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Defined hydrodynamic shear stresses influence the adhesion behaviors of marine *Bacillus* sp. on stainless steel in artificial seawater

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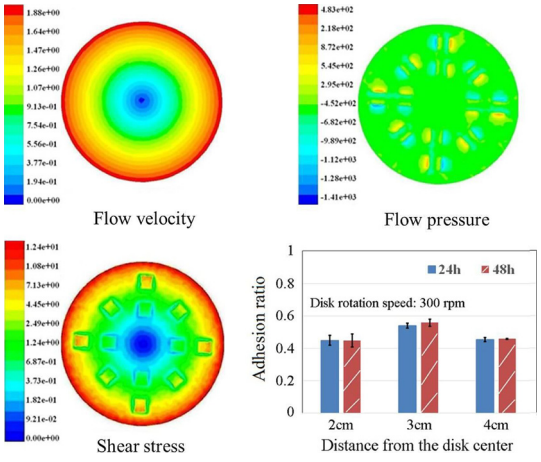
GRAPHICAL ABSTRACT



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ABSTRACT

Formation of microbial biofilm on marine structures is crucial for subsequent attachment of macro fouling species and understanding the impact of fluid conditions on formation and growth of the biofilm is essential for preventing biofouling. Here we report the effect of shear stresses generated by laminar and weakly turbulent flow on the adhesion of typical marine bacterium *Bacillus* sp. biofilm on stainless steel. Numerical simulation was carried out to calculate the distribution of pressure and shear stress applied on adhered bacteria. The shear stress was proportional to both the distance from the center of rotating disk and rotation speed. The stress reached 18 Pa as the flow velocity was 2.4 m/s and the rotation speed was 600 rpm. Further adhesion testing revealed that development of the bacterial biofilm in early stage was unaffected by the shear stresses. However, shear stress regulated the adherence orientations of the bacteria on the steel surface. Turbulent flow obviously shaped development of the architecture of the biofilm, as revealed by CLSM and SEM characterization. Biofilm growth

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patterns were clearly observed under weakly turbulent flow, however, they were not seen under laminar flow. The microcolonies assumed elongated forms, possibly triggered by exerted pressure drag force. The results give insights into possibly controlling the formation of marine biofilm on marine infrastructures for desired anti-fouling performances.

1. Introduction

Marine foulers have a predilection for attaching and growing on marine infrastructures. The settlement of foulers on submerged marine surfaces causes some well-known problems like corrosion, drag-related speed loss of ship hulls and so on [1,2]. Understanding the mechanisms of microorganisms' adhesion and growth of associating biofilm especially under defined flow conditions would facilitate the research efforts for minimizing or even preventing biofilm formation.

It is established that steady state 3D structure of microbial aggregates could be shaped by hydrodynamic shear forces. The forces in turn influence biofilm development [3,4]. The interactive strength between hydrodynamic shear force and aggregates was believed to determine the outer shape and size of the microbial aggregates [5,6]. Detachment, erosion and sloughing could also be guided by hydrodynamic parameters [7]. Microbial settlement was found at high shear stresses of up to 84 Pa [8]. High shear stresses were reported to deter and increase adhesion of typical marine microorganisms [9,10]. It has been reported that biofilms with smaller thickness and denser structure or consisting of streamers were formed under higher fluid-flow velocity [10]. It was suggested that high shear stresses might reduce microbial diversity of a biofilm and slow down biofilm maturation [11]. As one of the major microorganisms that are responsible for formation of biofilm, bacteria behave distinctively during their involvement in biofilm formation. The behavior is predominately dependent on the surrounding conditions, among which shear stress is the most pronounced one that decides the fate of the bacteria as to whether they attach on or detach from marine structures [12]. To date, biofilm-related research mainly involves static culture media or the media with low flow velocity. This study aims to investigate the effect of shear stresses created by different rotational speeds on the adhesion of biofilm on marine structures.

We already realized that colonized bacteria accelerated the corrosion of stainless steel by triggering pitting in early growth stage of the *Bacillus* sp. biofilm under no-flow conditions [13]. Equivalent circuit models were proposed to provide direct electrochemical information about the liquid/surface interfaces. This work further investigated the effect of shear stresses created by two typical rotational speeds (300 and 900 rpm) on biofilm formation of *Bacillus* sp. in artificial seawater. Distribution of the stresses along the radial direction parallel to substratum surface was calculated by finite element analysis. By developing the simplified model that represents laminar and weak turbulent flow conditions, the role of shear stress on *Bacillus* sp. biofilm structure was further characterized and elucidated.

2. Materials and methods

Generation of and variations in shear stress were accomplished by using a rotating disk in artificial seawater (ASW). A polypropylene disk (Zhejiang Gongdong Medical Technology Co., Ltd, China) with 12 cm in diameter and 5 mm in thickness was rotated in a glass container with 20 cm in diameter and 15 cm in height. The drive motor (Hangzhou Gengyu Instrument CO., Ltd, China) used was a DW-2-90 W stirrer. Mirror-polished 316 L stainless steel coupons with the dimension of 1 × 1 × 0.2 cm were used as the substrates for adhesion testing of *Bacillus* sp. For the testing, the steel coupons were fixed to the disk by screws at the distance of 2, 3 and 4 cm away from the center of the disk to acquire various shear stresses simultaneously. Prior to the testing, the surfaces of the steel coupons were degreased ultrasonically in acetone. The samples contained vessel was immersed in a water bath to ensure a constant temperature in the culture medium. The distance between the disk and the bottom of the vessel was 5 cm. All experiments were carried out at 30 °C. ASW was prepared according to the ASTM D1141-98 (2003). Peptone (3 g/l) was added to the media. The culture media were refreshed every 48 h. The media with the pH value of 7 were firstly sterilized at 121 °C for 20 min.

To accurately determine the shear stress values, the three-dimensional fluid flow of the rotating disk was analyzed by numerical calculation using CFD software FLUENT. The rotation of the disk was simulated by the dynamic mesh computing method. The geometry of the rotating disk is schematically shown in Fig. 1a, where R is diameter of the glass container, r is diameter of the disk, H is height of the glass container, h is the distance between the disk and the bottom of the container, d_1, d_2, and d_3 are position parameters of the steel coupons. Since the flow is incompressible, the governing differential equations for continuity, momentum, and energy were used to describe the fluid flow. The mass and momentum conservation can be described by Eqs. (([1])–([4])) [14].

The mass conservation equation is:

$$\frac{\partial u}{\partial x} + \frac{\partial v}{\partial y} + \frac{\partial w}{\partial z} = 0 \tag{1}$$

The momentum conservation equations are:

$$\frac{\partial u}{\partial t} + \frac{\partial (uu)}{\partial x} + \frac{\partial (uv)}{\partial y} + \frac{\partial (uw)}{\partial z} = -\frac{1}{\rho_l}\frac{\partial p}{\partial x} + \vartheta\left(\frac{\partial^2 u}{\partial x^2} + \frac{\partial^2 u}{\partial y^2} + \frac{\partial^2 u}{\partial z^2}\right) \tag{2}$$

$$\frac{\partial v}{\partial t} + \frac{\partial (uv)}{\partial x} + \frac{\partial (vv)}{\partial y} + \frac{\partial (vw)}{\partial z} = -\frac{1}{\rho_l}\frac{\partial p}{\partial y} + \vartheta\left(\frac{\partial^2 v}{\partial x^2} + \frac{\partial^2 v}{\partial y^2} + \frac{\partial^2 v}{\partial z^2}\right) \tag{3}$$

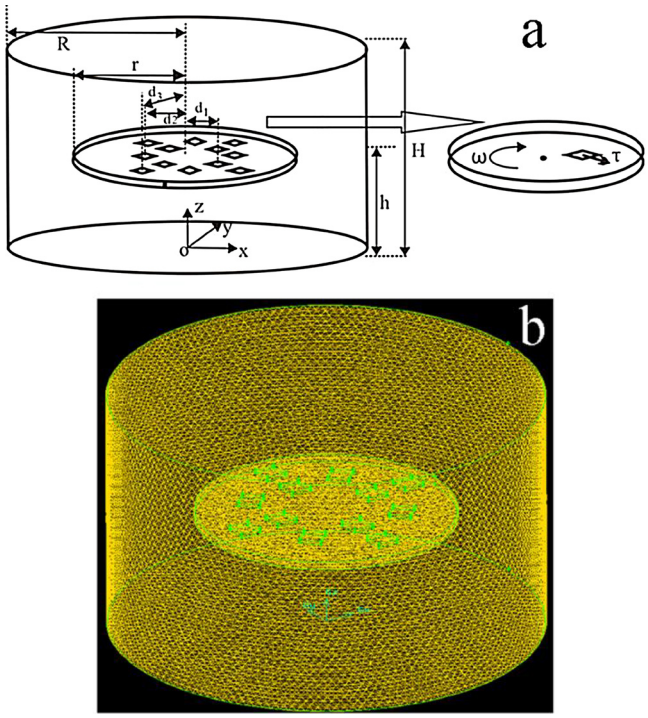

Fig. 1. (a) Schematic depiction of the geometry of the rotating system for generating turbulent flow, and (b) the unstructured mesh for the simulation.

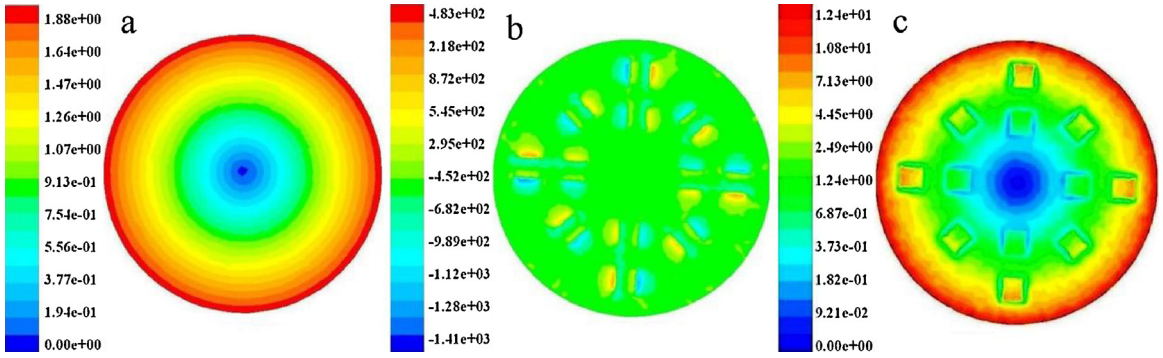

Fig. 2. Distribution of the flow velocity (a), flow pressure (b), and shear stress (c), the simulation was made with the rotation speed of 300 rpm.

$$\frac{\partial w}{\partial t} + \frac{\partial (uw)}{\partial x} + \frac{\partial (vw)}{\partial y} + \frac{\partial (ww)}{\partial z} = -\frac{1}{\rho_l}\frac{\partial p}{\partial z} + \vartheta\left(\frac{\partial^2 w}{\partial x^2} + \frac{\partial^2 w}{\partial y^2} + \frac{\partial^2 w}{\partial z^2}\right) \quad (4)$$

where ρ is density, p is pressure, u, v, w is the speed along the direction x, y, z, respectively, ϑ is kinematic viscosity coefficient. For the simulation, density of the liquid was 1023 kg/m^3 at 30 °C, and the viscosity was 8.6 × 10^{-4} Pa.s. The hydrodynamic boundary conditions were also defined: at the wall surface of the disk and the tank (no-slip): $u = v = w = 0$; at the open point of the tank: $p_{in} = 0$. Three rotational speeds of the disk, 300, 600, and 900 rpm, were empirically investigated. In addition, unstructured mesh (a tessellation of the Euclidean space by simple triangle shape in an irregular pattern) was adopted for the simulation domain (Fig. 1 b). The dynamic mesh technique was employed to simulate the rotation of the disk. The irregular grid finite-volume method was used along with the SIMPLEC algorithm that served to enhance the well-known SIMPLE algorithm. After the numerical computation, the shear stress was calculated by the equation $\tau = \mu\frac{dU}{dl}$.

The marine bacteria *Bacillus* sp. (MCCC1A00791, Marine Culture Collection of China) were employed in this study. The bacteria were cultured in ASW-based culture media (1.0% of yeast extract and 0.5% of peptone) shaken at 30 °C for 24 h. The *Bacillus* sp. suspension with an initial concentration of 10^6 cells/ml was prepared at 30 °C under aerobic conditions.

Biofilm was formed by the *Bacillus* sp. on the stainless steel fixed on the rotating disk. All components were firstly sterilized. Before the experiments, the vessel containing 2500 ml of sterile ASW with peptone being added was inoculated with the bacterial suspension (10^6 cells/ml). During the testing, the disk was rotated in the *Bacillus* sp. suspension at adjustable rotation speeds (300 and 900 rpm in separate trials). After 24 h, 48 h, and 1 week incubation under the rotating conditions, the stainless steel were washed with distilled water for three times and then the consequently formed biofilm was fixed by 2.5% glutaraldehyde (AR, Aladdin Chemistry Co., Ltd, China). Staining of the samples was done for 30 min using 150 μl of propidium iodide (95%, Alfa Aesar Co., Ltd, China) at room temperature in dark followed by PBS washing for three times.

Morphological characterization of the samples was conducted by using confocal laser scanning microscope (CLSM, Leica TCS SP5 II, Germany) and field emission scanning electron microscope (FESEM, FEI Quanta FEG 250, the Netherlands). The samples stained by propidium iodide were excited at 535 nm spectral line of argon-ion laser and observed with 615 nm emission filter. For FESEM observation, dehydration was performed through the critical point drying by 25, 50, 75, 90, and 100% ethanol solution. The student's t-test was used for the statistical analyses of the data acquired and differences were considered significant if $p < 0.05$.

3. Results

It is established that the structure of typical microorganism biofilm is highly dependent on the shear force applied on the biofilm during its growth [4,6,15]. For the steel coupons fixed on the rotating disk, the shear stress values are directly proportional to both the rotation speed and the distance of the testing point away from the rotation center. Numerical simulation revealed the distribution of the flow velocity, flow pressure, and shear stress at the top surface of the plate. The values are typically shown in Fig. 2 (the rotation speed is 300 rpm). It is not surprising that, since the disk rotates at a constant speed, the flow velocity along the radial direction of the disk becomes gradually larger as the testing point is farther away from the disk center. Positive pressure is seen at the front side of the disk due to the incoming flow. Negative pressure is caused by the eddy near the small block. In addition, higher rotation speed results in larger shear stress values, for faster speed gives rise to greater velocity gradient. As the rotation speed is 300, 600, and 900 rpm, increase in both flow velocity and shear stress is revealed (Fig. 3). Typical shear stress values are listed in Table 1. It is clear that increase in rotation speed gives rise to significantly enhanced shear by the flow media.

Fluid flow could be simply characterized by Reynolds number (*Re*), which can be defined here for the rotating disk as $\rho\omega r^2/\mu$, where ρ is fluid density (kg/m^3), ω is angular velocity (rad/s), r is radius of the disk (m) and μ is fluid viscosity (Pa.s). Under current simulation conditions, turbulent flow is anticipated as velocity of fluid increases. This triggers breaking down of the orderly laminar streaks, forming small eddies and fluid oscillations and turbulent spots at random locations. For a while, eddies keep on mixing the fluid layers, but fluid tends to remain laminar. The simulation results give clear insight into evolvement of flow velocity and shear stresses applied on the surfaces of the disk. In fact, further bacterial adhesion testing suggests the significant effect of the changes in shear stresses on the bacterial behaviors (Fig. 4). Three distances away from the center of the disk (2, 3 and 4 cm) for fixing the samples and two different rotation speeds, 300 and 900 rpm, during the first 48 h of incubation were typically investigated. After the early stage of the testing (24 h), rotation speed of the plates showed minor influence on the adhesion ratio of the bacteria. This therefore indicates that shear stress, which is altered by the different rotational speed, applies negligible impact on formation of the bacterial biofilm at early stage. After 48 h of the testing, however, it is noted that the adhesion ratios are remarkably different depending on the distances away from the disk center of the 316 L plates (Fig. 4). Significant differences are seen for the adhesion behaviors of the bacteria on the 316 L plates placed at 4 cm from the disk center (*Re* ≈ 194,000, transitional or weakly turbulent flow), in comparison to the samples fixed at the same position but with different rotational speed (Fig. 4). There is no significant difference in biofilm growth after 24 h and 48 h at 300 rpm,

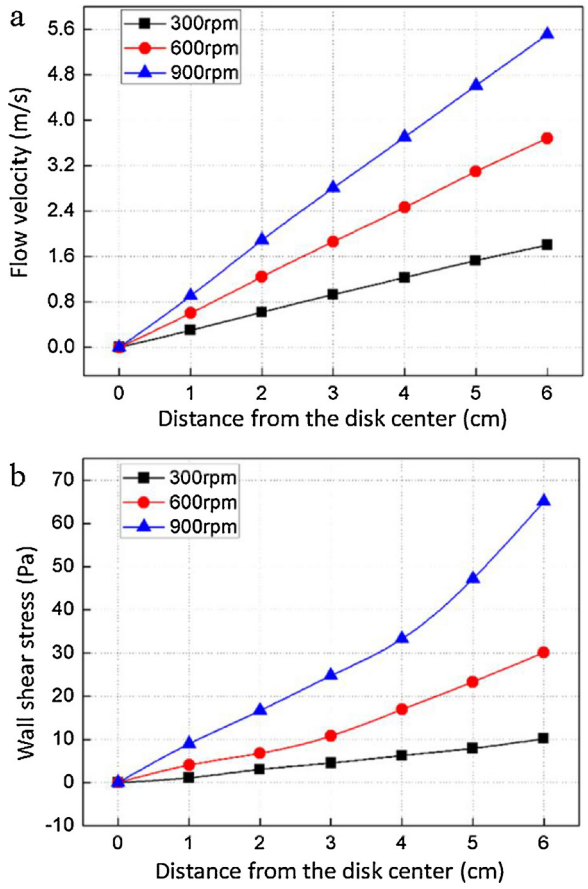

Fig. 3. Flow velocity (a) and wall shear stress (b) versus distance away from the rotation center under different rotation speeds of the disk.

Table 1
Shear stresses applied on the adhered bacteria.

Rotational speed (rpm)	Distance away from disk center (cm)	τ(Pa)
300	2	3.045
	3	4.523
	4	6.267
900	2	17.658
	3	24.745
	4	33.268

regardless of the distance to the disk center. However, as the rotation speed increases to 900 rpm, significant differences in the adhesion ratios are realized for the bacteria adhered on the plates located at 2, 3 and 4 cm away from the disk center.

In addition, it is also noted that during the initial growth stage (48 h), biofilm was distributed randomly under laminar and weak turbulent flow (data not shown), but followed as spherical or hemispherical mound-shaped in laminar and streamer patterns in the weak turbulent flow treatment after 1 week (Fig. 5). The bacteria encountering higher shear stress values (after 48 h and 1 week) show promoted adhesion on the surface, which agrees well with the prediction proposed by other researchers [16].

CLSM and SEM images (Figs. 5 and 6) show markedly different morphological features of the *Bacillus* sp. biofilm developed under laminar and transitional flow. The biofilm formed in laminar flow conditions consists of hemispherical microcolonies. Individual bacteria could be seen attaching to the stainless steel surface between the microcolonies (Fig. 5b, c). The biofilm microcolonies grown in weak turbulent flow, however, are elongated to form filamentous streamers (Fig. 5d–f). Similar result has also been previously reported [17].

4. Discussion

According to Astrid's theory [18], particle flux from bulk fluid to the wall increases with increasing fluid velocity and Reynolds number, which results in faster attachment of marine microorganisms owing to higher mass transport, although higher fluid shear stimulates microorganisms detachment. At higher flow velocities, as calculated shown in Fig. 2, convection begins to play a key role in regulating mass transport. Accordingly, the mass flux into the biofilm obviously increases. It has been reported that mass transport was strongly affected by hydrodynamic conditions like liquid flow velocities [19].

Accumulation of adhered bacteria at initial testing stage usually leads to the formation of individual microcolonies firmly attaching to substrate surface. As water particles flow toward the leading edge of each microcolony, the back (downstream) side of the microcolonies forms a wake-like morphology. A regular pattern of vortices can be observed in the wake and is the source of the effect called vortex-induced vibration [20]. In biofilm it may cause the development of downstream pressure drag. The biopolymers holding the microcolonies together are viscoelastic and are subject to external force which would be expected to assume an elongated shape. The dynamics of such action is directly related to the flow velocity, the shear stress, and magnitude of the pressure drag. It is evident that drag force contributes to the different dynamics of biofilm formation. The microcolonies in the biofilm formed under high shear stress condition are longer along the flow direction (Fig. 5 d, e, f). While the biofilm grown under laminar

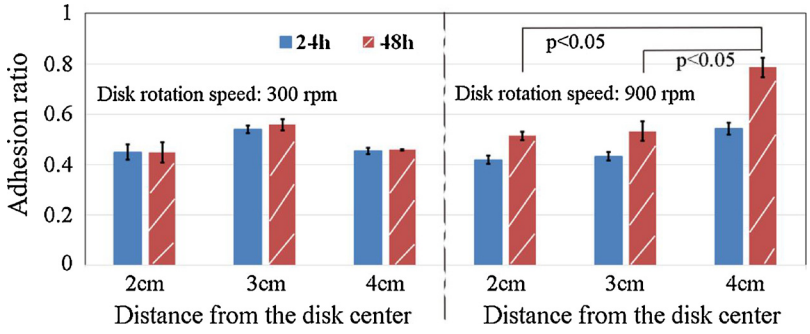

Fig. 4. Adhesion ratios of the bacteria on the stainless steel after 24 h and 48 h of exposure to the bacterial suspensions. The disk was rotated with the rotation speed of 300 rpm and 900 rpm.

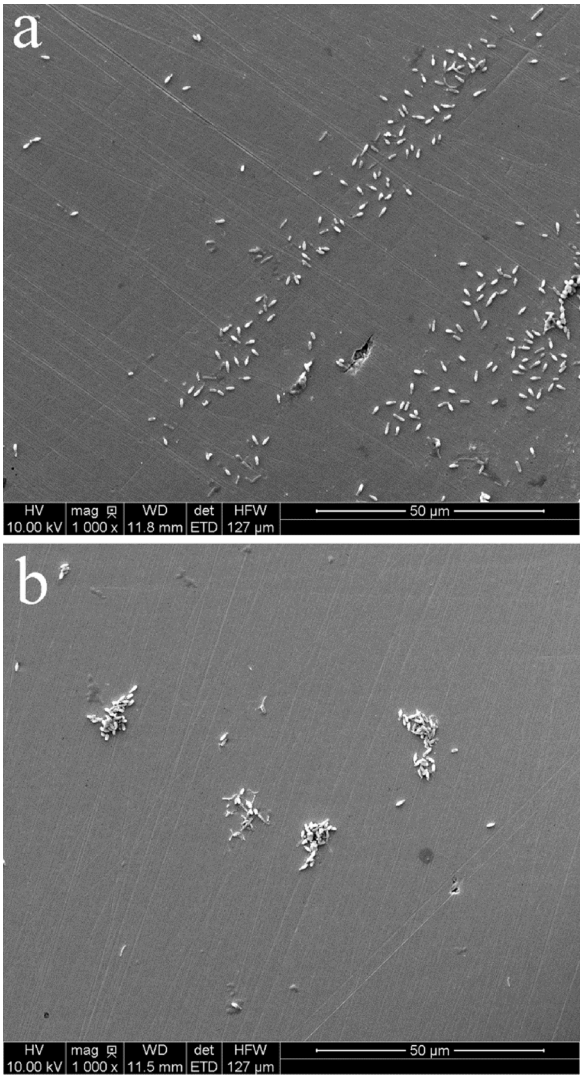

Fig. 6. SEM images of the *Bacillus* sp. biofilm formed on stainless steel after 1 week incubation with the rotation speed of 300 rpm (a) and 900 rpm (b).

condition consisted of microcolonies and single bacteria that demonstrates no relation with the flow direction (Fig. 5 a, b, c). Similar results have also been previously reported [17,21].

Under high shear stresses, bacterial growth in either y- or z- direction is partly constrained because of their exposure to high fluctuation and thereby forced detachment [22]. Therefore, a desirable path for the microorganisms to spread out would be along the flow direction, x axis in this case (the axes were defined in Fig. 1), as they can be effectively protected from shear stresses. Chen et al. suggested that biofilms formed under higher shear stresses are more strongly adhered than those formed under lower shear stresses [23]. One assumption would be that higher shear stresses conditions produce stronger biofilms. The distinguishable physical properties of biofilm may lead to different biofilm strength, but it is also possible that microbial biofilms adjust their strength in response to external environment. It could be attained by increasing extracellular polymeric substances (EPS) production [24] or by metabolic regulation in response to shear stresses [25]. It was also reported that the biofilm strength could be adjusted by altering the structure of polymers in bacteria biofilm [26]. Possible application of the above mentioned mechanisms to the adhesion of *Bacillus* sp. to stainless steel in this research is to be further explored in future.

5. Conclusions

Simplified fluctuations in ASW were made using a rotating disk apparatus and the effect of hydrodynamic shear stress on the adhesion behaviors of *Bacillus* sp. on stainless steel was investigated. Pressure and shear stresses on the top surface of the plate that is in intimate contact with the bacteria were determined by numerical simulation. Shear stress plays crucial roles in deciding structure of the biofilm on the surfaces. The bacterial biofilm generally consists of hemispherical mound-shaped microcolonies under laminar flow and the biofilm is elongated to form filamentous streamers under weakly turbulent flow. Within short time period of exposure to laminar and transitional flow, structure of the bacterial biofilm is independent of shear stress. Further elongated exposure results in enhanced impact of shear stress on the adhesion of bacteria onto the surface. The results would give insight into understanding and regulating the formation and development of bacterial biofilm in response to variations in fluid environment.

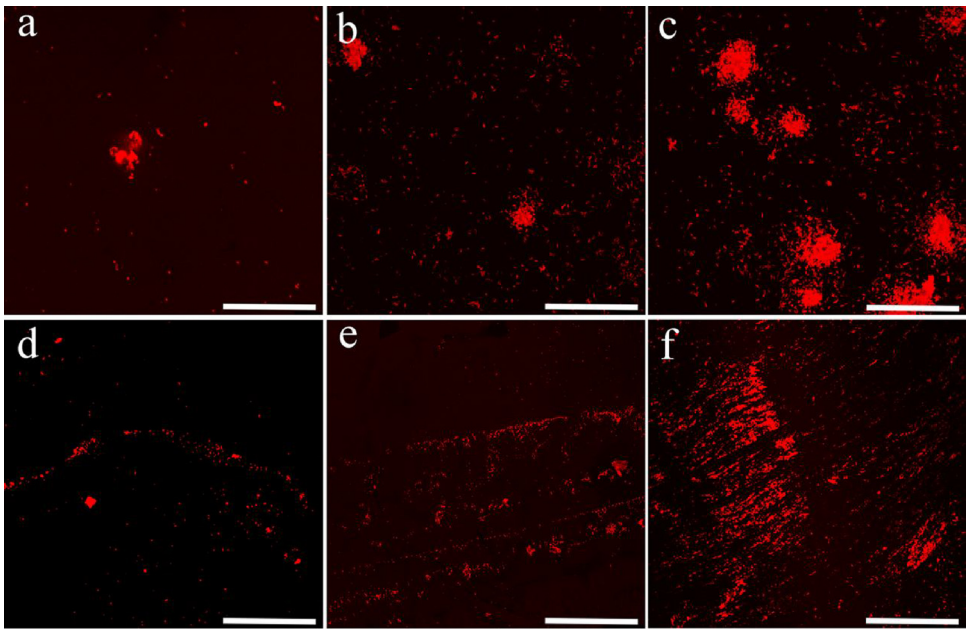

Fig. 5. CLSM images of the *Bacillus* sp. biofilm formed on the stainless steel after 1 week incubation. The rotation speed of the disk is 300 rpm (a: 2 cm, b: 3 cm, c: 4 cm, the length is the distance of the steel plate away from the disk center) and 900 rpm (d: 2 cm, e: 3 cm, f: 4 cm). The scale bar is 25 µm.

Acknowledgements

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