



An investigation on the sterilization of berry fruit using ozone: An option to preservation and long-term storage

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ABSTRACT

In the present study, the effect of ozone treatment on the bacteria affecting fruits was investigated. The process of application of ozone for treatment was termed as OTRE technique. Ozone is a trioxxygen inorganic molecule having a pungent smell and formed from the atmospheric oxygen (O₂) by the action of electrical discharge. Ozone was generated adopting Ozonizer through corona discharge technology. The bacteria isolated from Kiwi fruits by microbiological technique were exposed to ozone in two approaches, firstly, the ozone discharged at various time from 10 to 60 min into the reactor vessel containing bacteria (1.5×10^8 CFU mL⁻¹). Secondly, use of OTRE in cycle basis, where the sample was treated 30 min and 5 min break and again repeated for 10 min, which continued to the discharge of 60 min. The absorbance of all these varied intervals showed decrease in absorbance, which implies that the number of colonies were decreasing as the OTRE continued. In addition, the growth of these colonies on the nutrient agar was also confirming that the OTRE is quite effective to prevent bacteria from kiwi viz., the bacterial count was decreased from 1.5×10^8 CFU mL⁻¹ to 2.0×10^2 CFU mL⁻¹ in 30 min and 1.5×10^8 CFU mL⁻¹ to 1.2×10^2 CFU mL⁻¹ in 60 min. Thusly, OTRE technique is a rapid, better solution to preserve fruits from disease causing bacteria and enhance fruits durability against spoilage during postharvest storage.

1. Introduction

Kiwi fruit is native to Asia and other few countries such as New Zealand and it is famous world-wide for its nutritional properties as it contains high levels of bioactive compounds such as flavonoids, vitamin C, vitamin E, minerals, carotenoids, antioxidants. Apart from the fact that kiwi fruit has a numerous health benefits but it is grown in very limited parts of the world like China, Italy and New Zealand and it takes 3–5 years for a kiwi plant to produce the fruit which makes it one of the expensive fruits all over the world. Internationally kiwifruit is the minor crop showing a mere representation of 0.2%–0.35% of total fresh fruit production. At the time harvesting fruits from the trees, they are flooded with the microorganisms. In kiwi these microorganisms are mostly bacteria where the milky exudates come from the infected part and these exudates are a good source of the inoculum. The bacterial canker caused by the *Pseudomonas syringae* was detected for the first

time on the green fleshed kiwi fruit on Jeju island on 1988. After that the disease was found to be spread to other parts of the Korea producing kiwi (Koh et al., 2010).

The ozone is effective in both ways whether given in gaseous form directly or using ozone in the aqueous solution, it will reduce the microbial counts on fresh produces. The use of ozone as a disinfectant of vegetables and fruits was studied and shown its efficacy earlier (Horvitz, 2014). A number of factors that may affect the efficiency of ozone are microbial populations, time of exposure, ozone concentration, type of produce, relative humidity and the material in which the produce was packed (Glowacz et al., 2015).

It is a process or a treatment to destroy various microorganisms by the exposure of these microorganisms to ozone. Ozone is a trioxxygen inorganic molecule having a chemical formula O₃. It is a pale blue gas with a pungent smell and it is an allotrope of oxygen much less stable than the diatomic allotrope oxygen. Ozone is formed from the

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atmospheric O₂ by the action of ultraviolet light and electrical discharges (Bennett et al., 2004). Ozone has the microbial activity that makes it a suitable candidate as the disease control agent of postharvest pathogens on fruits and a good alternative to the chemical agents as it has a greater advantage of absence of residues on the treated products (Food and Drug Administration, 1997). The kiwi fruit (*Actinida deliciosa*) is mainly affected by bacterial diseases. These diseases are known for their devastating effect on kiwi fruit industries around the world causing huge economic losses (Scortichini et al., 2012). When the kiwi fruit is infected by the bacteria such as *Pseudomonas syringae* and *P. actinidae*, milky bacterial ooze flows out of the fruit (Loreti, 2011).

Ozone can be an effective agent in the food industries with regard to the food preservation and its shelf life as it's a powerful sterilizing agent. European regulatory authorities have affirmed to qualify the status of the use of ozone as 'Generally Recognised as Safe' (GRAS) which prompted various food industries to look at the possibilities to use ozone for many aspects (Horvitz, 2014). Copper based compounds and streptomycin to treat the diseases in kiwi especially in Korea and New Zealand but unfortunately, both copper based compounds like copper sulphate and streptomycin show problems of phytotoxicity, bacterial resistance and residues on fruits. Thus, it is attempted to see how these bacterial diseases can be controlled using ozone or ozone treatment, so-called OTRE technique by the collection, isolation and identification of bacteria from berry fruits of high commercial value, assessment of OTRE technique on bacteria causing food spoilage and an investigation of effective inhibitory value of OTRE on the infected berry fruits.

2. Materials and methods

2.1. Collection of fruit

Fruit sample for this work were collected from Kasapapuram, Chennai, India. The sample was taken to the laboratory in a small container in order to avoid the contamination during transport. (Fig. 1a,b).

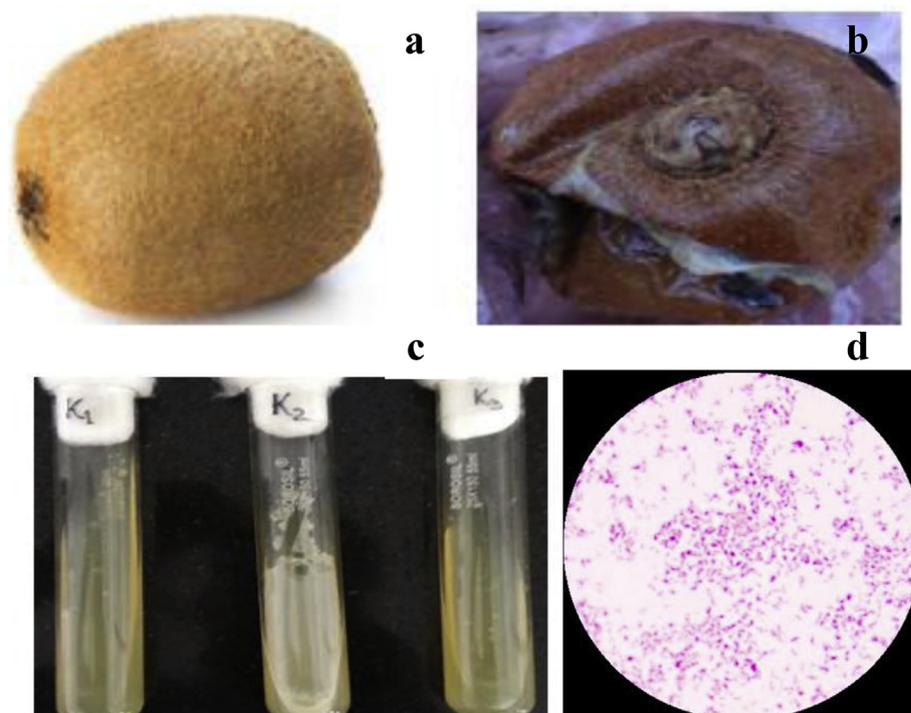


Fig. 1. Isolation of bacterial culture from Kiwi fruit: Healthy fruit (a); spoiled fruit (b) and three different bacteria isolates named K₁, K₂ and K₃ (c) and Gram staining showing gram negative bacteria (d).

2.2. Medium and broth

The nutrient broth and or agar was prepared using Beef extract/ yeast extract (0.3%), peptone (0.5%), NaCl (0.5%), and Agar (1.5%), suspended in 1 L of distilled water in a conical flask and autoclaved at 121 °C for 15 min. Phosphate buffer saline was used for the experiments, it mainly contained 1.26 g of sodium hydrogen phosphate and 0.34 g of disodium hydrogen phosphate in 1000 mL distilled water and the pH is maintained at 7.4.

2.3. Isolation and characterization of bacteria from kiwi

The LB agar was prepared (25 g of LB agar dissolved in 1 L of distilled water) and then it was autoclaved at 121 °C for 20 min. The plates were poured with the LB agar and inoculated with the bacterial samples and then kept in incubator at 37 °C for 24 h. The morphologically varied colonies were isolated and stored in the slants (MubarakAli et al., 2015). A loop full of isolates was taken from the slants and inoculated into the LB broth in the test tubes and again incubated for the 24 h at 37 °C. These isolates were performed for Gram's staining to understand the gram's pattern of the isolates.

2.4. Effect of OTRE on the bacteria at various discharge time

Briefly, the bacterial culture was taken and investigated for the concentration of cells by obtaining absorbance at 600 nm using spectrophotometer (Type 117 of Systronics (India) Ltd). The cells were diluted with PBS buffer to obtain cell concentration of 0.5 McFarland i.e., (1.5×10^8 CFU mL⁻¹) of bacterial cells (MubarakAli et al., 2016). One ml of the vessel contain bacterial cells was subjected to OTRE and its absorbance was also assessed spectrophotometrically. OTRE was discharged at various times such as 10, 20, 30, 40, 50 and 60 min using ozonizer (PX-902C, ASIN, B078MSYFKF, India). After the OTRE, the treated cells were spread over nutrient agar to investigate the bacterial reduction. All the agar plates were incubated at 37 °C for 24 h.

2.5. Effect of OTRE at cycle basis discharge

The bacterial cells in a reactor vessel containing PBS and 0.5 McFarland (1.5×10^8 CFU mL⁻¹) of cells was subjected to the OTRE at a stretch of continuous 30 min. This is followed by a break of 5 min and the OTRE was again started for 30 + 1 (10) min then 5 min break and again OTRE for 30 + 2 (10) min followed by a 5 min break and then the discharge of ozone for 30 + 3 (10). All the treated broth was spread on the nutrient agar plate and incubated at 37 °C for 24 h.

3. Results and discussions

The frequent occurrence of growth of bacteria, K₂ isolate found predominant growth and occurrence among the three isolates such as K₁, K₂ and K₃ (Fig. 1c). All the isolates found to be gram negative bacteria showing rod shaped bacilli (Fig. 1d). Fruits are very important for the health as they contain the essential nutrients required for a healthy life such as vitamins, minerals and fiber. Kiwi fruit is known as the Chinese gooseberry as it is the national fruit of china. It is mainly known for the antioxidant properties and the fiber content especially in its peel. Consumption of kiwi fruit is very beneficial as it has been found to have a preventive effect against certain cancers and cardiovascular diseases (Tsubota et al., 2011). Different cancers especially the stomach cancer has been treated with kiwi fruit prescriptions due to its cytotoxic and antioxidant activities. Hence, there is a dire need to minimize the harmful effects affecting the useful fruit.

Generally, ozonation proves to be very effective against microorganisms and it can even degrade the pesticide residues in the after harvest by its oxidation property (Artes et al., 2009). *Pseudomonas ac-tinida* related to the kiwi fruit destruction was identified and studied to be the cause of kiwi fruit disease in china (Huang et al., 2012). The effect of ozone mixed with atmospheric air was tested on raspberries under non refrigerated conditions for a specific period of time (Balawejder et al., 2015). It was stated that the bacterial spores are seen to be very resistant to toxic chemicals and other environmental stresses. These resistances are being contributed by many factors like the multi-layered spore coats on fruits (Young et al., 2012). The components of food have also a significant effect on the bactericidal power of ozone treatment against bacteria (Guzel et al., 2004). It has been reported that the effect of ozone on oranges and lemon and a significant inhibition of the pathogens affecting these fruits was documented (Ong, 2015). Generally, the ozone treatment that can inhibit the pathogens affecting one type of fruit cannot be same as the one affecting some other (Alva, 2014).

The discharge time (min) of ozone and its corresponding concentration (mg.mL⁻¹) for OTRE of the bacteria is shown in Table 1. It was estimated that 10 min of discharge is providing to 3.3 mg mL⁻¹ and 60 min of discharge showed 19.8 mg mL⁻¹. The bacterial isolates were first seen to have formed the dense round colonies on the agar plates but after the OTRE, the colonies very less dense and small in size and much scattered. The yellowish colour of the isolate was faded as the

Table 1

The discharge time (min) corresponding to the concentration of ozone (mg.mL⁻¹).

Discharge time (min)	Conc. of ozone (mg.mL ⁻¹)
10	3.3
20	6.6
30	9.9
40	13.2
50	16.5
60	19.8
70	23.1
80	26.4
90	29.7

Table 2

The discharge of ozone and the CFU (cells.mL⁻¹).

Discharge time (min)	CFU (cells.mL ⁻¹)
0	TNTC
10	3.6×10^3
20	2.3×10^3
30	1.7×10^3
40	1.3×10^3
50	8.1×10^2
60	3.1×10^2

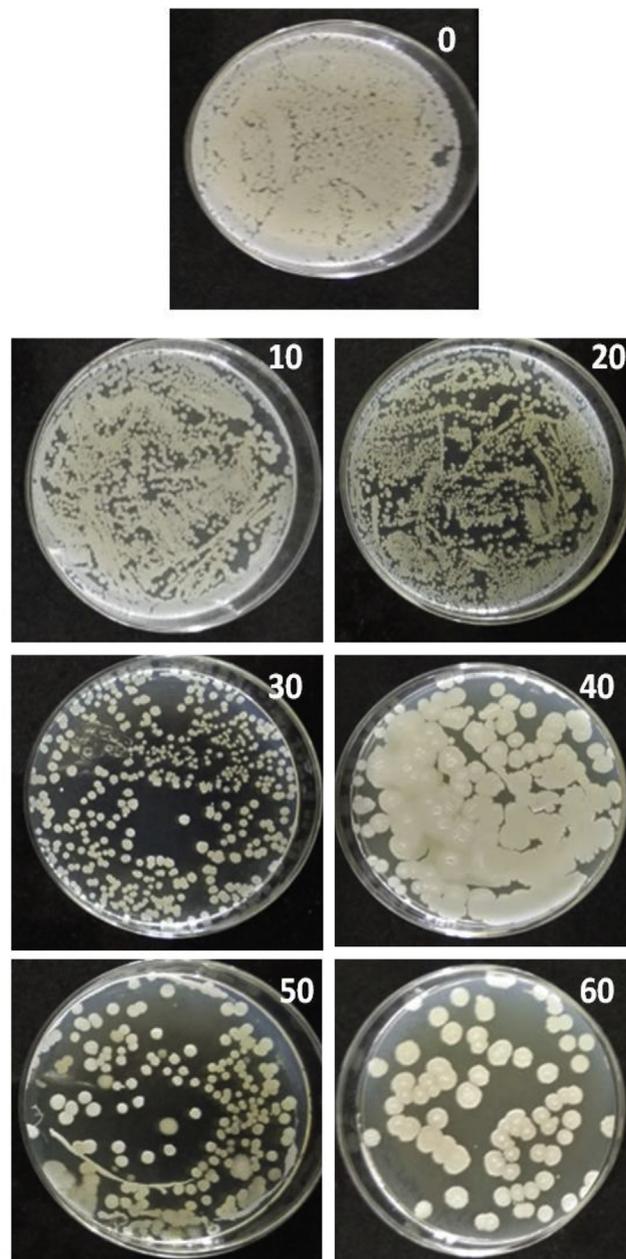


Fig. 2. Effect of ozone on the bacterial count: Control (0 min), 10 min, 20 min, 30 min, 40 min, 50 min, and 60 min of ozone exposure showed substantial reduction of bacterial count and observed morphological abrasions.

OTRE was continuously discharged. The discharge time (min) of ozone and the colony forming unit (cells.mL⁻¹) after each time of the exposure to ozone showing how the bacterial cells were reduced after OTRE treatment is shown in Table 2. This shows a decrease in the

viability of the pathogen and its effect on the media as shown in Fig. 2. The control (0 min) showed too numerous to count (TNTC) cells corresponds to 1.5×10^8 CFU mL⁻¹. Whereas, 10 and 60 min of discharge showed 3.6×10^3 CFU mL⁻¹ and 3.1×10^2 CFU mL⁻¹ respectively.

The decay and spoilage control can be enhanced by using the Ozonation of the kiwi which will destroy the effecting pathogens. When ozone molecule makes contact with the cell wall of the bacteria, a reaction known as an oxidative burst occurs which makes a tiny hole in the cell wall occurs. This hole in cell wall injures the bacteria and eventually, bacteria losing its shape as the ozone molecules continue to form pores in the cell wall and the bacterial count was also decreased. The maintenance of the firmness of ozone-exposed kiwi was associated with the inhibition of cell wall degrading enzyme activity that is polygalacturonase (Singleton, 2016). Efficiency of the ozone treatment in reducing the microbial counts was seen depending on the ozone dose such as ozone concentration versus time of discharge and on the initial microbial counts (1.5×10^8 CFU mL⁻¹). This data can help to give a specific dose at specific time if the pathogen to be targeted is known, this way it will save a lot of time and excessive dosage can be prevented. Ozone is industrially applied in the preservation of vegetables and fruits and the fruits which were ozonised include apples, fresh cut salads (McCann et al., 2013). Ozone was also spread on onions and potatoes to prevent the fungal growth. Effect of ozone application during cold storage of kiwifruit on the development of stem-end rot caused by *Botrytis cinerea* was studied (Minas et al., 2012). The Centre for Disease Control and Prevention shows that approximately 12.3% of the food borne diseases from 1990 to 2007 were linked with the use of vegetables and fruits and 21.9% were associated with all food borne illnesses (Dalton et al., 1997).

The OTRE on the sample, the absorbance was gradually decreasing as the time of the ozone discharge increased. This was done in two aspects, first the ozone was given continuously for 60 min and the sample was taken after every 10 min. The second time the ozone was given at a stretch for 30 min. This is followed by a pause of 5 min and the ozone treatment was again started for 10 min (30 + 10) then 5 min pause and again ozone treatment for 30 + 2 (10) followed by a 5 min break and then the exposure of ozone for 30 + 3 (10). This time the ozone treatment was much effective as the number of colonies of the pathogens were much less as compared to the first one (Fig. 3). The control (0 min) showed too numerous to count (TNTC) cells corresponds to 1.5×10^8 CFU mL⁻¹. Whereas, 30 and 60 (30 + 3 (10)) min of discharges showed 2.0×10^2 CFU mL⁻¹ and 1.2×10^2 CFU mL⁻¹ respectively (Table 3).

Ozone is FDA approved as a safe agent for decontamination of fruits and powerful disinfectant and has the ability to filter through to the target it needs to reach. OTRE is the best alternative which has a lot of scope if applied on a measurable scale in orchards with proper care to eradicate the bacterial outbreaks of kiwi. It was estimated that 44% diseases were caused by bacteria, 49% by viruses, 6% by chemicals and remaining one percent by parasites. This observation led the scientific community to think for the feasible technology which can be done the process of sanitation of the fruits and vegetables with a promising quality and standards. Then they came up with the application of ozone as it can increase the shelf life of the fruit by the reduction of enzymatic activity and by destruction of pathogens and spoilage microorganisms and their toxins (Guzel et al., 2004). The ozone dosages are given in two forms mainly: the gaseous ozone which can be added intermittently or continuously to the place where the after harvest is kept and the product can be washed or dipped in the water treated with ozone. The effect of chlorine, UV light and ozone was studied on ascorbic acid content of the fruits and other (Bermudez et al., 2013). The possible bacterial diseases of kiwi are caused by the *Pseudomonas* species mostly for example bacterial blight caused by *P. syringae* and *P. viridiflava*. The bacterial canker is caused by *P. syringae* P.v. actinidiae. Crown gall of kiwi is caused by the *Agrobacterium tumefaciens*. The identification of the *Pseudomonas* species especially the *P. syringae* was done on various

parts of the kiwi plant such as twigs during its disease (Gallelli et al., 2011). All the data reviewed shows that ozone treatments can play a vital role for fruits and vegetable's preservation and decontamination from the pathogens.

A handful of studies showed that the ozone reactivity is too tough to predict in the presence of organic matter and it is reported that ozone may oxidize or ionize a substrate or will decompose it to free radicals and oxygen (Manousaridis et al., 2005). The excessive usage of ozone is not good and sometimes it causes discoloration or even development of undesirable smell (Khadre et al., 2013). In comparison with other traditional disinfectants, ozone has very minimal side effects and in fact, no side effects if used properly and in a proper dosage. The studies have shown that the level of microbial inactivation by ozone varies as per pH, relative humidity, temperature, additives (surfactants, sugars etc.) and of course amount of the organic matter surrounding the cells (Restaino et al., 1995).

The atmosphere enriched with 4 mg h^{-1} of gaseous ozone was tested on kiwi fruit. Through this exposure of ozone, it is seen that the pathogen was controlled during the storage period (Barboni et al., 2010). In the other experiment, it was reported that a delay of a month was seen in the detection and appearance of stem-end rot disease of the kiwi enriched with ozone ($0.3 \mu\text{L.L}^{-1}$). This is a fungal disease and the effect on bacterial diseases is yet to be studied fully. After the treatment with ozone, the disease incidence was reported to be reduced by 56%. The results of pathogen inhibition were better when the ozone was used for 144 h which showed that there was an accumulation of phenolic compounds in the kiwi (Minas et al., 2012). The report of bacterial canker being found in Korea and studied to be caused due to the *Pseudomonas* species (Butler et al., 2015). The post-harvest treatment of apples with ozone showed a good results of the pathogen destruction (Yaseen et al., 2015). Ozone also helps in preventing the over ripening of kiwi due to the ethylene of packets in which they are stored, thus a great agent in preventing the kiwi from decaying (Vasilakakis et al., 2014).

4. Conclusion

We summarize, OTRE technique has showed positive results in treating the kiwi affected with pathogens. OTRE is rapid and free from residues for fresh fruit supply and the processing of kiwi without affecting the natural components of the fruits and this treatment will improve the shelf life of the products with a good hygiene. Three bacterial culture isolated from kiwi (denoted as K₁, K₂ and K₃) subjected for OTRE to determine the appropriate discharge time and concentration. The isolated strains are Gram negative bacteria with bacilli and found that *P. syringae* based on the cultural and biochemical characteristics. The based on the occurrence and frequency, K₂ isolate was used from initial bacterial count was 1.5×10^8 CFU mL⁻¹ substantially reduced to 1.2×10^2 CFU mL⁻¹ after 60 min of OTRE corresponding to 19.8 mg. mL^{-1} of O₃. Very importantly, cycle based OTRE showed effective disinfection time than that of continuous OTRE. It is rapid as compared to other conventional methods of fruit disinfection and with no harmful residues. The prominent side effects of ozone can be avoided by following the specificity of the threshold of ozone needed to reduce a specific amount of pathogens. It can be a solution for the food industries to treat the postharvest at a large scale with ozone.

Conflicts of interest

There is no conflict of interest to this work.

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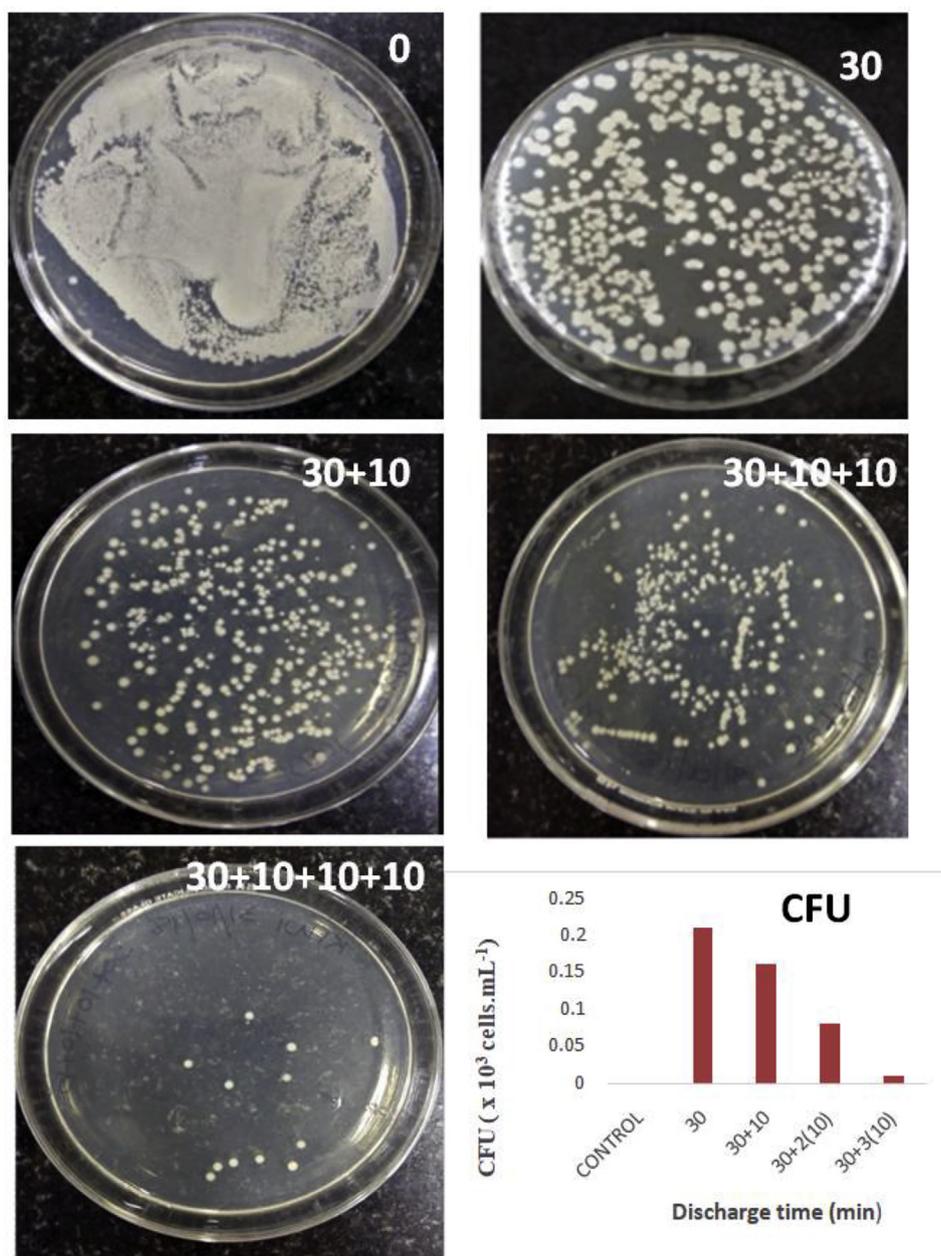


Fig. 3. Effect of ozone on the bacterial count: Control (0 min), 30 min, 30 + 1(10) min, 30 + 2 (10) min, 30 + 3 (10) min, after ozone exposure. The effect of ozone can be seen at varies intervals of time and the response of the pathogen to the dosage after each specified interval.

Table 3

The discharge of ozone and the CFU (cells.mL⁻¹) during cycle based discharge.

Discharge time (min)	CFU (cells.mL ⁻¹)
Control	TNTC
30	2.0×10^2
30 + 10	1.6×10^2
30 + 2 (10)	8.0×10^2
30 + 3 (10)	1.2×10^2

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://>

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