Treatment of Microencapsulated β-Galactosidase with Ozone : Effect on Enzyme and Microorganism

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ABSTRACT : The present study was designed to examine the effect of ozone treatment in microencapsulated β-galactosidase on inactivation of the enzyme and sterilization of microorganism. The efficiency was the highest as 78.4% when the ratio of polyglycerol monostearate (PGMS) was 15:1. Activities of lactase remaining outside the capsule were affected by ozone treatment. With the increase of ozone concentration and duration of ozone treatment, the activity reduced significantly. In sensory aspect, with 2% microcapsule addition, no significant difference in sweetness was found compared with a market milk during 12 d storage. Above result indicated that the additional washing process of lactase was not necessary to inactivate the residual enzyme. In a subsequent study, the vegetative cells of microorganisms were completely killed with 10 ppm for 10 min treatment by ozone. The present study provides evidence that ozone treatment can be used as an inactivation and a sterilization process. In addition, these results suggest that acceptable milk products containing lactase microcapsules made by PGMS can be prepared with ozone treatment. *(Asian-Aust. J. Anim. Sci. 2002. Vol 15, No. 4 : 596-601)*

Key Words : Ozone Treatment, Microencapsulation, Lactase, Milk

INTRODUCTION

Milk is a universal and nutritious food, but a large majority of various ethnic population groups cannot properly digest it because they lack sufficient quantities of lactase (β-galactosidase, EC 3.2.1.23) (Harris, 1972; Kretchmer, 1972; Simmons, 1978). To solve this problem, lactase was added into milk during processing (Scrimshwa and Murray, 1988; Kwak et al., 2001). However, the hydrolysis of lactose by the enzyme makes the milk about four times sweeter than normal milk (Onwulata et al., 1989; Solomon et al., 1985). The enzyme treated milk has been rejected by most of consumers in the world due to the sweetness. To prevent the high levels of sweetness in the milk, the technique of microencapsulation of the enzyme was adopted (Bersen'eva et al., 1990; Jackson and Lee, 1991). Therefore, we investigated the optimum coating material for a high microencapsulation efficiency and the degree of microcapsule stabilization retaining lactase in milk during storage (Kwak et al., 2001). With this procedure, another process, such as washing and centrifugation, is required to inactivate the remaining uncapsulated lactase in the dispersion fluid (Kwak et al., 2001) and to keep the same level of sweetness in milk.

In 1990s, minimally processed, fresh-like products with reduced thermal inputs during sterilization and preservation have become commonplace in the food industry (Mertens and Knorr, 1992). In addition, fatty acid ester PGMS, the coating material used in our study, is soluble over 55°C, and so thermal input is hardly acceptable. Thus, among

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nonthermal food preservation processes including electric or magnetic fields, microwave radiation, ionizing radiation, light pulses, high isobaric pressure, chemical agents such as carbon dioxide (Graham, 1997), ozone treatment was chosen for sterilization of the contaminated microorganisms and inactivation of residual lactase.

Ozone is polymerized oxygen with a molecular weight of 48. Since molecular ozone is highly effective for inactivating many microorganisms, such as bacteria and viruses (Driedger et al., 2001), ozone has been usually used in water and waste water disinfection and in the preservation of food products to provide microbiologically safe (Broadwater, 1973; Graham, 1997; Xu, 1999; Yang and Chen, 1979). Also, ozonation is recognized by the Food and Drug Administration as a disinfectant or sanitizer for foods (Graham, 1997).

However, since limited commercial skill and regulation in the world dealing with use of ozone in foods is available, there has been no opportunity for commercial vendors to develop skills with food products. Therefore, the objectives of this study were to examine the effects of ozone treatment in microencapsulated lactase on inactivation of the enzyme and on sterilization of microorganisms during storage of milk.

MATERIALS AND METHODS

Materials

For microencapsulation of lactase, PGMS (polyglycerol monostearate) was used as a coating material. It was purchased from Il-Shin Emulsifier Co., Ltd. (Seoul, Korea). As a core material, lactase originated from *Kluyveromyces lactis* in the form of liquid was provided from Culture

Systems. Inc. (Mishawaka, IN, USA). The specific activity of the enzyme was 504 units/g. One unit will hydrolyze 1.0 μmol of p-nitrophenyl α-D-galactosidase to p-nitrophenol and D-galactose per min at pH 6.5 at 25°C.

Microencapsulation

Microcapsules of lactase were made with polyglycerol monostearate (PGMS) as described in previous study (Kwak et al., 2001). Distilled water was additionally added because PGMS is highly viscous. PGMS and distilled water were mixed with the ratio of 5:4 (w/v), heated to 55°C for 20 min, and stirred with 1.200 rpm for 1 min for spraying. The ratio of coating material to core material was 10:1 to maximize lactase content and stability of microcapsules, and mixed at 1.200 rpm for 1 min with a stirrer. An airless paint sprayer (W-300, Wagner Spray Tech. Co., Markdorf, Germany) nebulized a coating material-lactase emulsion at 45°C into a cylinder containing a 0.05% polyethylene sorbitan monostearate (Tween-60) solution at 5°C. The diameter of the nozzle orifice was 0.4 mm. The chilled fluid was centrifuged at 450×g for 10 min to separate unwashed microcapsule suspension. Microcapsules were formed as the lipid solidified in the chilled fluid.

Efficiency of lactase microencapsulation

The dispersion fluid was assayed for untrapped enzyme in microencapsulation according to a modified procedure of Shin et al. (1995). Two mL of the dispersion fluid was filtered by Whatman No. 540, followed by membrane filtration (di. 1.0 μm. Whatman International Limited. Maidstone, England). The 2 mL of 5 mM o-nitrophenol β-galactopyranoside (ONPG) (Sigma Chemical Co. St. Louis. MO. USA) heated to 37°C for 15 min was added to 0.5 mL of the dispersion fluid and incubated at 37°C in a water bath for 20 min. The reaction was stopped by adding 0.5 mL of 500 mM Na₂CO₃. The color intensity was read at 420 nm using a Beckman DU 650 Spectrophotometer (Beckman Instruments Inc.. Fullerton. CA, USA). The efficiency of microencapsulation was calculated as followed:

$$1 - \frac{\text{Specific activity of residual in the dispersion fluid}}{\text{Initial specific activity of enzyme in spray solution}} \times 100$$

The dispersion fluid was centrifuged at 200×g to remove the intact capsules from the fluid. Sample measurements were run in triplicate.

Ozone treatment

To inactivate residual enzyme adhering to the outside walls of microcapsules. unwashed microcapsules were treated by ozone generator. To find an optimum conditions for ozone treatment of lactase microcapsules. different concentrations (1. 5 and 10 ppm) and different times (1. 5 and 10 min) were applied to the dispersion fluid of microcapsules. Ozone was produced from purified. extra dry oxygen by using an ozone generator (Korea Ozone Tech.. Seoul. Korea). The ozone generator was connected to a 200 mL washing bottle. which contained 100 mL of the dispersion fluid collected from capsulation procedure. The washing bottle containing the lactase microcapsules was kept at 25°C. The ozone generator was operated by using UV light with 187 nm wavelength. Dry oxygen pressure was 0.5 kg/cm and flow rate was 10 L/min. The control samples were not ozone-treated. All treatments were triplicated.

Sensory evaluation

For the storage test, different concentrations (2 and 4%) of lactase microcapsules with PGMS as a coating material were added into the milk and stored for 1. 3. 5. 8. and 12 d. An eight-person panel, semi-experienced in judging dairy products evaluated the milk samples throughout the study. Sweetness was scored on a five-point scale (1=none and 5=strong).

Microbial test

As a preliminary study, each of 6 different microorganisms was added into the dispersion fluid to examine the effect of ozone treatment on sterilization. *Escherichia coli. Bacillus subtilis, Staphylococcus aureus. Salmonella typhimurium, Pseudomonas aeruginosa, and Enterobacter aerogenes* were obtained from Korean Culture Center of Microorganisms (Seoul, Korea) and stored in broth 50% glycerol at -70°C until used. Five different strains of microorganisms except *S. aureus* were propagated in nutrient broth (NB) and *S. aureus* was in tryptic soy broth (TSB) for 24 h at 37°C. One mL of the treatment suspension was removed and serial dilutions were made using nutrient broth. Plate counts were conducted in duplicate employing standard method agar. Plates were streaked and incubated in nutrient agar for 24-72 h at 32 and 37°C. All solutions were autoclaved and stored until used.

Subsequently. E. coli. S. aureus and total microbial counts of dispersion fluid containing microcapsules were made immediately before and after ozone treatment (10 ppm for 10 min). Procedures were applied as mentioned above.

Statistical analysis

Data from each experiment were analyzed by analysis of variance (ANOVA) using a SAS program (1985) and differences among treatments were determined by LSD at $p < 0.05$, unless otherwise stated.

RESULTS AND DISCUSSION

Microencapsulation

Since polyglycerol monostearate (PGMS) is a solid at room temperature, additional procedures were applied based on the method for medium-chain triacylglycerol (MCT) microencapsulation described in previous study (Kwak et al., 2001): firstly a heating process was applied for easy spraying, and secondly distilled water was added to reduce the viscosity of spray solution for encapsulating lactase. Then the mixture of coating material and distilled water was treated at 55°C for 20 min.

The efficiency of microencapsulation is shown in table 1. Efficiency of microencapsulation increased proportionally to the increase of coat to core ratio. The efficiency was the greatest (78.4%) when the ratio of coat to core was 15:1. Significant differences were found between those of 10:1 (65.7%) and 5:1 (53.2%) (p<0.05); therefore the optimum ratio of PGMS to lactase was found to be 15:1.

Similar studies (Kwak et al., 2001; Kim et al., 1996; Jackson and Lee, 1991; Magee and Olson, 1981) have reported the optimum ratios of coating (fatty acid esters, agar, gelatin, soluble starch, and milk fat) and core material (lactase, ω3 fatty acid, iron, flavor etc.) for an efficient microcapsule formation. When ω3 fatty acid was microencapsulated by milk fat, the ratio of coating to core material was 8:2 and the efficiency was 95.6% (Kim et al., 1996). In addition, Sankarikutty et al. (1988) indicated that the 7:3 ratio of cardamon oil to the mixture of gum acacia and maltodextrin showed the highest efficiency among other ratios. Those studies indicated that the optimum conditions, including the ratio of coating and core materials, the viscosity of spray solution, the method of microencapsulation, varied with kinds of coating, core materials and food to be applied.

In our previous study (Kwak et al., 2001) when the ratio of PGMS to distilled water was 5:4, the optimum ratio of PGMS to lactase (5:1, 10:1, 15:1 and 20:1) was examined. The highest efficiency (72.8%) was found with 15:1 (PGMS to lactase), which appeared to be lower than that for MCT microcapsules.

Effect of ozone treatment

During microencapsulating lactase with polyglycerol monostearate (PGMS), some of the enzyme could not be encapsulated. The activity of the non-capsulated enzyme was observed after treating with ozone as shown in figure 1. Activities of lactase were affected by ozone treatment (1, 5, and 10 min) at 24°C. Ozone treatment was divided into 1) 1 ppm, 2) 5 ppm, and 3) 10 ppm. In all treatments, the activity of lactase was adversely affected during ozone treatment. When 1 ppm ozone was applied for 1 min, the initial activity (100%) of lactase was reduced to 94.3%. When 5 and 10 min were applied, the enzyme activity reduced to 56.4% and 26.5%, respectively. With 5 ppm treatment, 82.0, 42.8 and 20.0% of activity remained on the outside of capsules at 1, 5, and 10 min treatments, respectively. Similar trend was found with 10 ppm treatment with lower levels of the enzyme activity.

In the previous study in our laboratory, we used the washing method by centrifugation to remove the residual lactase which was not encapsulated. In that experiment, although we did not examine the residual activity outside or in the dispersion fluid of microcapsules, we found the existence of activity outside microcapsules by measuring the lactose content in milk during storage. As we expected, unwashed microcapsules showed a significant decrease of lactose in milk. This indicated that the residual lactase hydrolyzed the lactose in milk during storage and resulted
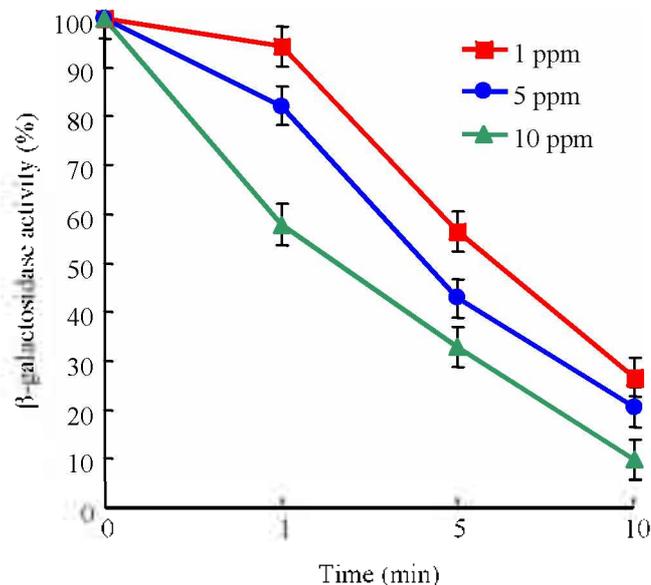
Table 1. Yield of microencapsulation for β-galactosidase with different ratios of coating material to core material[1]

Ratio (W/W)		Yield (%)
PGMS[2]	β-galactosidase	
15	1	78.4[a]
10	1	65.7[b]
5	1	53.2[c]
SEM		7.3

[1] Means of triplicate. Means in a column without the same letter are not significantly different (p<0.05). The mixture was heated at 55°C for 2 min.
[2] PGMS (polyglycerol monostearate) : distilled water = 5:4.

Figure 1. Effect of ozone treatment on the activity of uncapsulated lactase made by polyglycerol monostearate in dispersion fluid[1]. Means of triplicates.
[1] 0.05% polyethylene sorbitan monosterate (Tween-60) solution.

in a sweeter taste than normal milk. However. two-time washed microcapsules did not show any significant decrease of lactase until 8 day storage and the result was the same as normal milk in sweetness. In this experiment, we did not measure the lactose content, but measure the lactase activity directly.

The residual activity outside of the microcapsule was reduced by ozone treatment, and we investigated whether the activity of lactase inside the microcapsule was decreased or not. Therefore, the activity of microencapsulated lactase treated by ozone was measured as shown in figure 2. When the initial activity was assumed as 100%, the remaining activity treated by 1 ppm ozone for 10 min was 74.3% of initial value.

With the increase in concentration of ozone and duration of treatment time, the loss of activity inside the microcapsules increased. A similar trend was also found with other treatments. Lactase activity which was not encapsulated was reduced the lowest value when ozone treatment was 10 ppm for 10 min. This study suggested that the additional washing process of microencapsulated lactase is not necessary to reduce the residual enzyme.

Sensory analysis

Milk containing 2 or 4% microcapsule of lactase which treated with 10 ppm ozone for 10 min was observed by sensory evaluation during 12 d storage at 5°C as shown in table 2. With 2% microcapsule addition, no significant difference in sweetness was found, compared with a market
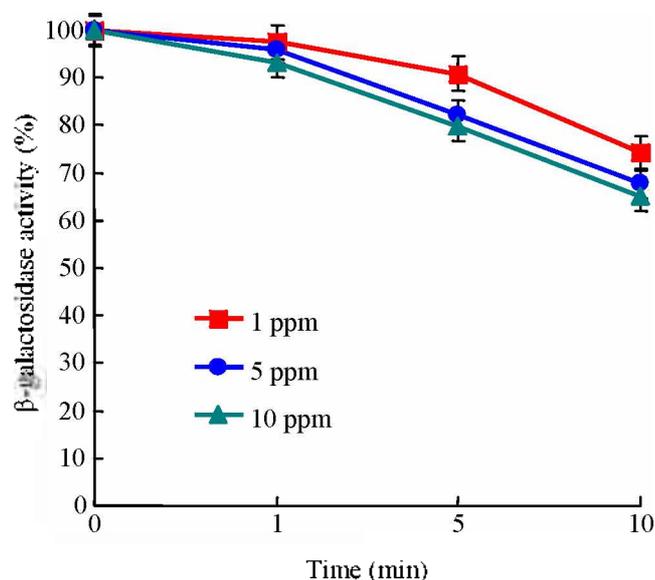

Figure 2. Effect of ozone treatment on the activity of microencapsulated lactase made by polyglycerol monostearate in dispersion fluid[1]. Means of triplicate.
[1] 0.05% polyethylene sorbitan monosterate (Tween-60) solution.

Table 2. Sensory score of sweetness in milk containing β-galactosidase microcapsule during storage for 12 d at 5°C [1]

Microcapsule concentration (%)	Storage period (day)				
	1	3	5	8	12
0[2]	1.0[a]	1.0[a]	1.0[a]	1.0[a]	1.0[a]
2	1.0[a]	1.0[a]	1.0[a]	1.12[ab]	1.38[b]
4	1.0[a]	1.0[a]	1.38[b]	1.63[bc]	1.75[c]
SEM			0.12		

[1] Sweetness scoring: 1: none, 2: slight, 3: moderate, 4: slightly strong, 5: strong.
Means of 8 replicates. Means in column without the same letter are not significantly different (p<0.05).
[2] Control: commercial market milk.

milk (control) during the storage period, though it increased slightly at 12 d. In 4% microcapsule addition, sweetness increased sharply at 5 d and thereafter. Even though microcapsules were treated with ozone, which was supposed to remove most of remaining lactase, about 9.75% of activity resulted in the sweetness after 8 day storage. However, this study suggested that the degree of sweetness in the treated milk is almost similar to normal milk.

Microorganisms

Because lactase microencapsulated by PGMS needs non-heated treatment for sterilization of contaminating microorganisms, various kinds of microorganisms were selected. treated with ozone (10 ppm), and tested as shown in table 3. Six different strains were added to dispersion fluid. Ozone reduced the counts of three of the microorganisms (*S. typhimurium, P. aeruginosa and E. aerogenes*) by about 70% from the initial values. In particular, 0.5 min treatment greatly reduced *E. coli* and *S. aureus* counts to very low values which were only 3.7% and 15.0%, respectively. of those before the treatment.

The same ozone treatments were applied to study microcapsule sterilization (table 4). Vegetative cells of microorganisms in the dispersion fluid of microbial spoilage. the initial microbial count was 2,400 and 79.1% (1900) survived 0.5 min treatment with 10 ppm ozone: neither *E. coli* nor *S. aureus* were present before or after the treatment.

Broadwater et al. (1973) indicated that ozone, in relatively low concentration, was an effective bacteriocide against both vegetative cells and spores of three bacterial species. In practical applications, ozone most probably could be applied at higher dosages (0.5-10 ppm) and for longer contact periods (2-10 min) because. as was shown in their research. organic matter present in the water will exert an ozone demand and prevent the full utilization of the applied dose as a disinfectant.

Table 3. Effect of ozone treatment on microorganisms added in dispersion fluid[1]

Strains	Time of ozone treatment (min)				
	0	0.5	1	5	10
	CFU. (%)[2]				
Escherichia coli	2.4×10^9 (100)	8.9×10^7 (3.7)	3.1×10^7 (1.3)	9.0×10^6 (0.3)	0 (0)
Bacillus subtilis	1.6×10^8 (100)	9.6×10^7 (60)	2.2×10^7 (14)	6.0×10^6 (3.7)	0 (0)
Staphylococcus aureus	6.8×10^8 (100)	1.0×10^8 (15)	6.4×10^7 (9.4)	1.2×10^7 (1.8)	1.1×10^5 (0.01)
Salmonella typhimurium	2.5×10^9 (100)	8.4×10^8 (34)	1.1×10^7 (0.4)	1.5×10^6 (0.06)	0 (0)
Pseudomonas aeruginosa	3.4×10^8 (100)	1.2×10^8 (35)	7.5×10^7 (22)	3.6×10^6 (1.0)	0 (0)
Enterobacter aerogenes	1.4×10^7 (100)	5.4×10^6 (38)	1.0×10^6 (7.1)	2.4×10^5 (1.7)	0 (0)

[1] Ozone concentration was 10 ppm with 10 l/min flow rate at 20°C.

[2] Survival percentage (%) was calculated counts after treatment×100/ counts before treatment.

Table 4. Effect of ozone treatment on microorganisms in dispersion fluid[1]

Strains	Time of ozone treatment (min)				
	0	0.5	1	5	10
	CFU				
Escherichia coli	0[2]	0	0	0	0
Staphylococcus aureus	0	0	0	0	0
Total bacteria	2.4×10^2	1.9×10^2	1.0×10^2	2.0×10	0

[1] Ozone concentration was 10 ppm with flow rate of 10 l/min at 20°C.

[2] Total cell counts (CFU/ml).

CONCLUSION

The present study indicated that an additional washing process of microencapsulated lactase was not necessary to inactivate the untrapped enzyme and the contaminated microorganisms were completely killed by ozone treatment with 10 ppm for 10 min. Therefore, we suggest that the results of this study provide evidence that ozone treatment can be applied to inactivate uncapsulated enzyme and to sterilize the dispersion fluid containing lactase microcapsules without any adverse sensory effect.

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REFERENCES

Bersen'eva, E. A., A. A. Inanov, T. P. Sansonova, E. M. Chernova and N. I. Oragvelidze. 1990. Microencapsulated aromatizers for tea. Pishchevaya Paomyshlennost, USSR 1:57-59.

Broadwater, W. T., R. C. Hoehn and P. H. King. 1973. Sensitivity of three selected bacterial species to ozone. Appl. Microbiol. 26:391-393.

Driedger, A., E. Staub, U. Pinkernell, B. Marinas, W. Koster and U. Gunten. 2001. Inactivation of *Bacillus subtilis* spores and formation of bromate during ozonation. Wat. Res. 35(12):2950-2960.

Graham, D. M. 1997. Use of ozone for food processing. Food Technol. 51:72-75.

Harris M. 1972. One man's food is another man's whitewash. Natural History 81:12-14.

Jackson, L. S. and K. Lee. 1991. Microencapsulated iron food fortification. J. Food Sci. 56:1047-1050.

Kim, C. H., W. Lee, S. C. Baick, H. S. Kwak and J. O. Kang. 1996. Studies on the microencapsulation of ω-3 polyunsaturated fatty acid. Korean J. Food Sci. Technol. 28:743-749.

Kretchmer, N. 1972. Lactose and lactase. In: Scientific American Food. (Ed. J. E. Hoff and J. Janick). Freeman Company, San Francisco. CA. USA. pp. 35-43.

Kwak, H. S., M. R. Ihm and J. Ahn. 2001. Microcapsulation of β-galactosidase with fatty acid esters. J. Dairy Sci. 84:1576-1582.

Magee, E. L. Jr. and N. F. Olson. 1981. Microencapsulation of cheese ripening systems: Stability of microcapsules. J. Dairy Sci. 64:611-615.

Mertens, B. and D. Knorr. 1992. Developments of nonthermal processes for food preservation. Food Technol. May, 124-133.

Onwulata, C. I., D. R. Rao and P. Vankineni. 1989. Relative efficiency of yogurt, sweet acidophilus milk, hydrolyzed-lactose milk and a commercial tablet in alleviating lactose maldigestion. Am. J. Clin. Nutr. 49:1233-1237.

Sankarikutty, B. M., M. Sreekumar, C. S. Nrayanan and A. G. Mathew. 1988. Studies on microcapsulation of cardamon oil by spray drying technique. J. Food Sci. Technol., India. 25:352-356.

SAS. 1985. User's Guide: Statistics, Version 5 Edition. SAS Institute, Inc., Cary, NC.

Scrimshaw, N. S. and E. B. Murray. 1988. The acceptability of milk and milk products in populations with high prevalence of lactose intolerance. Am. J. Clin. Nutr. 48:1083-1159.

Shin, M. G., H. S. Kwak, P. S. Jang, B. K. Min, M. R. Yoo and D. C. Kim. 1995. Manufactures of spray solution containing microcapsulated lactase by milk fat. Korean patent. No.

088465.

Simmons, F. J. 1978. The geographic hypothesis and lactose malabsoprtion: A weighing of the evidence. Digest Dis. 23:963-967.

Solomon, N. W., A. M. Guerro, and B. Torun. 1985. Dietary manipulation of postprandial colonic lactose fermentation. II. Addition of exogenous microbial β-galactosidase at meal time.

Am. J. Clin. Nutr. 41:209-211.

Xu, L. 1999. Use of ozone to improve the safety of fresh fruits and vegetables. Food Technol. 53:58-62.

Yang, P. P. W. and T. C. Chen. 1979. Stability of ozone and its germicidal properties on poultry meat microorganisms in liquid phase. J. Food Sci. 44:501-504.