THE EFFECTS OF ACTIVATED CHLORINE DIOXIDE (ClO_2) ON SELECTED BACTERIAL PURE CULTURES

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Abstract. A series of experiments were done to determine the effects of activated chlorine dioxide (ClO_2) on selected bacterial pure cultures related with food-borne diseases and those that serve as indicators of proper hygiene and handling in seafood, particularly black tiger shrimps. These bacterial cultures were *Escherichia coli* (ATCC 11775), *Salmonella typhimurium* (ATCC 14028), *Staphylococcus aureus* (ATCC 25923), as well as three *Vibrios: V. parahaemolyticus* (AQ3815, Kyoto University, Japan), *V. cholerae* and *V. vulnificus* taken from the Universiti Kebangsaan Malaysia's pure culture collection. Pure cultures were exposed to five ClO_2 concentrations (20, 40, 60, 80, and 100 parts per million (ppm)) for 15, 30 and 45 min. Zero ppm ClO_2 at 0 min exposure served as the control. Activated ClO_2 was found successful in reducing bacterial population. At 20 ppm, the halophilic *V. parahaemolyticus* and *V. vulnificus* were eliminated, while *V. cholerae*, was eliminated only at 60 ppm. *E. coli* and *S. typhimurium* were totally eliminated after 30 min of exposure with 100 ppm activated ClO_2 while *V. cholerae* was eliminated after 30 min at 20 and 40 ppm ClO_2. Further studies will involve spiking bacterial cultures into tiger shrimps and other sea foods to see whether ClO_2 can exhibit the same reducing action on bacterial population in a food matrix.

Abstrak. Suatu siri kajian telah dijalankan bagi menentukan kesan klorin dioksida teraktif (ClO_2) terhadap kultur bakteria terpilih yang berkaitan dengan penyakit bawaan makanan serta bertindak sebagai penunjuk kebersihan semasa mengendalikan makanan laut, terutamanya udang harimau. Kulture bakteria tersebut adalah *Escherichia coli* (ATCC 11775), *Salmonella typhimurium* (ATCC 14028), *Staphylococcus aureus* (ATCC 25923), serta tiga sepsis *Vibrios: V. parahaemolyticus* (AQ3815, Kyoto University, Japan), *V. cholerae* and *V. vulnificus* yang diambil daripada kultur simpanan Universiti Kebangsaan Malaysia. Kultur tulin tersebut telah didedahkan kepada lima kepekatan ClO_2 (20, 40, 60, 80, dan 100 bahagian per juta (ppm)) selama 15, 30 dan 45 min. Sifar ppm ClO_2 pada pendedahan 0 min bertindak sebagai kawalan. ClO_2 teraktif didapati berjaya mengurangkan populasi bakteria tersebut. Pada 20 ppm, *V. parahaemolyticus* dan *V. vulnificus* yang halofilik telah dimusnahkan, manakala *V. cholerae*, telah dimusnahkan hanya pada 60 ppm. *E. coli* dan *S. typhimurium* dimusnahkan selepas 30 min pendedahan dengan 100 ppm ClO_2 teraktif, manakala *V. cholerae* dimusnahkan selepas 30 min pendedahan pada 20 dan 40 ppm ClO_2. Kajian selanjutnya akan melibatkan penginokulatan kultur-kultur bakteria ke dalam udang harimau bagi menentukan samada ClO_2 berupaya memberi tindakan pengurangan terhadap populasi bakteria di dalam suatu matrik makanan.

Keywords: Chlorine dioxide; *E.coli; S. typhimurium; S. aureus; V. cholerae; V. parahaemolyticus; V. vulnificus*

Introduction

The risk of foodborne illness has increased markedly over the last 20 years, with nearly a quarter of the population at higher risk for illness today. Consequently, preventing foodborne illness and death remains a major public health challenge [1].

Escherichia coli and *Salmonella typhimurium* are classified as organisms causing food-borne infections while *Staphylococcus aureus* is considered one that causes food-borne intoxication [2]. Roberts and Greenwood [2] further state that *E. coli* is a microorganism that can be used as a marker to demonstrate that faecal pollution may have occurred at some stage during the production of food. The virulence of *S. typhimurium* grown in different foods varies. The possibility exists, therefore, that ingestion of foods containing the same number of salmonellae may not constitute a similar health hazard [3].

Foods that require considerable handling during preparation and that are kept at slightly elevated temperatures after preparation are frequently involved in staphylococcal food poisoning [4]. This microorganism is frequently introduced into food by food handlers and indirectly by equipment [2] and can therefore be considered indicative of proper food handling and hygiene.

Strains of the genus *Vibrio* are ubiquitous in the estuarine environment and as might be expected, given their environmental distribution, vibrios are a frequent isolate from seafood [5]. Only a few have been found to be responsible for food-borne infection, and of these, *V. cholerae, V. parahaemolyticus,* and *V. vulnificus* occur most frequently [6].

Chlorine dioxide (ClO_2), is a chemical compound which has been discovered to possess great potential in reducing microbial load. Junli et al. [7] confirms that the killing effect of ClO_2 on bacteria is similar to or better than that of liquid chlorine, at a relatively wider pH range, making it an excellent disinfectant substitute. Chlorine dioxide is registered with the Environmental Protection Agency (EPA Registration No.74986-1) [8], and is considered an excellent bactericide, fungicide and antimicrobial agent. The EPA also confirms that a post oxidant dose of 0.2-1 mg/l and a residual of 0.1-0.5 mg/l of ClO_2 effectively inactivate pathogenic viruses, bacteria and protozoa. The use of chlorine dioxide on food has been approved by the Food and Drug Administration [9].

Information on the utilization of ClO_2 as a bacteriocide in seafood products is limited [10]. This study hopes to provide technical data that may, in the future, be useful as basis for the use of ClO_2 on different food and food systems. The objective of this study was to determine the effectivity of different ClO_2 concentrations at different exposure times in reducing pure culture populations of the above mentioned pathogens.

Experimental

Preparation of Working Cultures

Working cultures of *E. coli*, *S. typhimurium*, *S. aureus*, and *V. cholerae* (Vc) were prepared by streaking a loopful onto Nutient Agar (Oxoid). Working cultures of *V. parahaemolyticus* and *V. vulnificus* were prepared by streaking a loopful onto NA with 3% NaCl (NA+3% S) content. All NA and NA+3% S plates were then incubated overnight at 37^{o}C.

After incubation, individual colonies of different cultures were inoculated into Tryptic Soya Broth (TSB). *V. parahaemolyticus* and *V. cholerae* were inoculated into TSB added with 3% salt (TSB+3% S). TSB and TSB+3% S were then incubated overnight at 37^{o}C.

Preparation and Activation of Chlorine Dioxide

Chlorine dioxide (Aqua 5) was obtained from Mitrol Technology Pte Ltd. The liquid stock solution had a 50,000 ppm concentration. Chlorine dioxide was prepared in glass bottles in an open area. The needed concentrations of ClO_2 were prepared based on the following equation:

$$c1v1=c2v2,$$

where,

c1 = Concentration of stock solution (Aqua 5) = 50,000 ppm
v1 = Volume ClO_2 needed to be prepared
c2 = Target concentration of Stabilized ClO_2 (20, 40, 60, 80, 100 ppm)
v2 = Target volume of distilled water

Chlorine dioxide was activated using food-grade citric acid. The amount of citric acid used was 10% of the amount of stock solution needed to prepare the different concentrations of ClO_2. A sample computation of both preparation and activation of ClO_2 is provided below:

E.g. To prepare 100 ml of 100 ppm ClO_2
v1 = c2v2/c1,
v1 = (100 ppm)(100 ml)/ 50,000 ppm
v1 = 0.2 ml ClO_2 needed
To activate, we get 10% of the amount of needed ClO_2:
(0.2 ml)(0.1) = 0.02 g of citric acid for activation
Therefore, 0.02 g of citric acid is to be added to 0.2 ml ClO_2 in order to prepare 100 ml of 100 ppm ClO_2 solution.

Citric acid was added to the ClO_2 and left to activate for 10-15 minutes or until a yellow color developed and gas evolved. The glass container was not covered to allow the escape of gas, a result indicative of the completion of the chemical reaction. The appropriate amount of water was then added. Preparation of ClO_2 concentrations was done right before application.

Application of Chlorine Dioxide

Prepared bacterial cultures inoculated in 10 ml portions of TSB and TSB+3% S were transferred aseptically into individual 15 ml-capacity centrifuge tubes and centrifuged at 3600 rpm at 27°C for 15 min to obtain cell pellets with clear supernatant. The supernatant was then decanted and, in place, 10 ml of freshly prepared activated ClO_2 was poured into the centrifuge tube, capped tightly, vortexed to allow dispersal of the cultures from its concentrated state, and kept for the desired amount of time.

Enumeration of Bacteria

After exposure to ClO_2 at different times, the surface-colony drop-plate method [2] on Plate Count Agar (PCA) was used for counting *E. coli*, *S. typhimurium*, and *S. aureus*. Plate Count Agar is a non-selective medium used for general viable counts of bacteria in food [11]. *Vibrio cholerae*, *V. parahaemolytius*, and *V. vulnificus* were enumerated using the spread plate method [12]. For *V. cholerae*, enumeration was done on thiosulfate-citrate bile salts sucrose agar (TCBS) while *V. parahaemolyticus* and *V. vulnificus* were enumerated on TCBS+3% S because of their halophilic nature. TCBS agar is a selective isolation medium for pathogenic vibrios [13]. The selective agents in the media are Sodium thiosulphate, sodium taurocholate and ferric citrate [6], making it suitable for growth of *V. cholerae*, *V. parahaemolyticus* and other vibrios [14]. A control sample (0 ppm or 0 min) was employed. All experiments were done in duplicate.

Statistical Analysis

The statistical analysis, one-way Analysis of Variance (ANOVA), with Turkey's family error rate comparison, was done using the MINITAB Release 12.1.

Results And Discussion

The Effect of Different ClO_2 Concentrations

Figure 1 shows the effects of various activated ClO_2 concentrations when applied to bacterial pure cultures for 15 min. A decreasing trend of colony counts as ClO_2 concentrations increase was seen. The effectivity of most ClO_2 concentrations, as per bacterial culture, significantly differs from one another (see Table 1). At 20 ppm, the halophilic *V. parahaemolyticus* and *V. vulnificus* were eliminated, while *V. cholerae*, was eliminated only at 60 ppm.

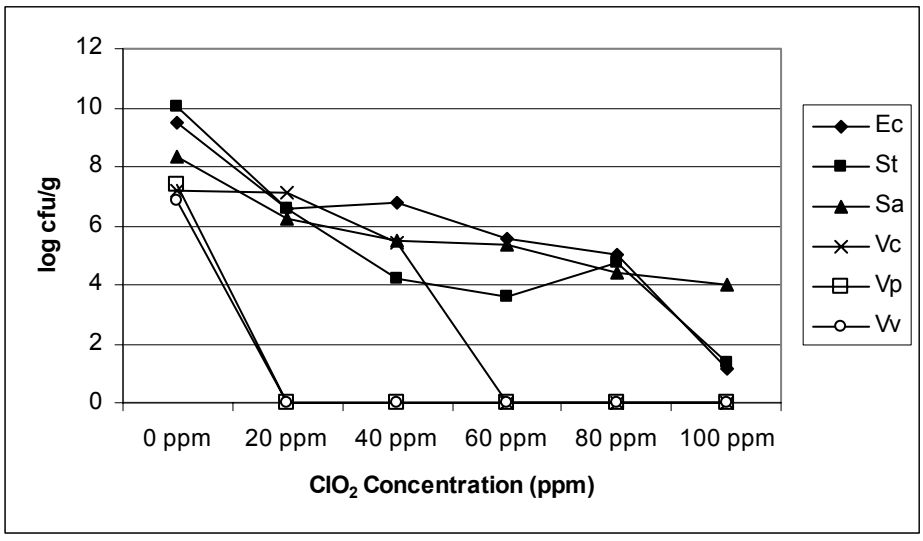

FIGURE 1. The Effect of ClO_2 on Bacterial Pure Cultures after 15 Min Exposure Time

The ability of ClO_2 to kill bacteria was studied by Junli et al. [7], who after finding similar results, stated that the bactericidal effect of ClO_2 is due to its 100 % existence in molecular form when dissolved in water. Further, Junli's study indicated that because of this molecular state, ClO_2 easily permeated through cell membranes and into the bacterial body, thus exhibiting its bactericidal effect. *E. coli*, *S. typhimurium*, and *S. aureus* were not eliminated even with treatments of up to 100 ppm at 15 min exposure time.

TABLE 1. Effects of ClO_2 on Log Reduction of Bacterial Pure Cultures at 15 Min Exposure Time

Pure	Log cfu/g					
Culture	0 ppm	20 ppm	40 ppm	60 ppm	80 ppm	100 ppm
Ec	9.52*[a]	6.59[b]	6.75[c]	5.59[d]	5.01[e]	1.19[f]
St	10.06[a]	5.58[b]	4.20[c]	3.62[d]	4.78[e]	1.37[f]
Sa	8.36[a]	6.22[b]	5.46[bc]	5.36[c]	4.43[d]	4.01[e]
Vc	7.19[a]	7.10[a]	5.39[b]	0.00[c]	0.00[c]	0.00[c]
Vp	7.37[a]	0.00[b]	0.00[b]	0.00[b]	0.00[b]	0.00[b]
Vv	6.83[a]	0.00[b]	0.00[b]	0.00[b]	0.00[b]	0.00[b]

*Results are the mean scores of 2 replications
[a-f] Means with two different lowercase letter superscripts for rows are significantly different ($p<0.05$)

The Effect of Extended Exposure Times

Figure 2 illustrates the result of extended exposure time (30 and 45 min) on colonies of bacterial pure cultures. *E. coli* and *S. typhimurium* were totally eliminated after 30 min of exposure with 100 ppm activated ClO_2 while *V. cholerae* was eliminated after 30 min at 20 and 40 ppm ClO_2. *S. aureus* was not eliminated even after 30 min of exposure with activated 100 ppm ClO_2. This may be due to the structure of *S. aureus*; it is gram-positive and forms a cross, three-dimensional spatial network structure of large mechanical strength in four-peptide side chain which is difficultly contacted by disinfectants, making it stronger than gram-negative bacteria [7]. *S. aureus* was totally eliminated after 45 min exposure with 100 ppm activated ClO_2.

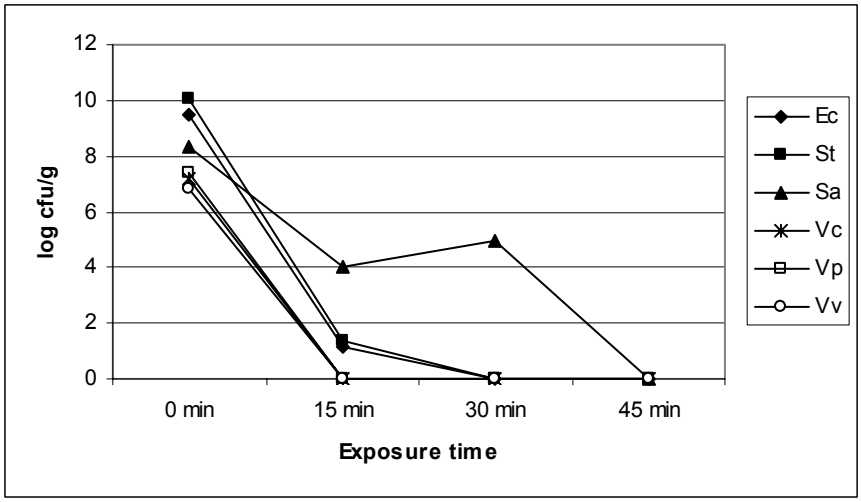

FIGURE 2. The Effect of Prolonged ClO_2 (100 ppm) Exposure Time on Bacterial Pure Cultures

Table 2 tabulates the effects of prolonged ClO_2-exposure on microbial population. No reduction trend was seen for the different exposure times. Although the number of microbial colonies does decrease as ClO_2-exposure time increases, not all these changes were significantly different from one another. Previous studies by Du et al. [15] on the efficacy of ClO_2 gas in reducing *E. coli* O157:H7 on apple surfaces suggested comparable results where the log reductions increase with the increase of treatment time at the 3.3-7.2 mg/l levels, as did similar studies by Han et al. [16] on green pepper surfaces. It is also noted that *S. aureus* was the microorganism least affected by prolonged ClO_2 exposure.

TABLE 2. Effects of ClO_2 at 15, 30 and 45 Min Exposure Times on Bacterial Pure Cultures

Microbes/ exposure time	Log cfu/g					
	0 ppm	20 ppm	40 ppm	60 ppm	80 ppm	100 ppm
E. coli						
15 min	9.52*^{Aa}	6.59^{Ab}	6.75^{Ac}	5.59^{Ad}	5.01^{Ae}	1.19^{Af}
30 min	9.52^{Aa}	6.63^{Ab}	5.77^{Bc}	5.72^{Ac}	0.49^{Ad}	0.00^{Be}
45 min	9.52 ^{Aa}	6.49^{Ab}	5.65^{Bc}	2.47^{Bd}	1.38^{Ae}	0.00^{Bf}
S. typhimurium						
15 min	10.06^{Aa}	6.58^{Ab}	4.20^{Ac}	3.62^{Ad}	4.78^{Ae}	1.37^{Af}
30 min	10.06^{Aa}	5.45^{Bab}	5.42^{Bab}	1.14^{Bb}	1.44^{Bb}	0.00^{Bc}
45 min	10.06^{Aa}	6.35^{Ab}	2.27 ^{Cc}	1.11^{Bd}	0.00^{Ce}	0.00^{Be}
S. aureus						
15 min	8.36 ^{Aa}	6.22^{Ab}	5.46^{Abc}	5.36^{Ac}	4.43^{Ad}	4.01^{Ae}
30 min	8.36 ^{Aa}	5.53^{Abc}	4.31^{Bbc}	4.83^{Abc}	4.20^{Ac}	2.94^{Ad}
45 min	8.36 ^{Aa}	4.65^{Ab}	3.87^{Cb}	3.77^{Ab}	3.68^{Ab}	0.00^{Ac}
V. cholerae						
15 min	7.19^{Aa}	7.10^{Aa}	5.39^{Ab}	0.00^{Ac}	0.00^{Ac}	0.00^{Ac}
30 min	7.19^{Aa}	0.00 ^{Bb}	0.00^{Bb}	0.00^{Ab}	0.00^{Ab}	0.00^{Ab}
45 min	7.19^{Aa}	0.00 ^{Bb}	0.00^{Bb}	0.00^{Ab}	0.00^{Ab}	0.00^{Ab}
V. parahemolyticus						
15 min	7.37^{Aa}	0.00^{Ab}	0.00^{Ab}	0.00^{Ab}	0.00^{Ab}	0.00^{Ab}
30 min	7.37^{Aa}	0.00^{Ab}	0.00^{Ab}	0.00^{Ab}	0.00^{Ab}	0.00^{Ab}
45 min	7.37^{Aa}	0.00^{Ab}	0.00^{Ab}	0.00^{Ab}	0.00^{Ab}	0.00^{Ab}
V. vulnificus						
15 min	6.83^{Aa}	0.00^{Ab}	0.00^{Ab}	0.00^{Ab}	0.00^{Ab}	0.00^{Ab}
30 min	6.83^{Aa}	0.00^{Ab}	0.00^{Ab}	0.00^{Ab}	0.00^{Ab}	0.00^{Ab}
45 min	6.83^{Aa}	0.00^{Ab}	0.00^{Ab}	0.00^{Ab}	0.00^{Ab}	0.00^{Ab}

* Results are the mean scores of 2 replications.

$^{A-C}$ Means with two different uppercase letter superscripts for columns and each bacterial culture are significantly different ($p<0.05$).

$^{a-e}$ Means with two different lowercase letter superscripts for rows and each bacterial culture are significantly different ($p<0.05$).

Conclusions

From the experiment conducted, the following can be concluded:

- As ClO_2 concentration increases, microbial population decreases.
- Different ClO_2 concentrations (0, 20, 40, 60, 80, and 100 ppm) have significantly different reducing effects on bacterial pure cultures.
- Longer exposure of pure cultures to ClO_2 further reduces colony counts but the differences observed were not significantly different.
- Activated ClO_2 is effective in reducing and eliminating certain microbial pure cultures, especially in halophilic *Vibrios* even at relatively low concentrations.

In continuation of this study, the effect of activated ClO_2 in reducing and ultimately eliminating microbiological population in food is being examined. The food of interest is black tiger shrimps (*Penaeus monodon*).

Experiments are being conducted to determine whether or not activated ClO_2 would exhibit the similar microbial-reducing actions when applied to a food matrix. The success of above mentioned will provide technical information that will further support the use of ClO_2 in the food, and particularly the seafood industry as an alternative anti-microbial agent, so as to meet safety measures dictated by consumers and the world market in general.

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