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Original article

Evaluation of sodium hypochlorite efficiency on the elimination of *Pseudomonas aeruginosa* biofilm using two methods

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Abstract: *Aims* — The purposes of the present work were to study the biofilm formation of *Pseudomonas aeruginosa* on stainless steel (316 and 304) and to investigate the effectiveness of sodium hypochlorite with different concentrations after 5 min of treatment, against *P. aeruginosa* biofilm formed on the same substrata.
Methods — The methods used in this study were plate count method (PCM) and atomic force microscopy (AFM).
Results — The results obtained using PCM showed that bacteria adhered more to stainless steel 316 than stainless steel 304. Furthermore, sodium hypochlorite was effective against cells of *P. aeruginosa* adhered to stainless steel 304 at the concentration of 0.5 %, but it was not effective against the ones adhered to stainless steel 316 until the concentration of 1%. The AFM results appeared that some bacteria still adhered to two types of stainless steel after sodium hypochlorite treatment at all concentrations.
Conclusion — The type of surface and the disinfectant concentration have an effect on the efficiency of the disinfectant against biofilm. In addition the PCM and AFM do not give the same results.

Keywords: biofilm, *Pseudomonas aeruginosa*, stainless steel, atomic force microscopy, plate count method.

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Introduction

Pseudomonas aeruginosa is aubiquitous environmental bacterium. It is considered as one of the important opportunistic human pathogens, causing serious problems in food sectors. This bacterium was isolated from different surfaces [1, 2]. It has the ability to attach to a variety of them, including the food processing environment, and form biofilm [3, 4]. This latter is defined as a biologically active matrix of cells and extracellular substances in association with a solid surface [5], and it is more resistant to sanitizing and antimicrobial agents [3]. The adhesion is considered the key step of biofilm formation and it is widely studied especially on the food contact surfaces [6-10]. This adhesion and consequently biofilm formation might be affected by physicochemical characteristics of the surfaces [11, 12], and by its topography and roughness [4, 13].

Cleaning and disinfection are the key way to prevent the contamination of the food contact surfaces and consequently food products. Moreover, the contamination of food-contact surfaces might lead to the persistence of pathogens in food processing environments [14], which must be a significant public health impact [15]. Several chemical agents were tested against biofilms of various microorganism cells such as peracetic acid [14, 16-19] and hydrogen peroxide [20]. The first one showed the ability to eliminate approximately 98% and 99% of viable *S. aureus* and *P. aeruginosa*, respectively, after 1 min as contact time but not the biofilm matrix [14], while the second one is considered an efficient disinfectant against biofilms [21]. In addition, Ozone was also evaluated against biofilm in many studies [22-26], which cannot lead to microorganism resistance [27].

The efficiency of disinfection operation depends on the microorganism, the disinfectant, the concentration of disinfectant, the contact time, the pH, the temperature, etc. The relationship between the concentration of the disinfectant and the contact time should be considered to achieve a determined reduction of the microorganism [28, 29]. Sodium hypochlorite is one of the chemical compounds used for bleaching or disinfecting different things including surfaces. Numerous works were done about its efficiency against biofilms [30-32] and contradictory results were obtained. Sodium hypochlorite was reported to be an effective disinfectant for biofilm inactivation [33].

Different methods are applied to quantify the number of adhered bacteria and the remained ones after treatment. These methods include McFarland turbidity test [18], swabbing method

[34], microplate absorbance measurement [18], and plate count method. For this latter many techniques are used for biofilm dislodging from a surface such as ultrasonic [34, 35], shaking with beads [18] and vortexing [37]. In addition to that, many other techniques are used for biofilm visualization including epifluorescence microscopy [9, 37-39], scanning electron microscopy [35, 36, 40] and atomic force microscopy [41, 42]. Based on our best knowledge no study has compared between plate count method and atomic force microscopy.

The goals of this work were to study the *P. aeruginosa* biofilm formation on two types of stainless steel (316 and 304), and to investigate the ability of sodium hypochlorite to eliminate biofilm from two types of stainless steels using AFM and PCM methods.

Material and Methods

Bacterium strain preparation of bacterial suspension

The bacterium used in this study was *Pseudomonas aeruginosa*. This bacterium was isolated from a stainless steel surface, after cleaning and disinfecting procedures. The bacterium was grown at 37°C for 24 hours on Luria Bertani agar. This medium was made using the following components; 10 g tryptone, 5 g yeast extract, 10 g NaCl and 15g agar, and one liter of distilled water. After 24 hours of incubation, bacterial cells were scraped off the agar plates and harvested by centrifugation at 6500 rcf for 15 minutes. Cells pellets were resuspended in KNO3 (0.1M) and adjusted by spectrophotometer to an optical density wave length angle of 600 nm of optical density approximately between 0.7 and 0.8 corresponding to 108 CFU/ml. .

Substratum preparation

The size of two types of stainless steel (316 / 304) surfaces was 1 cm per 1 cm. These surfaces were immersed in the solution of absolute ethanol for 15 minutes. Then rinsed three times with distilled water, and they were autoclaved at 121°C for 20 minutes [43, 44].

Disinfectant tested

The disinfectant selected for this study was sodium hypochlorite (NaCLO), at four different concentrations which are 0.5 %, 1 %, 1.5 %, and 2 %.

Biofilm formation test

The sterilized surfaces were immersed in the Petri dishes containing the bacterial suspension, for 3 hours at 25°C. After, the surfaces were rinsed three times by distilled and sterilized water to remove the non-attached bacteria. Then the surfaces were placed in Luria Bertani broth and incubated at 25°C for 24 hours [18, 39, 45, 46]. Three replicates were carried out for each experiment and only the numbers of colonies between 30 and 300 are included in the calculation of the number of the bacteria adhered to two stainless steels.

Efficiency of sodium hypochlorite against biofilm cells

The surfaces colonized by *P. aeruginosa* were rinsed three times to remove the non-adhered cells. Then, they were placed in Petri dishes containing sodium hypochlorite already prepared at diverse concentrations (0.5%, 1%, 1.5% and 2%). After 5 min of contact, the surfaces were rinsed 3 times. They were after placed in the glass tubes containing 10 mL of sterile physiological saline, and sonicated at 35 kHz for 10 min and vortexed [4, 35]. To quantify viable cells, bacteria were resuspended, serially diluted 10-fold with sterilized physiological saline and cultured on nutrient Agar at the temperature of 37°C for 24 hours [45, 47, 48]. Three replicates were carried out for each experiment.

Atomic force microscopy analysis

The topography images and roughness of both stainless steels colonized by *P. aeruginosa* biofilm and treated with sodium hypochlorite at different concentrations (0.5 %, 1 %, 1.5 %, and 2 %) were measured using Atomic Force Microscopy (Nanosurf flex AFM). The measurement was carried out with an easy scan 2 controller from Nanosurf. The tapping mode (Dynamic) in an ambient air environment was used for scanning and measuring. The Ra value, which is the arithmetic mean deviation of profile, is the most commonly used descriptor of surface roughness [49]. The Ra value was determined using the software easy scan 2 (three replicates).

Statistical analysis

Statistical analyses were performed using Software STATISTICA version 6. Newman-keuls test was used to compare the means $\log_{10}$UFC/cm^2 of each surface before and after disinfection treatment ($P<0.05$). The means presented in the figures with the same letters are not significantly different and the ones with distinct letters are significantly different.

Results

P. aeruginosa biofilm formation on two types of stainless steel

In the present work, the ability of *P. aeruginosa* to form biofilm on stainless steel 316 and stainless steel 304 was studied. *Figure* 1 presents the numbers of biofilm cells adhered to two substrata. The results obtained show that a number of $\log_{10}$ 6.78 CFU.cm^{-2} of cells was adhered to stainless steel 304, whereas, a number of $\log_{10}$7.61 CFU.cm^{-2} adhered to stainless steel 316. Based on statistical analysis there is a highly significant difference ($p<0.01$) in the number of adhered bacteria to two stainless steel. In addition to this, *Figure* 2 shows the AFM images of *P. aeruginosa* biofilm formed on stainless steel 304 (*Figure* 2: C, D) and stainless steel 316 (*Figure* 2: G, H). These images appeared that the bacterial cells colonized different parts of two substrata by forming a mass of bacteria embedded in a matrix of exopolysaccharides (EPS).

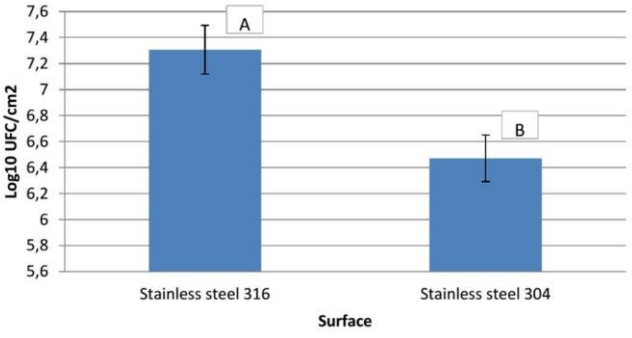

Figure 1. Numbers of biofilm cells adhered to stainless steel 304 and stainless steel 316.

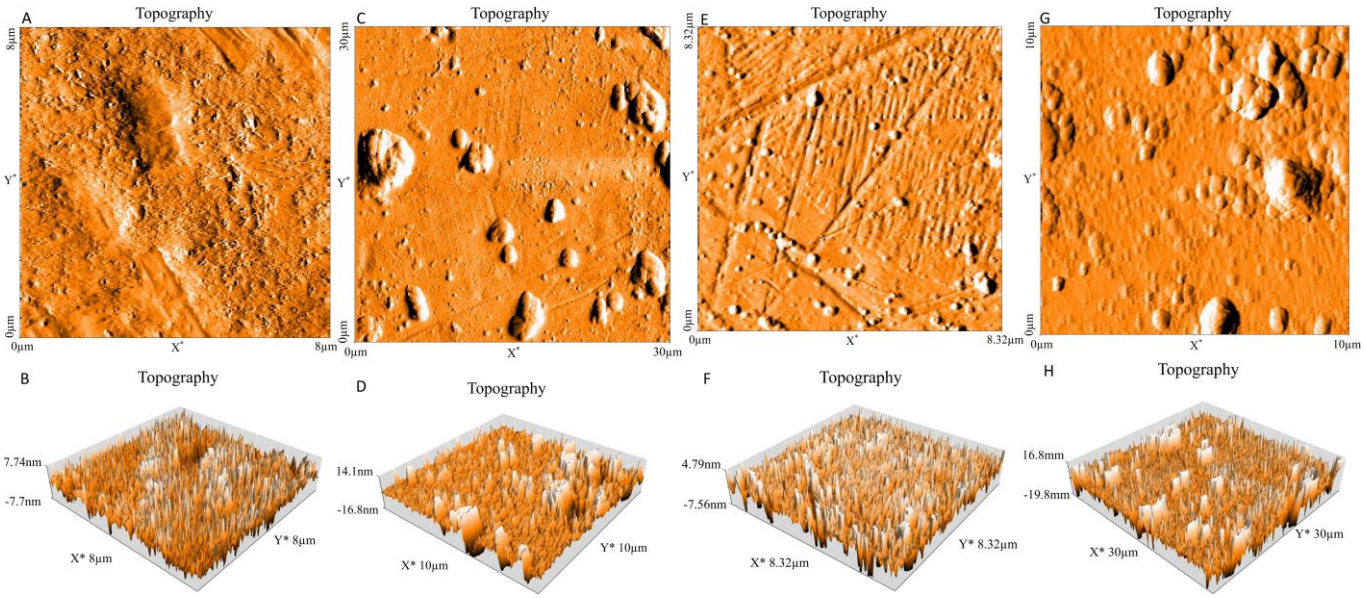

Figure 2. Topography (Three dimensional images) of stainless steel 304 (bare substrate) (A, B), stainless steel 304 (biofilm) (C, D), stainless steel 316 (bare substrate) (E, F) and stainless steel 316 (biofilm) (G, H).

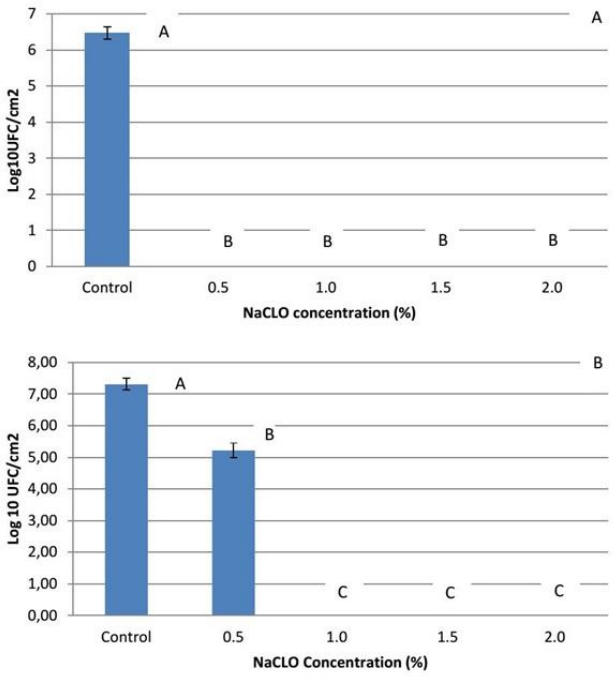

Figure 3. Number of biofilm cells adhered to stainless steel 304 [A] and 316 [B] after sodium hypochlorite treatment. The Control represents the surface colonized with Biofilm.

Evaluation of Sodium Hypochlorite Ability to Eliminate Biofilm from Surfaces by using AFM and PCM methods

The ability of sodium hypochlorite to eliminate *P. aeruginosa* biofilm formed on stainless steel 304 and stainless steel 316 was investigated by using plate count method (PCM) and atomic force microscopy (AFM). The results presented in *Figure* 3 showed a highly significant difference in the number of biofilm cells attached to both stainless steel ($p<0.01$) after treatment compared with the control (Sodium hypochlorite concentration equal 0%). Sodium hypochlorite was efficient against biofilm cells installed on stainless steel 304 at the concentration of 0.5 % after 5 min of treatment, whilst it was not efficient against biofilm cells formed on stainless steel 316 under the same condition until the concentration of 1% (*Figure* 3). Whereas, the results obtained when evaluated using AFM (*Figure* 4), showed that the bacteria were left on both stainless steel (316 and 304) after sodium hypochlorite treatment especially at the concentrations of 0.5% and 1%. But, the numbers of the persisted bacteria decreased at the concentrations of 1.5% and 2.0%, indicating that the two methods (AFM and PCM) do not give the same results.

Discussion

The *P. aeruginosa* biofilm formation on the stainless steel 316 and stainless steel 304 was performed and the results demonstrated that a high number of *P. aeuginosa* cells have been adhered to stainless steel 316 than stainless steel 304. This difference could be explained by the surfaces roughness as reported by Azelmad et al. [4] which showed that stainless steel 316 (6.1±1.4 nm) is rougher than stainless steel 304 (2.7±0.1 nm). Other works also reported that the bacterial adhesion increases by the increasing of the surface roughness [4, 40, 50]. In contrast, several studies showed that surface roughness has no effect on the bacterial adhesion [51, 52]. In addition to this, the bacterial adhesion to both stainless steel could also be explained by physicochemical characteristics of surfaces. According to Azelmad, Hamadi [4], stainless steel 316 has a hydrophilic surface, while stainless steel 304 has a hydrophobic one. Based on the physicochemical approach, the hydrophobic cells tend to attach to the hydrophobic substrate and the hydrophilic cells tend to attach to the hydrophilic substrate. This could explain the higher adhesion of *P. aeruginosa* which is also hydrophilic [53, 54] to stainless steel 316 than stainless steel 304. Our results underlined that the bacteria adhere to a large extent on stainless steel 316 compared with stainless steel 304 which means that the surface type influences the bacterial adhesion. This is in agreement with studies of numerous researchers who also found that the rate of the adhered bacteria is influenced by the type of the surface [39, 55-58].

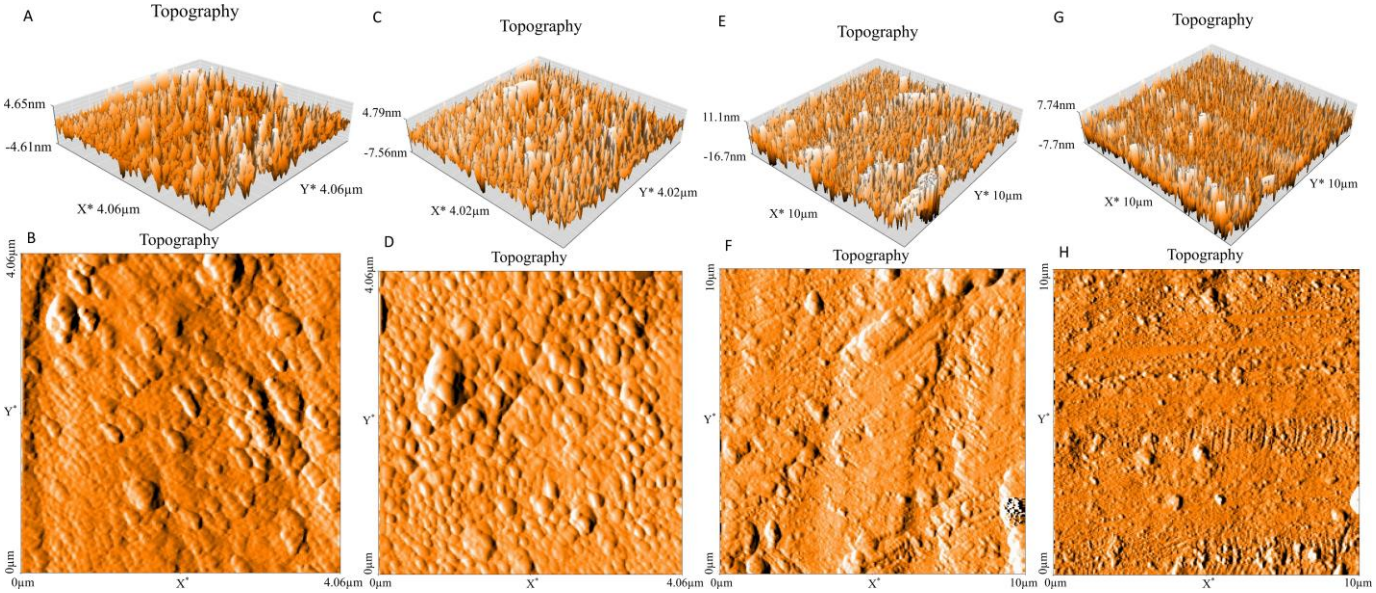

Figure 4. Topography (Three dimensional images) of stainless steel 304 colonized by *P. aeruginosa* biofilm and treated with sodium hypochlorite at concentration of 0.5% [A, B], 1% [C, D], 1.5% [E, F], 2% [G, H].

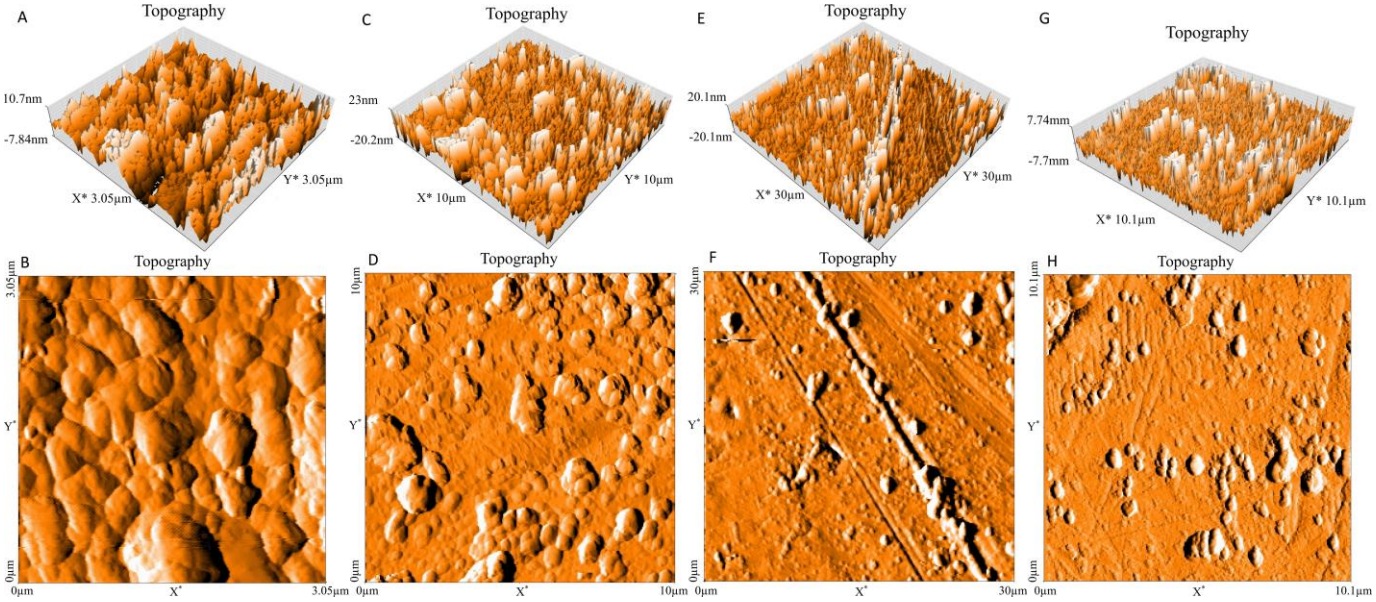

Figure 5. Topography (Three dimensional images) of stainless steel 316 colonized by *P. aeruginosa* biofilm and treated with sodium hypochlorite at concentration of 0.5% [A, B], 1% [C, D], 1.5% [E, F], 2% [G, H].

The *P. aeruginosa* biofilm formation was also assessed using AFM which confirms that this bacterium has the capacity to stick and form a biofilm on both stainless steels. This result is in agreement with the ones reported by other authors using AFM [42, 59] and scanning electron microscopy [20].

The efficiency of sodium hypochlorite against *P. aeruginosa* biofilm formed on stainless steel surfaces was evaluated using AFM and PCM which pronounced opposite results. The absence of bacterial cells after sodium hypochlorite treatment in the case of the plate count method could be explained by the fact that bacteria might be dead, injury or none cultivate after treatment. Cabeça et al. [35] evaluated five disinfectants against biofilms cells by using plate count method and scanning electron microscopy, and they showed that sodium hypochlorite was the most effective disinfectant against these biofilms compared to others such as biguanide and peracetic acid. While, their results obtained by scanning electron microscopy observations revealed that the bacteria still adhered to stainless steel after all concentrations treatment which is in agreement with our findings. Wirtanen et al. [60] reported that chlorine-based disinfectant was efficient against biofilms of *Pseudomonas aeruginosa* and *Pseudomonas fragi* formed on stainless steel 304, by using conventional cultivation, impedimetry and epifluorescence microscopy. In addition to that, the coverage of this surface by the biofilm was decreased better after this treatment. Other studies also investigated the efficiency of disinfectants such as peracetic acid, chlorhexidine, benzalkonium chloride, alkyldiaminoethyl glycine and sodium hypochlorite against biofilm cells [18, 61-63]. Takeo et al. [62]

reported that eradication of *P. aeruginosa* biofilm cells by disinfectants required more time than that in suspension and that the increasing concentration of disinfectant raised the antimicrobial effects against biofilm cells. Whilst, Martin-Espada et al [18] evaluated the peracetic acid by microplate absorbance measurements and McFarland turbidity. Their results showed that peracetic acid kills 100% of *Pseudomonas aeruginosa* biofilm installed on polystyrene at the concentration of 1.61% after 15 min of treatment.

Conclusion

The results of the present work reveal that *P. aeruginosa* has the ability to form the biofilm on stainless steel 316 and stainless steel 304. The maximum of adhered cells was observed on stainless steel 316 compared with stainless steel 304. In addition, sodium hypochlorite is more efficient against viable cells adhered to stainless 304 at 0.5% of sodium hypochlorite after 5 min of treatment, whilst this disinfectant was not efficient against one installed on stainless steel 316 until the concentration of 1% at the same time of treatment. In contrast, AFM images appeared that some bacterial cells still attached to two types of stainless steel after treatment at all concentrations applied.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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