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Effects of Sarawak local salts and commercial sodium chloride on biofilm formation of *Vibrio cholerae*

Elexson Nillian*, Valarie Han, Samuel Lihan and Grace Bebey

Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, 94300 Kota Samarahan, Sarawak, Malaysia.
Email: nelexson@unimas.my

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ABSTRACT

Aims: Bacterial biofilms can be defined as a community of microorganisms in which cells adhere to one another on a surface and are embedded in a protective matrix of lipids, nucleic acids, proteins and polysaccharides. Biofilm produced by *Vibrio cholerae* represents a significant threat to food safety, as they can lead to the transmission of diseases. Hence, the purpose of this study is to review the effect of different types of sodium chloride on minimum biofilm eradication concentration (MBEC) and morphology of biofilm formation of *Vibrio cholerae.*
Methodology and results: In this study, *V. cholerae* biofilm was treated with four different types of sodium chloride; 'Bario' salt, 'Bakelalan' salt, commercial sodium chloride and laboratory sodium chloride. By using MBEC test, the concentration of sodium chloride needed to eradicate the biofilm of *V. cholerae* was determined. Based on the result obtained, commercial sodium chloride and laboratory sodium chloride showed the highest anti-biofilm activity against the biofilm of *V. cholerae* at 500 mg/mL concentration while no complete eradication of *V. cholerae* biofilm was achieved when treated with Sarawak local salts ('Bario' salt and 'Bakelalan' salt). However, noticeable inhibitions of bacterial growth were seen at the highest concentration of local salts.
Conclusion, significance and impact of study: Commercial sodium chloride and laboratory sodium chloride showed a better anti-biofilm activity towards the *V. cholerae* biofilm formation as compared to the local salts. Thus, commercial sodium chloride and laboratory sodium chloride can be an effective anti-biofilm agent to mitigate the biofilm formation of *V. cholerae.* Further studies can be done to determine the MBEC values of other pathogenic bacteria against commercial and laboratory sodium chloride.

Keywords: Bacterial biofilms, sodium chloride, minimum biofilm eradication concentration (MBEC), anti-biofilm, industrial process

INTRODUCTION

Recent studies have reported that more than 99% of microorganisms in this world live in form of biofilms (Prakash *et al.*, 2013). Bacterial biofilms can be defined as communities of microorganisms, which are attached on a substratum and work together to form a protective extracellular polymer in order to survive from environment stressors. The first evidence and discovery of bacterial formation was observed by Antonie van Leeuwenhoek. He discovered the bacterial biofilm adhered on his own teeth and described it as "animalcules" (Percival *et al.*, 2011). Bacteria in form of biofilm can survive in unexpected environment stressors such as antimicrobial agents, disinfection treatment, temperature changes, pH changes, ultraviolet rays to mention a few (Costerton *et al.*, 1999). These bacterial biofilms are resistant against antimicrobial agents because they can resist the phagocytic activity and host immune mechanism (Costerton *et al*., 1999). Most bacteria are capable of forming biofilms. Therefore, bacterial biofilm has become a major concern and causes a lot of problems to food industries.

Sodium chloride can be described as ionic compound added in various foods and medicines. It is also known as common salt or table salt. Sodium chloride is often used for cooking to enhance the taste of food and to improve food texture. It also has been viewed as food preservative by inhibiting the growth of microorganisms in food that spoil the food products and reduce their shelf life (Marjorie and Kathleen, 2010).

Biofilms produced by bacteria create significant threats to food safety and public health. Bacterial biofilms contribute to cross-contamination of food products and serious hygienic problems which can lead to foodborne diseases. They also can cause economic losses to food

*Corresponding author

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industries due to food spoilage, breakdown of equipment and high maintenance of contaminated equipment (Giaouris *et al.*, 2013). Since bacterial biofilms are resistant against common disinfection treatments and antimicrobial agents, food industries need to invest a lot of money in order to find an effective way for biofilms removal. Giaouris *et al.* (2013) stated that various approaches had been used to control the biofilms formation in food industries, however it raises a concern over the effectiveness of these approaches and their safety towards consumers and environment. Thus, more studies should be done in order to find effective approaches for biofilms removal which could cause foodborne illness.

Sodium chloride can affect the biofilms formation of foodborne pathogens (Lee *et al.*, 2013). Hence, the purpose of this study is to review the effect of different types of sodium chloride on minimum biofilm eradication concentration (MBEC) and morphology of biofilm formation of *V. cholerae.*

MATERIALS AND METHODS

Sample preparation

Four different types of salt used in this study are 'Bario' salt, 'Bakelalan' salt, laboratory sodium chloride and commercial sodium chloride. 'Bakelalan' salt was collected from local market of Lawas, Sarawak while 'Bario' salt was collected from Bario, Sarawak in September 2016. Commercial sodium chloride and laboratory sodium chloride (NaCl) were obtained from UNIMAS Microbiology laboratory.

Bacteria strain and morphology confirmation

A total of nine strains of *V. cholerae* were used in this study. These strains of *V. cholerae* were isolated from different sources such as contaminated water, sewage and from clinical sample of patient (Sarawak General Hospital). The isolated *V. cholerae* were further confirmed based on the visible growth of the bacteria on the selective agar, Thiosulfate Citrate Bile Salt Sucrose Agar (TCBS) and CHROMAgar Vibrio. Colony of *V. cholerae* was picked using sterile swabs and transferred into tubes that contained 1 mL of tryptone soy broth (TSB). Further adjustment was done to ensure the turbidity was corresponding to 10^5 CFU (colony forming unit) turbidity of McFarland standard No. 5. In order to ensure the final concentrations of cells in each well (96-well, U-shaped microtiter plate) was standardized to 10^5 CFU, a serial dilution was done (Lee *et al.*, 2013).

Minimum biofilm eradication concentration (MBEC)

To form biofilm layers, about 100 µL of isolated *V. cholerae* were pipetted into the each well of 96-well, U-shaped microtiter plate. Negative control containing only fresh broth was pipetted into column 1, while positive control containing only *V. cholerae* inoculum was pipetted into column 2. The biofilm formations of *V. cholerae* were compared with these two controls. The plate was then sealed with parafilm and incubated without agitation for 24 h at 37 °C. After discarding the overnight medium, about 100 µL of fresh TSB were filled into each wells of 96-well microtiter plate.

Then, sodium chloride was added into the wells in two-fold dilutions serially from 500 mg/mL to 0.15625 mg/mL. The plate was then incubated for 24 h at 37 °C. After incubation, the medium was discarded. To remove the non-adherent cells, the biofilm was washed with sterile distilled water three times. The plate was inverted and left for 30 min in laminar flow to air-dry. Minimum biofilm eradication concentration (MBEC) was proceeded by scratching the biofilm formed at the bottom of wells using a metal loop, and then streaked over the surface of TSA. The MBEC values were determined by observing the growth of *V. cholerae* on the streaked plate. The $MBEC_{50}$ value defined as the 50% eradication of biofilm formation were achieved while $MBEC_{90}$ defined as 90% eradication of biofilm formation were achieved.

Morphological analysis of cells via scanning electron microscopy (SEM)

The change in morphology of *V. cholerae* was observed after treated with several concentration of sodium chloride using the scanning electron microscopy (SEM). The *V. cholerae* biofilm was formed at the bottom of the microtiter plate and the bacterial pellet was collected after 8 h of incubation at 37 °C. The bacterial pellet was fixed in solution of 2.5% of glutaraldehyde at 4 °C, overnight. After fixation, bacterial pellet was washed with PBS and left to dry in 15 min. Then, the pellet was rehydrated using absolute ethanol and left for 15 min of drying. The dried biofilm samples were subsequently undergone critical point drying method. The sample was coated with gold using sputter coating system. The morphology of *V. cholerae* was then observed using scanning electron microscopy (SEM).

RESULTS

Minimum biofilm eradication concentration (MBEC)

In this study, the minimum biofilm eradication concentration (MBEC) of nine different strains of *V. cholerae* was tested on four different types of salt. Four different types of salt used in this study are 'Bario' salt, 'Bakelalan' salt, laboratory sodium chloride and commercial sodium chloride.

Based on the result obtained (Table 1), no complete eradication of *V. cholerae* biofilm was achieved when treated with 'Bario' salt and 'Bakelalan' salt. The visible growth of *V. cholerae* biofilm was slightly reduced by treatment using concentration ranging from 125 mg/mL to 500 mg/mL. At 50 mg/mL and 5 mg/mL of 'Bario' and 'Bakelalan' salt, visible growth of biofilm was observed which indicated that both salts were not effective as an anti-biofilm agent because no eradication was achieved.

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Table 1: Summary of minimum bactericidal eradication concentration (MBEC) (mg/mL) of nine different strains of *V. cholerae,* treated with four different types of salt.

Strain no.	Age of patients	Source of *V. Cholerae*	MBEC (mg/mL) value of *V. Cholerae* treated with sodium chloride			
			Bario salt	Bakelalan salt	Commercial NaCl	Laboratory NaCl
NA31	21	Rectal swab	No eradication	No eradication	500	500
NA25	75	Rectal swab	No eradication	No eradication	500	500
NA6	24	Rectal swab	No eradication	No eradication	500	500
NA7	34	Rectal swab	No eradication	No eradication	500	500
THUR3	24	Stool swab	No eradication	No eradication	500	500
MON18	33	Stool swab	No eradication	No eradication	500	500
MS001	-	Moore swab	No eradication	No eradication	500	500
MS002	-	Moore swab	No eradication	No eradication	500	500
SA001	-	Water sample	No eradication	No eradication	500	500

However, *V. cholerae* biofilm eradication was achieved after being treated with 500 mg/mL concentration of commercial salt and laboratory sodium chloride respectively. From Figure 1, no visible growth of bacteria was observed at 500 mg/mL concentration of commercial salt and laboratory sodium chloride which indicated that the *V. cholerae* biofilm was completely eradicated. Besides that, the growth of bacteria was reduced at 125 mg/mL concentration of commercial salt and 250 mg/mL laboratory sodium chloride, respectively. There was no complete eradication of *V. cholerae* seen at 5 mg/mL and 50 mg/mL of both commercial and laboratory NaCl. However, it was clearly observed that the growth of bacteria was reduced by half when treated with 50 mg/mL (Figure 2) and 25 mg/mL concentration of both commercial salt and laboratory sodium chloride. About 50% of biofilm formation ($MBEC_{50}$) of *V. cholerae* was eradicated from 125 mg/mL until 250 mg/mL concentration (Figure 1) of commercial salt and laboratory sodium chloride, respectively. Meanwhile, eradication at 90% of biofilm formation ($MBEC_{90}$) was achieved by using 500 mg/mL of both commercial salt and laboratory sodium chloride.

Figure 3 showed the biofilm formation of *V. cholerae* after it had been treated with laboratory sodium chloride. Noticeable inhibition of biofilm formation of *V. cholerae* was observed from microtiter plate well (k) to well (a) which contained untreated *V. cholerae.*

Morphological analysis of cell via scanning electron microscopy (SEM)

The change in morphology of *V. cholerae* was observed using scanning electron microscopy (SEM). The results were obtained by comparing the morphology of untreated *V. cholerae* and their morphology after been treated with laboratory sodium chloride. In Figure 4 (A), no bacteria cell was observed as it is the negative control (fresh broth) in this study. Figure 4 (B) showing the rod-shaped structure of bacteria cells, which was the positive control in this study (untreated *V. cholerae*). The morphology of *V. cholerae* has changed in Figure 4 (C) as they lost the original shape after being treated with 500 mg/mL concentration of laboratory sodium chloride. Based on the observation, the bacteria cells of *V. cholerae* was ruptured as it clearly showed the irregular cell wall structure at Figure 4 (C). The morphology of *V. cholerae* in Figure 4 (D and E) was still unchanged even after it had been treated with 5 mg/mL and 50 mg/mL concentration of laboratory sodium chloride.

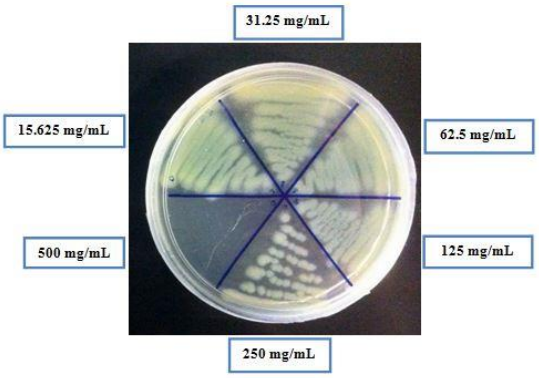

Figure 1: Representative of minimum biofilm eradication concentration (MBEC) of *V. cholerae* after treated by 500 mg/mL of laboratory sodium chloride (NaCl).

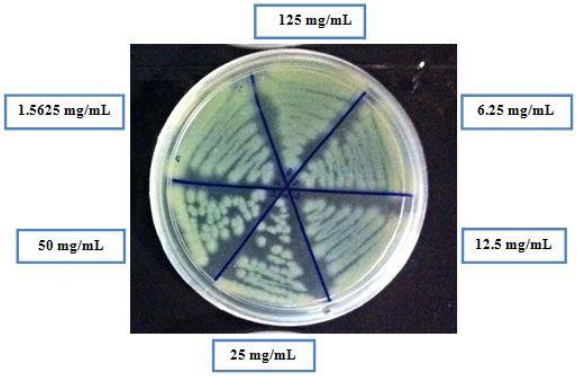

Figure 2: Representative of minimum biofilm eradication concentration (MBEC) of *V. cholerae* after treated by 50 mg/mL of laboratory sodium chloride (NaCl).

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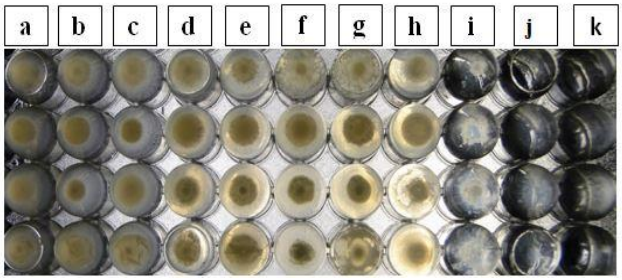

Figure 3: Representative of biofilm formation of four strains of *V. cholerae* at the bottom of the treated wells using laboratory sodium chloride. Well (k), 500 mg/mL, well (j), 250 mg/mL, well (i), 125 mg/mL, well (h), 6.25 mg/mL, well (g), 31.25 mg/mL, well (f), 15.625 mg/mL, well (e), 7.813 mg/mL, well (d), 3.906 mg/mL, well (c), 1.953 mg/mL, well (b), 0.977 mg/mL, well (a), untreated *Vibrio cholerae* (positive control).

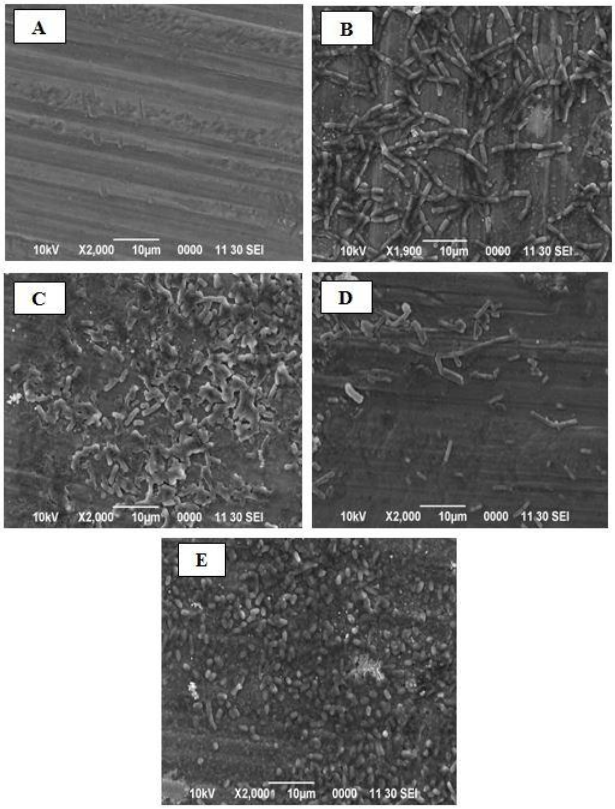

Figure 4: Scanning electron microscopy (SEM). The changes of morphology of *V. cholerae was* observed under SEM after been treated with different concentration of laboratory sodium chloride; (A) Negative control; Fresh broth, (B) Positive control; Untreated *Vibrio cholerae,* (C) treated with 500 mg/mL, (D) treated with 50 mg/mL, (E) treated with 5 mg/mL.

DISCUSSION

The ability of bacteria to form biofilm contributes to serious hygienic problems, cross contamination of food product and lead to transmission of diseases (Giaouris *et al.*, 2013). Having more anti-biofilm agent alternatives is vital to alleviate this issue. Hence, the present study focuses on using sodium chloride of different types and of varying concentration to understand its effect on *V. cholerae* biofilm formation by observation under scanning electron microscope (SEM). Minimum biofilm eradication concentration (MBEC) carried out in this research was for the treatment dose that is suitable to disrupt the biofilm formation of *V. cholerae*.

Based on the result obtained, no complete eradication of *V. cholerae* biofilm was achieved when treated with 'Bario' salt and 'Bakelalan' salt. At 500 mg/mL concentration of 'Bario' salt and 'Bakelalan' salt, the *V. cholerae* biofilm was reduced from concentration 125 mg/mL to 500 mg/mL for both salts. The visible growth of *V. cholerae* colonies on the streaked plate indicated that 'Bario' salt and 'Bakelalan' salt was not an effective anti-biofilm agent as no eradication was achieved. Since these two salts are made by local Kelabit people using traditional method, these two salts may not be undergoing a proper industrial process during packaging (without sterilization) or unhygienic packaging environment.

During the salt packaging and processing, there are high possibilities of some other factors contaminated or attached to the surface of equipment which lead to accumulation of biofilm layers. Based on previous study, if there was more than one species of bacteria form a biofilm layers together, it will alter the gene expression, bacterial behaviour and pathogenicity of the *V. cholerae* (Annous *et al.*, 2009). Thus, the formation of *V. cholerae* biofilm becomes more resistant towards local salts. Even though *V. cholerae* has been treated with the highest concentration of these two salts, no complete biofilm eradication was achieved. It indicated the strength of the *V. cholerae* biofilm is strong and a higher concentration of local salts is needed to kill or inhibit the biofilm formation. According to Kovac *et al.* (2013), natural salts was said to have a high natural minerals content such as natural iron, potassium, and magnesium and more salty than commercial sodium chloride. In addition, there were no additive was added into the natural salts.

On the other hand, *V. cholerae* biofilm eradication was achieved as the biofilm was completely eliminated after been treated with 500 mg/mL concentration of commercial sodium chloride and laboratory sodium chloride while at 125 mg/mL and 250 mg/mL concentration, the growth of bacteria was reduced. About 50% of biofilm formation ($MBEC_{50}$) of *V. cholerae* was able to eradicate from range of 125 mg/mL and 250 mg/mL concentration of commercial sodium chloride and laboratory sodium chloride while 90% of biofilm formation ($MBEC_{90}$) was eradicated with 500 mg/mL concentration. This is because these two salts have undergone the proper industrial process before it been commercialized.

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If compared to the Sarawak local salts, commercial sodium chloride and laboratory sodium chloride has undergone multiple process of evaporation to purify the salt to a higher level and several treatments to remove any of insoluble impurities. Besides that, some other compounds such as potassium iodide, magnesium carbonate, calcium silicate, calcium phosphate and calcium carbonate are added to make the salt become free-flowing. As mentioned by Dennis (2010), about 0.01% potassium iodide was added into commercial sodium chloride as additive which is needed for oxidation processes in our body.

Based on the result obtained, it illustrated that nine different isolated of *V. cholerae* give the same value of minimum bactericidal eradication concentration (MBEC) of 500 mg/mL after it been treated with commercial salt and laboratory sodium chloride.

Fu *et al.* (2014) stated that *V. cholerae* have a high salt tolerance and it can survive in a condition of high pH. Sodium chloride can act as additional nutrients for the bacteria; about 0.5% to 2% concentration of sodium chloride is needed for optimum growth. He also claimed that the growth of *V. cholerae* will stop in range of 6% to 7% concentration of sodium chloride. This is because sodium chloride promotes the rate of bacterial attachment, hydrophobicity and virulence of bacterial biofilms. *V. cholerae* change their surface properties from smooth to rough and causing the increase of hydrophobicity. Therefore, these changes will lead initial bacterial adhesion, colony establishment and biofilm formation (Dickson *et al.*, 1992). Sodium chloride also can increase the resistance of pathogen towards heat and acid due to changes of gene expression of pathogens. Thus, to mitigate the biofilm formation, *V. cholerae* need to be exposed to high concentration of sodium chloride. Caly *et al.* (2009) claimed that biofilm formation will be inhibited due to repressed of flagellum expressions, which affected the adhesion capability of *V. cholerae.*

In this study, the results obtained indicated that commercial sodium chloride and laboratory sodium chloride can inhibit or mitigate the biofilm formation of *V. cholerae* from cholera patients.

The change in morphology of *V. cholerae* was observed using the scanning electron microscopy (SEM). The morphology of *V. cholerae* was still unchanged even though it has been treated with 5 mg/mL and 50 mg/mL concentration of sodium chloride. Based on the observation, changes can be seen on the morphology of *V. cholerae* after treatment with 500 mg/mL concentration of sodium chloride as they lost their original shape. The bacteria cells of *V. cholerae* was ruptured as it clearly showed the irregular cell wall structure. This is because the *V. cholerae* cell experienced osmotic shock which result in loss of water from cell, therefore causing cell death and inhibition of the bacteria growth. Moreover, *V. cholerae* have a thin peptidoglycan layer. Therefore, when it is exposed to a high concentration of sodium chloride, the bacterial shape undergone a rapid alteration. Based on previous studies, exposure of high concentration of sodium chloride caused flagellum expressions to repress, therefore it affected the adhesion capability of *V. cholerae* (Caly *et al*., 2009).

CONCLUSION

There is a link between different salt types and salt concentration used to the morphology of *V. cholera* biofilm. These findings suggest that commercial sodium chloride and laboratory sodium chloride exhibit a better anti-biofilm activity towards the biofilm formation of *V. cholerae* as compared to Sarawak local salts; 'Bario' salt and 'Bakelalan' salt. Therefore, both salts (commercial sodium chloride and laboratory sodium chloride) can be potential anti-biofilm agents to mitigate the biofilm formation of *V. cholerae.* It is recommended that further studies to be done to determine the MBEC values of other pathogenic bacteria against commercial sodium chloride and laboratory sodium chloride.

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