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Inactivation of *Shewanella putrefaciens* by Plasma Activated Water

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Abstract Plasma activated water (PAW) generated by atmospheric-pressure air microplasma arrays is a solution containing a variety of reactive species. Here we investigate the effects of different applied voltage and water-activated time on bactericidal activities against *Shewanella putrefaciens* (*S. putrefaciens*). Our measurements showed that the sterilization efficiency of *S. putrefaciens* by PAW could be up to 2.0 Log Reduction. Scanning electron microscopy image and DNA concentration measurement showed that the *S. putrefaciens* cells were damaged and deformed due to the PAW treatment. The physicochemical properties of PAW treated by different applied voltage and water-activated time were evaluated, including pH value, initial PAW temperature, and the concentrations of plasma-activated species, such as H_2O_2, NO_3^-, NO_2^-, and O_3. Analysis indicates that the sterilization efficiency of *S. putrefaciens* treated by PAW was mainly determined by H_2O_2 concentration and pH value of PAW. This study provides a basis for the PAW potential applications in the disinfection of rotten food.

Keywords Plasma activated water · Inactivation · Hydrogen peroxide · Nitrate · *S. putrefaciens*

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Introduction

Disinfectant pollution has caused wide public concern over the world. Widespread use of chemical disinfectant is a terrible hazard for the environment. Plasma activated water (PAW) has gained increasing attention as a novel and very efficient disinfectant [1–4]. PAW can be generated by non-thermal plasma and is currently under study as potential alternatives to conventional sterilization techniques applied for agriculture [5, 6], food [4–7] and medicine [8, 9]. The main advantages of using PAW for sterilization include less adverse impact on the environment and no risk during storage and transportation [10].

Depending on the type of discharge, long-lived reactive species, such as H_2O_2 and O_3 can be transferred from the plasma into the liquid, and they are believed to play dominant roles in the inactivation process [2, 11]. The contribution of nitrogen-containing species, such as NO_2^-, ONOOH, and O_2NOOH [12, 13], also needs to be considered. These species are highly cytotoxic and can react with cellular tissue [14, 15]. Therefore, the antimicrobial properties of PAW were temporarily attributed to the synergetic effect of H^+ ions,the H_2O_2 and NO_2^-/NO_3^- remaining in noticeable concentrations in the solution [16].

However, few investigations focus on the effective inactivation of Spoilage bacteria by PAW. Spoilage bacteria has gained an increasing attention [17, 18], and the decay of meat products is mainly caused by Spoilage bacteria. Rotten meat products can emit some gases with peculiar smells, for instance hydrogen sulfide (H_2S) and trimethylamine (TMA). TMA is easy to resolve into dimethylamine and formaldehyde. Formaldehyde that has been identified as carcinogen by the World Health Organization can seriously affect the activities of cells and cause great damage to cells. *S. putrefaciens* is a major spoilage bacterium of meat, and it emits gas with a peculiar smell [19]. Therefore, there is an urgent need to search an effective and natural way to inhibit the bacterial activity.

In this study, an atmospheric-pressure plasma device consisting of 28 air microplasmas was used to generate PAW. PAW was used to sterilize *S. putrefaciens* cells. The sterilization efficiency of *S. putrefaciens* cells was evaluated via colony forming unit (CFU) count on Petri dish. In order to analyze the inactivation process of *S. putrefaciens* cells, the scanning electron microscopy (SEM) was used to observe their structural changes. After PAW treatment, DNA concentration of *S. putrefaciens* cells has been measured by UV/VIS-spectrophotometer. Moreover, the physicochemical characteristics of PAW, including initial PAW temperature, pH value, and the concentrations of H_2O_2, NO_3^-, NO_2^- and O_3 were measured. Finally, the sterilization efficiency of *S. putrefacien* cells treated by PAW was compared to the one of *S. putrefacien* cells treated by the H_2O_2/HNO_3 mixture solution.

Experiment Setup

Plasma Device and PAW Generation

A schematic diagram of the microplasma array is shown in Fig. 1. Previously, we have reported a similar structure [20]. The plasma device is mainly composed of 28 microplasma jet units housed in an equilateral triangle quart cup. In each microplasma jet unit, a quartz tube with its inner diameter of 700 μm and its outer diameter of 1000 μm is used to generate the microplasma. The 300 μm thick tungsten wires, acting as a high-voltage electrode, are inserted into the quartz fiber, and the distance between the nozzle and tungsten wire is 3 mm. The 28 microplasmas jet units are uniformly distributed inside the
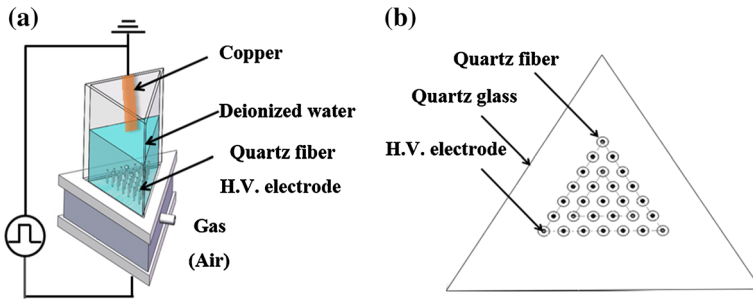
Fig. 1 Schematic diagram of the experimental setup used in this study **a** mimic diagram of microplasma triangle array device, **b** top view of the plasma device

equilateral triangle reactor. The air is fed into the reactor at a flow rate of 1.0 standard liter per minute (SLM). The air flows from the quartz tubes into the water. One copper electrode immersed in the water is connected to the ground. The power supply consists of a signal generator, a power amplifier and a transformer and can generate the pulse voltage with its peak voltage of 6.0–12.0 kV and its frequency of 7.0 kHz. The 40 mL deionized water was activated by changing the water-activated time from 0–30 min.

Antimicrobial Activity

The isolate of Shewanella putrefaciens (China General Microbiological Culture Collection Center, CGMCC number 1.3667) was used in this study. Fresh solution of Shewanella putrefaciens (*S. putrefaciens*) was cultured overnight in 100 mL Luria–Bertani (LB) by shaking with 180 rpm at 37 °C for 12 h. *S. putrefaciens* cells were cultured in LB medium at 37 °C until its density reached 1×10^8 cfu/mL. In order to obtain a countable number of colonies on control plates, *S. putrefaciens* cells were diluted to 1×10^5 cfu/mL with normal saline solution. As shown in Fig. 2, 40 mL PAW was generated by changing water-activated time and applied voltage. After the discharge for 3–5 s, 20 μL *S. putrefaciens* solution diluted was immediately added into 300 μL PAW. 20 μL *S. putrefaciens* solution diluted was transferred to 300 μL sterile water as the control. After 5 min incubation time, the 320 μL mixed solution was spread over the LB agar.
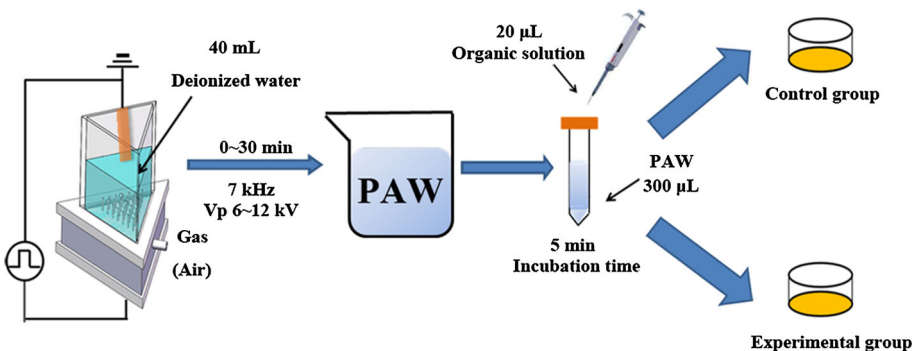

Fig. 2 Schematic diagram of the sterilization way

The effects of initial water temperature and pH values on sterilization efficiency were studied to analyze the inactivation process of *S. putrefaciens* cells by PAW. Thermostat water bath and ice were used to control initial sterile water temperature. The initial temperature of sterile water value was in the range of 5–100 °C. Nitric acid was used to preset the pH value of water. The pH value of the water was in the range of 2–5.9. 20 μL *S. putrefaciens* solution diluted was added into 300 μL sterile water. After 5 min incubation time, the 320 μL mixed solution was spread over the LB agar. The sterilization efficiencies of *S. putrefaciens* cells treated by H_2O_2, HNO_3 solutions and their mixture solution were also evaluated. 20 μL *S. putrefaciens* diluted solution was added into 300 μL H_2O_2, HNO_3 solutions, and H_2O_2/HNO_3 mixture solution. After 5 min incubation time, the 320 μL mixed solution was spread over the LB agar. Plate colony-counting method was used to count the survival amount microflora. It should be pointed out that all the bacterial-inactivated experiments reported here were repeated three times. The sterilization efficiency of solution was evaluated by Log Reduction, as follows:

$$\text{Log Reduction} = \text{Log}\left(\frac{\text{CFU}_{\text{control}}}{\text{CFU}_{\text{treated}}}\right) \tag{1}$$

SEM Analysis

After *S. putrefaciens* cells were treated by PAW, scanning electron microscopy (S-4800, HITACHI, Japan) was used to observe the morphological changes of the *S. putrefaciens* cells. To perform SEM measurements, *S. putrefaciens* cells were diluted to 1×10^7 cfu/mL with normal saline solution. 100 μL *S. putrefaciens* solution was mixed with the 1.5 mL sterile water or PAW. 10 μL the mixed solution was transferred to sterile conductive silicon wafers. The *S. putrefaciens* solution was washed by PBS and fixed by 2.5% glutaraldehyde solution at 4 °C. The samples were washed twice by using the PBS and fixed by 1% osmic acid. Subsequently, they were eluted by changing concentrations of alcohol dehydration. After coated with a gold layer, they were visualized with SEM [21].

DNA Released from *S. putrefaciens* Cells Analysis

The concentration of DNA molecules released from *S. putrefaciens* cells was measured to evaluate the integrity of *S. putrefaciens* cells. The DNA concentration was calculated by monitoring the absorbance peak of samples at 260/280 nm by UV/VIS-spectrophotometer [22]. Nucleic acids (DNA or RNA) contain conjugated double bonds in their purine and pyrimidine rings, which have a specific absorption peak at 260 nm. The intensity of UV absorbance is proportional to the concentration of nucleic acid [22]. 3 mL of the *S. putrefaciens* solution was transferred to 10 mL sterile water or PAW. To obtain a clarification solution, their mixture solution was centrifuged at 6000 g for 10 min. Then, 6 mL supernatant was filtered by 0.22 μm filtration membrane. Subsequently, 3 mL solution filtered was used to measure DNA concentration by using UV/VIS-spectrophotometer (TU-1950, PERSEE, China).

pH and Temperature Measurement of PAW

PAW was generated by changing water-activated time and applied voltage. After discharge, both the pH value and temperature of PAW were immediately measured. The pH

value and initial temperature of PAW were measured by a pH meter (model Lab-850; SI Analytics Co., Germany) and a mercury thermometer, respectively.

Chemical Species in PAW

The atmospheric-pressure air microplasma contains reactive oxygen species (ROS), such as H_2O_2, HO_2, O_3, NO_X, and O and OH radicals in the gas phase. Long-lived species, such as H_2O_2, NO_3^-, NO_2^- and O_3 were formed during the chemical reaction between the plasma and water, and their concentrations were measured by UV/VIS-spectrophotometer (TU-1950, PERSEE, China). For the measurements of H_2O_2 concentration, 2 mL $TiSO_4$ (5%) solution was immediately transferred to 2 mL PAW after it was treated by the atmospheric-pressure microplasma. 4 mL H_2SO_4 (4 mol/L) solution was added to the mixed solution. The 3 mL PAW containing $TiSO_4$ and H_2SO_4 was measured by using UV/VIS-spectrometer at 407 nm [16, 23, 24]. To measure the NO_3^- concentration, 1 mL PAW was immediately added to 5 mL Nickel Sulfamate solution (5%). Then, 3 mL of the mixed solution was measured by using UV/VIS-spectrophotometer at 219 nm. NO_2^- concentration was also measured by using UV/VIS-spectrophotometer. Sulphanilamide was used as the diazotizing reagent and N-(1-Naphthy1)-ethylenediamine hydrochloride was used as the coupling reagent. 40 μL of 10 g/L sulphanilamide was added to 2 mL PAW. Then this solution was incubated at room temperature for 2 min. Subsequently, 40 μL of 1.0 g/L N-(1-Naphthy1)-ethylenediamine hydrochloride was added into the mixture solution. After reaction for 20 min at room temperature, the NO_2^- concentration was measured by using UV/VIS-spectrophotometer at 540 nm [16, 23, 24]. The concentration of O_3 dissolved in liquid was determined by the Indigo method. 2 mL sodium indigotin-disulfonate solution (279 mg/L) was transferred to 5 mL phosphate buffer solution (7 g/L KH_2PO_4, 7 g/L NaH_2PO_4, pH = 2). Then, 5 mL PAW was added to the mixed solution, then their mixture was diluted to 50 mL by ultrapure water. The O_3 concentration was measured by using UV/VIS-spectrophotometer at 610 nm [25, 26].

Results

Sterilization Efficiency

Figure 3 shows the sterilization efficiency as a function of water-activated time and applied voltage. The water-activated time was varied from 0 to 30 min. It can be clearly seen that both the water-activated time and applied voltage have a significant effect on the sterilization efficiency. After 30 min of water-activated time, the average sterilization efficiency can reach 0.13 Log Reduction at 6.0 kV, 0.38 Log Reduction at 7.0 kV and 2.0 Log Reduction at 8.0 kV. With increasing the applied voltage to 10.0 kV, the average sterilization efficiency can reach as high as 2.0 Log Reduction at the water-activated time of 25 min. When applied voltage is 12.0 kV, the average sterilization efficiency can be up to 2.0 Log Reduction at water-activated time of 20 min.

The morphological changes of S. putrefaciens cells were observed after the PAW treatment. Figure 4 shows SEM images of S. putrefaciens cells treated by PAW at voltage of 12.0 kV. The S. putrefaciens cells treated at the water-activated time of 5, 15 and 20 min were defined as 5-min PAW, 15-min PAW and 20-min PAW, respectively. As shown in Fig. 4a, before S. putrefaciens cells are treated by PAW, their shapes are
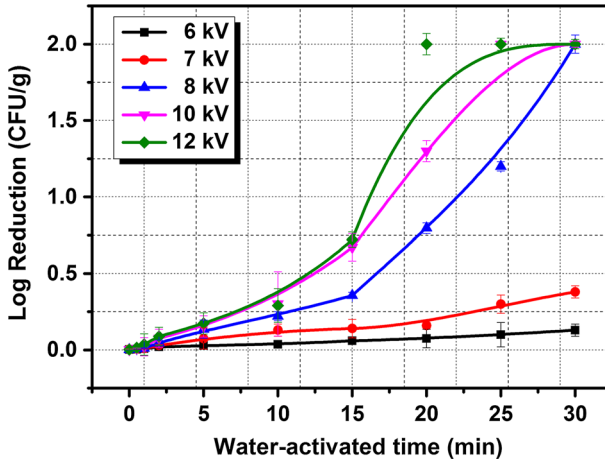
Fig. 3 The sterilization efficiency of PAW as a function of applied voltage and water-activated time. The incubation time of *S. putrefaciens* with PAW is 5 min. The data points and error bars in all figures represent the averages and standard deviations from three independent measurements
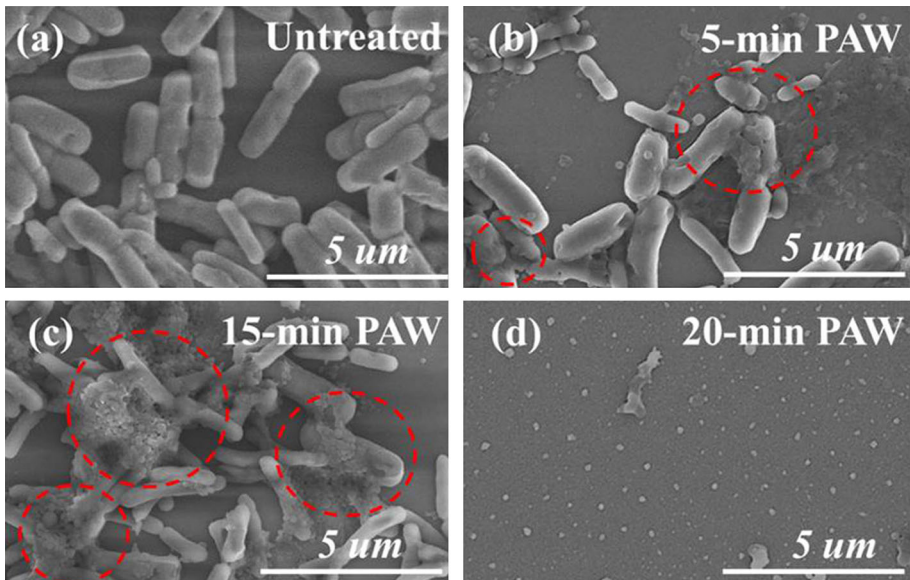

Fig. 4 Morphological changes of *S. putrefaciens* cells after PAW treatments

relatively complete, long and narrow with smooth surface. There is no cell damage or overflow of the content. After PAW treatment, the *S. putrefaciens* cells exhibit different degrees of damage. The *S. putrefaciens* cells underwent a transition from initially smooth surfaces to severely deformed surfaces. It was clear that *S. putrefaciens* cells are destroyed, as shown in the red circles in Fig. 4b, c. Damage of the *S. putrefaciens* cells walls could be caused by ROS in PAW, which could oxidize unsaturated fatty acids in lipid bilayer of cell wall, cleave peptide bonds and oxidize amino acid side chains [4]. After 20 min PAW

treatment, it is difficult to find the relatively complete cells structure, as shown in Fig. 4d. It can be seen that PAW has a detrimental effect on *S. putrefaciens* cells. *S. putrefaciens* cells walls or membrane could be destroyed, resulting in the leakage of cell protoplasm and electrolyte, which is considered to be lethal to *S. putrefaciens* cells.

The concentration of DNA molecules released from *S. putrefaciens* cells was measured to evaluate the integrity of *S. putrefaciens* cells. The DNA concentration was measured after PAW treatment. As shown in Fig. 5, the DNA concentration is significantly dependent on the water-activated time and applied voltage. The DNA concentration significantly increases at applied voltage of 7.0 kV. The DNA concentration firstly increases with increasing water-activated time and eventually decreases with further increasing water-activated time at voltage of 8.0–12.0 kV. Moreover, the DNA concentration is beyond the detection limit at applied voltage of 6.0 kV. The DNA leakage could be attributed to the disruption of the cell membrane of *S. putrefaciens* cells during the PAW inactivation process.

As shown in Fig. 6a, the pH value of PAW is significantly dependent on the water-activated time and applied voltage. The pH value of PAW is typically in the range of from 5.8 to 2.5, depending on the water-activated time and applied voltage. NO_x is formed inside the atmospheric-pressure air microplasma. The chemical reactions between NO_x, air and H_2O lead to a decline in the pH value of PAW, as shown below [27–30].
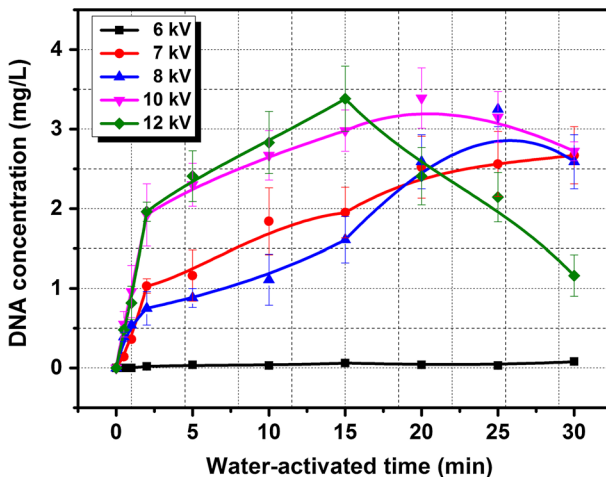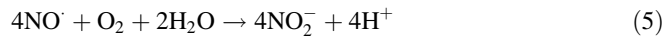
$$NO(aq) + NO_2(aq) + H_2O \rightarrow 2NO_2^- + 2H^+ \tag{2}$$

$$2HNO_2 \rightarrow NO^{\cdot} + NO_2 + H_2O \tag{3}$$

$$2NO_2 + H_2O \rightarrow NO_3^- + NO_2^- + 2H^+ \tag{4}$$

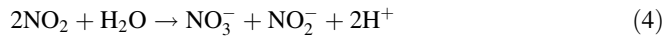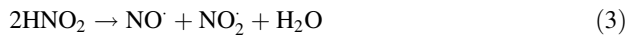$$4NO^{\cdot} + O_2 + 2H_2O \rightarrow 4NO_2^- + 4H^+ \tag{5}$$

Fig. 5 The DNA concentration of mixed solution as a function of treatment time and applied voltage. The incubation time of *S. putrefaciens* with PAW is 5 min. The data points and error bars in all figures represent the averages and standard deviations from three independent measurements

Fig. 6 a The pH values of PAW as a function of water-activated time and applied voltage, **b** the sterilization efficacy of water as a function of pH value. The incubation time of *S. putrefaciens* with PAW is 5 min. The data points and error bars in all figures represent the averages and standard deviations from three independent measurements


$$4NO_2^- + O_2 + 2H_2O \rightarrow 4NO_3^- + 4H^+ \tag{6}$$
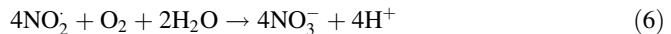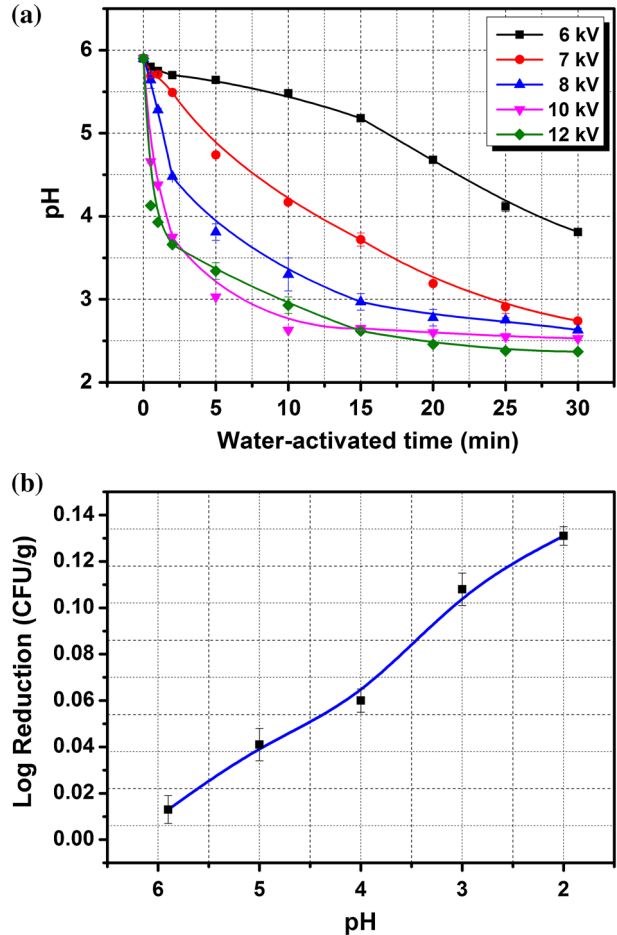
The pH value of PAW can have an impact on bacteria growth since the optimum pH value for *S. putrefaciens* growth is from 6 to 9. The pH value of PAW can affect a series of biological substance of bacteria, such as protein and nucleic acid [29]. However, some bacteria can not be affected by liquid acidity unless a hazardous pH value is reached. For example, for Staphylococcus aureus a hazardous pH value of 2 is needed to initiate inactivation [31]. Figure 6b shows the sterilization efficiency of *S. putrefaciens* cells as a function of pH value in water. The sterilization efficiency can reach as high as 0.13 Log Reduction at pH value of 2.

The initial PAW temperature was measured immediately by using mercury thermometer after plasma treatment. Clearly, the initial PAW temperature is significantly dependent on the water-activated time and applied voltage, as shown in Fig. 7a. The effect of water temperature on sterilization efficacy was studied. Figure 7b shows the sterilization efficiency of *S. putrefaciens* cells as a function of initial sterile water temperature. The sterilization efficacy can reach as high as 0.10 Log Reduction at temperature of 100 °C.

Fig. 7 **a** The temperature of PAW as a function of water-activated time and applied voltage, **b** the sterilization efficiency of water as a function of different temperature value. The incubation time of *S. putrefaciens* with PAW is 5 min. The data points and error bars in all figures represent the averages and standard deviations from three independent measurements
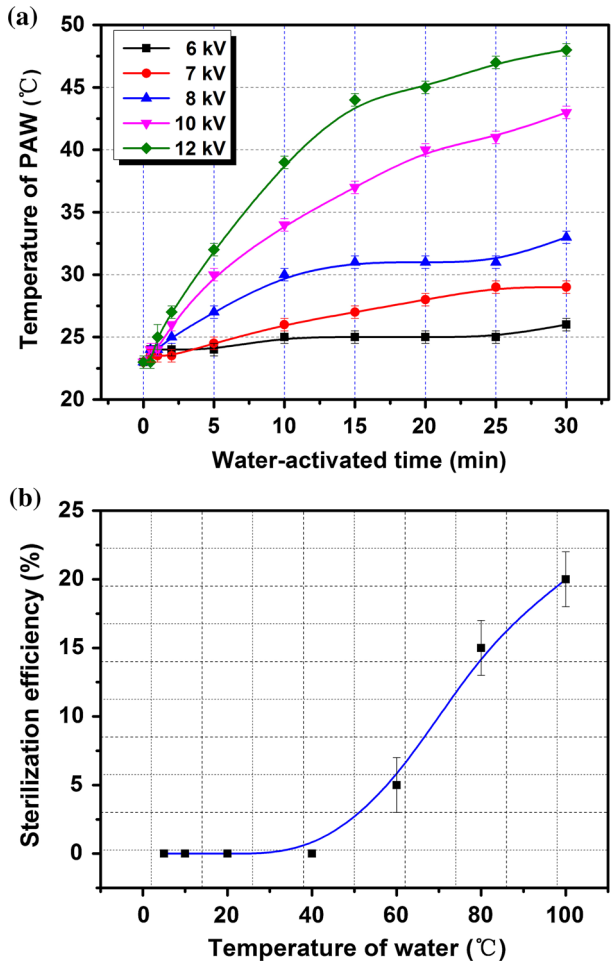

This result may be due to the fact that sterile water temperature changed to room temperature during the process of incubating bacteria. Thus, sterilization efficiency at temperature of 100 °C is not so high as expected. However, the sterilization efficacy of *S. putrefaciens* only is 0.02 Log Reduction at water temperature of 60 °C. This indicates that the initial PAW temperature is not an important parameter for antimicrobial activity. Thus, the temperature of PAW below 50 °C does not significantly result in the inactivation of *S. putrefaciens*.

Chemical Properties of PAW

H_2O_2 is thought to be an important oxidant in PAW and a steady product. Depending on water temperature, its half-life period ranges from 8 h to about 20 days [31]. To avoid the effect of holding time on the H_2O_2 concentration of PAW exposed to the air, the H_2O_2 concentration was immediately measured by using the UV/VIS-spectrophotometer. Figure 8 shows the H_2O_2 concentration of PAW as a function of water-activated time and applied voltage. The H_2O_2 concentration can be up to 4.6 mmol/L at applied voltage of
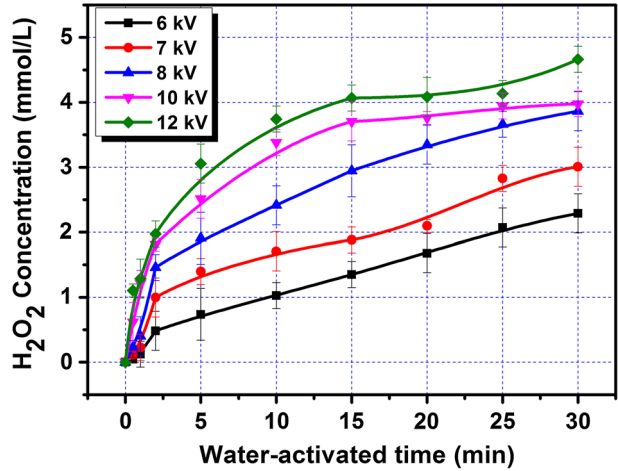
12 kV, when water-activated time is 30 min. The H_2O_2 concentration in the PAW was related to the sterilization efficiency of *S. putrefaciens*. The H_2O_2 molecule may act as one oxidant in the solution, and they can react with organisms [32].

In addition, reactive nitrogen species (RNS) in PAW, such as nitrites and nitrates, were believed to play crucial roles in the sterilization process [16, 31]. The NO_3^- concentration was immediately measured by using the spectrophotometer. Figure 9a shows the NO_3^- concentration of PAW as a function of the water-activated time and applied voltage. The NO_3^- concentration of PAW is significantly dependent on the applied voltage ranging from 6.0 to 12.0 kV and the water-activated time ranging from 0 to 30 min. The NO_3^- concentration can be up to 1.6 mmol/L in the PAW at applied voltage of 12.0 kV when water-activated time is 30 min. The chemical reactions related to NO_2^- and NO_3^- can be described by reactions (2) and (4)–(9).

In this study, NO_2^- concentration was also measured, as shown in Fig. 9b. The NO_2^- concentration initially increases with increasing water-activated time and eventually decreases with further increasing water-activated time at applied voltage of 6.0–12.0 kV. The NO_2^- concentration of PAW can be up to 8.9 μmol/L. This result indicates that the NO_2^- concentration decreases with increasing applied voltage from 6.0 to 12.0 kV. Due to the increasing of H_2O_2 and O_3 concentration at applied voltage of 6.0–12.0 kV, NO_2^- can be oxidized into NO_3^-, ONOOH, O_2NOOH, etc. These complicated oxidation reactions can occur in the plasma zone. The relevant chemical reactions can be described by reactions (7)–(11) [16, 27, 33, 34]. The decay mechanisms of NO_2^- under this experimental condition are still under study.
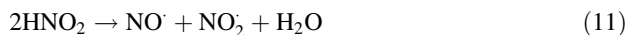
$$NO_2^- + H_2O_2 + H^+ \rightarrow ONOOH + H_2O \tag{7}$$

$$NO_2^- + O_3 \rightarrow NO_3^- + O_2 \tag{8}$$

$$NO_2^- + H_2O_2 + H^+ \rightarrow NO_3^- + H_2O \tag{9}$$

$$HNO_2 + H^+ \rightarrow H_2NO_2^+ \rightarrow NO^+ + H_2O \tag{10}$$

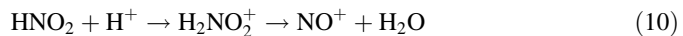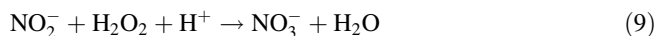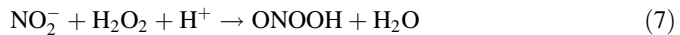$$2HNO_2 \rightarrow NO^{\cdot} + NO_2^{\cdot} + H_2O \tag{11}$$

Fig. 9 a The NO_3^- concentration of PAW as a function of water-activate time and applied voltage, **b** the NO_2^- concentration of PAW as a function of water-activate time and applied voltage. The data points and error bars in all figures represent the averages and standard deviations from three independent measurements
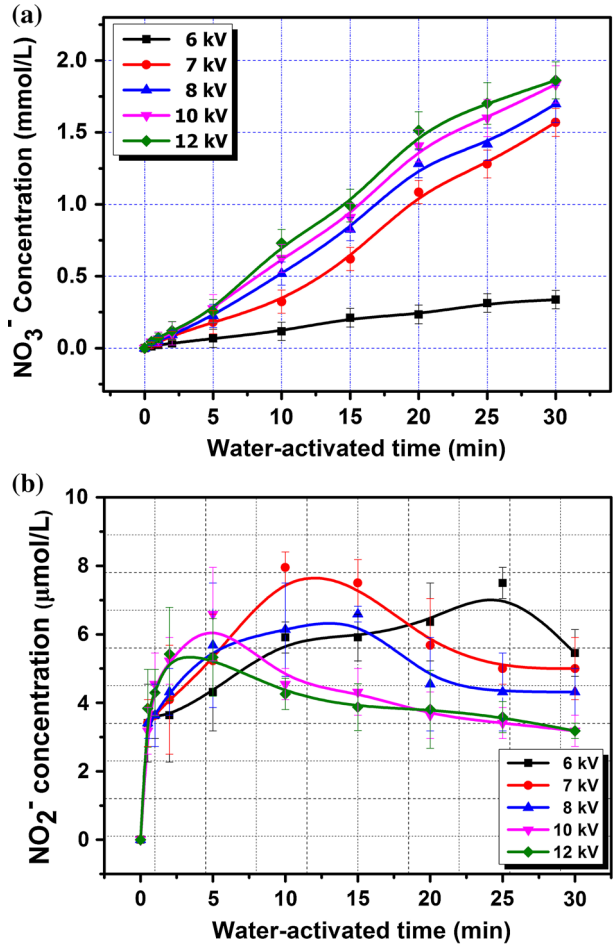

Figure 10 shows the O_3 concentration of PAW as a function of the water-activated time and applied voltage. The O_3 concentration of PAW is significantly dependent on the applied voltage ranging from 6.0 to 12.0 kV and the treatment time ranging from 0 to 30 min. The concentration of O_3 dissolved in PAW increases rapidly in the first minute of water-activated time, and then shows a much slower increase. When water-activated time is 30 min, the O_3 concentration of PAW can reach 0.13 mmol/L at 12.0 kV, 0.11 mmol/L at 10.0 kV, 0.09 mmol/L at 8.0 kV, 0.07 mmol/L at 7.0 kV and 0.04 mmol/L at 6.0 kV. O_3 as a long-lived species is produced in the gas phase. O_3 can transfer from the gas phase into the liquid and participate in the chemical reaction process and inactivation of bacteria in the PAW. In this work, the concentration of O_3 in the effluent gas reaches almost 200 PPM. However, the O_3 concentration of PAW was relatively lower compared with the one of the effluent gas. O_3 can react with $NO^.$ and $NO_2^.$ species in the gas phase or react with nitrites in the liquid, and residual O_3 can dissolve into PAW [27, 35, 36].

The sterilization efficiencies of *S. putrefaciens* cells by H_2O_2, HNO_3 and H_2O_2/HNO_3 mixture solution were measured to analyze the inactivation process of *S. putrefaciens* cells. The solution of *S. putrefaciens* was mixed with sterile water as control group, and their

Fig. 10 The O_3 concentration of PAW as a function of treatment time and applied voltage. The data points and error bars in all figures represent the averages and standard deviations from three independent measurements
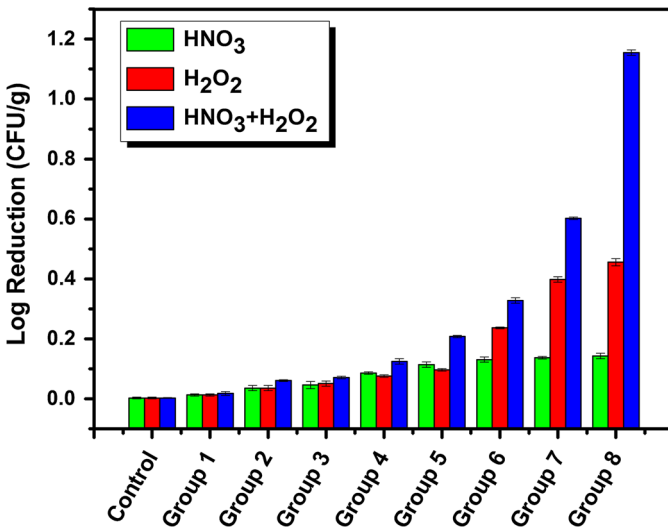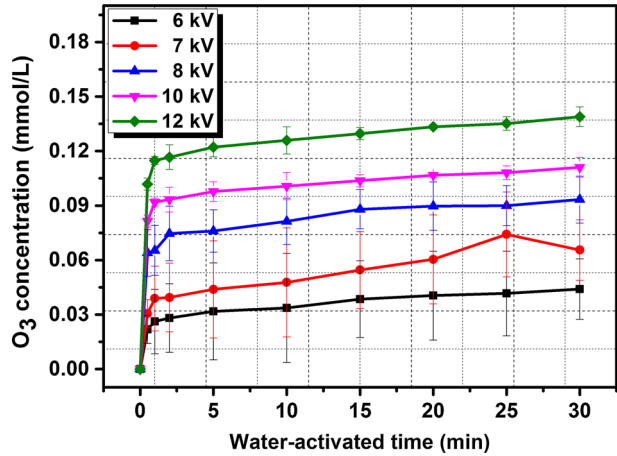

	HNO₃ (mmol/L)	pH	H₂O₂ (mmol/L)	pH	HNO₃/H₂O₂ mixture solution (mmol/L)	pH
Control	0	5.86±0.15	0	5.78±0.21	0	5.71±0.13
Group 1	0.02	4.82±0.32	0.03	5.67±0.16	0.02+0.03	4.65±0.12
Group 2	0.04	4.26±0.31	0.07	5.41±0.12	0.04+0.07	4.13±0.32
Group 3	0.09	4.11±0.13	0.14	5.33±0.25	0.09+0.14	4.18±0.18
Group 4	0.18	4.01±0.21	0.26	5.31±0.11	0.018+0.26	3.93±0.26
Group 5	0.35	3.50±0.22	0.52	5.29±0.10	0.35+0.52	3.44±0.14
Group 6	0.71	3.10±0.34	1.04	5.27±0.13	0.71+1.04	3.13±0.11
Group 7	1.41	2.65±0.12	2.09	5.23±0.16	1.41+2.09	2.41±0.29
Group 8	2.82	2.25±0.21	4.18	5.23±0.21	2.82+4.18	2.20±0.23

Fig. 11 The inactivation efficiencies of *S. putrefaciens* cells as a function of the concentration of H_2O_2, HNO_3 and H_2O_2/HNO_3 mixture solutions. The incubation time of *S. putrefaciens* with the solution is 5 min. The data points and error bars in all figures represent the averages and standard deviations from three independent measurements

concentration is shown in Fig. 11. As shown in Fig. 11, the measurements indicate that HNO_3 solution does not significantly contribute to the inactivation of *S. putrefaciens* cells. The sterilization efficiencies of *S. putrefaciens* cells treated by HNO_3 and H_2O_2 solutions
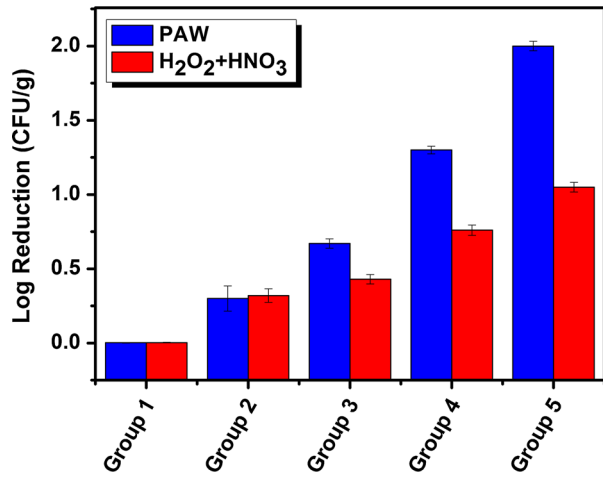
	PAW(V_p=10.0 kV)	H_2O_2/HNO_3 mixture solution	
		H_2O_2(mmol/L)	HNO_3(mmol/L)
Group 1	0	0	0
Group 2	10 min	3.18	0.62
Group 3	15 min	3.65	0.91
Group 4	20 min	3.76	1.41
Group 5	30 min	3.98	1.83

are 0.14 Log Reduction and 0.46 Log Reduction, respectively. Compared to the HNO_3 and H_2O_2 solutions, the H_2O_2/HNO_3 mixture solution is more effective in killing *S. putrefaciens* cells. The sterilization efficiency can reach as high as 1.16 Log Reduction. This indicates that H_2O_2 and HNO_3 mixture in the solution is important for the sterilization in the PAW.

In order to compare the sterilization efficiency of H_2O_2/HNO_3 mixture solution with the one of PAW, H_2O_2 and HNO_3 were added into sterile water. Both the HNO_3 and H_2O_2 concentrations of their mixture solution are similar to the ones of the PAW used to sterilize *S. putrefaciens* cells. As shown in Fig. 12, the sterilization efficiencies of PAW produced at water-activated time of 15, 20 and 30 min are higher than the ones of the H_2O_2/HNO_3 mixture solution. This result indicates that more reactive species in PAW can participate in the inactivation process of bacteria, compared to the H_2O_2/HNO_3 mixture solution. The sterilization efficiency of PAW produced at water-activated time of 5 min is similar to the one of H_2O_2/HNO_3 mixture solution. This result indicates that the synergistic effect of H_2O_2 and H^+ ions plays a crucial role in the sterilization process at water-activated time of 5 min.

Discussion

To evaluate the sterilization efficiency of *S. putrefaciens* cells by PAW, deionized water treated by atmospheric-pressure microplasma arrays was used to sterilize *S. putrefaciens* cells. Reactive species formed in PAW were measured by UV/VIS-spectrophotometer. Other physicochemical properties, such as pH value and initial PAW temperature were

analyzed. Antimicrobial ability of PAW was improved at an increasing applied voltage and water-activated time, as shown in Fig. 3. The ROS and RNS in PAW can play important roles in controlling the sterilization efficiency of *S. putrefaciens* cells by PAW treatment.

As shown in Fig. 6a, the pH values of PAW are typically in the range of 2.5–5.8, depending on the applied voltage and the water-activated time. Antimicrobial activity of acidic solution was analyzed. As shown in Fig. 6b, the inactivation process of *S. putrefaciens* cells is affected by the pH value of culture when it is varied from 2 to 5.9. Change in pH value appears to affect the activity of microorganisms by inducing changes both in the cell walls and the structure of the compound [31]. Low pH value is more favorable for the reactive species to penetrate cell walls. The presence of reactive species reduces the resistance of bacteria in acidic solution [32, 33]. Moreover, pH plays a significant role in the chemical reaction of PAW. The ROS in acidic solution may provide a warranty of relatively good germicidal efficacy [34].

The reactive species have been formed in PAW, and the pH value can have an obvious effect on the chemical reactions during the inactivation process of *S. putrefaciens* cells in PAW. Specifically, a linear increase in the H_2O_2 concentration of PAW was observed with increasing applied voltage from 6.0 to 12.0 kV and water-activated time from 0 to 30 min, as shown in Fig. 8. Meanwhile, a linear increase in the NO_3^- concentration of PAW was observed with increasing water-activated time from 0 to 30 min at applied voltage of 7.0–12.0 kV, as shown in Fig. 9a. However, the NO_3^- concentration was relatively lower at voltage applied of 6.0 kV, compared with the one at applied voltage of 7.0–12.0 kV. The anomalous behavior is ascribed to non-uniform plasma at 6.0 kV, and the amount of NO_2^- oxidized to NO_3^- is much less at 6.0 kV, due to the fact that the pH value of PAW is higher than 4 at voltage applied of 6.0 kV [27, 37]. NO_2^- concentration is highly related to water-activated time and applied voltage, as shown in Fig. 9b. The behavior of NO_2^- in PAW is ascribed to NO_2^- reduction to NO and its oxidation to stable NO_3^-. Moreover, the chemical reaction between H_2O_2 and NO_2^- occurs in acidic solution, leading to the formation of ONOOH [27, 37–39]. ONOOH is a strong oxidant which reacts with biological molecules through various mechanisms. ONOOH is particularly toxic to cells due to its ability to diffuse through cell walls. ONOOH is also able to initiate lipid peroxidation, causing cell wall damage [13].

H_2O_2, as a stronger oxidant, can oxidize molecules of cell walls. And H_2O_2 can be decomposed into ˙OH radical, which promotes the sterilization effect of *S. putrefaciens* cells [31]. HNO_3, as a long-lived secondary product, is not only a stronger oxidant, but also a reactive species in acidic solution. They are considered to be important constituents in the sterilization process. To evaluate antimicrobial activity of HNO_3 and H_2O_2 species in PAW, H_2O_2, HNO_3 solutions and H_2O_2/HNO_3 mixture solutions were used to inactivate *S. putrefaciens* cells. As shown in Fig. 11, H_2O_2/HNO_3 mixture solutions exhibit much higher sterilization efficiency compared with the H_2O_2 or HNO_3 solution. The sterilization efficiency of H_2O_2 was obviously improved due to the presence of nitric acid. Cords et al. [40] studied the effects of pH on bactericidal and sporicidal action of H_2O_2 and indicated a greater H_2O_2 activity in the acidic solution. Alzamora [41] reported the effect of H_2O_2 solutions on the inactivation of *E. coli* in acidic solutions. Therefore, the synergistic effect of H_2O_2 and H^+ ions may play a main role for sterilization process in the PAW. Figure 12 shows that with increasing water-activated time, the sterilization capacity of PAW is higher than the one of H_2O_2/HNO_3 mixture solution. This result indicates that HNO_2, ONOOH and O_2NOOH, etc. can be produced by microplasma in PAW, and these reactive species may be able to participate in the inactivation process of *S. putrefaciens* in PAW.

Pavlovich et al. [42] reported that O_3 is the dominant species for bacterial inactivation under these conditions. However, in this current study, the contribution of O_3 to the inactivation of bacteria is likely negligible. As shown in Fig. 10, the concentration of O_3 dissolved in the PAW is relatively low. Similar results have been reported in the literature [27]. Therefore, O_3 cannot play an important role for antimicrobial effect of PAW in this study.

Those species (H_2O_2, NO_3^-, NO_2^- and ONOOH) may be in contact with cell wall and cell membrane of *S. putrefaciens* cells. They have a direct impact on the cells of microorganisms and especially on their outermost membrane, which can cause membrane damage and cell leakage [18]. Montie et al. [43] suggested that membrane lipids of Gram negative and Gram positive bacteria may be the most vulnerable macromolecule of the cell because of their location near the cell surface and their sensitivity to reactive oxygen species. As shown in Fig. 4, the integrity of the *S. putrefaciens* cells is damaged. Hence cell permeability is destroyed by those species. The broken cell membrane and cell wall led to the leakage of cell protoplasm, electrolyte and DNA, etc. As shown in Fig. 5, the DNA concentration firstly increases with increasing water-activated time and eventually decreases with further increasing water-activated time at voltage of 8.0–12.0 kV. It was reported that oxidants such as free radicals and hydrogen peroxide resulted in the multiple forms of damage, including base modifications of guanosine and double-strand breaks [44]. With increasing water-activated time, the reactive oxygen and nitrogen species (RONS) accumulation could exceed antioxidant ability of DNA and result in adverse effects on DNA [45]. Thus, the DNA concentration is reduced. However, it is difficult to detect the DNA in the solution at applied voltage of 6.0 kV. This result indicates that the RONS concentration at applied voltage of 6.0 kV is relatively lower than the RONS concentration at applied voltage of 7.0–12.0 kV. Although the sterilization efficiency of *S. putrefaciens* cells by PAW can be up to 0.13 Log Reduction at applied voltage of 6.0 kV, the cellular structure cannot be destroyed by RONS. Therefore, it is difficult to detect DNA in the solution at applied voltage of 6.0 kV.

Conclusion

In summary, we demonstrate that the sterilization efficiency of *S. putrefaciens* cells by PAW is dependent on applied voltage and water-activated time, and the sterilization efficiency of *S. putrefaciens* cells can be up to 2.0 Log Reduction. The synergistic effect of H_2O_2 and H^+ ions in the solution plays an important role in sterilization process in PAW. After PAW inactivation, the cell membrane of *S. putrefaciens* is disrupted, resulting in the release of the cytoplasm into the surrounding medium. The reactive species in the PAW can attack the protein and DNA molecules in cytoplasm and inactivate the *S. putrefaciens* cells. This study shows that the PAW generated by atmospheric-pressure air plasmas has enormous potential in the application as an antimicrobial agent.

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