STUDY OF PLASMA-INDUCED INACTIVATION OF A REFERENCE MICRO-ORGANISM

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We present a result of an ongoing European initiative (European COST Action MP 1101) that aims to create a reference plasma system and a biological reference protocol for the study and comparison of plasma treatments across laboratories. Endospores of *Bacillus subtilis* ATCC 6633 are proposed as reference microorganism as they can be stored for long periods without loss of viability, serving as a common starting point for experiments performed in different locations and at different times. To avoid problems related to shadowing effects, a monolayer of spores are deposited onto filter membranes using a vacuum filtration technique and these are then exposed to different plasma systems. In this study, sample preparation protocols and bactericidal properties of a dielectric-barrier surface discharge are reported upon.

1 Introduction

Research related to non-thermal atmospheric pressure plasma sources and technologies is currently focused on emerging applications in medicine and biology. As in any other plasma application, the efficacy of the plasma treatment depends on various discharge operating conditions such as discharge geometry, dissipated power and feed gas composition, all of which influence chemical and physical processes in the discharge. In addition, in biomedical applications attention needs to be paid to the various methods in which biological 'targets' are prepared and presented to the plasma as these can have a profound influence on the treatment efficacy. Currently, different laboratories around the world use a wide variety of plasma devices and microbiological techniques, making a direct and quantitative comparison of experimental results virtually impossible. For example, pipetting a bacterial solution onto a substrate is one of the techniques most commonly used in the study of bactericidal properties of plasmas. Yet, the microbial distribution that results from this technique is heterogeneous and not very reproducible.

The objective of this work was to create a reference biological protocol for the study and comparison of plasma treatments across laboratories and use it to evaluate the bactericidal properties of dielectric-barrier discharge (DBD) chamber.

2 Experimental

Dielectric Barrier Discharge chamber device

The device incorporates a chamber where that can be evacuated by means of a pump and refilled with a gas mixture of any desired composition (Fig. 1). Synthetic air was used as working gas for this study and the plasma was operated under static conditions, i.e. with no gas flow, and at atmospheric pressure. The plasma was generated along the top cover of the chamber by means of a dielectric barrier discharge that consisted of a high voltage electrode, a 5.8 mm thick Perspex sheet and a mesh ground electrode. The plasma was operated at 36 kV and its operation could be modulated.

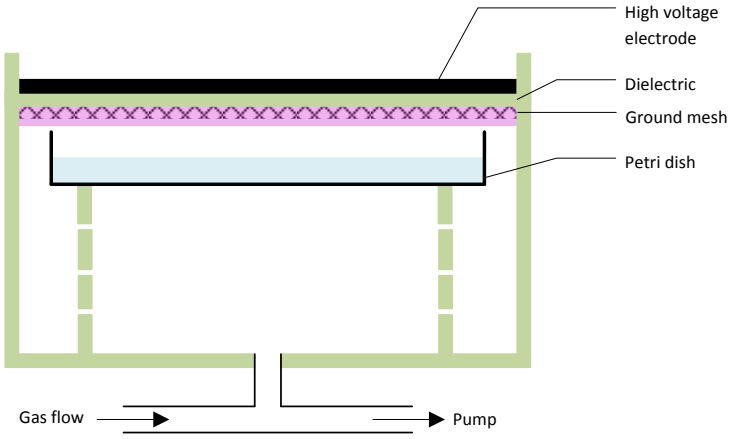

Figure 1: Dielectric barrier discharge chamber device

Preparation of *B. subtilis* membranes

25 mm filter holders were used to create a monolayer deposition of spores on the membranes (0.22 µm, Nuclepore Polycarbonate, Whatman). The filter holders were prepared for each experiment as follows. Membranes were placed onto the moistened bottoms of the filter holders (these had been deposited with 30 µl of sterile deionised water). An O-ring was then placed on the membrane and the upper side was screwed on top. A small amount of non-absorbent cotton wool was then used to seal the holes in the filter holders. The filter holders were then autoclaved in a beaker covered in an aluminium foil at 121 °C for 15 minutes before use.

The filtration was carried out through a rubber bung on a top of a Büchner flask using a vacuum pump. The filter holder was placed into a sterile needle which perforated the rubber bung. The cotton wool was removed and a syringe containing 1.5 ml of the spore solution was inserted in the top of the filter holder. Once the spores were deposited on the membrane, the filter holder was unscrewed and the membrane was removed using tweezers, care was taken to only touch the edge of the membrane in the area where the seal had been in contact with the membrane so as not to disrupt the spore monolayer [1]. The membrane was then placed on a Petri dish containing Technical Agar (1.2%).

Plasma treatment

The Petri dish which held the deposited membrane was then treated inside the DBD chamber. After treatment, spores were recovered by transferring the membrane into a

sterile universal bottle containing 13.5 ml of PBS and 6 ballotini beads; the tube was then pressed onto an Epperndorf shaker for 45 seconds. Spore suspension was diluted to desired concentration and 100 µl was spread on TSA plate with a disposable spreader. We recommend the use of the latter as we found that spores of *B. subtilis* resisted dipping in alcohol and flaming on glass spreaders. Plates were incubated at 30 °C for 24 hours.

3 Experiments and results

Preparation of *Bacillus subtilis* spore stock suspension

A freshly delivered lyophilised sample of *Bacillus subtilis* ATCC 6633 was re-suspended in 100 ml of TSB and a loop was spread onto TSA. After overnight incubation, the culture was re-inoculated onto several deeply filled TSA Petri dishes and left in the incubator at 30 °C. The plates were checked on a weekly basis to see the progress of the sporulation process. It was observed that bacteria need a minimum of three weeks to transfer predominantly to the spore state under these conditions. Sterile PBS (5 ml) was added to the Petri dishes in order to harvest the spores. The surface was carefully scraped with a sterile inoculation loop to separate the spores from the surface of the agar [2]. Inevitably small pieces of agar are generated by this procedure and these were removed by transferring the resulting suspension into centrifuge tubes and centrifuging at various conditions: 4000 g for two minutes, 2000 g for 30 seconds and final centrifugation of 8000 g for 30 seconds, in order to determine those resulting in maximal removal of agar debris. The supernatant was poured away and 10 ml of fresh sterile phosphate buffered saline (PBS) was added followed by vigorous shaking in order to re-suspend the spore pellet. This process was then repeated a further two times in order to remove the majority of the remaining debris. The resulting spore suspension contained in a sterile Universal glass bottle was transferred to a water bath at 70 °C for 30 minutes. It is recommended to follow the same procedure for heat shocking for the preparation of spores, as the conditions under which the shock is applied determines the resistance of the spores to inactivation. Once the spores were produced, they could be stored for long periods without loss of viability. The concentration was determined by serial dilution for future plasma experiments.

Recovery of *B. subtilis* spores from the membranes

Several recovery techniques were carried out to find the most efficient recovery rate of spores from the membrane. The study compared the type and time of shaking, and the addition of ballotini beads and addition of detergent (0.05 % (w/v) Tween-80) into the PBS (Fig. 2). The effect of addition of Tween-80 was found to be negligible and the recovery of spores from membranes increased with more vigorous shaking. The effect of a conventional orbital shaker and an Eppendorf vortex were also compared. A significant increase in the spore recovery was observed with the addition of six glass ballotini beads (dia. 3 mm). The beads improved the recovery by striking the membrane as it was shaken. Although recovery increased with shaking time, there was no further increase after 45 seconds of vigorous shaking.

Before each plasma-decontamination experiment, the inoculated membrane was placed onto a Technical Agar and left to dry in the safety cabinet for one hour. A slight

reduction in the spore recovery efficacy was observed after the drying process and this is attributed to enhanced spore adhesion to the membrane. After plasma exposure, bacteria were recovered in PBS using 6 ballotini beads and 45 seconds of shaking with an Eppendorf vortex.

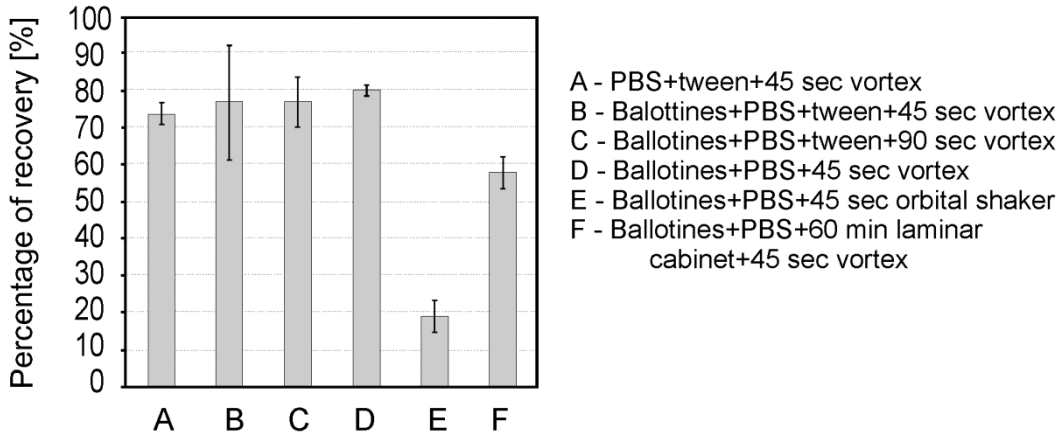

Figure 2: *B. subtilis* spore recovery from membranes (3 replicates) at various conditions without plasma treatment.

Inactivation caused by DBD chamber plasma device

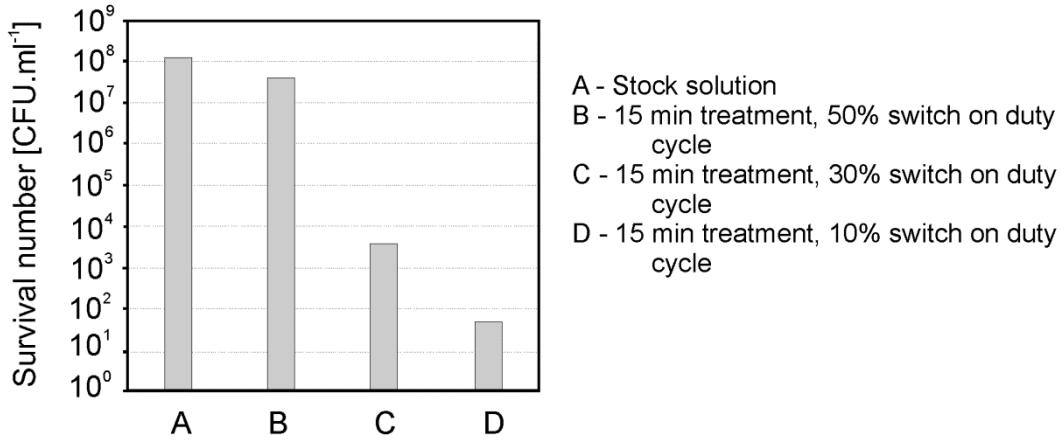

Figure 3: *B. subtilis* spore inactivation after exposure of DBD generated in air gas mixture for 15 minutes of treatment. Each experiment was done in duplicate.

Membranes inoculated with *B. subtilis* spores were exposed to plasma for 15 minutes with two 5 minutes breaks (Fig. 3). A slight effect of plasma-decontamination was noticed after 15 minutes of treatment employing plasma generated at 50% duty cycle. A larger than 4 log reduction was observed after plasma treatment generated under a 30% duty cycle. And the most effective plasma treatment occurred when plasma was generated with a 10% duty cycle. The increase inactivation with decreasing duty cycle is attributed to a lower temperature operation of the device, which favors the production of ozone, a

well-known bactericidal agent produced in low temperature air plasmas. It is noted, however, that in all cases the agar plates remained close to room temperature and no thermal inactivation was observed.

4 Conclusions

In this work, we focused on development of a biological reference protocol for the study and comparison of plasma treatments across laboratories and used it to characterize a DBD plasma chamber.

Our findings and recommendations are summarised as follows:

- Endospores of *Bacillus subtilis* ATCC 6633 are proposed as reference microorganism as they can be stored for long periods without loss of viability. Protocols for *B. subtilis* spore suspension and sample preparation have been developed.
- For reproducibility, the procedures described in Section 2 should be followed strictly. It was observed that gentle centrifugation for up to two minutes is required to obtain clean spore suspension without growth medium debris. The resistance of spores depends on the conditions leading to sporulation and here we recommend the use of a 30 minutes 70°C thermal shock.
- Disposable spreaders are recommended to prevent cross-contamination when performing serial dilutions.
- For the inoculation of the membranes, a volume of 1.5 ml of 10^8 spore suspension should be filtered through the membrane to ensure deposition of a monolayer of spores (25 mm diameter membranes).
- The best conditions for recovering spores from membranes were found to be a PBS suspension containing ballotini beads and 45 seconds of shaking on an Eppendorf vortex. It was observed that the percentage of recovery is sensitive to the speed of agitation and the presence of ballotini beads.
- The DBD chamber device was able to inactivate *B. subtilis* spores, even though spores were extremely resilient
- The enhanced bactericidal effects observed at low duty cycles highlight the importance controlling the operational temperature of the device and not only of the exposed target.

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References

[1] D. L. Bayliss, J. L. Walsh, F. Iza, G. Shama and J. Holah, M. G. Kong, *Plasma Process. Polym*, 9(6): 597-611, 2012.

[2] C. M. Shaw, Inactivation of *Clostridium difficile* spores in the Healthcare Environment using hydrogen peroxide vapour, Doctoral thesis, Loughborough, 2013.