Comparative Study on the Use of Different Metal Electrodes in Low-Pressure Glow Discharge Plasma Sterilization

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ABSTRACT: The sterilizing efficacy of low-pressure direct current glow discharge hydrogen peroxide (H2O2) plasma generated by a planar parallel plate plasma source using the plasma enhanced chemical vapor deposition (PECVD) facility was tested. This study compares the effect of using different metals (copper, stainless steel, and aluminum) as electrodes in plasma sterilization of stainless steel dishes. Test samples were exposed to H2O2 plasma under different sets of discharge currents and exposure times. Bacillus subtilis was used as the test organism and microbial analysis was made by means of the standard plate count method of serial dilution and pour plating. Evaluation of microbial death was done using survival curves, percent reduction, and decimal reduction value. Results showed that sterilization using copper electrodes exhibited the highest decimal reduction value (D-value) and percent reduction among the three electrodes. However, statistical analysis using multivariate analysis of variance (MANOVA) at 0.05 significance level assessed that the type of electrode material is not a significant factor in the H2O2 plasma sterilization of Bacillus subtilis cells.

KEY WORDS: plasma sterilization; hydrogen peroxide plasma; electrodes; Bacillus subtilis

I. INTRODUCTION

The viability of nonthermal or cold plasmas to inactivate microorganisms without the presence of toxic residues at low temperatures and low penetration depth thus preserving the bulk properties of the material has proven to be a great alternative to conventional methods.1,2 Cold plasma sterilization can be achieved either at low pressure or atmospheric pressure.3,4 Low-pressure plasma is advantageous due to its stability. It requires low breakdown voltages and electron temperature able to dissociate molecules (1–5 eV) while maintaining low neutral temperature. It contains high concentrations of ions and radicals and has a uniform glow over a large gas volume. In contrast, atmospheric plasma can be generated without the need of vacuum operation which is time-consuming, expensive, and high maintenance. However, atmospheric plasmas lack stability and it is difficult to obtain a uniform glow discharge. In addition, atmospheric plasma systems...
require higher operating power and plasma temperature is greater compared to low-pressure plasma sources.\textsuperscript{5}

Several gases have been used to evaluate the efficacy of plasma sterilization such as argon (Ar), oxygen (O\textsubscript{2}), nitrogen (N\textsubscript{2}), carbon dioxide (CO\textsubscript{2}), and hydrogen (H\textsubscript{2}) and its mixtures.\textsuperscript{1,6-8} Hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) vapor and water (H\textsubscript{2}O) vapor were also employed.\textsuperscript{3,9} It was determined that O\textsubscript{2} and H\textsubscript{2}O\textsubscript{2} were the most effective gases and that H\textsubscript{2}O\textsubscript{2} plasma is more effective than O\textsubscript{2} plasma.\textsuperscript{3}

Investigations of the mechanism and factors affecting plasma sterilization have been done by several researchers.\textsuperscript{1,10,11} In theory, the main factors would be charged particles, ultraviolet radiation, and chemically active plasma species (free radicals or neutral particles). Soloshenko and coauthors\textsuperscript{7} determined that charge particles are not key influences in plasma sterilization. Conversely, a study by Laroussi et al.\textsuperscript{10} observed an electrophysical process by the charge particles responsible for the destruction of gram-negative bacteria. However, gram-positive bacteria are not affected by this electrophysical process due to a much thicker bacterial cell wall. It has also been widely known that ultraviolet radiation, like ionizing radiation, can destroy microorganisms. The UV spectrum ranges from 100 to 400 nm in wavelength with 240–280 nm the most germicidal region. UV can also be generated in plasma when electrons make a transition from a highly energetic state. This damages the DNA of the microorganism by irradiation and by breaking chemical bonds in the microorganism material by intrinsic photodesorption.\textsuperscript{11} Chemically active plasma species such as the reduction of O\textsubscript{2} to form superoxide radicals (O\textsubscript{2}\textsuperscript{-}) and peroxides (O\textsubscript{2}\textsuperscript{2-}) and hydroxyl free radicals (OH), which are highly reactive, proved to be a major influence in plasma sterilization. The formation of radicals which are strong oxidizing agents can inactivate enzymes and destroy the DNA structure of the microbial cell.\textsuperscript{12}

Lerouge et al.\textsuperscript{13} pointed out the role of different plasma parameters such as flow rate, pressure, power, and geometrical factors in influencing the efficacy of plasma microbial inactivation. The materials making up the device and the substrate holder can significantly influence the discharge characteristics of the plasma and its active species. This study aimed to compare the effects of using different metals as electrodes for the PECVD facility in reducing counts of Bacillus subtilis inoculated in stainless steel test samples. Metals used as electrodes are stainless steel, copper, and aluminum. The antimicrobial properties of copper have been well-known\textsuperscript{14-16} while stainless steel and aluminum have no identified antimicrobial properties.

II. MATERIALS AND METHODS

The sterilization process is conducted using the plasma enhanced chemical vapor deposition facility shown in Fig. 1. It is a type of low-pressure glow discharge plasma system that utilizes direct current (DC) for operation.

The main chamber encloses two planar parallel plate electrodes, the cathode and the anode. The 40-mm-diameter cathode also serves as the sample holder. The anode
consists of a small hollow tube fitted with a 40-mm-diameter and 16-mm-thick stainless steel shower cap with holes 1 mm in diameter and spaced 1 mm apart for a uniform and homogeneous feedthrough. Three different types of materials were used as electrodes for the sterilization experiments: stainless steel, copper, and aluminum, shown in Fig. 2. The gap between the cathode and anode is approximately 10 mm. A solution of 3% concentration of hydrogen peroxide was used to supply the \( \text{H}_2\text{O}_2 \) vapor to the chamber since it was proven effective in sterilizing \textit{Bacillus subtilis}.

Stainless steel plates, measuring 12.7 mm ×25.4 mm, served as test samples. Stainless steel plates were washed and air-dried. Dry plates were cleaned and pretreated based on the work of Lunden.\textsuperscript{17} Pretreated stainless steel plates were placed inside a Petri dish for autoclaving and throughout the experiment process until plasma exposure.

Pure cultures of \textit{Bacillus subtilis} UPCC 1295(ATCC 6633) obtained from the Microbiological Research and Services Laboratory (MRSL), Natural Sciences Research
Institute (NSRI), University of the Philippines Diliman, was used as the test organism. All microbiological procedures were done using aseptic techniques.

A cell suspension of a 24-h nutrient agar (NA) culture of *B. subtilis* was prepared using 0.1% peptone water as diluent. The cell density was based on the McFarland standard no. 2 which corresponds to \(6 \times 10^8\) cells/ml. Stainless steel test samples were inoculated by dispensing 0.1-ml aliquots of the cell suspension into the surface of the sample. The inoculated test samples were incubated at 37°C for 24 h to dry the suspension. After 24 h, the samples were exposed to plasma. Unexposed inoculated stainless steel plates served as the control. The microbial load of the unexposed plates was counted to determine the initial bacterial population. Counting was done using serial dilution and pour plate method.

Colony-forming units (CFUs) were plotted in semilogarithmic scale as a function of the treatment time. Quantitative assessment of the efficacy of the sterilization can be done by determining the decimal reduction time (\(D\)-value) which can be obtained from the survival curve. The \(D\)-value is the time to kill 90% of the initial cell population (or to reduce the cell population by one decimal log). This is due to the exponential decrease of the population of the microorganisms in a sterilization process given by

\[
N_s(t) = N_o \times e^{-kt}
\]

where \(N_o\) is the initial cell density at \(t=0\) and the time constant, \(k\), is also denoted as the cell kill rate. For a particular time, the sterilization efficiency (\(ns\)) can be directly determined as

\[
ns = \log N_o - \log N_s
\]

where \(N_o\) is the initial cell population and \(N_s\) is the population of surviving cells. The greater the value of \(ns\), the more efficient the sterilization method is. The efficacy of sterilization can also be investigated by determining the percent reduction of the microorganism present in the sample. This is calculated as

\[
\% \text{ reduction} = \frac{\text{initial population} - \text{surviving population}}{\text{initial population}} \times 100\%
\]

Multivariate analysis of variance (MANOVA) was used to determine whether there are group differences regarding response variables considered simultaneously. In MANOVA, the group effects on the combination of parameters are being considered. The null hypothesis in MANOVA is that the effect of the parameters is zero with respect to the combinations of response variables. Analysis such as Wilk’s lambda statistic, Pillai’s trace, and Hotelling-Lawley trace are commonly used to test the null hypothesis. If the calculated \(p\)-value, the probability that the null hypothesis is true, is less than the assigned significance level \(\alpha\), the null hypothesis is rejected and the treatment variables are statistically significant. The commonly used \(\alpha\) in statistical
analysis is 0.05. MANOVA calculations are done using the R programming language for statistical computing.

III. RESULTS AND DISCUSSION

A. Effect of Vacuum Condition and Heat

The effect of vacuum pumping was investigated by an earlier study by Pineda\textsuperscript{18} wherein a test sample was placed inside the chamber but was not exposed to plasma. The test samples were counted and results showed that the microbial counts of the test samples were essentially similar to that of the original count. Thus, the vacuum process does not contribute to the sterilization. Also, since the samples used in the experiments are dried and incubated for 24 h, problems regarding suctioning of the microorganisms are addressed.

Another concern is the effect of temperature in the sterilization process. The stainless steel test sample would not have a problem regarding heat but if this method of sterilization will later on be applied to other types of medical and food processing materials, such as plastics, rubbers and other heat sensitive objects, a temperature profile should be obtained. The temperature profile of the plasma at 2 Torr using a thermocouple is shown in Fig. 3 for the 10- and 20-mA discharge currents, respectively.

The temperature of the plasma did not exceed 80°C after 10 min, which is much lower than that used in autoclaves (usually around 120°C) and dry heat sterilization (at 160–170°C). With regards to the resistance of the microorganism or test organism used in the experiment, \textit{Bacillus subtilis} is a well-studied type of bacteria that forms an endospore. Due to the presence of the endospore, \textit{B. subtilis} can withstand extreme conditions such as heat, desiccation, chemical stress, and nutrient depletion.\textsuperscript{19}

B. Comparison of the Efficacy of Cu, Al, and Stainless Steel in Sterilization

Table 1 gives the comparison of using stainless steel, copper, and aluminum electrodes in the 10-mA (5-W discharge power) H\textsubscript{2}O\textsubscript{2} plasma sterilization by means of the obtained

![Temperature profile for 10mA H2O2 plasma at 2 torr](image1)

![Temperature profile for 20mA H2O2 plasma at 2 torr](image2)

FIG. 3: Temperature profile for the hydrogen peroxide plasma
percent reduction and efficiency. It can be seen that 8 min plasma treatment using copper electrodes has the highest percent reduction of 95.3% and efficiency of 1.32.

The survival profile for *Bacillus subtilis* exposed in the 5-W discharge power plasma is given in Fig. 4. The interpolated trend line for the plot and its equation are also shown.

The equation of the trend line gives the cell kill rate and hence the $D$-values for the three types of electrode material summarized in Table 2.

Copper electrodes have the highest cell kill rate among the three types of metals used as electrode material. The $D$-value obtained for copper is 8 s faster than that of stainless steel and almost 40 s faster than aluminum.

Further increasing the discharge current to 20 mA or approximately 10-W discharge power gives a higher efficiency and percent reduction as can be seen in Table 3. This is expected because at higher discharge power, more energetic electrons and active plasma species are able to interact with the microorganism. Results showed that 99.99% of the *B. subtilis* cells were killed at 8 min exposure to plasma using any of the three electrode materials.

**TABLE 1**: Comparison of stainless steel, aluminum, and copper electrodes used in a 10-mA, 5-W discharge power H$_2$O$_2$ plasma sterilization

<table>
<thead>
<tr>
<th>Electrode material</th>
<th>Exposure time</th>
<th>Percent reduction</th>
<th>Efficiency (ns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stainless steel</td>
<td>5 min</td>
<td>92.5%</td>
<td>1.12</td>
</tr>
<tr>
<td></td>
<td>8 min</td>
<td>94.3%</td>
<td>1.24</td>
</tr>
<tr>
<td>Copper</td>
<td>5 min</td>
<td>93.4%</td>
<td>1.18</td>
</tr>
<tr>
<td></td>
<td>8 min</td>
<td>95.3%</td>
<td>1.32</td>
</tr>
<tr>
<td>Aluminum</td>
<td>5 min</td>
<td>73.8%</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td>8 min</td>
<td>94.5%</td>
<td>1.26</td>
</tr>
</tbody>
</table>

**FIG. 4**: Survival plots of *B. subtilis* exposed in H$_2$O$_2$ plasma at 5-W discharge power
The survival profile of *B. subtilis* exposed at 10-W discharge power plasma using the three different electrodes together with the interpolated trend lines is shown in Fig. 5. A summary of the cell kill rates and *D*-values is given in Table 4.

For the 10-W discharge power plasma, copper has the highest cell kill rate of 1.085 and the fastest *D*-value of 55.3 s. From the results, sterilization using copper as electrodes for the planar parallel plate plasma device has the fastest decimal reduction value and highest efficiency. A further and thorough investigation on the effect of each type of metal electrodes in the sterilization mechanism of the plasma is still needed to determine if the antimicrobial properties of copper provided a significant improvement on the sterilization efficacy of the plasma device.

### C. Multivariate Analysis of Variance (MANOVA)

The dependent variables for the MANOVA calculations are the percent reduction and sterilization efficiency obtained. The effects of exposure time, discharge current, and electrode material were investigated. Results showed that the discharge current and time are both statistically significant in the sterilization process. This just confirms that at higher discharge current, the presence of more energetic electrons and active plasma species enhances the sterilizing efficacy of the plasma. Also, at a higher discharge current, the temperature also increases. Similarly, a longer exposure time means a higher

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**TABLE 2**: *D*-values for different electrode material at 5-W discharge power plasma

<table>
<thead>
<tr>
<th>Electrode material</th>
<th>Cell kill rate (s⁻¹)</th>
<th>D-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stainless steel</td>
<td>0.403</td>
<td>148.8 s</td>
</tr>
<tr>
<td>Copper</td>
<td>0.428</td>
<td>140.4 s</td>
</tr>
<tr>
<td>Aluminum</td>
<td>0.336</td>
<td>178.8 s</td>
</tr>
</tbody>
</table>

**TABLE 3**: Comparison of stainless steel, aluminum and copper electrodes used in a 20-mA, 10-W discharge power H₂O₂ plasma sterilization

<table>
<thead>
<tr>
<th>Electrode material</th>
<th>Exposure time</th>
<th>Percent reduction</th>
<th>Efficiency (ns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stainless steel</td>
<td>5 min</td>
<td>97.29%</td>
<td>1.57</td>
</tr>
<tr>
<td></td>
<td>8 min</td>
<td>99.99%</td>
<td>3.93</td>
</tr>
<tr>
<td>Copper</td>
<td>5 min</td>
<td>98.63%</td>
<td>1.86</td>
</tr>
<tr>
<td></td>
<td>8 min</td>
<td>99.99%</td>
<td>4.08</td>
</tr>
<tr>
<td>Aluminum</td>
<td>5 min</td>
<td>92.77%</td>
<td>1.01</td>
</tr>
<tr>
<td></td>
<td>8 min</td>
<td>99.99%</td>
<td>3.98</td>
</tr>
</tbody>
</table>
 sterilization efficacy since more active plasma particles interact with the microorganisms for longer exposure time. On the contrary, MANOVA results showed that the electrode material is not statistically significant in the sterilization process. The \( p \)-values of the different parameters using the three statistical MANOVA tests, Wilk’s lambda, Pillai’s trace, and Hotelling-Lawley trace are tabulated in Table 5. Note that the significance level is 0.05 and a \( p \)-value less than 0.05 means the parameter has a significant effect in the sterilization.

Because copper is antimicrobial, an immediate hypothesis is that it is more advantageous to use as an electrode material than aluminum and stainless steel. The inherent antimicrobial properties of copper have been observed in several studies.\(^ {14-16} \) The interaction of copper with radicals most especially with molecular oxygen makes it very toxic to microorganisms. However, multivariate analysis of variance showed that the obtained percent reduction and efficiencies for the three electrode materials are statistically the same. This can be due to the lower operating discharge power used in this study (5 and 10 W). The power might not be enough to sputter out the Cu ions; hence, Cu ions did not have any interaction with the plasma radicals and also with the cellular membrane of the bacterial cells. The antimicrobial mechanisms of copper have not taken effect. Neverthe-
less, there is no need to increase the discharge power since all the three materials have 99.99% reduction of bacterial cells in 8 min exposure to $\text{H}_2\text{O}_2$ plasma. Also, an increase in power has a corresponding increase in temperature due to plasma heating. Thus, for lower discharge powers, the materials making up the substrate holder (in our case, the electrodes) did not significantly affect the efficacy of the plasma sterilization process.

### IV. CONCLUSION

Comparison of the effects of using different metal electrodes in the efficiency of plasma sterilization of *Bacillus subtilis* spores in stainless steel plates sterilized using the PEC-VD device of the Plasma Physics Laboratory was conducted. Results showed that sterilization using copper electrodes exhibited the highest decimal reduction value ($D$-value) at 10-mA, 5-W discharge power plasma of 140.4 s compared to stainless steel and aluminum which is 8 and almost 40 s slower, respectively. The 20-mA, 10-W discharge power plasma gave similar results with copper having a faster $D$-value of 55.3 s compared to stainless steel and aluminum with $D$-values of 59 and 61.8 s, correspondingly. Statistical analysis using MANOVA was conducted to assess if the variations in the percent reduction, efficiency, and hence the cell kill rate and $D$-values are due to the type of metal electrodes used. It was found that the electrode material is not a statistically significant factor in the sterilization process. On the other hand, exposure time and discharge current proved to be a major factor in the plasma sterilization efficacy.

### ACKNOWLEDGMENTS

The authors would like to acknowledge the Philippine Department of Science and Technology Science Education Institute for making this research possible.

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**TABLE 5: MANOVA calculated $p$-values**

<table>
<thead>
<tr>
<th>$p$-Value</th>
<th>Electrode material</th>
<th>Exposure time</th>
<th>Discharge current</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wilk's lambda</td>
<td>0.46295</td>
<td>0.02454</td>
<td>0.01126</td>
</tr>
<tr>
<td>Pillai's trace</td>
<td>0.46179</td>
<td>0.02454</td>
<td>0.01126</td>
</tr>
<tr>
<td>Hotelling-Lawley trace</td>
<td>0.48663</td>
<td>0.02454</td>
<td>0.01126</td>
</tr>
</tbody>
</table>
17. Pineda P. Destruction of Bacillus subtilis cells using a low-pressure glow discharge plasma, [undergraduate thesis in applied physics]. Quezon City, Philippines: National Institute of Physics, University of the Philippines, Diliman; 2004.