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Inactivation of *Escherichia coli* O157:H7 by treatment with different temperatures of micro-bubbles ozone containing water

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Abstract

This study investigated the effect of micro-bubbles ozone containing water at 13°C and 28°C on inactivation of *Escherichia coli* O157:H7. Oxidation reduction potential (ORP) and pH were measured for oxidation reaction of micro-bubbles ozone which resulting in an increase of ORP and decrease of pH with reaction times. The cell suspension of *E. coli* O157:H7 exposed to micro-bubbles ozone concentration of 0.01, 0.03, 0.05, and 0.02 mg O_3/L at 28ºC and the concentration of 0.14, 0.17, 0.14, and 0.18 mg O_3/L at 13ºC for 5, 10, 15 and 30 min, respectively and subsequently incubated at 35°C for 48 hr. The results showed that micro-bubbles ozone exhibited the most effectiveness to inactivate the growth of *E. coli* O157:H7 after exposure at 13°C for 30 min, compared to micro-bubbles and the control (distilled water). Therefore, the application of micro-bubbles ozone may be useful method for surface sanitation to control postharvest disease.

Introduction

Contamination of microorganism during the fresh-cut process is a major problem in fresh-cut fruits which could lead to foodborne disease (Monaghan *et al.*, 2010). There are many microorganisms which cause the foodborne disease (i.e. *Escherichia coli* and *Salmonella* spp). An enteropathogenic, especially *E. coli* has been considered as a major microorganisms causing foodborne disease. The Department of agriculture Thailand (January 2013) regulates the amount of this bacteria in food must be less than 10 CFU/g. Furthermore, European Union has reported the concerning about the infection of pathogens (*E. coli*) on imported fruits and vegetables from Thailand (International Institute for Trade and Development, 2014). Chlorine is one of sanitations, which had been widely used in food industry (Gil *et al.*, 2009). Sanitation of fresh-cut fruits is normally washed using chlorinated solutions to reduce the number of microorganisms. However, the use of chlorine has been raised by the formation of by-products such as trihalomethanes (THMs) and haloacetic acids (HAAs) (Focus on chlorine science, 2013). The high level of chlorination by-product affect in environmental and human health ;THMs increased a risk of cancer and halogenated by-product (HBPs) occurred natural halogenation process (Graham, 1997).

Alternatively, ozone is a well-known efficiency method to control the disease from pathogens. It has high disinfectant activity and widely be used in sterilization. The effect of ozone is to inactivate bacteria, fungi and viruses, and it was confirmed to be Generally Recognized As Safe (GRAS) (Graham, 1997; Kim *et al.*, 1999; Ikeura *et al.*, 2011). It has been reported that gaseous ozone inhibited the growth of disease incidence of green mold caused by Penicillium digitatum in tangerine during storage at 25°C (Boonkorn *et al.*, 2012). However, ozone has a limitation in water dissolution, which reduces its oxidation property. Micro-bubbles ozone (MBO) is a novel technology, which converts the ozone gas to the small size of bubbles (less than 50 µm) in water (Marui, 2013). These small bubbles increase dissolved potential and expand the oxidizing efficiency of ozone which enhance the microorganism collapsing. MBO generated more hydroxyl radical in water and it was efficiency to the log reduction of *Bacillus subtilis* spore (Takahashi *et al.*, 2007; Zheng *et al.*, 2013). As for microorganism, Li *et al.* (2012) reported that micro-bubbles technique reduced water pollutant, which was observed in phenol degradation

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(approximately 60%). Inatsu *et al.* (2011) found that ozone micro-bubbles reduced *E. coli* O157:H7 of viable cell (5.0-7.4 log) better than those of the ozonated water, and resulted in decontamination on the surface of leafy vegetables. In addition, temperature is one of the most important factors that affect the solubility of ozone in water. Lower temperature could be increasing the solubility of ozone concentration as reported by Kobayashi *et al.*, (2011) that the concentration of ozone micro-bubbles water rapidly increased at 15ºC after exposure time for 5 min when compared with 25 and 30ºC. This study aimed to determine the effect of different temperatures of MBO water on inactivation of *E. coli* O157:H7.

Materials and Methods

Preparation of micro-bubbles ozone

Micro-bubbles Ozone (MBO) was generated by ozone generator (Ozonizer, Model SO5AE) which connected to micro-bubbles water generator (Model 15KED02S, NIKUNI CO.,LTD.,Japan). That machine had a flow rate at 7L/min with an internal pressure at 0.25 MPa. The MBO water generated from micro-bubbles nozzle and circulated in the micro-bubbles bath (Figure 1). The concentration of dissolved ozone in water was measured by Indigo colorimetric method (Hoigne and Bader, 1980). The oxidation-reduction potential (ORP) and pH were measured by a pH/ORP meter (Sartorios, PB-20) for 5, 10, 15 and 30 min of MBO exposure time.

Preparation of E. coli *O157:H7 suspension*

E. coli O157:H7 DMST 12743 was obtained from the Department of Medical Science, Bangkok, Thailand. Cell suspension of *E. coli* O157:H7 was prepared from stock culture, which inoculated into trypticase soy broth (TSB) and incubated at 37°C for 10 hr. Cell suspension turbidity was measured by using spectrophotometer at OD 600 nm. After that, the dilution of serial in TSB and spread plate technique on trypticase soy agar (TSA) which were evaluated cell suspension at OD 600 nm (approximately 10^8 CFU/ml).

Effect of micro-bubbles ozone on in vitro inhibition of E. coli *O157:H7*

Cell suspension of *E. coli* O157:H7 was prepared from stock culture, that inoculated into tryticase soy broth (TSB) and incubated at 37°C for 10 hr. Cell suspension turbidity was measured by using spectrophotometer at OD 600 nm (approximately 10^8 CFU/ml). One ml of cell suspension was filled into

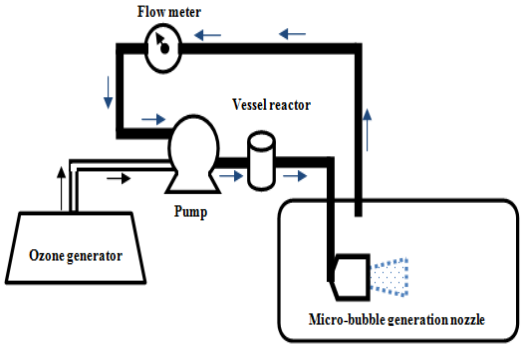

Figure 1. Schematic diagram of the Micro-bubbles Ozone system

the micro-bubbles ozone water (9 ml) for 0, 5, 10, 15 and 30 min and incubated at room temperature for 10 min. Then, 0.1 ml of cell suspension from each MBO were evaluated by spread plate technique on TSA and incubated at 37°C for 48 hr. The colony of *E. coli* O157:H7 was presented as the mean number of colony forming units (CFU/ml).

The different temperature of micro-bubbles ozone on inhibition of E. coli *O157:H7*

Micro-bubbles ozone water was determined at different temperature for 13 and 28 °C, which were controlled in water bath (Model BS 402, NÜVE). Each temperature water was investigated on oxidation-reduction potential (ORP), pH and dissolved O_3 concentration in water. For the growth inhibition; 1 ml of *E. coli* O157:H7 cell suspension (10^8 CFU/ml) was filled into MBO (9 ml) for 0, 5, 10, 15 and 30 min at different water temperatures, and incubated for 10 min. After that 0.1 ml of cell suspension from each MBO were evaluated by spread plate technique on TSA and inoculated at 37°C for 48 hr. The colony of *E. coli* O157:H7 was presented as the mean number of colony forming units (CFU/ml). All experiments were three replicated and evaluated with regression procedure using SPSS version 17. Differences among treatments performed using Duncan's Multiple Range test ($P \leq 0.05$).

Results and Discussion

Oxidation reduction potential and pH of micro-bubbles ozone at 13 and 28ºC

The oxidation efficiency of MBO at several exposure times were affected by extension of hydroxyl radical in water (•OH). Oxidation reduction potential (ORP) were measured for oxidation reaction of all treatments at 13 and 28ºC as represented. The highest value of ORP occurred in MBO treatment at 30 min compare with gas ozone and micro-bubbles (MB) (Table 1). The ORP showed the more oxidation

Table 1. Effect of micro-bubbles ozone at 13 and 28ºC on pH, and ORP (mV)

Temperature	Parameters	Treatment	Treatment time (min)				
			0	5	10	15	30
13 ℃	pH	Control	8.17±0.00a	8.17±0.00a	8.17±0.00a	8.17±0.00a	8.17±0.00a
		O_3	8.66±0.10c	7.90±0.10bc	7.86±0.13bc	7.63±0.29ab	7.52±0.11a
		MB	8.01±0.00d	7.57±0.12c	7.61±0.01c	7.34±0.06b	7.32±0.02a
		MBO	8.17±0.00d	7.96±0.42c	7.80±0.07c	7.57±0.09b	7.24±0.03a
	ORP(mV)	Control	-62.53±7.43a	-62.53±7.43a	-62.53±7.43a	-62.53±7.43a	-62.53±7.43a
		O_3	-46.18±5.54a	-44.40±1.07a	-36.73±0.03a	-45.23±2.97a	-42.26±4.08a
		MB	-21.40±7.86e	-12.90±6.59de	-6.43±1.70c	-5.16±2.31b	15.33±5.83a
		MBO	-22.67±11.23d	-15.40±6.81cd	-4.13±1.78bc	2.48±2.62ab	14.73±7.18a
28 ℃	pH	Control	8.17±0.00a	8.17±0.00a	8.17±0.00a	8.17±0.00a	8.17±0.00a
		O_3	8.14±0.00c	7.95±0.11bc	7.94±0.18bc	7.67±0.06ab	7.52±0.11a
		MB	8.01±0.00c	7.56±0.11b	7.61±0.01b	7.50±0.11b	7.12±0.23a
		MBO	8.17±0.00d	7.91±0.13c	7.91±0.72c	7.60±0.19b	7.14±0.52a
	ORP(mV)	Control	-62.53±7.43a	-62.53±7.43a	-62.53±7.43a	-62.53±7.43a	-62.53±7.43a
		O_3	-63.00±0.55a	-64.40±5.72a	-60.70±0.32a	-57.46±2.08a	-63.66±0.87a
		MB	-27.46±6.67c	-10.10±3.60b	-14.33±2.83b	-7.43±3.21b	4.63±2.25a
		MBO	-23.63±1.76c	-20.43±6.56bc	-12.80±9.29b	6.60±1.00a	7.26±0.46a

Data followed by the same letter within the column are not significantly different (*P>0.05)

efficiency which promoted the rising of oxidation efficiency by the reaction time. Suslow (2004) reported that an ORP had the activity to eliminate the pathogens that promoted the rising of ORP. An ORP at 650-700 mV reduced the sterilization time of *Escherichia coli*, *Salmonella* sp. and *Listeria* sp. when comparatively with the ORP lower than 485 mV. Therefore, high value of ORP in MBO might provide a high microbial disinfection. In addition, pH at 13 and 28ºC were determined in all treatments. The result showed that the pH of gas ozone, MB and MBO decreased with reaction times (Table 1). The lower pH showed that the slowly ozone decomposition in water which affected a little change of pH in water (Ozone, 1999). The concentration of dissolved ozone in water was measured by Indigo colorimetric method. MBO treatments at 28ºC obtained the concentration of 0.01, 0.03, 0.05, and 0.02 mg O_3/L and at 13ºC obtained the concentration of 0.14, 0.17, 0.14, and 0.18 mg O_3/L at 5, 10, 15 and 30 min, respectively (Figure 2). All treatments at 13ºC obtained the concentration of dissolved ozone more than at 28ºC. However, the concentration at 28ºC was not increased when exposure time increase to 30 min because of the measurement of concentration of dissolved ozone in water by Indigo method was not sufficiently accurate, and ozone concentration could change in a few minutes to lower concentration (Jacek, 2012). At 13ºC, the low temperature could increase the dissolved ozone in the water (Ozone, 1999). Micro-bubble has a high stability and surface area, which can maintain the ozone for a long time. Marui (2013) reported that the small bubbles increased the dissolved potential and free radicals during generated by the cracking of the bubbles.

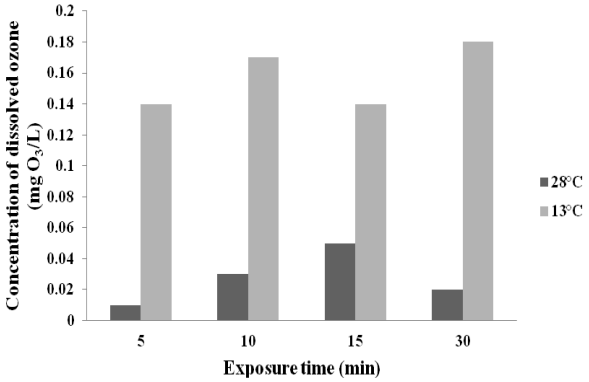

Figure 2. Concentrations of dissolved ozone in water was measured by Indigo colorimetric method at 28 and 13ºC

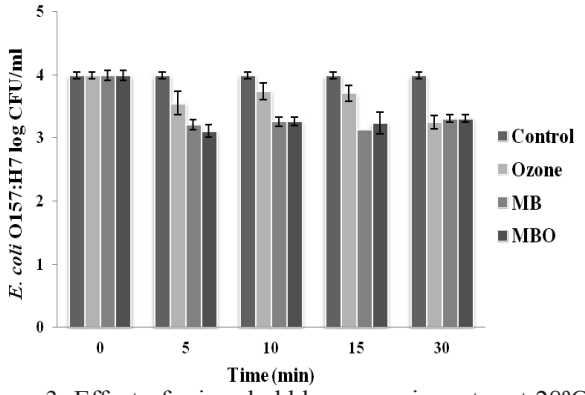

Figure 3. Effect of micro-bubbles ozone in water at 28ºC on growth inhibition of *E. coli* O157:H7 after incubated for 48 h.*Error bar represented standard deviations

The different temperatures of micro-bubbles ozone on growth inhibition of E. coli *O157:H7*

The effects of MBO at different temperatures on the inactivation of *E. coli* O157:H7 were shown in Figures 2 and 3. After 48 h of incubation, the results showed that ozone gas, MB and MBO with reaction times reduced cell suspension of *E. coli* O157:H7 at 28ºC (Figure 3) but not significantly differences

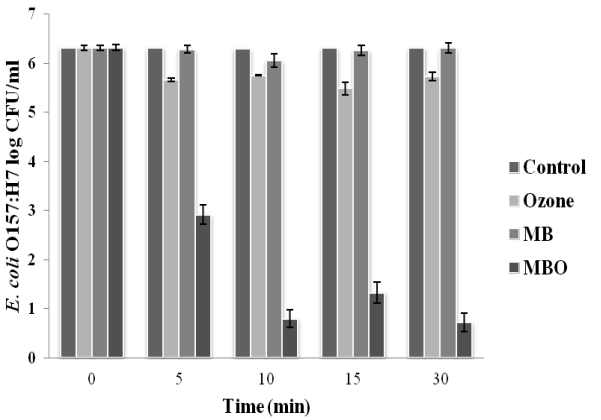

Figure 4. Effect of micro-bubbles ozone in water at 13ºC on growth inhibition of *E. coli* O157:H7 after incubated for 48 h.*Error bar represented standard deviations. Values (log CFU/ml) with the same letter on the same color bars represented no significance differences (*P>0.05)

among all treatments. At 13ºC, MBO treatments significantly inhibited the cell suspension of *E. coli* O157:H7 after exposure time for 5 min, and the exposure time for 30 min obtained the best result in inactivate cell suspension of *E. coli* O157:H7 by 0.72 $\log_{10}$ CFU/ml (Figure 4). *E. coli* O157:H7 was observed with gram stain technique under microscope (data not shown). The change of gram stain should be the effect of the ozone to be attacking the cell wall and cell membrane (Ozone, 1999). Curtiellas *et al.* (2005) reported that ozone had the effective to damage in the cytoplasmic space and loss of intercellular contents of *E. coli* when examined with microscopic. This research showed that the disinfectant efficiency of MBO depended on the increasing of the exposure times and temperature of water. Temperature of water related to the ability of dissolved ozone in water. Therefore, the low temperature could be increase the ability of dissolved ozone in water. Kobayashi *et al.* (2011) reported that the concentration of ozone microbubbles water rapidly increased at 15ºC after exposure time for 5 min. As the result, the highly concentration of dissolved ozone in water affect on bacterial membrane and disorder enzyme activity (Ozone, 1999). The result of this study conformed to other reports that MBO had a high efficiency on the reduction of *Bacillus subtilis* spore (Zheng *et al.*, 2013) as well as inactivation the growth of bacterial withering disease of tomato, brassica and strawberry with no harmful to the postharvest quality (Fukumoto *et al.*, 2010).

Conclusions

MBO water at different temperatures had effects on inactivation of *E. coli* O157:H7. The exposure times of MBO at 13ºC for 30 min provide the best result on the inactivation of *E. coli* O157:H7 which related to the highest value of ORP. Therefore, MBO at low temperature of water was suitable for microorganism inactivation and it has a high stability that can keep the ozone for long-term. This technique is the promising method for using as a sterilization technique.

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