Effects of pH on Bacterial Inactivation in Aqueous Solutions due to Low-Temperature Atmospheric Pressure Plasma Application

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Low-temperature atmospheric pressure plasmas applied to the surface of an aqueous solution have been shown to be efficient bactericides for bacteria suspended in the solution, if the solution is sufficiently acidic. Especially of interest is the finding that there is a critical pH value of about 4.7 for the bactericidal effects, below which the bacteria are efficiently inactivated and above which the bacteria are hardly affected by the plasma application. It has been also found that the presence of superoxide anion radicals $\text{O}_2^\cdot$ in the solution is essential for bacterial inactivation by the plasma application. Therefore, the critical pH value may arise from the $pK_a$ of the equilibrium reaction between $\text{O}_2^\cdot$ and hydroperoxy radicals $\text{HOO}^\cdot$, which is known to be approximately 4.8. The present experiments, where plasmas are not directly applied to bacterium surfaces and it has been confirmed that neither UV light nor heat from the plasma is the cause of bacterial inactivation, suggest the importance of highly reactive species generated in the solution via plasma–liquid interaction for the bactericidal effects.

Introduction

An electrical discharge, i.e., plasma, can be an efficient source of electrons, ions, heat, UV light, electric field, and/or free radicals, depending on the discharge conditions. Such attributes of plasmas have been known to be capable of inactivating bacteria or other microorganisms and therefore plasmas have been widely used for sterilization.

Many studies of plasma-based sterilization have been on the inactivation of microorganisms immobilized on a material surface under dry conditions, to which plasmas are directly applied.$^{[5-10]}$ Under such conditions, microorganisms are often physically destroyed by impinging energetic ions and/or electrons or their DNAs are severely damaged by UV from the plasma.$^{[6,7]}$ Reactive oxygen and other free radicals in the gas phase have been also reported to directly damage the microorganisms.$^{[5,8-10]}$ For example, Perni et al.$^{[10]}$ have reported that, under dry conditions, the dominant contributors for $\textit{Escherichia coli}$ ($\textit{E. coli}$) inactivation are plasma-generated O atoms (i.e., atomic oxygen) and $\text{O}_2^\cdot$ in the gas phase. Microscopic observations by Yu...
et al.\textsuperscript{9} have shown that \textit{E. coli} cells are destructed and their morphologies are completely changed when they are exposed to exited oxygen species generated by plasmas.

For many practical applications, however, sterilization under wet conditions is also important. When bacteria are immersed in a liquid or imbedded in a gel-like material with sufficient water content, neither ions nor electrons can interact directly with the bacteria as they are strongly absorbed by a liquid when applied through the gas–liquid interface. Nevertheless, as we shall show in this paper, exposure of the bacteria containing liquid to a low-temperature atmospheric pressure plasma (APP) can also inactivate bacteria suspended in the liquid. Therefore, the mechanism of bacterial inactivation under dry conditions and wet conditions can be significantly different.

For plasma sterilization under wet conditions, especially of importance is the use of nonequilibrium (i.e., nonthermal) APPs with low gas temperature, which are typically less harmful to animal tissues (and therefore human bodies as well) but can nevertheless cause some chemical reactions (especially sterilization) on the surface of an object or a material to which the plasmas are exposed. Such plasmas may be designed to emit very little UV but still to act as a strong source of free radicals. With the use of such low-temperature APPs, one can inactivate bacteria and other microorganisms only by free radicals, using neither UV nor heat. Since the penetration depth of free radicals on or near a solid surface is very much limited, such plasmas may be used to sterilize living animal tissues (including human skins) without leaving permanent damages to the tissues.

One of the goals of the present work is to develop a new method to inactivate bacteria in a liquid with the use of an APP whose UV or heat is harmless to living cells. For this purpose, we have performed plasma inactivation experiments for bacteria suspended in an aqueous solution, which we shall present in this paper.

One of the most important findings in this study is the presence of a critical pH of the solution, i.e., pH \( \approx 4.7 \), below which the bactericidal effect by low-temperature plasma application becomes markedly strong. This critical value is nearly universal and the same critical value applies to all the bacteria that we have found the plasma application can inactivate. In this article, we shall only show the experimental results for \textit{E. coli} and \textit{Leuconostoc citreum} (\textit{L. citreum}), but similar results have been found for other bacterial species including an anaerobic bacterium. The results for other bacteria will be presented in separate publications due to the limited space of this paper. The universality of the critical pH value suggests the strong association of superoxide anion radicals \( \text{O}_2^- \bullet \) with the bactericidal effect.

The rest of the paper is organized in the following manner. In the next section, we shall briefly discuss the plasma sources used for the experiments presented in this paper. In the Section \textit{Experimental Setup for Bacterial Inactivation}, details of the sterilization experimental procedures for bacteria suspended in aqueous solution will be presented. Details of the experimental findings are given in the \textit{Experimental Results} and the interpretations of the data as well as the conclusions of this study are presented in the final section.

Outline of the Plasma Systems

Our experiments were conducted with the plasma injection system shown in Figure 1. In this system, bacteria are suspended in a liquid (whose volume is typically 500 \( \mu \text{l} \) unless otherwise indicated) and low-frequency (LF) plasma

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\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{(A) A schematic diagram of a LF plasma jet generation system. LF/HV pulses are applied to the electrode, which is wound around the glass tube. A plasma is generated in a He gas flowing in the glass tube and a plume-like structure of the plasma (which we call a “plasma jet”) is extended toward the surface of a liquid in which bacteria are suspended. (B) A typical wave form of voltage applied to the electrode. The same system was also used to apply plasmas to bacterial suspensions contained in a microplate, as in Figure 2.}
\end{figure}
Plasmas are generated in the same manner as that of Figure 1, but the ambient air is controlled by a completely enclosing discharge chamber made of poly(propylene). As shown in Figure 2, the liquid in which bacteria are suspended, are exposed only to the gas introduced from the two incoming gas inlets. A He gas is always supplied as a discharge carrier gas from one of the inlets. From the second gas inlet, different types of gases (such as air) may be supplied independently in a controlled manner. An exhaust tube is also equipped as shown there, from which the supplied gases are removed after they are used for plasma generation. The outer and inner diameters of the poly(propylene) chamber are 16.2 and 14.5 mm. The high voltage electrode is a thin metal sheet wound around the poly(propylene) chamber. As in the system of Figure 1, low temperature plasmas are generated and in contact with the liquid surface in an ambient air-tight environment, when high voltage pulses are applied to the electrode.

In this paper, the plasma system of Figure 1, i.e., the system for He discharges in ambient air, is always used for sterilization experiments except for a few experiments for which the use of the system given in Figure 2 is explicitly indicated.

**Experimental Setup for Bacterial Inactivation**

In the experiments presented in this paper, we have used the following bacteria strains. *E. coli* IFO 3301, which was distributed from Institute for Fermentation, Osaka, was cultivated in Luria–Bertani (LB) broth. *L. citreum* was isolated from fermented milk and cultivated in 100 × 10⁻³ mm glucose containing LB broth. In each case, the bacteria were cultivated for 18 h at 30 °C with 110 rpm rotary agitation. They were grown to an optical density at 600 nm (OD₆₀₀) of 2.5 ± 3.0, harvested by centrifugation (8 000 × g, 2 min), and then suspended in an appropriate volume of distilled water (DW). The bacterial suspension was diluted with DW to OD₆₀₀ = 0.1, which contains approximately 7 × 10⁷ cells·ml⁻¹ of the bacteria.

With the use of the bacteria above, bacterial inactivation assays by LF plasma jets were performed in the following manner. The bacterial suspension (OD₆₀₀ = 0.1) prepared in the above manner, was diluted 10-fold with DW or a 2.0 × 10⁻³ M citrate-Na buffer (pH = 6.5 ± 3.7) and then 500 μl aliquots were distributed into 24-well microplates. The plasma jet was applied to the surface of the bacteria-containing solution in each well, for a predetermined period in ambient air. The tip of the tube that spews plasma jets is located approximately 2 cm above the solution surface. The He gas flow rate used for the experiments was 2.01·min⁻¹ (i.e., 2 000 sccm) and the applied voltage varies from −3.5 to 5.0 kV with a fixed frequency of 13.9 kHz. As
mentioned earlier, the neutral gas temperature of LF plasma jets used in this study is almost room temperature and therefore the plasma has no significant thermal effect on the solution. However, the He flow still evaporates some water from the solution, so that care was taken to separate the effect of evaporation from bactericidal effects by plasmas.

The number of live cells after plasma application was determined by colony forming unit (CFU) assay, in the following manner. After plasma application, the solutions were recovered from the wells and DW was added to each recovered solution to replenish water lost by evaporation during the process. The solutions were then serially diluted further with DW and 100 μl of each dilution was spread on an LB agar plate for E. coli or Plate Count Agar with BCP “Nissui” (Nissui Pharmaceutical) for L. citreum. These plates were incubated for 16 h at 30 °C, so that the colonies can be developed and become countable. The minimum detection value of the CFU assay in this study was 10 CFU/ml.

Experimental Results

One of the most notable chemical effects that plasmas have on the solutions, is acidification. A plasma exposure to the solution can significantly decrease its pH\(^{13,14}\), as shown in Figure 3(A). In the experiments shown in Figure 3, the plasma jet obtained under the same conditions as those for the bacterial inactivation assay (the results of which will be shown later in this section), was exposed to DW without bacteria. The initial pH of DW was 5.7.

Ion chromatography analysis (see Appendix A) was used to evaluate the amount of nitrogen oxides in the solution. Figure 3(B) shows that the sum of the numbers of NO\(_x^2\) and NO\(_x^3\) ions in units of moles in DW contained in the well, increases as a function of plasma exposure time. (Note that part of DW in the well, continues to evaporate as the exposure time increases. Although the data is not shown here, the exact amount of DW remaining in the well, is also recorded as a function of the exposure time.) On the other hand, no significant increase of other inorganic negative ions is detected in ion chromatography in the same plasma exposure process. Therefore, from the charge neutrality, the concentration of the hydronium ions must be equal to that of the observed NO\(_x^x\) (with x = 2 or 3) ions, i.e., [H\(^+\)] = [NO\(_x^x\)]. The pH values evaluated from this assumption (i.e., \(-\log[\text{NO}_x^x]\)) are plotted in Figure 3(A) as a function of time. It is seen that the pH values evaluated from the NO\(_x^x\) concentration, is in good agreement with those directly measured by a pH meter. It may be concluded therefore, that acidification by plasma exposure is caused by the dissolution of nitrogen oxides generated from ambient air by the plasma.

The data given in Figure 3 are averages over the data obtained from triplicated experiments. The maximum and minimum values of each data set obtained under the same conditions, are indicated by the error bar. The pH values evaluated from NO\(_x^x\) concentrations, which are evaluated only from the average values given in (A), have no error bars. If the size of the error bar is smaller than the symbol size in the figure, the error bar is not depicted for simplicity.

We have confirmed that hydrogen peroxide, H\(_2\)O\(_2\), is also produced in the solution up to 50 mg l\(^{-1}\) in concentration, during the same plasma exposure process. The concentration of H\(_2\)O\(_2\) in the solution was evaluated by semi-quantitative test papers (MACHEREY-NAGEL GmbH & Co. KG).

Plasma jets were similarly exposed to DW aliquots that contain E. coli. Figure 4 shows the concentration of bacterial cells (represented in units of CFU/ml) that remain in the aliquot and the pH value of the solution as a function of
the plasma exposure time. After 120 s of plasma exposure, the cell concentration is shown to have decreased more than $10^6$ times. Since $10$ CFU/ml is the detection limit of the present study, the result indicates that the solution was completely sterilized by this process. The time needed to reduce the number of live cells 10-fold, i.e., D value, is estimated to be 0.10 min during the period from 90 to 120 s. The bactericidal effect by plasma exposure is shown to have increased drastically after 90 s exposure or after the pH of the solution dropped approximately below 5.

To separate the effects of acidification from other possible effects due to plasma exposure, we examined the bactericidal effects by plasma exposure using pH controlled solutions with $2.0 \times 10^{-3}$ M citrate-Na buffers. Figure 5 shows the change in the number of surviving $E. coli$ cells when the solutions are exposed to He gas flows of the same fluence at the given pH values for 120 s with or without plasma jet exposure. When the plasma jet was on, the $E. coli$ suspension was exposed to the plasma jet in the same way as in the experiments given in Figure 4. When the plasma jet was off, no voltage was applied to the electrode and the $E. coli$ suspension was exposed only to the He flow. The number of surviving cells was obtained from the CFU assay. It is clearly seen that the combination of low pH ($pH = 3.5$) and plasma jet exposure, is essential for bacterial inactivation and the lack of either factor led to no bacterial inactivation. We performed the same experiments with $L. citreum$, which is a strain of lactic acid bacteria and therefore highly resistant to acidity, and obtained similar results. These experiments have clearly demonstrated that acidity up to $pH$ 3.5 without plasma exposure, hardly affects the survival of $E. coli$ or $L. citreum$ and the bacterial inactivation arises only from the combined effect of acidity and plasma exposure.

To examine the combined effect more clearly, we have examined inactivation of $E. coli$ and $L. citreum$ with various pH ($pH = 6.5 \approx 3.7$), using citrate-Na buffers. Figure 6 shows how the concentrations of surviving (A) $E. coli$ and (B) $L. citreum$ cells decrease during plasma exposure when the pH of the solutions are maintained at different constant values. The plasma conditions are the same as those in Figure 4 and 5.

The D values of these processes may be determined in the following manner. At the very early stage (which may be called the “shoulder” in a figure of time-dependent bacterial inactivation assay), the cell reduction can be exceptionally slow. This time delay may correspond to the time needed for a sufficient amount of bactericide provided by plasma to be accumulated, and diffuse uniformly in the solution. Another possibility is that the cell may exhibit strong resistance to the bactericide if its concentration is below a certain value. In the case of Figure 6(A), the first 60 s in the cases of pH 5.2 and 4.7 may be construed as their shoulders. Therefore, in the cases of pH 5.2 and 4.7 for $E. coli$, the shoulders may be considered very short and negligible but we must avoid CFU counts at the detection limit, i.e., 10 CFU/ml. Therefore,
for pH of 4.2 and 3.7 for *E. coli*, their D values are evaluated over the periods from 0 to 180 s, and from 0 to 60 s, respectively, with linear fitting to the data shown in Figure 6(A). The D values thus obtained for pH of 5.2, 4.7, 4.2, and 3.7 of Figure 6(A) are 1.92, 0.96, 0.59, and 0.21 min, respectively. No bactericidal activity was found at pH 6.5 in this case.

For Figure 6(B), the D values obtained in a similar manner are 1.44, 0.12, and 0.15 min at pH 4.7, 4.2, and 3.7, respectively. No bactericidal activity was found at pH 5.2 and 6.5 in this case, either.

It is clearly seen in Figure 6 that the pH of the solution strongly affects the effectiveness of plasma-based bacterial inactivation. Under neutral or weakly acidic conditions (pH = 6.5), plasma exposure hardly inactivates *E. coli* or *L. citreum*. On the other hand, under acidic conditions (pH < 4.7), bacterial inactivation occurs very rapidly by plasma exposure, for not only *E. coli* but even acidophilic *L. citreum*. In addition, it is clearly seen that pH 4.7 is an approximate “critical value,” below which bacterial inactivation is extremely efficient and above which bacterial inactivation is relatively weak or hardly occurs. It is interesting to note that these critical pH values are similar for both *E. coli* and *L. citreum*.

Figure 7 shows morphologies of *E. coli* bacteria obtained from phase-contrast microscopy (A) before and (B) after plasma application, similar to those in Figure 5 and 6. In this process, an *E. coli* suspension of approximately 1 x 10^9 CFU/ml in a citrate buffer of pH 3.7, was exposed to the standard plasma jets used in our experiments for 300 s. No particular difference in bacterial morphology is seen between (A) and (B). In other words, inactivated *E. coli* cells show no observable physical damage on their external structures. This indicates that bacterial inactivation of cells shown in Figure 7(B) was more likely to be caused by some damages to exterior structures that are unobservable macroscopically or to their interior structures, possibly including damages to some specific molecules of the cells.

It has been reported that superoxide anion radicals (O_2^−•) contribute to bacterial inactivation. In order to check whether superoxide anion radicals also play some roles in our plasma-based bacterial inactivation, we added superoxide dismutase (SOD) to *E. coli* suspensions in a citrate buffer of pH 3.7 before they are exposed to plasma jets. The SOD (obtained from bovine erythrocyte, MP Biomedicals Inc.) was first dissolved in DW (2.0 mg/ml) and then added to an *E. coli* suspension, so that the SOD concentration used in our experiments was 40 µg/ml. Bovine serum albumin, BSA (WAKO Pure Chemicals) was used for a control.

It is shown in Figure 8 that the number of living cells in the presence of SOD (denoted by solid circles) were 50 ± 25×10^6 times larger than that of BSA (denoted by solid triangles) at an early stage, i.e., 60 ± 120 s of plasma jet exposure. In other words, SOD, which is known to consume superoxide anion radicals, clearly reduces the bactericidal efficiency of plasma exposure. It indicates that superoxide anion radicals are likely to be the essential contributors to bacterial inactivation in this process.

It is not clear how superoxide anion radicals are generated in the liquid phase when the solution is exposed...
to the plasma jets generated by the system shown in Figure 1. Superoxide anion radicals may be generated in the solution by plasma exposure or come directly in the gas phase. More generally, other chemical compounds that originate from air may play an essential role for bacterial inactivation. In order to clarify whether the presence of air, in addition to He plasmas, is essential in the plasma-induced sterilization processes that have been discussed in this paper, we have used the plasma system of Figure 2 to perform plasma sterilization experiments with controlled gas flows.

In the experiments shown in Figure 9, the He gas flow rate was set to be 3.0 l · min⁻¹. The air-flow rate was either none (denoted by solid circles) or 15 ml · min⁻¹ (denoted solid triangles). Note that the amount of air added to the discharge experiments is significantly lower than that of He, which is the essential gas medium for plasma generation. As in the experiments discussed earlier, the plasma jets were applied to 500 ml E. coli suspensions (1 × 10⁷ CFU/ml) in a citrate buffer of pH 3.7 for 180 s.

It is clearly seen in Figure 9 that all the suspended bacteria were essentially inactive after 60 s exposure of He and air mixed plasma jets. On the other hand, the discharge of He gas only was shown to be very ineffective for bacterial inactivation. (In the He-only plasma jet experiments here, we cannot rule out the possibility that a very small amount of air leaked into the chamber during the experiments, which may have caused some bacterial inactivation.) Figure 9 thus clearly shows that the presence of air is essential for bacterial inactivation by plasma exposure, which also suggests that free radicals essential for sterilization (such as superoxide anion radicals) are likely to be originated from air.

Discussion on the Experimental Observations

As we discussed in the previous section, inactivation of E. coli or L. citreum suspended in a solution, was most efficient when the solution was maintained sufficiently acidic, during plasma jet exposure. In this section, based on the experimental evidences available to us so far, we will discuss possible causes of bacterial inactivation in a solution exposed to the plasma jets.

Ions and electrons of plasma jets cannot penetrate into a solution. Therefore, those which are responsible for bacterial inactivation must be some free radicals, oxidants, or peroxides generated by the plasmas. For example, as we have mentioned earlier, hydrogen peroxide (H₂O₂), nitric acid (HNO₃), nitrous acid (HNO₂) exist in the solution exposed to plasma jets. However, their concentrations were too low to sterilize the bacteria in solutions. For example, we have confirmed that 250 mg · l⁻¹ of H₂O₂ is needed to reduce the number of bacteria down to 1/10. However, as we mentioned earlier, only up to 50 mg · l⁻¹ of H₂O₂ was observed in the 500 µl aliquot of DW exposed to the plasma jets we used in our experiments. Such a low concentration of H₂O₂ is certainly insufficient to inactivate bacteria in the solution. As to NO⁻x, we have confirmed that E. coli bacteria were not inactivated at all when they were immersed in a 0.1 × 10⁻³ m nitric acid (HNO₃) solution (at pH = 4.0) for 2 h, the condition of which is similar to that of DW exposed to the plasma jets for 180 s, as shown in Figure 4.
Furthermore, if either NO\textsubscript{x} or hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) was the cause of bacterial inactivation, the level of bacterial inactivation would not depend on pH as the concentration of either NO\textsubscript{x} or H\textsubscript{2}O\textsubscript{2} depends essentially only on the plasma exposure time and not on the pH of the solution. The fact that the rate of bacterial inactivation strongly depends on pH, as shown in Figure 5 and 6, also indicates that the production of neither NO\textsubscript{x} nor H\textsubscript{2}O\textsubscript{2} alone can inactivate the bacteria.

We have also added bacteria to the solution 10 min after it was exposed to the same plasma for the same exposure time. In such solutions, even after 10 min, sufficiently high concentrations of both NO\textsubscript{x} and H\textsubscript{2}O\textsubscript{2} are observed. However, under such process, no bacteria were inactivated. This also corroborates our claim that neither NO\textsubscript{x} nor H\textsubscript{2}O\textsubscript{2} alone can inactivate bacteria in the solution.

Under strong UV radiation, DNA of bacterial cells can be severely damaged. However, UV is also unlikely to affect bacterial activities in the plasma sterilization experiments presented in this paper. First of all, our optical emission spectroscopy has shown that UV components (whose wavelength is shorter than 300 nm) are hardly observed in the emission from the plasma jets. Second, if the UV light from the plasma jets were the cause of sterilization, the number of \textit{E. coli} bacteria, when exposed to the plasma jets, should have been much more reduced even under the condition of pH 6.0 shown in Figure 5 as DNA damage by UV radiation is hardly influenced by the pH of the solution.

Free radicals such as hydroxyl radicals (OH•) and superoxide anion radicals (O\textsubscript{2}•\textsuperscript{-}) have been reported to contribute to bacterial inactivation in aqueous solutions.\textsuperscript{[16]} For example, hydroxyl radicals are known to be so highly reactive that they could inactivate bacteria very efficiently. However, due to their high chemical reactivity, hydroxyl radicals have a very short lifetime (200 μs at 1.0 × 10\textsuperscript{-6} M) in an aqueous solution.\textsuperscript{[17]} It is unlikely that they will be able to diffuse into the bottom of the microplate well, to inactivate all the bacteria used in our experiments. Convocation of the solution in the well, although it is directly exposed to a He flow, hardly takes place; so possible bacterial inactivation that could take place on the top thin surface layer of the solution due to direct exposure to hydroxyl radicals generated in the gas phase by the plasma, is unlikely to account for the full bacterial inactivation shown in Figure 4–6.

In contrast, superoxide anion radicals (O\textsubscript{2}•\textsuperscript{-}) have a relatively long lifetime (5 s at 1.0 × 10\textsuperscript{-6} M)\textsuperscript{[18]} in aqueous solutions, which allows the sufficient diffusion of superoxide anion radicals in the 500 μl aliquot. Although superoxide anion radicals are reasonably reactive, they are considered to be incapable of penetrating the cell membrane due to their charges and therefore unlikely to cause damages inside cells. However, the pH of the solution is sufficiently low, superoxide anion radicals are converted to hydroperoxy radicals (HO\textsubscript{2}•\textsuperscript{-}), which can penetrate the cell membrane and damage intercellular components.\textsuperscript{[19]}

The equilibrium reaction between superoxides and hydroperoxy radicals are given by

\[
O_2^{•-} + H^+ \leftrightarrow HO_2^{•-}
\]  

(1)

The acid dissociation constant (pK\textsubscript{a}) of this equation is 4.8,\textsuperscript{[19]} which means that a large number of superoxide anions will be converted to hydroperoxy radicals when the pH of the solution is lower than 4.8.

It is interesting to note that the critical pH for bacterial inactivation for both \textit{E. coli} and \textit{L. citreum} is observed in Figure 6, which is approximately pH 4.7, and is very close to the pK\textsubscript{a} of Equation (1). As we have already discussed in the previous section, the SOD experiments of Figure 8 also indicate the importance of superoxide radical anions for bacterial inactivation. Therefore, the fact that the critical pH and the pK\textsubscript{a} of Equation (1) are nearly equal, may suggest that the presence of hydroperoxy radicals, rather than superoxide anion radicals, is essential for bacterial inactivation and this may be due to the ability of hydroperoxy radicals to penetrate the cell membranes. This is similar to the mechanism that a phagosome of a macrophage inactivates microorganisms and viruses with acidophilic proteases,\textsuperscript{[20]} i.e., hydroperoxy radicals converted from superoxide anion radicals in a phagosome in which the pH is maintained below 4.8 or a lower value, contribute to the inactivation.

So far we have found a few circumstantial evidences that superoxide anion radicals are likely to be one of the key elements for bacterial inactivation by plasma application. However, it does not mean that the presence of a large number of superoxide anion radicals alone suffice the efficient bacterial inactivation in solutions that we have discussed so far in this paper. Indeed, our preliminary experiments have indicated that the combination of He plasma jets with oxygen only (instead of air) in the plasma system shown in Figure 2 results in somewhat weaker bacterial inactivation. This suggests that the presence of nitrogen may also be needed to account for the full bacterial inactivation in a short period that we have observed in our experiments.

If the presence of nitrogen in the gas phase as well as superoxides in the solution, is necessary for bacterial inactivation, it is possible that antimicrobial peroxynitrite anion (ONOO\textsuperscript{-}), which can be formed by the reaction of nitric oxide (NO•) generated by the plasma with superoxide anion radicals as

\[
O_2^{•-} + NO^{•} \rightarrow ONOO^{•-}
\]
may contribute to bacterial inactivation. The equilibrium reaction between peroxynitrite anions and peroxynitrous acid is given by

\[
\text{ONOO}^- + \text{H}^+ \rightleftharpoons \text{ONOOH}, \quad (2)
\]

whose acid dissociation constant \( \left( K_a \right) \) is 6.8.\[21\] In other words, under our experimental conditions with a neutral or acidic pH, peroxynitrite anion radicals should always exist and their concentrations hardly depend on the pH, although we have no direct experimental evidence of the presence of peroxynitrite anions in the solutions used in our experiments. Therefore, the strong dependence of bactericidal effects on pH shown in Figure 6 under neutral or acidic conditions may not be accounted for, by Equation (2).

Although peroxynitrite anions \( \text{ONOO}^- \) alone may not contribute to bacterial inactivation, they may be still needed for hydroperoxy radicals \( \text{HOO}^- \) to effectively inactivate \textit{E. coli} or \textit{L. citreum}. Determination of the essential factors for plasma-based bacterial inactivation would require further experiments and therefore will be the subject of a future study.

**Conclusion**

In conclusion, it has been found that He-based low temperature atmospheric plasma jets (i.e., LF plasma jets) in ambient air can efficiently inactivate \textit{E. coli} and \textit{L. citreum} bacteria in aqueous solutions, if the solution is kept sufficiently acidic. We have also found that there exists a critical pH for bactericidal effects, which is approximately 4.7. Below the critical pH, bacterial inactivation occurs by the plasma jet application to the solution surface whereas, above the critical pH, bacteria remain intact even with the same plasma application. The critical value seems to be the same for both \textit{E. coli} and \textit{L. citreum}, and our preliminary study for inactivation of other bacterial species also indicates that, for most bacteria that can be inactivated by plasma application discussed in this paper, the critical pH value exists and takes the same value of about 4.7.

Furthermore, we have shown that the presence of superoxide anion radicals \( \text{O}_2^- \) is essential for the bacterial inactivation. Since the critical pH for bactericidal effects agrees well with the \( pK_a \) of the equilibrium reaction between superoxide anion radicals \( \text{O}_2^- \) and hydroperoxy radicals \( \text{HOO}^- \) given in Equation (1), we postulate that hydroperoxy radicals \( \text{HOO}^- \) formed in the solution may directly interact with bacterial cells to inactivate them. On the other hand, we have confirmed that either \( \text{H}_2\text{O}_2 \) or UV irradiation generated by the plasmas has little or no effect on the sterilization.

Our preliminary experiments have also shown that the combination of He plasma jets with oxygen only (instead of air) results in somewhat weaker bacterial inactivation. In other words, for highly efficient bacterial inactivation that we have achieved by He plasma application to the solution surface in ambient air, the presence of nitrogen may also be needed. Although peroxynitrite anion (\( \text{ONOO}^- \)) are considered to be antimicrobial, a question whether peroxynitrite anions or other nitrogen related reactive species contribute to the efficiency of bacterial inactivation due to the plasma application, will be the subject of a future study.

**Appendix A: Ion Chromatography for Concentration Measurement of NO\(_x\) in a Solution**

The experimental procedure for ion chromatography is the following. One milliliter of DW set in each well of a 24-well microplate, was exposed to a LF plasma jet for a predetermined period in ambient air. Plasma applied DW was recovered from each well and DW was added to the recovered solution, to replenish water lost by evaporation during the process. The consequent samples were analyzed by an ion chromatography system (Ion chromatograph DX320; Nippon Dionex K. K.) that connects the guard column (IonPac AG4A-SC; Nippon Dionex K. K.), the separation column (IonPac AS4A-SC; Nippon Dionex K. K.), and the suppressor [ASRS-ULTRA 4 mm (40 mA); Nippon Dionex K. K.]. A carbonate buffer \( \text{Na}_2\text{CO}_3 \) was used as an eluent \( \text{Na}_2\text{CO}_3 + 2.1 \times 10^{-3} \text{M NaHCO}_3 \) was used as an eluent and flowed at the rate of 1.5 ml min\(^{-1}\). Negative ions in the samples were estimated with the use of DW as a control.

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