Germicidal effects of surface and aqueous ozone exposures on *Escherichia coli* O157:H7

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Abstract

Pathogenic bacteria present as contaminants in food and water, may survive during processing, grow during storage and cause spoilage of food or contamination of water leading to diseases to consumers. Hence, this research investigated the bactericidal action of gaseous ozone in both surface and aqueous exposure techniques for the elimination of *Escherichia coli* O157:H7. The ozone generated by Dielectric Barrier Discharge (DBD) using oxygen gas as a source of ozone. *E. coli* O157:H7 exposed to ozone by surface technique at concentration of 9.8 mg L^{-1} for 2.5-7.5 min and subsequently incubated at 35 °C for 24 h. The results showed that surface ozone treatments exhibited the most effectiveness to inactivate and cause complete reduction (100% in colony forming units (CFU) mL^{-1}) of *E. coli* O157:H7 growth after exposure times used. On the other hand, aqueous ozone treatments, zero plate counts were observed of *E. coli* O157:H7 at ozone concentrations of 20.3 and 21.6 mg L^{-1} after 18 and 10 minutes, respectively. This study pointed to a good candidate for decontamination of *E. coli* O157:H7 in both surface and aqueous ozone treatments. Therefore, the application of ozone may be useful method for surface and aqueous sanitations to control some of *E. coli* O157:H7 diseases.
Keyword: *Escherichia coli* inactivation, Germicidal, Ozone.

Introduction

Ozonation was found to be an effective method to eliminate some of pathogenic microorganisms of great public health concern (Kotp and Afifi, 2009). We cannot avoid the process of fresh-cut fruits because it is an essential process, contamination by microorganism already start during this process which could easily lead to food-borne disease (Monaghan and Huchison, 2010). The foodborne diseases are caused by many microorganisms like *E. coli* and *Salmonella spp*. *E. coli* one of the famous and/or the major enteropathogenic bacteria that causes foodborne diseases. Also, many researchers have reported their concerning about the infection of imported fruits and vegetables by *E. coli* (Ali *et al.,* 2002; Omole and Longe, 2008; Abd El-Aziz, 2011; Muhamad *et al.,* 2015). It has been reported that gaseous ozone prevented the growth of disease occurrence in tangerine known as "green mold" caused by *Penicillium digitatum* during storage at 25 °C (Boonkorn *et al.,* 2012). However, because the reduction in its oxidation property, ozone has a limitation in water dissolution. As for microorganism, micro-bubbles ozone (MBO) technique reduced water pollutant, which was observed in phenol degradation (Li *et al.,* 2012). Micro-bubbles ozone reduced *E. coli* O157:H7 of viable cell (5.0-7.4 log) better than those of the ozonated water, and resulted in decontamination on the surface of leafy vegetables (Inatsu *et al.,* 2011). Ozone was effective at inactivating *E. coli* O157:H7 (Sharma *et al.,* 2002; Ngadi *et al.,* 2004), and affect some major physiological and biochemical propertiesof some plants (Abdel Nasser, 2002; Bonjar, 2004; Koseki and Isobe, 2006; Yuan *et al.,* 2006; Zhang *et al.,* 2006; Baafi and Safo-Kantanka, 2008; Hasanuzzaman *et al.,* 2010). This study aimed to determine the effective concentrations and treatment times of both surface and aqueous ozone exposure techniques on inactivation of *E. coli* O157:H7 to be applied in both food and water contamination, respectively.

Materials and Methods

Ozone generator

In the present work the ozone generator, used to generate the ozone at different concentration values, by using an oxygen gas, consisted of coaxial electrodes. The inner electrode was made of brass rod, the length of the inner electrode was 500 mm and it`s diameter was 9.0 mm. The inner electrode was putted in cylindrical tube from Pyrex glass, it`s length was 700 mm, the inner diameter of tube was 11.5 mm, the thickness of the glass tube is 1 mm the

outer diameter of glass tube was 13.5 mm. The outer electrode was made of a graphite coated on the outer wall of glass tube, which has been used as a dielectric material. The gap space between the inner electrode and the inner wall of glass tube was equal to1.25 mm. The length of the generator region was equal to 500 mm and the pressure of gas could be controlled by the needle valves which enabled not only controlling the pressure gas but also the flow rate in the generator.

The values of applied voltage on the discharge tube of ozone generator and the ozone concentration were measured by using the analyzer. The conditions of measurements were, the flow rate of oxygen gas (0.45 L min^{-1}), gas pressure (1008.8 mbar), and the temperature of gas was at 37 ^{o}C. These values were used for the treatments of *E. coli* O157:H7.

The pressure inside the discharge generator tube was measured by using a mercury manometer and a gauge model P200-H (RS-497-606) which enabled the pressure to be measured in the range from 1-2.25 bar within 1 mbar experimental error. The flow rate of the gas was measured by using a flow meter (Cale Parmer) which has a range from 0 to 15.7 L min^{-1} for oxygen gas used (Fig. 1). The inner and outer electrode connected to the electric cruciate. Other details of the electric circuit were mentioned by Garamoon *et al.* (2002).

Electric circuit

The electric circuit of the discharge is consisting of AC power supply (0-220 volt, 50 Hz) connected to a high voltage transformer with variable output, 0-15 kV, maximum output current was 30 mA, in order to protect the transformer, a limiting resistance of 1.32 M is connected in series with a transformer, which protect the high voltage transformer from a short circuit current. In order to measure the applied potential across the discharge ozone generator tube, a potential divider has been built, consisting of two resistors in the ratio 1:450 connected in parallel of the discharge tube. Only the potential difference across the lower part of the divider was measured, and then the applied potential across the tube could be estimated. The electrical circuit of discharge is shown in Fig. 2.

Ozone analyzer

The concentration of ozone was measured by using an ozone analyzer, model H1 (AFX-instrumentation) using the absorption in Hartley band (Molina and Molina, 1986). The ozone concentration was calculated from Lambert-beer absorption law (Kogelaschatz, 1988). The strongest absorption was found in the ultraviolet region of the spectrum between 200-300 nm (Fig. 3).

Effect of different exposure times on inhibition of *E. coli* O157:H7

The bacterial Cell suspension of *E. coli* O157:H7 ATCC: 43889 was prepared using the stock bacterial culture inoculated into trypticase soy broth (TSB) and incubated at 37°C for 10 h. One milliliter of bacterial suspension was added to TSB (9 mL) to carry out the serial dilutions. For the growth inhibition, 0.1 mL of bacterial cell suspension for each dilution was determined by spread plate technique on trypticase soy agar (TSA) then exposed directly to ozone by surface exposure technique (Kotp and Afifi, 2009) at concentration of 9.8 mg L^{-1} for 2.5, 5, and 7.5 min. and incubated at 37°C for 24 h. The colony of *E. coli* was shown as the mean number of colony forming units (CFU mL^{-1}) and the reduction in colony were counted by % reduction in CFU mL^{-1} of *E. coli* O157:H7 (Jyoti and Pandit, 2004). All the experiments were carried out in triplicates.

Effect of different surface ozone concentrations on inhibition of *E. coli* O157:H7:

This experiment was carried out on both, the shortest exposure time shown complete reduction (CFU mL^{-1}) in *E. coli* O157:H7, and on the available count of cells in serial dilution. Thus, different surface ozone concentrations (mg L^{-1}) 5.6, 9.8, 13.9, 18, 22.2, and 24 for 2.5 min were evaluated. The growth inhibition measured as previously mentioned.

Effect of different aqueous ozone concentrations/exposure times on inhibition of *E. coli* O157:H7

All the aqueous ozone treatments were conducted using 100 mL preparation in conical flask (250 mL) of *E. coli* O157:H7 containing approximately (10^{8} CFU mL^{-1}). The flasks were exposed directly to ozone by inserting the ozone canal inside the cell suspension. For the growth inhibition, 0.1 mL of cell suspension was evaluated by spread plate technique on trypticase soy agar (TSA) (Kotp and Afifi, 2009). The colonies of *E. coli* O157:H7 were counted as previously mentioned. The first was studied the effect of different ozone concentrations (9.8, 13.4, 18, 21.6, and 22.8 mg L^{-1})/different exposure times (4, 10, 20, and 30 min), the second attempt was carried out using different ozone concentrations (9.8, 13.4, 18, 21.6, 22.8, 23.2, 23.5 and 24 mg L^{-1}) for exposure time (4 min), the third was carried out using different exposure times (0.5, 1, 2, 4, 6, 8, 10 and 33 min) at ozone concentration (9.8 mg L^{-1}), and the last attempt included the effect of different exposure times (1, 3, 8, 18, 33 and 45 min) and ozone concentrations (12.2, 20.3, and 23.2 mg L^{-1}).

Results and Discussion

Preparation of ozone

Efficacy of surface and aqueous ozone was evaluated for the purpose of decontaminating with *E. coli* O157:H7 to be applied in food and water

contamination, respectively. Ozonation is a well-known effective method for controlling diseases caused by pathogens. Ozone is a highly active disinfectant material and can be widely used in surface sterilization method (Ibeto *et al.,* 2010; Okpara *et al.,* 2011; Abdellah *et al.,* 2012; Atefe *et al.,* 2016). The role of ozone in growth inhibition of bacteria, fungi as well as viruses was confirmed to be generally recognized as safe (GRAS) (Graham, 1997; Kim *et al.,* 1999 and Ikeura *et al.,* 2011). The values of applied voltage on the discharge tube of ozone generator and the ozone concentration were measured by the analyzer as represented in Fig. 4.

The relation between the ozone gaseous concentration and the applied voltage can be divided into three parts, which can be observed clearly at the lowest pressure used. In the first part (I), no ozone was measured (concentration equal zero) because the applied voltage was not sufficient enough for breakdown in the gas to occur. The onset voltage for breakdown to occur depends mainly on the gas pressure, gap space, dielectric material and its thickness. Part (II) began at the onset voltage was breakdown in oxygen gas occur and separation of oxygen producing atomic oxygen, which recombines with oxygen molecule to produce ozone, and thus ozone can be detected and measured. The ozone concentration could be detected to increase at a slow rate up to a certain applied voltage and then aimed to increase at high fixed rate. Ozone was being produced through different mechanisms in pure oxygen (Eliasson *et al.,* 1987). In part (III), the rate of raising the ozone concentration aimed to slow down, which could be clearly noticed at lower pressures under high applied voltage. As the ozone concentration rose, the probability of its separation increased. Ozone could be destroyed by collision with electron and atomic (Gurevich *et al.,* 1995). The self-emitted light (200-300 nm) in the oxygen discharge, could cause the ozone to be separated through significant photo-dissociated reactions (Slanger *et al.,* 1988). Also, the amount of energy wasted in a micro-discharge led to a minute instantaneous local temperature rise. This rise in temperature could cause a significant dissociation of the ozone molecules too.

In this study, the ozone generated by DBD using oxygen gas as a source of ozone (Kotp and Afifi, 2009). In other researches, the oxidation efficiency of MBO was tested at different exposure times and it was found that, it was affected by extension of hydroxyl radical in water. For evaluating the oxidation reduction potential (ORP) we measured the oxidation reaction of all treatments at 13 and 28 ºC. The highest value of ORP recorded in MBO treatment at 30 min compare with gas ozone and micro-bubbles (MB) (El Genidy and Ali, 2016; Juric *et al.,* 2016; Chuajedton *et al.,* 2017). The ORP showed the more oxidation effect which enhanced the elevation of oxidation efficiency by the reaction time. An ORP had the activity to eliminate the pathogens that promoted the rising of ORP (Suslow, 2004a). An ORP at 650-700 mV minimized the time of sterilization for *E. coli*, *Salmonella sp.* and *Listeria* sp. comparing with the ORP less than 485 mV. Therefore, we could get high microbial disinfection via high value of ORP in MBO (Chuajedton *et al.,* 2017). The lower pH reduced the ozone decomposition in water which led to a little change of pH in water (Internet, 1999). Gram stain was changed due to the effect of the ozone which attacked the bacterial cell wall and membrane (Internet, 1999). Ozone had the power to damage the bacterial cytoplasmic space as well as the intercellular contents of *E. coli* when applied (Curtiellas *et al.,* 2005).

Effect of different exposure times on inhibition of *E. coli* O157:H7

Efficacy of surface ozone was evaluated for the purpose of decontaminating with *E. coli* O157:H7. Maximum reductions (100%) in CFU mL^{-1} of *E. coli* O157:H7 of surface ozone exposure at ozone concentration of 9.8 mg L^{-1} was obtained after, 2.5 min in serial dilutions from 10^7 to 10^9 CFU mL^{-1}, and after 5 and 7.5 min in serial dilutions from 10^5 to 10^9 of CFU mL^{-1} (data not shown). The numbers of colony forming unit (CFU mL^{-1}) in serial dilutions were not detected in dilutions 10^0 to 10^5. The available count of cells in serial dilution was determined at dilution 10^7 that equal to approximately (10^8 CFU mL^{-1}) which applied in all experiments in this study.

Effect of different surface ozone concentrations on inhibition of *E. coli* O157:H7

Efficacy of surface ozone was evaluated for the purpose of decontaminating with *E. coli* O157:H7. In an experiment that carried out on both, the shortest exposure time (2.5min) shown complete reduction in *E. coli* O157:H7 CFU mL^{-1}, and the available count of cells. Reduction in *E. coli* O157:H7 reached to 59.05 and 66.14% of CFU mL^{-1} at ozone concentrations of 5.6 and 9.8 mg L^{-1}, respectively, followed by reduction of 89.76 % at ozone concentration ranged from 13.9-18 mg L^{-1} and 98.42% at ozone concentration 22.2 mg L^{-1}. Complete reduction (100%) in CFU mL^{-1} of *E. coli* O157:H7 of surface ozone exposure was obtained at ozone concentration of 24 mg L^{-1} (Fig. 5).

The effect of gaseous ozone on pathogenic bacteria like *E. coli* O157:H7 and *Salmonella* inoculated on the plant surfaces like blueberries was estimated at treatment interval times of 2, 4, 8, 16, 32 and 64 min. Reductions in *Salmonella* colony forming units ranged from 0.3 to 1.0 log10 CFU g^{-1} for treatment times of 4 and 64 min, respectively. The treatment time of 64 min showed a significantly high reduction in *Salmonella* CFU than the lower treatments except for 32 min. While, reductions in *E.*

coli O157:H7 CFU was observed between 0.4 and 2.2 log10 CFU g^{-1} for the treatment times of 4 and 64 min, respectively. The treatment time 64-min led to significantly high log10 reduction than the lower treatment times. After continuous treatment, great log10 reductions for *E. coli* O157:H7 CFU was detected compared to reductions in *Salmonella* CFU (Bialka and Demirci, 2007). A comparison study between the lethal effects of continuous ozone exposure with other gaseous treatment methods, like chlorine dioxide, showed that it did not perform as well. The blueberries plant samples were inoculated with pathogenic *Salmonella* with 6.2 mg L^{-1} of chlorine dioxide for 60 min a reduction in 3.56 log10 CFU g^{-1} was observed (Sy *et al.,* 2005).

E. coli, *Salmonella* and *Shigella* can tolerate stressful environmental conditions like temperature and survive on the plant surface like "cantaloupe plant" (Suslow, 2004b). Observation of an inactivation case on treated samples for 7 days at 5 ^{O}C could point out to the presence of a sub-lethal damage on treated cells, which were finally inactivated during 5 °C storage. Some researchers observed that survival of *E. coli* was unaffected when exposure to ozone gas for short-term but membrane permeability was affected (Komanapalli and Lau, 1996). These results could be produced by combination between the effect of ozone treatment with the low refrigeration temperature. Micro-bubble had good properties of high stability and surface area, which could keep the ozone for a long time. The presence of small bubbles increased the dissolved potential while the cracking of the bubbles generated the free radicals. Apples fruits were inoculated with *E. coli* O157:H7 and treated with ozone (Marui, 2013).

Sterilization treatments were more effective when ozone was bubbled during apple washing than by dipping apples in pre-ozonated water. The corresponding decreases in counts of *E. coli* O157:H7 during 3 min treatments were 3.7 and 2.6 log10 CFU on apple surface, respectively. Optimum conditions for decontamination of whole apples with ozone included a pretreatment with a wetting agent, followed by bubbling ozone for 3 min in the wash water, which decreased the count of *E. coli* O157:H7 by 3.3 log10 CFU g^{-1} (Achen and Yousef, 2001).
Formation of ozone bubbles during apple washing was more effective in sterilization treatments process than by dipping apples in pre-ozonated water. The corresponding decreases in counts of *E. coli* O157:H7 during 3 min treatments were 3.7 and 2.6 log10 CFU on apple surface, respectively. Optimum conditions for decontamination of whole apples with ozone included a pretreatment with a wetting agent, followed by bubbling ozone for 3 min in the wash water, which decreased the count of *E. coli* O157:H7 by 3.3 log10 CFU g^{-1} (Achen and Yousef, 2001).

Effect of different aqueous ozone concentrations/exposure times on inhibition of *E. coli* O157:H7

In case of aqueous ozone, many experiments were evaluated for inhibition of *E. coli* O157:H7. The efficacy of aqueous gaseous ozone inoculated in TSB was evaluated at ozone concentrations ranged from 9.8-22.8 mg L^{-1} for exposure times 4, 10, 20, and 30 min. A treatment time of 4 min resulted in undetected counts of *E. coli* O157:H7 at all ozone concentrations tested (data not shown), followed by zero plate counts at treatment times of 20 and 30 min at all ozone concentrations tested (data not shown). However, a treatment time of 10 min resulted in, high reductions in *E. coli* O157:H7 reached to 85.03 and 89.00% reduction in CFU mL^{-1} at ozone concentrations of 9.8 and 13.4 mg L^{-1}, respectively, followed by higher reduction percentages reached to 94.48 and 97.63% of CFU mL^{-1} at ozone concentrations of 18.0 and 21.6 mg L^{-1}, respectively. However, the highest reduction (100%) was obtained at ozone concentrations of 22.8 mg L^{-1} (Fig. 6).

The efficacy of gaseous ozone on *E. coli* O157:H7 inoculated in TSB was evaluated at, ozone concentrations ranged from 9.8-24 mg L^{-1} for treatment time of 4 min, and at ozone concentration of 9.8 mg for treatment times ranged from 0.5- 33 min, plate counts were not detected for *E. coli* O157:H7 in two evaluations. In addition, in case of aqueous ozone experiment that conducted at exposure times ranged from 1-45 min at ozone concentrations of 12.23, 20.3, and 23.2 mg L^{-1}. The results confirmed that at ozone concentrations of 12.23 mg L^{-1} all *E. coli* O157:H7 counts were not detected at all treatments.

The efficacy of gaseous ozone on *E. coli* O157:H7 inoculated in TSB was evaluated at treatment times ranged from 1-45 min at ozone concentrations of 12.23, 20.3, and 23.2 mg L^{-1}. The results confirmed that at ozone concentrations of 12.23 mg L^{-1}, all *E. coli* O157:H7 counts were not detected at all treatment times tested (data not shown). A treatment time of 1 min resulted in low reductions in *E. coli* O157:H7 reached to 13.38 and 37.90, followed by, high reductions in *E. coli* O157:H7 reached to 58.26 and 70.86 after treatment time of 3 min, and higher reductions in *E. coli* O157:H7 reached to 98.42 and 99.21% reduction in CFU mL^{-1}, at ozone concentrations of 20.3 and 23.2 mg L^{-1}, respectively. However, treatment times of 18, 33, and 45 min resulted in a complete reduction (100% in CFU mL^{-1}) of *E. coli* O157:H7 at ozone concentrations of 20.3 and 23.2 mg L^{-1} (Fig. 7).

The concentration of dissolved ozone in water was estimated by Indigo colorimetric method. MBO treatments at 28 ºC acquired the concentration values of 0.01, 0.03, 0.05, and 0.02 mg L^{-1}, and at 13 ºC acquired the concentration values of 0.14, 0.17, 0.14, and 0.18 mg L^{-1} at 5, 10, 15 and 30 min, respectively (Chuajedton *et al.,* 2017). All treatments at 13 ºC acquired the concentration values of dissolved ozone

more than at 28 ºC. However, the concentration values at 28 ºC was not increased when exposure time increase to 30 min because of the measurement of concentration values of dissolved ozone in water by Indigo method was not enough accurate, and ozone concentration could change in a few minutes to lower concentration (Jacek, 2012). At low temperature values like 13 ºC, researchers observed the increasing of the dissolved ozone in the water (Puyate and Rim-Rukeh, 2008).

The resulted data of our study conformed to other reported data that the growth of *E. coli* O157:H7 was greatly reduced when treated with ozone (Chuajedton *et al.,* 2017), also, treatment by ozone inactivated the bacterial growth of withering disease of brassica, strawberry and tomato without harmful effect to the postharvest quality (Fukumoto *et al.,* 2010).

An interesting study was carried out for investigating the impact of micro-bubbles ozone containing water at different temperatures 13 °C and 28 °C for inhibition of *E. coli* O157:H7 treated by different micro-bubbles ozone concentrations of 0.01, 0.03, 0.05, and 0.02 mg L^{-1} at 28 °C and the concentrations of 0.14, 0.17, 0.14, and 0.18 mg L^{-1} at 13 °C for time intervals 5, 10, 15 and 30 min, respectively then incubated for 48 h at 35 °C. The results presented that micro-bubbles ozone displayed the highest effect of inactivation the growth of E. coli O157:H7 when exposure at 13 °C for 30 min, compared to micro-bubbles and the control (distilled water) (Chuajedton *et al.,* 2017).

In a previous study, the bactericidal action of gaseous ozone for the elimination of *Bacillus cereus, Salmonella typhi, Klebsiella pneumoniae* and *Serratia marcescens* has been investigated by surface exposure technique. Under identical treatment conditions, 4.9 and 9.7 mg L^{-1} ozone concentrations decreased bacterial counts by 0.27-1.27 log10 CFU mL^{-1} depending upon the bacterial species tested (Kotp and Afifi, 2009). Moreover, at ozone concentration of 12 mg L^{-1} up to 20 min, a complete log reduction took place in the population of all bacteria species tested. Moreover, at 14.3 mg L^{-1} ozone concentration, a complete log reduction in the number took place at the end of 2.5 min of *Salmonella typhi*, 5 min of *B. cereus* and *S. marcescens* and 10 min of *K. pneumonia* (Kotp and Afifi, 2009). Temperature of water has an important effect on the ability of dissolved ozone in water. Therefore, when the water temperature being low the ability of dissolved ozone could be increased. Kobayashi *et al.* (2009) observed a rapid increase in concentration of ozone micro bubbles water at 15 ºC when the exposure time was 5 min. As the result, the highly concentration of dissolved ozone in water disordered the bacterial enzyme activity and effected on bacterial cell membrane (Internet, 1999).

Ozone is an effective sanitizer with superior disinfecting properties when applied for the treatment of water and wastewater (Kessel *et al.,* 1943; Scarpino *et al.,* 1972; Korich *et al.,* 1990; Kobayashi *et al.,* 2009). Moreover, rapid decomposition of ozone to oxygen and lack of toxic residues make it a favorable environment-friendly sanitizer. Ozone was tested in lettuce processing and bubbling ozone reduced counts of natural microflora in the range of 2 to 3 log10 CFU g^{-1} (Kim *et al.,* 1999). However, use of ozone to sanitize apples has not been explored.

Conclusion

The objectives of this study were to define conditions for effective ozonation processes of solid and liquid cultured with *E. coli* O157:H7, and enhance the effectiveness of ozone through selected pretreatments. This research presented and confirmed that the disinfectant efficiency of *E. coli* depended on the exposure times and ozone concentrations of both surface and aqueous exposures.

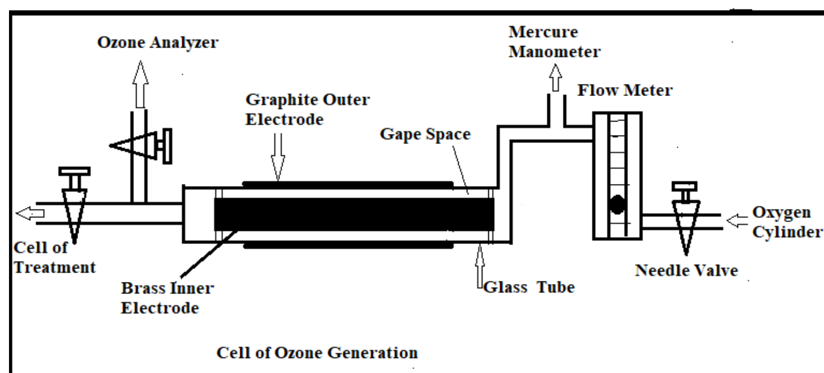

Fig. 1: The structural of the ozone generator.

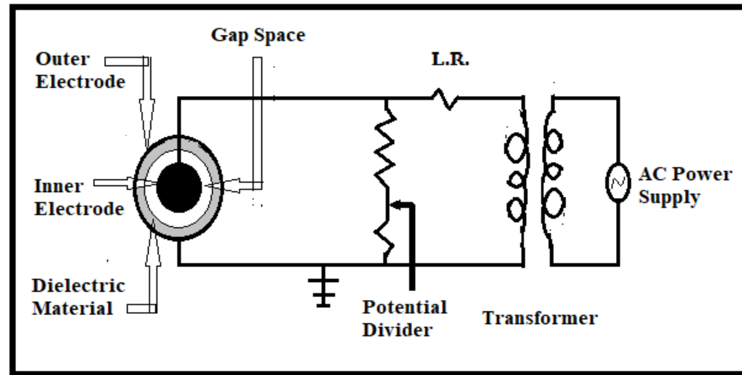

Fig. 2: The electric circuit of discharge.

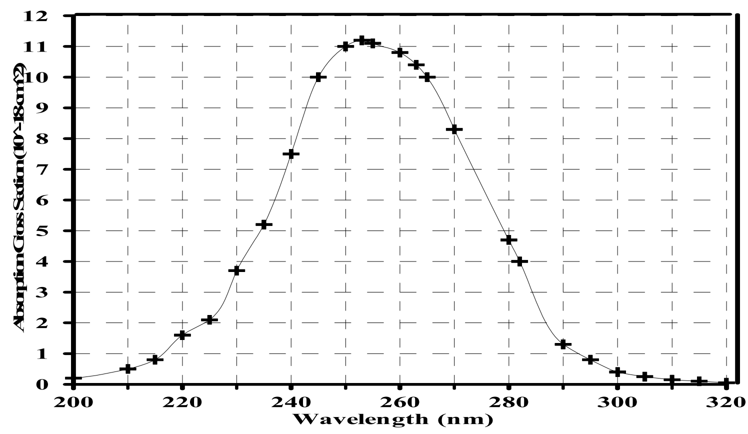

Fig. 3: The absorption curve of ozone.

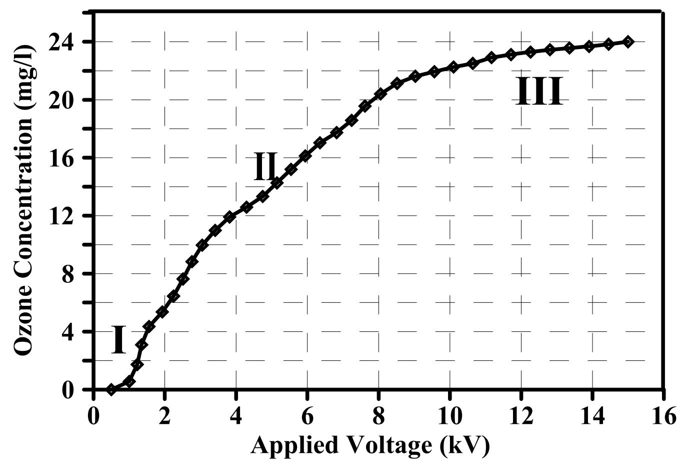

Fig. 4: The relation between the ozone concentration and the applied voltage.

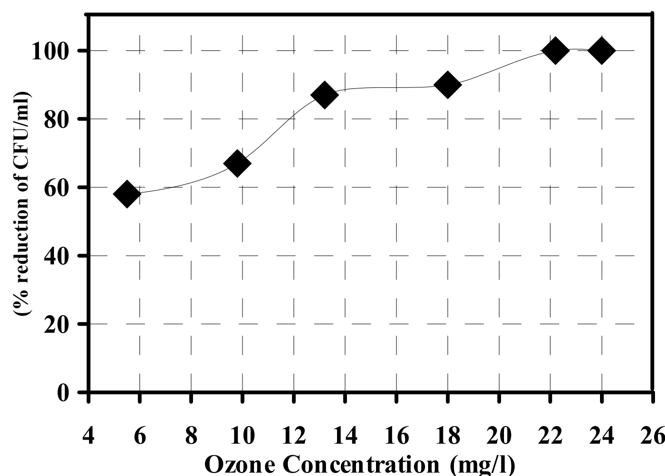

Fig. 5: Effect of surface ozone on inhibition of *E. coli* O157:H7.

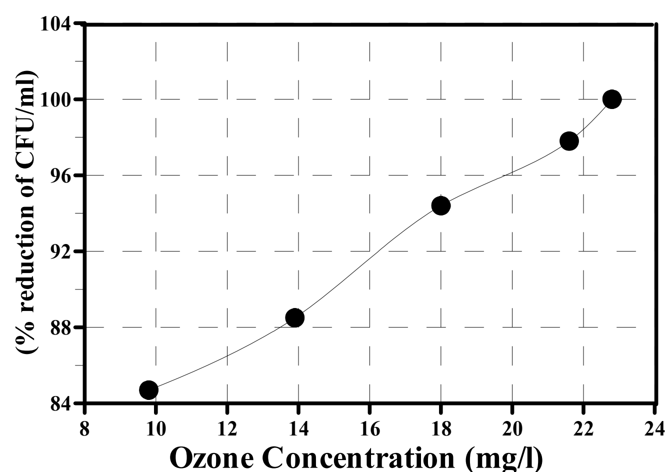

Fig. 6: Effect of aqueous ozone concentrations for 10min on growth inhibition of *E. coli* O157:H7.

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