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| Author(s) | Singh, M. K.; Ogino, Akihisa; Nagatsu, Masaaki |
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Inactivation factors of spore-forming bacteria using low-pressure microwave plasmas in an N₂ and O₂ gas mixture

M K Singh, A Ogino and M Nagatsu

Graduate School of Science and Technology, Shizuoka University, Johoku 3-5-1, Hamamatsu 432-8561, Japan
E-mail: tmnagat@ipc.shizuoka.ac.jp

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Abstract. In this study, we investigated the inactivation characteristics of Geobacillus stearothermophilus spores under different plasma exposure conditions using low-pressure microwave plasma in nitrogen, oxygen and an air-simulated (N₂ : O₂ = 4 : 1) gas mixture. The microwave-excited surface-wave plasma discharges were produced at low pressure by a large volume device. The directly plasma-exposed spores, up to 10⁶ populations, were successfully inactivated within 15, 10 and 5 min of surface-wave plasma treatment using nitrogen, oxygen and an air-simulated gas mixture, respectively, as working gases within the temperature of 75 °C. The contribution of different inactivation factors was evaluated by placing different filters (e.g. a LiF plate, a quartz plate and a Tyvek® sheet) as indirect exposure of spores to the plasma. It was observed that optical emissions (including vacuum UV (VUV)/UV) play an important role in the inactivation process. To further evaluate the effect of VUV/UV photons, we placed an evacuated isolated chamber, inside which spores were set, into the main plasma chamber. The experimental results show that the inactivation time by VUV/UV photons alone, without working gas in the immediate vicinity of the spores, is longer than that with working gas. This suggests that the VUV/UV emission is responsible not only for direct UV inactivation of spores but also for generation of reactive neutral species by photoexcitation. The scanning electron microscopy images revealed significant changes in the morphology of directly plasma-exposed spores but no change in the spores irradiated by VUV/UV photons only.

1 Author to whom any correspondence should be addressed.
1. Introduction

Low-temperature plasma processing is a potential means to sterilize exposed as well as wrapped medical instruments, because many medical instruments and wrapping materials are made of polymers. Such materials can be easily damaged by thermal treatments, e.g. dry heat and steam autoclave techniques. Other low-temperature techniques such as ethylene oxide (EtO) and gamma irradiation pose the problem of formation of toxic by-products and material degradation. The plasma sterilization technique overcomes many inherent limitations of conventional techniques because of low-temperature treatment of the heat-sensitive objects, short treatment time, safe operation, no toxicity after processing, etc. So far, depending on the discharge conditions, many low-pressure plasma devices have been developed and tested as an alternative technology for sterilization [1]–[9]. Also, atmospheric pressure discharges are being extensively investigated because they do not need a costly vacuum system [10]–[15]. To date sterilization of directly exposed instruments has been mainly performed, although it is customary to sterilize wrapped medical instruments in medical facilities. However, few studies have been done in this regard.

The mechanism of the plasma sterilization technique, either at low pressure or at atmospheric pressure, needs to be understood clearly to improve sterilization efficiency as a whole. In the low-pressure plasma, it is postulated that depending on the different plasma parameters, plasma-generated heat, optical radiations (especially vacuum UV (VUV)/UV) [1]–[3], [6], [16]–[19], charged particles and neutrals [4, 9, 11, 15], [20]–[22] play important roles in different proportions to inactivate the spores. In order to distinguish these inactivation factors, different optical filters are used [3]–[6], [14, 17, 18, 20]. In this way, the working gas is always filled under the filter-confined space at plasma processing pressure. When we assess and discuss the effect of VUV/UV radiations on spores inactivation through the filter media, the important consideration is the contribution from the interaction of VUV/UV photons with the working gas in the immediate vicinity of the spores. Apart from damaging the strand in
the deoxyribonucleic acid (DNA) of the spore’s cell, these high-energy VUV/UV photons are able to dissociate/ionize the working gas and produce radicals/species, which further play an important role in the inactivation process. Another approach to isolate and study the effect of individual species is the use of a particle beam. Recent results reveal that the combined impact of H or O atoms and argon ions at around 100–200 eV resulted in a very effective etching and perforation of the spore coat that leads to inactivation [23, 24].

In the present study, we investigated the inactivation characteristics and contribution of different inactivating factors generated in a low-temperature and low-pressure nitrogen, oxygen and air-simulated plasma for the inactivation of *Geobacillus stearothermophilus* spores. We used two optical filters, i.e. thin quartz ($\lambda_{\text{cut-off}} \sim 180$ nm) and lithium fluoride (LiF; $\lambda_{\text{cut-off}} \sim 120$ nm) plates, to identify the most efficient wavelength range. The effect of optical radiations alone was studied by placing a small isolated and evacuated chamber with a spore sample inside the plasma chamber.

2. Experiments

2.1. Experimental set-up

The experimental set-up consisting of a stainless steel chamber of 40 cm diameter and 40 cm height used for plasma processing in this study is shown in figure 1. The 2.45 GHz microwave guided by a rectangular waveguide was fed through a slot antenna into the chamber [4, 25]. The input microwave power could be varied from 0.2 to 3 kW. The working gases were fed to the chamber through a mass flow controller after evacuation to the order of $10^{-3}$ Pa by a turbo-molecular pump. In this study, we used pure nitrogen (200 sccm), pure oxygen (200 sccm) and an air-simulated gas mixture (160 sccm of nitrogen and 40 sccm of oxygen) as working gases. The surface-wave mode plasmas were produced at a net microwave power of about 700 W and at an operational pressure of 12 Pa for all the experiments. The operational pressure was controlled by a bleed valve. Thermosensitive sheets of different temperature ranges (Thermo Label 5E-50, 5E-75 and 5E-100, Nichiyu) were used to monitor the temperature during inactivation. The color change from white to black of the thermosensitive dots was considered as the temperature at that period. These sheets were attached to glass slides and kept just beneath the spore samples. For low-temperature inactivation, the time-modulated plasma discharges were produced using a remote control on/off timer module with the microwave system. Except under the Tyvek® condition, all the experiments were performed with the plasma on time and off time as 30 and 60 s, respectively (i.e. about 33% duty cycle). In the case of Tyvek® wrap, the plasma on time and off time were 20 and 60 s, respectively (i.e. 25% duty cycle) to avoid damage to the Tyvek® sheet. The net plasma on time has been described as the treatment time for a particular condition.

To investigate the contribution of different inactivation factors, we placed an isolated chamber (a metal cylinder of height 120 mm and diameter 35 mm) inside the plasma processing chamber, as shown in figure 1. The biological indicators are set inside the isolated chamber on the holder at about 15 cm below the quartz window of the plasma chamber. They are treated in different sets: at first without any filter so the radiations and active species can irradiate the spores simultaneously. Then, different filters (LiF plate, quartz plate and Tyvek® sheet) are placed successively at 5–10 mm above the spores. The thickness of quartz and LiF plates was 2 and 3 mm, respectively. For only the optical radiation (VUV/UV) contribution, the isolated chamber was evacuated to the order of $1.3 \times 10^{-4}$ Pa to exclude the role of any other radicals produced due to radiation and working gas interaction.
2.2. VUV/UV emission spectroscopy

Optical emission spectroscopy was performed using the VUV to infrared (IR) monochromator system VM-502 (Acton Research Corporation, Inc.), which was evacuated to a vacuum by using a turbo molecular/rotary pump. The spectrometer was directly installed in the side port of the plasma vacuum chamber by separating with a MgF$_2$ window plate. The operating wavelength range was from 115 to 800 nm and the wavelength accuracy was ±0.1 nm. The spore samples were set in about the line of sight of the spectrometers.

2.3. Biological test procedure

The sterilization experiments were performed using non-pathogenic spore-forming bacteria *G. stearothermophilus* (ATCC no. 12980, Raven Biological Laboratories, USA) as the sterility indicator. The population of spores pasted on an oblong polished stainless steel disc (SUS type) was in the range of 1.9–2.3 × 10$^6$. Different plasma conditions and irradiation periods were applied to treat the biological indicators. After treatment, one set of treated samples was put in a tryptic soy broth (TSB) culture solution and incubated at 55–60 °C for 7 days as the standard for *G. stearothermophilus*. A daily check of the color of TSB solution during the incubation process was used for monitoring the spores’ mortality. If the color changes, it is not sterilized;
otherwise it is sterilized. The other set of treated samples was washed with 1.5 ml of brain-heart infusion (BHI) solution in a test tube for colony counts. Test tubes containing the bioindicator carriers were stirred for 1 min at room temperature. After appropriate dilution, 0.1 ml of the spore suspension was inoculated onto nutrient agar medium, and after 2 days of incubation at 55°C, the colony forming units (CFUs), each representing a surviving cell, were counted. The reduction in CFUs of the treated sample was compared with that of the untreated sample and plotted as the survival curve.

3. Experimental results

The inactivation characteristics of G. stearothermophilus biological indicators under different experimental conditions were investigated and evaluated to understand the mechanism of inactivation of spore-forming microorganisms in low-pressure plasma.

3.1. VUV/UV optical emission spectra

VUV/UV optical emission spectra were measured using optical emission spectroscopy. In the present measurement, the minimum observable wavelength is 115 nm due to the limitation of grating reflectivity of the monochromator, so that we mainly study the wavelength region between 115 and 500 nm. Figure 2 shows the spectra in the cases of pure N₂, pure O₂ and air-simulated N₂/O₂ gas mixture discharge plasmas. In the pure N₂ case, several N atomic (N I) lines in the wavelengths at 120.3, 149.0 and 174.3 nm were observed in addition to several strong lines at 282.0, 293.5, 315.9, 337.1 and 357.7 nm originating from the second positive systems (C₃Πᵤ → B¹Π₉) of N₂ molecules. In the pure O₂ case, we also observed a strong VUV emission at 130.5 nm due to the O atomic line, as shown in figure 2(b). In N₂/O₂ gas mixture discharge plasma, there exist strong peaks in the wavelengths at 214.1, 226.9, 237.0, 247.9 and 258.8 nm originating from the NO γ system (A₂Σ⁺ → X₂Π). To examine the effect of these VUV emission lines on the sterilization, we carried out a comparison experiment using a LiF plate and a fused quartz plate as optical filters. We placed a LiF thin plate (thickness: 3 mm) and a quartz thin plate (thickness: 2 mm) on the sample holders for BIs, as shown in figure 1. The lower limit of wavelength at 50% transmittance is ∼120 nm for the LiF plate and ∼180 nm for the fused quartz plate, respectively.

3.2. Inactivation of directly exposed spores

The survival curves measured with the colony count method for directly exposed spores treated with the pure N₂, pure O₂ and air-simulated N₂/O₂ gas mixture discharge plasmas are shown in figure 3. The results showed that the (1.9 × 10⁶ population) spores were inactivated within 15, 10 and 5 min of treatment with N₂, O₂ and air-simulated N₂/O₂ gas mixture plasma discharges, respectively. It is noted here that the survival curves show a multi-slope, where the first turning points are observed at CFUs of roughly 10²–10³ for all the cases. Thus, it is considered that some amount of spores form so-called clumps in the present BI samples, so a longer time is required to inactivate the underlying spores. This might be a reason for the multi-slope phenomenon observed in the survival curves. The calculated D-values in the first phase are about 40, 45 and 35 s in the case of O₂, N₂ and N₂/O₂ gases, respectively.
Figure 2. Optical emission intensity with different plasma conditions of (a) N$_2$, (b) O$_2$ and (c) air-simulated gas mixture at 700 W input power and 12 Pa pressure.
The treatment temperature in all the cases was maintained below 70°C by on/off and intermittent pauses of the plasma discharges. Here, the plasma on time and off time are 30 and 60 s (i.e. a duty cycle of about 33%). The inactivation time is relatively longer when compared with the previous results [4, 6] because the spore samples were set inside a metal case, whereas samples were placed in a Petri dish in the previous experiment. This difference of inactivation rate may be explained by the difference of plasma density distributions in both the cases. When a metal case was used, the plasma was terminated at the metal surface. On the other hand, the probability for the surface loss of oxygen ions, electrons and radicals in the Petri dish may be lower than that when the metal acts as the plasma-facing surface. Thus the low surface loss of radicals assures us of its higher density and higher inactivation rate [26].

3.3. Inactivation of indirectly exposed spores

The significant role of VUV/UV in inactivation has been emphasized by many researchers. To study the effect of optical emissions, we blocked the plasma particles from interacting with spores by placing the thin LiF and quartz plates on top of the spores in the isolated chamber. The Tyvek® sheet was also used as the shielding sheet to simulate the practical sterilization experiment of Tyvek®-wrapped materials. In these cases, the working gases were present in the immediate vicinity of the spores at the same pressure as the main plasma chamber.

3.3.1. Inactivation with LiF and quartz plates. The inactivation characteristics after eliminating the plasma particles’ contribution by placing the LiF plate (with a cutoff wavelength of roughly 120 nm) or quartz plate (with a cutoff wavelength of roughly 180 nm) in the N₂ plasma are shown in figure 4, together with the result without any filter. It is clear from figure 4 that a longer time is required in the case of the quartz plate compared with the cases of no filter and LiF plate. Here the D-values in the case of LiF and quartz filters are 45 and 50 s,
respectively, a little more than the directly exposed condition. This result clearly suggests that
the VUV emissions in the wavelength domain 120–180 nm in our experimental conditions play
a significant role in the inactivation process as it has high-energy photons. Pollak et al [3]
and Munakata et al [21] have also shown in their specific conditions that the inactivation rate
is highest in the VUV range (112–190 nm). In the case of the N₂/O₂ gas mixture plasma,
comparatively short inactivation times were also observed, as shown in figure 5. Here the
D-values with LiF and quartz filters are about 30 and 75 s, respectively. This may be attributed

Figure 4. Survival curves of spores treated, under an LiF window and a quartz
window, with N₂ plasma discharges.

Figure 5. Survival curves of spores treated, under an LiF window and a quartz
window, with air-simulated plasma discharges.
to the presence of UV lines of the NO\textsubscript{\gamma} system and high-intensity VUV emissions, i.e. a higher number of photon emissions, when compared with N\textsubscript{2} plasma as observed in the optical emission spectroscopy measurement shown in figure 2.

3.3.2. Inactivation with Tyvek\textsuperscript{®} sheet. Inactivation experiments with a Tyvek\textsuperscript{®} sheet, a high-density polyethylene used for medical packaging, were performed by placing it on top of the spores. This experiment was performed keeping in view that it is usual to inactivate contaminated instruments already wrapped for safe handling and to protect them from external contamination. In this experiment, no visible damage to the Tyvek\textsuperscript{®} sheet was observed after the plasma treatment as low treatment temperatures were maintained by setting plasma on time and off time as 20 and 60 s, respectively (i.e. a duty cycle of about 25\%). Figure 6 shows the comparison of survival curves in N\textsubscript{2} and air-simulated gas plasmas with and without a Tyvek\textsuperscript{®} sheet. The results for Tyvek\textsuperscript{®}-wrapped spores clearly showed that the inactivation efficiency was significantly affected by the Tyvek\textsuperscript{®} sheet, as it partially blocked the active species as well as reduced the UV emission intensity. From the optical emission spectroscopy measurement, it was confirmed that roughly 10\% of UV emission can transmit through the Tyvek\textsuperscript{®} sheet.

3.4. Effect of optical emission in a vacuum

A further investigation to study the contribution of optical radiation (including VUV/UV) alone was performed with an evacuated isolated chamber carrying spores inside it by placing the chamber in the plasma discharges. The survival curve observed with a quartz window in the case of N\textsubscript{2} plasma is shown in figure 7. It was found that the spores were not inactivated even after 80 min by optical emissions (>180 nm) of N\textsubscript{2} plasma discharges. Here, it is important to mention that exposed spores were kept in a vacuum to exclude the contribution of reactive species excited by any VUV/UV emissions. When the N\textsubscript{2} gas was introduced into the isolated
chamber at the same pressure as the background gas, the spores were inactivated after 50 min. This result showed that optical emissions (VUV/UV) dissociate/ionize the working gas in the immediate vicinity of the spores and produce radicals/species, which further play an important role in the inactivation process. An analogous result was observed in the case of air-simulated gas plasma, as shown in figure 8. The survival curves were compared between the case where the isolated chamber was evacuated to a vacuum and the case where it was filled with air-simulated gas at the same pressure as the background gas.

Figure 7. Survival curves of spores treated, under a quartz window and evacuated conditions, with N\textsubscript{2} plasma discharges.

Figure 8. Survival curves of spores treated, under a quartz window and evacuated conditions, with air-simulated plasma discharges.
3.5. Morphological studies

The shapes of spores were investigated by analyzing the scanning electron microscopy images. The images of untreated (figure 9(a)) and treated *G. stearothermophilus* spores (figures 9(b)–(d)) were taken with the SEM (S-3000N, Hitachi) at accelerating voltages of 10 and 20 kV. It is obvious from the images that the shapes and size of spores directly exposed to O$_2$ or air-simulated plasmas were changed as compared with untreated spores, whereas there were no significant changes in the indirectly exposed, i.e. Tyvek$^\circledR$-wrapped, spores and spores placed under the LiF filter, as shown in figure 10. The optical emission of atomic oxygen in the optical emission spectrum shown in figure 2 may suggest the existence of oxygen radicals and may be attributed to the significant change in the morphology of directly exposed spores due to chemical etching.

4. Discussion

Although there are many reports about the dependencies of VUV/UV efficacy on wavelength, the photochemistry and photoreactivity of DNA also change notably in spores that are UV irradiated in a low or high vacuum [27]. Munakata et al [21] investigated *Bacillus subtilis* spores exposed to monochromatic radiations (from a synchrotron radiation source) at 13 wavelengths.
from 50 to 300 nm in a vacuum and reported two main regions of high efficacy: one in the VUV (around 170 nm) and the other in the mid-UV (220–270 nm). They explained the action spectra by three factors: the penetration depth of each radiation in a spore, the efficiency of producing DNA damage that could cause inactivation and the repair capacity of each type of spore. If we refer to the emission spectra in our experimental conditions, we find that in the air-simulated gas mixture the photon flux is in the same regions of high efficacy. UV light at wavelengths longer than 254 nm may also inactivate spores, but is much less effective than 254 nm radiation. The DNA's photochemistry at longer wavelengths is also somewhat different from that at 254 nm [27]. Moreau et al [1] showed that UV radiation in a N₂/O₂ post-discharge plasma (which has intense emission in the 300 < λ < 400 nm region) plays a significant role in inactivation of microorganisms. They also mentioned the responsibility of VUV radiation at λ = 104.8 and 106.6 nm in a pure argon discharge for the sterilization of B. subtilis spores. In a recently reported work, Pollak et al [3] have shown in their specific conditions that the inactivation rate is highest in the VUV range (112–190 nm), attributing this emission to impurities present in the argon plasma. Halfmann et al [19] have observed that for sterilizing
Bacillus atrophaeus spores in the double inductively coupled plasma (DICP) setup with Ar : N\textsubscript{2} : O\textsubscript{2} (100 : 4 : 1 sccm) discharge, the efficiency of inactivation of the radiation in the cases (with a wavelength higher than 112 nm) and (at 235 nm and higher) is rather constant. Lerouge et al [28] reported a relatively low sporidical efficacy of VUV radiation (between 115 and 170 nm) emitted by a hydrogen MW plasma, whereas Khomich et al [29] and Soloshenko et al [30] have observed the large efficacy of VUV/UV emission of direct current (dc) air plasmas (highest in the range 160–220 nm).

In our experiments, we first filtered wavelengths higher than around 180 nm by a quartz filter and investigated the inactivation effect. In the second filter with LiF, the effect of wavelengths higher than 120 nm as a whole was taken; hence, the significant contribution of VUV (120–180 nm) is deduced from the broad spectrum including UV (\(\lambda > 180\) nm), whereas many studies performed in photobiology and photochemistry either are from monochromatic radiation produced by excimer lamps, low-pressure mercury lamps, synchrotron sources, etc and concentrated in a narrow band or are performed in ambient condition where VUV, below 200 nm, is absorbed strongly by air.

In the oxygen plasma, in our experiments, where there is negligible UV emission, the etching/protein degradation by chemically reactive species such as atomic oxygen, molecular singlet oxygen, etc due to their versatility in covalent bonding with many different compounds is supposed to be the main cause of enhanced sterilization efficiency [4, 6, 9, 31, 32]. It can be seen from the SEM pictures that the spores’ morphology was significantly changed in the case of oxygen sterilization. On the other hand, in the nitrogen plasma, the VUV/UV emissions are playing an important role and no significant change occurs in the spore morphology. Hence, even though from figures 2(a) and (b) it seems that more UV emissions are irradiated in nitrogen plasma, this does not result in higher efficiency. However, in \(\text{N}_2/\text{O}_2\) plasma, the lethal damage is caused by both effects, i.e. etching/protein damage and VUV/UV photons, with highest efficacy. This can be understood from the fact that to reach the DNA with minimal attenuation, VUV/UV or other factors such as atomic oxygen must first etch the protective outer layers of the core of the spore. While the mechanism of spore inactivation is demonstrated for the DNA damage by UV-C (180–280 nm), the mechanisms for VUV are still not fully known. The absorption coefficient of DNA is high in the case of VUV photons as compared with UV (\(\lambda > 200\) nm), but it is also absorbed by other spore constituents such as proteinous outer layers, dipicolinic acid (DPA), water content in the core, etc. Even after such an attenuation, it is supposed that some VUV photons can penetrate into the core and induce the DNA lesion or dehydrate it [21]. Several studies have shown that a reduction in DNA hydration increases spore photoproduct (SP) production [33]. This lethal damage to DNA by very energetic VUV photons emitted in \(\text{N}_2/\text{O}_2\) plasma is further enhanced by the presence of UV photons, which may help explain their greater effectiveness, as observed in our experiments. Typically, over 99% of the microbial population succumbs to initial exposure but a remaining fraction survives, sometimes for prolonged periods [34]. This effect may be due to clumping [35, 36], dormancy or other factors. The resistant fraction of most microbial populations may be about 0.01–1%, but some studies suggest that it can be a large fraction for certain species. Here, in our experiments, although the intensity of a particular gas discharge varies across different filters, it is supposed that the dose for inducing the initial lethal damage is not drastically varying, thereby causing little difference in the first phase of inactivation.

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5. Conclusion

The inactivation characteristics of *G. stearothermophilus* spores were investigated in different plasma discharge conditions and using different filters (e.g. a LiF plate, a quartz plate and a Tyvek® sheet) as an indirect exposure of spores to the plasma. It has been demonstrated that the spores inside a Tyvek® sheet, mimicking the inactivation of wrapped medical instruments, took a long treatment time when compared with directly exposed spores, which may be attributed to the attenuated VUV/UV emission.

With the use of optical filters the dominant role of optical radiations (VUV/UV photons) was established. Moreover, we observed that the wavelength range of 120–180 nm (VUV) is more biocidal in the presence of working gases in the immediate vicinity of spores. However, the effect of VUV/UV was reduced in vacuum conditions, which demonstrates that the radicals/active species generated by VUV/UV photons’ interaction with working gases may also be playing an important role in the inactivation process. Although the morphology under such filter conditions was not changed compared with that of untreated spores, it is supposed that the inactivation occurred mainly by some chemical way such as protein degradation and lethal damage to the DNA of the spores by energetic VUV/UV photons. For fully understanding this phenomenon, this will need to be figured out in future study.

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