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Effective bacterial inactivation using low temperature radio frequency plasma

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ABSTRACT

Staphylococcus aureus is one of the most common pathogens responsible for hospital-acquired infections. In this study, *S. aureus* was exposed to 13.56 MHz radiofrequency (RF) plasma generated by two different gases namely nitrogen and nitrogen–oxygen mixture and their sterilization efficacies were compared. Nitrogen plasma had a significant effect on sterilization due to generation of ultraviolet (UV) radiation. However, the addition of 2% oxygen showed enhanced effect on the sterilization of bacteria through nitric oxide (NO) emission and various reactive species. The presence of these reactive species was confirmed by optical emission spectroscopy (OES). Scanning electron microscopy (SEM) analysis was carried out to study the morphological changes of bacteria after plasma treatment. From the SEM results, it was observed that the bacterial cells treated by N_2–O_2 mixture plasma were severely damaged. As a result, a $\log_{10}$ reduction factor of 6 was achieved using N_2–O_2 plasma after 5 min treatment with 100 W RF power.

1. Introduction

Sterilization is a physical and/or chemical process by which the microorganisms are inactivated or killed (Moisan et al., 2002). The commonly used sterilization methods are heat (either wet or dry), and chemicals like ethylene oxide and gamma radiation. Though these methods are commercially available, each method has its own limitations. For instance, heat sterilization or autoclaving cannot be used for sterilizing heat-sensitive materials as it may cause irreversible damage to the product. Ethylene oxide may be compatible for a low temperature operation for such materials to be sterilized at or near ambient temperature. However, the safety of the operating personnel has to be ensured since ethylene oxide is highly inflammable and toxic. Further, certain products sterilized by ethylene oxide forms carcinogenic residues over the surface (Rutala, 1996). This shows the limitation of using ethylene oxide as a sterilizing agent. Radiation sterilization can be achieved using gamma rays. But it requires an isolated site and complete protection of the operators. These unique requirements are beyond the scope of common healthcare facilities (Pollak et al., 2008). Also, certain polymers by which many medical devices are being designed nowadays, particularly those containing methyl groups, are not compatible towards gamma rays (Yoshida et al., 2003).

All these requirements collectively made to search for a new route of sterilization which should operate at low temperature, non-toxic, high efficiency and compatible to a wide range of materials. Plasma sterilization is considered to be a promising alternative for the commercial sterilization methods (Kelly-Wintenberg et al., 1999; Lerouge et al., 2001). Research on plasma sterilization is being investigated using various configurations such as dielectric barrier discharge (Choi et al., 2006; Tanino et al., 2007), radio frequency discharge (Akitsu et al., 2005; Lassen et al., 2006), microwave discharge (Moisan, 2003; Chau et al., 1996; Feichtinger et al., 2003; Sato et al., 2007), corona discharge (Scholtz et al., 2007), plasma needle (Stoffels et al., 2002), and plasma jet (Lim et al., 2007). The most important advantage of plasma sterilization is that it operates at or near ambient temperature which is more suitable for sterilizing heat-sensitive materials. Since it does not form any toxic products throughout the process, it is considered to be an eco-friendly process. During plasma treatment, the microorganisms are exposed to high energy ions, electrons, reactive species and UV rays (Lee et al., 2006). It is worth mentioning that any system in which sterilization is achieved using gases or vapors those have no disinfecting ability by themselves and their sterilizing property is generated only under the application of electric field is called a "real plasma sterilizer" (Laroussi, 2008). Commercial sterilizers use gases which already have a disinfecting ability and plasma is just being used as a degasifying agent to remove the toxic residues formed after the completion of sterilization process. Since plasma acts as an adjunct, such sterilizers may be called as "plasma-based sterilizers" (Krebs et al., 1998). In the present study, a non-toxic gas mixture has been used for determining the efficacy of sterilization. N_2–O_2

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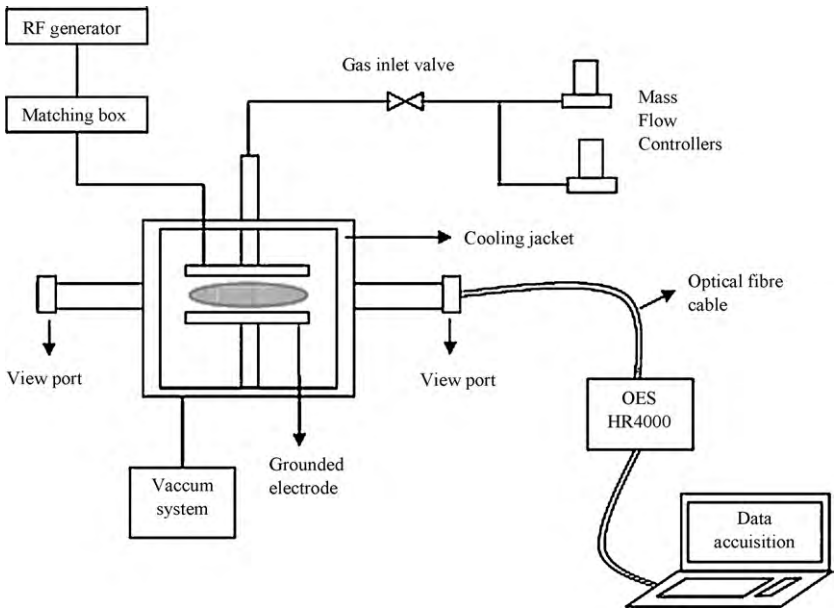

Fig. 1. Schematic diagram of the experimental setup.

plasma are excellent sources of reactive oxygen and nitrogen-based species such as O, O_2^*, NO, and NO_2 (Laroussi, 2005). This inspired us to select N_2–O_2 gas mixture for the sterilization study.

The present study focuses on the low temperature sterilization of the bacterial strain *Staphylococcus aureus* using N_2 and N_2–O_2 plasma and their efficacies were compared in terms of plasma exposure time. The efficiency of sterilization was expressed in terms of germicidal effect (GE). OES studies were performed before and after plasma treatment to understand the effect of various active species. The sterilization results were supported by SEM and absorbance measurements at 260 nm.

2. Materials and methods

2.1. Plasma sterilization chamber

The parallel plate capacitively coupled plasma reactor used for the current sterilization study was procured from M/s. Milman Thin Films Pvt. Ltd., Pune, India (Model: M-PECVD-1A[S]) as shown in Fig. 1. The sterilization chamber consists of a stainless steel cylindrical chamber of 300 mm diameter × 200 mm height which is cascaded with a rotary pump through a roots pump. The chamber can be evacuated to a maximum of 0.1 Pa. The periphery of the cylinder consists of a water jacket in which cooling water was supplied by a chiller. Two identical metal electrodes of 200 mm diameter separated by a distance of 20 mm were fixed inside the sterilization chamber. The top electrode was connected to the RF power supply whereas the bottom electrode was grounded. The top electrode has uniform perforations throughout the inner side of the plate for uniform gas distribution. Substrates were placed over the bottom electrode. Gas was supplied through a mass flow controller at a preset flow rate. The pressure inside the sterilization chamber was measured by a capacitance gauge attached to it. A thermocouple was attached to measure the temperature inside the chamber during plasma generation. Nitrogen and oxygen gases (BOC, 99.9% purity) used for this study were supplied by M/s. Tapaswi Enterprises, Kharagpur, India. For plasma treatment, N_2 gas was allowed to enter the sterilization chamber at a preset flow rate of 70 sccm while maintaining the chamber pressure at 8–9 Pa. By tuning the RF generator, the input power was set as 100 W and the reflected power was minimized as 0–1 W for all experimental runs. After the gas pressure inside the chamber reaches the set value, RF plasma was ignited and the slide glasses containing dried bacterial samples were exposed to the plasma for a definite period of time. In the case of N_2–O_2 plasma, 2% oxygen was added while maintaining the total flow rate of the gas mixture at 70 sccm.

2.2. Bacterial culture

The Gram-positive bacterium *S. aureus* NCIM 2079 procured as a lyophilized powder (NCIM, Pune, India) was used for this sterilization study. It is one of the common pathogens responsible for hospital-acquired infections. Microbes were sub-cultured in nutrient agar media (Hi Media Labs, Mumbai, India). Microbes were stored at −20 °C after supplementing 20% glycerol until further use. Simultaneously agar slants were maintained at 4 °C for experimental purpose. A loopful of colony was inoculated in nutrient broth medium (Hi Media Labs, Mumbai) and incubated at 37 °C in a rotary shaker at a constant speed of 175 rpm. For sterilization studies, the cells were harvested from the late exponential phase in which the cells were said to be more resistant. The cells were centrifuged at 3000 × *g* for 10 min at 4 °C and washed twice with sterile 0.02 M phosphate buffer solution (PBS) at pH 7.0. Glass microscopic slides of size 25 mm × 25 mm × 1 mm were used as substrates. Slides were surface sterilized over the flame for 1–2 min. One hundred microlitres of the bacterial suspension was deposited over the sterile glass slides and placed in the laminar flow hood for drying. After drying for 1 h, the slides containing dried bacterial samples were placed inside a sterile Petri dish, sealed with a parafilm and further wrapped by aluminum foil around its boundary without any air passage and transferred for plasma treatment. During plasma treatment, the slide glasses were placed inside the sterilization chamber. The bacterial strain was exposed to different plasma treatments by varying exposure time as 1, 2, 3, 4 and 5 min. One of the samples, not exposed to plasma, was kept as a control sample. The maximum temperature for all the sterilization experiments carried out in this study was fixed as 50 °C. The temperature at the end of each plasma treatment was measured. Three samples were prepared in a similar way, from which one of the samples was taken for SEM analysis and the remaining samples were used for plasma treatment.

The treated samples were eluted and serially diluted using sterile 0.02 M PBS. One hundred microlitres of the serially diluted bacterial suspension from each test tube was spread on the nutrient agar (NA) plates using conventional spread plate method. The NA plates were incubated at 37 °C for 24 h and monitored for colony formation. The colonies grown over the agar plates were counted manually and the number of colony forming units (CFUs) per ml were reported (Tessler et al., 1992; Ekem et al., 2006; Sureshkumar and Neogi, 2009).

2.3. Optical emission spectroscopy (OES)

OES is one of the preferred tools for plasma diagnostics since it is a non-invasive technique (Chiang and Hon, 2008). OES studies were carried out using high resolution optical emission spectrometer supplied by Ocean Optics Inc., USA (Model: HR 4000) with a spectral resolution of ±0.1 nm, and spectral range of 200–1100 nm. It is accompanied by a special software (Mikropack Specline, Version 2.0) for spectral analysis.

2.4. Absorbance measurements

The absorbance measurements of the bacterial samples were performed in UV spectrophotometer (Model: Lambda 45, Perkin Elmer, USA) at a wavelength of 260 nm.

2.5. Scanning electron microscopy (SEM)

SEM pictures made before and after plasma treatment provide a visual proof to study the morphological changes of bacteria (Yang et al., 2009). Both untreated and treated samples were centrifuged at 3000 × *g* at room temperature to concentrate the cells. Primary fixation was done using 2% glutaraldehyde and the samples were incubated at 37 °C in the dark. After fixing, the samples were washed thrice with 0.02 M PBS at pH 7.0. Osmium tetroxide was used as a post-fixating agent and the samples were allowed to fix by placing them in an incubator at 37 °C in the dark. After secondary fixation, they were washed thrice with 0.02 M PBS. The samples were then dehydrated using ethyl alcohol in increasing concentrations up to 100% for 15 min. Finally they were dried using hexamethyldisilazane (HMDS). Before analyzing by scanning electron microscope, the samples deposited over the slide glasses were gold coated under vacuum. They were analyzed using secondary electrons through a scanning electron microscope (JEOL JSM-5800, Japan).

3. Results

3.1. Effect of plasma treatment

During plasma treatment, the maximum temperature remained nearly 45 °C for 5 min continuous exposure while applying an input power of 100 W. A slight increase in the final temperature values of N_2–O_2 plasma compared to N_2 plasma alone was observed. This may be associated with the excess heat released by the dissociation of oxygen. Since the bulk temperature was well below the lethal range of bacteria (60 °C), the role of temperature for sterilization of bacteria using low pressure plasma was eliminated.

A plot between the plasma exposure time and germicidal effect (GE) was drawn. GE was determined by $GE = \log N_o - \log N_t$, where N_o and N_t are the number of CFUs in control and sterilized respectively (Liu et al., 2008). In the control plate, 4.0×10^8 CFUs/ml were counted and denoted as N_o.

It can be observed from Fig. 2 that increasing the plasma exposure time enhanced the germicidal effect in case of both N_2 and N_2–O_2 plasmas. After 1 min treatment, there was no significant

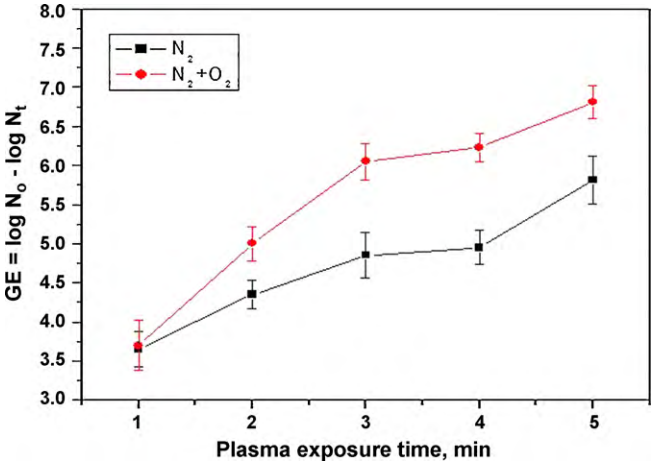

Fig. 2. Germicidal effect of *S. aureus* after plasma treatment.

difference between the GE for N_2 and N_2–O_2 plasmas. However, prolonged treatment showed distinguishable effect. After 5 min exposure, the GE value reached 5.8 for pure N_2 plasma whereas the addition of 2% oxygen, led to GE value greater than 6.5. This shows that 6 $\log_{10}$ reduction of the original bacterial population can be achieved using N_2–O_2 plasma at input power of 100 W for 5 min continuous exposure, which is close to the sterility assurance level (10^{-6}) of the medical industry.

3.2. Optical emission spectroscopy

OES studies were carried out for both N_2 and N_2–O_2 plasmas. Initially, each spectrum was collected without any sample for background correction. Later, a series of spectra were collected when the bacterium was treated by plasma. The plasma spectra for both N_2 and N_2–O_2 plasma are shown in Fig. 3a and b. In the N_2 spectrum, the presence of neutral, atomic and molecular nitrogen species were observed. An intense peak for neutral nitrogen species was observed at 425.34 nm. Atomic reactive nitrogen species (RNS) were found to be at 404.4 and 599.9 nm. Spectral lines of undissociated nitrogen were also found to occur at 234.75, 245.51 and 311.86 nm. The presence of various peaks in the wavelength range of 200–400 nm shows the presence of UV radiation in the spectra for both the plasmas. In case of N_2–O_2 plasma spectrum, after the addition of 2% O_2, new peaks for atomic and neutral oxygen species were formed at 235.10, 412.15 and 425.39 nm. The presence of oxygen emission lines in the spectrum captured by nitrogen plasma alone might be due to the presence of impurities.

3.3. Absorbance measurements at 260 nm

After plasma exposure, the samples were washed with PBS and the suspension was centrifuged at 3000 × *g* for 15 min. The resultant supernatant was analyzed in a UV spectrophotometer for changes in the absorbance values. The absorbance values were plotted for both N_2 and N_2–O_2 plasma treated samples (Fig. 4). From the absorbance results, it is understood that absorbance values increased from 0.0866 (control) to 0.2249 (N_2 plasma treated sample) and the value increased further with the addition of oxygen as 0.2521. It was observed that the absorbance values of the treated samples were increasing with exposure time.

3.4. Scanning electron microscopy

The images captured by scanning electron microscope are shown in Fig. 5. From the SEM images, it was observed that the

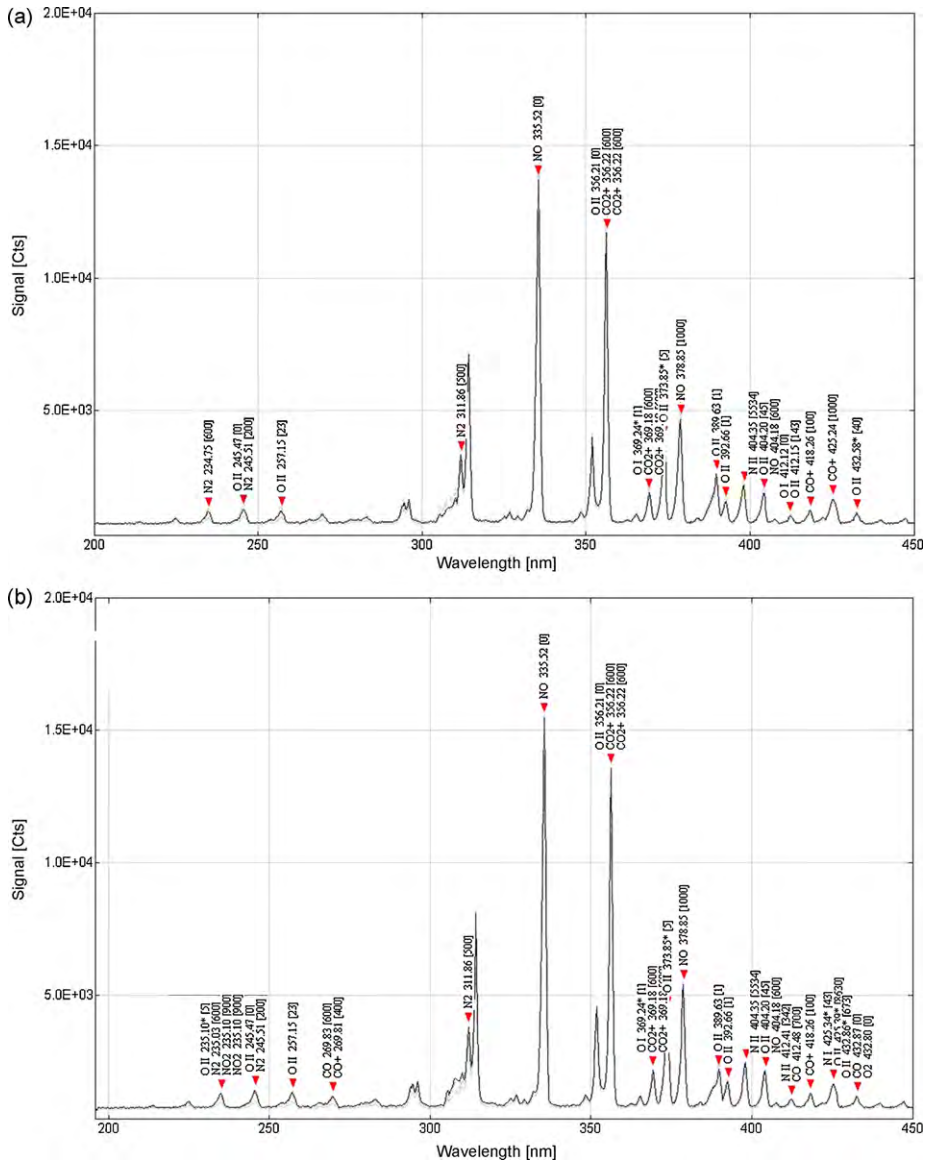

Fig. 3. OES images. (a) N_2 plasma after 5 min exposure (200–450 nm). (b) N_2–O_2 plasma after 5 min exposure (200–450 nm).

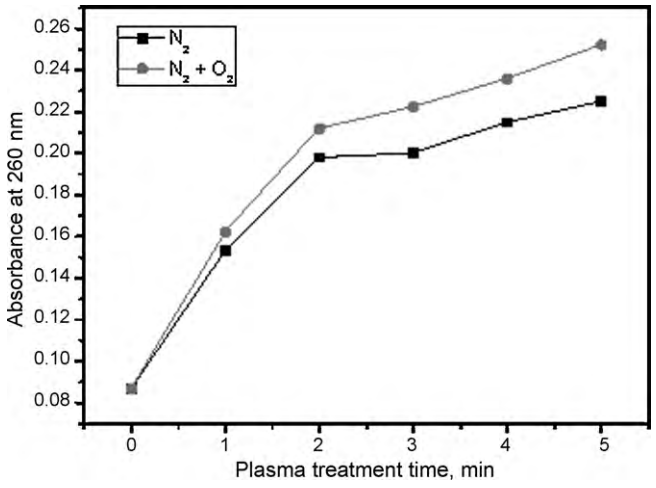

Fig. 4. Absorbance values measured at 260 nm.

treated cells resulted in a structural damage when compared to that of control sample. The untreated cells appeared as clusters (Fig. 5a) whereas the spherical shape of *S. aureus* found to be distorted after plasma treatment. The surface of the cells was found to be severely damaged as plasma exposure time increased. When the bacterial cells were treated by N_2 plasma alone for 5 min, the extent of damage was less (Fig. 5b). However, after the addition of oxygen followed by plasma exposure for the same treatment time, the cells were found to be heavily damaged (Fig. 5c).

4. Discussion

4.1. Action of plasma on bacteria

Since the substrate is being exposed to a direct plasma discharge, it is expected that all the species present in plasma encounter the bacterial cells. Depending on the gas or gas mixture present, the nature of the plasma species may vary according to the discharge conditions. It was realized from the results that N_2–O_2 plasma showed greater bacterial reduction than pure N_2 plasma. This enhanced bacterial reduction may be due to the presence

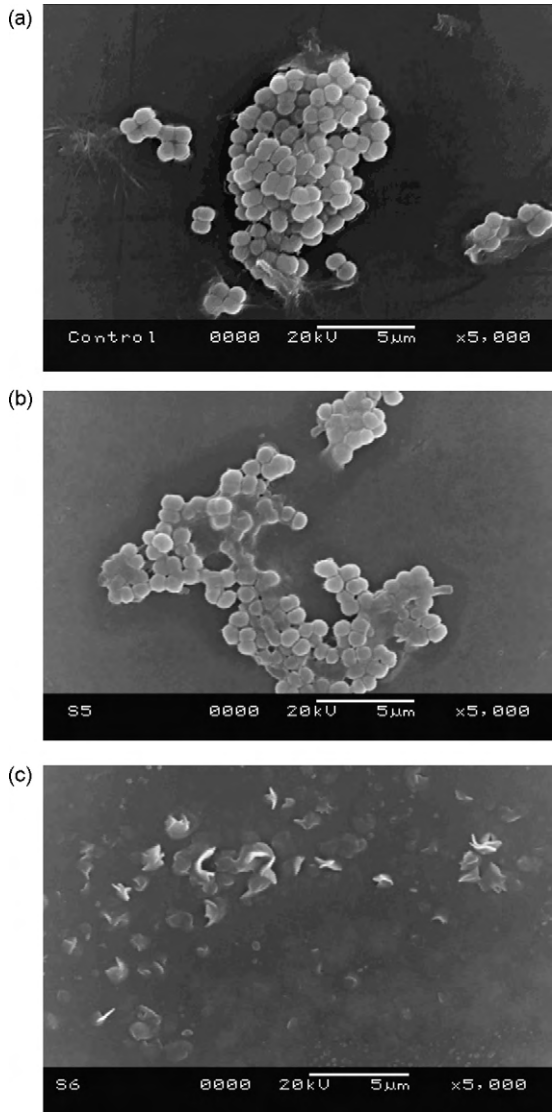

Fig. 5. SEM images of *S. aureus* after N_2–O_2 plasma treatment. (a) Untreated cells. (b) After 5 min N_2 plasma exposure. (c) After 5 min N_2–O_2 plasma exposure.

of atomic oxygen and other reactive species like NO which is evident from the OES studies. It has been already demonstrated (Moisan, 2003) that N_2–O_2 plasma produce large amount of UV radiation capable to inactivate cells. Scanning electron microscopy results confirms the effect of etching by reactive nitrogen (RNS) or oxygen species (ROS) after the addition of oxygen into the N_2 plasma. The etching effect may also be enhanced due to the UV irradiation through intrinsic photodesorption, by which UV radiation reacts with the cellular materials and results in the formation of small molecular species such as CO, OH which are constantly removed by the vacuum system (Moisan et al., 2001). The mechanism of sterilization using N_2/O_2 plasma is presumed as follows:

1. UV radiation (200–400 nm) is absorbed by the DNA of the bacterial cells when directly exposed to plasma. UV rays inhibit the bacteria to reproduce by dimerization of thymine base in the DNA strands. In particular, UV rays in the wavelength range of 220–280 nm are known to have greater germicidal effect (Norman, 1954). The presence of UV radiation in both N_2 and N_2–O_2 plasma was confirmed from OES.
2. CO peak formation may be due to the chemical reaction between the UV radiation and the organic components present in the biological system.
3. Due to the emission of various atomic oxygen species which is evident from spectrograph, cells have a high possibility of damage by reactive oxygen radicals or oxygen-containing species like NO. These species not only etched the outer cell membrane but also have diffused through it causing damage likely by oxidation of cytoplasmic membrane, proteins and DNA strands (Gallagher et al., 2007).
4. The structural integrity of the cells was lost due to the direct exposure of the active species present in the plasma.

4.2. Optical emission spectroscopy

The presence of UV emission during plasma treatment was confirmed by the occurrence of NO and NO_2 species in the wavelength range 200–400 nm. These peaks were formed by the recombination of atomic nitrogen and oxygen species (Ricard, 2005). Oxygen atoms were produced in oxygen-containing plasmas through electron impact dissociation and dissociative attachment (Cvelbar et al., 2007). The presence of CO peak at 412.5 and 269.8 nm (Fig. 3b) may be due to the reaction between UV radiation and the biological cell components. Though the presence of OH species at 666.5 nm was observed in the presence of bacteria, we could not confirm that these species were emitted from the reaction between the UV radiation and the biological materials.

4.3. Absorbance measurements

This assay shows the leakage of the cellular contents such as proteins and/or nucleic acids into the extracellular fluid. The increase in OD values was observed as a result of leakage of cellular contents in the supernatant solution (Kelly-Wintenberg et al., 1999). Similar to other results, OD values were relatively higher for N_2–O_2 plasma treated bacterial cells than for pure N_2 plasma alone.

4.4. Scanning electron microscopy

From the SEM images, the visual effects of plasma treated bacteria were observed to be puncturing and partial damage of cells, which may be due to etching of reactive species from oxygen and/or nitrogen. Since the bacterial cells are exposed to direct plasma exposure, it can be presumed that sterilization is achieved by a synergetic effect of UV radiation and reactive species which might have diffused into the cell. These reactions could either compromise the integrity of the cell wall, leading to its death, or may leave the cell unculturable (Gallagher et al., 2007). Some of the treated cells were observed to be damaged by a lesser extent like cells treated by nitrogen plasma (Fig. 5b). This effect may be due to the stacking of cells and the UV radiation or reactive nitrogen radicals which have to diffuse further into the sublayers for complete sterilization action whereas those were lying only over the top surface were affected by plasma (Philip et al., 2002).

5. Conclusions

The effect of RF plasma on *S. aureus* was studied using N_2 and N_2–O_2 gases. Results indicated that addition of 2% O_2 into the N_2 plasma resulted in improved sterilization than using pure N_2 plasma alone. Since the bacterial cells were exposed to direct plasma, the highly reactive species like atomic nitrogen, oxygen radicals and UV photons were considered to be the possible species responsible for sterilization. Hence, it was demonstrated that the current experimental setup can sterilize the tested bacteria by a factor of 10^6 within 5 min of plasma exposure using a non-toxic gas

mixture. Further investigation is required to measure the efficiency of spore-forming bacteria like *Bacillus subtilis*, *Bacillus stearothermophilus*, etc. which are highly resistant by nature.

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