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Sterilization by pure oxygen plasma and by oxygen–hydrogen peroxide plasma: An efficacy study

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Abstract

Plasma is an innovative sterilization method characterized by a low toxicity to operators and patients, and also by its operation at temperatures close to room temperatures. The use of different parameters for this method of sterilization and the corresponding results were analyzed in this study. A low-pressure inductive discharge was used to study the plasma sterilization processes. Oxygen and a mixture of oxygen and hydrogen peroxide were used as plasma source gases. The efficacy of the processes using different combinations of parameters such as plasma-generation method, type of gas, pressure, gas flow rate, temperature, power, and exposure time was evaluated. Two phases were developed for the processes, one using pure oxygen and the other a mixture of gases. *Bacillus subtilis* var. *niger* ATCC 9372 (*Bacillus atrophaeus*) spores inoculated on glass coverslips were used as biological indicators to evaluate the efficacy of the processes. All cycles were carried out in triplicate for different sublethal exposure times to calculate the *D* value by the enumeration method. The pour-plate technique was used to quantify the spores. *D* values of between 8 and 3 min were obtained. Best results were achieved at high power levels (350 and 400 W) using pure oxygen, showing that plasma sterilization is a promising alternative to other sterilization methods.

Keywords: Gas plasma; Inductively coupled plasma; *Bacillus subtilis* (*Bacillus atrophaeus*); Oxygen; Hydrogen peroxide

1. Introduction

The term sterilization implies complete inactivation of microorganisms. Spores of certain bacterial species that are highly resistant to sterilization are used as biological indicators to evaluate the extent to which effective sterilization is achieved by different sterilization processes (Philips, 1975). Sterilizing irradiation is a process that can be used with heat-sensitive materials. However, this process is very costly, not only because of the equipment required but also because of the strict safety precautions that must be observed. Furthermore, although irradiation is carried out at low temperatures and stabilizers are incorporated into the process, the formation of free radicals, cross-linking reactions, and double bonds cause mechanical and visual alterations that very often prevent the viable application of this process (Pinto et al., 2003). Another widely used sterilization method uses ethylene oxide, which also works at low temperatures, is commonly used for dental, medical and hospital equipment. Its mutagenic properties, however, make strict control of the process essential (Alfa et al., 1997).

Plasma is an innovative alternative method, and appears to have advantages over other sterilization methods currently in use. Advantages of plasma include the fact that the technique is effective in inactivating the required microbial load (safety assurance level), can be performed at room temperature, and do not use toxic gases (Moore, 2004). It has the characteristics of an ideal sterilant, namely, a high degree of efficacy, fast action, penetrability, lack of toxicity, compatibility with different materials, and cost-effectiveness. In the present case, plasma is sustained by applying a radio-frequency electric field to precursor gases, which in turn produce highly reactive species that lead

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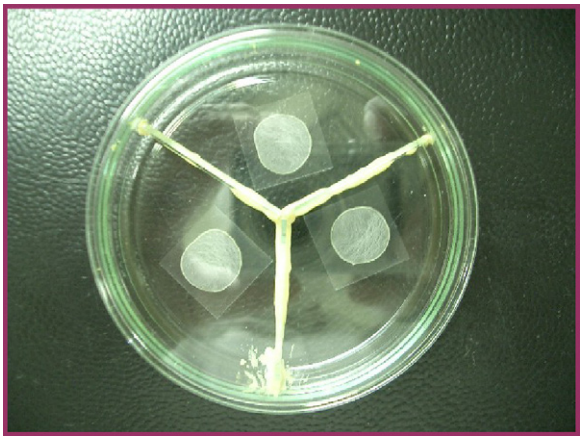

Fig. 1. *Bacillus subtilis* var. *niger* ATCC 9372 inoculated on a Petri dish in the form of three independent deposits.

to microbial inactivation. The radio frequency for the generation of plasma is 13.56 MHz; at this frequency, strong ion bombardment and high electric fields are typical plasma characteristics (Moreira et al., 2005).

The objective of this study was to investigate the efficacy of sterilization using inductively coupled plasma with the aid of biological indicators to monitor the processes.

2. Materials and methods

Bacillus subtilis var. *niger* ATCC 9372 (it is a specific microorganism used for sterilization validation because its spore form is very resistant, and some works with plasma already use this microorganism), inoculated on carriers made up of 18 mm × 18 mm glass coverslips and kept in glass Petri dishes, were used as biological indicators to study the processes (Fig. 1). The low inductively coupled plasma (ICP) system consisted of a horizontal fused silica tube of 15 cm diameter, and 120 cm length. The radio-frequency (RF) power was applied to a coil with eight spirals. The RF cable was coupled in the center of the coil (Fig. 2), and the coil was cooled off by water circulation. The vacuum system consisted of a fore pump with 28 m^3/h flow. The 13.56 MHz radio frequency generator was manufactured by Advanced Energy. At this frequency, strong ion bombardment and high electric fields are typical plasma characteristics.

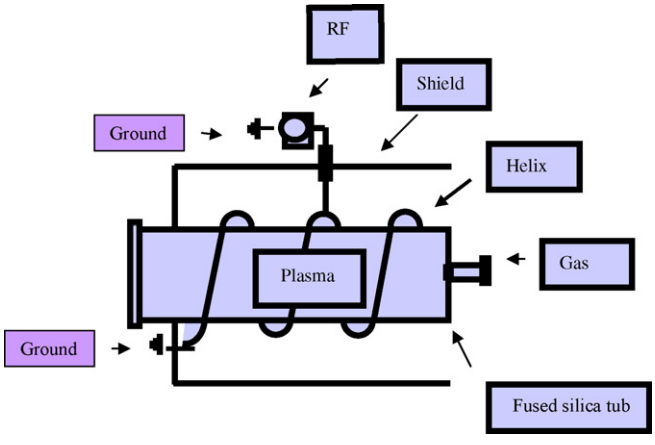

Fig. 2. Inductively coupled plasma arrangement.

2.1. Preparation of spore crops

Microorganisms were initially transferred in test tubes containing Merck® antibiotic agar no. 1, and incubated at 35 ± 2 °C for 24 h. After incubation, a loopful was transferred from the test tube to another tube containing 100 mL of soybean-casein broth (Difco), and incubated again at 35 ± 2 °C for 24 h. To collect spores, 5 mL of the suspension were inoculated in Roux bottles containing 200 mL of sporulation agar consisting of yeast extract, nutrient broth, manganese sulfate tetrahydrate, calcium chloride hexahydrate, agar, and distilled water.

Four Roux bottles inoculated with suspension from a single tube were used simultaneously for incubation, which was carried out at 35 ± 2 °C; the microbial growth was removed daily to monitor the degree of sporulation. This procedure was replicated six times to produce spore suspension groups I to VI.

To monitor the degree of sporulation, stainings with malachite green and aqueous safranine solution were performed. The percentage of spores in relation to the total number of vegetative cells was determined, and incubation was interrupted when the degree of sporulation reached 95%. Spore suspensions were harvested separately from the different groups when sporulation reached at least 95%, which was carried out within 12–15 days of incubation.

The cellular mass resulting from bacterial growth in the agar was harvested from each bottle using 30 mL of distilled water, and the suspension was passed through a sterile filter. The cells were then washed in distilled water and centrifuged at 3000 rpm (15 min). The centrifuged material was resuspended in approximately 40 mL of distilled water, and transferred to Erlenmeyer flasks that were maintained in a freezer at −10 °C. The standardization of spore suspensions occurred according to the previous group division (I, II, III, IV, and VI). All spore suspensions were then collected from the Erlenmeyer flasks to produce a pool of spores. The resulting suspension was transferred to 125 mL sterile flasks in 10 mL aliquots for each flask, yielding 25 flasks with the same contents.

Spore counts were performed in three randomly chosen flasks (labeled 1, 2, and 3), and the results were extrapolated for the remaining 22 flasks. The suspensions were first subjected to heat shock (70 °C for 15 min), followed by 10^{-1} to 10^{-11} dilutions in distilled water. Among them, only the 10^{-6} to 10^{-11} dilutions were plated by the pour-plate technique (incubation at 35 ± 2 °C for 72 h), and followed by CFU counts (USP, 2002).

To prepare the inocula in glass coverslip carriers, each previously obtained standardized 10 mL fraction was defrosted and diluted at a ratio of 1 mL of suspension to 9 mL of distilled water, giving 10 new tubes containing the solution diluted 10 times. These were used to simultaneously inoculate a group of biological indicators, giving a total of approximately 800 inoculates, with a spore concentration of 1.0×10^7 CFU's per glass coverslip. The spores were kept in 3 in. glass Petri dishes and used as test groups in the plasma sterilization process.

Table 1
Operating conditions for testing the plasma inactivation efficacy, using biological indicators (*Bacillus subtilis* var. *niger* ATCC 9372)

Experimental phases	Gas/mixtures	Pressure (mTorr)	RF powers	Gas outflow (sccm)	Exposure times	Total cycles number
Phase I	O_2 pure	330	300, 350, 400	100	3–15 min intervals 3 min, 20–60 min interval 20 min	72
Phase II	$O_2 + H_2O_2$ (5,10,20%)	330	300, 350, 400	95 O_2 + 5 H_2O_2, 90 O_2 + 10 H_2O_2, 80 O_2 + 20 H_2O_2	3–15 min interval 3 min, 20–40 min interval 20 min	189

2.2. Laboratory plasma-exposure cycles

The experiment described here allowed the effects of sterilization by inductively coupled plasma on biological indicators to be assessed and led to a greater understanding of the operation of the equipment. The sample was placed inside the chamber on a glass support so that it was in direct contact with the plasma.

Table 1 shows the experiment parameters considered that allow various operative conditions to be tested. Combinations of the following variables were used: power, gases (pure oxygen and oxygen–hydrogen peroxide gas mixture), pressure, exposure times and gas flow rate.

2.3. Counts of surviving microorganisms

After plasma exposure, three Petri dishes, each containing three biological indicators, were analyzed using a test tube with 10 mL of distilled water for each glass cover slip. First, each carrier was added to a 10 mL test tube, which was duly labeled. The samples were then vortexed in an MS1 Minishaker (IKA®, Sao Paulo, Brazil) at 3000 rpm for 1 min. Next, the samples were subjected to heat shock in a water bath at 70 °C (the temperature was monitored rigorously during the process to prevent damage to the microorganisms). Additional 10-fold dilutions were prepared for future plating.

Each diluted sample (from 10^{-1} to 10^{-6}) was plated using the pour-plate technique (incubation at 35 ± 2 °C for 24 h), followed by a CFU count. All ICP results were analyzed in order to plot the survival curves and calculate respective *D* values. In parallel, a Philips SEM 515 scanning electron microscope (SEM) was used to observe the morphology of the spores before and after the process.

3. Results and discussion

In the cycles carried out with pure oxygen gas (the first phase of the study), the results varied from a marked logarithmic drop to an absence of microorganisms. In oxygen plasma sterilization, degradation of the microorganisms is caused by slow combustion with the oxygen atoms and/or radicals present in the plasma (Hury et al., 1998). At higher temperatures, this combustion produces CO_2 and H_2O.

In the 300 W process, the number of surviving microorganisms was significantly reduced after 40 min, and no surviving microorganisms were observed after 60 min (Fig. 3). In a previous study of sterilization using inductively coupled plasma, a power of 300 W and biological markers with spore concentrations of 10^6 to 10^7 CFU were also used by Gans et al., 2005.

In the 350 and 400 W processes, there was a significant drop in the microbial load after 15 min, and after 20 min, no microorganisms were found to survive (Fig. 3). Between 3 and 9 min at a power of 400 W, there was a slightly greater growth than for a power of 350 W, but the logarithmic difference was small, and for the remaining time better results were obtained at a power of 400 W (Fig. 3). For each condition, a survival curve and its respective *D* value were drawn, where the *D* value is the exposure time needed to reduce the microbial population by a factor of 10 (90%) (Pinto et al., 2003).

Survival curves for the three cycles in the pure oxygen processes show that the *D* value for the lowest power (300 W) is greater than that for a power of 350 W, and also greater than that for a power of 400 W. However, there was no significant difference between the *D* values for powers of 350 and 400 W (3.1 and 3.0, respectively), whereas the *D* value for the 300 W cycles was 8.27 min (Fig. 3).

In the analysis of the results obtained with the gas mixture, it was found that the lower the hydrogen peroxide gas flow concentration, the lower the remaining microbial load (the more effective the process); the logarithmic decay rate after 40 min was satisfactory for all concentrations and powers (Figs. 4–6).

In the processes with 5% hydrogen peroxide, the microbial count was lower than in those with 10% and 20% hydrogen peroxide, for which the logarithmic values were highly correlated. For higher powers, the *D* values were lower, but there was no significant difference between them; *D* values for the different concentrations and powers ranged from 5 to approximately 7 min (Figs. 4–6).

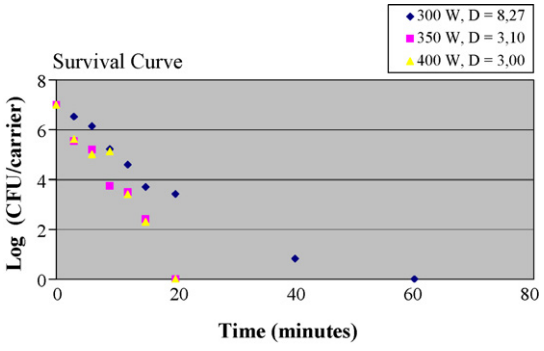

Fig. 3. Survival curve for the process using pure oxygen gas (first phase) at 300, 350 and 400 W (and respective *D* values), under 330 mTorr pressure and 100 sccm gas outflow.

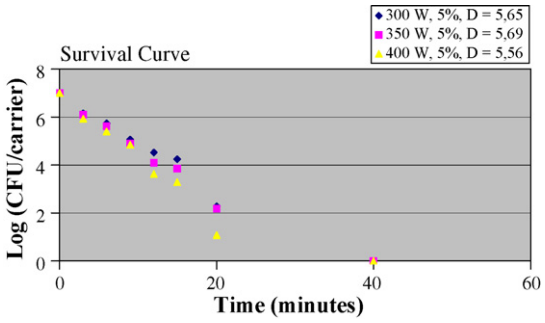

Fig. 4. Survival curve for the process using the oxygen–hydrogen peroxide gas mixture (second phase) at 300, 350 and 400 W (and respective *D* values), under 330 mTorr pressure and 95 sccm gas outflow for oxygen and 5 sccm for hydrogen peroxide.

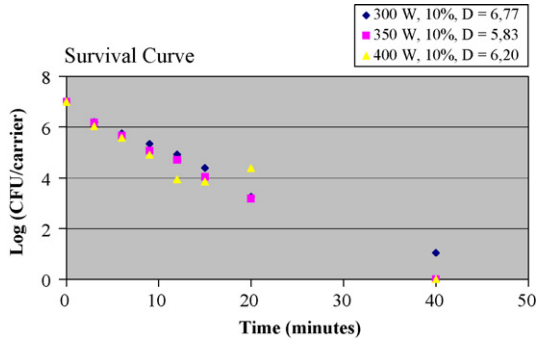

Fig. 5. Survival curve for the process using the oxygen–hydrogen peroxide gas mixture (second phase) at 300, 350 and 400 W (and respective *D* values), under 330 mTorr pressure and 90 sccm gas outflow for oxygen and 10 sccm for hydrogen peroxide.

The results obtained using hydrogen peroxide as a sterilizing gas to form plasma were disappointing since the greater the concentration of this gas, the greater the microbial count observed and *D* value obtained. Hydrogen peroxide is an effective bactericide, but the results achieved in this study using pure oxygen gas were better than those obtained using a mixture of gases. The reduction of microbial load, when hydrogen peroxide was used, has been comparatively smaller than that obtained in oxygen processes due to the low hydrogen peroxides diffusion on the sample. The hydrogen peroxide used in these processes was diluted in water at 30% concentration and presented low dissociation and consequently low diffusion in the samples when it was carried into the chamber though vacuum. This fact made the gas in the chamber be diluted, thus reducing the antimicrobial action of the hydrogen peroxide and oxygen mixture. According to Krebs et al. (1998), after the first 30 min of the diffusion phase the sporicide efficiency ceases, becoming the hydrogen peroxide plasma phase such as destroying all toxic residual present in medical devices, causing their sterilization. Therefore, a good initial diffusion is fundamental to obtain better results in plasma application. Because of the novel and almost random nature of both parameters (pure oxygen and oxygen–hydrogen peroxide gas mixture), and the results obtained in this study, further investigation is required to confirm the results for the gas mixture.

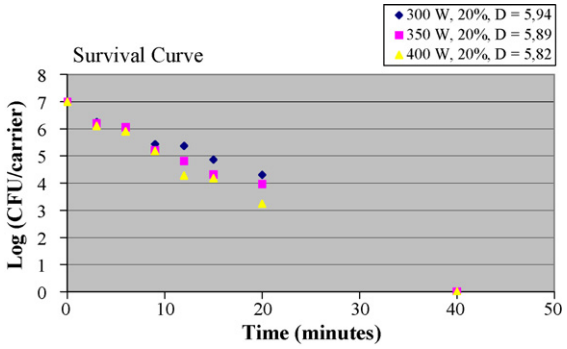

Fig. 6. Survival curve for the process using the oxygen–hydrogen peroxide gas mixture (second phase) at 300, 350 and 400 W (and respective *D* values), under 330 mTorr pressure and 80 sccm gas outflow for oxygen and 20 sccm for hydrogen peroxide.

The Sterrad® sterilization system has been used in a similar study (Vickery et al., 1999). In this equipment, hydrogen peroxide is effective since it is the main active agent and has a diffusion time of 50 min after injection of an 18 mL volume of the gas, at a concentration of 59% followed by 15 min of plasma at 400 W. In this case, it was not the hydrogen peroxide plasma but the precursor gas that was responsible for the death of the microbes. However, in the current paper the percentage of hydrogen peroxide was reduced and the plasma did the sterilization action.

During the sterilization processes using inductively coupled plasma, it could be observed that, as expected, the process temperature remained close to room temperature at 300 and 350 W. At 400 W (high power), an increase in temperature was observed with limited heating of the equipment (measured by a thermometer connected to the equipment cover).

The morphology of *Bacillus subtilis* var. *niger* ATCC 9372 spores is seen in SEM micrographs taken both before and after the processes, in isolation and in colonies (Fig. 7). Scanning electron micrographs show the destruction of the microorganism cell walls during the various processes for the exposure periods that gave the best results. Park et al. (2003) also used the biological indicator *Bacillus subtilis* in their work on plasma sterilization and took scanning electron micrographs of samples after the processes, in which damaged and broken cell membranes can be clearly seen.

On closer examination, the electron micrographs clearly reveal morphology, details of erosion and topography of the microorganisms (when observed individually), and the extent to which they have been destroyed (the membrane degraded) as a result of ionic attack by the plasma. Panels A and B (Fig. 7) show the structure of the cellular wall of the microorganisms before they were exposed to sterilization. In some processes (Fig. 7F), the presence of spores with cell walls that have not been destroyed can be observed, indicating that a certain amount of microbial were survive. However, some photographs show that all the microorganisms in a particular region have been destroyed, particularly in those processes that used higher power levels and pure oxygen gas. The greater the power used, the greater the ionic attack and, consequently, the better the result in terms of sterilization.

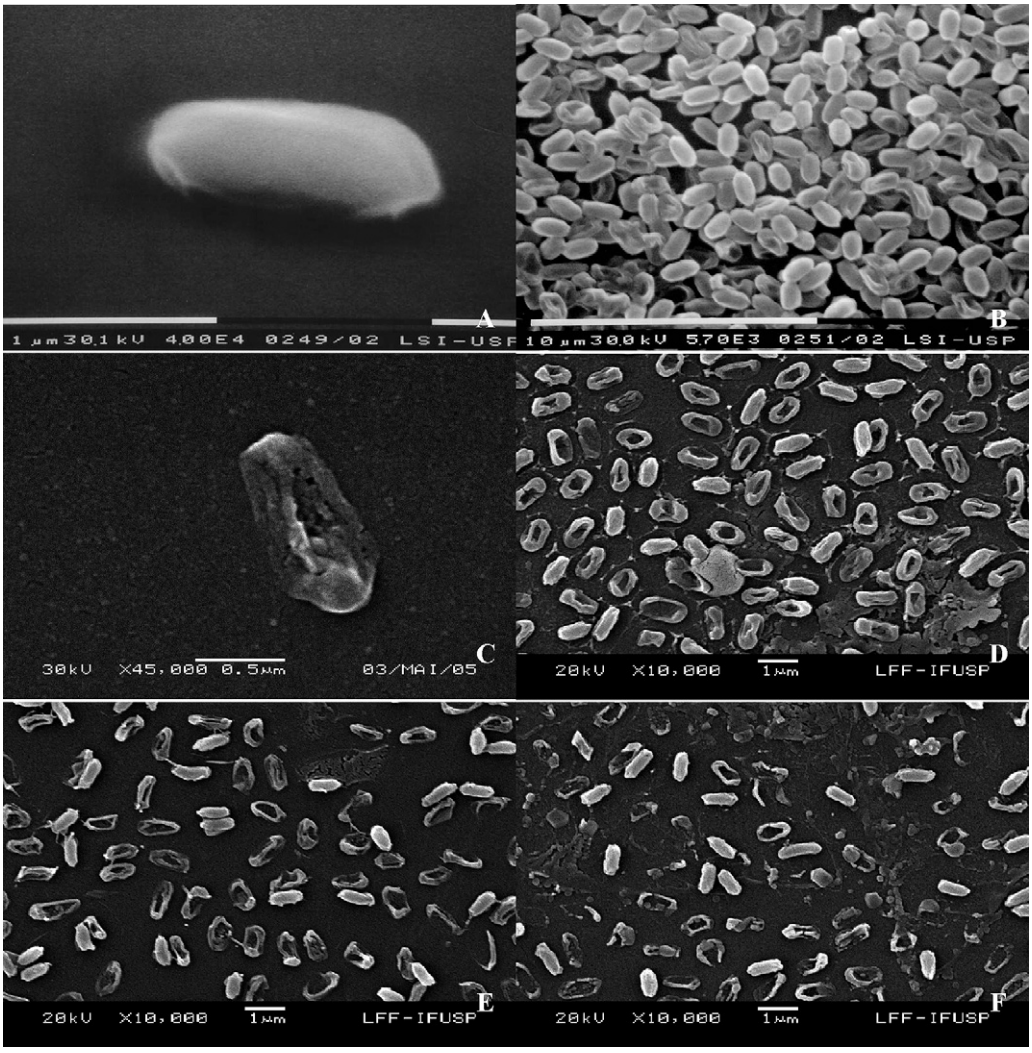

Fig. 7. Scanning electron micrographs of *B. subtilis* spores: (A) unexposed microorganisms (control); (B) unexposed microorganism population from SEM micrographs; (C) after process at 300 W and period of 60 min with pure oxygen gas; (D) after process at 350 W and period of 40 min with pure oxygen gas; (E) after process at 400 W and period of 40 min with pure oxygen gas; (F) after process at 350 W and period of 20 min using 80 sccm of oxygen and 20 sccm of hydrogen peroxide.

According to Moisan et al. (2001), there are three basic mechanisms involved in the inactivation of microorganisms by plasma: (A) direct destruction of the microorganisms genetic material by UV irradiation; (B) atom-by-atom erosion of the microorganisms by photodesorption caused by UV irradiation to form volatile compounds from atoms intrinsic to the microorganism; (C) atom-by-atom erosion of the microorganisms by etching to form volatile compounds as a result of slow combustion. In some cases, etching is also activated by UV photons, thus increasing the microorganism inactivation rate. Due to these mechanisms, plasma sterilization is distinctly different from classic sterilization techniques, and is potentially suitable for inactivating unconventional infectious agents such as prions. In our studies, UV low emission intensity was observed, which shows us that this was not a preponderant sterilization agent in this process. It has been verified that the main mechanism which had sterilizing effect in these processes was microorganisms chemical corrosion, having the cell membrane been firstly destroyed, with posterior destruction of all microorganisms. For comparison, all results can be seen in a single graph in Fig. 8.

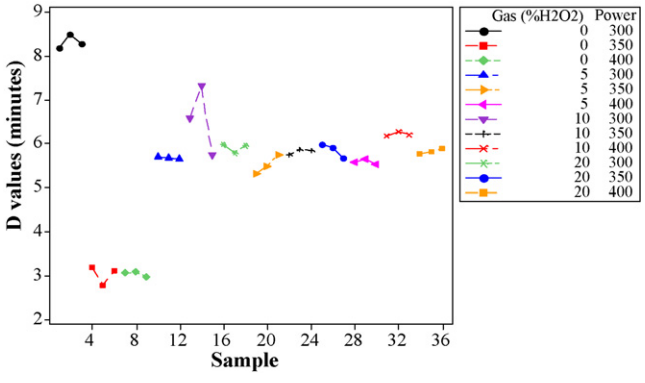

Fig. 8. Individual *D* values (three samples each) for various gas combinations: pure oxygen, various hydrogen peroxide gas percentages in the oxygen–hydrogen peroxide mixtures.

4. Conclusions

The investigation of sterilization by inductively coupled plasma (ICP) showed that results with pure oxygen were bet-

ter than those with a mixture of oxygen and hydrogen peroxide gases. For better results regarding the use of the mixture of hydrogen peroxide and oxygen the concentration of the former should be higher. With a low hydrogen peroxide vapor pressure, the dissociation of this gas would be increased as well as its efficiency without the process parameters alteration. The plasma that was formed showed bactericidal activity when it came in contact with the surface of the biological indicator samples, which had a standardized initial load.

The 350 W processes gave the best result, since the microbial load fell quickly and the *D* value was the second lowest at 3.1 min.

In the 400 W processes, however, there was substantial heating of the equipment, particularly during longer cycles, and the *D* value was only slightly lower than that for the 350 W processes.

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References

Alfa, M.J., Degagne, P., Olson, N., 1997. Bacterial ability of ethylene oxide plus 90% hydrochlorofluorocarbon sterilizing gas. Infect. Control Hosp. Epidemol. Thorofare 18, 641–645.

Gans, T., Osiac, M., O'connell, D., Kadetov, V.A., Czarnetzki, U., Schwarz-Selinger, T., Halfmann, H., Awakowicz, P., 2005. Characterization of stationary and pulsed inductively coupled RF discharges for plasma sterilization. Plasma Phys. Control. Fusion 47, A353–A360.

Hury, S., Vidal, D.R., Desor, F., Pelletier, J., Lagarde, T., 1998. A parametric study of the destruction efficiency of bacillus spores in low pressure oxygen-based plasmas. Lett. Appl. Microbiol. France 26, 417–421.

Krebs, M.C., Bécasse, P., Vrejat, D., Darbord, J.C., 1998. Gas–plasma sterilization: relative efficacy of the hydrogen peroxide phase compared with that of the plasma phase. Int. J. Pharm. 160, 75–81.

Moisan, M., Barbeau, J., Moreau, S., Pelletier, J., Tabrizian, M., Yahia, L.H., 2001. Low-temperature sterilization using gas plasmas: a review of the experiments and Na analysis of the inactivation mechanisms. Int. J. Pharm., Amsterdam 226, 1–21.

Moore, G.L., 2004. Introduction to Inductivelly Coupled Plasma-Atomic Emission Spectrometry. Elsevier Science, Amsterdam, pp. 3, 65–89, 265–283.

Moreira, A.J., Mansano, R.D., Pinto, T.J.A., Ruas, R., Zambon, L.S., Silva, M.V., Verdonk, P.B., 2005. Sterilization by oxygen plasma. Appl. Surf. Sci. 235, 151–155.

Park, B.J., Lee, D.H., Park, J.C., 2003. Sterilization using a microwave-induced argon plasma system at atmospheric pressure. Phys. Plasmas. Korea 10, 4539–4544.

Philips, G.B., 1975. Sterilization. In: Remington's Pharmaceutics Sciences, 15th ed. Mack, Pennsylvania.

Pinto, T.J.A., Kaneto, T.M., Ohara, M.T., 2003. Controle Biológico de Qualidade de Produtos Farmacêutico, Correlatos e Cosméticos, second ed. Atheneu, São Paulo, pp. 110–125.

US Pharmacopeia, 2002, 25th ed., United States Pharmacopeial Convention, Rockville, MD, pp. 200–206, 1846–1849, 1976–1981.

Vickery, K., Deva, A.K., Zou, J., Kumaradeva, P., Bisset, L., Cossart, Y.E., 1999. Inactivation of duck hepatitis B virus by a hydrogen peroxide gas plasma sterilization system: laboratory and "In Use" testing. J. Hosp. Infect. 41, 317–322.