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Sanitation of plastic bottles using ozonated water

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Abstract

Ozone is a powerful sanitizer that does not generate hazardous residues. Thereby it can be used as an alternative for traditional chemical sanitizers on surfaces, vegetables and packaging decontamination. This study aimed to characterize the ozone decay kinetic in water and to evaluate the ozonated water efficacy for decontamination of plastic bottles. The ozone concentration decay was determined by ozone measurement in water after ozone generation. The ozone efficacy was studied using PET bottles contaminated with spores of *Bacillus subtilis* and then washed with ozonated water at concentrations of 1 and 4 mg.L^{-1} for 3, 7 and 10 minutes. The maximum ozone concentration solvated in water was 4 mg.L^{-1} and the ozone decays was described by an exponential equation. The half-life of the ozone in water was calculated as 9.2 minutes. Based on this data, the times for bottle treatments were chosen. The results of *B. subtillis* spores reduction showed that ozonated water at 1 mg.L^{-1} was not effective. On the contrary, around 2 decimal reductions were reached by applying ozonated water at 4 mg.L^{-1} for 3-10 minutes. The results showed that ozonated water is a potential sanitation to be applied for bottle surface decontamination, but the process efficacy is dependent on the ozone concentration, temperature and time of exposure.

Introduction

Ozonation is an emerging technology applied to reduce the microbial load of surfaces and liquids (Fuhrmann *et al.*, 2010). The ozone is a high oxidative compound with a broad antimicrobial spectrum; it is able to inactivate bacterial vegetative cells and spores, yeast, molds, and viruses and to kill insects in stored grains and degrade mycotoxins (Tiwari *et al*., 2010).

Ozone inactivates microorganisms by the progressive oxidation of vital cellular components. The microbial surface is the primary target of ozonation with the oxidation of the polyunsaturated fatty acids and consequent loss of selective permeability and cell disruption. Additionally, ozone causes the oxidation of sulfhydryl groups and amino acids of enzymes, peptides, and proteins including nucleic acids and vital enzymes (Khadre *et al*., 2001; Guzel-Seydim *et al*., 2004).

In general, Gram negative bacteria are more sensitive to ozone treatment than Gram positive ones (Khadre and Yousef, 2001). However, several other factors affect ozone efficacy including the strain of the microorganism, age of the culture, density of the treated population, and presence of ozone-demanding medium components and method of applying ozone (Khadre and Yousef, 2001). In spores, ozone degrades the coat layer components thus exposing the cortex and the core to oxidation by ozone (Khadre and Yousef, 2001; Tiwari *et al.*, 2010).

The ozone application stands out for its high efficacy (low concentration and short contact time are commonly enough to achieve the desired microbial inactivation) and to be totally degradable into oxygen with no waste/toxic products (Tiwari *et al.*, 2008; Tiwari *et al*., 2010). Thus, ozone is generally recognized as safe (GRAS) for food applications (Guzel-Seydim *et al.*, 2004; Dhillon *et al.*, 2009; Tiwari *et al*., 2010). An additional advantage is the lower cost of ozone equipment (Guzel-Seydim *et al.*, 2004).

Ozone has been previously used in food industry to inactivate microorganisms in packages and stainless steel surfaces (Khadre and Yousef, 2001a), for microbial decontamination of eggs (Fuhrmann *et al.*, 2010) and poultry carcasses, on chilled water treatment and recycling (Khadre and Yousef, 2001; Guzel-Seydim *et al*., 2004), for meat (Novak and Yuan, 2004), fish (Pastoriza *et al*., 2008), milk (Cavalcante *et al*., 2013a) and cheese shelf-life extension (Cavalcante *et al.*, 2013b), for fruit juice processing (Tiwari *et al*., 2009; Choi *et al*., 2012), for sanitation of fruit and vegetables (Achen and Yousef, 2001; Patil *et al.*, 2010; Kim and Hung, 2012), and

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for decontamination of dry products as seeds, grains, and pepper (Akbas and Ozdemir, 2006; 2008; Emer *et al*., 2008; Dhillon *et al*., 2009; Tiwari *et al.*, 2010). Taking into account that ozone is a chemical sanitizer, *Bacillus subtilis* is an ideal target, due to its high resistance to chemical compounds (Moreau *et al.*, 2008).

Considering the expected efficacy of ozonation, this study was performed to evaluate the use of ozonated water in PET bottles sanitation, aiming to inactivate *B. subtilis* spores.

Material and Methods

Bacillus subtilis

Freeze dried *Bacillus subtilis* ATCC 6633 was donated by André Tosello Research and Technology Foundation – FAT (Campinas, Brazil – www.fat.org.br). The microorganism was activated using nutrient broth (Difco®) and kept under refrigeration at 7ºC on five nutrient agar (Difco®) slants.

The *B. subtilis* spores suspension was prepared as described by Amorim *et al.* (2013): *B. subtilis* sporulation was carried out in 20 Roux bottles using nutrient agar (Difco®) supplement with 20 $mg.L^{-1}$ of manganese sulphate. The incubation was carried out at 30°C for 14 days.

The harvesting procedure was carried out using a 0.9% saline solution (20 mL per bottle) and a glass rod to help the spore removal from the bottle. After harvesting, spores were centrifuged three times at 4000*g* for 10 minutes at 10°C. A heat shock at 70°C for 20 minutes was carried out before *B. subtilis* count. After, the microorganisms were plated on nutrient agar and incubated at 30°C for 48 h.

Bottles

For these experiments it was used PET bottles (polyethylene terephthalate bottles) of 360 mL, commonly used for water and juice packaging in Brazil (Figure 1). The bottles caps were made of polypropylene. The bottles have 17.7 cm of height, 22 g of weight and 0.295 ± 0.003 mm of wall thickness.

Ozone generation and ozonated water

The ozone was produced from pure oxygen using an ozone generator from Panozon (model PNZ 714) coupled to an oxygen concentrator (90-95% of purity). In order to produce small bubbles and improve ozone dissolution into the water a hose with a porous plate made of ore sand was attached (aerate volume: 0.6 $L.min^{-1}$; size: 12 x 20 mm) at the equipment outlet. The water was ozonated at 8ºC using a glass beaker (2 L) without mechanical agitation. The time for ozonation was around 5 minutes (time enough to reach a stable final concentration of ozone in the water). The water was ozonated at the concentration of 1 and 4 $mg.L^{-1}$ (maximum concentration able to be reached in the system). Additionally, the system had a gasification tank to separate the ozone produced as gas, which posteriorly was degraded in oxygen.

The ozone concentration for each process was controlled by a colorimetric test, which is based on the ozone reaction with N-dietil-P-Fenilenodiamin. The reaction produces a pink color that has intensity proportional to the ozone concentration. The final color was measured using a colorimetric test (Brand HACH, model OZ-2) able to evaluate the ozone concentration at the range of 0-2.3 $mg.L^{-1}$ (Cavalcante *et al.*, 2013b). If the ozone concentration was higher than 2.3 $mg.L^{-1}$, the sample of ozonated water was diluted with deionized water before ozone concentration measurement.

Decay of the ozone concentration in ozonated water

The decay of the ozone concentration in water was measured for 20 minutes after water ozonation, using the colorimetric method with N-dietil-P-Fenilenodiamin. A curve was plotted and a mathematical model was adjusted in the data of ozone decay using a software Excel (Microsoft®, USA).

Bottles sanitation

The bottles and its caps were sanitized by immersion in ethanol at 70% (v/v) for 2 minutes (Petrus (2000) showed that ethanol at 70% reduced more than 5 log cycle of microbial load in PET packages). After this, the bottles and caps were placed in a laminar flow cabinet to dry, avoiding contamination. Then, each bottle was inoculated using 0.5 mL of *Bacillus subtilis* spore suspension at $2x10^7$ $CFU.mL^{-1}$, following the methodology described by Petrus (2000) for tests of surface asepsis. This volume was enough to homogeneously distribute the microorganism load on the package surface and also to guarantee the complete water evaporation after inoculation (avoiding that the microbial load stayed deposited in the bottom of the bottle). After the inoculation, the bottles were manually agitated for 2 minutes to improve the homogeneity of microorganism distribution in the bottle surface (The agitation was stopped when it was not more possible to visually observe drops inside the bottles). The bottles were capped with a cotton cap (to allow the water evaporation) and incubated at 37ºC for 16 h to guarantee the complete dry of the bottles and adhesion *Bacillus subtilis* in the package surface (adherence process).

In sanitation tests, the bottles were completely filled with ozonated water at 1 mg.L^{-1} for 2 minutes and at concentration of 4 mg. L^{-1} for 3, 7 and 10 minutes. The contact times were defined by using the curve of ozone decay in water. After the sanitation process, the bottles were washed with sterile solution of 10% sodium thiosulphate to neutralize any possible residual ozone or derivate of ozone oxidation (Tribst *et al*., 2009). The effect of micro-organisms removal by water drag out was evaluated using bottles completely filled with sterile water for 10 minutes, following by bottles washing with sterile solution of 10% sodium thiosulphate (to emulate the process with ozonated water). The sanitation process was carried out in duplicate. A triplicate of bottles was evaluated at each studied condition.

Microbiological analysis

Following sanitation and neutralization, the bottles were filled with rinse solution (20 mL of 0.9% saline solution and capped with their original cap). After, the bottles were rinsed vigorously by manual agitation for 2 minutes. Then, the rinse solution of each bottle was subjected to heat shock at 70ºC/20 minutes to active the *B. subtilis* spores. After, serial dilutions were performed; samples were plated in nutrient agar and incubated at 35ºC for 48 h. The results were expressed as number of decimal reductions (NDR), calculated using the equation 1:

NDR = log (*B. subtilis* count after sanitation/ *B. subtilis* initial count) (Equation 1)

The initial count (control sample) was determined by washing the bottle with 20 mL of saline solution immediately after the adherence of *B. subtilis* (samples not washed with ozonated or sterile water).

Statistical analysis

Data were statistically evaluated by ANOVA and the differences between the results were determined by Tukey test, considering 95% of confidence level ($p < 0.05$). The statistical analyses were performed using software STATISTICA 7.0.

Results and Discussion

The first attempt of *Bacillus subtilis* inactivation was carried out using ozone at the concentration of 1 mg.L^{-1} for 2 minutes of contact time. However, no significant reductions on the *Bacillus subtilis* were observed (final count of 4.9 x 10^5 CFU.mL^{-1}, with reduction of around 0.5 log cycles). Previous results of *Bacillus subtilis* inactivation in lettuce showed that the sanitation process using ozonated water at 1 mg.L^{-1} for 1 minute reduced 5.3 log cycles of this microorganism (Cavalcante, 2007). The difference in the microbial inactivation caused by ozone at the same concentration/contact time can be attributed to the step of spores adherence used in this work and the absence of humidity in the package. Possibly, the spore adherence increases the spores resistance to the sanitizer. This is an important step to be performed in test of microbial resistance to sanitizer, since the microorganism is normally adhered to the food surface and packaging.

Figure 1. Bottles used in the experiments

Considering that no efficacy of the ozonated water was observed at concentration of 1 mg.L^{-1}, the study was carried out using the maximum ozone concentration able to be reached in the water by the equipment (4 mg.L^{-1}). The decay of ozone concentration in water was measured to determine the ideal contact time to be applied in sanitization process. Figure 1 shows the decay of ozone concentration.

The ozone concentration rapidly decayed in ozonated water. This fast reduction of the ozone concentration was described previously by Gurol and Singer (1972) and occurred due to the decomposition of ozone into gaseous oxygen, since ozone is a highly unstable molecule.

An exponential model was that better fitted in the data of ozone concentration decay (Figure 2). This highlights that the decomposition of ozone in water is higher in the first minutes after solvation. Moreover, lower reductions in ozone concentration were observed after 10 minutes of ozone solvation in water. By the equation, the half-life of ozone in water was calculated as 9.2 minutes. Considering the decay of ozone concentration in the ozonated water, the assays of *Bacillus subtilis* inactivation in PET bottles were performed at contact times of 3, 7 and 10 minutes, which represent, respectively, ozone concentrations of 3, 2.3 and 2 mg.L^{-1} at the end of the sanitation process for each contact time.

Table 1 shows the counts of *B. subtilis* spores recovered from the bottles (control and ozonated samples using ozone concentration of 4 mg.L^{-1}). The count of control sample showed that the procedure was able to recover almost 100% of the inoculated spores, showing that the procedure adopted was

Table 1. Counts of *B. subtilis* spores recovered from the bottles

Sample	Count (CFU. mL^{-1})
Control	5.6 x 10^5 (~99% of the inoculated spores)
Water	4.9 x 10^4
4 mg.L^{-1} of ozone / 3 minutes	1.7 x 10^3
4 mg.L^{-1} of ozone / 7 minutes	3.4 x 10^3
4 mg.L^{-1} of ozone / 10 minutes	2.2 x 10^3

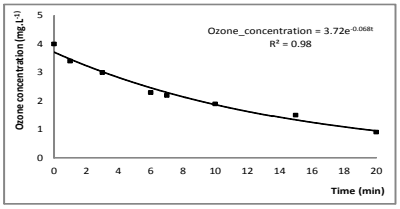

Figure 2. Decay of ozone concentration in ozonated water

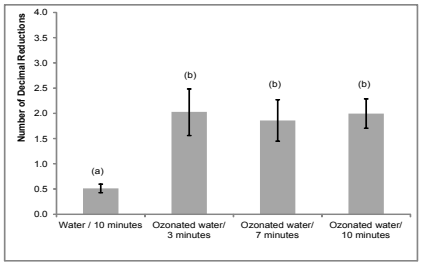

Figure 3. Number of decimal reduction after PET bottles sanitation with ozonated water at 4 mg.L^{-1} for different time. Different letters indicates significant differences between the results ($p < 0.05$)

efficient to remove the adhered spores in the package surface. On the contrary, when the bottles were just filled with water (without mechanical agitation), the counts of *B. subtilis* spores was kept high, indicating that the water drag out in the bottles inoculated was minimum in this process. For samples subjected to ozonated water, lower counts were reached after process using ozone at 4 mg.L^{-1}.

Figure 3 shows the number of decimal reductions obtained for bottles washed just with water and sanitized with ozonated water for different contact time. The bottles washed just with water showed around 0.5 decimal reductions, indicating a minimal removal of spores by water. On contrary, the use of ozonated water (4 mg.L^{-1}) resulted on around 2 decimal reductions of *B. subtilis* independent on the contact time applied (3-10 minutes). These results shown that ozonated water is effective just at concentrations up to 3 mg. L^{-1} (concentration reached after 3 minutes of contact time) when the target is *B. subtilis* spores adhered in plastic bottles. Therefore, 3 minutes was enough to reach the maximum decimal reduction caused by ozonated water at 4 mg.L^{-1}.

Cavalcante (2007) showed 5.3 decimal reductions of *B. subtilis* in lettuce washed with 1 mg.L^{-1} ozonated water for 3 minutes and Amorim *et al.* (2013) observed more than 5 decimal reductions of *B. subtilis* in cassava starch using gaseous ozone. Macedo (2004) observed 3 decimal reductions of this same microorganism in water after ozonation with 0.7 mg.L^{-1}. Additionally, higher concentration of ozone in ozonated water (8-13 mg.L^{-1}) was described as able to inactivate 8 log cycles of *Bacillus subtilis* in multi-layer laminated package and stainless steel (Khadre and Yousef, 2001).These results showed that ozonated water efficacy is normally higher than the observed in the bottles.

The differences can be attributed to the initial load of *Bacillus subtilis* adhered in the bottles, the adherence process, the ozone concentration, the packaging surface and the absence of water in the package. Similarly to the results of bottles decontamination, 3 decimal reductions were obtained after sanitation of stailess steel surface contamined with *B. subtilis* spores using gaseous ozone at 1 mg.L^{-1} (Aydogan and Gurol, 2006).

Although the inactivation reached in PET bottles were just 2 log cycles, this could be enough to guarantee a safe packaging, when associated with good practices in packaging production and storage. This can substitute the application of chlorine for bottles sanitation, reducing the water consumption in the food industry (ozonated water not requires rinse process after sanitation, since the ozone is degradable) and the need of wastewater treatment.

Conclusion

The ozonated water at 4 mg.L^{-1} caused 2 decimal reduction of *B. subtilis* spores in PET bottles, being an alternative for packaging sanitation with low level of contamination. Additionally, ozonated water with ozone concentrations below than 3 mg.L^{-1} was ineffective for *B. subtilis* spores inactivation.

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