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Inactivation of Yeast cells using Dielectric Barrier Discharge

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Conventional sterilization methods pose certain disadvantages such as long exposure times, deposition of dangerous residues on objects being sterilized and limited versatility. Prior literature has proved that plasma sterilization trumps these conventional methods in terms of exposure time, operating temperature, versatility and safety. This paper focuses primarily on investigative efforts spent in trying to understand the underlying mechanisms involved in the sterilizing effect of plasma on yeast. Samples of yeast cells were exposed to RF induced Dielectric Barrier Discharge (DBD) plasma. While complete sterilization was noted within 60-120s, it was also seen that the sterilization process was affected by factors such as dielectric material and number of sterilization cycles.

I. Introduction

1.1 Sterilization

Sterilization is defined as any process that destroys all micro-organisms clinging to a surface, especially bacterial endospores. Endospores are tough, dormant and non-reproductive organisms produced by most bacteria to tide over environmentally stressful conditions. When conditions start to become stressful, the bacterium replicates its DNA and cordons it off with a double membrane, thus forming an endospore. Once conditions become favourable again, the endospore regerminates thus ensuring survival. Examples include B.subtilis and B.stearothermophilus. Such endospores are extremely resistant to normal household cleaning agents and require rigorous sterilizing procedures. Such extraordinary measures might seem drastic in our typical every-day lives, but are critical in clinical settings or even on the battlefield wherein instantaneous sterilization techniques ensure quicker response times.

Plasma Sterilization is also being investigated in the context of space-craft sterilization. Types of bacteria that can be found in extra-terrestrial atmosphere have been studied thoroughly in order to understand their resistance mechanisms and thus enforce effective sterilization procedures. Cooper et.al[15] state that it is important that extraplanetary-bound landers and probes have ≤ 30 bacterial spores on the free surfaces of a landed system to prevent contamination from terrestrial bacterial sources. Such relevant research makes understanding the fundamentals of plasma sterilization even more important.

The ideal sterilant, according to Moisan et.al. (2001)[2] should provide (a) shorter sterilization times, much less than 60 minutes (autoclaving) (b) processing temperatures equal to or lower than 55^{o}C (achieved during EtO sterilization) (c) versatility of operation and (d) harmless operation for patients, operators and materials.

Conventional Sterilization techniques make use of a number of agents- dry heat, moist heat, chemical sterilants and even γ-radiation[1]. Autoclaving uses high pressure and temperature to achieve complete

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sterilization. Objects are exposed to damp heat at 121°C for 15 minutes or at 134°C for 3 minutes. This process is used in hospital settings either to sterilize medical waste before its disposal or to sterilize large batches of surgical instruments. Autoclaving provides a cleaner alternative to incineration and is one of the most effective sterilization techniques. However it requires long sterilization times (~20-40 minutes), longer standing times and large infrastructure. New strains of bacteria, that have proved resilient towards autoclaving, have also been discovered. Moreover, most instruments in hospitals nowadays are made mainly of polymers. Using heat-intensive techniques might easily damage these instruments.

Dry heat ovens also use intense thermal energy to effect sterilization. However, steam is often preferred due to its higher heat capacity. Additionally dry heat ovens require operational times of 3-8 hours depending on the temperatures attained.

Another popular sterilization technique uses chemical agents such as Ethylene Oxide (EtO), formaldehyde and glutaraldehyde. Their high toxicity ensures complete inactivation but also provides an operating disadvantage. Objects need to be soaked in formaldehyde or glutaraldehyde for 8-10 hours for effective sterilization. Sterilization by EtO is a rigorous process that needs to be controlled carefully. Due to its extreme flammability, EtO can only be pumped into the sterilization chamber after all the air has been pumped out. It takes as long as four hours for all the EtO vapors to dissipate. Hence vent times, often longer than sterilization times are required. There is always the omni-present threat of toxicity to handlers and patients from toxic residues that can be deposited on the surface of the object during sterilization. These disadvantages make for a very cumbersome process.

Other processes like γ-irradiation and UV sterilization act by bombarding the object with γ-rays or UV rays irrespectively. The irradiation is a very effective sterilant. However γ-irradiation requires expensive infrastructure and an isolated site for safe operation. Also it affects the bulk properties of certain polymers which makes it undesirable for sterilization of medical equipment. UV-radiation on the other hand requires a reliable source of electricity, is effective only when directly striking the micro-organisms and accidental exposure to it can burn the skin and eyes.

Plasma sterilization is a faster, low-temperature, safer and more versatile option to all the methods described above. There is an abundant cache of literature on experimental methods of plasma sterilization, wherein different plasma sources using different electrical parameters have been used to sterilize different standard bacterial samples[3],[4]. However before this topic can be discussed further, a brief introduction to plasma, specifically Dielectric Barrier Discharge (DBD) plasma needs to be provided.

1.2. Dielectric Barrier Discharge Plasma (DBD)

Plasma, or the fourth state of matter is strictly composed of ions and electrons only. However commercially, plasma is just 'ionized gas' comprised of charged radicals, neutrals and UV photons. The simplest way of producing plasma is to apply an electric field to a neutral gas. Non thermal plasmas in dc discharges are usually created in closed discharge vessels using interior electrodes. Different types of discharges and plasmas can be obtained depending on the applied voltage and the discharge current. Fig.1 shows this dependence.

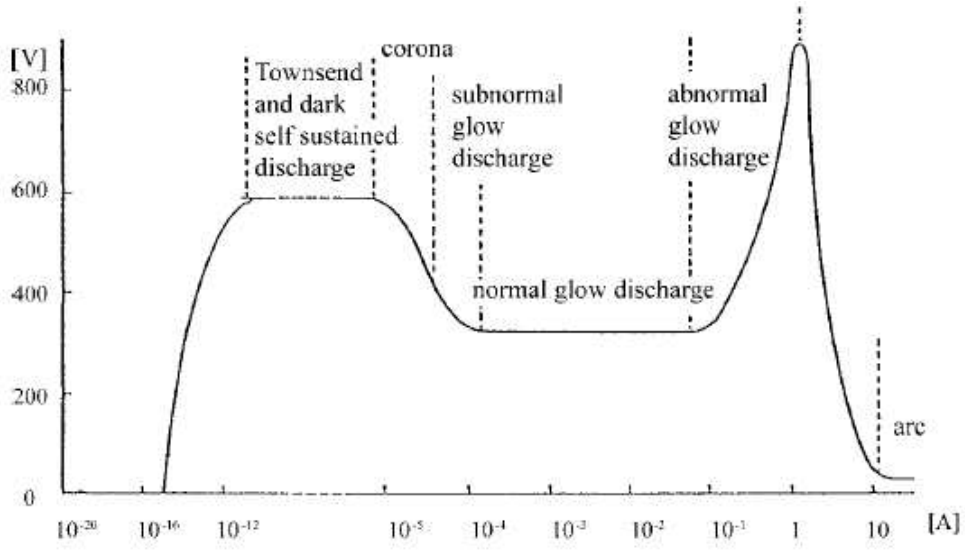

Fig.1: Dependence of voltage upon current for various kinds of dc discharges[5]

As seen in Fig.1, the Townsend discharge is a self-sustained dark discharge. As the transition to a sub-normal and normal glow discharge occurs, it is seen that the voltage decreases accompanied by an increase in discharge current. An abnormal glow discharge occurs as current is further increased finally transitioning irreversibly into the arc. This can be prevented by operating a resistor in series with the glow discharge. The silent discharge or the dielectric barrier discharge (DBD) occurs in the transition between the corona and the normal glow discharge

DBDs have been known as early as 1857, when Werner Von Siemens[6] reported experimental investigations wherein a flow of O_2 or air was subjected to the influence of a DBD maintained in a narrow annular gap between two coaxial electrodes, to which an alternating electric field was applied. For a long time, ozone generation was the major industrial application utilizing DBDs. For this reason, they were also sometimes known as 'ozonizer discharges'. Apart from that, DBDs are also implemented in surface modification, plasma chemical vapor deposition, pollution control, excitation of CO_2 lasers and plasma display panels.

DBD discharge is generated between two electrodes with a dielectric barrier in between them. The gap width is of the order of a few millimetres. The planar DBD configuration used in this paper is shown sketched in Fig.2. Since the dielectric layer in between cannot pass dc current, these devices require alternating voltages for their operation. The dielectric also acts as ballast- it imposes an upper limit on the current density in the gap. Typically DBDs are operated at a voltage of 1-100 kV and frequencies of 50Hz-1MHz. At higher frequencies, it becomes tougher to effect the dielectric limitation on the current density. When electric field is sufficiently high to cause breakdown of the discharge gas, a large number of micro-discharges can be observed emanating from the electrodes. These can be clearly seen when the pressure is of the order of 10^5 Pa or at atmospheric pressure, which is why procedures such as ozone generation are carried out at atmospheric pressure.

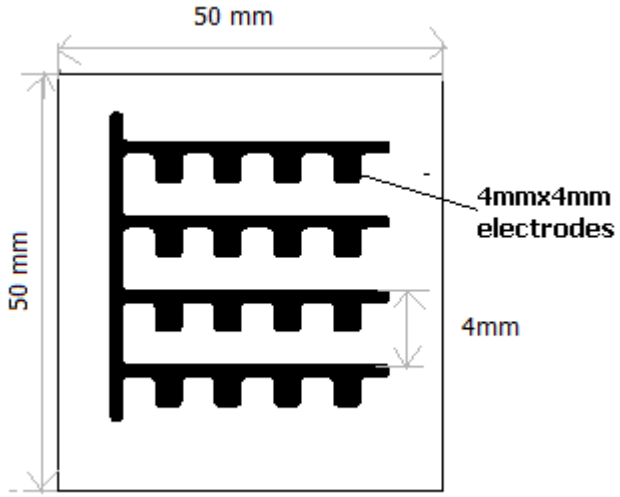

Fig.2: Schematic of the top electrode layout(sawtooth design) for the DBD device used

The schematic of the DBD shown in Fig.2 warrants a description. This device has been milled out using the Quick Circuit prototyping system (a product of T-Tech, Inc.). The board material used here is standard FR4 overlaid with copper and is roughly 0.78 mm thick. FR4 (or Flame Retardant 4) is commonly used for making Printed Circuit Boards (PCBs). The cross-section shown in Fig.2 measures 50mmx50mm. The top layer, which consists of a sawtooth design, is connected to AC input power while the bottom layer, which consists of a sheet of copper, is grounded. Batch A, consisting of seven such identical devices, were made using this mill.

Before we delve into our experimental results involving DBD plasma and its sterilization efficiency, a brief review about plasma sterilization is in order. This review helped identify the chief parameters that needed to be investigated in our study.

1.3. Plasma Sterilization- A Review

Plasma sterilization dates all the way back to 1968, when Menashi reported experiments wherein he used a pulsed RF field to sterilize 10^6 spores in times less than 1 second. These spores were deposited on the inner walls of a vial which was wrapped in coil, connected to a power source. A patent was filed for this high

temperature/high pressure plasma sterilization process. A separate patent was later filed in 1972 for a low-temperature plasma sterilization process. Further patents by Boucher (Gut) in 1980 and Bithell in 1982 established firmly that sterilization could indeed be achieved by subjecting bacteria to an electrical discharge in an appropriate gas. Further experiments concentrated on refining the experimental setup used. Most early experiments used inert gases such as He and Ne but scientists started experimenting with H_2O_2 and halogens. Also the type of electric field applied was varied with some researchers using microwaves and lasers to generate plasma. Numerous early research efforts such as these cemented an earnest interest in plasma sterilization.

While the bulk of literature on plasma sterilization has mainly been reported in volume plasma, a considerable amount of research in using surface plasma such as DBDs for sterilization has also been reported as recently as 2008. Tanino et.al[3] in 2007 used 34 kHz, 230W DBD plasma with Teflon as the dielectric to sterilize wet and dry sample of B.subtilis spores spread on PET film. Complete bacterial inactivation was achieved in 4 minutes (for the dry sample) and 40s for the wet sample. Roth et.al[7] in 2000 reported sterilization experiments by glow discharge plasma operating at atmospheric pressure (OAUGDP). Choi et.al[8] in 2005 reported using a pulsed DBD system employing an alumina dielectric to achieve complete inactivation of Escherichia Coli(E.coli) in a little more than 60s. The D-value, which is defined as the time taken to reduce the initial number of cells by 90%, was 15.2 s in this experiment.

Starting in the late 20th century, research efforts in plasma sterilization also began to focus on determining the mechanism of plasma sterilization. Plasma sterilization involves a plethora of parameters at each stage. Factors such as gas flow rate, power density, type of discharge gas determine the type of plasma generated. Factors such as the geometry of the surface being sterilized and type of substrate material used determine the effectiveness and practicality of the sterilization process. For instance, a curved geometry is harder to sterilize than a flat surface. Finally another set of factors also determine the efficacy of the sterilization process. These include the type of species being killed, the number of micro-organisms and the amount of organic residues on the surface. By organic residue, we mean blood and other superficial impurities which might shield the micro-organisms from the plasma. Arriving at an optimal mix of parameters can be a daunting task, although that has not deterred scientists from trying.

Hury et.al[9] in 1998 conducted a parametric study wherein they tested the destruction efficiency of oxygen-based plasmas on B.subtilis spores. They concluded that the O_2 based plasmas achieved more killing than pure argon plasmas, with H_2O_2 and CO_2 plasmas showing higher destruction efficiencies. They also concluded, using CO_2 plasmas, that a lower surface density contributed to higher destruction efficiency. Another observation made, which might seem counter-intuitive was that a higher temperature contributed to more killing. Similarly Lerouge et.al[10] in 1999 conducted research studies wherein different gas compositions (O_2, O_2/CF_4, O_2/Ar etc.) were compared in terms of destruction efficiency. Moreau et.al[11] in 2000 used the flowing afterglow of a plasma to inactivate B.subtilis spores. The afterglow, as the name suggests, is the region immediately after the glow-discharge region. Reactive species produced in the discharge region sometimes migrate over to this region thus providing sterilization without the electric field. Certain advantages have been cited in using the afterglow. Moreau et.al[11] explore this more thoroughly in their paper.

Researchers have also tried pursuing an alternative line of research- if the mechanism of the process is better known, the working efficiency of select phases in the process could be improved in order to increase overall efficiency. Moisan et.al[2] in 2001, published an extensive review wherein they analyse prior literature on parameter analyses in plasma sterilization and arrive at a tentative conclusion regarding the mechanism involved.

While conventional sterilization methods show a linear survival curve, plasma sterilization shows non-monotonic behaviour. Typically survival curves for plasma sterilization have been shown to be tri-phasic with a short initial phase, a secondary lag-phase and a final phase, similar to the first phase. Moisan et.al. [2] explain this phasic behaviour in the following manner. When electric field is applied to a discharge gas, the gas ionizes producing UV photons, charged radicals and neutrals. The UV photons initially irradiate the top layer of spores, thus producing a sharp drop in concentration. The subsequent layers are then eroded by the combined action of UV photons and reactive species, wherein both react with atoms intrinsic to the organism to produce volatile compounds that can be flushed away. Hence the second phase has a longer D value. The third phase starts at the very end, just before complete sterilization, wherein reactive radicals etch away the remaining spores. Hence the D values of the first and third phases are often similar.

While many hypotheses have been proposed to explain the mechanism of killing, not enough research has been invested into isolating the action of each individual agent involved in killing. For instance, filters designed especially to absorb UV wavelengths could be employed to allow only a certain band of radiation to reach the surface being sterilized. Another method would be to use a gas-detection system to detect certain neutrals and filter them out before they reach the surface of the object being sterilized. In doing so, one or two of the three potential parameters are eliminated allowing us to concentrate only on the killing efficiency of the one remaining parameter. This method would help identify the dominant killing agent, the respective roles of the three killing agents and so on. Such fundamental research is the objective of the present paper.

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II. Experimental Setup

The schematic used for plasma generation is also shown in Fig.3. A function generator (HP 33120A) is used to generate a 14 kHz RF sine wave at an amplitude of 2.1 V. The power of this signal is then amplified through using a Crown CDi4000 amplifier. This amplified signal is then passed through a step-up transformer which steps up the input voltage to the order of kV. The final signal being fed into the device has an input voltage of 11.4 kV (p-p).

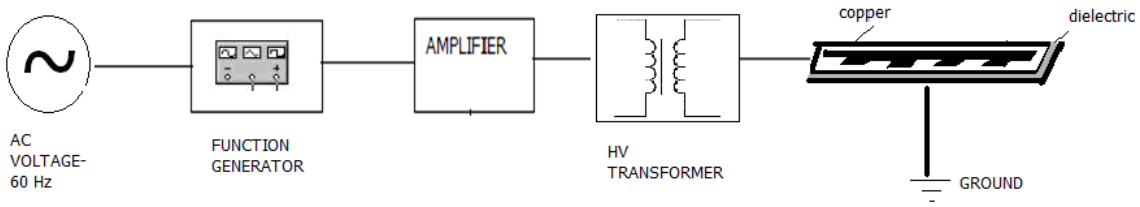

Fig. 3: Schematic of the experimental setup used for plasma generation

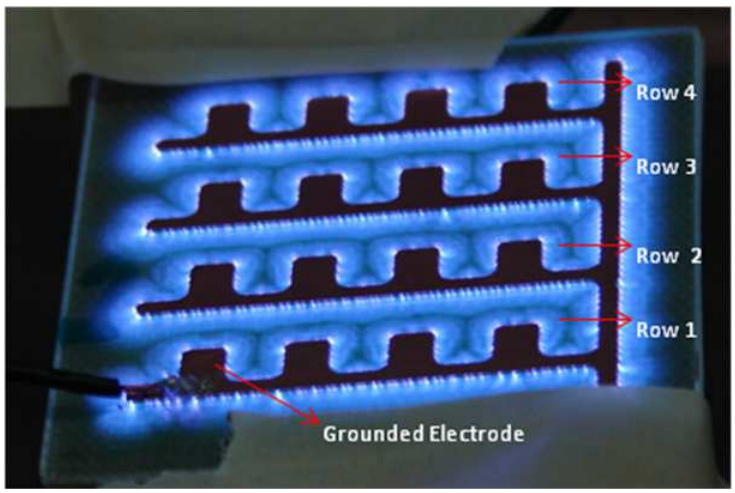

Fig.4: The DBD device used

The yeast sample was prepared by dissolving dry yeast substrate in warm water and agitating it in an incubator overnight. An appropriate amount of this solution containing 10^7 cells ml^{-1} was then cultured in LB broth and used as the deposition sample. For each of the devices, 80 µl of this solution was then deposited between any two of the rows as seen in Fig.4. Once these devices were exposed to plasma for the required time interval, they were treated with 5 ml of LB broth and vigorously shaken in order to detach any cells clinging to the devices. 0.1 ml of this solution was then suspended in 0.9 ml of PBS solution and a dilution series was prepared. 0.1 ml of each dilution was then overlaid on fresh brain-heart infusion and these culture plates were then incubated for 24-48 hours at 37°C. Each experiment was replicated thrice.

III. Results & Discussion

A feasibility study was first performed wherein samples of yeast, containing 10^7 CFU/ml were cultured overnight. 100 µl of this yeast solution was then deposited on the device shown in Fig.4. Two devices from Batch A were used. One served as a control while the other was fired for a minute. Both were then stamped onto separate agar plates and incubated at 37°C for 48 hours. After 48 hours, the control showed an appreciable yeast distribution while the other device showed no yeast at all. Although the stamp test serves only as a qualitative predictor, it proved that the plasma was killing yeast cells.

Encouraged by these results, we graduated to performing quantitative experiments, wherein we ran different devices for different lengths of time and attempted to obtain a survival-curve i.e., a plot of the logarithm of the number of survivors versus exposure time. Before that, we performed another preliminary study. This study was aimed at comparing the difference between plasma effects on a single drop of yeast solution versus a uniformly spread drop of yeast solution. Two identical devices from Batch A were compared, one on which 80 µl of yeast solution was deposited and spread evenly over the whole row (Fig.4) and one on which the yeast solution was deposited as a single drop. After running plasma (firing) for 60s, it was noticed that while complete inactivation was obtained in 60s for the first device, only a 3 $\log_{10}$ reduction was obtained in the case of the second device. Based on this study, for all devices subsequently tested, care was taken to spread the yeast evenly over the entire surface.

Based on feasibility results obtained, a time curve test was done, wherein, different devices were fired at different time intervals. These devices were treated with yeast as described in the experimental setup. The time intervals tested were 15s, 30s, 45s, 60s, 75s and 90s with one device acting as the control (not fired). Results obtained showed a 3 $\log_{10}$ reduction at 30s and complete inactivation at intervals above 60s, as seen in Fig.5.

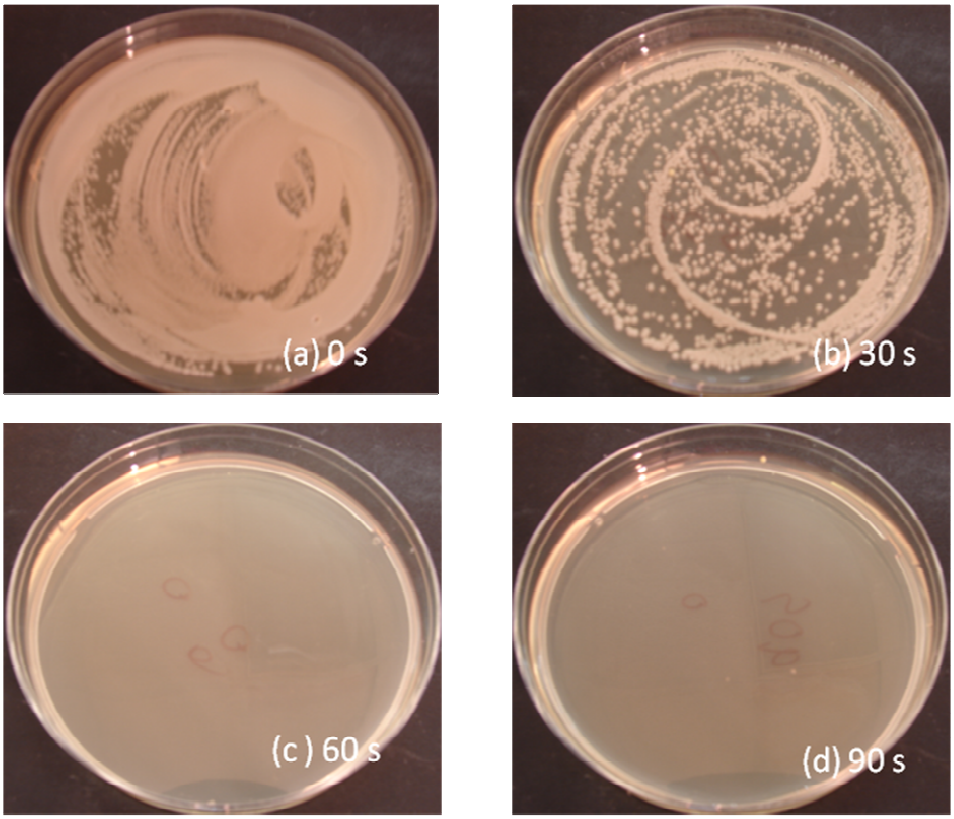

Fig.5: Yeast samples after exposure to plasma for (a) 0s (b) 30s (c) 60s (d) 90s

Jin Ying et.al (2006)[12] conducted a comparative study of the inactivation of yeast using N_2, air and He DBD plasma. Their DBD plasma was generated using a frequency of 6.5 kHz and an input voltage of 10 kV. Starting from an initial concentration of 10^6 CFU/ml, they were able to achieve a reduced yeast concentration of 10^3 CFU/ml in 5 min. They also concluded that N_2 DBD plasma achieved inactivation in the fastest time while He DBD plasma took the longest time for inactivation. Fig.6. given below compares the survival curve obtained from Jin Ying et.al. (Air plasma against yeast) with the survival curve obtained from our experimental data. It is observed that, in our case, complete inactivation was achieved under 100s.

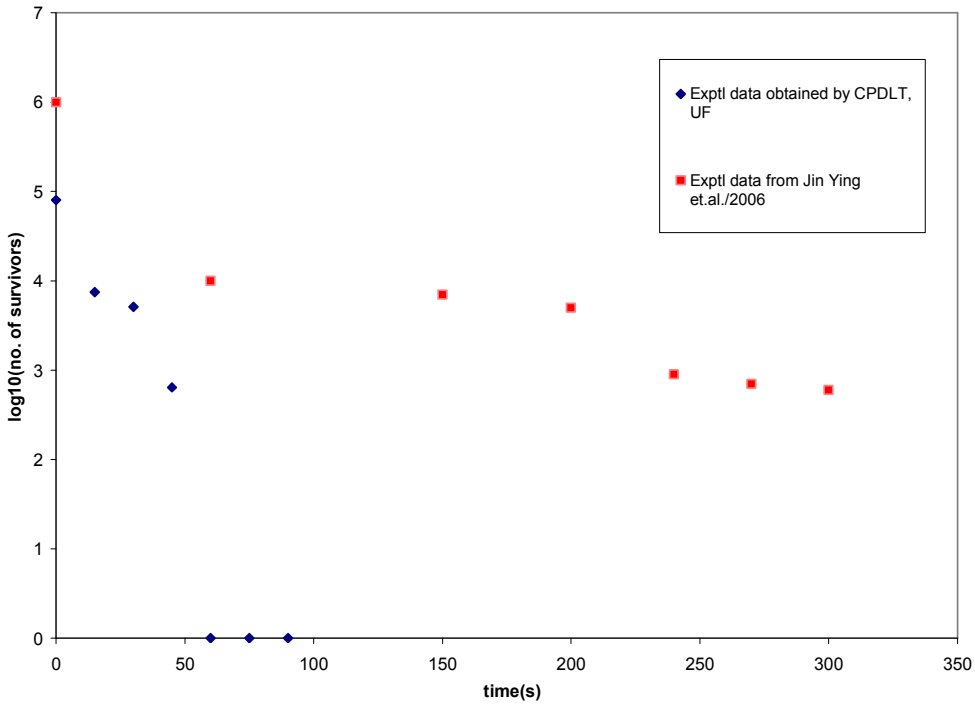

Fig.6: Comparison of experimental data obtained with that from Jin Ying et.al(2006)[12]

These tests were triplicated to confirm our observations. All devices in Batch A were subjected to at least 7-10 sterilization cycles. One *sterilization cycle* comprised of dipping the device in 95% proof alcohol and storing it in a sterile bag overnight, depositing yeast on it, running plasma, post-processing (as described in Section 3) and finally dipping the device in alcohol for the next experiment.

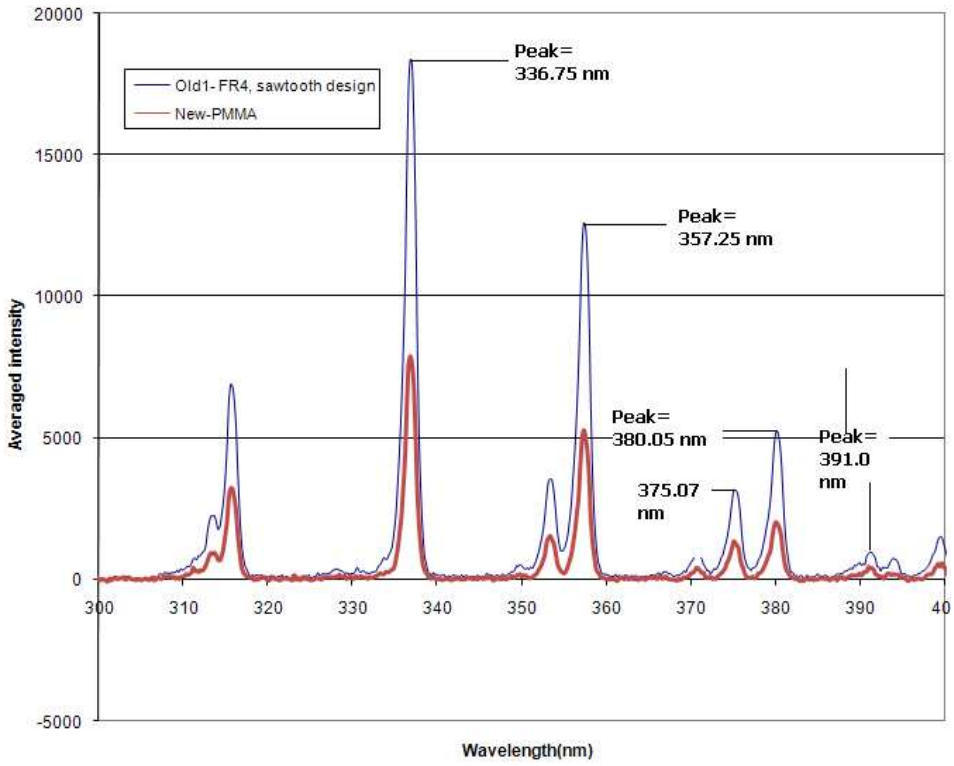

Fig.7. Plot of Intensity Vs Wavelength obtained from spectral analysis

In an attempt to determine what kind of species were being generated in the plasma, a spectral analysis of the DBD glow discharge was conducted using an Ocean Optics USB 4000 spectrometer. The fibre optic cable used to detect and transmit wavelengths to the spectrometer was held about 5 mm above the DBD surface. Time averaged data samples were taken and a plot of intensity versus wavelength (Fig.7) was obtained. In Fig.7, the blue line represents the intensity Vs wavelength plot obtained for the device shown in Fig.4. Emission intensity was plotted by arbitrary unit. The important thing to note here is the pronounced intensity peak at wavelengths predominantly characteristic of N_2 and N_2^+ atoms. The peak at 336.75 nm depicts energy transition from $C_3\pi$-$B_3\pi$ energy levels for N_2 atom. The peak at 391 nm depicts the same energy transition for N_2^+ atoms. What is surprising is that we see no intensity peaks at wavelengths characteristic of O_2 or O_3 or any of the associated oxygen radicals. Choi et.al.[8] in 2005 performed a similar spectral analysis in which they analyzed the spectrum of a pulsed DBD discharge. While they saw the same intensity peaks for N_2 and its related radicals in the 300-400 nm range and one peak at 661 nm, they noted most intensity peaks for O atoms at wavelengths higher than 394 nm. The absence of oxygen radicals in our spectral analysis can be explained by one of two reasons: the radicals could have diffused out very rapidly and hence their wavelengths could not be detected by the spectrometer or the excitation wavelength for these radicals could not be achieved in the sampling interval.

Another notable point worth mentioning is the performance of sterilization tests on designs other than the one shown in Fig.4 as well as designs using PMMA as dielectric instead of FR4. Poly (methyl methacrylate) or PMMA, also commonly known as plexiglass, is a well known transparent thermoplastic. A layer of PMMA, twice as thick as the FR4 layer used in the device shown in Fig.4 was taken. Sawtooth Electrodes (as shown in Fig.4) were milled out on copper adhesive tape, using the T-tech mill. These electrodes were then adhered onto the PMMA slab. Since the thickness of this PMMA slab was greater than that of the FR4 slab used, twice the voltage as well as previously used firing voltages were used in generating plasma. In spite of this, it was generally noted that the FR4 devices functioned better than the PMMA devices i.e. Complete inactivation of yeast cells was noted in 60s for the FR4 devices, whereas only three orders of reduction was seen in 60s for the PMMA devices.

Two other designs were also tested out. One had the top electrode designed as shown in Fig.7(a) while the bottom electrode was a sheet of copper. The other design employed a single straight copper electrode embedded on the top of the dielectric, while another copper electrode was embedded into the bottom of the dielectric layer. As shown in Fig.7(b), the top electrode is powered while the bottom electrode is grounded. The design shown in 7(a) used the same firing voltage as our sawtooth design, while the design shown in 7(b) used almost double the firing voltage. Yeast was deposited on both devices and both devices were run for 60s. When the incubated agar samples were examined, it was noticed that the design in 7(a) and 7(b) showed complete inactivation in 60s also, proving that both designs were equally efficient.

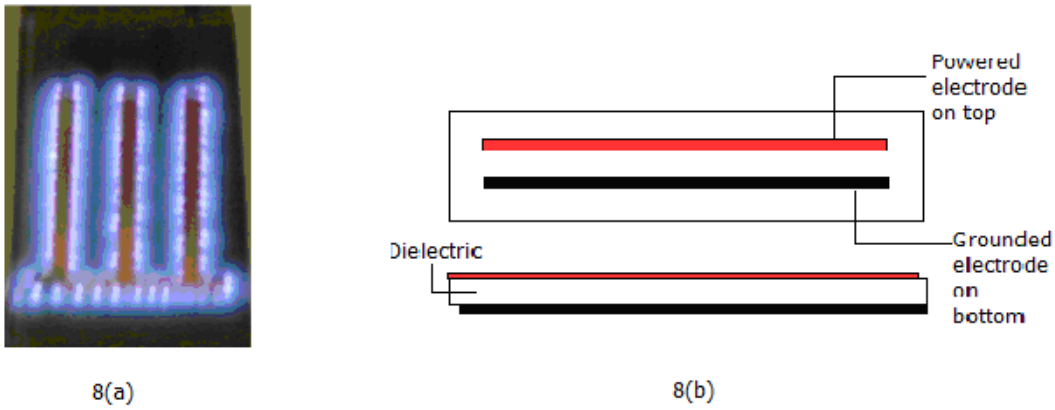

Fig. 8(a) The three-pronged electrode design 8(b) The second design tested- Has one straight copper electrode (powered) on top and one on bottom(grounded)

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More tests on the fundamental level need to be conducted to identify the physics of the mechanism involved in plasma sterilization. There are three agents whose roles need to be evaluated in plasma sterilization: charged particles (ions, electrons), neutrals and the UV photons. Literature offers a confused overview on the roles of each. Khomich et.al (1997) investigated sterilization using a d.c. discharge at pressures in the range of 0.05-0.2 Torr. They concluded that charged particles did not play an essential role. However Lisovskiy et.al. (2000) proved,using a capacitive RF discharge operated at RF voltages, that if the charged particles could reach high enough energies (eV), ion bombardment could play a major role in sterilization. Similarly, Khomich et.al.(1998) and Soloshenko et.al.(1999) speculated that plasma sterilization needed to rely on neutral particles more than UV photons, since the action of UV photons is limited by shadowing effects and the fact that in the atmospheric pressure range, most UV photons emitted at such a high pressure are very likely reabsorbed by their emitting gas, as mentioned by Kelly-Wintenberg et.al. (1998)

Hence in order to distil these facts more stringently, we need to use tools such as spectral analysis, physical and chemical filters and numerical simulation. Spectral analysis helps identify those chemical species that emit energy, during plasma generation. For instance, commercially available mercury-vapor lamps emit about 86% of their light at 254 nm, which is a germicidal wavelength. This is because UV light at this wavelength causes adjacent thymine molecules on the DNA of the micro-organism to dimerize, hence rendering it harmless. Similarly, a spectral analysis can help identify such germicidal wavelengths.

In order to help decide whether UV photons or neutrals play a greater role in sterilization, we can use filters that allow UV radiation to reach the yeast sample but prevent neutrals from acting upon it and compare the inactivation results from this experiment with those shown in Fig.6. If neutrals do indeed play a pivotal role in the killing mechanism, inactivation time recorded in this experiment should be longer than previous results.

Before we could go ahead with such tests, an anomaly was noticed in experimental results that needed to be investigated further. While each device in Batch A had been subjected to at least 10 sterilization cycles, it was noticed that at the 9th sterilization cycle, inactivation time for yeast was taking longer than 60s. This was noticed with all the devices from Batch A. Each device was now taking 120s for complete inactivation of yeast.

Fig.9(a) Microscopic images of a section of the copper electrode from a used device(Fig.4.). This device was milled out on the T-tech mill as mentioned before 9(b). Microscopic images from a new unused device, milled out on a different mill

Fig.9(a) shows microscopic images taken of the copper electrodes embedded into these devices. Fig.9(b) shows microscopic images of the copper electrodes from new, unused devices. The new unused devices were milled using a different mill. After being subjected to numerous sterilization cycles, the electrodes from Fig.9(a) show a lot of corrosion as well as 'pitting'(damaged due to bombardment by plasma species). The electrodes in Fig.9(b), as compared to 9(a), show a smoother appearance instead. The smoother appearance is also in part due to the different mills used. In each sterilization cycle, each device is layered with yeast solution for the experiment, dipped in broth for post-processing and finally in 95% ethyl alcohol for storage. All three media are liquid media and could induce corrosion of the copper electrodes. It is possible that due to such intense corrosion, the electrodes were no longer producing as strong an electric field as before, which could be affecting the inactivation rate of yeast. Another possible reason could be that the dielectric(FR4) had also become pitted due to repeated use, and therefore now harboured many crevices and minute holes that might be hiding some yeast. This issue needs to be investigated further.

IV. Conclusion

This paper details a preliminary experimental analysis of the effect of 14 kHz, 11.4 kV DBD plasma on yeast cells. This experimental analysis has been conducted as part of a larger effort aimed at determining and understanding the fundamental processes involved in plasma sterilization.

Using DBD plasma generated at an input voltage of 11.4 kV(p-p), complete inactivation of yeast cells)was achieved in 60s. Different electrode designs were tested in order to determine whether this played a role in inactivation rate. It was observed that the two alternate designs that were tested (Fig.9) achieved inactivation in the same time as the initial design used (Fig.4). A spectral analysis was also conducted using a OceanOptics Spectrometer (USB 4000) in order to identify the chemical species emitted during plasma generation. N_2 and N_2^+ species were noticed in the spectral analysis but oxygen species were conspicuous by their absence.

An experimental anomaly concerning the devices used was also observed during the course of these studies. It was observed that after a number of sterilization cycles, the device started to fail i.e. it was no longer achieving complete inactivation of yeast in 60s. Such a device now needed 120 s to achieve inactivation. The reasons for these have been speculated upon but definite quantitative tests such as a 'stress' tests, wherein we subject each device to numerous sterilization cycles and pinpoint exact point of failure, are required. Once this failure point is known, an analysis of experimental conditions before and after this point, will help zero in on the exact cause of failure. This stress testing is critical in determining the functional lifetime of each plasma device as a sterilization tool.

Future experimental efforts need to be focused on two parallel paths: (i) identifying the reason for the experimental anomaly observed (ii) Conducting experiments described above in order to analyse the roles of the different agents involved in plasma sterilization (neutrals, UV photons and charged particles).

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