

Sporicidal Effect of Ozone on Fungal and Bacterial Spores in Water Disinfection

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Abstract: The sporicidal effects of high ozone concentrations were tested against an endospore forming bacterial strain (*Bacillus subtilis* ATCC 6633) and a fungal strain (*Aspergillus brasiliensis* ATCC 16404) as a method of water disinfection. We compared the sporicidal action of ozone against these fungal and bacterial strains. Under identical treatment conditions, ozone showed a sporicidal effect on bacterial and fungal spores in water. Our present results showed that ozone concentrations at 7.0 and 9.0 g/m³ have a sporicidal effect against bacterial and fungal spores respectively. Electron microscopic study of ozone-treated *B. subtilis* and *A. brasiliensis* spores mentioned above suggests the outer spore coat layers as a probable site of action of ozone. Our present study on ozone supports the notion that oxidizing agents including ozone probably kill spores by degrading the outer spore components and exposing the spore core to the action of the sanitizer. The ozone was generated using coaxial dielectric-barrier-discharge (DBD) technique. The coaxial DBD cell consists of two cylindrical coaxial electrodes separated by a gap distance and dielectric barrier (glass). AC (50 Hz) high voltage (2-5 kV) was applied on the DBD cell to generate filamentary discharge. The DBD cell is fed by oxygen gas. The basic mechanism of ozone generation simply consists of dissociation of oxygen molecules by the discharge electrons that are formed in the discharge filaments inside the discharge gap. The atomic oxygen, which is produced due to the dissociation, reacts with the oxygen molecules to form ozone. In the discharge, the oxygen molecules are dissociated prior to ozone formation. The concentration of the generated ozone was controlled by the discharge current and the gas flow rate. The generated ozone was used to treat the spores under investigation.

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1. Introduction

Water arrives in many ways: simple rain, floods, tornados, hurricanes, monsoons, snow, hail and sleet. These bring to organisms precious water needed for life. Without water, there would be no life. With water, there is life and among the living are molds, yeasts and bacteria. The molds, yeasts and bacteria can form spores. Spores are important for survival and dissemination (Donald, 2009).

Microbial life is abundant, tenacious, and is often very difficult to control. Organisms including viruses, bacteria, and fungi are often characterized by an ability to spread easily, reproduce rapidly, and thrive under conditions that can destroy higher life forms. Many of these organisms are completely harmless to humans and play an important role in many eco-systems and natural processes. Some organisms, though, cause human diseases, and exclusion or destruction of these organisms is important to prevent or block the spread of disease (Cross *et al.*, 2003).

Bacterial spores are of considerable interest due to their remarkable resistance to physical agents and regular antiseptics. Their tolerance to heat and the fact that they can survive a number of years in a dried state are of great importance in medicine as

well as in food preservation (Mathias *et al.*, 2010). Microbiological spores are among the most resilient forms of dormant life known to man. Although formed by many different types of microbiological organisms, the most commonly studied spores are from various *Bacillus* or *Clostridium* species. In particular, *Bacillus* spores are amazingly resistant to common sterilizing techniques. For example, most vegetative bacteria die quickly when subjected to temperatures in excess of 80 °C, but bacterial spores often survive boiling water at 100 °C for two hours or more. Spores have survived for 20 years in 70% alcohol solutions. Drying has little effect on spores, as demonstrated by spores surviving in the intestines of Egyptian mummies for thousands of years (Alcamo, 2001).

Fungi are ubiquitous in nature (Goncalves *et al.*, 2006). They are present in and have been recovered from a wide range of aquatic habitats including lakes, streams, distribution systems, drinking water and also on the surface of drinking water reservoirs and distribution pipes (Bitton, 2005). Fungi may cause some problems in drinking water. They are involved in the production of taste and odors in water (Bitton, 2005; Goncalves *et al.*, 2006). Some of them form humic-like substances that may

act as precursors of trihalomethanes (Bitton, 2005). Health problems are also possible. *Aspergillus* is a group of filamentous fungi that account among the most frequently isolated fungi in water environments.

Aspergillus spp. could produce aflatoxins, a group of acutely toxic and potentially carcinogenic and immunosuppressive mold metabolites. Although aflatoxins are often associated with food but aflatoxins and *Aspergillus flavus* were detected from stored water (Goncalves *et al.*, 2006). There are also evidences showing nosocomial aspergillosis, a life threatening infection in immunocompromised patients, that is thought to be primarily airborne may be waterborne in hospital environments (Anaissie and Costa, 2001; Anaissie *et al.*, 2002). Waterborne *Aspergillus* species can aerosolize and their concentration is highest near water activity (Anaissie and Costa, 2001). Moreover fungal spores appear to be more resistant to chlorine and ozone than coliform bacteria and can remain viable for extended periods of time (Bitton, 2005).

An understanding of mold spores is important for control. Without control, mold spores can cause extensive damage of materials and goods. Mold spores are reproductive, they are like fertilized eggs; each spore can generate a new mold. One mold colony can produce millions of spores in a day. Mold spores are resistant to drying (desiccation) and heat, but they may be killed by boiling, ozone, and fungicides containing phenolics and quaternary ammonium treatments. Mold spore walls are chemically similar to their thread-like hyphae. However, spore walls are much thicker more than mold hyphae. The thick spore walls resist drying and protect against heat. Some molds like *Rhizopus* form spores inside a sporangium (Donald, 2009).

Ozone is a strong, fast and broad-spectrum antimicrobial agent that works effectively against bacteria, bacterial spores, viruses, fungi, fungal spores and protozoa. Unlike many other sterilizing agents ozone is easy and fast to remove after the process and does not leave any remaining chemicals, odor or taste. Inactivation of bacteria is thought to occur by ozone oxidizing the fatty acids in the cell-membrane and macromolecules, like proteins and DNA. The damage caused by ozone is irreversible and causes lysis of the cell wall and the death of the bacteria. It also kills spores and viruses as it oxidizes DNA and proteins in the spore as well as in viruses. The effect of ozone in water is well known and seems to be more effective than ozone in air. It has also been indications that ozone needs a higher humidity in the air to be really effective (Ronny, 2005).

Significant advantages of ozone in water are that it decomposes quickly to oxygen, leaving no residue of itself and few disinfection by-products,

and it has more potency against bacteria, cysts of protozoa, viruses, and fungal spores than hypochlorite (White, 1999). Ozone can oxidize many organic compounds, particularly those with phenolic rings or unsaturated bonds in their structure (Razumovski and Zaikov, 1984), and can therefore have a role in reducing pesticide residues in process water (Nickols and Varas, 1992) and mycotoxins in durable commodities (McKenzie *et al.*, 1997). Ozone attacks and dissolves the spore's coat, and an overly long exposure time eliminates any trace of the spore.

2. Material and Methods

2.1. The test microorganisms

2.1.1. Bacterial and fungal strains

Bacterial and fungal strains used in the course of this study were *Bacillus subtilis* ATCC 6633 and *Aspergillus brasiliensis* ATCC 16404. The strains were obtained kindly from the Department of Microbiology of Memphis Pharmaceutical Company (Cairo, Egypt). The bacterial strain was cultivated on Nutrient agar slants, while the fungal strain was cultivated on Malt extract agar slants.

2.1.1.1. Bacterial spores Preparation

To prepare *B. subtilis* ATCC 6633 spores (Lijie Li, 2004), a loopfull of *B. subtilis* was inoculated onto nutrient agar slants and incubated at 37°C for 10 days to sporulate. The spores were collected by rinsing the slants with sterile phosphate buffer and centrifuging at 4000 rpm for 4 minutes. Then the supernatant was withdrawn and washed twice with buffered water and centrifuged at 6000 rpm for 10 minutes. The final sporulated suspension was pasteurized in a 75°C water bath for 15 minutes with the purpose of killing all vegetative cells and to activate spore germination. The spore suspension was stored at 4°C for no more than one week until they were needed.

2.1.1.2. Cultivation of treated *Bacillus subtilis* ATCC 6633 spores

The treated spores were diluted, inoculated onto the surface of nutrient agar plates and incubated at 37°C for 24 hours (Lijie Li, 2004). At the end of incubation period, the number of colonies in the plates was counted (the number of colonies to be counted was between 20 and 80 colony/plate).

2.1.1.3 Determination of viable spores concentration (CFU/ml)

The viable spores (bacterial or fungal) concentration was computed and expressed (Lijie Li, 2004) as colony forming units (CFUs)/ml as shown in the following equation:

$$\text{CFU/ml} = \sum Ni / \sum Vi$$

Where Σ is the summation taken overall plates of the particular sample, N_i is the colonies counted in a plate, V_i is the actual volume (ml) of the sample in a plate.

2.1.1.4. *A. brasiliensis* ATCC 16404 spores Preparation

Fungal spore preparation was carried out as described by Mahnaz and Hossein (2008). Indigenous spores were prepared by streaking on malt extract agar medium. The pure culture was inoculated into 10 ml malt extract broth tubes and after 5 days incubation at 37°C, spores were collected and harvested by washing with sterile deionized water containing 1% Tween 80. Spores densities were determined microscopically by haemocytometer and controlled by spreading plate of 200 μ l serially diluted suspensions on malt extract agar. The suspension of concentrated spores was stored at 4°C until they were needed.

2.1.1.5. Cultivation of treated *A. brasiliensis* ATCC 16404 spores

The treated spores were diluted, inoculated onto the surface of malt extract agar plates and incubated at 37°C for 5 days. At the end of incubation period, the number of colonies in the plates was counted (the number of colonies to be counted was between 20 and 80 colony/plate).

2.2. Ozone generation

The ozone was generated using coaxial dielectric-barrier-discharge (DBD) technique. The coaxial DBD cell consists of two cylindrical coaxial electrodes separated by a gap distance and dielectric barrier (glass). AC (50 Hz) high voltage (2-5 kV) was applied on the DBD cell to generate filamentary discharge. The DBD cell is fed by oxygen gas. The basic mechanism of ozone generation simply consists of dissociation of oxygen molecules by the discharge electrons that are formed in the discharge filaments inside the discharge gape. The atomic oxygen, which is produced due to the dissociation, reacts with the oxygen molecules to form ozone. In the discharge, the oxygen molecules are dissociated prior to ozone formation. The concentration of the generated ozone was controlled by the discharge current and the gas flow rate was adjusted to 5 L/min (Garamoon *et al.*, 2009). Ozone was applied directly into the tubes containing the bacterial and fungal spores under investigation.

2.3. Ozone treatment and analysis

Bacterial and fungal spores were exposed to different concentrations of ozone viz. 0.0, 1.0, 3.0, 5.0, 7.0 and 9.0 g/m³ for 1 minute. Microbial survival was expressed as the log spores per ml.

2.4. Electron microscopy

The samples were coated by gold sputter coated (SPI-Module) and examined by Scanning electron microscopy (JEOL-JSM-5500 LV) by using high vacuum mode at the Regional Center of Mycology and Biotechnology, Cairo, Egypt.

3. Results

3.1. Effect of ozone on *Bacillus subtilis* ATCC 6633 spores

Treatment of spore suspension with different ozone concentrations, of 1.0, 3.0, 5.0, 7.0 and 9.0 g/m³ for 1 minute, reduced spore counts by 5.4×10^7 until become undetected at ozone concentrations of 7.0 and 9.0 g/m³, respectively. Results in table (1) illustrate the sporicidal effect of ozone, since the total count of *B. subtilis* ATCC 6633 spores decreased obviously when the concentrations of ozone increased (Fig.1).

Table 1. Treatment of *B. subtilis* (ATCC 6633) spores with different Ozone concentrations for 1 min. at 37 °C

Ozone Conc. (g/m ³)	Viable spores (CFU/ml)	Log spores/ml
0.0	5.4×10^7	7.7
1.0	2.2×10^5	5.3
3.0	4.3×10^3	3.6
5.0	5.8×10^2	2.7
7.0	0.0	0.0
9.0	0.0	0.0

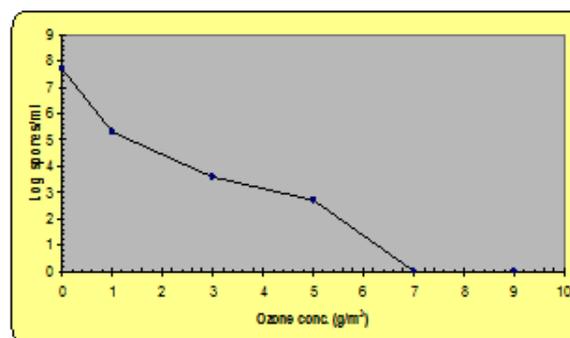


Figure 1. Inactivation of *B. subtilis* ATCC 6633 spores, when treated with different Ozone concentrations for 1 min. at 37 °C.

3.2. Mechanism of action of ozone on *Bacillus subtilis* ATCC 6633 spores

Inactivation of bacteria by ozone is a complex process because ozone attacks numerous cellular constituents including proteins, unsaturated lipids and respiratory enzymes in cell membranes, peptidoglycan in cell envelopes, enzymes and nucleic acids in the cytoplasm, proteins and peptidoglycan in

spore coats. Correlation between susceptibility of *B. subtilis* ATCC 6633 spores to ozone gas may reflect the mechanism of spore inactivation. Spores, treated and untreated with ozone, were examined by scanning electron microscope (SEM). Obviously, these micrographs revealed damage to the surface layer of ozone-treated spores (Fig.2).

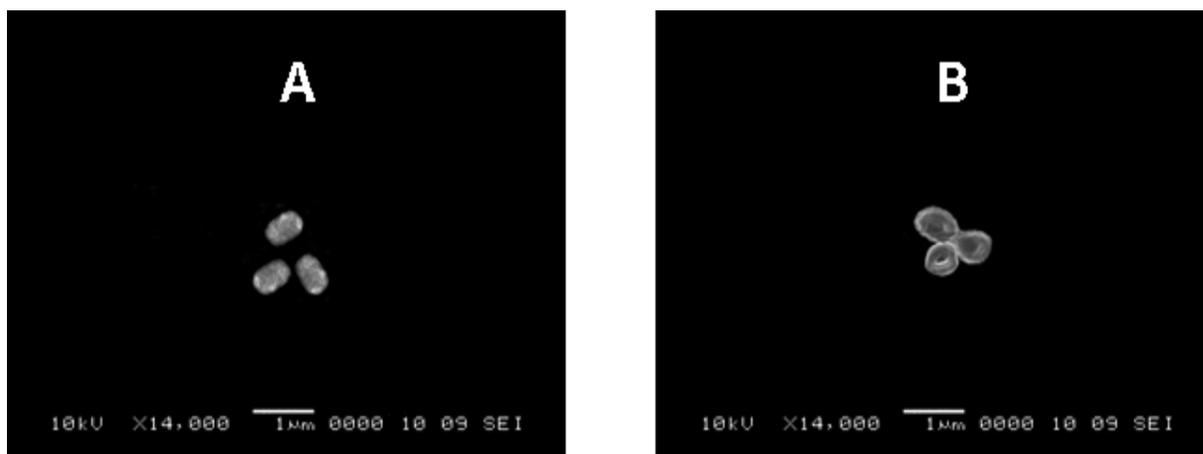


Figure 2. Scanning electron microscopic micrograph of *B. subtilis* ATCC 6633 spores; untreated (A) or treated (B) with ozone conc. (5.0 g/m^3) at 37°C for 1 min. Note that the surface layer and the outer spore coat are the structures most apparently damaged by ozone treatment.

3.3. Ozone effect on *Aspergillus brasiliensis* ATCC 16404 spores

The survival rate of *Aspergillus brasiliensis* ATCC 16404 was decreased as ozone concentrations increased. Increasing ozone concentrations are more effective against spores of *Aspergillus brasiliensis* ATCC 16404 as shown in table (2). It was found that ozone concentration of 9.0 g/m^3 has a lethal effect on *A. brasiliensis* ATCC 16404 spores during 1 minute exposure (Fig.3). From the table it can be seen that moderate doses of ozone are sufficient to achieve significant microbial reductions.

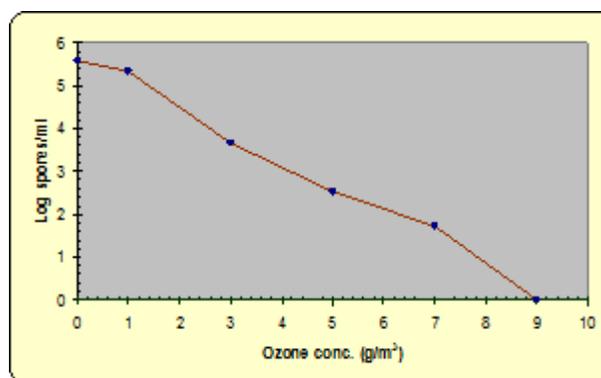


Figure 3. Effect of different ozone concentrations on the spores of *A. brasiliensis* ATCC 16404 during 1 min exposure at 37°C .

Table 2. Reduction of the survival rate of *Aspergillus brasiliensis* ATCC 16404 at different ozone concentrations for 1 min at 37°C

Ozone conc. (g/m^3)	Viable spores (CFU/ml)	Log spores/ml
0	3.7×10^5	5.57
1	2.2×10^5	5.34
3	4.8×10^3	3.68
5	3.4×10^2	2.53
7	0.5×10^2	1.71
9	0.0	0.0

3.4. Mode of action of ozone on *A. brasiliensis* ATCC 16404 spores

Scanning Electron micrograph appearance of the spores (Fig.4) showed observable changes in the fine structure of the fungal spores. Ozone, in our study, damaged the outer layer of spore as well as the inner layer. It is suggested that the vast majority of these spores lost their viability. Ozone and its produced free radicals play a part in this inactivation mechanism but there is no consensus on which of

them is more decisive. As shown in fig.(4) the fungal spore is damaged by spore disruption or disintegration leading to leakage of the spore contents. Our present study on ozone supports the

notion that ozone can kill spores by degrading outer spore components, and exposing the spore core to the action of the oxidizing agent.

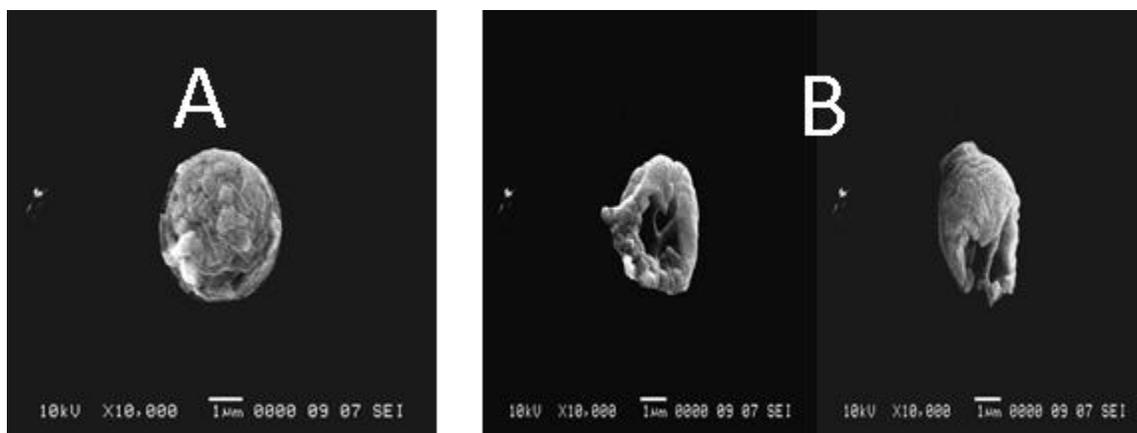


Figure 4. Scanning electron microscopic micrograph of *Aspergillus brasiliensis* ATCC 16404 spores; untreated (A) or treated (B) with ozone. Ozone-treated spores were exposed to ozone conc. (7.0 g/m^3) at $37 \text{ }^\circ\text{C}$ for 1 min. Note that the fungal spore is damaged by spore disruption or disintegration leading to leakage of the spore contents.

4. Discussions

The purpose of this study was to investigate the potential of using ozone gas to kill microbial (fungal and bacterial) spores. The results achieved during this study show that ozone may work as a sanitizing agent for disinfection and may be even for sterilization, although a higher ozone concentration was needed to get a major killing effect on *Aspergillus brasiliensis* ATCC 16404. An experiment to see the effect of ozone on fungal and bacterial spores in a short time (1 min.) was performed, and the killing effect was high. In just 1 min. almost all of the tested microorganisms including the very tolerant *Aspergillus brasiliensis* spores were inactivated. The experiment performed to see how well ozone at different concentrations could be used for eliminating microbial spores in water. This result is significant in terms of the fact that the used ozone gas has a sporicidal effect on the bacterial and fungal spores under investigation. This work is quite complete and agrees with that of other workers. Foegeding, (1985) found that *Bacillus cereus* spores, with removed coat, were rapidly inactivated by ozone, compared to intact spores.

Bacterial spores are known to be resistant to common antimicrobial physicochemical agents (Mathias *et al.*, 2010). As compared to vegetative cells, spores are highly resistant to a wide range of toxic chemicals (Setlow, 2006). The spore's first line of defense is the coat, the multiple protein layers of which act as a chemical filter. The researcher concluded that the spore coat is a primary protective

barrier against ozone. Recently, Khadre and Yousef (2001b) found that spores of *Bacillus subtilis* treated with aqueous ozone showed heavily disrupted outer spore coats.

Researches that are carried out on fungi indicated that the mode of action of ozone on fungi is not certain. Since ozone attacks cellular membranes of higher plants, perhaps fungal membranes could be similarly affected. If that is true, exposed conidial membranes could experience decreased differential permeability. Perhaps ozone increases conidiophore respiration, resulting in prematurely formed and nonviable conidia. Ozone exposure of conidia to 0.30 ppm for two 6-hr periods totally inhibited their ability to infect detached leaves (McKeen, 1974). Ozone inactivation of pathogenic fungi (*Aspergillus niger*) was studied by Coronel *et al.* (2002). Ozone at certain doses may inhibit, directly or indirectly, enzyme activity of the fungus thus resulting in less maceration of cells and possibly decreased infection. A comprehensive study has shown the effectiveness of ozone as a germicidal agent against a wide range of pathogenic organisms like bacteria, protozoa, fungal and bacterial spores (Kim and Yousef, 1999). Kottapalli *et al.* (2005) indicated significant reduction in *Fusarium* survival rates upon treatment with gaseous ozone.

Ozone is a potent oxidizing agent that can be used for disinfection in the food industry (Rice *et al.*, 2000). Low concentrations of ozone and shorter contact times are necessary compared to other weaker

oxidizers such as chlorine, mono-chloramine and chlorine dioxide (DeMers and Renner, 1992). Ozone also is a potent sanitizer with promising applications in the modern food industry. The sanitizer is effective against a wide spectrum of microorganisms, and it can be used in an environment-friendly manner. Currently, ozone is the most likely alternative to chlorine and hydrogen peroxide in food applications.

Knowledge of the disinfection mechanism of pathogens is important for optimizing kill efficiency and minimizing undesired effects on surroundings and on higher-level multicellular organisms. Disinfectants can be classified into two groups according to whether the kill mechanism originates from inside the pathogen or outside. Ozone may oxidize various components of cell envelope including polyunsaturated fatty acids, membrane-bound enzymes, glycoproteins and glycolipids leading to leakage of cell contents and eventually causing lysis (Khadre *et al.*, 2001).

RNA of microorganisms is degraded into protein subunits by ozonation (Kim *et al.*, 1980). Roy *et al.* (1981) indicated that the primary mode of fungal spore inactivation by ozone appears to be nucleic acid damage. Ozone degradation of nucleic acids was also studied by Shinriki *et al.* (1981). Several authors referred to enzyme inactivation as an important mechanism by which ozone kills cells. Takamoto *et al.* (1992) observed that ozone decreased enzyme activity in *E. coli* at a greater degree in case of cytoplasmic α -galactosidase than in case of the periplasmic alkaline phosphatase. More recent work has shown that ozone treatment does not destroy spores by causing DNA damage, but affects spore germination by damaging the inner membrane of the spore's coat (Young, 2000). Ozone is shown to have produced single and double-strand breaks in plasmid DNA and to open up circular plasmid DNA (Hamelin, 1985). Ozone treatment also decreased transcription activity of plasmid DNA (Mura and Chung, 1990). Ozone has also been shown to cause mutation in *E. coli*, however, ozone was considered to be a weak mutagen (Dubeau and Chung, 1982). Franco (2005) found that ozonation of RNA causes its denaturation. Also, ozone showed antifungal activity against *Aspergillus fumigatus* (Geweely, 2006).

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