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MICROBIOLOGICAL STUDIES ON GERMICIDAL EFFECT OF OZONE GAS ON SOME PATHOGENIC BACTERIA

(With 4 Figures)

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دراسات ميكروبيولوجية على التاثيرات القاتلة لغاز الأوزون على بعض البكتيريا الممرضة

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بسبب البكتيريا الممرضة الممثلة كملوثات في الغذاء والمقاومة للعمليات الغذائية والتي تنمو أثناء فترة التخزين والمسببة لفساد الأغذية والأمراض للمستهلك لذلك هذا البحث يفحص الفعل القاتل لغاز الأوزون للقضاء على *Bacillus cereus, Salmonella typhi, Klebsiella pneumoniae and Serratia marsecence* بواسطة طريقة التعرض المباشر للغاز. ظروف المعالجة باستخدام تركيزات الأوزون 4.9 و 9.7 جم/م3 أظهرت قلة في عدد الأنواع البكتيرية ($\log_{10}$ CFU/ml) إلى المدى من 0.27- 1.27 وذلك يعتمد على أنواع البكتيرية المختبرة. في تطبيق تركيز 12جم/م3 لغاز الأوزون لمدة 20 دقيقة اظهر إزالة كاملة لجميع الأنواع البكتيرية المختبرة وهذه الإزالة تمت أيضا بعد (2.5 دقيقة في حالة *S. typhi*) و(5 دقيقة في حالة كل من *B. cereus* and *Serratia marsecence*) و (10 دقيقة في حالة *K. pneumoniae*) عند تركيز 14.3جم/م للغاز المستخدم. وجد أن تطبيق غاز الأوزون كانت طريقة فعالة في القضاء على البكتيريا المسببة للأمراض في مجال الصحة العامة.

SUMMARY

Due to pathogenic bacteria, present as contaminants in food, may survive processing, grow during storage, and cause spoilage of food or diseases to consumers. Hence, this research investigates the bactericidal action of gaseous ozone for the elimination of *Bacillus cereus, Salmonella typhi, Klebsiella pneumoniae and Serratia marsecence* by

surface exposure technique. Under identical treatment conditions, 4.9 and 9.7 g/m^3 ozone concentrations decreased bacterial counts by 0.27 to 1.27 $\log_{10}$ cfu/ml depending upon the bacterial species tested. Ozone concentration of 12 g/m^3 up to 20 min, a complete log reduction took place in the population of all bacteria species tested. Moreover, at 14.3 g/m^3 ozone concentration, a complete log reduction in the number took place at the end of, 2.5 min of *S. typhi*, 5 min of *B. cereus* and *Serratia marsecence*, and 10 min of *K. pneumoniae*. Ozonation was found to be an effective method to eliminate some of pathogenic microorganisms of great public health concern.

Key words: *Ozone application, elimination, pathogenic bacteria.*

INTRODUCTION

Gaseous ozone treatment has bactericidal effect on *Salmonella enteritidis*, inoculated on the surface of the tomatoes and can be used for surface sanitation of *S. enteritidis* on tomatoes before storage at different conditions (Elif *et al.* 2006). *Bacillus cereus* survives adverse environmental conditions, adapts and eventually multiplies in foods (Meer *et al.* 1991). Some strains of *B. cereus* grew to $\sim 10^6$ cfu/g and produced toxin in refrigerated foods (Dufrenne *et al.* 1995).

Jankowski and Doroszkiewicz (1990), studied on analysis of the bactericidal influence of an ozonized blood serum on gram-negative bacilli. They mentioned that, a considerable differences were observed concerning the effect of ozonized normal human serum on *Klebsiella, Pseudomonas* and *Salmonella* strains. Inactivation of *Klebsiella terrigena* (6-log), *E. coli* (6-log), MS2 (6-log) and poliovirus 1 (>3-log) was observed after 1 min of ozonation in a 1 L batch reactor (Tanner *et al.* 2004).

Ozone has been approved as an antimicrobial agent in foods in the US (FDA 2001). The use of ozone gas has been suggested as an alternative to reduce microbial populations on melon before cutting (Suslow 2004a,b; Selma *et al.* 2008). *Salmonella* and other foodborne pathogens attached to the external surfaces of cantaloupes are of great public health concern because they can be transferred to the edible flesh during cutting (Suslow *et al.* 2000; Ukuku and Sapers, 2001).

Akbas and Ozdemir (2006) studied the effect of different ozone treatments for decontamination of pistachios contaminated with *B. cereus* and *E. coli*. They found that, at 1.0 ppm ozone concentration

for 360 min of ozonation, *B. cereus* counts in pistachio kernels and shelled pistachios were reduced 3 log numbers, while *E. coli* counts were decreased by 3.5 log numbers. Moreover, the spoilage microorganisms of vegetables such as onions, potatoes, and sugar beets are also reduced after storage in an atmosphere containing low ozone concentrations (Kim *et al.* 1999a,b; Gil and Selma 2006).

Therefore, the aims of this study were to determine the effect of ozone concentrations and exposure times for reducing/eliminating *B. cereus, S. typhi, K. pneumoniae* and *S. marsecence* to be applied in food and medical microbiology fields.

MATERIALS and METHODS

1. Ozone

Dielectric barrier discharge (DBD) laboratory scale cell, designed and prepared as described previously was used. The equipment consists on a gas-tight glass chamber (at temperature of 22±0.5, Relative humidity (RH) of 82–86%). The equipment provides the supply of O_2 gas to feed the ozone generator from a compressed gas cylinder. Gaseous ozone concentration in the chamber was measured with an ozone gas analyzer (HI-AFX-Instrument, USA), as indicated in a previous study (Afifi and Kotp 2004).

Ozonation of inoculated plates continued until the targeted ozone concentration was attained. Gaseous ozone concentrations were chosen based on preliminary experiments on the sensitivity of *B. cereus, S. typhi, K. pneumoniae* and *S. marsecence*, to varying concentrations of ozone (4.9 to 14.3 g/m^3). Excess ozone was neutralized by diverting the gas stream into a reservoir containing 2% potassium iodide solution. Protective masks and ozone-resistant gloves were worn during the experiments.

2. Bacterial cultures

Four bacterial strains were tested in this study. These strains were; *Bacillus cereus*, IFR/NL94/25, obtained from the milk laboratory, school of Agriculture at Alex university; *Salmonella typhi*, MSA, obtained from the department of Microbiology, school of Medcine at Assuit University; and both *Klebsiella pneumoniae*, MCIB-9111, and *Serratia marsecence*, IMRU-70, were obtained from fermentation biotechnology center at AL-Azhar University. Stock cultures of these bacteria were grown in nutrient broth at 37 °C for 24 h.

3. Microbiological inoculation

Prior to ozonation treatments, bacterial cells were prepared and obtained by scraping overnight cultures from nutrient agar plates, diluted with sterile peptone water solution (0.1%, w/v), and inoculated at a level of 10^6 microorganism ml^{-1} by the surface spread plating technique.

Inoculated plates were divided into six separate Petri plates (half for control and half for treatment). The plates were, placed into the ozonation chamber after the desired ozone concentrations were attained, and were incubated for 48h at 37 °C. Colonies were counted by colony forming units technique. Results obtained for counting were expressed as log cfu ml^{-1}.

4. Statistical analysis

Initially, microbial population of control (untreated plates) averaged 2.39, 2.35, 2.40, and 2.41 log cfu/ml for *B. cereus* (Fig. 1), *S. typhi* (Fig. 2), *K. pneumoniae* (Fig. 3), and *S. marsecence* (Fig. 4), respectively. Each ozonation trial was performed with three replicates. The data obtained for each treatment evaluated were submitted to analysis of variance (F test) to analyze data. The mean value was compared at 5% level of significantly using the last significance differences (LSD) test (Gomez and Gomez 1998).

RESULTS

The efficacy of 9.7 g/m^3/20 min ozone treatment on microbial inactivation was somewhat improved as compared to 4.9 g/m^3/20 min ozone treatment on both *S. typhi* (Fig. 2) and *K. pneumoniae* (Fig. 3), being especially more effective on both *B. cereus* (Fig. 1) and *S. marsecence* (Fig. 4). Thus, 9.7 g/m^3/20 min ozone treatment produced high significant reduction in, *S. typhi* counts by 0.27 log cfu/ml, *K. pneumoniae* counts by 0.73, *B. cereus* counts by 1.27 log cfu/ml and *S. marsecence* counts by 1.26 log cfu/ml.

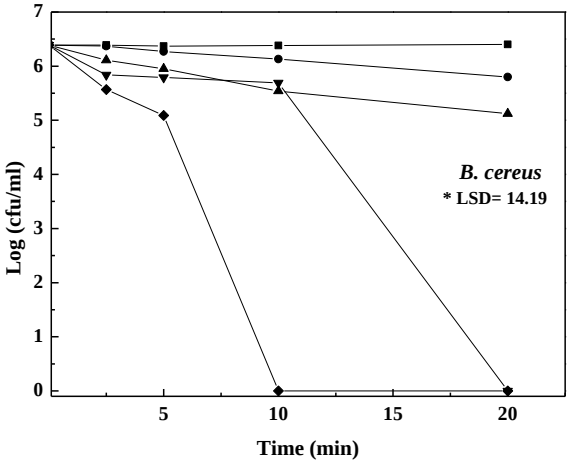

Fig. 1: Comparative effects of ozone concentrations (•) 4.9 g/m^3, (▲) 9.7 g/m^3, (▼) 12 g/m^3 and (♦) 14.3 g/m^3 on population of *B. cereus* (■) control. Values are means of three experiments with triplicate determinations per experiment. Asterisks represent LSD at P≤0.0001. (Kotp, E. F. and Afifi, M. M.).

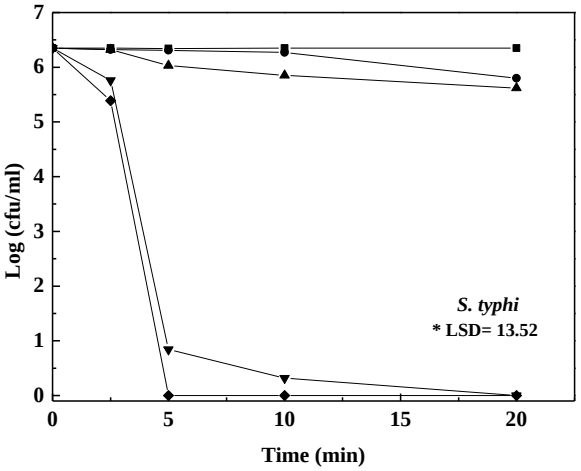

Fig. 2: Comparative effects of ozone concentrations (•) 4.9 g/m^3, (▲) 9.7 g/m^3, (▼) 12 g/m^3 and (♦) 14.3 g/m^3 on population of *S. typhi* (■) control. Values are means of three experiments with triplicate determinations per experiment. Asterisks represent LSD at P≤0.0001. (Kotp, E. F. and Afifi, M. M.).

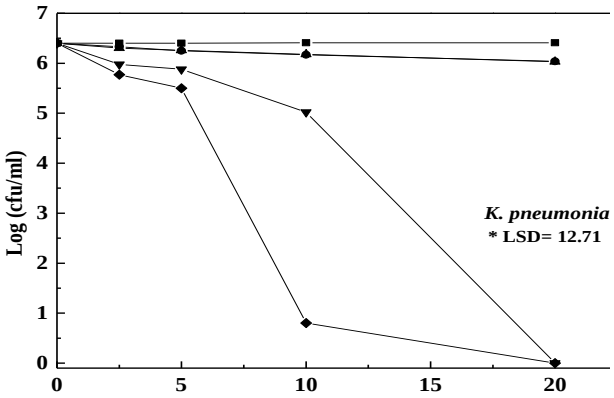

Fig. 3: Comparative effects of ozone concentrations (•) 4.9 g/m^3, (▲) 9.7 g/m^3, (▼) 12 g/m^3 and (♦) 14.3 g/m^3 on population of *K. Pneumoniae*(■) control. Values are means of three experiments with triplicate determinations per experiment. Asterisks represent LSD at P≤0.0001. (Kotp, E. F. and Afifi, M. M.).

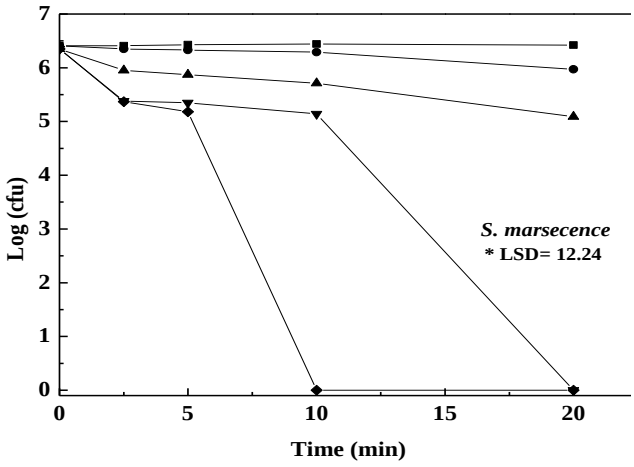

Fig. 4: Comparative effects of ozone concentrations (•) 4.9 g/m^3, (▲) 9.7 g/m^3, (▼) 12 g/m^3 and (♦) 14.3 g/m^3 on population of *S. marsecence* (■) control. Values are means of three experiments with triplicate determinations per experiment. Asterisks represent LSD at P≤0.0001. (Kotp, E. F. and Afifi, M. M.).

DISCUSSION

Results of ozone treatment on microbial activation were consistent with the findings reported by Akbas and Ozdemir (2006). They found that, at 1.0 ppm ozone concentration for 360 min of ozonation, *B. cereus* counts in pistachio kernels and shelled pistachios were reduced 3 log numbers.

Selma *et al.* (2008) demonstrated that, Gaseous ozone (10,000 ppm for 30 min under vacuum) reduced viable, recoverable *Salmonella* from inoculated physiologically mature non-ripe and ripe melons with a maximum reduction of 4.2 and 2.8 log CFU/rind-disk (12.6 cm^2), respectively. Moreover, Rowan et al (2007) revealed that, a pulsed-plasma gas-discharge (PPGD) treatments at 4 degrees C produced significant reductions (> or = 3 log CFU/ml) in recalcitrant *B. cereus* NCTC 11145 endospore numbers within 30 s.

Similarly, *Escherichia coli* and *B. cereus* counts were decreased by 3.5 log numbers at 1.0 ppm ozone concentration for 360 min ozone treatment in dried figs (Akbas and Ozdemir 2008). *Bacillus cereus* reduction may be beneficial to quality retention due to this microorganism's contribution to spoilage of perishable, refrigerated product.

At ozone concentration of 14.3 g/m^3 was most effective in slowing down microbial growth at the end of, 2.5 min of *S. typhi*, 5 min of both *B. cereus* and *S. marsecence*, and 10 min of *K. pneumoniae* being high significant reduction (0.96, 1.30, 0.23 and 5.60) log cfu/ml, respectively. After these treatments, and at 12 g/m^3 for 20 min, the population of all bacterial species tested completely eliminated too.

Similarly, Heindel *et al.* (1993), studied the microbicidal effect of ozone in air was tested at concentrations between 50 and 600 micrograms/m3 against the species: *Staphylococcus epidermidis, Micrococcus luteus, Arthrobacter citreus, Bacillus subtilis* (veg.), *E. coli, S. typhimurium, S. marcescens, Pseudomonas fluorescens* and *Candida albicans*. They found that, concentrations of 50 to 100 micrograms (0)3/ m^3 for 1 h resulted only in little reduction, whereas 500 to 600 micrograms/m^3 for one hour led to 99% reduction in all bacterial species tested. Ozone was tested against *Pseudomonas fluorescens, Escherichia coli* O157:H7, *Leuconostoc mesenteroides,* and *Listeria monocytogenes*. When kinetic data from a batch reactor were fitted to a dose-response model, a 2-phased linear relationship was observed. A continuous ozone reactor was developed to ensure a

uniform exposure of bacterial cells to ozone and a constant concentration of ozone during the treatment (Kim and Yousef 2000).

In contrast, Gehring *et al.* 1990, studied the germicidal influence of various antiseptics on the bacterial and fungal flora of leg ulcers in vitro. They demonstrated that, the treatment with ozone resulted in sufficient inactivation of germs with the only exceptions of *Serratia* and *Klebsiella* species. Moreover, Smilanick (2003), demonstrated that, doses of ozone required to rapidly kill post harvest microorganisms in a few minutes, or even hours of exposure, are very high. Therefore, it is important to recognize that technique aimed at eliminating the pathogenic microorganisms of great public health significance.

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