CHAPTER 3

Effect of EO Water and US Waves on the Control of Microorganism Contamination in Pineapple Fruit

3.1 Introduction

Pineapple (*Ananas comosus* (L.) Merr) is an economically important fruit in Thailand. Domestically, consumption is in the form of fresh fruit, rather than canned fruit. In 2010, only 5 % of the pineapple produced (3,294 metric tons) was exported as fresh fruit to England, Canada, Iran, Singapore, Ireland, Japan, and other countries, but the value of the exported fruit was about 75 million Baht (Office of Agricultural Economics, 2011). The postharvest loss of pineapple fruit remains a substantial problem for foreign markets. Fungal decay is the main cause of pineapple postharvest loss. Fungal decay depends on weather conditions and the postharvest handling system. The high incidence of postharvest disease in pineapple is primarily due to the fungus *Ceratocystis paradoxa* and *Fusarium* sp. The fungi preferentially penetrate wounds caused by de-crowning that occurs in the postharvest handling systems prior to export. Postharvest disease control methods of fresh fruit vary and depend on the requirements of target markets. Washing pineapples with clean water is recommended in many countries (Kader, 2009; Paull, 1992; Department of Agriculture Kuala Lumpur Malaysia, 2004). In Thailand, harvested pineapples are distributed to markets without washing. Therefore, washing pineapple with electrolyzed water and ultrasound, as a physical disease control method, offers an attractive alternative to the use of fungicides.

EO water is one of the potential alternatives for environmentally friendly broad spectrum microbial decontamination that has been proven to exhibit strong bactericidal activity for inactivating many pathogens (Al-Haq *et al*., 2002; Venkitanarayanan *et al*., 1999; Hong *et al*., 1998). Several studies have demonstrated that EO water has application in postharvest disease control. Deza *et al*. (2003) reported that electrolyzed

water, used to treat tomato peels, resulted in a decline of bacteria such as *Escherichia coli* 0157:H7, *Salmonella enteritidis* and *Listeria monocytogenes*, without any effect on the environment. Hung *et al*. (2010) reported that EO water treatment of strawberries and broccoli significantly reduced the *E. coli 0157:H7* counts. Paola *et al*. (2005) found that washing lettuce with electrolyzed water for 5 minutes significantly inhibited the growth of *L. monocytogenes*. Jane *et al*. (2008) also reported that electrolyzed water, used as a microbial decontamination agent, for structural surfaces that contain mixed populations of heterotrophic bacteria and as a rinse treatment, reduced bacterial populations on spinach and lettuce. Whangchai *et al*. (2009) found that electrolyzed water treatment of tangerine cv. Sai Nam Pung, at a free chlorine concentration of 215 ppm for 120 and 240 seconds, completely inhibited growth and development of *Penicillium digitatum*. In addition, Whangchai *et al*. (2010) found that washing orange with electrolyzed water with continuous ozone exposure for 2 h day^{-1} significantly controlled *P. digitatum* disease during storage.

US technology has a bactericidal effect, caused by the occurrence of the cavitation phenomenon, which consists of the formation, growth and collapse of air bubbles. These bubbles generate localized mechanical and chemical energies that are capable of inactivating microorganisms (Adekunte *et al*., 2010; Gogate & Kabadi, 2009; Piyasena *et al*., 2003; Valero *et al*., 2007). US has been used in postharvest treatments to reduce decay, and to maintain the quality of fruits and vegetables. Yang *et al*. (2011) reported that using ultrasonic waves (40 kHz, 10 min) and salicylic acid (SA) (0.05 mM) on peach fruit resulted in significant control of *Penicillium expansum*, which causes blue mold.

The objective of this research was to determine the effects of EO water and US waves or their combination on the control of spore germination and mycelial growth of *Fusarium* sp. and microorganism contamination on pineapple fruit after treated.

3.2 Materials and methods

3.2.1 Isolation of fungal pathogens

Isolation of the pathogen was done using the method of Amadioha, (1998). In this, diseased test plants were collected from the Muang mai market, Chiangmai Thaland, in May 2011. The infected de-crowned pineapple fruit were washed in different changes of sterile distilled water and then surface sterilized with 70% ethanol. Using a sterile scalpel, the infected spots were cut into sections and plated on potato dextrose agar (PDA). The plates were incubated at 27°C until visible growth was observed, and pure cultures of the pathogens were maintained as stock culture at 4°C. Fungi were identified conventionally according to their macroscopic and microscopic features.

3.2.2 Pathogenicity Test

To test pathogenicity of the isolated fungi, some pieces of mycelia were re-inoculated into healthy de-crowned pineapple fruit. A piece of mycelium of each fungus was inoculated on a center of de-crowned pineapple fruit and thereafter covered with a transparent polyethylene bag and incubated at room temperature. In the control, wound was created and only a piece of gelled PDA was introduced. The experimental set-up was replicated 5 times and observed daily for symptoms development.

3.2.3 Fungal culture

Fusarium sp. was obtained from the isolated methods that describe above. The fungi were grown on potato dextrose agar (PDA) for 7 days at 27 ◦C. A spore suspension was prepared by flooding a 1 week old culture of *Fusarium* sp. with 10 ml sterile distilled water and transferring the spores to 100 ml of sterile distilled water in a 250 ml conical flask. The suspension was shaken for 10 min on an orbital shaker at 27 ◦C, and filtered through two layers of sterile muslin cloth. Spores (conidia) were counted with a hemacytometer and the concentration was adjusted with sterile distilled water to a final concentration of 10^5 conidia/ml.

3.2.4 Preparation of treatments

Electrolyzed oxidizing water was generated by electrolysis in a cell with positively and negatively charged titanium electrodes coated with TiO_2, separated by a polypropylene membrane. The electrodes were then subjected to a direct current of 8 A and 8 V using a DC power source. A 5% NaCl solution was simultaneously introduced into the system. The pH was recorded with a pH/ion meter, and ORP (oxidation-reduction potential) was measured by a pH/ ORP meter. The amount of free–chlorine concentration was determined by using a DPD (N,N-diethyl-P-phenylene diamine) test (Pailin, 1967). The EO water with initial concentration of 660 ppm was diluted with distilled water to concentrations of free-chlorine at 100, 200, and 300 ppm, and used for the microbiological study, within 2 hour after generation. Sterilized distilled water was used as a control for this experiment. In our experiments the EO generator model (Figure 3.1B) to generate EO water was used.

An ultrasonic bath with an input power of 24 watts and a frequency of 1 MHz with eight transducers was made by Honda Electronics Company (Toyohashi, Aichi, Japan) (Figure 3.2). Ultrasonic polyethylene cylinder reactors, 10 cm in diameter, equipped with a transducer and input power 3 watt at the lower part were used (Figure 3.1A).

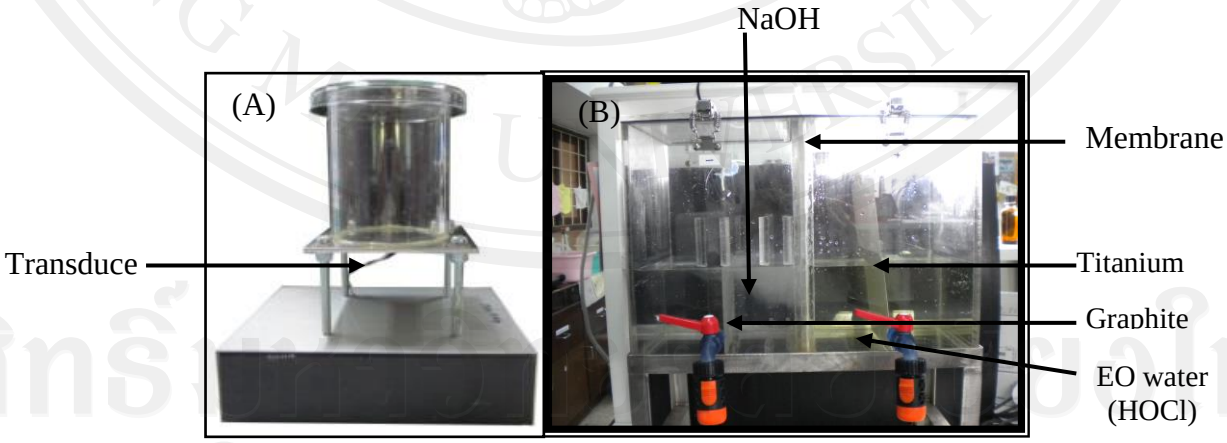

Figure 3.1 Ultrasonic devices (A) and EO water generator model (B)

Figure 3.2 Ultrasonic water bath made by Honda Electronics Company, Japan

3.2.5 Effect of EO water and US waves on *in vitro* spore germination inhibition of *Fusarium* sp.

The effect of EO water on spore survival of *Fusarium* sp. was studied. Spread plate technique was applied. For EO water treatment, one ml of the spore suspension (10^5 conidia/ml) was added into 9 ml of EO water containing 100, 200 and 300 ppm free chlorine. All treatments were incubated at room temperature for 0, 10, 30 and 60 minutes. After treatments, each 0.1 ml of treated spore suspension was added to 0.9 ml. of 0.1 N sodium thiosulfate. After well mixing, 0.1 ml. of solution was spread on PDA and incubated at 27 °C for 48 hours. Survival of the fungus was expressed as the mean number of colony-forming-units (cfu/ml). Each treatment consisted of 9 replicates and the experiment was repeated twice independently. The control treatment consisted of a 1 ml of spore suspension and 9 ml of distilled water.

The effect of ultrasonic wave on spore survival of *Fusarium* sp. was studied. Spore suspension (10^5 conidia/ml) was treated by adding 1 ml of spore suspension to 9 ml of sterile water. Those suspensions were subjected to ultrasonic waves at frequencies of 108, 400, 700 KHz and 1 MHz. for 0, 10, 30 and 60 minutes. After treatments, all

treated samples were counted colony-forming-units by spreading plate technique as described above.

3.2.6 Effect of EO water and US waves on *in vitro* mycelial growth inhibition of *Fusarium* sp.

The effect of EO water and US wave on mycelial growth of *Fusarium* sp. was studied. For EO water treatment, 1 cm of mycelium disc was added into 9 ml of EO water containing 100, 200 and 300 ppm free chlorine. All treatments were incubated at room temperature for 0, 10, 30 and 60 minutes. For US wave treatment, 1 cm of mycelium disc was added into to 9 ml of sterile distilled water and subjected to ultrasonic waves at frequencies of 108, 400, 700 KHz and 1 MHz for 0, 10, 30 and 60 minutes. Thereafter, the mycelium disc was placed onto a PDA plate and incubated at 27 °C. Mycelium growth was recorded daily. Each treatment consisted of 9 replicates and the experiment was repeated twice independently. One cm of mycelium disc and 9 ml of distilled water were used as the control.

3.2.7 Effect of EO water combined with US waves on *in vitro* spore germination and mycelial growth inhibition of *Fusarium* sp.

The effect of EO water combined with US wave on spore survival of *Fusarium* sp. was studied. The spread plate technique was applied. One milliliter of a $1x10^5$ spore suspensions or 1 cm of mycelium disc was added into the ultrasonic chamber containing EO water with a free-chlorine concentration of 100 ppm and subjected to simultaneous and continuous MS at 1 MHz. Treatments were carried out at room temperature for 10 min. Thereafter, each 0.1 ml of treated spore suspension was added to 0.9 ml of 0.1 N sodium thiosulfate mixed well and 0.1 ml of solution and spread onto a PDA plate. The mycelial disc was placed onto a PDA plate and incubated at 27°C. Survival of the fungus was expressed as mean numbers of colony-forming-units (cfu/ml) after incubation for 48 h and mycelial growth was recorded daily. Each treatment consisted of 9 replicates and the experiment was repeated twice independently. The control treatment consisted of a 1ml spore suspension and 1 cm of mycelium disc and 9 ml of distilled water.

3.2.8 Effect of EO water and US waves on ultrastructure changes of *Fusarium* sp.

The cell morphological characteristics of *Fusarium* sp. as affected by treatments will be observed under a light microscope. For EO water, 1 ml of spore suspension was added into a tube containing 9 ml of EO water at a concentration of 100 ppm free chlorine. For US wave, 1 ml of spore suspension was added into an ultrasonic chamber containing 9 ml of distilled water with a frequency of 1 MHz. All of treatments were run for 10 min. After treated, 0.1 ml of the suspension was mixed with 0.9 ml of 0.1 N sodium thiosulfate. Then, the mixture was immediately poured on the glass slide and observed under a light microscope or 0.1 ml of the mixture was spread on PDA, spore germination characteristics were observed under a light microscope after incubated at 27 °C for 24 h.

3.2.9 Effect of EO water in combination with US wave on the reduction of microorganism contaminants of pineapple

Pineapple fruits (*Ananas comosus* cv. Phu Lae) were harvested, at the green mature stage, from a commercial orchard in Chiang Rai Province, Thailand. After harvesting, the fruits were transported immediately to the Postharvest Biology and Technology laboratory, Chiang Mai University. The peduncle was cut with a knife to leave 2 cm peduncle on the fruit. The crown was trimmed to a length of 3 - 4 cm. Samples of 12 fruits were used for each replicate. The treatment which shown good results in the control of spore germination from previous experiment was used for this experiment. In the first treatment, fruits were subjected to ultrasonic waves of 3 watts and at a constant frequency of 1 MHz. The experiments were carried out in an ultrasonic water bath (Honda Electronics Company (Toyohashi, Aichi, Japan) dimensions: 44.5 × 51.5 × 35 cm). The capacity of the device was 50 litres which was able to wash 10 Kg. of pineapple fruits. In the second treatment, pineapple fruits were treated with EO water with a concentration of free-chlorine at 100 ppm. In the third treatment, pineapple fruits were immersed into the ultrasonic chamber containing EO water with a free-chlorine concentration of 100 ppm. and subjected to simultaneous continuous US at 1 MHz.

Pineapple fruits, treated with tap water, were used as controls. All treatments were run for 10 min. After the treatments, fruit samples were placed in a basket and air-dried. Fruit were then covered with a commercial plastic bag and maintained at 13°C for 20 days. Samples were taken initially and at 5-day intervals during storage for decay evaluation and other analysis.

3.2.9.1 Microbiological analysis

From each treatment, 25 g samples of pineapple were put into 225 ml of aseptic physiological saline with 0.2% Tween 80, shaken in a shaker for 5 min. and serially diluted (1:10).

3.2.9.2 Aerobic mesophilic microorganism counts (AC)

For AC determinations, 1-ml aliquots of each sample dilution were plated with duplicates on Petrifilm AC plates following the method recommended by the manufacturer. After incubation at 30 ± 1◦C for 48 ± 3 h, plates containing 25–250 red colonies were selected, the colonies were counted, and the average number of cfu/ml was determined.

3.2.9.3 Yeast and mold counts (YM)

For YM determinations, 1-ml aliquots of each sample dilution were plated with duplicates on Petrifilm YM plates following the manufacturer's instructions. These plates were incubated at 25 ± 1°C for 72 ± 3 h for acid tolerant mold and yeast counts. Plates containing 25–100 colonies were selected and counted, and the average number of cfu/ml was calculated.

3.2.9.4 Estimation of fruit decay

Fruit decay was visually estimated using 12 fruits from each replicate after storage. De-crowned pineapples with visible mold growth were considered to be decayed. The severity of fruit decay was expressed as a percent of the fruit showing fungal symptoms.

3.3 Statistical analysis

The Statistical Package for the Social Science (SPSS version 17) software for Windows was used for the Analysis of Variance (ANOVA) and least-significant difference (LSD) at the 95 % confidence level of each variable value under completely randomized design (CRD). Each experiment had tree replicates and all experiments were run two times with similar results.

3.4 Results and discussion

3.4.1 Isolation of fungal pathogens and pathogenicity test

3.4.1.1 Isolation and pathogenicity test

The fungi species isolated from the infected of de-crowned pineapple fruit are presented in the left hand of Figure 3.3 (A, B, C). Then, all of these were inoculated into de-crowned pineapple fruit for pathogenic tested. Our results indicated that all fungus could be growth on de-crowned pineapple fruit especially Figure 3C was markedly observed. This fungus completely disintegrated the affected tissue (100% infection) with extensive mycelial growth covering the de-crowned pineapple fruit within 5 days. Therefore, this fungus was chosen to use for next experiments and identification.

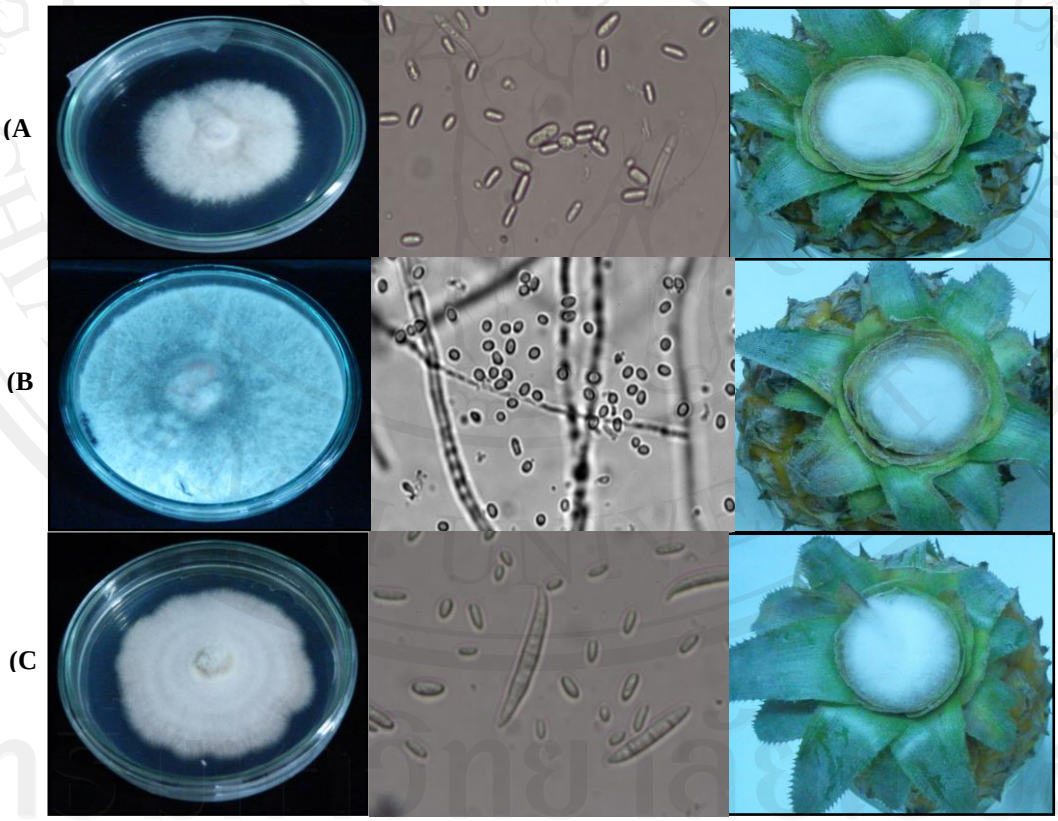

Figure 3.3 Morphological structure of fungus after inoculated of *Collectrotrichum* sp. (A), unknown (B) and *Fusarium* sp. (C) on de-crowned pineapple fruit at 5 days of incubated at room temperature.

3.4.1.2 Fungal identification

The extensive network of fungal hyphae was observed on the surface of PDA after incubation (Figure 3.4A). On the basis of microscopic examination and morphologic characteristics, the fungal strain was identified as *Fusarium* sp. It grew rapidly on PDA and produce woolly to cottony, flat, spreading colonies. Hyaline septate hypae and phialides, macroconidia and microconidia were observed microscopically (Figure 3.4B).

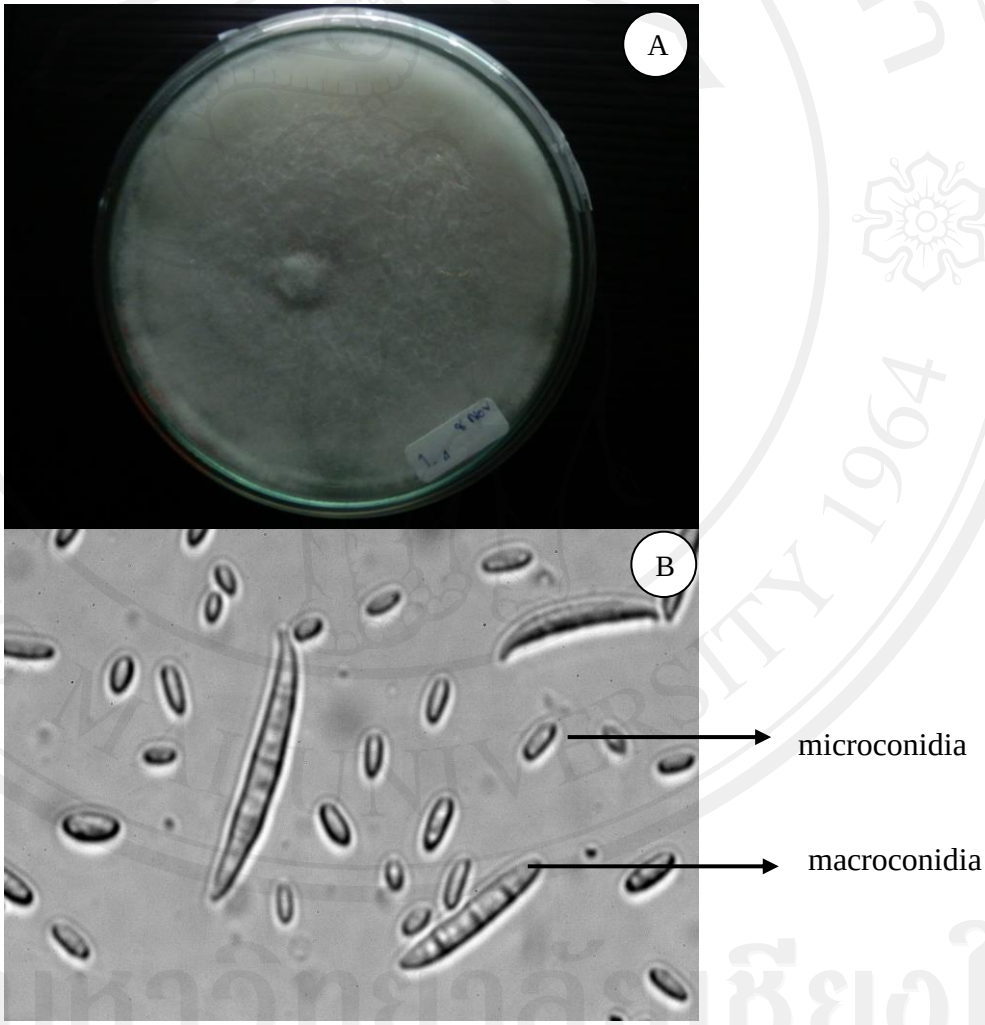

Figure 3.4 Morphological characteristic of *Fusarium* sp on PDA(A). Microscopic examination of morphologic characteristics of macroconidia and microconidia of *Fusarium* sp. (B) (40x).

3.4.2 Effect of EO water and US waves on *in vitro* spore germination inhibition of *Fusarium* sp.

The effects of the EO water and US wave on the inhibited spore germination of *Fusarium* sp. are presented in Table 3.1. All the EO concentrations completely inhibited the spore germination of the fungi (Figure 3.5). The concentration of free chlorine in EO water was sufficient to reduce fungal growth. Hypochlorous acid is the most effective form of chlorine compound. It damages the microbial cell by oxidizing nucleic acids and proteins, causing lethal damage (Acher *et al*., 1997). Low pH in EO water sensitizes the outer membrane of the cells, thereby allowing hypochlorous acid to enter the cells more efficiently. This study showed similar results with the effect of EO water on the inactivation of *Penicillium digitatum* offer 1 min exposure in EO water (Whangchai *et al*., 2010). Buck *et al*. (2002) also reported that it took about 30 s or less to inhibit the thin-walled fungi and at least 2 min for the thicker-walled species. In addition, US treatment significantly reduced the spore survival of *Fusarium* sp. on the PDA plates (Figure 3.6). All four exposure frequencies (108, 400, 700 KHz and 1 MHz) significantly reduced spore survival compared with the control group (Table 3.1). The inhibition percentage was increase with increasing treatment time. Also, there was a slight reduction of survival at less than 10 min exposure time, but an increased level of reduction occurred after longer durations, and was not difference among ultrasound treatments. However, the ultrasound treatment with 1 MHz was more effective at reducing survival of *Fusarium* sp. Reduction of spore survival by ultrasound is mainly due to free- radical attacks; hydroxyl radical attack and the physical disruption of cell membranes (Phull *et al*., 1997). These results are similar to the effects of US on the reduction of fungi achieved after longer periods (Dehghani *et al*., 2007). Sagong *et al*., (2011) also suggested that the reduction of *E. coli* O157:H7, *S. typhimurium* and *L. monocytogenes* on lettuce was greater after exposure to ultrasound for 20 min compared with a 5 min treatment.

Table 3.1 Mean percentage inhibition of spore survival by various concentrations of available free chlorine of EO water or a frequencies of US wave.

Treatments		Time (min)		
		10	30	60
EO water	100 ppm	100[a]	100[a]	100[a]
	200 ppm	100[a]	100[a]	100[a]
	300 ppm	100[a]	100[a]	100[a]
US wave	108 KHz	1.77[b]	3.43[b]	6.86[b]
	400 KHz	14.23[a]	13.87[a]	15.93[a]
	700 KHz	13.62[a]	13.14[a]	14.05[a]
	1 MHz	14.96[a]	14.69[a]	16.96[a]

Mean of 6 replicates. Means followed by the same letter(s) do not differ significantly at 5% (LSD).

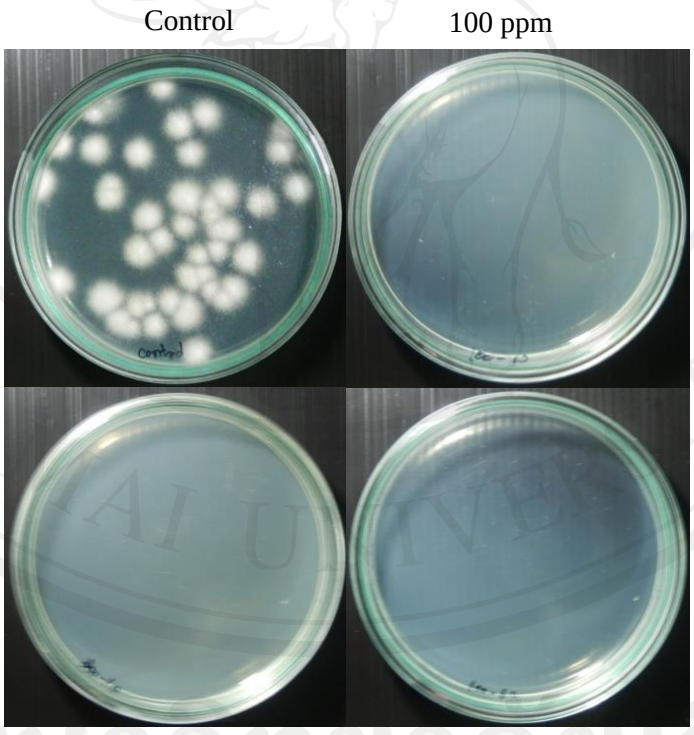

Figure 3.5 Spore survival populations of *Fusarium* sp. on PDA plates, after being treated with EO water at concentrations of 100, 200 and 300 ppm for 10 min and incubated at 27 °C for 48 hr.

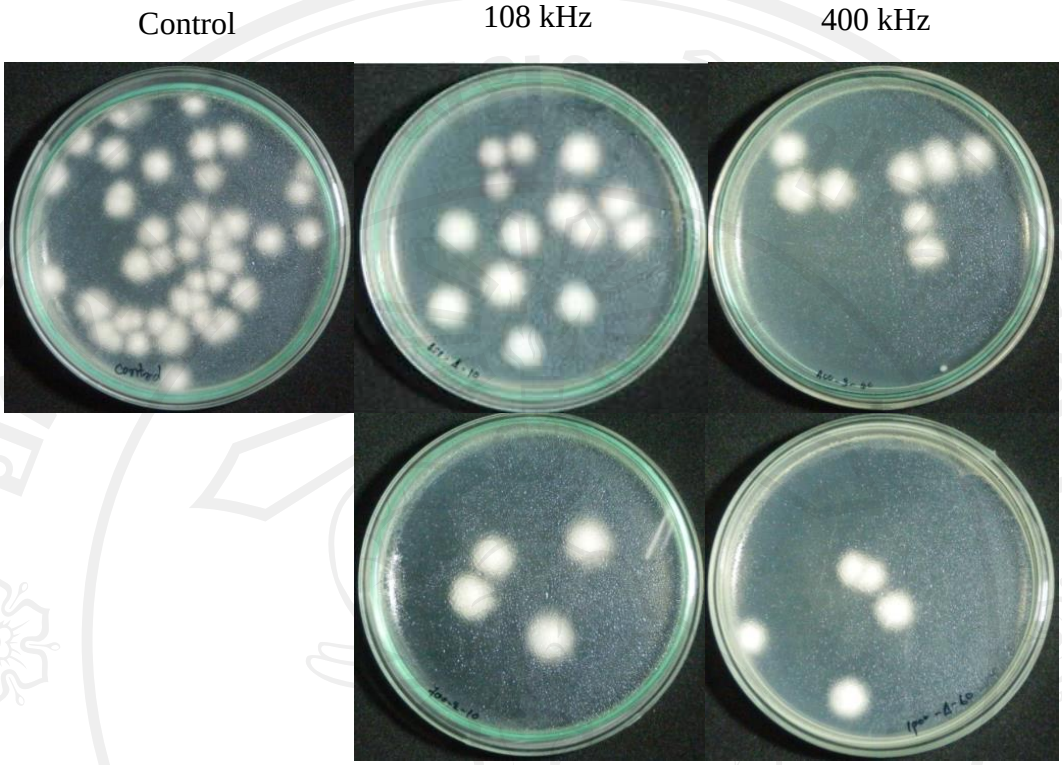

Figure 3.6 Spore survival populations of *Fusarium* sp. on PDA plates, after being treated with US waves at frequencies of 108, 400, 700 KHz and 1 MHz for 60 min and incubated at 27 °C for 48 hr.

3.4.3 Effect of EO water and US wave on *in vitro* mycelial growth inhibition of *Fusarium* sp.

Mycelial growth was significantly inhibited by all three concentrations of the EO water used. Concentration effects for *Fusarium* sp. mycelial were not significant between 100 and 200 ppm (Table 3.2). At concentrations of 300 ppm had a highest suppressed the fungi tested (36.22% inhibition) in the 5 days of storage. Moreover, at a concentration of 300 ppm and 60 min exposure time showed the strongest mycelium growth inhibition of *Fusarium* sp. (Table 3.3 and Figure 3.7). In addition, the level of inhibitory effect of US wave showed poor fungitoxicity effect on mycelial growth of fungi (0.9 – 14.9% inhibition), in all frequencies and all times exposure had no inhibitory effect on mycelial growth (Table 3.2 and 3.3). Because the mycelium structure is composed of a compact mass of hyphae, ultrasound wave could not penetrate to the inner mycelium matrix, leaving some parts protected from high pressure and temperature effect of ultrasound wave.

Table 3.2 Mean percentage inhibition of mycelial growth by various concentrations of available free chlorine of EO water or frequencies of US wave.

Treatments		Days after treated			
		1	3	5	7
EO water	100 ppm	40.68a	9.09b	15.56b	11.43b
	200 ppm	40.68a	14.72b	17.78b	10.08b
	300 ppm	40.68a	20.35a	36.22a	34.29a
US wave	108 KHz	0.00b	4.46b	1.00b	0.99b
	400 KHz	0.00b	9.55a	3.59a	1.81a
	700 KHz	2.61a	12.10a	8.38a	2.63a
	1 MHz	2.61a	14.97a	9.98a	4.60a

Mean of 6 replicates. Means followed by the same letter(s) do not differ significantly at 5% (LSD).

Table 3.3 Mean percentage inhibition of mycelial growth by concentrations of 100 ppm of EO water and frequencies of 1 MHz US wave, with a different time exposure.

Treatments		Days after treated			
		1	3	5	7
EO water	10 min	41.67a	10.16b	7.88b	14.50b
300 ppm	30 min	41.67a	15.85b	20.76a	24.67ab
	60 min	41.67a	25.20a	31.50a	34.09a
US wave	10 min	3.42a	0.95b	1.55a	1.29a
1 MHz	30 min	0.85b	9.52a	5.35a	1.93a
	60 min	4.27a	9.84a	10.02a	3.86a

Mean of 6 replicates. Means followed by the same letter(s) do not differ significantly at 5% (LSD).

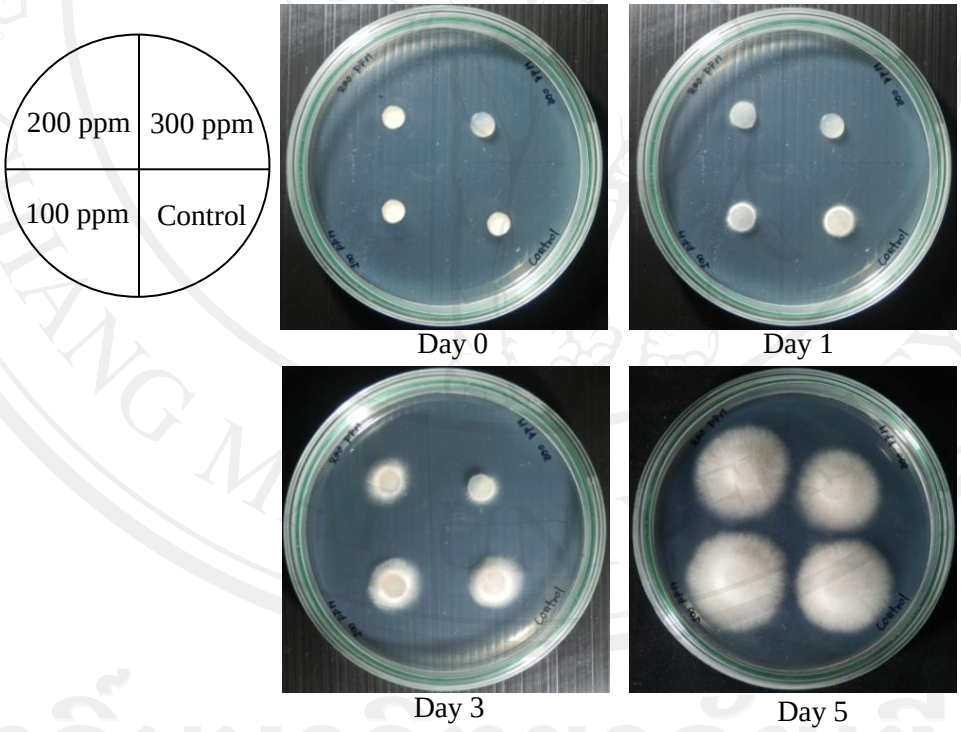

Figure 3.7 Mycelial disc growth diameter of *Fusarium* sp. on PDA plate after treated with EO water (100, 200 and 300 ppm) for 60 min and during incubated at 27 °C for 5 days.

3.4.4 Effect of EO water combined with US waves on *in vitro* spore germination and mycelial growth inhibition of *Fusarium* sp.

The results shown in Figure 3.8 indicate that the combination and EO water treated completely inhibited the spore germination of *Fusarium* sp. when compared with the control and US wave group. At 72 hr after treated, germinated spores were visually noticeable in the control and in US wave treatments with recorded fungal concentration of 8.1 and 6.4 log cfu/ml, respectively. However, spores treated with EO water and combined treatments did not germinate even after 72 hr of incubation at 25°C. The effects of treatments on mycelium growth are shown in figure table 3.4. The mycelial diameter in the treated fungus was significantly lower than that of the control group. Among all treatment at the 5 days of storage, combined treatment of US wave with EO water gave the best inhibition of mycelial growth (67.74 % inhibition) followed by EO water(23.04 % inhibition) and US wave(6.11 % inhibition) treatments, respectively. Strongly acidic EO water has antifungal properties. Hypochlorous acid damages the microbial cell by oxidizing nucleic acids and proteins, causing lethal damage (Acher *et al*., 1997). Low pH in EO water sensitizes the outer membrane of the cells, thereby allowing hypochlorous acid to enter the cells more efficiently. EO water has wide fungicidal activity, which may facilitate its use as a contact fungicide on aerial plant surfaces (Al-Haq, 2005). Some researcher used EO water by spray or as irrigation water, for arresting fungal growth on horticultural crops (Grech and Rijkenberg, 1992). Our results agree with those of Whangchai *et al*., 2010, who found that the effect of EO water on the inactivation of *Penicillium digitatum* offer 1 min exposure in EO water. Buck *et al*. (2002) treated 22 fungal species with EO water *in vitro* and reported that it took about 30 sec or less to inhibit the thin-walled fungi and at least 2 min for the thicker-walled species. In addition, US treatment significantly reduced the spore germination of *Fusarium* sp. on the PDA plates compared with the control group. Reduction of spore germination by US is mainly due to the cavitation phenomenon, which consists of the formation, growth and collapse of air bubbles. These bubbles generate localized mechanical and chemical energies that are capable of inactivating microorganisms (Adekunte *et al*., 2010; Piyasena *et al*., 2003). Our results related to

Palacios *et al*. (1991), who found that the heat resistance of spores of *Bacillus stearothermophilus* was reduced when subjected to US treatment. Scherba *et al*. (1991) also reported that US treatment had a significant reduction growth of *Trichophyton mentagrophytes* when compared with the control on PDA plates.

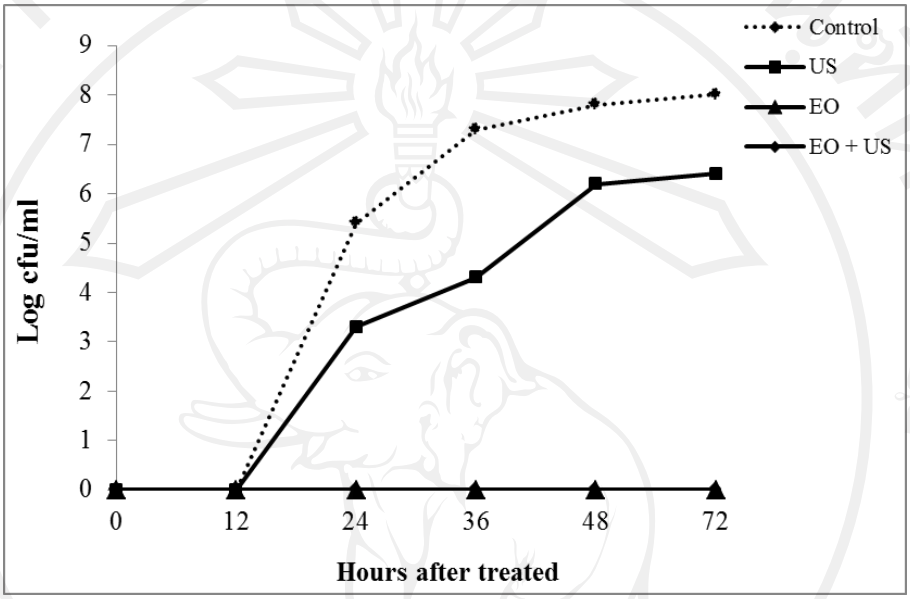

Figure 3.8 Colony-forming units of *Fusarium* sp. (log cfu/ml) on PDA plates after treat with US wave at frequency of 1 MHz and EO water at available free chlorine of 100 ppm and their combination for 10 min. during incubation for 48 hr at 25°C

Table 3.4 Mean percentage inhibition of mycelial growth by concentrations of 100 ppm of EO water and frequencies of 1 MHz US wave

Treatments	Days after treated		
	3	5	7
US	16.44c	6.11b	0.68b
EO	37.02b	23.04a	11.17a
EO + US	69.72a	67.74a	58.38a

Mean of 6 replicates. Means followed by the same letter(s) do not differ significantly at 5% (LSD).

3.4.5 Effect of EO water and US waves on ultrastructural changes of *Fusarium* spore.

The Microscopic images of fungal spores after treated are shown in Figure 3.9. Figure A is a normal spore and figure B is abnormal spores from EO water treated and figure C from US wave treated. It revealed that direct EO water and US wave to fungal spores for caused abnormalities and cell damage. Contents from damaged cells are shown to have leaked out in the EO water treated, while some parts of spore were damage in the US wave treated. After incubated for 24 h the normal germination of *Fusarium* sp. was shown in Figure 3.10A. The spore germination were completely inhibited when treated with EO water as shown in Figure 3.10B while the hyphal of germinated spores exhibited abnormal swelling at the tip when treated with US wave as shown in Figure 3.10C.

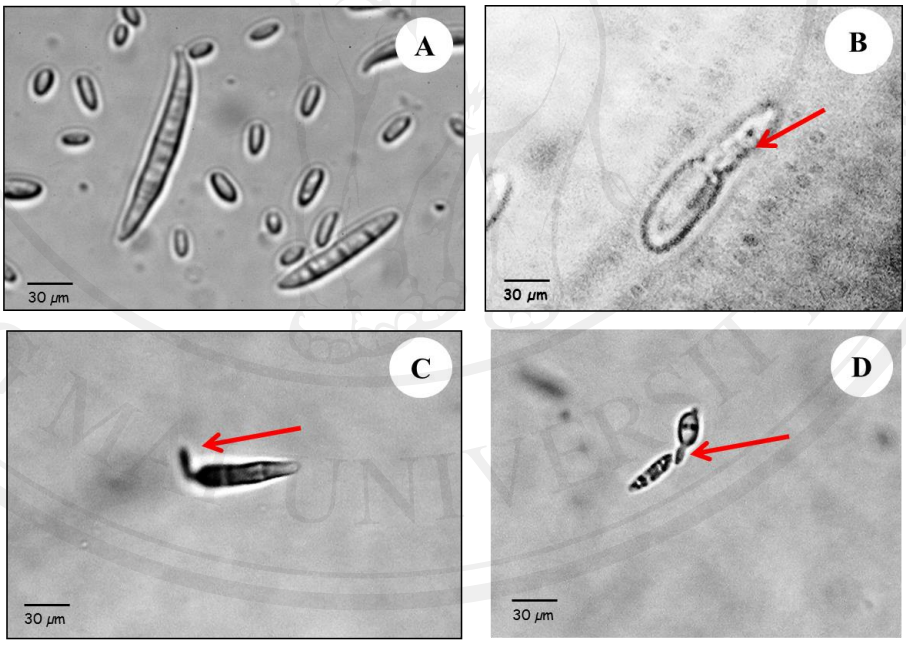

Figure 3.9 Microscopic photographs showing the normal (A) and abnormal of *Fusarium* sp. spores after treated with EO water at a concentration of free chorine of 100 ppm (B) and US wave at a frequency of 1MHz (C), both were run for 60 min (Arrows indicate the damage site of cell wall).

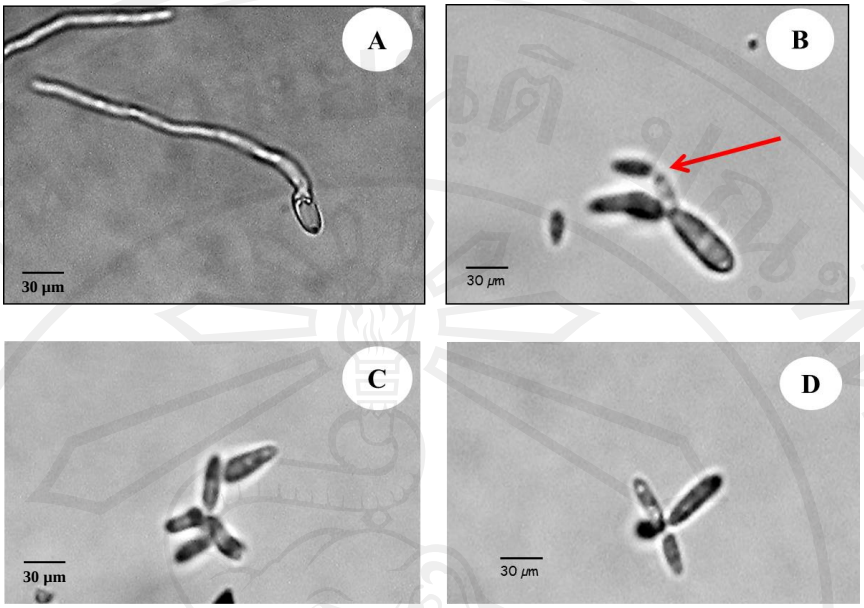

Figure 3.10 Microscopic photographs showing normal (A) and abnormal of the germination of *Fusarium* sp. spores after treated with EO water at a concentration of free chorine of 100 ppm (B) and US wave at a frequency of 1 MHz (C) then placed on PDA plate for 24 hr. (Arrows indicate hyphal tip abnormalities).

3.4.6 Effect of EO water in combination with US wave on the reduction of microorganism contaminants of pineapple

The effects of US and EO water on aerobic microorganism (AC) and yeast and Mold (YM) are shown in figure 3.11, 3.13 and 3.12, 3.14 respectively. For AC, tap water-washed samples (control) presented the highest microbial count during storage at 13 ˚C. Pineapple washed with US or EO water (before storage) had the highest AC reduction,1.55 and 0.25 log cfu/ml, for the combination of US with EO water and EO water individually respectively. During storage, these two washing treatments resulted in pineapple with lower AC, when compared to the results obtained with the US or control. In relation to YM, tap water-washed samples presented significantly the highest number of YM compared with all other treatments. The combination of US with EO water (before storage) was the most effective at reducing the initial contamination of pineapple, in terms of YM, with an average–reduction of 0.34 log cfu/ml observed (Figure 3.12). The effectiveness of the EO water was greater with ultrasonication and achieved an average of 0.09 log cfu/ml greater reduction than without ultrasonication. Kim *et al*. (2003) found that the application of EO water in conjunction with ultrasonication enhanced the bactericidal effectiveness of EO water on alfalfa sprouts by 80%. Hung *et al*, (2010) also reported that dipping inoculated strawberries into chlorinated water or EO water with ultrasonication reduced *E. coli* O157:H7 cells by 0.7 to 1.9 log cfu/g depending on the treatment time and treatment solution temperature. Brilhante São José and Dantas Vanetti (2012) indicated that the combined treatment of US for 10 min and peracetic acid resulted in greater removal of microorganisms from the surface of tomatoes. Sagong *et al*. (2011) found a synergistic effect in the use of organic acids combined with US (40 kHz) in the inactivation of *E. coli* O157:H7, *Salmonella typhimurium* and *Listeria monocytogenes* inoculated on organic lettuce without significantly affecting the color and texture.

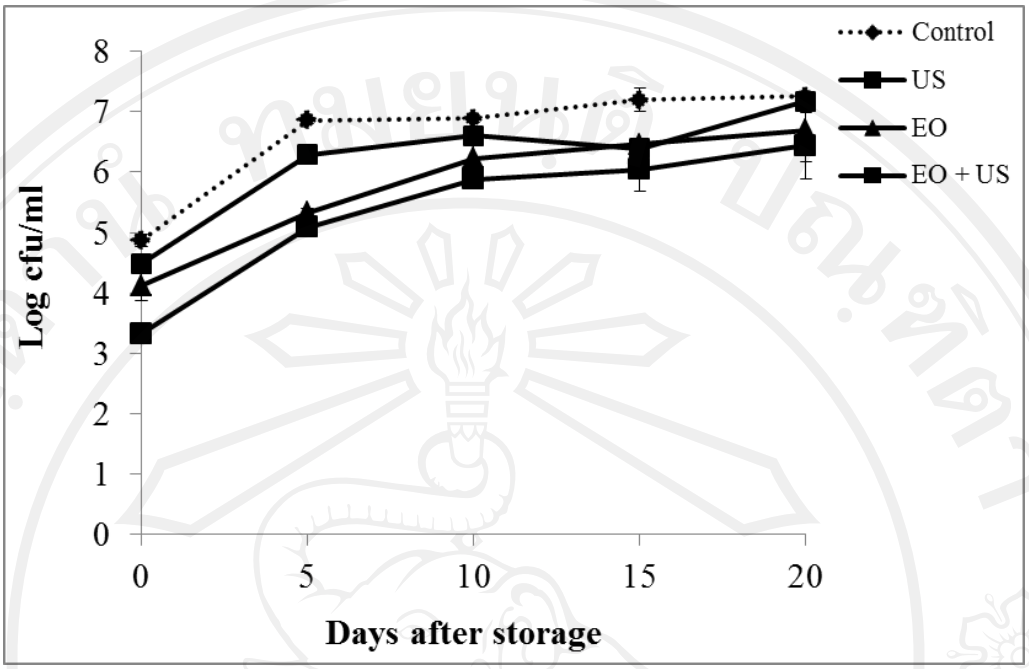

Figure 3.11 Effect of EO water and ultrasonic wave on total aerobic plate count (log cfu/ml) in pineapple fruit during storage at 13 °C for 20 days.

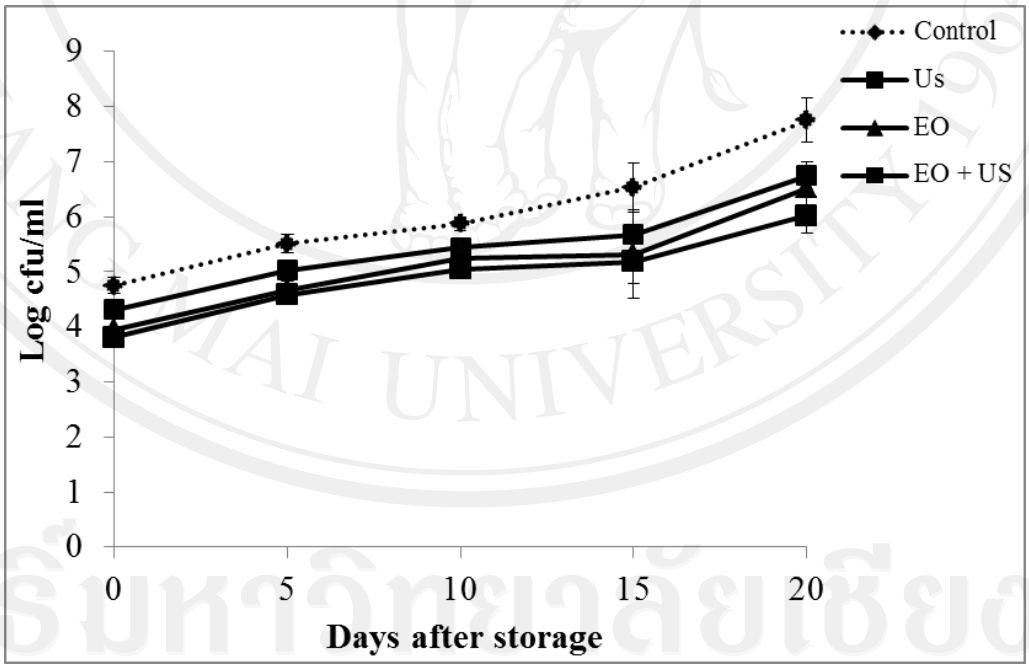

Figure 3.12 Effect of EO water and ultrasonic wave on yeast and mold count (log cfu/ml) in pineapple fruit during storage at 13 °C for 20 days.

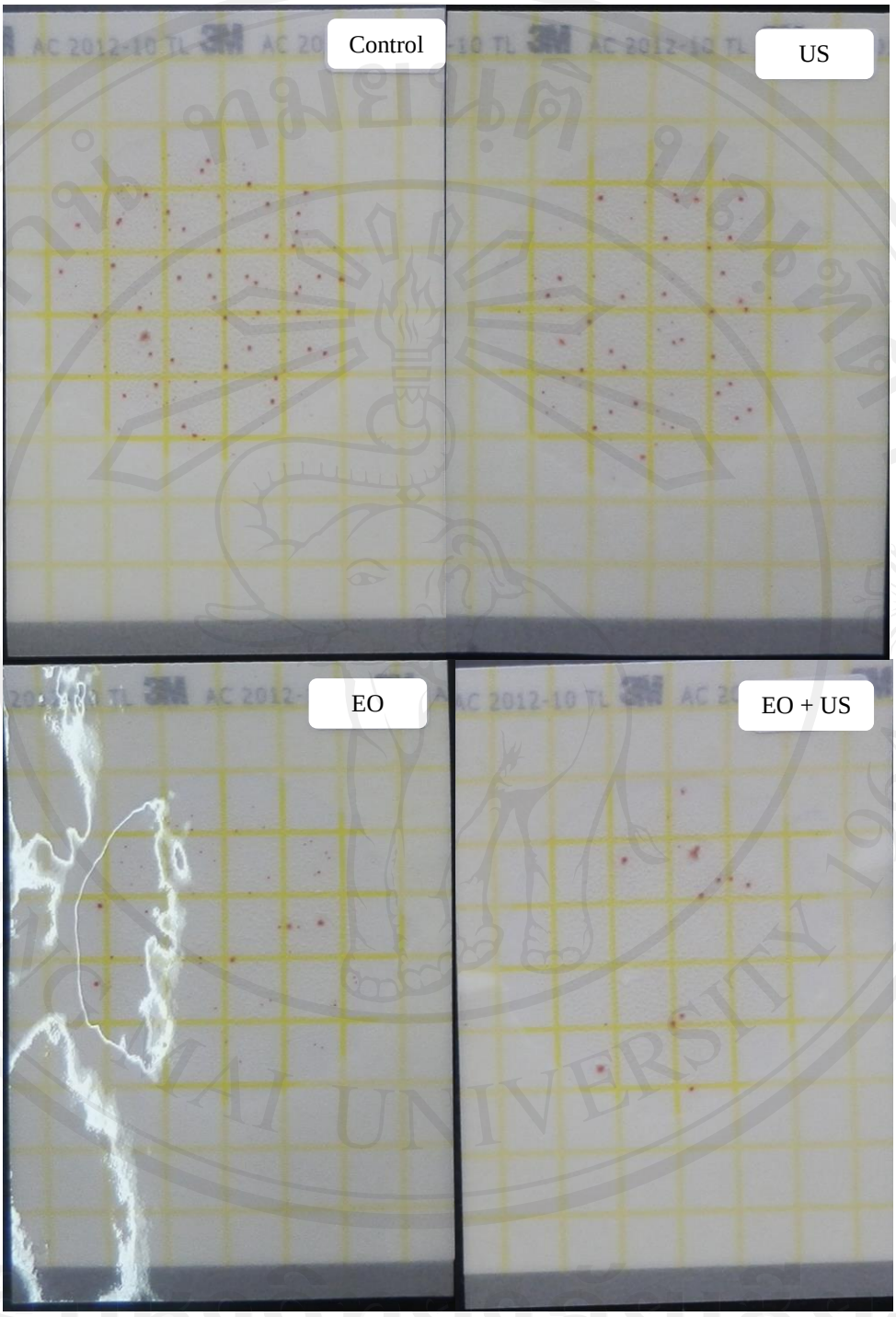

Figure 3.13 Total aerobic plate counts on petrifilm after being treated with EO water and US wave and their combination, and then incubated at 30 ± 1°C for 48 ± 3 hr.

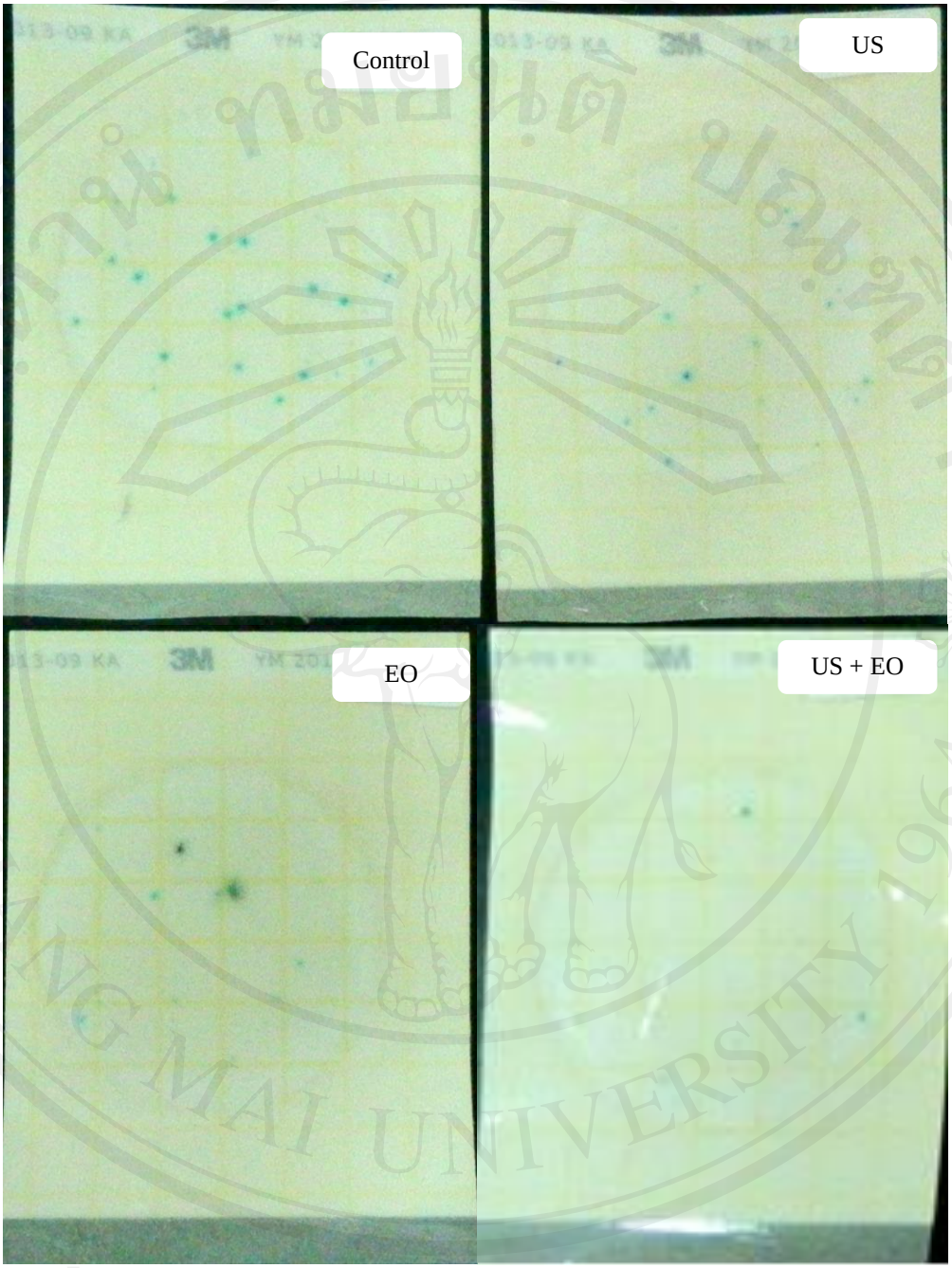

Figure 3.14 Yeast and Mold count on petrifilm after being treated with EO water and US wave and their combination, and then incubated at 25 ± 1°C for 72 ± 3 hr.

3.4.7 Effect of EO water and ultrasonic waves on the control of postharvest decay of pineapple fruit

The incidence of decay of pineapple fruit increased with storage time (Figure 3.15-3.18). The incidence of decay was significantly higher for the control compared with other treatments. However, the combination treatment with EO water and US significantly ($p < 0.05$) inhibited decay incidence for the 5 and 10 days of storage at 25 and 13 °C, respectively. Seven days after storage at 25 °C, the combination treatment reduced (80 %) of de-crowned decay incidence of pineapple fruits, followed by EO water and US wave treated alone, which reduced decay incidence by 60 and 20 %, respectively. De-crowned pineapple fruit treated with combined treatments caused a highly significant reduction of 75%, while EO water treated alone was reduced decay incidence by 37.5 %, but US wave not reduce the decay incidence after storage for 20 days at 13 °C. The combined treatment with EO water and US was more effective at reducing fruit decay. This was probably because the combined treatment effectively disinfected the fruit from pathogenic fungi through the mechanical disruption of the microbial cell, caused by the direct exposure to EO water disinfectants. Hung *et al*., (2010) also concluded that a combination of EO water and US resulted in a greater reduction of the bacterial contamination of broccoli. Huang *et al*., (2006) observed that US, combined with sanitization, enhances removal of *Salmonella* and *Escherichia coli* O157:H7 inoculated on apples and lettuce. The compression pressure, generated during the use of ultrasound, may contribute to the penetration of the chemical oxidants through the cellular membrane and the cavitation process may assist in the disaggregation of the microorganisms, which culminates in an increased efficiency of the sanitization treatment (Gogate and Kabadi, 2009). Thus, the potential of EO water, in combination with US, might be applied to pineapple handling systems, due to its marked synergistic effect against fungal decay on de-crowned pineapple fruit during storage.

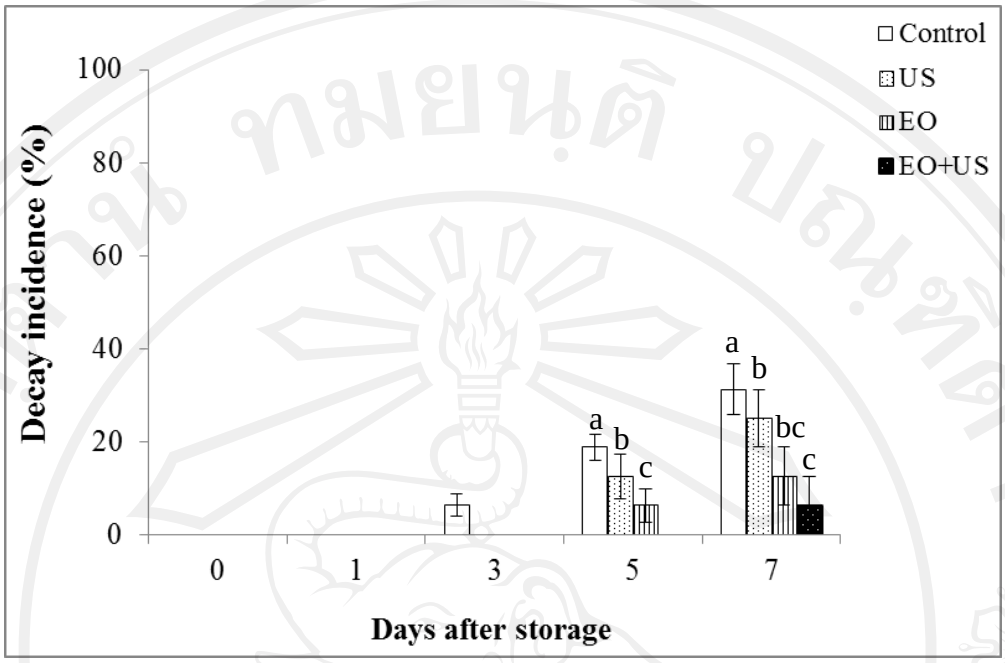

Figure 3.15 Effect of EO water and US wave on the incidence of decay in pineapple fruit during storage at 25 °C.

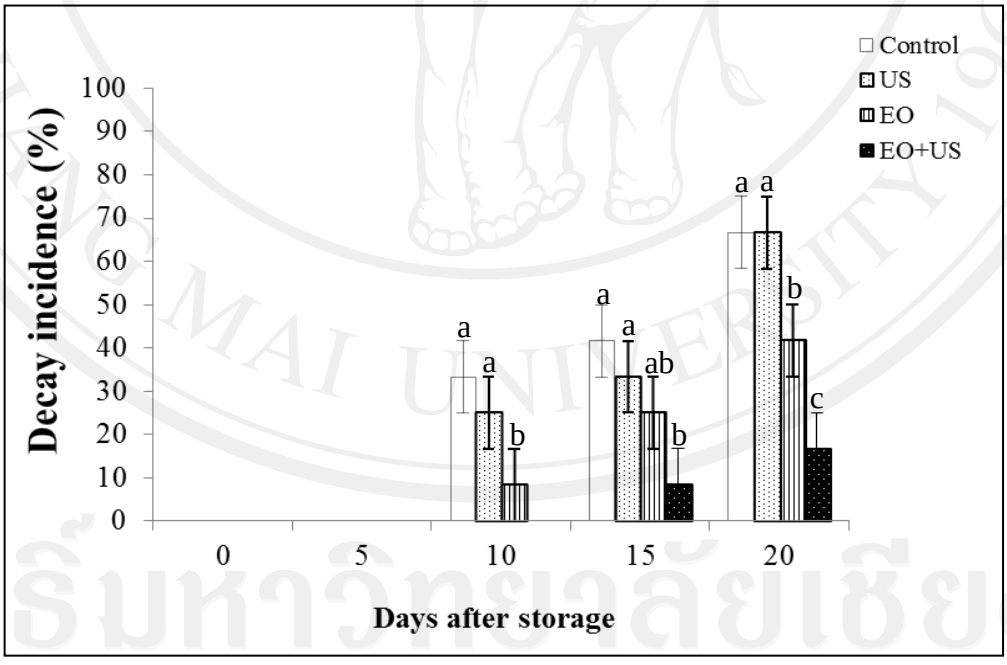

Figure 3.16 Effect of EO water and US wave on the incidence of decay in pineapple fruit during storage at 13 °C.

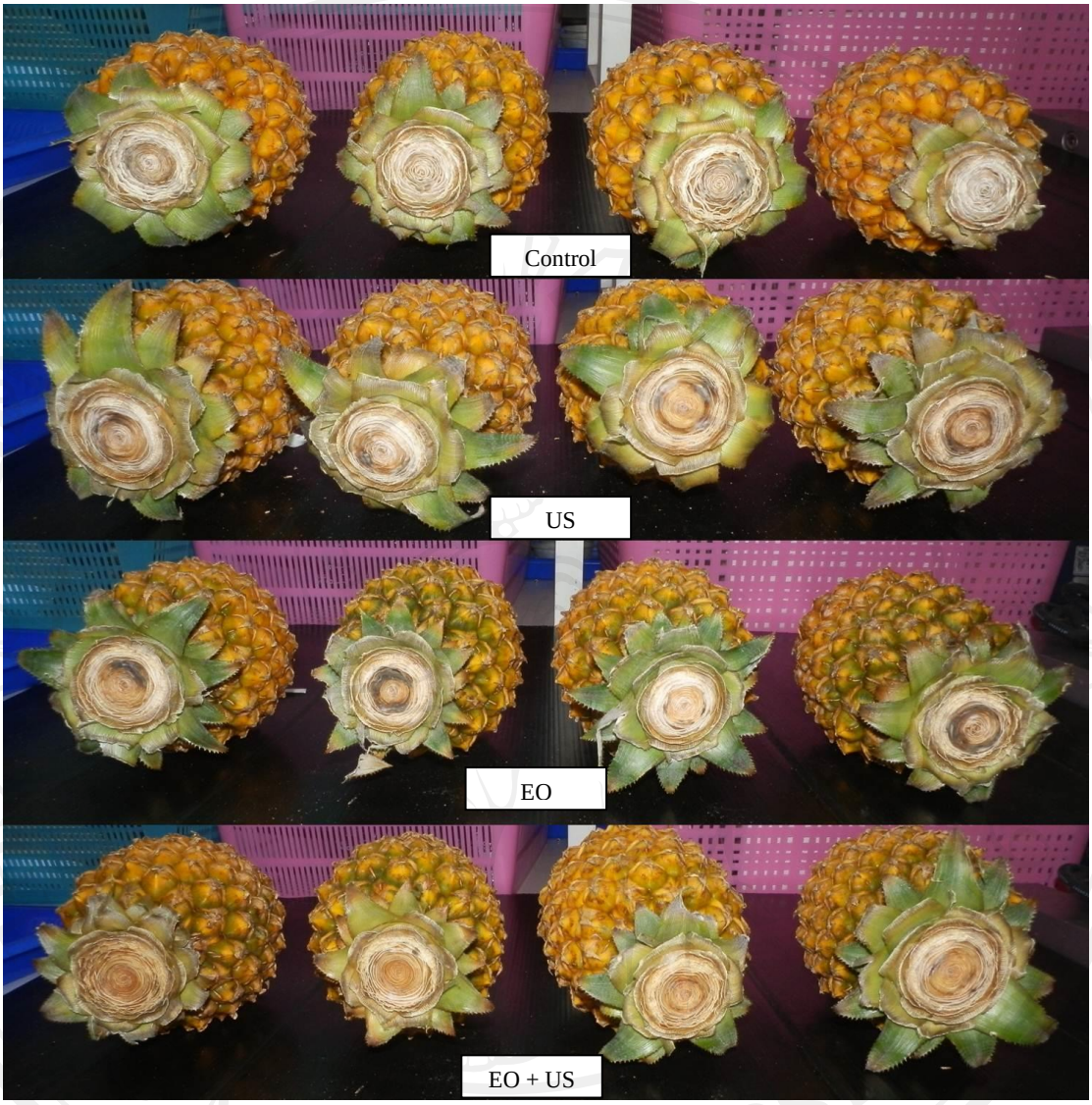

Figure 3.17 The disease development on the 7 days of de-crowned pineapple fruits those treated with EO water and US wave and then the fruit were kept at 25 ◦C.

Figure 3.18 The disease development on the 20 days of de-crowned pineapple fruits those treated with EO water and US wave and then the fruit were kept at 13 ◦C.

3.5 Conclusion

One MHz US irradiation and all of the EO water treatments significantly inhibited spore survival of *Fusarium* sp. The combined treatments gave the best inhibition of mycelial. The application of combined EO water and US wave for controlling microorganisms on de-crowned pineapple has not previously been reported. Therefore, the synergistic effect of EO water and US wave may provide valuable insight into the reduction of microorganism on pineapple fresh produce. This combined treatment was the most effective in reducing natural decay of the fruit and prolonged shelf life for 20 and 7 days after storage at 13 and 25 °C respectively.