การลดปริมาณ *Listeria monocytogenes* ปนเปื้อนบนผักด้วยสารละลายคลอรีนไดออกไซด์
Elimination of *Listeria monocytogenes* Contaminated on Vegetables by Chlorine Dioxide

ณัฐวุฒิ มาสกรานต์[1] วราภา มหากาญจนกุล[1] และ ธนะบูลย์ สัจจาอนันตกุล[1]
Nattawut Maskran[1], Warapa Mahakarnjanakul[1] and Tanaboon Sajjaanantakul[1]

Abstract

Concerning of nutrition value of fresh produce urged consumers increase consumption of the products. However inappropriate washing step cannot eliminate foodborne pathogens contaminated on produce, consequently in many western countries it becomes the cause seriously outbreaks. Soaking and washing lettuce, that artificially contaminated with *Listeria monocytopenes* 3.6 $\log_{10}$CFU/mL, in chlorine dioxide solution concentration of 3, 4, 5, 6 and 10 ppm at 30 ($\pm$2 $^{\circ}$C) for 10 min could completely eliminate the bacteria. While soaking and washing lettuce at lower contamination of 2.6 $\log_{10}$CFU/mL at concentration of 3 ppm only 5 min could destroy all cells. At the same condition total microorganisms were reduced to 2.2 $\log_{10}$CFU/mL (or 99.4%). Similarly baby corn contaminated at 3.4 and 2.4 $\log_{10}$CFU/mL were conducted. At concentration of 3, 4, 5, 6 and 10 ppm could completely eliminate *L. monocytogenes* cells and reduced total microorganisms by 1.9 $\log_{10}$CFU/mL (or 98.9%) in baby corn. However, at high concentration of 10 ppm caused strong smell and off-flavor in both vegetables. Using chlorine dioxide solution at concentration of 3 and 5 ppm for soaking and washing natural contaminated lettuce were done. Washed lettuce were kept in different temperature at 30($\pm$2) $^{\circ}$C, 4($\pm$1) $^{\circ}$C and 10($\pm$1) $^{\circ}$C. Results showed that the total aerobic count were not significantly different from washing lettuce with tap water. Population decreased about 1-1.8 $\log_{10}$CFU/mL since the chlorine dioxide solution could not eliminate all spoilage bacteria. It could be concluded that using chlorine dioxide solution at the appropriate conditions for washing and soaking produce obviously destroy foodborne pathogen and increase wholesomeness for consumption.

บทคัดย่อ

การตื่นตัวทางด้านโภชนาการทำให้ผู้บริโภคนิยมรับประทานผักและผลไม้สดเพิ่มขึ้น แต่ผักผลไม้ที่ล้างอย่างไม่ถูกต้องอาจมีจุลินทรีย์ที่ทำให้เกิดโรคปนเปื้อน จึงเพิ่มความเสี่ยงต่อการเกิดโรคเนื่องจากอาหารเป็นพาหะได้ การล้างและแช่ผักกาดหอมที่สร้างการปนเปื้อนด้วย *Listeria monocytogenes* 3.6 $\log_{10}$CFU/mL ด้วยสารละลายคลอรีนไดออกไซด์ความเข้มข้น 3, 4, 5, 6 และ 10 ppm ที่อุณหภูมิ 30($\pm$2 $^{\circ}$C) เป็นเวลา 10 นาที สามารถทำลายเซลล์แบคทีเรียชนิดนี้ได้หมด หากผักปนเปื้อนด้วยเซลล์ *L. monocytogenes* ปริมาณต่ำกว่าคือ 2.6 $\log_{10}$CFU/mL สารละลายคลอรีนไดออกไซด์ความเข้มข้น 3 ppm สามารถทำลายได้หมดในเวลา 5 นาที ที่ความเข้มข้นเดียวกันนี้ทำลายจุลินทรีย์ทั้งหมดได้ 2.2 $\log_{10}$CFU/mL (หรือ 99.4%) การล้างและแช่ข้าวโพดผักอ่อนที่สร้างการปนเปื้อน 3.4 และ 2.4 $\log_{10}$CFU/mL นั้นพบว่าสารละลายคลอรีนไดออกไซด์มีความเข้มข้น 3, 4, 5, 6 และ 10 ppm สามารถทำลายแบคทีเรียชนิดนี้ได้หมดในเวลา 5 นาที และลดปริมาณจุลินทรีย์ทั้งหมดได้สูงสุด 1.9 $\log_{10}$CFU/mL (หรือ 98.9%) สารละลายคลอรีนไดออกไซด์ความเข้มข้นสูง เช่น 10 ppm มีกลิ่นฉุนและผักมีกลิ่นผิดปกติ ส่วนการล้างและแช่ผักกาดหอมที่ปนเปื้อนจุลินทรีย์ตามธรรมชาติด้วยสารละลายคลอรีนไดออกไซด์ที่เข้มข้น 3 และ 5 ppm เป็นเวลา 10 นาที แล้วเก็บผักที่อุณหภูมิ 30$\pm$2 $^{\circ}$C, 4$\pm$1 $^{\circ}$C และ 10$\pm$1 $^{\circ}$C ปรากฏว่าปริมาณจุลินทรีย์ทั้งหมดลดลงไม่แตกต่างจากการล้างด้วยน้ำประปา ลดปริมาณจุลินทรีย์ทั้งหมดได้ 1-1.8 $\log_{10}$CFU/mL ในที่นี้การล้างผักด้วยสารละลายคลอรีนไดออกไซด์ไม่ยับยั้งการเจริญของจุลินทรีย์ที่ทำให้ผักเน่าเสีย แต่สามารถทำลายจุลินทรีย์ที่ก่อให้เกิดโรคได้

Introduction

In recent years, the frequency of foodborne illness outbreaks associated with consumption of fresh fruits and vegetables has increased, partly as a result of an increased demand for minimally processed (ready-to-eat) produce (Beuchat, 1999). Since fruits and vegetables are nomally contact with soil, insects and animals during growing and harvesting in the field. Consequently, their surfaces are not free from natural contaminants (Pao, 2001). The list of pathogenic bacteria which associated with produce have been reported as *Salmonella spp., Eschericia coli, Listeria monocytogenes, Shigella spp.* and

[1] ภาควิชาวิทยาศาสตร์และเทคโนโลยีการอาหาร คณะอุตสาหกรรมเกษตร มหาวิทยาลัยเกษตรศาสตร์
Department of Food Science and Technology, Faculty of Agro-Industry, Kasetsart University

Staphelococcus aureus which present in many type of vegetables and foods including lettuce, cabbage, tomato, carrot, coleslaw and salad, (Beuchat, 1999)

Listeria monocytogenes is a gram-positive, nonsporeforming bacterium that is commonly found in soil, water and decaying plant material (Soriano *et al*., 2001). It was reported as the cause of listeriosis in human (Lovette, 1985). The organism was also known as environmental pathogen and usually presented in the processing environment such as drainage and wet area (Mazzotta, 2001). Washing produce with water can effectively remove sand, soil and other debris from fresh fruits and vegetables, however that could not completely remove microorganisms. Many type of sanitizers become widely used in food processing to reduced undesirable microorganisms (Cherry, 1999). Chlorine dioxide (ClO_2) has been recognized as a bactericidal, viricidal and fungicidal agent. Since ClO_2 Produces very little of no Trihalomethanes (THMs) in treated water, therefore, it is a potential substitute for other chlorine compounds. Moreover, the bactericidal activity of ClO_2 is not affected by alkaline conditions or organic compounds comparable to other chlorine compounds (Kim et at., 1999). However, the efficiency of ClO_2 depends on some factors such as concentration, temperature, type of microorganisms and type of fresh produce. The appropriate condition to use ClO_2 in washing fresh fruits and vegetables needs to be determine and will assist industry to produce the wholesomeness foods for customers.

This study was designed to examine the potential of ClO_2 concentrations to reduce *Listeria monocytogenes* that artificially inoculated in lettuce, baby corn and cabbage. Storage study at different temperatures was also conducted to determine the shelf life of washed and sanitized vegetables comparable to the washing condition by tap water.

Materials and Methods

Preparation of inoculum

Listeria monocytogenes (NCTC11994) were obtained from Bangkok MIRCEN. A stock culture was maintained on Trypticase Soy Agar Slant (TSA; Merck, Darmstadt, Germany). Cell was cultured in Trypticase Soy Broth (TSB; pH 7.3; Merck, Darmstadt, Germany) at 37 °C. Loop inocula were transferred to TSB at two consecutive 24 h intervals then transferred by one milliliter to TSB 100 ml at 37 °C for 185 h served as inocula for all experiments.

Preparation artificially inoculated vegetables

Lettuce, baby corn and cabbage were purchased from local market. Two or three outer of lettuce leaves were used then the core were removed and discarded. Baby corn that removed the cap were used. Removed two or three cabbage leaves divided for a quarter, cut the core out and slice it to 0.5 inch by piece. Prepared vegetables veighed about 10-12 g, rinsed with tap water and dried for 1 h at room temperature (30±2 °C). By rinse method enumerated the natural contamination by culturing on Trypticase Soy Agar (TSA) and counted as total aerobic plate count (APC). Lettuce, baby corn and sliced cabbage were inoculated with *L. monocytogenes* to achieve final population 10^4 and 10^6 CFU/mL in culture suspension for 2 min, then dried for 30 min under laminar flow hood at room temperature (30±2 °C). Then a vegetables were treated with ClO_2 solution (Bello Zon®) of concentration at 3, 4, 5, 6 and 10 ppm for 5 and 10 min. Enumerated *L. monocytogenes* and aerobic plate count on Oxford agar (OX agar) and TSA.

Storage study was conducted at room temperature (30±2 °C) compared to low temperature (4±1 °C) and 10±1 °C). Lettuce was taken at 0, 12, 24 and 48 h at room temperature (30±2 °C) and 0, 2, 4, 6 and 8 days at low temperature (4±1 °C and 10±1 °C) then enumerated for survivor by rinse method. While baby corn and sliced cabbage were taken after storage for 0, 1, 2, 3, 4, 5 and 6 days at room temperature (30±2 °C) and 0, 2, 4, 6 and 8 days at 4±1 °C and 10±1 °C then analyzed for *L. monocytogenes* and APC.

Results and Discussion

Efficiency of ClO_2 solution to reduce *L. monocytogenes* on lettuce and baby corn

Lettuce After soaking and washing inoculated lettuce with high contamination of *L. monocytogenes* at 3.6 $\log_{10}$CFU/mL at room temperature (30±2 °C) by tap water at 10 min, total microorganisms were reduce to 3.44 $\log_{10}$CFU/mL (Table 1). Applying ClO_2 solution at concentration of 3 ppm at 30±2 °C for 5 min could reduce *L. monocytogenes* cells by 1.0 log and reduce total aerobic plate count by 2.3 log (or 99.5% reduction), while extending washing time for 10 min could completely eliminated *L. monocytogenes* (100% reduction) and reduced total microorganisms by 1.3 log (94.7% reduction).

Low contaminated lettuce had initial cell loads and *L. monocytogenes* at 4.7 $\log_{10}$CFU/mL and 2.6 $\log_{10}$CFU/mL, respectively, it was noticed that *L. monocytogenes* cells became 50% of total aerobic bacteria count (table 1). After soaking and washing lettuce with ClO_2 3, 4, 5 and 6 ppm at 30±2 °C for 5 and 10 min was done, results showed that applying ClO_2 in washing step completely destroyed *L. monocytogenes* cells. The best condition was found at 3 ppm, total microorganisms were reduced by 2.3 log (99.4% reduction) and 1.3 log (94.6%) after washing and soaking 5 and 10 min.

Noticeably, the longer extend washing period for (5 min to 10 min) could not be assure the better aerobic count reduction. Effectively of ClO_2 may be loss over the period of washing time. The ability of each microorganisms to attached tightly to pieces of vegetable may be better at longer exposure time. At concentration of 3-5 ppm, the effectiveness of ClO_2 solution to destroy aerobic microorganisms did not relevant with the concentration except at very high concentration such as 6 ppm. However soaking and washing lettuce with higher concentration of ClO_2 at 6 ppm for 5 min at 30±2 °C could not completely reduced aerobic microorganisms but to assure the safety of foods both high and low contaminated lettuce could be soaking and washing with ClO_2 3 ppm for 10 min respectively in order to eliminate *L. monocytogenes* cells on produce. In addition at 10 ppm, the highest concentration, although it could completely eliminate both *L. monocytogenes* and total aerobic microorganisms within 5 min, however it caused strong and off-flavor in leaves.

Table1 Population of *L. monocytogenes* and aerobic plate count (APC) on high and low inoculated lettuce (3.6 and 2.6 $\log_{10}$CFU/mL) before and immediately after ClO_2 treatment at 5 and 10 min.

ClO_2 (ppm)	Time (min)	High inoc. lettuce (Log_{10}CFU/mL)		Low inoc. lettuce (Log_{10}CFU/mL)	
		APC[a]	LM[b]	APC	LM
0	5	4.74±0.02	3.58±0.01	4.68±0.01	2.65±0.04
	10	4.73±0.01	3.58±0.01	4.68±0.01	2.63±0.00
3	5	2.45±0.01	2.57±0.01	2.44±0.01	ND
	10	3.44±0.01	ND[c]	3.41±0.00	ND
5	5	2.88±0.01	ND	2.89±0.00	ND
	10	3.88±0.01	ND	2.47±0.01	ND
6	5	3.45±0.01	ND	3.47±0.01	ND
	10	ND	ND	ND	ND

[a]APC = Population of total microorganisms on TSA

[b]LM = Population of *L. monocytogenes* on OX agar

[c]ND = Not detected (<25 CFU/mL)

Baby corn artificially contaminated of *L. monocytogenes* 3.4 and 2.4 $\log_{10}$CFU/mL on baby corn was conducted. Total aerobic microorganisms was found 4.4 $\log_{10}$CFU/mL while after soaking and washing with ClO_2 3 ppm at room temperature (30±2 °C) for 5 min could completely eliminate *L. monocytogenes* and reduced total microorganisms by 2 log (99% reduction) (Table2).

Table 2 Population of *L. monocytogenes* and aerobic plate count (APC) on high and low inoculated baby corn (3.4 and 2.4 $\log_{10}$CFU/mL) before and immediately after ClO_2 treatment at 5 and 10 min.

ClO_2(ppm)	Time (min)	High inoc. baby corn (Log_{10}CFU/mL)		Low inoc. baby corn (Log_{10}CFU/mL)	
		APC[a]	LM[b]	APC	LM
0	5	4.41±0.00	3.41±0.00	4.43±0.00	2.43±0.03
	10	4.43±0.03	3.42±0.01	4.43±0.01	2.44±0.00
3	5	2.48±0.02	ND[c]	2.41±0.01	ND
	10	3.61±0.00	ND	3.56±0.02	ND
5	5	3.79±0.01	ND	3.87±0.06	ND
	10	3.97±0.02	ND	4.08±0.01	ND
6	5	3.62±0.00	ND	3.51±0.03	ND
	10	3.65±0.01	ND	3.41±0.00	ND

[a]APC = Population of total microorganisms on TSA

[b]LM = Population of *L. monocytogenes* on OX agar

[c]ND = Not detected (<25 CFU/mL)

As we expected at high concentration level of ClO_2 as 3-6 ppm could completely eliminate *L. monocytogenes* cells, however it could not eliminate all aerobic microorganisms which included spoilage microorganisms. Completely elimination of both *L. monocytogenes* and total microorganisms was success at 10 ppm for 10 min, but the limitation was strong smell and off-flavor left in baby corn similarly to lettuce.

The best condition to wash baby corn with high inoculated *L. monocytogenes* (3.4 $\log_{10}$CFU/mL) is ClO_2 3 ppm 5 min. This treatment could reduce total aerobic microorganisms by 1.9 log even better than those of higher ClO_2 treated and any changes in flavor and color.

Similarly to previous results that ClO_2 solution at concentration of 5 and 6 ppm could completely destroy *L. monocytogenes*, however this treatment could reduce total aerobic microorganisms lower than those of at concentration of 3 and 4 ppm. Population was reduced only 0.5-0.9 log or 55.6-90.7% reduction.

Storage of washed and sanitized vegetables at room temperature (30±2 °C), 4±1 °C and 10±1 °C

All vegetables treated by soaking and washing ClO_2 3 or 5 ppm at 5-10 min, then stored at room temperature (30±2°C), low temperature at 4±1°C and 10±1°C. Initial population counted in leaves after soaking and washing with tap water, with ClO_2 solution 3 and 5 ppm for 10 min at room temperature (30±2°C) wre 5.4, 4.4 and 3.7 . Total aerobic microorganisms in all samples increased during the storage time at 12, 24 and 48 h. Although ClO_2 solution 3 and 5 ppm could reduce total microorganisms by 1 and 1.8 log from the initial loads but after stored leaves at room temperature for 12 h total microorganisms was no difference in numbers (5.4-5.6 log). At 24 h all samples high population at 6-7 log and off-odor also appeared (Table 3).

Table 3 Changes in bacterial numbers over time on treated lettuce with different concentration of ClO_2 solutions. Storage at 30±2 °C.

Sample	ClO_2 treatment (ppm)	Log_{10}CFU/mL			
		0 h	12 h	24 h	48 h
Lettuce	Tap water	5.40±0.23	5.39±0.56	6.43±0.28	Spoilage
	3 ppm 10 min	4.44±0.01	5.57±0.45	5.54±0.39	Spoilage
	5 ppm 10 min	3.65±0.15	5.46±0.29	5.93±0.42	Spoilage

Results were similarly in baby corn and slice cabbage, the initial total microorganisms were higher as 5.0-5.5 $\log_{10}$CFU/mL although treated them with tap water, ClO_2 solution 3 and 5 ppm in 10 min. The changes of total aerobic microorganisms in baby corn were not difference in numbers comparable to tap water and ClO_2 solution treated (table 4). Noticeably the sensory quality of the ClO_2 treated baby corn was still favorable after following storage at 30±2°C for 4 days. On the other hands in sliced cabbage, the quality accepted within a day at 30±2°C either in ClO_2 treated samples and tap water treated (Table 4).

Table 4 Changes in bacterial numbers over time on treated baby corn with different concentration of ClO_2 solutions. Storage at 30±2 °C.

Sample	ClO_2 treatment (ppm)	Log_{10}CFU/mL			
		0d	2d	4d	6d
Bagy corn	Tap water	5.30±0.55	6.18±0.03	6.73±0.39	Spoilage
	3 ppm 10 min	5.09±0.73	5.88±0.03	6.50±0.31	Spoilage
	5 ppm 10 min	5.15±0.42	6.15±0.40	7.07±0.98	Spoilage
Sliced cabbage	Tap water	5.27±1.04	Spoilage	Spoilage	Spoilage
	3 ppm 10 min	5.17±0.58	Spoilage	Spoilage	Spoilage
	5 ppm 10 min	5.22±1.09	Spoilage	Spoilage	Spoilage

Low temperature as 4±1 °C and 10±1 C ClO_2 could extend shelf-life all treated vegetables up to 8 days. The total aerobic microorganisms slowly increased and likely similar in number (Table 5 and 6). The total aerobic microorganisms in ClO_2 treatedlettuce, baby corn and sliced cabbage were ranged 4.5-5.1, 4.5-6.7 and 5.7-6.9 log similarly to tap water treated (4.5-6.6, 4.9-7.3 and 6.9-8.4 log) at 6 days. Since low temperature could delay the growth rate of the spoilage bacteria therefore

the numbers of total aerobic microorganisms at low temperature was gradually increased compare to those stored at room temperature which rapidly increased.

Table 5 Change in bacterial numbers over time on treated vegetables with different concentration of ClO_2 solutions. Storage at 4±1 °C.

Sample	ClO_2 treatment (ppm)	Log_{10}CFU/mL			
		0d	2d	6d	8d
Lettuce	Tap water	5.40±0.23	5.77±0.81	4.53±0.01	6.42±0.44
	3 ppm 10 min	4.44±0.01	4.86±0.78	4.49±0.06	5.90±0.05
	5 ppm 10 min	3.65±0.15	4.70±0.81	4.53±0.18	5.87±0.09
Baby corn	Tap water	5.30±0.55	4.95±0.33	4.93±0.03	6.30±0.72
	3 ppm 10 min	5.09±0.73	4.67±0.15	4.48±0.03	6.43±0.66
	5 ppm 10 min	5.15±0.42	3.90±0.15	4.57±0.22	6.26±0.85
Sliced cabbage	Tap water	5.27±1.04	4.37±0.24	8.41±0.29	5.20±1.01
	3 ppm 10 min	5.17±0.58	4.20±0.29	6.98±0.08	6.24±0.64
	5 ppm 10 min	5.22±1.09	5.55±0.89	6.94±0.02	5.05±0.83

Conclusion

Results demonstrated that ClO_2 in step of washing and soaking fresh produce was very effective in eliminating pathogenic bacteria on produce. Significantly, type of vegetables, exposure time and concentration are influenced on efficiency of ClO_2. Recommended that leafly vegetables should soak and wash longer comparable to baby corn due to the higher surface area. The appropriate concentration of ClO_2 solution at 3 ppm for 10 min could completely eliminate *L. monocytogenes* and reduced total microorganisms loads and therefore, extend the shelf-life. ClO_2 solution may be considered as the sanitizers of choice to use as a chlorine alternative in food process.

Table 6 Change in bacterial numbers over time on treated vegetables with different concentration of ClO_2 solutions. Storage at 10±1 °C.

Sample	ClO_2 treatment (ppm)	Log_{10}CFU/mL			
		0d	2d	6d	8d
Lettuce	Tap water	5.40±0.23	4.76±0.07	4.06±1.72	6.62±0.60
	3 ppm 10 min	4.44±0.01	4.46±0.98	5.14±0.56	5.56±0.78
	5 ppm 10 min	3.65±0.15	3.64±0.15	5.04±0.59	5.64±0.42
Baby corn	Tap water	5.30±0.55	4.62±0.54	7.36±0.13	6.73±0.54
	3 ppm 10 min	5.09±0.73	4.70±0.14	6.20±0.29	6.44±1.40
	5 ppm 10 min	5.15±0.42	3.95±0.13	6.76±0.02	6.16±1.44
Sliced cabbage	Tap water	5.27±1.04	4.19±0.32	6.90±0.05	6.25±1.19
	3 ppm 10 min	5.17±0.58	3.94±0.49	5.84±0.08	6.33±1.16
	5 ppm 10 min	5.22±1.09	4.21±0.65	5.75±0.01	5.98±1.45

คำขอบคุณ

งานวิจัยนี้ได้รับทุนสนับสนุนจาก โครงการพัฒนาบัณฑิตศึกษาและวิจัยเทคโนโลยีหลังการเก็บเกี่ยว

References

Beuchat, L.R. 1999. Pathogenic microorganisms associated with fresh produce. J. Food Prot. 59(2): 204-216.

Cherry, J.P. 1999. Improving the safety offresh produce with antimicrobials. Food Technol. 53 (11): 54-57, 59.

Kim, J., T.-S. Huang, M.R. Marshall and C.-I. Wei. 1999. Chlorine dioxide treatment of seafood to reduce bacterial loads. J. Food Sci. 64(6): 1089-1093.

Lovette, J. 1985. Isolation and identification of *Listeria monocytogenes* in ready-to-eat seafood products. J. Assoc. Off. Anal. Chem. 71: 658-660.

Mazzotta, A.S. 2001. Heat resistant of *Listeria monocytogenes* in vegetables: evaluation of blanching processes. J. Food Prot. 64(3): 385-387.

Pao, S., C.L. Davis and M.E. Parish. 2001. Microscopic observation and processing validation of fruit sanitizing treatments for the enhanced microbiological safety of fresh orange juice. J. Food Prot. 64(3): 310-314.

Soriano, J.M., H. Rico, J.C. Molto and J. Manes. 2001. *Listeria* species in raw and ready-to-eat foods from restaurants. J. Food Prof. 64(4): 551-553.