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A Novel Sterilized Method for *Escherichia coli* Infected Eggs: Atmospheric Arc Discharge Technology

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In order to avoid environmental microorganism pollution of fresh eggs, a novel arc discharge equipment was built up, which can be worked in air environment at room temperature. As a case, clean and fresh eggs were inoculated with *Escherichia coli* (*E. coil*, $10^6 \sim 10^8$ Colony-Forming Units (CFU)/mL) suspension, then eradiated under the atmospheric arc discharge for different durations. Surface and cross section morphologies of irradiated *E. coli* collected from the eggs respectively by Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) analysis indicate that arc plasma phase could effectively and efficiently inactivate *E. coli* in a very short duration time through etching effect and protein leakage. Subsequently, the analysis of chamber diameter was measured to assess the storage and freshness preservation performances of eggs. Our results indicated that this technology could effectively inactivate *E. coli* suspended on the surface of eggshell and extend egg shelf-life, which open the possibility of industrial applications of atmospheric arc discharge in sterile area.

Keywords: Inactivation Mechanism, Atmospheric Arc Discharge, *E. coli* Inactivation.

1. INTRODUCTION

As total production of eggs in the world increases up to nearly 80,000 kT in recently five years, the total production of China with partial ratio of 33.5% is also increasing synergistically from 27022 kT in 2008 to 28612 kT in 2012 by increment rate of 1.2% per year, indicating a larger domestic consumption market.[1] However, this large domestic consumption is arriving in a constant level and being saturated as the production increment rate is increasing gradually, leading to a higher and higher pressure on the export trade and secondly deep processing of egg product. At the same time, newly built trade regulations from both Europe and the United States further enhance the import requirements and standards of outside commodity by establishing a series of safety and health standards, especially for eggs, which means that the imported ones must be clean and fresh in a higher degree.[2]

According to traditional China's consumption habits, most of fresh eggs are directly consumed by domestic customers, which occupied nearly more than 90% of total production.[3] With the formation of newly published food safety law, safety and health standards of eggs are paid more and more attention from farm households, related companies and consumers.[4] Studies indicate that the number of bacteria per egg from the general market will reach more than 14 million while this number will reduce less than 4.0 thousand after cleaning and inactivation treatment. Therefore, it can be concluded that clean and sterilization treatment of eggs after initial birth and before market is becoming more and more indispensable to avoid the potential anxiety and doubt from consumer and healthy problems.

During recent years, research on the storage and fresh preservation technology has been extensively studied, such as heat treatment, chemical sterilization, ultra high pressure sterilization and ultraviolet sterilization,[5] etc. However, the current packaging technology and equipment is difficult to meet the requirement of clean packaging technology,[6] such as damage to the product composition, environment and biological pollution, chemical residues, etc.[7] As for eggs, which is very sensitive to thermal inactivation process, such as steam treatment and joule heating, and always lose its nitrate compound and odor when maintained during a certain time at high temperatures. So it is

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easy to deduce that the key point to sterilize the fresh egg is to avoid thermal effects while keep its nutrient compound safe and healthy.

Compared with common cold sterilization methods, such as hydrogen peroxide solution and the ozone gas, cold atmospheric air plasma discharge as an alternative non-thermal sterilization method since initially appeared at the beginning of the 90's,[8] could effectively inactivate pathetic microorganism and planktonic biofilms originated from the surface of materials or apparatus,[9, 10] which has wide applications, such as physical chemises, medicine science, wound healing and tissue regeneration, bio-decontamination.[11, 12] This project will build up a novel type of sterilization technology-arc discharge technology to overcome above disadvantages. Based on sterilizing theory, inactivation effect is verified by sterilizing *E. coli* bacterial on the surface of fresh eggs, and subsequently assessment on the health and nutrition is executed with the aim of prolonging the shelf-life of fresh eggs. Our results will provide a preliminary exploration and pave the way for the application of new sterile packaging equipment.

2. MATERIALS AND METHODS

2.1. Atmospheric Arc Discharge Equipment

The atmospheric arc discharge equipment located in the ceramic tube consists of parallel copper electrodes, which is connected with AC high-voltage power supply, as shown in Figure 1. Once the power is supplied, the entranced air flow from the top side driven by air compressor can be ionized to form arc discharge. Eggs can be located on a conveyor belt under the direct exposure to the plasma plume. With rotating supporter, testing eggs can be self-rotated meanwhile passed through plasma plumes area by the conveyor belt, realizing a 360° sterilization in 100×50 mm^2 area with a treatment yield of 10,000 per hour. Meanwhile the distance between the nozzle and the conveyor belt can be adjusted freely, achieving different levels of sterilizing duration, as the air flow and discharge power do.

Treatment parameters in our study can be listed as: exposure duration: 0, 30, 60, 120 and 300 s, and the distance between plasma nozzle and eggs surface is set at 3.0 cm, the air flow is 40 L/min with a gas pressure of 11.76 MPa. After treatment process stage, each group was cultured for 5 days at 37 °C in constant temperature and humidity box.

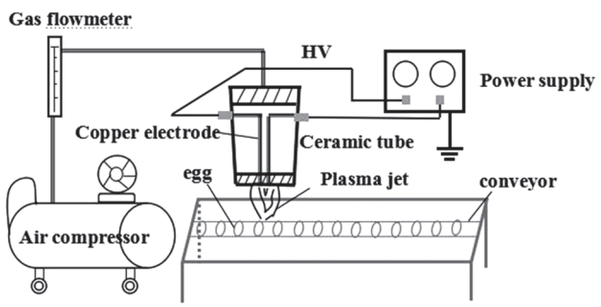

Fig. 1. Equipment schematic diagram of sample handling and packaging.

2.2. Preparation of Bacterial Suspension

E. coli (ATCC8099) frozen strains used in this study was purchased from China Center of Industrial Culture Collection. The strain was conserved in sterile glycerol at −80 °C after being resuscitated by three times. At the beginning of sterilization experiment, 1 mL of the *E. coli* solution were inoculated into 50 mL of Luria-Bertani (LB) broth (1% tryptone; 0.5% yeast extract, 1% sodium chloride, pH = 7.4) at 37 °C for 12∼16 h at a shaking state at speed of 180 rpm.[13] Under sterile conditions, original liquid was diluted by 0.85% sterile saline solution until the concentration of bacterial suspension reaches $10^6 \sim 10^8$ CFU/mL, which can be estimated by the classical plating counting technique on LB agar.

2.3. SEM/TEM/Protein Leakage Analysis

Atmospheric arc discharge treated or untreated samples collected from the eggs were fixed by 2.5% glutaraldehyde solution overnight at 4 °C.[14] After washed with 0.1 M phosphate buffer solution (PBS) three times, the samples were fixed in 1% osmic acid and 0.1 M PBS for 2 h. Then the cells were washed with the same buffer three times followed by a series of dehydration, using step by step increasing concentrations of ethanol (10%, 30%, 50%, 70%, 90%, 95% and 100%).[15] After dehydration, the samples were washed with tert-butyl alcohol three times and dried to powder in VFD-21S freeze drier for SEM analysis (FEI, Quanta 200, America).

Similarly, the samples prepared for TEM also firstly went through steps of fixation, washing and ethanol gradient dehydration. Then infiltration and embedment of Epon 812 was performed, which polymerized at 60 °C for 48 h.[16] Finally, the samples were processed into ultrathin sections by 60∼80 nm, then stained with 2% uranyl acetate saturated solution and Lead citrate for 10 min respectively to observe the micrographs obtained from TEM (FEI, TECNAI G2 20 TWIN, America).

E. coli suspension, which was used for inoculating onto egg, was centrifuged at 4500 rpm for 15 min, and then the sedimentation was spread onto sterile glass slides followed by drying out under sterile conditions. Then the samples were treated with arc discharge for different times. Afterwards, the samples were washed with 4.0 mL of sterile saline followed by centrifugation at 10,000 rpm for 10 min. The combination of supernatant liquid and Coomassie brilliant blue was used to determine the absorbance with 722N ultraviolet visible spectrophotometer under 595 nm,[17] and the value of protein leakage depends on the changes of absorbance.

2.4. Assessment of Egg Quality Traits

In order to assess possible negative effects of the arc discharge on egg quality traits, the gas chamber diameter (as an indirect method to evaluate the shell membrane integrity) was measured. The diameter of gas chamber was measured with a vernier caliper from three different angles with the help of light sources.[18]

3. RESULTS AND DISCUSSION

3.1. Microbiological Analysis

The inactivation effect and its influence on the number of *E. coli* were clearly observed as a function of storage days, as shown in Figure 2, all total number of colonies sampled from the eggshell surface in the same size decreases with storage days. Comparing with original samples, exposed ones decrease sharply from 70 to 1 CFU/cm^2 after arc discharge eradication, event down to a sterile state if the discharge duration is long enough. Fatherly, if the storage duration is postponed enough to more than five days, then all the treated samples come into a sterile state, which could also be reached faster by arc discharge exposure in a shorter time. So it can be theoretically deduced that by arc discharge treatment the bacterial from the surface of eggs can be cleared out and left the samples in a sterile environment instead of longer storage times, which open up a novel method to avoid the biological pollution from environmental bacterial, such as *E. coli*.

Compared with other inactivation methods, such as thermal and dielectric barrier discharge (DBD) technology, arc discharge technology in our study will be more efficient and effective within several seconds, since the exposure duration for DBD technology will spend several minutes.[19] In fact, the inactivation mechanism of arc plasma discharge owns some different characteristics, as can be elucidated by SEM and TEM results shown in Figure 3. Comparing with non-treated samples, holes were observed on the treated *E. coli* collected from the surface of eggs at short exposure durations of 30 s. If the exposure duration is longer enough of more than 300 s, irradiated area will increase into a local collapse as a fragment. *E. coli* surface can be obtained through cross section by TEM measurement as shown in Figures 3(d)–(f). Comparing with untreated one, wrinkles in the treated surface will exist and grow up into an etched line, resulting in the scattered fragments as shown in Figure 3(f), which is consist with SEM results in Figure 3(c). As is well known that, arc plasma phase will accelerate electrons up to a certain energy level that required driving a reactive environment through excitation, ionization and dissociation processes. Then, a reactive environment arise, including reactive oxidized species of OH, O and ON, which will directly react with the cell membrane, resulting in the intense bombardment of micro-organisms, as is called etching effect.[20, 21] After all, plasma sources of different designs but of very similar properties are known to be able to interact not only with the cell membranes, but the plasma effects can also penetrate the cell interior, such as protein leakage.[22]

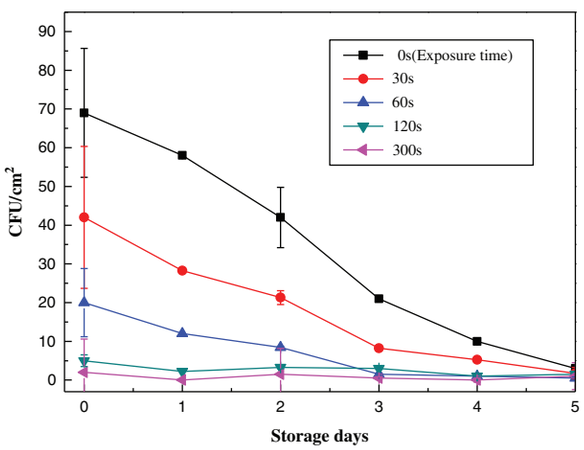

Fig. 2. Variation of total number of colonies sampled from the eggshell surface under different exposure times.

As a frequent consequence of free radicals and ROS species, membrane lipid peroxidation effects happens in biological membrane systems, leading to protein leakage, as shown in Figure 4. As the exposure duration increases up to 100 s, measured protein leakage from the intercellular phase keeps in a constant level of less than 20 μg/mL, and then grow faster into a high level of more than 40 μg/mL when the exposure duration excess 100 s.

Although the inactivation mechanism of arc plasma discharge will be more complex for different storage durations, such as, protein leakage, etching effect and so on,[23] due to the fact that physical, chemical and microbiological reactions happened in the treatment process and had a mutual influence on the inactivation effect, arc discharge used in the sterilization process of fresh eggs is proved to have a huge potential commercial applications in the future, such as some fruits and vegetables for the inactivation method in blueberries.[24, 25]

3.2. Assessment of Egg Quality Traits

In order to assess the potential application probability of arc discharge technology in agricultural products processing and storage industries, characteristics of egg freshness such as chamber diameter was measured under different exposure times as a function of storage days, as well be depicted as following.

Figure 5 shows the variation of chamber diameter under different exposure times as a function of storage days. Whether exposure duration is longer or shorter, the increment of entire chamber diameter treated by arc discharge plume increases with storage days with comparison to original ones, which even increased up to 9.0 mm after 4 storage days. It is easy to deduce that the internal water of eggs would evaporate naturally through surface pores of egg shells, which is in the scale of micrometers and thus synergistically play a positive contribution

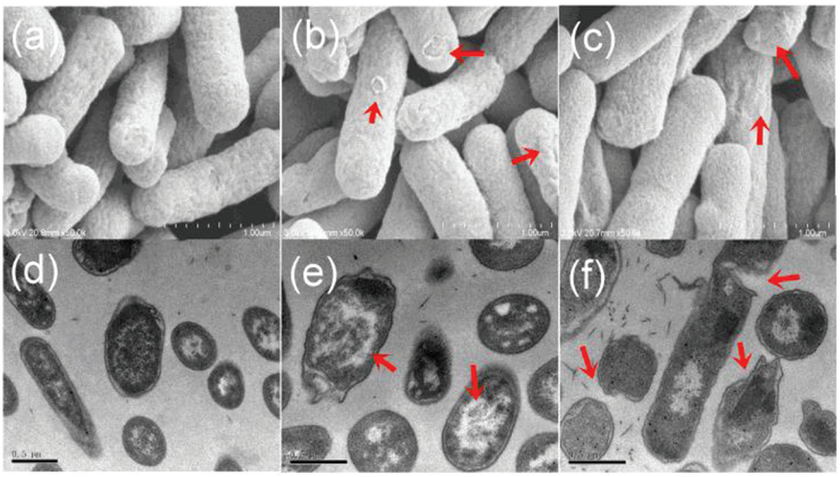

Fig. 3. SEM and TEM images of various types of *E. coli* cell samples from the eggshell with plasma treatment of 0 s (a, d) 30 s (b, e), 300 s (c, f), respectively. The scale bars are 1.0 μm and 0.5 μm respectively.

to the expansion of chamber diameter increments. Essentially, protein membrane out of the eggshell surface at birth will effectively protect the water evaporation and keep the size of chamber diameter constant. Once processed under the arc discharge, this protein membrane was damaged or born into gases though oxidation reaction, leaving the surface pore naked and thus leading to the higher increasing rate of chamber diameter.

Whether its water loss offset the sterilize state will be studied further from the viewpoint of rotting rate as shown in Figure 6. We carry out spot check on the treated eggs at the middle 15th and final 21st days with the objective of investigating the influence of plasma treatment on the shelf life of eggs. The rotting rate of eggs treated under different plasma treatment times from 0 s to 300 s increases as storage duration until the yolk part of egg is scattered under light irradiation. It can be also seen that part of egg under 60 s plasma treatments has lower rotting rate, which is only 30% at 15th storage duration and 75% at final days. Thus, it can be concluded that longer plasma treatment time is not always positive for egg preservation from the viewpoint of sterilization effects, 60 s treatment is better to keep egg quality and extend shelf-life, as more than 60 s will pose a negative effect by dissolve or damage protein membrane from the egg surface.

In the future, the reaction mechanism between the plasma irradiation and egg surface is still needed to pay more attention meanwhile further studies are required to rectify and ensure the effectiveness of fresh preservation and storage of eggs, such as nutrition integrity, shelf-life time, freshness quality and freshness, our technology will supply a new preservation method for fresh egg preservation and transportation.

Moreover, nanomaterials[26–45] and nanotechnology[46–63] have been paid more and more attention,[64–75] and many applications[76–93] including antibacterial application[93–99] of them have been reported, we will try to introduce

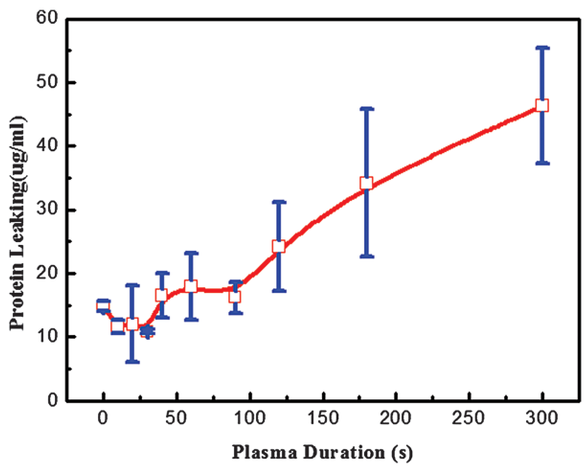

Fig. 4. Protein leakage of intercellular compounds as a function of exposure duration.

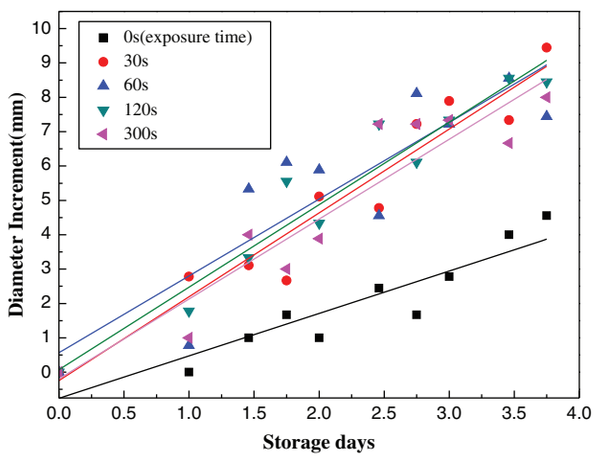

Fig. 5. Variation of egg gas chamber diameter after exposure from 0 to 300 s during storage days.

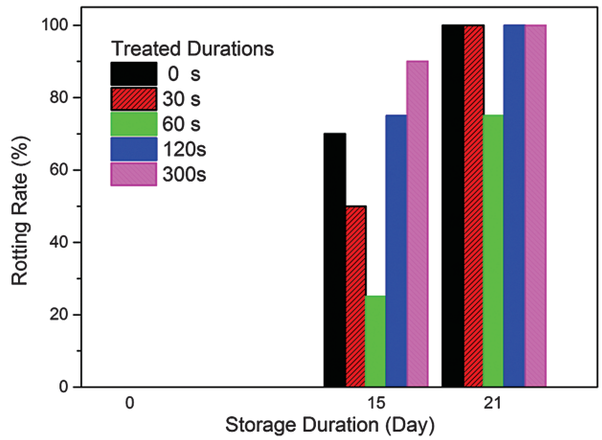

Fig. 6. Rotting rate trend of plasma treated eggs with different storage times.

nanomaterials into our coming research to further improve the results.

4. CONCLUSIONS

Novel inactivation equipment in clean egg production line was developed in this study, which includes both arc discharge and dynamic transport system to preserve egg freshness and prolongs shelf-life. The inactivation measurements indicate that total number of colonies on eggshell surface could be reduced significantly, even into a sterile state if the eradication duration is more than hundred seconds, as a novel method to avoid the biological pollution from environmental bacterial. The inactivation mechanism was also studied by morphology analysis that arc plasma discharge will create a reactive environment to interact with cell membranes, which cause etching effect and cellular protein leakage. Finally, egg chamber diameter was assessed to forecast the potential application probability in agricultural product processing and storage industries. Although this arc discharge technology was initially used to preserve the egg freshness and its inactivation mechanism was also needed to be well understood, this potential application in fresh produce would be verified in our study and should become alternative for fresh egg transportation and shelf-life prolongation.

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