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Efficacy of Ozonated Water against Various Food-Related Microorganisms

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The antimicrobial effects of ozonated water in a recirculating concurrent reactor were evaluated against four gram-positive and four gram-negative bacteria, two yeasts, and spores of *Aspergillus niger*. More than 5 log units each of *Salmonella typhimurium* and *Escherichia coli* cells were killed instantaneously in ozonated water with or without addition of 20 ppm of soluble starch (SS). In ozonated water, death rates among the gram-negative bacteria—*S. typhimurium*, *E. coli*, *Pseudomonas aeruginosa*, and *Yersinia enterocolitica*—were not significantly different ($P > 0.05$). Among gram-positive bacteria, *Listeria monocytogenes* was significantly ($P < 0.05$) more sensitive than either *Staphylococcus aureus* or *Enterococcus faecalis*. In the presence of organic material, death rates of *S. aureus* compared with *L. monocytogenes* and *E. coli* compared with *S. typhimurium* in ozonated water were not significantly ($P > 0.05$) affected by SS addition but were significantly reduced ($P < 0.05$) by addition of 20 ppm of bovine serum albumin (BSA). More than 4.5 log units each of *Candida albicans* and *Zygosaccharomyces bailii* cells were killed instantaneously in ozonated water, whereas less than 1 log unit of *Aspergillus niger* spores was killed after a 5-min exposure. The average ozone output levels in the deionized water (0.188 mg/ml) or water with SS (0.198 mg/ml) did not differ significantly ($P < 0.05$) but were significantly lower in water containing BSA (0.149 mg/ml).

"The treatment of domestic water to provide a microbiologically safe, aesthetic, potable end product has been normal practice since before the turn of the twentieth century" (5). Chlorine at low concentrations has been the usual agent of choice in drinking-water purification. At the low concentrations of chlorine used, however, its limitations as a bactericide (3, 4) as well as limitations in its effectiveness against certain eukaryotic pathogens and viruses (18, 20, 21, 25, 27) have been recognized. Furthermore, chlorine at low concentrations may alter certain organic compounds in water producing off tastes and odors as well as forming chloro-organic compounds with carcinogenic potential (22).

As an alternative to chlorination in drinking-water disinfection, ozonation of water supplies, which was done first a century ago (29), has become an established means of disinfection and has been reviewed by Rice et al. (25). Bacteria, including *Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus*, *Bacillus megaterium*, *Salmonella typhimurium*, *Shigella flexneri*, and *Vibrio cholerae* are sensitive to ozonated water under various conditions (5, 6, 13, 17). Limited information on ozone's effectiveness against bacterial endospores (5, 17) and viruses (6, 20) as well as against eukaryotic pathogens including *Cryptosporidium parvum* (11, 21, 24) and *Giardia lamblia* and *Giardia muris* (12, 31, 32) also exists.

The use of ozone in the food industry has been investigated for food preservation, shelf life extension, equipment sterilization, and improvement of food plant effluents (9, 14, 19, 28).

The purpose of this investigation was to determine the antimicrobial efficacy of ozonated deionized water (with and without added organic material) generated by a recirculating ozone reactor (Lifex EV 2000R) against various heterotrophic bacteria and fungi important in the food industry as pathogens, spoilage organisms, and indicators of fecal contamination.

Pseudomonas aeruginosa ATCC 15442, *E. coli* ATCC 25922, *S. typhimurium* ATCC 6994, *S. aureus* ATCC 6538, *B. cereus* (food isolate), *Listeria monocytogenes* 4b (courtesy of Silliker Laboratories, Chicago Heights, Ill.), *Enterococcus faecalis* (food isolate), *Yersinia enterocolitica* ATCC 27729, *Candida albicans* ATCC 22572, *Zygosaccharomyces bailii* (purchased from Silliker Laboratories; isolated from mayonnaise), and *Aspergillus niger* (courtesy of James Grosklags, Northern Illinois University, DeKalb) were used in this investigation. The bacterial cultures were maintained on Difco (Detroit, Mich.) brain heart infusion agar slants except for *E. faecalis*, which was stored on Difco APT agar slants. All the bacterial cultures were stored at 4 to 6°C and transferred every 4 weeks. The two yeast cultures and *A. niger* were maintained on Difco potato dextrose agar slants, stored at 4 to 6°C, and transferred every 12 weeks. Loopfuls of cells from each of the bacterial strains except *E. faecalis* were aseptically transferred into 40 ml of brain heart infusion broth in 250-ml Erlenmeyer flasks and incubated at 35°C for 24 h, whereas *B. cereus* cells were incubated at 30°C for 24 h. *E. faecalis* cells were transferred to 40 ml of Difco APT broth in 250-ml Erlenmeyer flasks and incubated at 30°C for 24 h. For *C. albicans* and *Z. bailii*, loopfuls of cells were transferred to 40 ml of Difco potato dextrose broth in 250-ml Erlenmeyer flasks, placed in a gyratory water bath shaker model G76 (New Brunswick Scientific, Edison, N.J.) (100 to 125 rpm), and incubated at room temperature (18 to 22°C) for 48 h. After incubation, 30-ml volumes of each culture were centrifuged at $1,450 \times g$ for 25 min at room temperature. The pellets were resuspended in 20 ml of sterile saline and centrifuged again at $1,450 \times g$ for 25 min. After the second centrifugation, the pellets were resuspended in 10 ml of sterile saline. For the tests with soluble starch (SS; Fischer Chemical, Fairlawn, N.J.) and bovine serum albumin (BSA; Fraction V 96 to 99% albumin; Sigma, St. Louis, Mo.), the pellets were

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resuspended in 10 ml of sterile saline containing 20 ppm (20 mg/liter) of the corresponding organic material.

For *A. niger*, the spores from the stock culture were aseptically transferred to two potato dextrose agar slants and incubated at room temperature (18 to 22°C) for 8 days. After incubation, 15 ml of sterile 0.05% Triton X-100 (Sigma) was added to each tube and the spores were resuspended by using a vortex mixer (Baxter, McGaw Park, Ill.). The spore suspensions were centrifuged as described above, and the final pellet was resuspended in 15 ml of sterile saline. All washed cells and spores were chilled before use.

Ozone levels (in milligrams per liter) were measured by the indigo colorimetric method (1). Ozonated water was generated by a Lifex ozone apparatus (model EV 2000 R; Life Ozone Corp., Southfield, Mich.) using a 115 to 120 variable alternating current power source.

Duplicate ozone determinations of uninoculated solutions were performed directly at the generator outlet faucet and in the reservoir near the inlet tube before each organism was passed through the ozone apparatus. A concurrent system in which the ozone gas and water flowed in the same direction was used in this investigation. According to Lev and Regli (23), ozone concentrations (in milligrams per liter) can be conservatively measured in a "cocurrent" (sic) system in two ways: (i) outlet faucet concentration (C_{out}) and (ii) average concentration (C_{avg}), calculated as (C_{out} + inlet C_0)/2, where C_0 is the concentration near the inlet. Since C_0 was substantially lower in our system, C_{out} was a better measure for determining overall ozone concentration versus antimicrobial efficacy (23).

The antimicrobial effects of ozone were determined in deionized water with or without added organic material in the form of 20 ppm of SS or BSA, which may simulate organic levels in treated wastewater (30). BSA was dissolved directly in the deionized water, whereas SS was first dissolved by boiling a concentrated solution in deionized water before diluting. All solutions were ozonated at room temperature (19 to 21°C). The pH levels of nonozonated deionized water and ozonated deionized water were 5.7 and 6.1, respectively, whereas before and after ozonation in the presence of SS the pH remained at 6.0. In the presence of BSA, the pH was reduced from 6.4 to 6.0 after ozonation.

To a previously sanitized (70% ethanol followed by air drying) polyethylene container (capacity, 3.785 liters), 1.5 liters of deionized water with or without 20 ppm of SS or BSA was added. Four milliliters of washed cells or spores was pipetted into the polyethylene container, and the contents were stirred, yielding approximately 10^6 cells or spores per ml. Triplicate samples were extracted from the container to determine the initial cell or spore concentrations before ozonation. The ozone apparatus was equipped with a peristaltic pump producing a constant flow rate of 660 ml/30.0 s and an outlet faucet with an on-and-off lever. The hoses attached to and contained inside the ozone apparatus had a total volume of 150 ml. The hose attached to the peristaltic pump was primed and placed in uninoculated water while the faucet in the open position was placed over an empty container. The ozone apparatus was turned on, and the uninoculated water was pumped through the machine for 15 s, allowing approximately 330 ml to flush through the system. While still on, the inlet hose was momentarily blocked aseptically, removed from the uninoculated deionized water, and immediately immersed into the inoculated menstruum. After the uninoculated deionized water was allowed to purge the system for 25 s, a minimum of 400 ml of the inoculated suspension was flushed through the system and a sample designated "time zero" was taken. Immediately after the time zero sample, the faucet was placed in the inoculated container just over the water level, forming a recirculation loop. During inoculum recirculation, samples were taken after 1, 2, and 5 min. Final volumes in the container and ozone apparatus ranged from 1.0 to 1.1 liter with a recirculation rate of 47 s. A minimum of two separate evaluations were made for each organism.

After each run, the inlet hose was immersed in 1.0 liter of deionized water and the ozone apparatus was completely cleansed and rinsed. The apparatus was disassembled, and all hoses, the polyethylene container, beakers, and screens were sanitized with 70% ethanol and air dried. The apparatus was reassembled, and 1 liter of uninoculated deionized water was pumped through the system. Samples taken to check for residual contamination showed <10 cells or spores per ml.

Samples were collected from the ozone faucet into sterile test tubes (16 by 125 mm) and 1.1 ml was immediately pipetted into 10 ml of Difco D/E neutralizing broth and subjected to vortex mixing. Sterile deionized water was used for all subsequent dilutions. The pour plate technique was used to enumerate the surviving cells and spores. All platings were performed in duplicate. For all bacterial strains, except *E. faecalis*, Difco tryptic soy agar was used, whereas Difco APT agar was used to enumerate *E. faecalis*. All tryptic soy agar plates were incubated at 35°C for 48 h except for *B. cereus* plates, which were incubated at 30°C for 48 h. Potato dextrose agar plates incubated at room temperature (18 to 22°C) for 4 to 7 days were used to enumerate surviving yeast and mold spores.

Data were converted to $\log_{10}$ values, and the geometric means of duplicate plates per organism from at least two separate experiments were calculated. The log number of cells or spores killed per milliliter equaled the geometric mean before exposure to ozone minus the corresponding geometric mean after treatment for the various time periods. By using general linear-model procedures, the means and ranges for the C_{out} and C_{avg} ozone levels in the deionized water with 20 ppm of SS or BSA and deionized water without SS or BSA were calculated.

Statistical analysis programs PROC GLM (26) were used to test the significance of the data. Repeated-measures analysis of variance (univariate tests of the hypotheses) was used to compare survivorship of *E. coli*, *S. typhimurium*, *S. aureus*, and *L. monocytogenes* cells in ozonated deionized water containing SS or BSA with survivorship in ozonated deionized water without SS or BSA. The survival comparisons for each cell wall type were made in ozonated deionized water without SS and BSA. The conservative Greenhouse-Geisser adjustments were used to modify the *F* values. Bonferroni adjustments were used for multiple pairwise contrasts (0.017). Tukey's studentized range test (95% confidence level) was used to test the ozone concentrations for all possible comparisons of deionized water with and without added 20 ppm of SS or BSA. Ozone levels in deionized water, deionized water plus SS, and deionized water plus BSA were compared by a one-way analysis of variance.

The antimicrobial effects of ozone against two gram-positive bacteria, *S. aureus* and *L. monocytogenes*, are shown in Fig. 1. Statistical analyses of the curves indicated that neither organism showed a significant difference ($P > 0.05$) in a paired death rate comparison between the antimicrobial effect of ozonated deionized water with SS and the effect of that without SS or in the comparison between ozonated deionized water containing SS and that containing BSA. When the death rates in ozonated deionized water and ozonated water containing BSA were compared, however, the antimicrobial effects with no added organic material were significantly ($P < 0.05$) greater than in the presence of BSA. Overall, more than 4 log units of *L. monocytogenes* cells per ml was killed at time zero (instanta-

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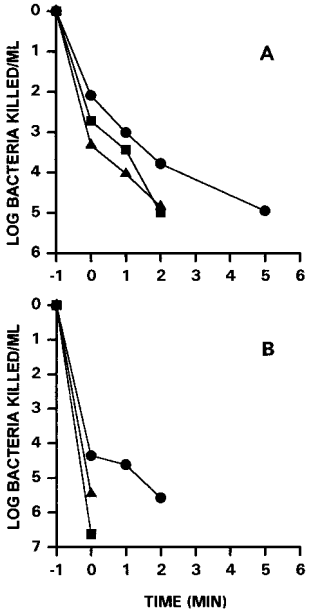

FIG. 1. Killing of viable cells of *S. aureus* (A) and *L. monocytogenes* (B) by ozone in deionized water containing no added organic material (▲) or 20 ppm of SS (■) or BSA (●). The data are means for at least two replicate experiments.

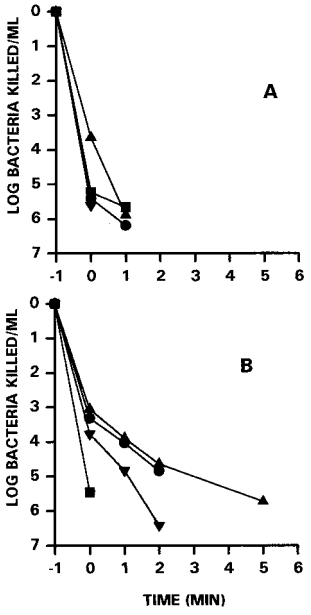

FIG. 3. Antimicrobial efficacy of ozone in deionized water against gram-negative bacteria *E. coli* (●), *S. typhimurium* (■), *P. aeruginosa* (▲), and *Y. enterocolitica* (▼) (A) and gram-positive bacteria *S. aureus* (●), *L. monocytogenes* (■), *B. cereus* (▲), and *E. faecalis* (▼) (B). The data are means for at least two replicate experiments.

neously) in the absence or presence of added organic material (Fig. 1A).

The death rate curves of ozonated deionized water with and without added organic material for *S. typhimurium* and *E. coli* are shown in Fig. 2. No significant difference ($P > 0.05$) between ozonated deionized water with SS and that without SS in the death rates or in the patterns of the curves for these two gram-negative bacteria was observed. For each bacterium, however, the death rates as well as the overall pattern of death obtained in ozonated water with or without SS were significantly greater ($P < 0.05$) than in ozonated water containing BSA. More than 5 log units each of *S. typhimurium* and *E. coli* cells per ml was killed instantaneously (time zero) after exposure to ozonated water or to ozonated water containing SS, whereas equivalent levels of death for *S. typhimurium* and *E. coli* in the presence of BSA occurred only after 2- and 5-min exposures, respectively (Fig. 2).

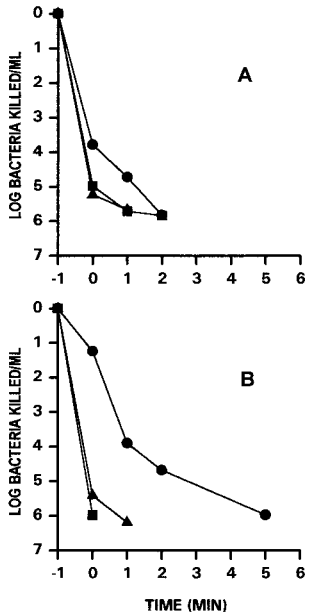

FIG. 2. Killing of viable cells of *S. typhimurium* (A) and *E. coli* (B) by ozone in deionized water containing no added organic material (▲) or 20 ppm of SS (■) or BSA (●). The data are means for at least two replicate experiments.

The antimicrobial effects of ozonated deionized water without added organic material against eight bacterial strains are presented in Fig. 3. With respect to the amount of death and the overall patterns of the death curves, no significant difference ($P > 0.05$) among the four gram-negative bacteria was observed (Fig. 3A). Among the gram-positive bacteria, the extent of death as well as the death rate curves for *S. aureus* and *E. faecalis* cells did not differ significantly ($P > 0.05$); however, *L. monocytogenes* displayed a significantly greater ($P < 0.05$) rate of death than *S. aureus* and *E. faecalis* (Fig. 3B). For *L. monocytogenes* cells, at time zero, more than 5 log units was killed by ozonated deionized water, whereas for *S. aureus*, *E. faecalis*, and *B. cereus*, 3 log units of bacteria or more was killed (Fig. 3B). Overall, except where instantaneous death occurred, most bacteria displayed biphasic death curves which were enhanced when effective ozone levels were reduced in the presence of BSA (Fig. 1 to 3).

The antimycotic effects of ozonated deionized water against yeasts of two genera and *S. niger* spores are shown in Fig. 4. Ozonated deionized water without added organic material caused more than 4.5 log units of killed cells per ml for the two yeasts, *C. albicans* and *Z. bailii*, at time zero. Less than 1 log unit of *A. niger* spores per ml was killed under the same conditions after 5 min of exposure.

Ozone concentrations in deionized water and deionized water containing 20 ppm of SS or BSA are presented in Table 1. Ozone concentrations were determined in two ways: at the outlet of the ozone apparatus (C_{out}) and over the whole system

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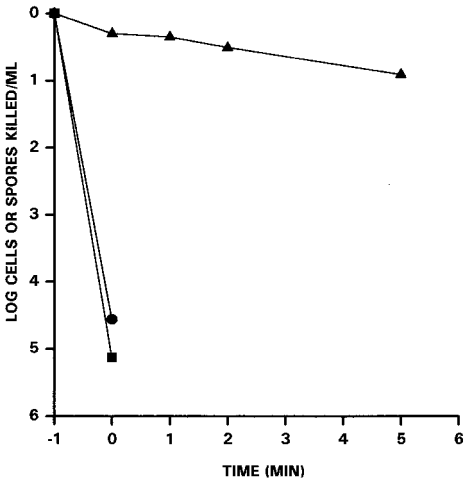

FIG. 4. Killing of fungal spores and viable yeast cells by ozone in deionized water with no added organic material. Symbols: ▲, *A. niger* spores; ●, *C. albicans*; ■, *Z. bailii*. The data are means for at least two replicate experiments.

(C_{avg}). No significant difference ($P > 0.05$) in the ozone levels (for C_{out} and C_{avg}) in deionized water and deionized water containing SS was observed. The ozone levels of C_{out} in deionized water and deionized water with SS added were 0.188 and 0.198 mg/liter, respectively, whereas C_{avg} levels were 0.064 and 0.068 mg/liter, respectively. The ozone levels for the deionized water containing BSA were significantly less ($P < 0.05$) than the ozone levels (C_{out} and C_{avg}) for deionized water and water containing SS. Average ozone concentrations in deionized water containing 20 ppm of BSA were 0.149 and 0.044 mg/liter for C_{out} and C_{avg}, respectively.

The results obtained in this study have shown that ozonated water is highly effective in killing both gram-positive and gram-negative food-associated bacteria. With the exception of *L. monocytogenes*, the gram-negative bacteria were substantially more sensitive to ozonated water than the gram-positive bacteria either in the absence or in the presence of added organic material. Yeast cells displayed sensitivity to ozonated water, whereas fungal spores were highly resistant.

Previous studies on the effects of ozone on microbes have involved the use of pure cultures and organisms naturally contaminating foods and water. Broadwater et al. (5), studying the effects of ozone on washed vegetative cells, reported that 0.12 mg/liter for *B. cereus* and 0.19 mg/liter for *E. coli* and *B. megaterium* were the minimal lethal threshold concentrations after 5 min of exposure. Fetner and Ingols (10) reported 0.4 to 0.5 mg/liter as the threshold concentration for *E. coli* after 1 min at 1°C. Both studies used varying ozone concentrations at a single time period and suggested that ozone killed microbes via an all-or-none phenomenon. Thus, in these studies either high ozone concentrations for a short period (1 min) or lower ozone concentrations for a single extended period (5 min) provided sufficient activity to reach the minimal threshold for complete killing. In the present study, however, ozone effectiveness determined by repeated sampling over a 5-min period at a single ozone concentration (0.15 to 0.20 mg/liter) indicated that an all-or-none killing phenomenon does not occur. Finch et al. (13) showed that *E. coli* in ozone demand-free phosphate water exhibited a disinfection rate consisting of an initial rapid stage followed by a slower inactivation stage. Several other studies using ozone have also shown that death rate kinetics for a variety of bacteria and viruses exhibit a biphasic process over an extended time period (6, 16, 20). In this study, biphasic death curves were observed for the majority of the gram-positive and gram-negative bacteria ozonated in the absence of added organic material (Fig. 3). This phenomenon was even more apparent wherever residual ozone levels were reduced by the presence of BSA (Fig. 1 and 2).

The sensitivity of microorganisms to ozone is profoundly affected by the organic nature of the medium, with protection caused both by physical factors, as in the case of agar, and by reduced ozone levels due to ozone demand of organic nutrients in the medium or in environmental waters (2, 8, 15, 16). This study shows that the type of organic material present during ozonation is more important than the amount present. Residual ozone levels in deionized water were significantly reduced only in the presence of 20 ppm of BSA, whereas 20 ppm of SS was without effect (Table 1). Consequently, death rates in the presence of BSA were reduced substantially but were not significantly affected in the presence of SS (Fig. 1 and 2).

The potential uses of ozone in the food industry include reduction of microorganisms on meat and poultry carcasses and in chilling water (19, 28), extension of shelf life of marine fish (14), reduction of atmospheric mold levels in meat and in small fruit storage rooms (9), and antimicrobial effects in bulk food items such as shelled eggs, bacon, beef, bananas, butter, mushrooms, cheese, and fruits (19). Additionally, ozone has been used synergistically with other antimicrobial agents (7, 14–16). The present study shows that ozonated water can effectively kill spoilage organisms (*P. aeruginosa* and *Z. bailii*), fecal contaminants (*E. faecalis* and *E. coli*), and food-borne pathogens (*L. monocytogenes*, *B. cereus*, *S. typhimurium*, *Y. enterocolitica*, and *S. aureus*). The sensitivity of the gram-negative pathogens and *L. monocytogenes* suggests that ozonated water might be especially applicable for killing these organisms on food surfaces such as fruits and vegetables where interference by organics may be minimal. Additionally, gaseous ozone might be effective in controlling *L. monocytogenes* in food environments. Although pure cultures of various food-borne pathogens and spoilage bacteria were used in this investigation, the ozone sensitivity data obtained on pure cultures used in this study should provide guidelines for further studies on ozone applications in the food and environmental industries.

TABLE 1. Mean concentrations and ranges of ozone in deionized water in the absence and presence of organic material

Additive (20 ppm)	Ozone (ppm) [mean (range)]	
	C_{out}	C_{avg}
None	0.188 (0.166–0.225)	0.064 (0.043–0.084)
SS	0.198 (0.166–0.246)	0.068 (0.052–0.092)
BSA	0.149[a] (0.131–0.168)	0.044[b] (0.036–0.053)

[a] Significantly less ($P < 0.05$) than the other C_{out} values.
[b] Significantly less ($P < 0.05$) than the other C_{avg} values.

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