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RESEARCH NOTE

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Sensitivities of *Salmonella typhimurium* and *Staphylococcus aureus* to Ozonation in the Presence of Soluble Starch and Metal Ion Complex

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Abstract This study was carried out to investigate the bactericidal efficacy of concentration (0.1, 0.2, and 0.4 ppm) and exposure time (10 and 30 min) of ozone on bacterial reduction rate of *Salmonella typhimurium* KCTC 2541 and *Staphylococcus aureus* ATCC 13515 in the distilled water (DW), and DW supplemented with 0.2% soluble starch (SS), and metal ion (MC) using argentums (Ag) and copper (Cu). The significant bactericidal differences of *S. aureus* were showed in the treatments of DW and SS, respectively, at the concentration of ozone above 0.1 ppm for 10 min, comparing the respective initial bacterial counts. The bacterial reduction of *S. aureus* was more sensitive than that of *S. typhimurum* at the same concentration of ozone. The bacterial reduction rate of SS treatment was slightly lower than that of DW treatment at the same concentration of ozone ($p<0.05$), however, the bacterial reduction rate of strains improved in the MC treatment compared to the DW treatment at the same concentration of ozone.

Keywords: ozonation, *Salmonella typhimurium*, *Staphylococcus aureus*, soluble starch, metal ion complex

Introduction

In the last decade, there have been many methods used for preservation and extending shelf life of food in food industry. The physical and chemical methods such as filtration, thermal treatment, and addition of chemical agents for the extending shelf life were used in sanitizing water and food system. Bacterial pathogens in food cause an estimated 76 million cases of human illness, 325,000 cases of hospitalization, and up to 5,000 deaths annually in the US. The chlorine-based have been often used to sanitize raw materials of foods and surfaces of the food manufacturing equipment, as well as reduce microbial populations of water using for cleaning equipment (1,2). However the productions of chlorinated organic compounds such as trihalomethanes are potential carcinogens (2), and this reason has created the need to investigate the efficiency of non-traditional sanitizers and other alternative technologies. Therefore, sanitizing methods for food lines are still under study. Ozone (O_3), a strong antimicrobial agent with numerous potential applications in the food industry, has been approved in the US to be classified as a food additive (3). Ozone has been extensively applied for sanitation of drinking water with efficacy against bacteria, molds, viruses, and protozoa (4,5). Furthermore, ozonated water has reduced microbial populations and extended the shelf life of some fresh-cut fruit and vegetables (5-7). The mode of inactivation by ozone appears to be by damage to DNA (8). Ozone reacts quickly, and therefore inactivation can occur by both gaseous ozone (through direct physical contact) and dissolved ozone (9,10). The decrease in pathogens including *Salmonella typhimurium, Yersinia enterocolitica, Staphylococcus aureus, Listeria monocytogenes*, and *Escherichia coli* O157:H7 have also been described earlier (11), however, ozonation level should be limited because of intensive and offensive odor originated from ozone. The aim of this study was to evaluate the bactericidal effect of low different kinds of ozone on *S. typimurium*, and *S. aureus* in distilled water (DW), and DW supplemented with soluble starch and metal ion. And we also compared the bactericidal effect of different exposure time against microorganisms in the ozonated solutions.

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Materials and Methods

Preparation of water components The efficacy of ozone to reduce bacterial populations in water was evaluated with soluble starch, $CuCl_2 \cdot 2H_2O$, and $AgNO_3$ (Sigma-Aldrich Chemical Co., St. Louis, MO, USA). Solution (0.2%) of starch was prepared by adding 2 g of each substrate to a total volume of 1,000 mL sterile high perdormance liquid chromatography (HPLC) grade DW. And the solution of metal ion mixture (1 mM $CuCl_2 \cdot 2H_2O$ and 0.1 mM $AgNO_3$) was mixed in 1,000 mL sterile HPLC grade distilled water. The metal ion mixtures required gentle heating with stirring to enter solution. All the components were stored overnight at 25°C until use.

Preparation of bacterial inoculation The strains of *S. typhimurium* and *S. aureus* were obtained from the Korea Food Research Institute (KFRI), Gyeonggi, Korea. Each isolated microorganisms were grown in brain-heart infusion

broth (Difco Lab., Detroit, MI, USA) supplemented with 0.2% yeast extract (BHIY). All cultures were grown at 37°C for 24 hr with 100 rpm agitation provided by a rotary shaker (New Brunswick Scientific, New Brunswick, NJ, USA). And then the cultivated cultures were washed 3 times by centrifugation (1,800×g, 10 min, 21°C) with 0.1 M sterile phosphate buffer (PBS), pH 7.0 and then the cell pellets were re-suspended in the same buffer. All the microorganisms were stored under refrigeration until before immediately use. A 1 mL microorganism of the suspension was transferred into 9 mL of serial dilution (1:10 diluent, 0.1% peptone-water, Difco Lab.). The diluted cultures were used for ozonation experiment. Fresh cultures were prepared for the replication of these experiments.

Ozonation of inoculated microorganisms The pilot plant consisted of an ozone generator CTS^{TM} (Samjung Tech Co., Ltd., Seoul, Korea) and ozone analyzer (Samjung Tech.). The output of the generator was 5 g per hr. Ozone from the injector of CTS^{TM} was injected for 30 and 60 min after the commencement of ozonation in sterile DW (dH_2O, HPLC grade). It was stored under dark, refrigerated conditions prior to use. Used ozone concentration in the stock solution ranged from 0.1 to 0.4 mg/L. Ozone residuals in the water were measured by the indigo colorimetric procedure (12,13). For making each concentration of ozone, the prepared ozonated water was diluted with distilled water in experimental solutions (DW, SS, and MC) inoculated with 1 mL of prepared microorganisms stock solutions. After the commencement of ozonation, the ozonated samples were maintained for 10 and 30 min to evaluate the bactericidal effect of ozone. This procedure was repeated 4 times for each bacterium.

Microbiological analysis Microbiological analysis was performed upon the individual samples. Treated or untreated inoculated broth were decimally diluted in sterile water and plated in duplicate on Luria Bertaini (LB) agar (Difco Lab.) to count *S. aureus*, and on Baird Parker agar (Difco Lab.) to count *S. typhimurium* at 37°C for 24 hr. Tested samples were plated in duplicate, and then counted the vial counts of each microorganism.

Statistical analysis The results were statistically analyzed using analysis of variance (ANOVA) and Duncan's multiple range tests. Statistical significance was accepted at a level of $p<0.05$ or $p<0.01$ (14).

Results and Discussion

In food production line, ozone level should be limited because of the intensive and offensive odor of ozone, therefore, the low level of ozone concentrations (below 0.4 ppm) were tested to reduce its odor and to evaluate antimicrobial effect of minimal dose of zone in the DW and DW supplemented with starch and metal ion. The results obtained from the inoculation method. The bacterial counts of *S. typimurium* and *S. aureus* in DW containing ozone of 0.1, 0.2, and 0.4 ppm for 10 and 30 min exposure in DW were shown in Table 1. There were no significant differences in the counts of *S. typhimurium* counts at the low levels of ozone (0.1 and 0.2 ppm) for 10 min exposure in DW. The microbial counts of *S. aureus* at the all tested ozone samples for 10 min were significantly lower than that of untreated sample in distilled water. These results indicated that the sensitivities of *S. aureus* to ozone were higher than that of *S. typhimurium* at the same concentration of ozone for 10 min. As the longer time (30 min) of ozone exposure, the reduction rates of *S.aureus* at the same concentration of ozone increased comparing those of 10 min exposure time in DW, however, there were no significant differences of *S. typhimurium* counts among different exposure time at the same concentration of ozone in DW. These results indicated that *S. aureus* were more

Table 1. The bacterial counts of *Salmonella typimurium* KCTC 2541 and *Stapylocuccus aureus* ATCC 13515 in distilled water (DW), and DW supplemented with soluble starch (SS), and metal complex (MC) treated with 0.1, 0.2, and 0.4 ppm of ozone for 10 and 30 min[1)]

		S. typhimurium				*S. aureus*			
		10 min		30 min		10 min		30 min	
	OC	BC	RE (%)	BC	RE (%)	BC	RE (%)	BC	RE (%)
DW	0.0	9.79^{a}	-	9.79^{a}	-	8.37^{a}	-	8.37^{a}	-
	0.1	9.71^{a}	0.61	9.44^{ab}	3.28	7.90^{Ab}	5.48	7.12^{Bb}	14.95
	0.2	9.21^{a}	5.38	8.64^{b}	10.95	6.27^{Ac}	25.02	5.88^{Bc}	29.83
	0.4	7.92^{b}	18.25	7.26^{c}	25.06	5.27^{Ad}	36.96	4.62^{Bd}	44.71
SS	0.0	9.66^{a}	-	9.66^{a}	-	8.44^{a}	-	8.44^{a}	-
	0.1	9.64^{a}	0.28	9.55^{a}	1.17	8.29^{a}	1.71	7.87^{b}	6.71
	0.2	9.25^{a}	4.15	8.70^{b}	9.49	7.00^{b}	16.95	6.60^{c}	21.78
	0.4	8.30^{Ab}	13.79	7.36^{Bc}	23.27	5.75^{Ac}	31.85	4.81^{Bd}	42.93
MC	0.0	10.00^{a}	-	10.00^{a}	-	8.27^{a}	-	8.27^{a}	-
	0.1	9.55^{a}	4.25	9.19^{a}	7.67	7.25^{Ab}	12.40	6.23^{Bb}	12.40
	0.2	8.14^{Ab}	18.29	7.07^{Bb}	28.67	5.61^{Ac}	32.13	4.74^{Bc}	32.13
	0.4	6.43^{Ac}	35.21	5.51^{Bc}	44.34	3.94^{Ad}	52.39	2.80^{Bd}	52.39

[1)]Letters a-d and A-B were significantly different in the same column and ozone concentration, respectively at $p<0.05$. OC=ozone concentration (ppm); BC=bacterial count; RE=reduction rate.

sensitive against ozonation than *S. typhimurium*. Reversely, these results estimated that the bacterial reduction of *S. typhimurium* was more subject to the concentration of ozone than the exposure time of ozone. These results were same the result of Mielcke and Ried (15), they reported that ozone concentrations as low as 0.01 ppm are toxic to bacteria and Gram-positive bacteria are more sensitive to ozone than Gram-negative bacteria. The bactericidal effects of ozone have been studied and documented on a wide variety of organisms, including both Gram-positive and negative bacteria as well as spores and vegetative cells (16). Two major mechanisms have been identified in ozone destruction of the target organisms (17). The first is that ozone oxidizes sulfhydryl groups and amino acids of enzymes, peptides, and proteins to shorter peptides. The second is that ozone oxidizes polyunsaturated fatty acids to acid peroxides (17). In Gram-negative bacteria, the lipoprotein and lipopolysaccharide layers are the first sites of destruction resulting in increases in cell permeability and eventually cell lysis (18). Many agricultural and food industrial wastes contain lots of starch and cellulose which are rich in carbohydrate contents. Starch containing solid wastes is easier to produce-carbohydrate and hydrogen gas, and can be hydrolyzed to glucose and maltose by acidic and enzymatic hydrolysis followed by conversion of carbohydrates to organic acids and then to be hydrogen gas (19). For these reason starch could affect the survival of microorganisms in water. There were no significant differences ($p>0.05$) in the both *S. typhimurium* and *S. aureus* counts at 0.1 ppm for 10 min. The bacterial reduction rates of SS treatments against *S. typhimurium* and *S. aureus* were lower than those of DW treatment in the same ozone concentration for the same exposure time (Table 1). These results assumed that addition of SS in ozonated water might have been the reason of reduction the bactericidal activity of ozone (20,21). However, there were no significant difference ($p>0.05$) between SS and DW in the same ozone levels and exposure time. These results were similar to the results of several researchs (22). Guzel-Seydim *et al.* (21) reported that statistically significant ($p<0.05$) log cycle reductions in the *E. coli* populations at 10 min were observed in buffer (6.10), and starch (6.11). For the *S. aureus*, statistically significant log reductions were detected at 10 min in buffer (6.48), and starch (6.47), and there were no significant differences between *S. typhimurium* and *S. aureus*. There were significant differences ($p<0.01$) in the *S. typhimurium* and *S. aureus* counts for 10 and 30 min exposure in all ozone levels (0.1, 0.2, and 0.4 ppm). In these results, these bactericidal effects of *S. typhimurium* and *S. aureus* were dramatically increased in the presence of metal ion complex compared with the reduction rate of the DW treatment. It was assumed that the addition of metal ion complex improved the bactericidal effect of ozone. Also, these results presented that the microbial reduction rates of *S. aureus* at the concentration of ozone of 0.2 and 0.4 ppm for 10 and 30 min exposure time in MC treatment were significantly higher than that of *S. typhimurium* at the same concentration of ozone for 10 and 30 min exposure time, respectively ($p<0.05$). Several studies have been conducted on the combined use of copper and silver ions to disinfect cold and hot-water systems at hospitals against *Legionella* bacteria, with most applications on recirculation hot-water systems (23,24). These ions are believed to interfere with enzymes involved in cellular respiration and bind to DNA at specific sites (23). A similar observation was made about the superiority of copper and silver ions over thermal treatment on a hot-water system by Mietzner *et al.* (18). In conclusion, as the ozone level increased, the microbial counts of *S. typhimurium* and *S. aurues* decreased. And these bactericidal effects of ozone were increased when the exposure time of ozonation was extended. The treatments of longer exposure time of ozone were more important to improve bacterial reduction rates of *S. typhimurium* and *S. aureus* than the treatments of higher ozone concentration ranging from 0.1 to 0.4 ppm of ozone in DW treatment. The bacterial reduction rate of SS treatment was slightly lower than that of DW treatment at the same concentration of ozone ($p<0.05$). And the bacterial reduction rate of *S. typhimurium* and *S. aureus* improved in the MC treatment comparing the DW treatment at the same concentration of ozone. In addition to, the reduction of *S. aureus* was more sensitive to ozone than that of *S. typhimurium* in all treatment.

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