The importance of superoxide anion for Escherichia coli biofilm removal using plasma-activated water
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Abstract: Plasma-activated water (PAW) exhibit powerful disinfectant activity. However, the optimal generating conditions, such as the choice of gas used to produce PAW, remain unclear. Here, a range of different PAWs were generated from argon, nitrogen, air, and oxygen in a plasma bubble spark discharge (BSD) reactor capable of directly treating Escherichia coli (ATCC 25922) (*E. coli*) biofilms in situ. PAW generated using oxygen was the most effective and completely removed E. coli biofilms on stainless steel surfaces.
Keywords: Plasma activated water, Bubble spark discharge reactor, Biofilm, Escherichia coli, Reactive species, Superoxide anion radicals.

1. Introduction

Contamination of surfaces in contact with biofilm-forming bacteria is a major problem for engineering industries, including drinking water supplies, cooling water systems, and food processing stainless-steel units [1-3]. Biofilms are particularly hard to eradicate with increased resistance to antimicrobials and disinfectants compared to planktonic cells [4, 5]. Therefore, a new decontamination method is critically needed that can effectively decontaminate the resistant biofilms, whilst also avoiding damage to the surface of the treated material or leaving toxic chemical residues.

One of the promising treatment methods that have these characteristics is plasma-activated water (PAW) generated via cold atmospheric plasma (CAP) processing. Plasma is the fourth state of matter and is generated when a gas is exposed to an electric field voltage, leading to the ionisation of gas molecules and the generation of diverse excited and reactive species [7]. PAW presents a broad range of antimicrobial activity[8-12], which are mainly attributed to the formation of reactive oxygen and nitrogen species (RONS)[8, 13-16].

Although, PAW exhibits powerful disinfectant activity, the optimal generating conditions, such as the choice of gas used to produce PAW, remain unclear. Therefore, in this study, our focus was to identify the reactive species formed in the PAW generated via the differing gas inputs and study their physical and chemical properties and the resulting antimicrobial efficacy.

2. Methodology

Bacteria biofilm formation and plasma treatment

E. coli with cell density of $1x10^{*5}$ CFU/mL was inoculated into wells of a 24-well plate containing sterile stainless-steel coupons (diameter: 12.7 mm and thickness: 3.8 mm from BioSurface Technologies, Bozeman, Montana, USA). Plates were incubated for 48 h at 30 °C with 110 rpm shaking to allow for cell attachment and subsequent biofilm formation.

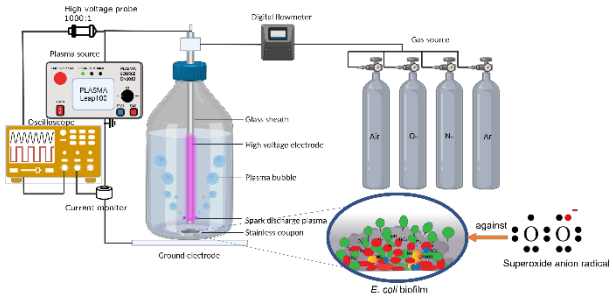

Fig.1 A schematic illustration of the in-situ biofilm treatment using plasma-activated water (PAW) generated by a bubble spark discharge (BSD) plasma reactor.

An E. coli biofilm was grown on a stainless-steel coupon and placed at the bottom of the Schott bottle containing 100mL of MilliQ water (Fig. 1). The plasma bubble reactor consists of a high-voltage electrode and a glass sheath. To generate spark discharge plasma, the high-voltage electrode was powered by PlasmaLeap100. The

voltage and current of the power source are monitored by the oscilloscope equipped with a high-voltage probe and a current monitor. Four different gases were tested, including argon (Ar), nitrogen (N_2), air, and oxygen (O_2). As a control, coupons were placed into 100 mL sterile MilliQ for 10min.

Cell viability

Immediately following the PAW treatment, coupons were extracted from the treatment bottle to determine the colony-forming units (CFU). Live/dead staining was performed on the biofilms formed on the coupon surfaces (Syto9 for viable cells and propidium iodide for dead cells, Invitrogen) observed with an inverted Nikon Ti-E confocal microscope.

PAW physical and chemical properties

Concentrations of hydrogen peroxide (H_2O_2), nitrate (NO_3^-), and nitrite (NO_2^-) along with pH, temperature, and electrical conductivity were assessed in the PAW generated using argon, N_2, air, and O_2 gases for 10 min. The concentration of H_2O_2 was measured by a titanium sulphate method (Zhou et al., 2021). pH, temperature, electrical conductivity (EC), and oxidation-reduction potential (ORP) were measured using a Hanna Instrument pH/ISE/EC meter (HI5522). To investigate the effect of specific active species that were generated in the plasma, a range of molecular scavengers were used as previously described (Rothwell et al., 2022).

Intracellular ROS measurement

Biofilms were stained with 2',7'-dichlorofluorescin diacetate (DCFDA) to assess intracellular ROS according to the manufacturers' instructions.

Statistical analysis

Experiments were performed 3 times and values are expressed as mean ± standard deviation ($\mu \pm \sigma$). A parametric, unpaired t-Test (2 tail, $p<0.05$) or a One-way ANOVA (with Tukeys multiple comparisons test, $p<0.05$) was performed where appropriate to identify significant differences in log reduction of each sample compared to the control.

3. Results

A significant reduction in CFU was observed for the PAW generated with all of the 4 gases when compared to the control (*Fig. 2*), with a 2-log reduction for the PAW-air, 1 log for the PAW-N_2 and 0.5 log for the PAW-Ar. A complete reduction in viability was only seen for biofilms treated with PAW-O_2 (6-log reduction).

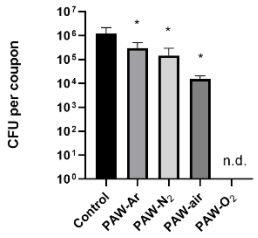

Fig. 2 Reduction of E. coli biofilms using PAW generated in argon, nitrogen, air, and oxygen assessed by CFU. ($P < 0.05$, unpaired t-Test).

A significant number of dead cells occurred in biofilms treated with the PAW-air. While there were not many cells left on the PAW-O_2 treated biofilms, most of the cells were dead. When Tiron (O_2^- scavenger) is added to the PAW-O_2 sample leading to a loss of efficacy of suspected superoxide species, more viable biofilm cells can be observed (*Fig. 3*).

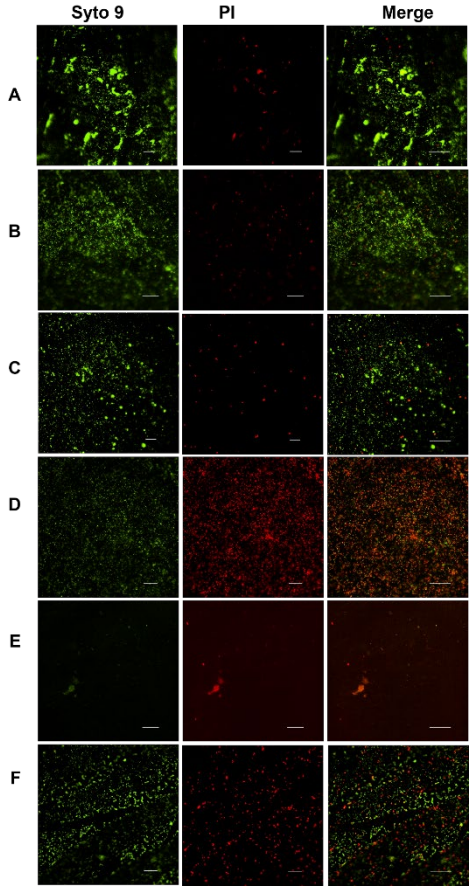

Fig. 3 PAW-induced E. coli biofilm removal. Biofilms were stained using live/dead kit and observed with an inverted Nikon Ti-E confocal microscope.

As shown in *Fig. 4*, H_2O_2 (a known antimicrobial RONS generated in PAW) was predominant in the PAW-Ar and the PAW-O_2. High amounts of NO_3^- and NO_2^- were detected in the PAW-air and lower amounts in the PAW-

N_2. PAW has been shown to have a low pH and this may further contribute to its antimicrobial activity. We have observed a significantly ($p<0.05$) higher EC of the PAW when air N_2 were used as the plasma-forming gas. Our data show an increase in ORP for all four PAW types compared to the MilliQ control. The highest ORP was measured for the PAW-O_2. This correlates with the highest CFU reduction of the PAW-O_2 compared to the other gases.

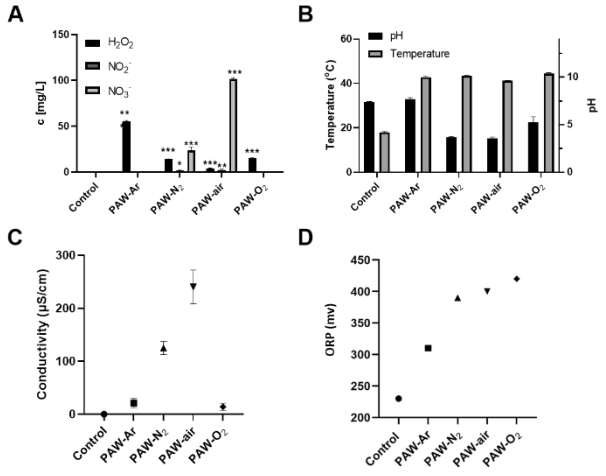

Fig. 4 Physical and chemical characterisation of PAW-Ar, PAW-N_2, PAW-air and PAW-O_2. A) reactive oxygen and nitrogen species of hydrogen peroxide (H_2O_2), (NO_3^-), and nitrite (NO_2^-), B) pH and temperature, C) electric conductivity, and D) oxidation-reduction potential (ORP) values of PAW using four different gas sources (argon, N_2, air, and O_2) at 10 minutes treatment time.

After treatment with the PAW-O_2 (*Fig. 5*), ROS levels were increased significantly by approximately 4.5-fold compared to the control. While the addition of 20 mM of Tiron (O_2^- scavenger) to the PAW-O_2 treated biofilms resulted in significantly less accumulation of ROS (Fig. 9). These data suggest that O_2^- radicals play an important role in intracellular ROS accumulation and may be responsible for the significant biofilms removal caused by PAW-O_2

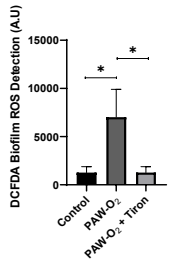

Fig. 5 Detection of intracellular ROS in E. coli biofilms following treatment with PAW-O_2, PAW-O_2 + Tiron, and Control.

4. Discussion and conclusions

In summary, a bubble spark discharge (BSD) with an input gas of oxygen generated plasma-activated water (PAW) can completely remove 48h *E. coli* biofilms grown on stainless-steel surfaces. The short-lived superoxide anion radical was found to be a decisive factor in the antimicrobial effect caused by direct exposure to PAW-O_2. Input gas sources of atmospheric air, argon, and nitrogen were used to generate PAW, and were found to generate a mixture of reactive species with variable biofilm removal efficacy.

This research presents new knowledge on the use of PAW to remove bacterial biofilms and points to the importance of short-lived reactive species in the observed antibacterial efficacy. This has implications for sterilising surfaces, as well as possible treatment of food products or medical devices where conventional disinfection or sanitation is neither suitable, nor effective. Utilising compressed atmospheric air (or oxygen) as a gas input source is an economically viable option when generating PAW. This further underscores its attractiveness for use. Lastly, the plasma source used in this work also has the advantage of being small and portable making it suitable for diverse applications in the chemical industry, food industry, clinical equipment sterilization, medicine, and environment.

5. Acknowledgements

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6. References

1. Lu, P., et al., Phylogenetic diversity of microbial communities in real drinking water distribution systems. Biotechnology and Bioprocess Engineering, 2013. 18(1): p. 119-124.

2. Wan, K., et al., Accumulation of antibiotic resistance genes in full-scale drinking water biological activated carbon (BAC) filters during backwash cycles. Water Research, 2021. 190.

3. Dula, S., T.A. Ajayeoba, and O.A. Ijabadeniyi, Bacterial biofilm formation on stainless steel in the food processing environment and its health implications. Folia Microbiologica, 2021. 66(3): p. 293-302.

4. Costerton, J.W., P.S. Stewart, and E.P. Greenberg, Bacterial biofilms: a common cause of persistent infections. Science, 1999. 284(5418): p. 1318-22.

5. Chakraborty, P., et al., Biofilm formation in the lung contributes to virulence and drug tolerance of

Mycobacterium tuberculosis. Nature Communications, 2021. 12(1).

6. Cámara, M., et al., Economic significance of biofilms: a multidisciplinary and cross-sectoral challenge. npj Biofilms and Microbiomes, 2022. 8(1): p. 42.

7. Mai-Prochnow, A., et al., Interactions of plasma-activated water with biofilms: inactivation, dispersal effects and mechanisms of action. npj Biofilms and Microbiomes, 2021. 7(1): p. 1-12.

8. Chen, T.-P., J. Liang, and T.-L. Su, Plasma-activated water: antibacterial activity and artifacts? Environmental Science and Pollution Research, 2018. 25(27): p. 26699-26706.

9. Jenns, K., et al., Inactivation of foodborne viruses: Opportunities for cold atmospheric plasma. Trends in Food Science & Technology, 2022.

10. Gilmore, B.F., et al., Cold Plasmas for Biofilm Control: Opportunities and Challenges. Trends in Biotechnology, 2018. 36(6): p. 627-638.

11. Renn, T.-Y., et al., Water composed of reduced hydrogen bonds activated by localized surface plasmon resonance effectively enhances anti-viral and anti-oxidative activities of melatonin. Chemical Engineering Journal, 2022. 427: p. 131626.

12. Xu, H., C. Liu, and Q. Huang, Enhance the inactivation of fungi by the sequential use of cold atmospheric plasma and plasma-activated water: Synergistic effect and mechanism study. Chemical Engineering Journal, 2023. 452: p. 139596.

13. Rothwell, J.G., et al., The antimicrobial efficacy of plasma‐activated water against Listeria and E. coli is modulated by reactor design and water composition. Journal of Applied Microbiology, 2022. 132(4): p. 2490-2500.

14. Zhao, Y., et al., Subcellular inactivation mechanisms of Pseudomonas aeruginosa treated by cold atmospheric plasma and application on chicken breasts. Food Research International, 2022. 160: p. 111720.

15. Guo, D., et al., Plasma‐activated water production and its application in agriculture. Journal of the Science of Food and Agriculture, 2021. 101(12): p. 4891-4899.

16. Thirumdas, R., et al., Plasma activated water (PAW): Chemistry, physico-chemical properties, applications in food and agriculture. Trends in food science & technology, 2018. 77: p. 21-31.