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FULL PAPER

Effect of surface roughness on susceptibility of *Escherichia coli* biofilm to benzalkonium chloride

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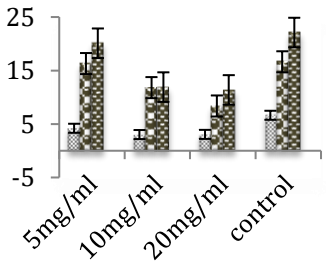
Graphical abstract



Abstract

The inherent natural tendency of bacteria to adhere and form biofilm on both biotic and abiotic surfaces and the consequential resistance to antimicrobial treatments remained a major concerned to humanity. The surface roughness of the sand blasted and cleaned stainless steel (type 304) substrates were measured using 3D measuring laser microscope before biofilm were developed on different surface roughness under continuous nutrient supply. The effect of benzalkonium chloride (BKC) as antibacterial agent on the biofilms was investigated. A concentration of 5 mg/mL BKC exert no pronounced effect on the biofilm formed on the three surfaces as compared to the 10 mg/mL and 20 mg/mL that removed approximately 50% of the cells from the respective surfaces. Conversely, the overall effect of the three concentrations tested were significantly higher ($p \leq 0.05$) on the stainless steel coupon with the least average surface roughness of 0.38 ± 1.5 µm. These observations support the hypothesis that surface profile is one of the factors that influence biofilm susceptibility to antibacterial agents and reinforced the wide spread observation that microorganisms living as biofilm tends to be resistance to antimicrobial treatment especially at lower concentrations of 5 mg/mL.

Keywords: Biofilm, benzalkonium chloride, surface roughness

INTRODUCTION

Microbial adhesion to surfaces and consequent biofilm formation has been documented in different environments. Biofilm is a natural tendency of microorganisms to attach to wet surfaces, multiply and embedded themselves in a slimy matrix composed of extracellular polymeric substances (EPS). The ability of microorganisms to form biofilms on the surfaces of utensils and other equipment used in domestic kitchen raises the possibilities that infection may occur following the cross contamination of freshly prepared foods in domestic kitchens. Outbreak of food borne pathogens such as *Escherichia coli* and *Listeria monocytogens*, *Yersiniaenterocolitica*, *Campylobacterjejuni*, *Salmonella* spp., *Staphylococcus* spp. and storage bacteria such as *Pseudomonas* spp. enhance resistance to antibiotic or sanitizers when cells are in biofilms [1-3].

Direct microscopic examinations and quantitative recovery techniques used to study biofilms revealed that more than 99.9% of the bacteria live as biofilms on a wide range of surfaces [4]. These solid surfaces become more vulnerable to biofilm formation when they are submerged in nutrient rich liquids or in contact with water for a long period of time. In order to combat biofilms, surface disinfection is normally carried out by applying liquid chemical disinfectants to food contact and non-food contact surfaces [5]. The microbicidal activity of commercial antimicrobial agents is largely based on quaternary ammonium compounds, phenolic compounds, organic acids, alcohol, chlorine and iodophores [2, 6].

Despite the use of disinfectants, significant number of researches has documented the persistence of some food borne pathogens on food contact surfaces especially when they are in biofilms [7], thereby affecting the quality and safety of the food products. The resistance of the biofilms to the antimicrobials have been linked to several mechanism including but not limited to little or no infiltration of antibacterial agent through the EPS [4]. This is due to binding action between the positively charged and negatively charged ions of the antimicrobial agents and the EPS [8, 9]. The retardation of the growth rate by the biofilm microorganisms consequently affects the action of antimicrobials which required vigorous microbial growth, physiological changes, development and transfer of resistance phenotypes among the organisms in biofilm. In addition, the alteration of microenvironment can also antagonized the effect of antimicrobials due to nutrient or waste accumulation [10-12].

Furthermore, to avoid contamination of freshly prepared food by pathogenic organisms there is need for efficient use of antimicrobial agents or sanitizers on domestic kitchen surfaces. Many of the commercial antimicrobials currently in use in domestic kitchens have been found to be effective against microbial suspensions. However their effectiveness against biofilm adherence to food contact surfaces and kitchen utensils has not been fully evaluated. Therefore, study of effect of commercial antimicrobial agents on biofilm formation is a necessary requirement in combating biofilm formation by the pathogenic organisms. The results of this study will go a long way in addressing the problems face in eradication of biofilms on domestic kitchen surfaces. It will also point out suggestions on the dose and

exposure time of commercial antimicrobial agents that should be applied to food contact surfaces.

EXPERIMENTAL

Preparation of the stainless steel

The stainless steel (SS) (Type 304 no. 4 finish) was selected as substratum for growing biofilm because it is the most widely used due to its high corrosion resistance in diverse environment. The SS was obtained from CK Stainless Steel work (Johor, Malaysia) and cut into 20 pieces of coupons with 10 mm diameter x 2 mm thickness. Three of the SS coupons were left unmodified while different roughness profiles were created on the other stainless steel coupons using a sandblasting machine (MHG Strhlanlegen, UTM). A sandblasting machine was generally used to clean and abrade surfaces of rusting, paints or any undesired surface materials using silica sand. Briefly, the coupons were mounted to a plier with the useful surface facing up and an air powered pressure gun was used to fire out the silica sand at high speed whilst directing it to the surface of the coupon held in the pliers. Different roughness was maintained on each sets of the stainless steel by sandblasting them for 3 min and five min respectively.

All the SS coupons were washed with a detergent solution for 20 min and rinsed three times in 15 mL of sterile deionised water while agitating using a vortex. The coupons were sterilized by exposing them to UV light for 60 minutes. Subsequently, the coupons were degreased in alcohol for 1 h and rinsed with deionised water using a vortex before they were finally dried in a laminar air flow cabinet following the procedure demonstrated by [28].

Measurement of the surface profile

The surface roughness of the sand blasted and cleaned SS substrates were measured using 3D measuring laser microscope (Olympus LEXT OLS4100, Crest Systems (M) Sdn. Bhd). The images were acquired using a 50X objective lens, covering a total area of 256 µm × 256 µm. The line roughness (R_a) which considers the average roughness was acquired in addition to the 3D image. The standard deviations of the mean (SD) from the data obtained were determined.

Effect of Benzalkonium Chloride on the biofilm

Benzalkonium chloride (BKC) (Fluka) was used to study the effect of commercial antimicrobial agents on biofilms. Its application in wide range of commercial disinfectant formulations, such as being the active component of Dettol and Lysol are commonly used for surface disinfections. In order to study the effect of BKC, the biofilms of *E. coli* DH5α was grown in continuous flow system. Initially, the *E. coli* DH5α colony from the slant agar bottle prepared from late log phase was grown on Luria Bertani (LB) agar for 24 h. A colony from LB agar was subsequently grown in 100 mL LB broth at 37 °C.The culture obtained was used in a volume of 10 % of the medium bottle as the inoculum for the biofilm formation as previously demonstrated by Jayaraman [13] and Soleimani *et. al.* [14]. The peristaltic pump (Watson marlow 120U/R) was used to pump the culture medium through the home-made flow cell at flow rate of 1 rpm for 72 h. Throughout the experimental period, the culture medium was stirred using a magnetic stirrer to ensure the efficiency of oxygen dispersion in liquid media [14]. After 72 h, the attached biofilm was rinsed by flowing PBS to remove loosely bound cells and the media. Then, the system was perfused with 5 mg/ mL of BKC whilst running the pump at 10 rpm for 30 min following the techniques demonstrated by Romanova *et. al.*[15]. After the exposure time, the system was further rinsed with sterilized PBS to wash out and neutralized the residual BKC. The number of attached organisms that survive the effect of the BKC was determined via cell viability on the coupons. The same procedure was repeating using 10 mg/mL and 20 mg/mL of the BKC. However, in the case of higher concentrations of BKC, 100 µL of the initial suspensions were plated on LB agar without dilution and incubated overnight at 37 °C. The control experiment was conducted by perfusing the system with sterilised distilled water without the BKC for 30 min after the initial rinsing with the PBS solution. The percentage bacterial removal was calculated using equation the equation below:

$$M\ (\%) = [(NTVC - NVC)/NTVC] \times 100$$

Where M is the percentage of bacteria removed, NTVC is a number of total adhered cells in CFU/mL and NVC is a number of viable cells after treatment with corresponding concentration of BKC in CFU/mL, respectively.

Statistical analysis

The data generated were subjected to statistical analysis using the popular SPSS version 18. P-Values less than 0.05 were considered significant.

RESULTS AND DISCUSSION

Stainless steel surface topography

The roughness images of each SS coupon were taken from four different points using 3D measuring laser microscope (Olympus LEXT OLS4100). Mean value of surface roughness of each of the three SS surfaces measured in triplicate are presented in the Table 1. The figures after ± represent the standard error of the mean (SEM) from three independent SS surfaces which give a total of 9 measurements. Figure 1 to 3 show the representative OLS4100 micrograph of each of the SS coupons and the corresponding 3D images. The major parameter normally used in comparison of surface profile is the average roughness (R_a). R_a represents the arithmetic mean deviation of the unconditional ordinate values obtained from a given sampling length[16]. It is also worth mentioning that surface roughness of less 0.8 µm (which is also the industrial threshold) is recommended to minimize fouling and microbial contamination of surfaces [17-19].

As shown in the figures, the roughness profile increases from SS-1 to SS-3. However, the SS- 2 and SS-3 contains some crevasses and cracks with high peaks. Such cracks and crevasses were hardly cleaned and therefore were capable of harbouring more bacterial cells than the SS1 that appear smoother except for little imperfections [20].

Effect of Benzalkonium chloride (BKC) concentration

The effect of BKC concentration on the biofilms was estimated as a percentage removal, M (%) based on the number of cells recovered from the coupons after the exposure to BKC. Figure 4 indicates that no pronounced effect was observed after exposure to 5 mg/mL of BKC. Pronounced effect of BKC on the biofilm was observed with 10 mg/mL and 20 mg/mL. However, these concentrations do not completely remove the biofilms from the SS surface. These observation reinforced the wide spread report that microorganisms living in biofilm are generally resistance to antimicrobial treatments and underscore the adequacy of lower concentrations of BKC and other antimicrobial agents in removing microbial biofilms.

The resistance of biofilm cells to antimicrobial concentration has been linked to several factors. For example, slow growth of biofilm organisms that lead to poor expression of antimicrobial binding proteins and insensitive to antimicrobials that require vigorous bacterial multiplication [11]. EPS has been reported to reduce the potency of antimicrobial agents on the biofilms by diluting the antimicrobial concentration there by allowing little or no concentration to reach the cells depending on the concentration of the antimicrobial agent [8, 21]. BKC, being positively charged Quaternary ammonium compound (QAC) can also interact with negatively charged extracellular material that surrounds the biofilm and consequently reduce the penetration of the BKC. Based on these observations, the insignificant effect of the lower concentration (5mg/mL) observed in this study may be attributed to the ability of the EPS to dilute this concentration thereby reducing their potency against the *E. coli* DH5α biofilms.

Furthermore, QACs have generally been demonstrated to exert only a bacteriostatic effect at lower dose. Whilst, the bactericidal activity can only be achieved at high concentrations [22, 23]. Therefore, the use of lower dose is capable of inducing development of resistance by the microorganisms most especially when they are entrapped in biofilms structure. Development of resistance by microorganisms including *E. coli* and *P. aeruginosa* as a consequential effect of continuous exposure to sub lethal doses has been reported in several literatures [24, 25].

In another development, there have been reports that QACs such as BKC are capable of disrupting the outer membrane and consequently

leak out the intracellular components of gram negative organisms [26]. Consequently, one may inferred that 10 mg/mL and 20 mg/mL BKC that shows significant effect on the *E. coli* DH5α biofilms in this study were able to overcome the barriers created by the EPS and thus able to disrupt the outer membrane of the cells within the biofilm.

Effect of Surface Roughness on the Effectiveness of the Benzalkonium Chloride

The effect of surface roughness on the efficiency of the BKC was determined by comparing the cell removal percentage for each concentration. Table 2 shows that high percentage of 36%, 54% and 53% were removed with 5 mg/mL, 10 mg/mL and 20mg/mL respectively from the substrate with the least surface roughness (0.38 ± 0.15µm) followed by 1.5 ± 0.18 µm and 2.0 ± 0.09 µm. Though the percentage of cell removal of the three concentrations from the SS-3 with the highest roughness of 2.0 ± 0.09 µm were slightly higher than those of the SS-2 with roughness of 1.5 ± 0.18 µm. Statistical comparison of the number of cells that remained attached to the three surfaces after their exposure to 20 mg/mL BKC also show a very significant effect ($p \leq 0.05$) on the SS with the least roughness. These observations reinforced the view that surviving bacteria might hide in cracks and fissures in the rougher surfaces [27] and hence one may conclude that surface topography does not only determined the extent of biofilm formation on stainless steel, but also influence biofilm susceptibility to antimicrobial treatment.

Table 1 Mean surface roughness of the three sets of stainless steel

Stainless steel sample	Surface roughness (µm) ± SEM
Stainless steel 1(SS1)	0.38 ± 0.15
Stainless steel 2 (SS2)	1.5 ± 0.18
Stainless steel 3 (SS3)	2.0± 0.09

Table 2 Percentage bacteria removal from stainless steel coupons with different BKC concentrations

Stainless steel surface roughness (µm)	Percentage (%) bacteria removal at different BKC concentrations		
	5 mg / mL	10 mg / mL	20 mg / mL
0.38 ± 0.15 (SS1)	36	54	53
1.5 ± 0.18 (SS2)	5	29	50
2.0 ± 0.09 (SS3)	9	46	49

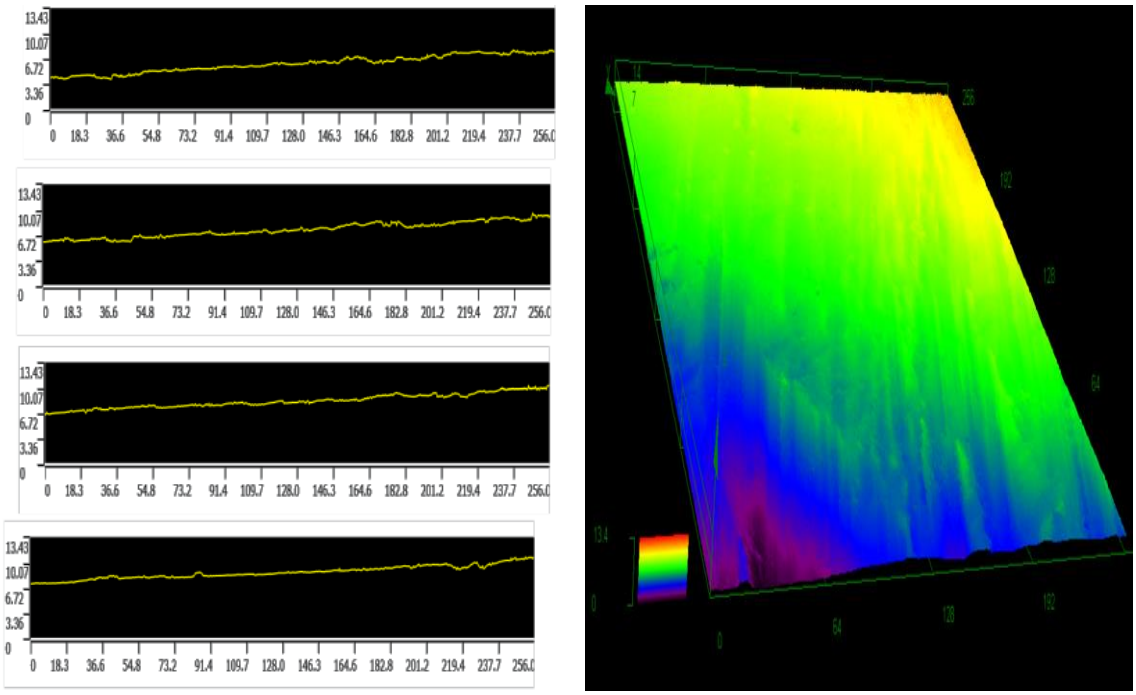


Figure 1 A representative micrograph of surface roughness profile of the SS-1 with the corresponding 3D image. The mean value of the surface roughness was found to be 0.38 ± 0.15 µm. The colours show height difference at each point with the red been the highest point and the purple been the inner most depth.

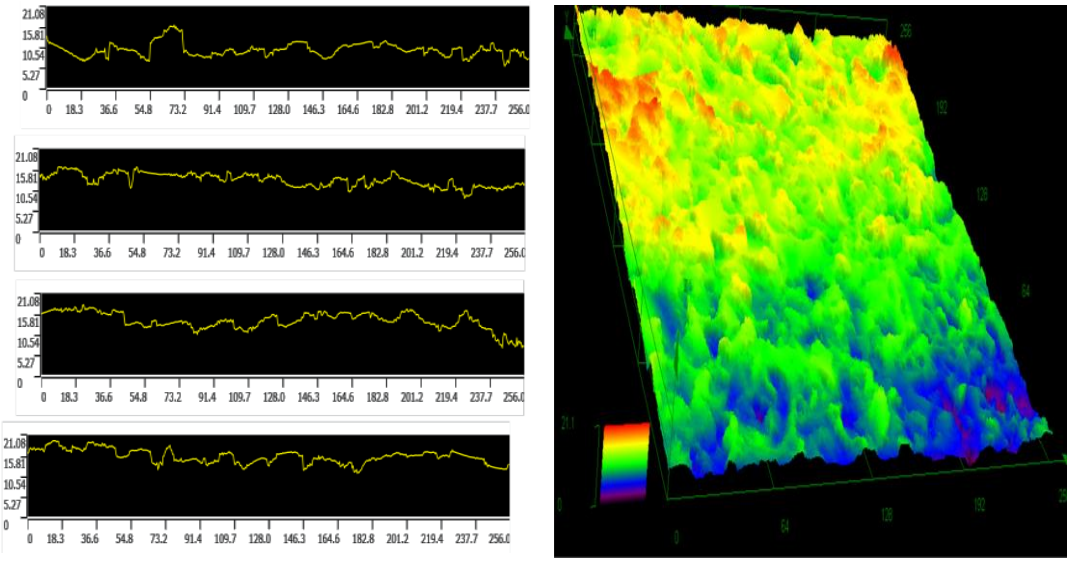


Figure 2: A representative micrograph of surface roughness profile of the triplicate SS-2 with the corresponding 3D images. The mean value of the surface roughness was found to be 1.5 ± 0.18 µm. The colours show height difference at each point with the red been the highest point and the purple been the inner most depth.

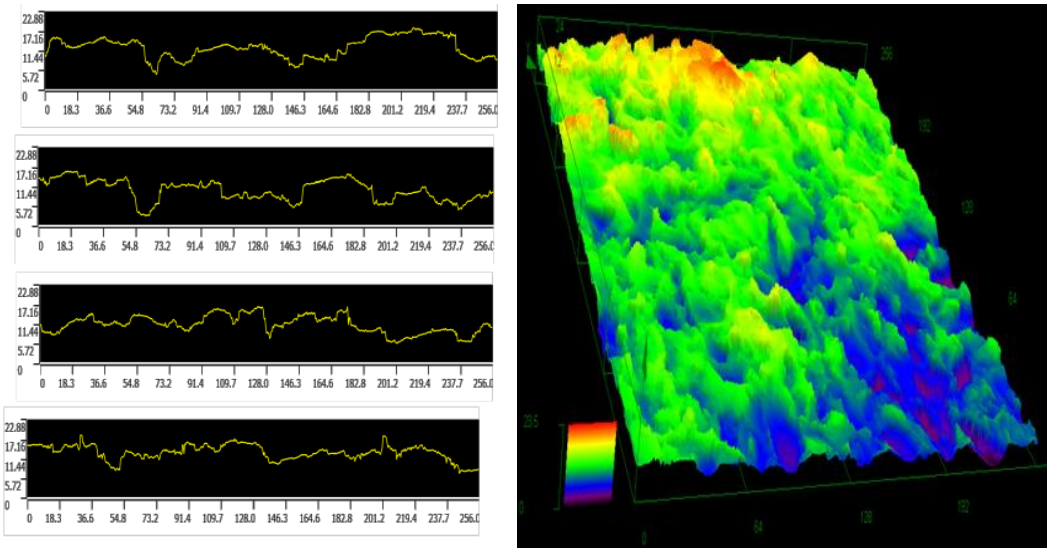


Figure 3: A representative micrograph of surface roughness profile of the triplicate SS-3 with the corresponding 3D image. The mean value of the surface roughness was found to be 2.0 ± 0.09 µm. The colours show height difference ateach point with the red been the highest point and the purple been the inner most depth.

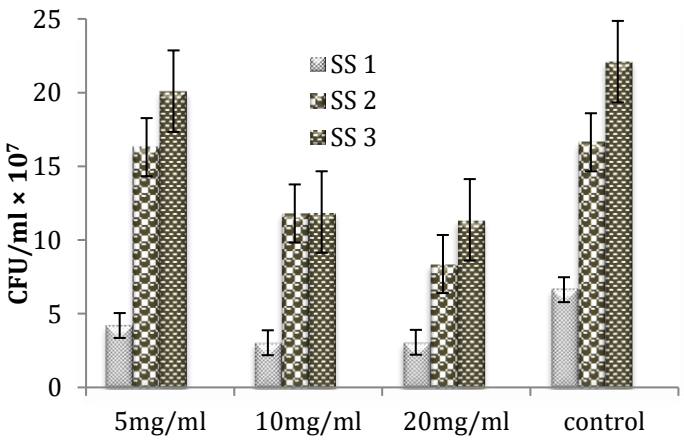


Figure 4 The bacteria recovered after exposure of the stainless steels to the corresponding concentration of BKC.

CONCLUSION

The investigation of biofilms sensitivity to BKC concentrations revealed that more biofilm can be removed with 10 mg/mL and 20 mg/mL. All the three concentrations of BKC tested were more effective on the biofilms grown on the coupon 1 with the least R_a value as compared to other two coupons indicating surface topography has influence on efficiency of antibacterial agents in removing biofilms. Overall, the results of this study indicate that surface roughness does not only determine the extent of biofilm formation of *E. coli* DH5α on stainless steel, but also influence biofilm susceptibility to antimicrobial treatment especially with benzalkonium chloride.

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