species of various plasmas are detected by OES. Among different working gases, Ar is well

known for its low breakdown voltage [25], O_2 is an electronegative gas which tends to form anions (negatively charged species, e.g., O_2^-), and N_2 produces more stable discharge compared to O_2 plasma. During discharge, operating parameters are maintained at applied voltage of 20 kV, current of 4 mA and frequency of 6 kHz for all working gases. The germicidal effects achieved with the plasma generated with various working gases are presented in Table. 1 The exposure times to the plasmas are all controlled at 240 seconds. The germicidal effects of *B. subtilis* are in the order of O_2 (5.0) > air (3.5) > N_2 (3.2) > Ar (3.0) (log reduction). O_2 plasma has the best germicidal effect due to ozone generation at a concentration higher than 900 ppm. Disinfection with ozone concentration of 100 ppm results in higher germicidal effect (3.4) than those plasmas without oxygen. In air plasma, NO_x including NO and NO_2 can be generated and NO_x also contributes for disinfection effects, resulting in higher germicidal effects of air plasma compared to N_2 or Ar plasmas [27],[31]. N_2 and Ar plasmas could not produce ozone and NO_x, thus, their germicidal effects are relatively lower if compared with that achieved with O_2 plasma. However, germicidal effects of those plasmas are close and reach 3, indicating there are other disinfecting species. The results demonstrated that incorporating oxygen in gas stream to generate ozone may enhance the germicidal effect. It is noted that the germicidal effect of *E. coli* is different from that of *B. subtilis*. The germicidal effects of *E. coli* achieved are in the order of O_2 (5.2) > Ar (4.1) > Air (3.9) > N_2 (3.8) (log reduction) as presented in Table. 1. Among them, O_2 plasma has the highest germicidal effect, showing that ozone produced in O_2 plasma is an important specie in disinfecting *E. coli*, however, air plasma has lower germicidal effect than that of Ar plasma, indicating that other active species produced by Ar plasma are also effective in disinfecting *E. coli*.

To elucidate the role of O_2 in the plasma and to enhance disinfection, working gases including N_2 and Ar were diluted with different percentage of O_2 and the total

flow rates are fixed at 5 lpm. The germicidal effects with the operation time of 240 seconds are presented in Table 1. As N_2 or Ar diluted with O_2 is applied as the working gas, both germicidal effects of *B. subtilis* and *E. coli* are increased, indicating that O_2 addition in feeding flow induces ozone and other reactive species (e.g., NO_x and O_3) production and enhances disinfection of both *B. subtilis* and *E. coli*. For N_2 plasma, adding 75% of O_2 in feeding gas increases germicidal effect of *B. subtilis* from 3.2 to 4.9 (log reduction). Likewise, the germicidal effect of *E. coli* is increased from 3.8 to 5.4 (log reduction). The results show that the feeding gas of 25% N_2 and 75% O_2 produces ozone and NO_x efficiently, resulting in good germicidal effects which are comparable to O_2 plasma. It is noted that ozone concentration measure in effluent for various N_2/O_2 plasmas has the identical trend: 25%N_2/75%O_2 (> 400 ppm) > 50%N_2/50%O_2 (~ 400 ppm) > 75%N_2/25%O_2 (< 100 ppm). For Ar plasma, adding 25% of O_2 increases the germicidal effects from 3.0 and 4.1 to 5.9 and 6.9 (log reduction) for *B. subtilis* and *E. coli*, respectively. With O_2 addition of 50%, germicidal effects of *B. subtilis* and *E. coli* decrease to 3.3 and 3.8 (log reduction), respectively, both are lower than the condition of 25% O_2. Results indicate that feeding gas composed of 75% Ar and 25% O_2 produces ozone and other disinfection species (e.g., Ar^* and Ar^+) most efficiently, due to abundant secondary free electrons produced by Ar plasma and is effective in disinfecting *B. subtilis* and *E. coli*. Besides, 50%Ar/50%O_2 and 75%Ar/25%O_2 plasmas have effluent ozone concentrations between 100 ~ 300 ppm, indicating that ozone also plays a significant role in Ar/O_2 plasmas.

Power consumptions are measured for various reactors. For Ar/O_2 plasmas, power is ranged from 120 ~ 140 Watt while for N_2/O_2 plasmas the power consumptions are between 80 ~ 100 Watt. The energy consumption of this study is comparable to other studies [33].

Table 1

3.4. Analysis of active species

Fig. 5 (A) shows the OES spectra of N_2 plasma and N_2 diluted with 50% of O_2, respectively. N_2 and N_2/O_2 plasmas have similar emissions between 300 and 450 nm, showing that these characteristics are N_2 plasma species since these working gases are of different N_2 contents. Introducing oxygen into the plasma reactor results in generation of NO (between 220 and 290 nm), which is recognized as active disinfectants. Thus, higher germicidal effect of 50%N_2/50%O_2 can be partly attributed to the generation of NO [27].

Fig. 5 (B) shows the OES spectra of Ar and 50%Ar/50%O_2 plasmas, respectively. It should be noted that 75%Ar/25%O_2 spectrum is close to 50%Ar/50%O_2 spectrum. Ar plasma has characteristic peaks located at two regions, one is between 300 and 400 nm, and the other is between 200 and 900 nm, indicating Ar plasma has strong UV (307.8 nm for Ar III) and visible-infrared region (from 650 to 850 nm). When Ar is diluted with 50% of O_2, the UV irradiation disappeared. Active oxygen species including O_2 and O are observed and are possibly principal active disinfectants. As shown in Table 1, Ar diluted with 25% of O_2 has the highest germicidal effect among all working gases, indicating that discharge under this operating condition produces disinfection species most efficiently, including ozone, and reactive oxygen species (ROS). However, as O_2 content is increased to 50% in working gas, the germicidal effects decrease to 3.27 and 3.77 (log reduction) for *B. subtilis* and *E. coli*, respectively, indicating that the production rates of disinfection species depend on Ar/O_2 ratio. The reason why germicidal effect depend on Ar/O_2 may be explained as follow: Ar concentration influences secondary electron production, then, secondary

electrons can collide with O_2 to induce ionization, dissociation, dissociative ionization and electron attachment of O_2 to form ozone, oxygen atoms and other active oxygen species (e.g., O_2^-) to enhance disinfection. As a result, sufficient amount of Ar is needed to induce disinfection.

Figure 5

3.5. Damage degree of cell wall

Brelles-Mariño (2010) applied fluorescent nucleic acid stains to check if cells are alive or dead, however, the method they applied is mainly for the examination of cell wall morphology change after plasma treatment. Instead, optical microscopy can be used to observe the cell morphology after plasma treatment [21]. Fig. 6 shows the microscopic images of *B. subtilis* after 0 second, 30 seconds, 60 seconds and 120 seconds of O_2 plasma treatment, respectively. It is obvious that bacterial cell number decreases with increasing discharge time. Moreover, the decreasing of bacterial cell number is uniform, indicating that disinfection is homogeneous and non-directional. Fig 7 shows the microscopic images of *E. coli* after 0 second, 30 seconds, 60 seconds and 120 seconds of O_2 plasma treatment, respectively. Disinfection of *E. coli* reveals similar trend to *B. subtilis* disinfection, showing that plasma disinfection is homogeneous and uniform. The uniformity of disinfection effect is attributed to the addition of acrylic barrier. After acrylic barrier is added, local convection is induced between two barriers, thus the gas residence time and the distribution of active species becomes uniform.

Tables 2 and 3 present sugar and protein release, respectively, achieved with various disinfection tools. Results show that UV has the lowest sugar and protein release, indicating that the disinfection power of UV is relatively low. Furthermore,

there is a strong relevance between sugar and protein release amount and germicidal effect, showing that ozone and plasma possess good capability to damage the cell wall of *B. subtilis* and *E. coli*.

The cell morphology of plasma treated sample is observed with cytoplasm leakage as presented in Fig. 8 (A). Gram-negative organisms are of a thinner peptidoglycan layer compared to Gram-positive organisms, thus, their peptidoglycan may be strongly damaged by the free electron, ozone or other active species produced by plasma. Another treatment is ozone disinfection and the microscopic view is presented in Fig. 8 (B). Optical microscopic image of the cell wall is foggy and hard to distinguish, indicating that ozone is effective in destroying cell wall. Moreover, ozone is a strong oxidant and can oxidize peptidoglycan once the cell wall is ruptured [34],[35].

Since cell morphologies of plasma treatment and ozone treatment are both vague to distinguish which treatment is more effective in disinfection, protein and total sugar analysis provide us with the information on the degree of cell wall damage. The sugar releasing analysis shows that plasma treatment releases significant amount of total sugar compared to ozone and UV irradiation, indicating that plasma can destroy cell wall effectively. Moreover, the cell fragmentations including coats and debris are observed after plasma, ozone and UV treatments. For protein analysis, ozone treatment releases a large amount of proteins, however, the amount of proteins released keeps relatively constant as the treatment time is increased. The releasing rate of proteins is partly ascribed to the conversion of protein into volatile molecules and then disappears [26]. Microscope result shows that ozone has strong oxidative capacity, therefore, ozone can oxide the outer membrane and thin peptidoglycan layer, leading to cell pyrolysis. Finally, plasma produces ozone if the working gas contains sufficient O_2 to destroy cell walls and peptidoglycans.

Figure 6

Figure 7

Figure 8

3.6. Mechanism of DBD disinfection

Fig. 9 illustrates the mechanisms of plasma disinfection. During discharge, reactive species including free electron, ions (e.g., O_2^- and N_2^+), UV photons, radicals (e.g., OH and O) and reactive molecules (e.g., ozone, NO) are generated by electron impact dissociation or electron impact attachment. Then, those species can collide with agar or cell wall to generate OH radical and volatile matters (CO or light hydrocarbons) [9]. These reactions can destroy or oxidize bacterial cell wall. Ozone produced by plasma can oxidize bacterial cell wall simultaneously with other oxidants including volatile matters, thus, the germicidal effects are enhanced.

Fig. 10 shows different damage degree of cells achieved with UV, ozone and plasma, respectively. UV irradiation can destroy the protein content of outer membrane, however, the energy of UV irradiation is insufficient to penetrate peptidoglycan layer. On the other hand, plasma and ozone can reach the peptidoglycan layer. Some studies indicated that ozone and UV do not destroy the structure of cell wall and only charge bombardment affects the cell structure effectively [16]. In addition to UV and ozone, plasma generates species such as reactive oxygen and nitrogen species (RONS) which play the crucial roles to destroy cell wall and hence inactivate microorganisms. As a result, agar plays an important role in disinfection since agar contains various nutrient sources and further influences the generation of RONS [36]. The existence of agar can increase or decrease germicidal

effect, depending on its composition. In this study, agar also plays a role as conductor which can influence discharge behavior including electron energy and density. Since plasma produces UV, ozone and other active species in a short period, it provides sufficient disinfecting species such as energetic electrons and ozone to attack deep peptidoglycan layer. Plasma can reach much deeper of peptidoglycan layer in a short discharge time [37]. Briefly, plasma can damage cell wall efficiently, especially for the microorganism with simple cell wall structure [17].

To sum up, degree of cell wall damage depends on the resistance of cell wall to different disinfectants. In other words, *B. subtilis* has higher resistance of disinfectant when compared with *E. coli*, resulting in lower germicidal effect.

Figure 9

Figure 10

4. CONCLUSIONS

This study aims to compare the effectiveness of different treatments including ozone, UV irradiation and plasma in disinfection. The plasma treatment reveals different degrees of germicidal effects as different working gases including N_2, O_2, Ar, and air are applied to generate plasma. Different amount of O_2 was also added into N_2 and Ar plasmas to investigate the germicidal effects. Since the working gas containing O_2 produces effective disinfection species including ozone, UV photons and other reactive oxygen-containing species, the germicidal effect of plasma is higher than those of ozone treatment and UV irradiation. Results show that O_2 plasma has a higher germicidal effect than Ar, N_2 and air plasma, indicating that O_2 plasma can disinfect microorganisms efficiently. Ar plasma diluted with 25% of O_2 shows the

highest germicidal effect, indicating higher secondary electron production is beneficial to disinfection. Moreover, ozone plays a key role in disinfection since all O_2 dilution promotes the germicidal effects whatever the working gas is. OES analysis indicates that UV irradiation with wavelength between 200 and 300 nm is enhanced with N_2/O_2 plasma, indicating that NO may have germicidal effect in N_2/O_2 plasma. Finally, ozone, UV irradiation and co-existing active species produced by Ar/O_2 plasma are effective disinfectant, resulting in synergistic effects for high germicidal effect.

Acknowledgements

The authors would like to thank Mr. Yang and Mr. Yan in the Institute of Nuclear Energy Research (INER) of Taiwan for the assistance in carrying out experiment and analysis.

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Figure Captions

Figure 1. Schematic diagram of disinfection system (A) experimental setup, (B) front view of DBD reactor and (C) top view of petri dish.

Figure 2. Influence of relative humidity on germicidal effect, operating time = 60 seconds, feeding flow rate = 5 lpm and air as working gas.

Figure 3. Influence of ozone concentration on germicidal effect (A) operating time = 60 seconds and (B) operating time = 240 seconds, feeding flow rate = 5 lpm and air as working gas.

Figure 4. Germicidal effect of UV radiation with wavelength of (A) UV 254 nm and (B) UV 356 nm (irradiance = 0.4 mW/cm^2).

Figure 5. Optical emission spectrum of (A) pure N_2 and N_2 diluted with O_2 and (B) pure Ar and Ar diluted with O_2 (feeding flow rate = 5 lpm, applied voltage = 20 kV, current = 4 mA, frequency = 6 kHz).

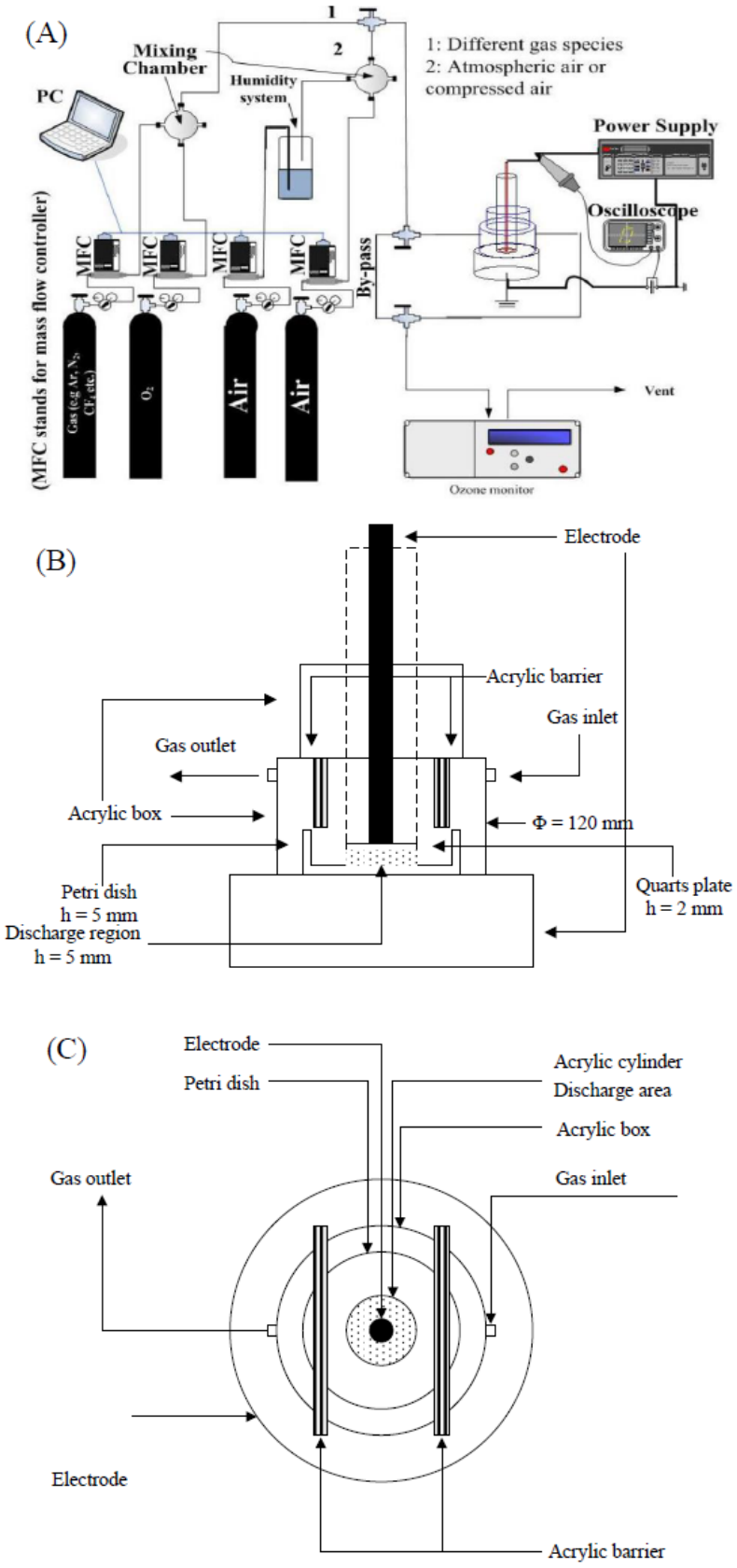
Figure 6. Microscopic images of O_2 plasma treated petri dish of *B. subtilis* after (A) 0 second, (B) 30 seconds, (C) 60 seconds and (D) 120 seconds s, magnification = 10,000.

Figure 7. Microscopic images of O_2 plasma treated petri dish of *E. coli* after (A) 0 second, (B) 30 seconds, (C) 60 seconds and (D) 120 seconds, magnification = 10,000.

Figure 8. Cell morphology of *E. coli* treated by (A) plasma and (B) ozone, magnification = 10,000.

Figure 9. Disinfection mechanisms of plasma treatment.

Figure 10. Damage degree of cell achieved with different treatments.



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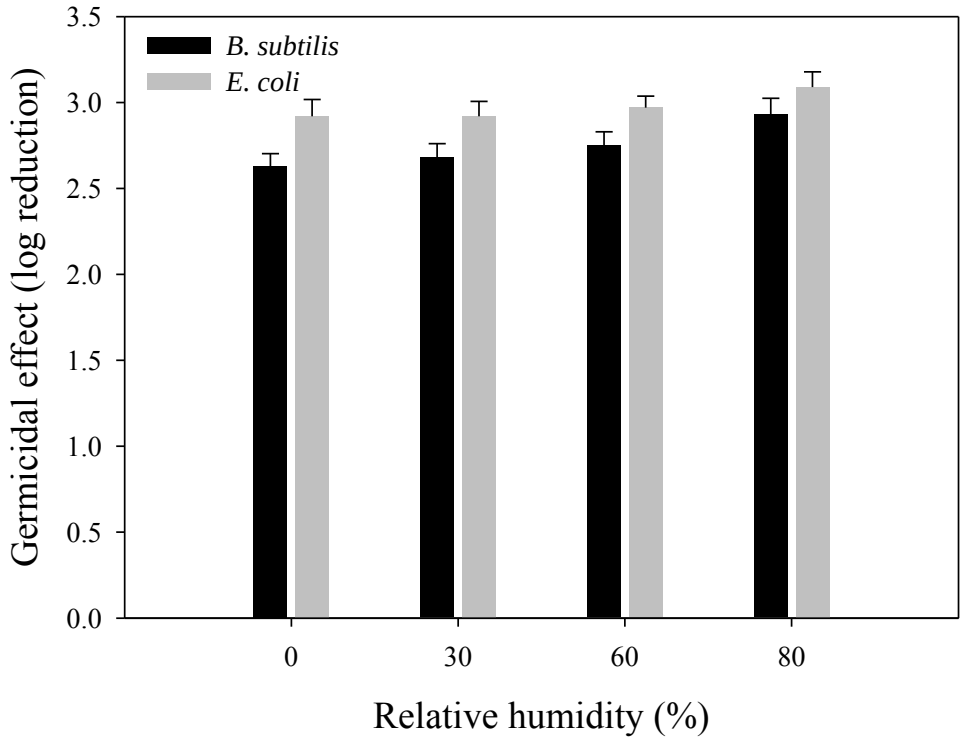
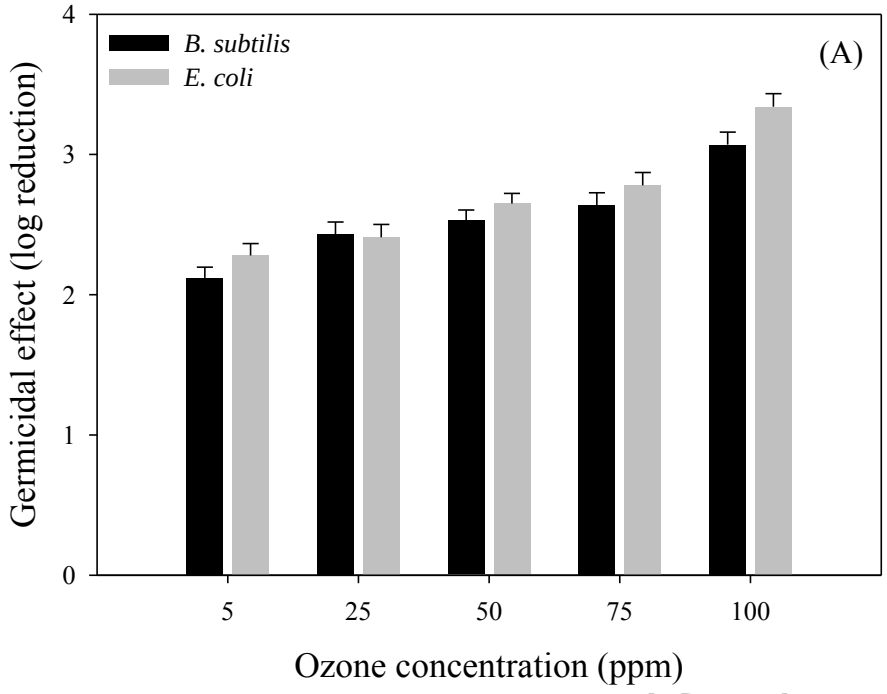


Figure 2. Influence of relative humidity on germicidal effect, operating time = 60 seconds, feeding flow rate = 5 lpm and air as working gas.



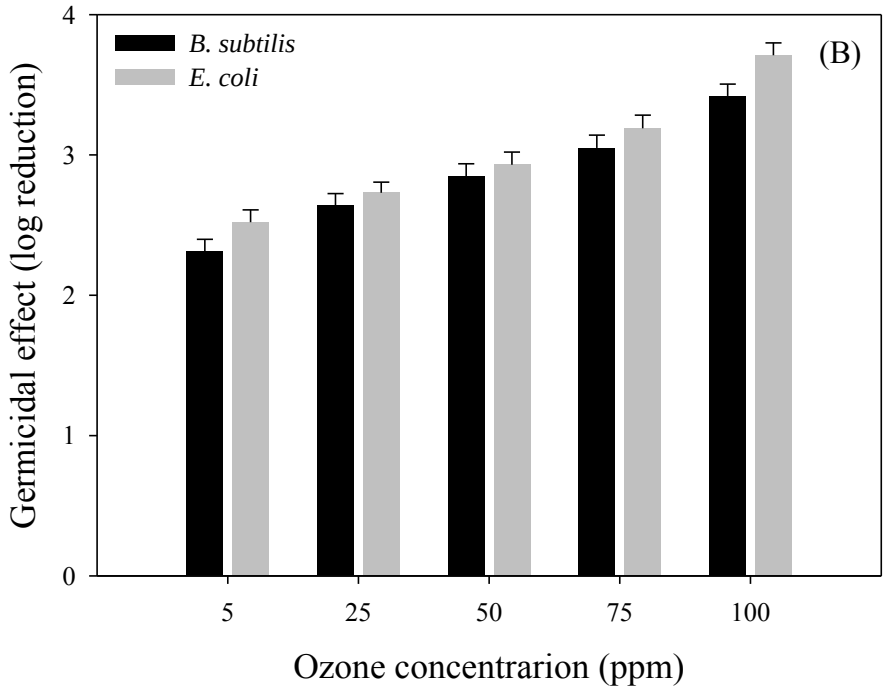
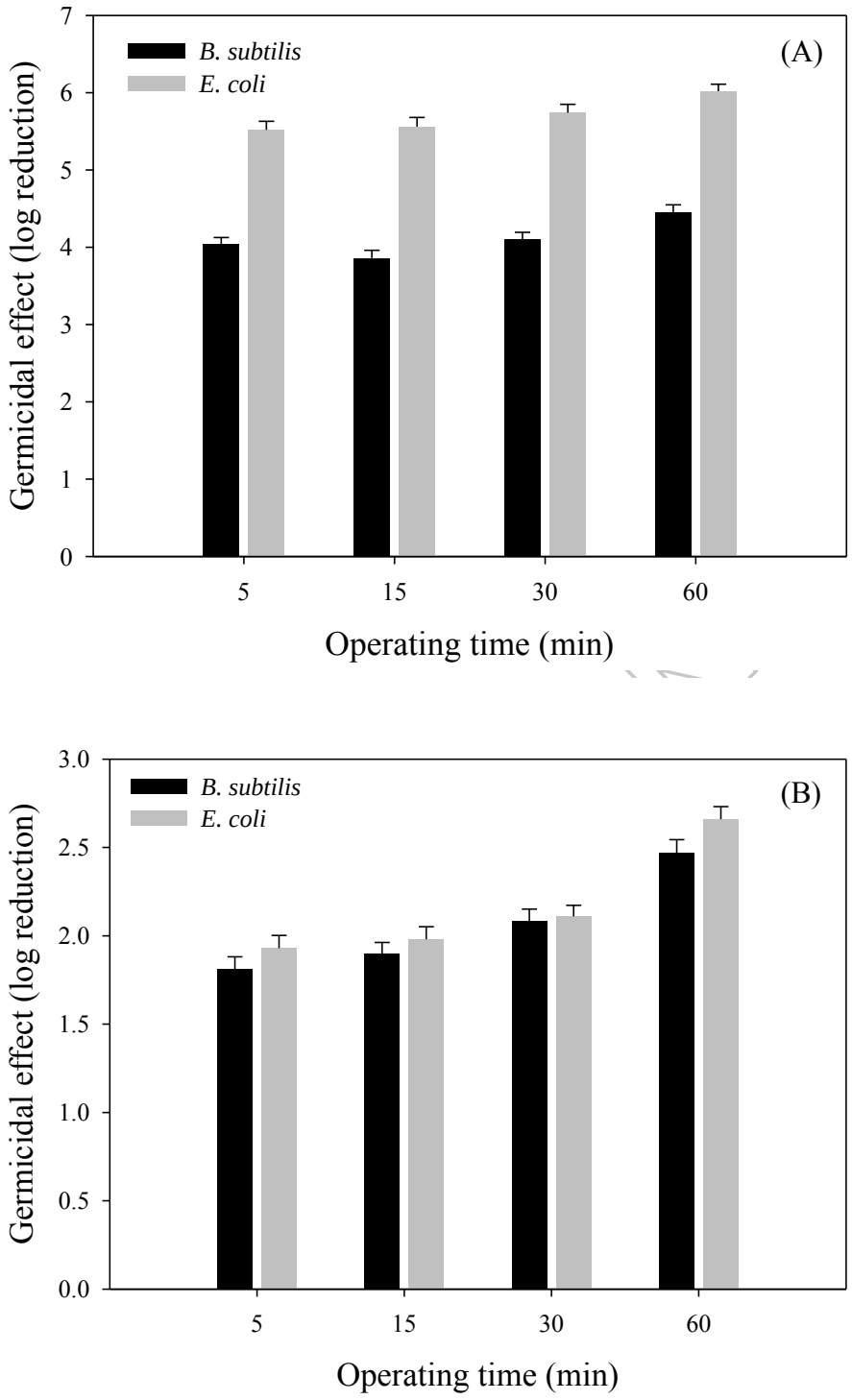


Figure 3. Influence of ozone concentration on germicidal effect (A) operating time = 60 seconds and (B) operating time = 240 seconds (feeding flow rate = 5 lpm and air as working gas).



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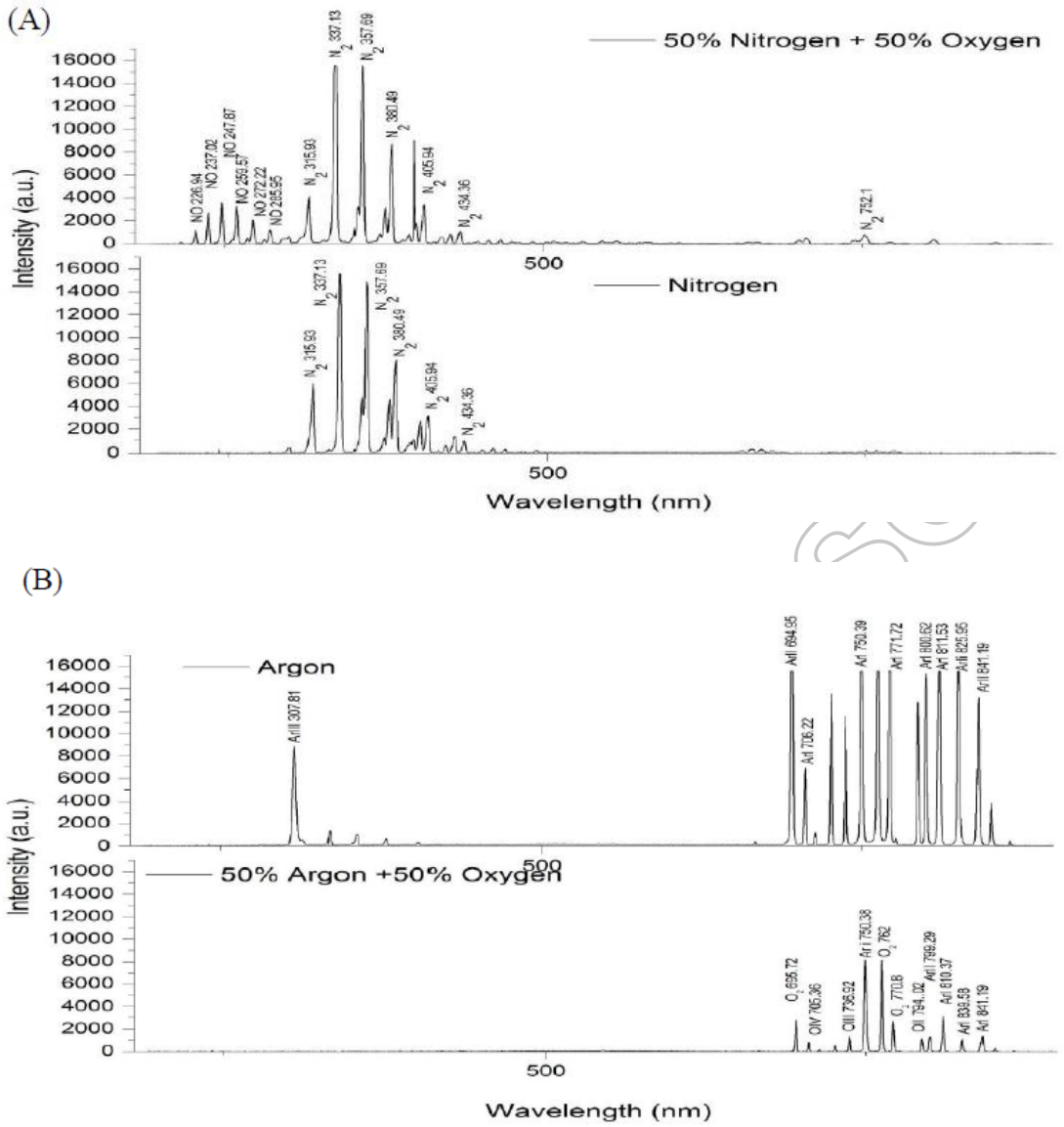

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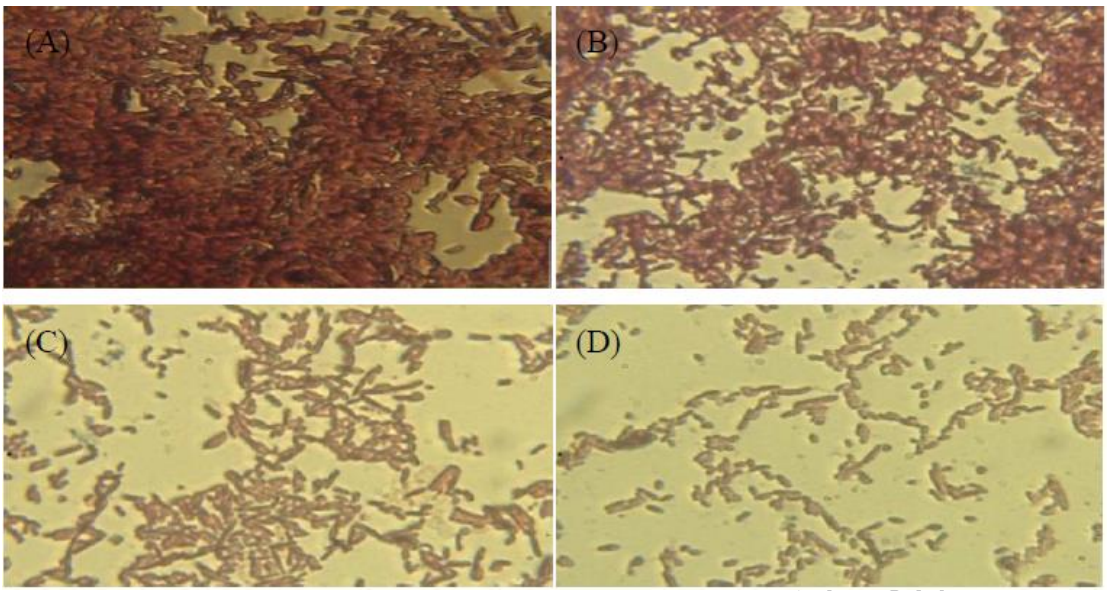

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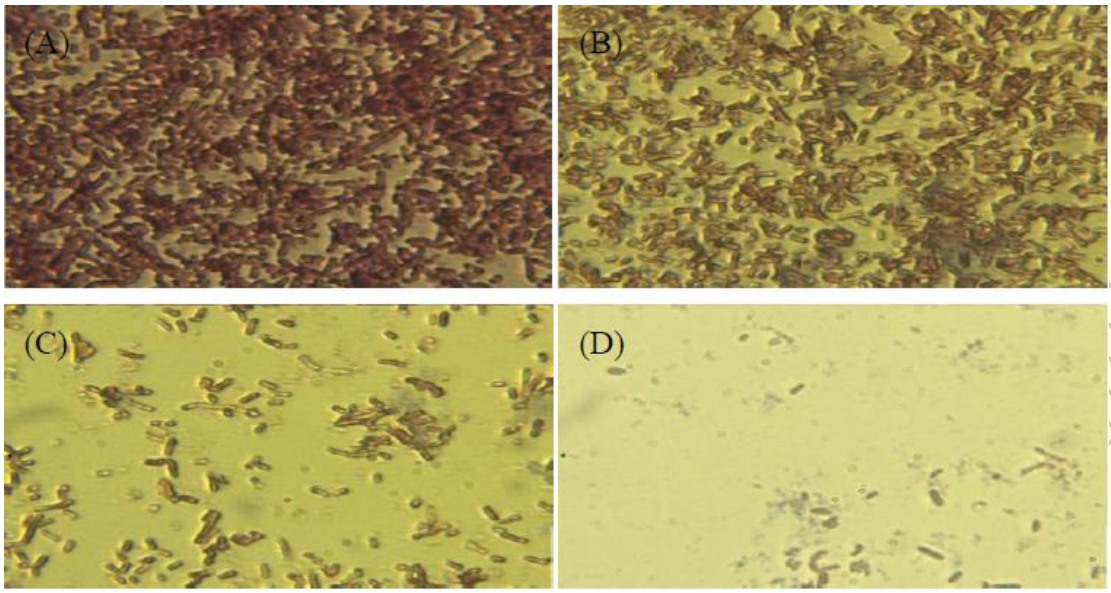


Figure 7. Microscopic images of O_2 plasma treated petri dish of *E. coli a*fter (A) 0 second, (B) 30 seconds, (C) 60 seconds and (D) 120 seconds, magnification = 10,000.

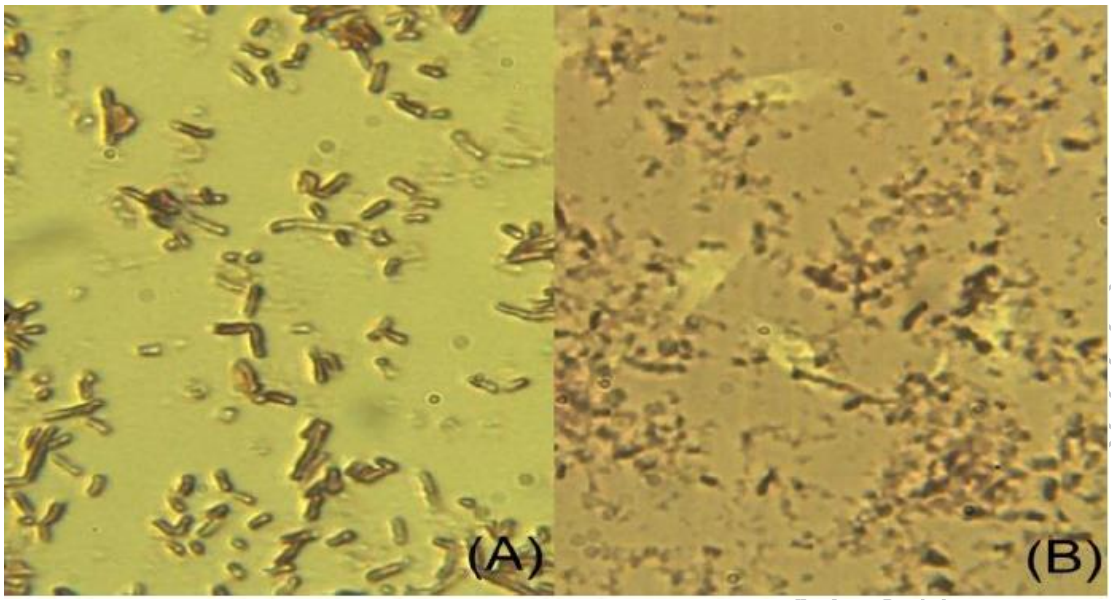
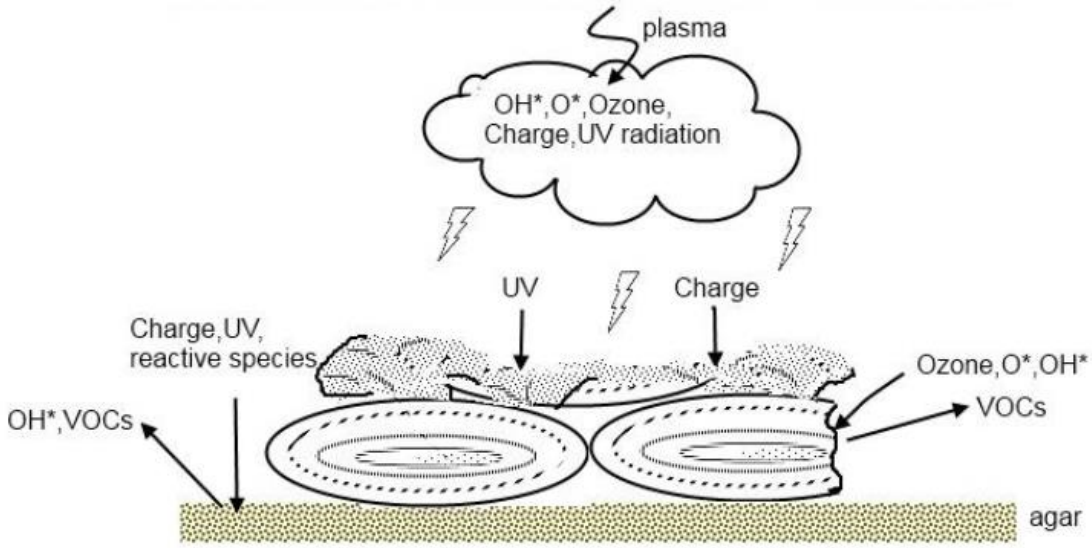


Figure 8. Cell morphology of *E. coli* treated by (A) plasma and (B) ozone, magnification = 10,000.



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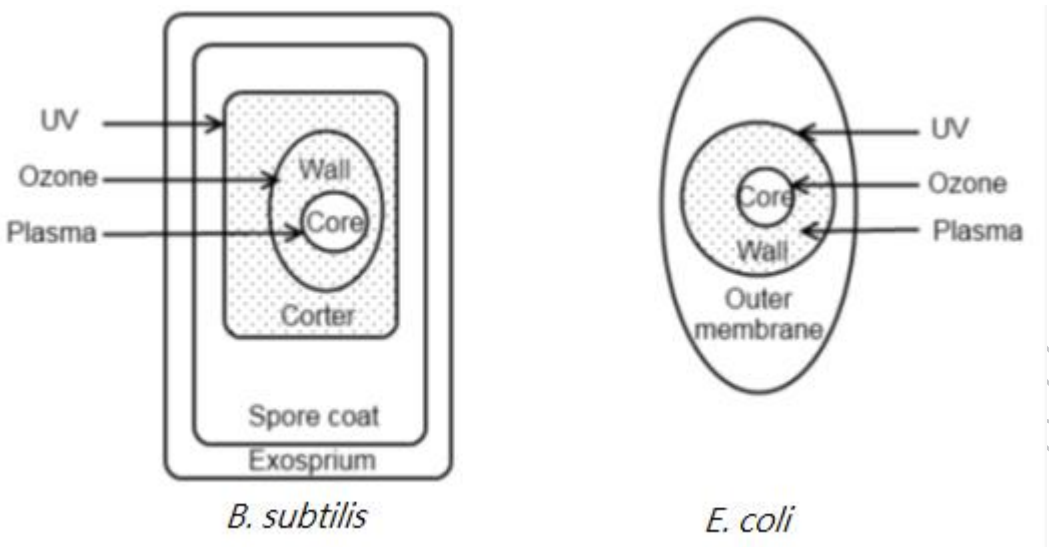

Figure 10. Damage degree of cell achieved with different treatments.

[38]

Table captions

TABLE. 1. Germicidal effects achieved with plasma of different working gases, operating time = 240 seconds, flow rate = 5 lpm (N = 5).

TABLE. 2. Amounts of sugar released, operating time = 240 seconds, flow rate = 5 lpm (N = 5).

TABLE. 3. Amounts of protein released, operating time = 240 seconds, flow rate = 5 lpm (N = 5).

TABLE. 1. Germicidal effects achieved with plasma of different working gases, operating time = 240 seconds, flow rate = 5 lpm (N = 5).

Gas composition	*B. subtilis in log reduction*	*E. coli in log reduction*
N_2	3.2 ± 0.1	3.8 ± 0.1
75%N_2/25%O_2	3.5 ± 0.1	4.0 ± 0.1
50%N_2/50%O_2	4.4 ± 0.2	5.2 ± 0.1
25%N_2/75%O_2	4.9 ± 0.2	5.4 ± 0.2
Ar	3.0 ± 0.1	4.1 ± 0.2
75%Ar/25%O_2	5.9 ± 0.2	6.9 ± 0.3
50% Ar/50%O_2	3.3 ± 0.1	3.8 ± 0.1
O_2	5.0 ± 0.2	5.2 ± 0.1
Air	3.5 ± 0.1	3.9 ± 0.1

TABLE. 2. Amounts of sugar released, operating time = 240 seconds, flow rate = 5 lpm (N = 5).

Treatment	*B. subtilis* $(S/S_0)^a$	*E. coli* $(S/S_0)^a$
Ozone	0.08 ± 0.01	0.09 ± 0.01
UV (254 nm)	0.06 ± 0.01	0.08 ± 0.01
N_2	0.15 ± 0.03	0.15 ± 0.02
25%N_2/75%O_2	0.21 ± 0.03	0.22 ± 0.02
Ar	0.14 ± 0.02	0.17 ± 0.02
75%Ar/25%O_2	0.24 ± 0.03	0.26 ± 0.03
O_2	0.22 ± 0.02	0.22 ± 0.02

[a]: S denotes sugar released while S_0 stands for initial sugar content

TABLE. 3. Amounts of protein released, operating time = 240 seconds, flow rate = 5 lpm (N = 5).

Treatment	*B. subtilis* $(P/P_0)^a$	*E. coli* $(P/P_0)^a$
Ozone	0.22 ± 0.02	0.20 ± 0.02
UV (254 nm)	0.14 ± 0.02	0.15 ± 0.02
N_2	0.31 ± 0.03	0.30 ± 0.04
25%N_2/75%O_2	0.36 ± 0.02	0.38 ± 0.03
Ar	0.28 ± 0.02	0.25 ± 0.02
75%Ar/25%O_2	0.41 ± 0.02	0.42 ± 0.03
O_2	0.30 ± 0.02	0.32 ± 0.02

[a]: P denotes protein released while P_0 stands for initial protein content

[39]