Research on killing *Escherichia Coli* by reactive oxygen species based on strong ionization discharging plasma

Y J Li ¹, Y P Tian ¹*, R H Li ², J Y Gao ³, L J Cai ², Z T Zhang ¹, ², ³

¹ Department of Physics, Dalian Maritime University, Dalian 116026, China
² Marine Engineering College, Dalian Maritime University, Dalian 116026, China
³ Environmental Engineering Institute, Dalian Maritime University, Dalian 116026, China

E-mail: yiping_tian@163.com

Abstract. Reactive oxygen species solution produced by strong ionization discharging plasma was used to kill *Escherichia coli* by spraying. Several effect factors such as pH value, solution temperature, spraying time and exposure time were observed in this study, and their effects on killing rate of *Escherichia coli* were discussed and analysed. Results show that the treating efficiency of ROS solution for *Escherichia coli* is higher in alkaline solution than that in acid solution. The killing rate of *Escherichia coli* increases while the spraying time and exposure time are longer and the temperature is lower. The effects of different factors on killing rate of *Escherichia coli* are as follows: spraying time > pH value > exposure time > solution temperature.

1. Introduction

Reactive oxygen species (ROS), also known as oxygen radicals, refer to ordinary molecules and oxygen radicals with stronger oxidizing capability, which mainly include: superoxide anion free radical (\(\cdot O^2-\)), hydroperoxyl radical (\(\cdot HO_2\)), hydroxyl radical (\(\cdot OH\)) and ozone (O₃). ROS are widely applied as strong oxidant and broad-spectrum bactericide [1, 2]. In this study, highly effective and non-selective ROS solution was produced by ionizing oxygen and water through a strong electric field discharge plasma generator with special dielectric layer and processing technology under the atmospheric pressure and temperature [3-5]. The disinfection effect of ROS solution was also investigated through the killing experiment of bacteria.

*Escherichia coli* (E. coli) as the simulated warfare agent were used in the killing experiment according to the Technical Standard for Disinfection. To well study the disinfection effect of ROS solution on E. coli, the impact factors like the solution pH value, temperature, spray time, exposure time and their interactions were observed and analyzed in the experiment. Therefore, the optimized technical parameters of ROS solution could be obtained and provided for practical use.

2. Materials and methods

2.1. Reagents

Sodium thiosulfate solution (1 %), 0.03 mol L⁻¹ phosphate buffer solution, pH=7.2.

* To whom any correspondence should be addressed.
2.2. Strains
*E. coli* (ATCC8099) using in the killing experiment is preserved in the laboratory strains, purchased from the Management Center of the China Industrial Microorganisms.

2.3. Experimental system
Figure 1 shows the ROS solution process system. According to Reference [6], the homemade plasma device was used to send O₂ and H₂O into the plasma reaction chamber after pre-treatment, where ROS is produced under the strong electric field ionization discharge condition. Then ROS solution went through a mixer with injector. The effective mass transfer efficiency could be obtained by the high-pressure injecting. After a series of complex plasma chemical reactions, the ROS solution was formed.

![Figure 1. Experimental flow chart.](image)

1. Oxygen 2. Filter 3. Ball Valve 4. Solenoid Valve 5. Flow Meter 6. Control Module 7. Cooling System 8. Sprinkler 9. Check Valve 10. Water 11. Pump 12. Booster Pump 13. Buffer 14. Mixer 15. Pressure Gage 16. Sample Connection 17. Altitude Controller 18. Sampling Unit

2.4. Experimental methods
The ROS solution preparation device was turned on first. When the system was stable (about 24 min later), the quantitative bactericidal spray experiment was conducted in laboratory.

2.4.1. Neutralizer experiment
1.0 % sodium thiosulfate was chose as the neutralizer to carry out the neutralizing experiment according to the Technical Standard for Disinfection with *E. coli* ATCC8099 as the test organism [7].

2.4.2. Carriers quantitative spray sterilizing
The concentration of bacterial suspension was diluted to 5×10⁷ cfu mL⁻¹. Bacterial infectant disc was prepared in accordance with the Technical Standard for Disinfection. Then put them in sterile glass petri dishes with the diameter of 55 mm. Each petri dish contained three pieces of disc arranging in a triangle. The petri dishes were placed in the sample positions of disinfection and then sprayed with ROS solution. After certain minutes, dose of neutralizer was dropped onto the disc immediately. 10 minutes later, bacterium suspension was diluted to proper concentration and the numbers of bacteria were counted. The average value of three paralleled samples in each group was adopted. The control group replaced the disinfectant with aqueous solution without oxygen active particles. The other operations were the same.
2.4.3. Effect of Bactericidal

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\text{killing rate} = \frac{\text{bacterium amount in the positive control group}}{\text{bacterium amount in the negative control group}} \times 100 \%
\]

Positive control group: results of the experiment when replacing disinfection agent solution with hard water; negative control group: the experimental group. Other conditions were equal.

3. Results and Discussion

3.1. The effect of pH value on killing E. coli by ROS solution

Experimental condition: ambient temperature \( T_1 = 23 \, ^\circ\text{C} \), ambient relative humidity \( D = 42.6 \% \); solution temperature \( T_2 = 20 \, ^\circ\text{C} \); spray time \( t = 2 \, \text{s} \); the oxygen ventilation volume of preparation device maintains 4 L min\(^{-1}\).

![Figure 2. The effect of pH on E. coli killing rate.](image)

Figure 2 shows the effects of ROS solution at different pH (from 3.4 to 10.4) on the killing rate of E. coli under the same spray time of 2 s. The results indicate that the killing rate keeps rising while the pH value of ROS solution increases, which mainly results from the rising content of \( \cdot \text{OH} \) in ROS solution. The reaction rate constant of \( \cdot \text{OH} \) is \( 10^9 \, \text{L/(mol s)} \). It could rapidly react with lipid, protein and nucleic acid of bacterial cells, which only last from 1 to 10 s [8]. The pH value falls while the increasing rate of sterilizing goes up significantly with the reaction continuing. That is mainly because that the ROS solution contains not only strong oxidizing and unstable chemicals such as \( \cdot \text{OH} \), but also stable active substance, such as ozone being the major one. The concentration of ozone water increases while the pH value of the solution decreases. To sum up, the disinfectant effect of ROS solution on E. coli strengthens with the solution pH value rising. The ROS solution is more effective in alkaline condition than in acid condition.

3.2. The effect of temperature on killing E. coli by ROS solution

Experimental condition: ambient relative humidity \( D = 42.6 \% \); pH=7.2; spray time \( t = 2 \, \text{s} \); the oxygen ventilation volume of preparation device maintains 4 L min\(^{-1}\).
The effects of ROS solution at different temperature (from 8~23 °C) on killing rate of *E. coli* are shown in figure 3, under the same spray time of 2 s and pH of 7.2. Results show that the killing rate of *E. coli* keeps rising while the water temperature falling. The killing rate maintains 100 % regardless of the temperature with the reaction continuing. There are two possible reasons for these results: the first one is that lower temperature helps to increase the mass transfer efficiency, therefore the initial concentration of ROS solution is relatively high; the second one is that the decay rate of active substance in ROS solution decreases with the temperature dropping, therefore when the ROS solution is sprayed out after efficient atomization, the lower temperature leads to higher killing rate. In addition, lower temperature may lead the bacteria at dormant state, which results in suspended animation. To sum up, lower temperature is advantageous to kill *E. coli*.

**Figure 3.** The effect of temperature on *E. coli* killing rate.

**Figure 4.** The effect of spray time on *E. coli* killing rate.
3.3. The effect of spray time on killing E. coli by ROS solution
Experimental condition: ambient temperature $T_1=23$ °C; pH=7.2; ambient relative humidity $D=42.6$ %; solution temperature $T_2=20$ °C; the oxygen ventilation volume of preparation device maintains 4 L min$^{-1}$.

Figure 4 shows the effects of spray time on killing E. coli by ROS solution with pH of 7.2. The killing rate keeps rising with the spray time extending (regardless of water loss). In addition, the longer spray time leads to shorter exposure duration of ROS solution. This is probably caused by higher density of ROS solution on bacterial contamination disc with longer spray time, which promotes biochemical reaction between ROS solution and bacteria, and results in higher killing rate. However, considering energy consumption, the dose and requirements in Technical Standard for Disinfection, it is economical and effective to maintain spray time for 2 s and allow the reaction lasting for over 2 s.

3.4. Orthogonal experiment
In order to find out the main factor influencing the killing rate of E. coli, and the optimum reaction condition, the following orthogonal experiments were conducted in the hope of providing technical parameter for massive biological contamination. The results are shown in table 1 and 2.

### Table 1. League table of experimental factors.

| Influencing factors | 1     | 2     | 3     | 4     |
|---------------------|-------|-------|-------|-------|
| Contents            | pH    | solution temperature (°C) | spray time (s) | exposure time (s) |
| Level               | 1, 2, 3 | 1, 2, 3 | 1, 2, 3 | 1, 2, 3 |
| Value               | 3.4, 7.2, 10.4 | 8, 16, 23 | 1, 2, 3 | 5, 60, 300 |

### Table 2. E. coli orthogonal experiment table.

| Number | A | B | C | D | Killing rate (%) |
|--------|---|---|---|---|------------------|
| 1      | 1 | 1 | 1 | 1 | 99.931           |
| 2      | 1 | 2 | 2 | 2 | 99.971           |
| 3      | 1 | 3 | 3 | 3 | 100.000          |
| 4      | 2 | 1 | 2 | 3 | 100.000          |
| 5      | 2 | 2 | 3 | 1 | 100.000          |
| 6      | 2 | 3 | 1 | 2 | 99.938           |
| 7      | 3 | 1 | 3 | 2 | 100.000          |
| 8      | 3 | 2 | 1 | 3 | 100.000          |
| 9      | 3 | 3 | 2 | 3 | 99.996           |

k1 99.967 99.977 99.956 99.976
k2 99.979 99.990 99.989 99.970
k3 99.999 99.978 100.000 100.000

K stands for the average value of parallel samples

| R      | 0.032 | 0.013 | 0.044 | 0.030 |

R stands for range

Optimum choice A$_3$ B$_2$ C$_3$ D$_3$ A$_3$ B$_2$ C$_3$ D$_3$

The range in experimental result shows that the effects of different factors on killing rate of E. coli are as follows: spraying time > pH value > exposure time > solution temperature. The optimum reaction conditions are: ambient temperature=19 °C, ROS solution temperature=16 °C, solution
pH=10.4, spray time=3 s, reaction time=5 min. In this case, the bacteria solution with original concentration of $1.0 \times 10^8$ cfu mL$^{-1}$ could be disinfected by 100%.

3.5. Resurrection experiment
were selected according to Technical Standard for Disinfection, i.e. In order to further test the effectiveness and feasibility of ROS solution in killing *E. coli*, the resurrection experiments were carried out in this study under the following conditions: Ambient temperature=23 °C; solution pH value=7.2; ambient relative humidity D=42.6%; solution temperature $T_2$=20 °C; the oxygen ventilation volume of preparation device maintains 4 L min$^{-1}$. After spraying for 2 s, we observed whether there was any survival *E. coli* in 24 h, 36 h, 48 h, 72 h, and 96 h. The observation result shows that no *E. coli* resurrects after disinfection by ROS solution, which confirms the effectiveness and feasibility of ROS solution in killing *E. coli*.

4. Conclusion
ROS solution prepared through strong electric field ionization and efficient immiscible can kill *E. coli* by spraying. This is a disinfection and epidemic prevention technology which is efficient and environmental friendly. The experiment results show that: ① Alkaline condition is advantageous to the killing of *E. coli* by ROS solution. ② The killing rate of *E. coli* by ROS solution increases with the solution temperature decreasing. ③ The killing rate of *E. coli* keeps increasing with the spray time extending and meets the requirements of *Technical Standard for Disinfection* when the spray time lasts for 2 s. ④ The effects of different factors on killing rate of *E. coli* are as follows: spraying time > pH value > exposure time > solution temperature.

Acknowledgments
This work is supported by The National High Technology Research and Development Program of China under Grant No 2012AA062609, The International Science and Technology Cooperation Project of China under Grant No 2010DFA61470, The National Science Foundation for Distinguished Young Scholars of China under Grant No 61025001, The Fundamental Research Funds for the Central Universities under Grant No 2012QN067 and 2012TD061.

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