

Dielectric barrier discharge plasma treatment on *E. coli*: Influence of CH₄/N₂, O₂, N₂/O₂, N₂, and Ar gases

Abhijit Majumdar,^{1,a)} Rajesh Kumar Singh,^{2,b)} Gottfried J. Palm,² and Rainer Hippler¹

¹*Institute for Physics, Ernst-Moritz-Arndt-University Greifswald, Felix-Hausdorff-Str. 6, 17489 Greifswald, Germany*

²*Institute for Biochemistry, Ernst-Moritz-Arndt-University Greifswald, Felix-Hausdorff-Str. 4, 17489 Greifswald, Germany*

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Atmospheric pressure dielectric barrier discharge (DBD) plasma has been employed on Gram-negative bacteria, *Escherichia coli* BL21. Treatment was carried out using plasma generated with different compositions of gases: CH₄/N₂ (1:2), O₂, N₂/O₂ (1:1), N₂, and Ar, and by varying plasma power and treatment time. *E. coli* cells were exposed under the DBD plasma in triplicates, and their surviving numbers were observed in terms of colony forming units. It has been observed that the CH₄/N₂ plasma exhibits relatively higher sterilization property toward *E. coli* compared to plasma generated by using O₂, N₂/O₂, N₂, and Ar gas mixtures. The time to kill up to 90% of the initial population of the *E. coli* cells was found to be about 2–3 min for CH₄/N₂ and O₂ gas mixture DBD plasma. A prolongation of treatment time and an increase in the dissipated power significantly improved the *E. coli* killing efficiency of the atmospheric pressure DBD plasma. © 2009 American Institute of Physics. [doi:10.1063/1.3236570]

I. INTRODUCTION

The interest toward the application of atmospheric pressure (AP) plasma in medicine and surgery increases day by day. Plasma sterilization and plasma interaction with microorganisms are two of the most interesting and ongoing research areas among the several features of AP plasma. Several sterilization methods are already well known, such as autoclaving, ethylene oxide, UV sterilization, γ -irradiation, and the newly evolving plasma sterilization technique.^{1–5} AP plasma can kill almost all kinds of bacteria since several ions and reactive species, such as oxygen atoms and ozone, are generated during the AP plasma treatment. AP plasma is also currently used in air cleaners and sterilizers.³ The application of atmospheric plasma technology is now more focused on the use of plasma in humans and animals. In this application we need to be careful that the plasma should specifically kill only the targeted microorganisms or tissues in our body.

AP plasmas have been developed for many applications such as surface modification of polymers,^{6–8} air purification,^{9–11} and sterilization.^{11–17} Plasma-tissue interaction is one of the most important issues in the biomedical applications of plasma.⁴ The first report on the usage of a plasma as a sterilizing agent was by Menashi in 1968.¹⁶ The apparatus used was a pulsed rf field to achieve argon plasma at AP, which sterilized the inner surface of vials. Laroussi *et al.*¹⁸ tested plasma treatment of *Escherichia coli* (*E. coli*) bacteria (strain PER 322) for 2–20 min exposure, and reported nearly complete killing of 10⁸ *E. coli* cells/ml cells by glow discharge at atmospheric pressure. Montie *et al.*¹⁹ re-

ported that the sterilizing factor of atmosphere uniform glow discharge plasma could be attributed to the attachment of hydroxyl (OH^{*}) radicals to fatty acids and induction of lipid peroxidation. Mendis *et al.*²⁰ stated that the electrostatic charge accumulation on the cell membrane induces an electrical stress that ruptures the cells. Similarly, it has been mentioned by Fridman *et al.*⁵ that charged particles in the plasma play an essential role in the interaction of a plasma with living organisms, although the synergy with longer living active molecules and atoms, also with the UV radiation generated in plasma, induced the intrinsic photodesorption, which breaks chemical bonds in the microorganism material and leads to the formation of volatile compounds.^{13,14} The complete killing of microorganisms depends on several factors such as the plasma power, density, the gas used, the type of bacteria, and the type of medium.

In this paper, we present new results on AP dielectric barrier discharge (DBD) plasma-induced killing of *E. coli* bacteria. The DBD plasma treatment on *E. coli* has been carried out at different time scales, power, and different gas compositions. The viability of plasma treated bacterial cells was determined in terms of colony forming units (CFUs) of plated cells, which were counted after an overnight incubation at 37 °C. The Gram-negative *E. coli* was used in these experiments as an easy to handle model organism.

II. EXPERIMENTAL PROCEDURE

A. DBD plasma setup

Figure 1 shows the schematic of the glass slide containing a thin layer of air dried *E. coli* and its placement on the ground electrode (glass). The experimental setup of the DBD has been described in detail elsewhere.^{21–23} Both stainless steel electrodes (from Fig. 1) were covered by dielectrics: The upper (powered) electrode was covered with alumina

^{a)} Author to whom correspondence should be addressed. Electronic mail: majumdar@physik.uni-greifswald.de. FAX: +493834864701.

^{b)} Present address: Biochemical Engineering Group, Chemical Engineering Division, National Chemical Laboratory, Dr. Homi Bhabha Road, Pune 411008, India.

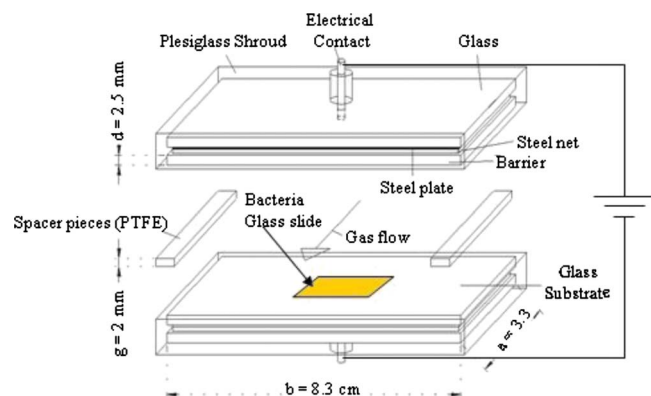


FIG. 1. (Color online) Schematic (two-dimensional) diagram of the placement of the glass slide with a thin layer of air dried *E. coli* (yellow) on ground electrode and the electrical configuration of the DBD plasma.

($\epsilon \sim 10$), and the lower (grounded) electrode with a glass plate ($\epsilon \sim 3.8$). Both electrodes were separated by 0.20 cm from each other. The upper electrode was connected to a homebuilt high voltage power supply, while the lower electrode was grounded. The chamber was evacuated by a membrane pump down to a base pressure of about 1 mbar. The high voltage power supply had a frequency generator delivering a sinusoidal output that was fed into an audio amplifier. The amplifier could be operated at up to 500 W. Experiments were performed in the range 8–9 kV (peak to peak) and at 5.5 kHz. The electrical power under these conditions was in the range 0.1–2 W.²¹

B. Bacteria sample preparation

First, *E. coli* colonies (*E. coli* BL21 with plasmid pT-Fred11 coding for ampicillin resistance and green fluorescent protein sg11)²⁴ were scraped from Luria–Bertini (LB)-agar medium containing ampicillin (100 $\mu\text{g}/\text{ml}$) and were homogeneously resuspended in 1 ml LB medium. From the resuspended culture, 100 μl was pipetted on to each glass slide, having the same surface area (area = $1.5 \times 2.5 \text{ cm}^2$ and thickness = 1 mm). These glass slides were then air dried under sterile conditions (class II biological safety cabinet) for about 1.5 h. Before starting experiments, it was also ensured that each batch of *E. coli* was viable after at least 3 h of air drying. Control experiments were also done to check the impact of the primary vacuum on the *E. coli* cell.

C. Sample treatment by plasma

The glass slides with the air dried bacterial cells were placed on to the ground electrodes (also made of glass) one by one for each subsequent experiment. Figure 1 shows the schematic of the glass slide containing a thin layer of air dried *E. coli* and its placement on the ground electrode (glass). The distance between the upper electrode and the bacterial plate was kept constant at 2 mm. The treatment time and power were varied from 10 to 240 s and from 100 to 500 mW, respectively. After the plasma treatment, the layer of dried *E. coli* culture on the glass slide was resuspended completely into a 10 ml LB medium in an Erlenmeyer flask. The resuspended culture was serially diluted 100

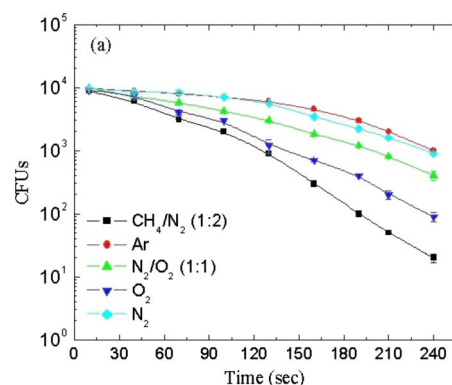


FIG. 2. (Color online) The viability of *E. coli* determined in terms of surviving number of CFUs as a function of time at AP DBD plasma treatment (dissipated power = 0.4 W) (logarithmic scale).

times. From the diluted *E. coli* suspension, 100 μl was spread onto a LB-agar plate containing ampicillin (100 $\mu\text{g}/\text{ml}$) and incubated overnight at 37 °C. Following incubation, the postplasma treatment viability was determined by counting the number of bacterial colonies on the plate in terms of CFUs.

D. Observations (DBD plasma treatment)

After the overnight incubation at 37 °C an untreated sample (control) shows an evenly distributed bacterial lawn on the Petri dish. Compared to the control, the treated sample shows significantly less or no CFUs depending on the treatment time, used gas mixture, and also the discharge pressure. Experiments were performed in triplicates, and the error bars represent “standard deviations.” All results were normalized with respect to the control, with the number of CFUs in the control taken as 100%, since all dilutions and cell counts of the bacterial culture for experiment and reference were the same.

III. RESULTS

Figure 2 shows the viability of *E. coli* determined in terms of surviving number of CFUs with respect to DBD plasma treatment time for CH₄/N₂, Ar, N₂/O₂, and O₂ gas mixtures at constant dissipated power of 0.4 W. We note that the sterilization efficiency is different for different gas mixtures of the DBD plasma. The sterilizing efficiency of CH₄/N₂ gas mixture plasma is relatively higher than for other gases (oxygen, Ar, N₂/O₂, and nitrogen). Figure 3 depicts the surviving number of CFUs with respect to the dissipated power during the plasma treatment by CH₄/N₂ (1:2), O₂, N₂/O₂ (1:1), N₂, and Ar gases’ plasma. In this case, the duration of the treatment time was kept constant at 150 s. The impact of the dissipated power on the killing of *E. coli* changes significantly as the power is increased from 100 to 500 mW. At an elevated dissipated power, the number of surviving colonies decreases significantly for the CH₄/N₂, O₂, and N₂/O₂ gas combinations, as is noted from Fig. 3. A significant and noticeable deactivation response was not observed for N₂ and Ar gas plasmas in either case.

A widely used kinetics measurement parameter referred to as the *D* value (decimal value) is the time required to

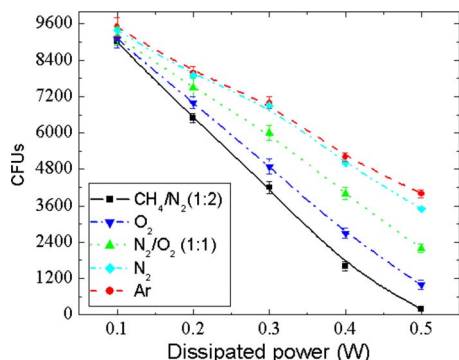


FIG. 3. (Color online) Surviving number of CFUs as a function of dissipated power at electrode distance of 2.0 mm, at AP discharge (duration of the treatment time=150 s) (liner scale).

reduce an original concentration of microorganisms by 90%, i.e., the time for a one log 10 reduction.^{25,26} It can be expressed as

$$D = \frac{t_s}{\log N_0 - \log N_s}, \quad (1)$$

where t_s is the time to destroy 90% of the initial population N_0 , where N_s is the surviving population.²⁶ From Fig. 4, it is observed that the D value is different for the different DBD plasmas. D values are 45, 70, 142, 218, and 240 s for CH_4/N_2 (1:2), O_2 , N_2/O_2 (1:1), N_2 , and Ar gases' mixture plasma, respectively. Ar and N_2 gas plasmas exhibit almost similar D values. There are noticeable changes in the D values for $E. coli$ for both oxygen and N_2/O_2 (1:1) gas mixture plasma. Figure 5 with the colonies (CFUs) of $E. coli$ on the LB-agar plate containing ampicillin visualizes the effect clearly: (a) control, i.e., without plasma treatment, (b) treatment with Ar plasma, and (c) treatment with CH_4/N_2 (1:2) plasma. The shown cultures were treated with 0.4 W (dissipated power) for 150 s. The comparison demonstrates the larger killing efficiency of the CH_4/N_2 compared to the Ar DBD plasma.

IV. DISCUSSION

From the presented experimental results, we can demonstrate the detrimental effect of the application of the DBD plasma on $E. coli$ cells as shown by the number of surviving

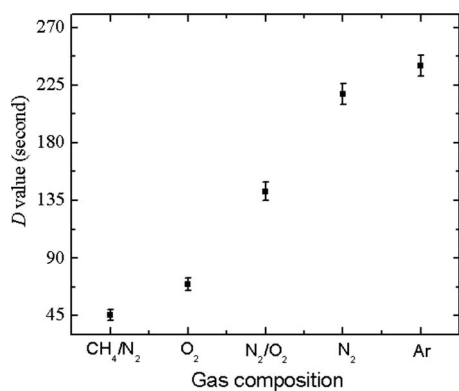


FIG. 4. D values (decimal values in s) for different gas mixtures at AP discharge.

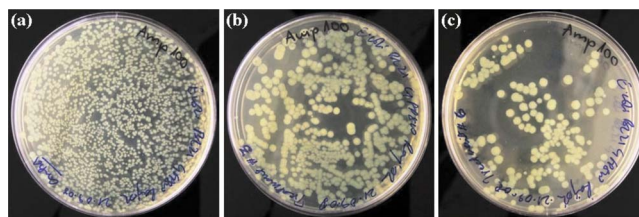


FIG. 5. (Color online) $E. coli$ growth represented in terms of CFUs on agar plates, where (a) control without plasma treatment, (b) Ar plasma, and (c) CH_4/N_2 (1:2) plasma. The dissipated power was set to 0.4 W and the treatment time was 150 s.

CFUs versus plasma treatment time, discharge power, and gas composition compared to the control. There are several advantages to use the CH_4 plasma, but it is highly unstable and difficult to stabilize. A certain amount of N_2 gas admixture greatly enhances the stability of the DBD plasma. Besides the conventional reactive gases, CH_4/N_2 gas mixture plasma has been employed directly on $E. coli$ cells to observe the detrimental or toxic effects on it. In our previous work, on the cytotoxic effects, it has been observed that the different mammalian cell lines were not able to survive after a certain time span on the aH-CN_x film deposited by CH_4/N_2 DBD plasma.²⁷ From Figs. 2–4 we can see that all gases exhibit sterilizing properties against $E. coli$, but the behavior of CH_4/N_2 gas plasma is quantitatively different than the other four gases (O_2 , CH_4/N_2 , N_2 , and Ar). At 1 atm pressure, the CH_4/N_2 gas mixture plasma only needs 45 s to reach the D value, whereas it takes 70 s for oxygen plasma. In the case of N_2/O_2 plasma the D value is 142 s, which thus exhibits a moderate sterilizing property among the five gases. In general N_2 gas is considered as an inert gas in most practical cases and like Ar it is mixed with oxygen gas in a certain ratio. This gas mixture results in reactive plasma species (O_2^* , OH^* , and HO^{2-}), which enhance the reactivity of the N_2/O_2 gas plasma processes. From Figs. 2 and 3, it is clear that the sterilization ability of the CH_4/N_2 (1:2), O_2 , N_2/O_2 (1:1), N_2 , and Ar gas plasmas is time, as well as discharge power dependent; i.e., the longer plasma treatment offers better sterilization/detrimental properties. Also the elevated power enhanced the bactericidal properties. Ar and oxygen gases are mostly used in sterilization processes (air purifications, dental bacterial infection treatment, etc). It has been classified in the present study that there is a noticeable difference in the sterilization ability for different gas mixture DBD plasmas. The sterilizing effect depends on the type of microorganism, the type of the medium supporting the microorganisms, and the method of exposure.^{4,5,19,28} The presence of some level of humidity inside the closed chamber (DBD reactor) leads to the generation of hydroxyl and oxygen radicals, OH^* and O^* that play a key role in killing the targeted microorganism. CH_4/N_2 gas mixture plasma could produce plasmas different to those with CO_2 , H, OH, and N_2 , and are effective on the surface of the bacteria. At the same time, CH_4/N_2 plasma can deposit a hydrocarbon film on the bacterial surface area, which is cytotoxic in nature.²⁷ Oxygen gas plasma is a well known reactive plasma that produces several reactive species (O_2^* and OH^*), which effectively react with the bacterial surface. The active radicals, excited

atomic oxygen, and anions such as O_2^* , OH^* , O^* , and HO_2^- play the most important role in the destruction of microorganisms in AP plasmas.^{29,30} In case of N_2 and Ar gases' plasmas, the generation of reactive species in the plasma is less than in the other gases' plasmas, and this could be the reason for the partial inertness of N_2 and Ar gases' plasmas. In Fig. 5, one can see the lesser effect of the Ar plasma compared to the CH_4/N_2 DBD plasma.

The plasma sterilization or bactericidal processes are supported by several mechanisms. The effect of the charged particles on the plasma plays an essential role in interaction with living organisms, although the synergy with longer living active molecules and atoms, as well as the UV radiation generated in the plasma and at the surface of tissues, may also play a role in the sterilization process. Following ignition of the plasma, the charges start to accumulate over the surface of the *E. coli* layer, and as the treatment proceeds the accumulated charges are sufficient to produce electrostatic fields surrounding the *E. coli* cells. This electrostatic field generates multiple mild electrical shocks as well as local heating. The electrical shocks (or electrical stress)^{5,20} rupture the bacterial wall and gradually increase the killing process of *E. coli*. In some cases, UV photons induce the etching mechanism and the intrinsic photodesorption, which breaks chemical bonds in the microorganism material and leads to the formation of volatile compounds.^{13,14}

Carrying out all experiments inside a closed chamber as in the current configuration has limitations. It is inconvenient for medical applications, the flexibility is low, and contamination can occur during sample transfer in and out of the reactor. Nevertheless, the fundamental aspect of the possible application of DBD plasma to reduce the bacterial load and enhance sterility has been shown in this study. The use of the DBD plasma jet³¹ will be the next step following our present work. In this study we cannot decide on the actual bactericidal agent, but we can put forward our observation that the DBD plasma generated using CH_4/N_2 or oxygen gas exhibits comparatively higher detrimental and sterilizing property toward *E. coli* compared to N_2/O_2 (1:1), N_2 , and Ar gas mixtures at AP. We can classify the five gas plasmas into three sections according to the sterilization property, i.e., highest, intermediate, and lowest or partially inert. CH_4/N_2 gas mixture exhibits the highest sterilization property since it produces electron, ions, UV radiation, as well as chemical reaction on the surface. Chemical reactions take place in the presence of hydrocarbons and nitrogen that produce hydrocyanogens gas as well as H-CN thin layer on the targeted microorganisms. Nearly similar case happens in case of oxygen plasma due to the presence of reactive oxygen species. N_2/O_2 plasma stands in the intermediate stage although it exhibits better sterilizing property than inert Ar and N_2 alone due to the presence of certain amount of oxygen. The use of N_2 plasma could be an alternative choice to the ongoing use of Ar plasma in current medical applications such as in blood coagulation treatments or gastric ulcer treatments using endoscopic methods.

V. CONCLUSION

CH_4/N_2 and O_2 DBD plasmas exhibit relatively higher sterilization properties toward *E. coli* compared to N_2/O_2 (1:1), N_2 , and Ar plasmas. It has been established that the best conditions correspond to medium power levels (around 200–400 mW) and a treatment time around 2–3 min using the corresponding gas mixture [CH_4/N_2 (1:2), O_2 , N_2/O_2 (1:1), N_2 , and Ar] at AP DBD plasma. The bactericidal properties of the plasma can most likely be assigned to the electrical shocks or electrical stresses that rupture the bacterial cell wall. Also, UV photons induce the intrinsic photodesorption that breaks chemical bonds, and interactions with short-living plasma species gradually enhance the bacterial death rate. Prolongation of treatment time and elevated dissipated power significantly improved the *E. coli* destruction efficiency at AP DBD plasma.

For the future prospect, N_2 gas plasma could be an alternative in blood coagulation (inside stomach) treatment instead of Ar plasma as it exhibits partial inertness toward *E. coli*. Having strong effects on the *E. coli* cells, a long-term follow-up is also required to assess the possibility of the use of CH_4/N_2 plasma even in the treatment of skin cancer.

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