Sterilization Efficiency of Inactivation Factors in a Microwave Plasma Device

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The low temperature plasma inactivation of *Geobacillus stearothermophilus* spores in a low pressure, large-volume microwave plasma device was studied. The experiments were carried out to understand the relative effectiveness of the inactivation factors contributing to plasma sterilization. The effect of ultraviolet photons produced along with plasma was studied using an evacuated isolated chamber with quartz cover, placing inside the main plasma processing chamber. It was observed that exposed spores were successfully inactivated within 40 min whereas the Tyvek(R) wrapped spores were inactivated after 60 min of the treatment by all inactivating factors of air-simulated plasma. From the observed multi-slopes structure of survival curves, we carried out a simple analysis of the relative inactivation rates of different inactivating factors, such as UV emission, plasma species and heat. The scanning electron microscopy images of Tyvek(R) wrapped spores revealed no significant changes in the size of the spores with that of untreated spores despite the survival curve showed that the spores were inactivated.

Keywords: plasma treatment, spore, sterilization, inactivation, surface-wave plasma

1. Introduction

In the recent years, plasma sterilization of medical devices has emerged as potential alternative technology to the available conventional sterilization methods such as physical (dry or moist heat) or chemical treatments (EtO, or H_2O_2). It has numerous advantages e.g. low temperature processing of the heat sensitive objects, short treatment time and no toxicity after processing etc. compared to conventional sterilization techniques. Several types of plasma regimes such as low pressure [1-11] and atmospheric pressure [12,13] discharges are utilized for sterilization. Depending on the discharge conditions (chamber geometry, gas composition and flow, pressure, power etc.) many plasma techniques are being employed and investigated, which leads to sterilization with different effects. These all plasmas are required to be uniform and high density, like many other plasma processing applications, to achieve high sterilization efficiency. With the understanding of plasma sterilization, high industrial and scientific interests are shown. In the plasma produced by electromagnetic fields in the precursor gas (e.g. O_2, N_2, Air, He, Ar etc.), the reactive species such as radicals, neutral particles as well as UV photons are produced and interact with the microorganisms. The mechanisms of their interaction with biological systems are studied by various researchers and still are continued, though many suppositions have been proposed. In the atmospheric pressure, the ozone plays an important role in the spores' inactivation compared to other factors while the sterilization mechanism in the low pressure regime is speculated as the process of destruction of genetic material of microorganism caused by UV/VUV radiation and erosion/etching by radicals. Rossi et al [7] found that UV/VUV radiation was the only mechanism to the spores' inactivation in the case of monolayer but in the case of stacked spores, the total sterilization efficiency was controlled by the etching and erosion of the spores and not by UV radiation. Opretzka et al [5] revealed the contribution of chemical sputtering to the efficiency of the plasma-induced inactivation of bacteria. They observed that the simultaneous impact of H atoms and low energy (at around 100 eV) argon ions, caused a very effective etching and perforation of the spore coat.

Keeping in view of the postulations it can be said that plasma sterilization is caused by three inactivation factors (i) optical radiation (UV/VUV), (ii) plasma produced reactive species/radicals and/or (iii) heating of the bacteria due to the energy release of the adsorbing species [6-11]. So far very few studies have been reported to assess the relative contribution of above mentioned factors in low temperature plasma sterilization.

In this paper we investigated to assess the relative effects of inactivation factors. We studied the sterilization characteristics of wrapped as well as exposed *Geobacillus stearothermophilus* spores at low temperature, with the air-simulated plasma discharges produced by a large-volume microwave plasma device. The spore samples were tested by placing them directly in the discharge, where losses of active particles at internal

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elements of the plasma chamber can be avoided. The effect of optical radiation produced along with plasma was studied using an isolated small chamber with quartz cover, placing inside the main plasma processing chamber of device. The optical emission spectroscopy measurement of the used gases was performed and morphology of treated spores was studied to understand the sterilization mechanism.

2. Experiment

The schematic drawing of experimental setup is shown in Fig.1. 2.45 GHz microwave, guided by a rectangular waveguide, was fed into a vacuum chamber filled with discharge gas, through slot antenna cut in the broad face of each waveguide. The vacuum chamber consisted of a top cylindrical section of 55 cm in diameter and of 21 cm in height for plasma source and a bottom one of a square cross section of 63×63 cm^2 and of 48 cm in height for sample treatment. The vacuum chamber was evacuated to the order of 10^{-5} Torr with a turbo-molecular pump. The introduced microwave power could be varied from 0.2 to 3 kW. The surrounding temperature of biological indicator (BI) samples during the plasma processing was measured using thermo-label sheets. These sheets were attached to glass slides and kept beneath the sample. To avoid the thermal damage from high temperature, the time-modulated plasma discharges were produced using a remote control on/off timer module with microwave system.

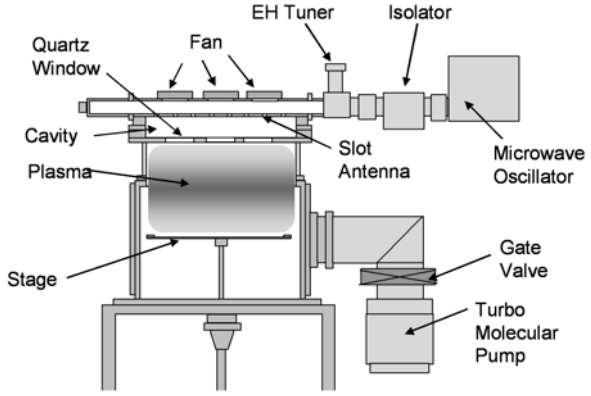

Fig.1 Schematic drawing of the experimental set-up.

The studies have been performed using non-pathogenic spore-forming bacteria: *Geobacillus stearothermophilus* (ATCC 12980) as the sterility indicator produced by Raven Biological, USA, with a spore population of 1.9~2.3x10^6. The spores were pasted on a small oblong stainless steel disc (SUS type) and wrapped in a Tyvek®/poly pouch. The BIs were placed on the moveable stage in the discharge chamber at about 35 cm away from the microwave launcher. The plasma treatment was done at 2.2 kW instantaneous power and a gas pressure of about 50mTorr. Here, we used air-simulated, N_2 and O_2 gas mixture to produce the surface wave plasma. The plasma-treated spores samples were incubated in the culture solution (Tryptic Soy Broth) for seven days at the temperature range of 55~60ºC. The color change of the Tryptic Soy Broth solution after the incubation process was used as initial guideline for spores' mortality. For colony forming units (CFU) count, the Bio-Indicator carriers that were not exposed to plasma treatments were used as a control. Spore survivors from both the experimental and the control bio-indicator carriers were recovered by plunging the carriers into 1.5 ml of BHI (Brain-Heart Infusion) solution in the test tube. Test tubes containing the bio-indicator carriers were vortexed for 1 min at room temperature. The spore suspension of 0.1 ml from the test tube was inoculated onto nutrient agar media with triple replication. The survivors were counted as colony forming units per BI carrier after incubation at 55ºC for 24 h. The results of the counted CFU were plotted to determine the survival curves.

3. Results and Discussion

The inactivation rate of the bacterial spores: *Geobacillus Stearothermophilus* by an inactivating agent of the plasma was analysed through the decrease in the number of surviving spores, which is commonly known as survival curve. This is a plot of the logarithm of the number of surviving spores versus treatment time.

In order to evaluate the respective contribution of the plasma inactivating agents, we performed the following set of experiments. In the first set of experiments, contribution of all inactivating factors was studied. For this purpose spores were completely exposed to the plasma. The effect of partial blocking of inactivating factors by means of Tyvek wrapping was also studied to understand the sterilization of wrapped medical devices. In the second set of experiments, the respective contribution of optical radiation (UV/VUV) and heat was studied through an isolated chamber placed inside the plasma.

The spore samples without any cover (exposed BI) were treated with air-simulated gas mixture (N_2/O_2: 200/50sccm) at 50mTorr pressure and 2.2 kW microwave power when on/off time was 30/60 second. With this discharge conditions, we found that exposed spores were inactivated after 40 min with all inactivation factors and the survival curve obtained is shown in Fig.2. Interestingly, the survival curve is of three-phases. The decimal reduction value (D-value), which is the time required for one log reduction of spore population, has been estimated and shown for each phase (Fig. 2). The inactivation in second phase (D-value: ~12min) is much slower than first phase (~4min) and third phase (~5min).

In order to study the effect of all inactivation factors of the plasma to sterilize the wrapped medical devices,

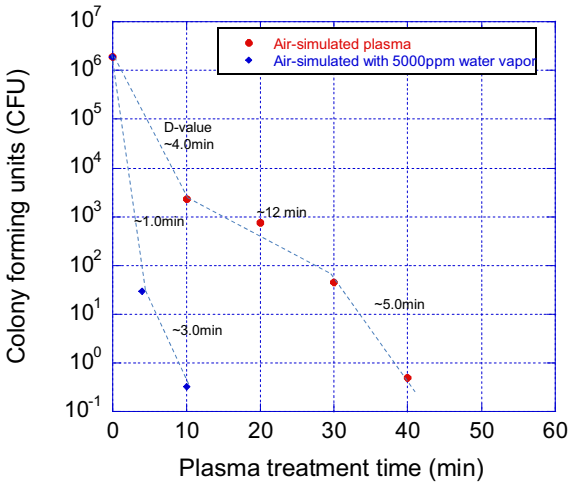

Fig.2 Survival curve of exposed BI treated with all inactivation factors of the plasma.

Tyvek wrapped BI were placed directly to the plasma. The discharge conditions were similar as treatment to exposed BI. It was observed that Tyvek wrapped BIs were inactivated within 60 min as survival curve and D-values are shown in Fig.3. This is obvious to infer that Tyvek cover has partially blocked the inactivation factors to interact with spores.

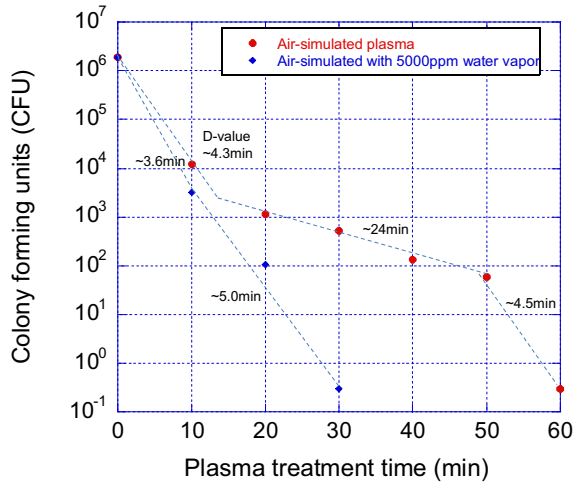

Fig.3 Survival curve of Tyvek(R) wrapped BI treated with all inactivation factors of the plasma.

The survival curves (shown in Figs. 2 and 3) of the inactivation experiments of exposed as well as wrapped spores performed with 5000 ppm water vapor addition to the air-simulated gas mixture and keeping other discharge conditions as similar are only indicative of the improved sterilization efficiency. It is considered that OH and other radicals produced with introduction of water vapor to the precursor gas mixture play an important role in inactivation process [1, 2]. The optical emission spectra indicated the relatively strong OH lines (i.e. generation of OH radicals) in water vapor added air-simulated plasma.

To evaluate the inactivation by optical radiation including UV produced with plasma, we placed an isolated chamber with quartz cover inside the main plasma-processing chamber of device as shown in Fig.4. The isolated chamber was evacuated to less than one mTorr to avoid any radical production due to UV photons. The isolated chamber dimensions were 125 mm in length and 110 mm in diameter. The quartz plate (128x11mm) was placed atop the isolated chamber to pass the optical radiation under cut off wavelengths up to 180 nm.

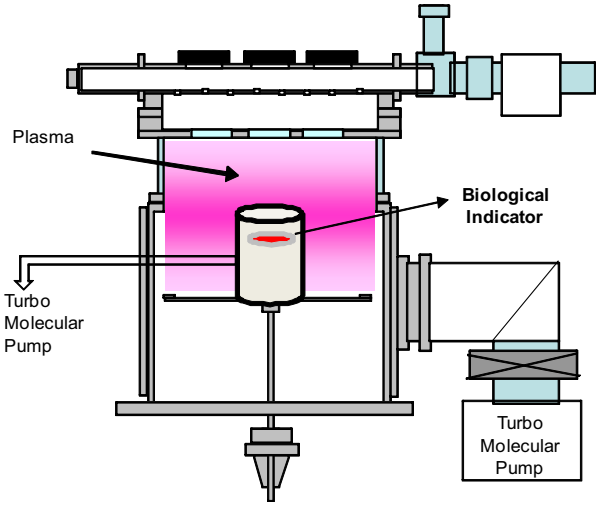

Fig.4 Schematic drawing of an isolated chamber inside the main plasma-processing chamber.

The spores kept inside the isolated chamber were treated in air-simulated plasma at previous discharge conditions. We found that the spores inside isolated chamber with quartz cover and at vacuum were inactivated within 60 min by the combined contribution of photons (optical radiation) and heat of the plasma. The survival curve and D-values found with this experiment are shown in Fig.5.

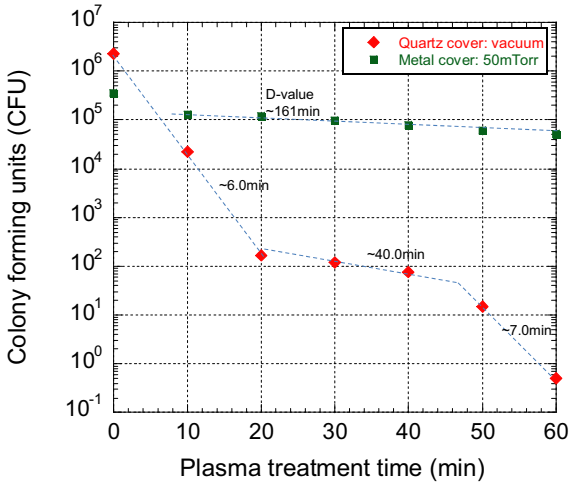

Fig.5 Survival curve of BI treated with quartz and metal cover.

Heating of the spores either by releasing the kinetic energy of ions on impact or radicals recombination is also thought an inactivating agent. To evaluate the only heat

effect we placed metal plate instead of quartz plate atop the isolated chamber and did not evacuated, hence blocked all radicals and optical radiations to interact. The survival curve obtained due to heat is shown in Fig. 5.

Since the experiments were performed at low temperature (<75ºC, by on/off cycle and intermittent pauses), it is worthy to consider that different inactivation factors i.e. heat, photons (optical radiation) and particles (plasma species) have acted simultaneously in each phase of the inactivation process. Hence, the assessment of the relative contribution of the inactivation factors was done separately for each phase by making use of the inactivation rate, k (i.e. inverse of the D-value) for each factor as explained by Vicoveanu et al [8]. The contents of the survival curves by air-simulated plasma only (Figs.2, 3 and 5) were analyzed and the relative inactivation rates of each factor in each phase were calculated. Here we assumed constant heat effect (D-value: 161 min) in each phase because of low-temperature treatment. Only photons effect was obtained by subtracting the heat effect from the combined heat and photons effects whereas only particles effect was found by subtracting the combined effect of heat and photons from the effects of all inactivation factors. Hence, it was found that in the first and third phases, photons have more contribution and are related to UV radiation breaking bonds in the outer coat of the spore and damage to the DNA. In the second phase the more contribution of plasma particles in combination with photons affected etching and erosion of the spores' debris hence the lowered inactivation rate [6,7]. The estimation of the relative effectiveness of different factors done in Tyvek$^{(R)}$ wrapped spores revealed a similar result.

The scanning electron microscopy images as shown in Fig.6 revealed that except exposed spores no significant changes in the actual size were observed compared to untreated spores though the survival curves indicate that the spores were inactivated. This shows that the spores were inactivated by lethal damage to the genetic material caused by UV photons.

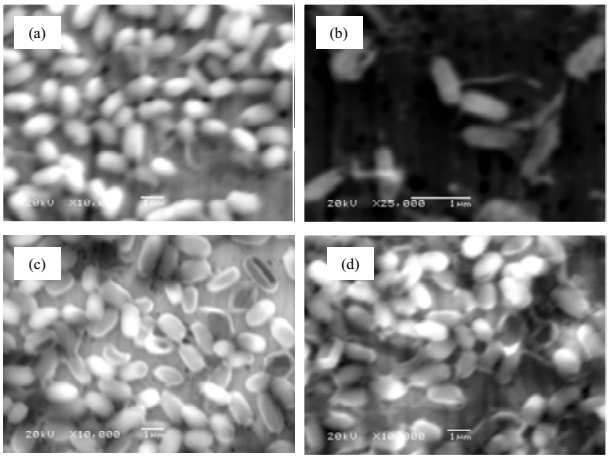

Fig.6 SEM images: untreated spores (a), and treated spores with different conditions as exposed spores with plasma (b), Tyvek(R) wrapped spores treated with air-simulated plasma (c), Tyvek(R) wrapped spores treated with water added air-simulated plasma (d).

4. Conclusion

In order to assess the inactivation effectiveness of different factors, inactivation of *G. Stearothermophilus* spores was investigated in low-pressure air-simulated microwave plasma at low temperature. The exposed spores were successfully inactivated within 40 min whereas the Tyvek$^{(R)}$ wrapped spores were inactivated after 60 min of the processing by all inactivating factors. The longer period of wrapped spores' inactivation may be attributed to the partial obstruction of inactivating factors by Tyvek$^{(R)}$ sheet. It was found that heat produced in the plasma had no significant effect, whereas the optical radiation had significant effect on the inactivation process. The synergetic effect of the optical radiation and radicals in the air-simulated plasma with water vapor had highest inactivation efficiency.

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