Fabrication of a Continuous Flow System for Biofilm Studies

Mohammed Jibrin Ndejiko*[1], Wan Rosmiza Txina Wan Dagang[2]

*Department of Microbiology, Ibrahim Badamasi Babangida University, **PMB** 11, **Lapai**, **Nigeria.***

*Fakulty of Biosciences and Medical Engineering, Universiti Teknologi Malaysia, **81310 Skudai, Johor Bahru,** Johor, Malaysia*

Abstract: Modern and current models such as flow cell technology which enhances a **non-destructive growth and** inspection of the sessile microbial communities revealed a great understanding of **biofilms. A continuous flow** system was designed to evaluate possibility of biofilm formation by *Escherichia coli* **DH5a on the stainless steel** (type 304) under continuous nutrient supply. The result of the colony iorming **unit** (CFU) **count shows that bacterial** attachment and subsequent biofilm formation on stainless steel coupons with average **surface roughness of 1.5 ± 1.8** urn and 2.0 ± 0.09 urn were both significantly higher ($p < 0.05$) than those of the **stainless steel coupon with lower** surface roughness of 0.38 ±1.5 urn. These observations support the hypothesis **that surface profile is one of the** factors that influence biofilm formation on stainless steel surfaces. The Scanning **electron microscopy and Field** emission scanning electron microscopy micrographs of the stainless steel coupons **also revealed the attached** *Escherichia coli* DH5a biofilm and dehydrated extracellular polymeric substance **on the stainless steel surfaces.** Thus the fabricated flow system represented a very useful tool to study biofilm formation **under continuous nutrient** supply.

Keywords: Biofilm, Coupon, Flow cell, Stainless steel

Introduction

The abundant form of life on earth is the microorganisms, they are numerically and phylogenetically diverse than every other living forms (Pamp *et al.*, 2008). Our basic knowledge of microorganisms is based on historical reasons that originate from empirical researches on microorganisms that live in suspension in liquid growth media. However, microbiologist and biotechnologist have generally found out that most of microbial cells live in spatially distinct communities, otherwise known as biofilms. 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 embed themselves in a slimy matrix composed of extracellular polymeric substances (EPS) that they produced. In comparison with the free living microorganisms, the functions and pathological properties of biofilms becomes altered.

Living as biofilms confer advantages on the microorganisms such as resistance to adverse conditions (low nutrient, antimicrobials and detergents), ability to acquire transferable genetic materials within short period of time (Mohammed and Dagang, 2017, Watnick and Kolter, 2000). The existence of microorganism in biofilms has direct or indirect influence on human life in terms of health, diseases, pipe water contamination, contamination of food contact surfaces and natural environment and hence it is important to have good understanding of their formation, development and response to various conditions.

*Corresponding author:

***Mohammed Jibrin Ndejiko*[1],**

Modern and current models such as flow cell technology which enhances a non-destructive growth and inspection of the sessile microbial communities revealed a great understanding of biofilms. Flow cells allow continuous growth of biofilms under hydrodynamic nutrient conditions and therefore make it a convenient model to study microbial biofilms (O'Sullivan *et al.*, 2009). Over the years static systems have been employed in the investigation and analysis of biofilms however, these systems give inconsistent results and observations that are rarely reproducible. Moreover, the static system of growing biofilm does not really represent the natural conditions of biofilm that mostly grow under liquid flow. Hence a convenient and reliable system is a needed requirement to fully understand biofilm formation.

The most widely used and recent convenient method of studying biofilm formation is the flow cell technology; however, the facilities used in flow cell technology is rarely available to underdeveloped and developing countries with low investment in research. In order to overcome these limitations, this study fabricated a flow system to investigate bacterial adherence to the surface of the stainless steel. The flow system adopted a continuous flow of the medium at a controlled flow rate through a completely sealed chamber, thereby avoiding possibility of any contamination, lack of shear and accumulation of toxic wastes that characterize the static systems

Materials and Method

Organism Used

A gram negative facultative aerobic bacterium; *Escherichia coli* DH5α was obtained from Promega PTE LTD. This strain was selected as model organism

to grow the biofilms based on its abundant community lifestyle, possession of a wide array of known genetic materials and its ability to form biofilm under low substrate concentration (Huang *et al.*, 1994; Beloin *et al.*, 2008). It is also a risk category 1 organism and can be easily grown in the laboratory with little or no risk to individual or the immediate community (Raya, 2009). It is a rod shape versatile bacteria capable of growing well in luria bertani broth (LB) using peptides and catalazeable amino acids as the carbon source (Sezonov *et al.*, 2007).

Culture Media and Growth Condition

A loopful of the *E. coli* DH5α from a stock culture kept in glycerol at -80°C was streaked on luria bertani (LB) agar plate (consisted of 5 g l^{-1} yeast extract, 10 g l^{-1} tryptone, 10 g l^{-1} sodium chloride, 15gl^{-1} agar and pH 7.0) and incubated at 37°C for 24 h. A colony from this agar plate was inoculated into 100 mL of LB broth (5 g l^{-1} yeast extract, 10 g l^{-1}tryptone, 10 g l^{-1} sodium chloride, pH 7.0) and grown overnight in a shaker incubator at 150 rpm and 37 °C. 750 µl of the resulting culture was used to produce a stock bacteria by adding 20 % (v/v) glycerol and kept at −80 °C for future use. *E. coliDH5α* from the agar plate was then grown in 100 mL of LB broth (5 g l^{-1} yeast extract, 10 g l^{-1} tryptone, 10 g l^{-1} sodium chloride) in duplicate. The bacteria were grown at 37 °C in a shaker incubator at 150 rpm. The optical density at 600 nm and colony forming unit (CFU) were measured every 2h until the culture reached a stationary phase. The CFU was carried out using a serial dilution technique. 0.1 mL of the various dilutions were aseptically spread on LB agar plates and incubated at 37°Cfor 16 – 24 h and consequently bacterial colonies were counted using a colony counter. The optical density readings over the growth period were determined with a Spectrophotometer (GENSYS 105 UV VIS) at a wavelength of 600 nm. 5 mL of the culture was centrifuged and the pellet was dissolved in 2 mL distilled water before the spectrophotometric measurement.

The Flow System

Fabrication of the Flow Cell

The flow cell (Figure 1) was fabricated using Epoxy glass obtained from Rainbow Malaysia. The glass was cut into 10 × 7 cm, 10 × 1 cm and 5 ×1 cm using Cutting edge precision machine (BAS 317 precision) The bench drilling machine (model ST- 16 A) was used to drill circular holes of 10 mm by 2 mm diameter into the piece of the epoxy glass (10 cm by 7 cm) to be used as base of the flow cell such that the stainless steel coupons fit stably into these holes. Another piece of the perpex glass (10 cm by 7 cm) was left unaltered such that it could be used as the cover plate for the flow cell. These pieces of epoxy glasses were gummed together with the 10 cm by 1 cm pieces and 5 cm by 1 cm pieces placed on the lengths and breadths of the base respectively. A hand drilling machine was used to drill two opposite holes at both breadths of the gummed glasses. Pippete tips were then inserted into the two holes to serve as the flow channel. The stainless steel coupons were prepared and aseptically put in to the drilled holes on the base of the flow cell and the cover plate placed on the flow chamber using silicone glue.

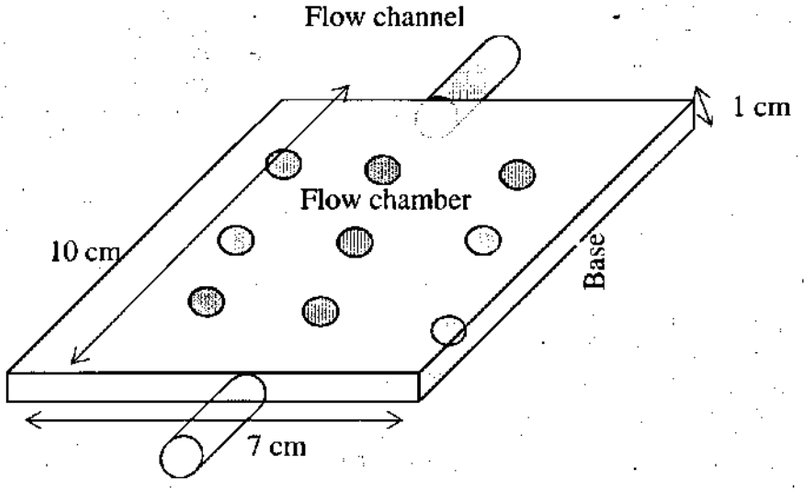


Figure 1: An illustration of the fabricated flow cell used to grow biofilm

Setting up the Flow Cell with other Components

The setup of the flow system consisted of diverse tubing, bubble trap, medium/effluent bottle, the peristaltic pump and flow cell was interconnected with connectors. All these components are reusable between the experiments. A masteflex tubing connected to the medium bottle was passed through the peristaltic pump (Watson marlow 120U/R) and connected to the bubble

trap that is capable of trapping any bubble that might go into the flow chamber to destroy the growing biofilm architecture. Another piece of tubing was connected to the outlet of the bubble trap and subsequently connected to the flow cell while the outlet tubing from the flow cell was connected to the medium bottle to ensure continuous collection of waste media as illustrated in Figure below:

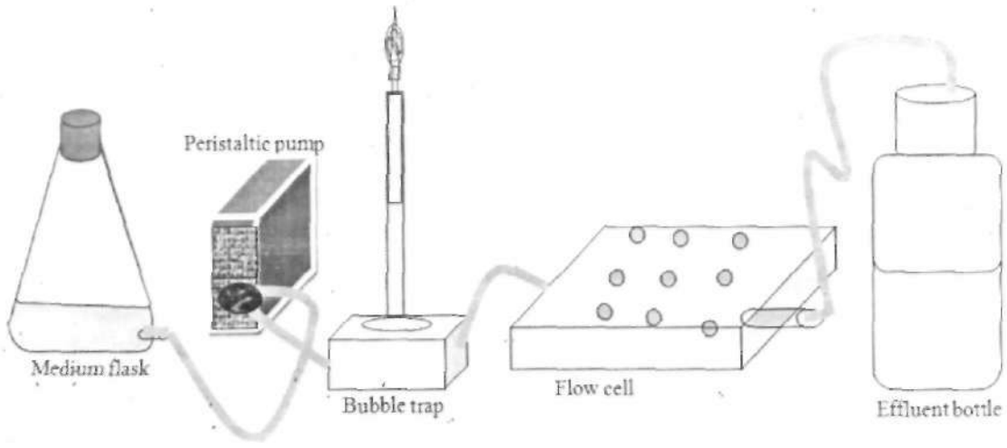


Figure 2: The continuous biofilm system set up used to grow biofilm under continuous nutrient supply

Verification of the Flow System

Prior to the implementation of the flow systems to study the bacterial attachment, some verification on the stability of the coupons in the chambers and absence of leakage in the entire system was carried out. The flow system was made to undergo flow behaviour analysis for three days using water and consistently checked before they were utilized to study the bacterial attachment.

Sterilization of the System

The medium bottle along with other autoclavable components (tubing, bubble trap, flow chamber) of the flow system were autoclaved at 121°C for 20 min before setting up the system. After the set up the entire system was further sterilized by flowing 2.5% (v/v) sodium hypochlorite through the system at 10 rpm for 3 h and then rinsed 3 times with distilled water at the highest speed of the peristaltic pump to wash away the residual sodium hypochlorite. This procedure was repeated before every new experiment.

Inoculation of the Flow System and Biofilm Formation

The *E. coli DH5α* colony from the slant agar bottle prepared from late exponential phase was streaked on LB agar plate and incubated for 24 h. A colony from the LB agar was further grown in 100 mL LB broth over night at 37°C. The resulting culture was inoculated to the medium bottle in a volume of 10 % of the useful volume of the medium bottle as the inoculum for the biofilm formation following the techniques previously described by Soleimani *et al* (2013), Simoes, (2005) and Jayaraman (1997). The peristaltic pump was subsequently used to circulate the medium with bacteria through the flow cell at the constant flow rate of 1 rpm for the period of 3 days and thus the organisms were allowed to form biofilm under continuous flow condition in order to produce biofilms that are resistance to flow and shear stresses. After each 24 h of growth, 5 mL of four times-concentrated LB broth was aseptically added to the medium bottle to serve as the nutrient source for the organisms. Throughout the experimental period, the medium bottle with the bacteria was stirred using a magnetic stirrer to provide oxygen for the microbial growth following the techniques demonstrated by Soleimani *et al.*, (2013). After 3 days, the flow cell was rinsed with saline solution at 32 rpm (20 mL/min) to remove loosely bound cells as previously demonstrated by (Nielsen *et al.*, 2011) and (Raya, 2009). The experimental runs were all conducted at temperature of 23°C. This temperature was chosen based on earlier reports that genes responsible for biofilm formation are well expressed at lower temperature of 23 °C (White-Ziegler *et al.*, 2008).

Quantification of Biofilms

Following three days of continuous medium flow to the flow chamber, the system was rinsed with sterile Phosphate Buffer Saline (PBS) solution containing 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24g of KH_2PO_4 in 1 L distilled water, to remove loosely bound cells and the medium from the coupon surfaces. The biofilm growth on each of the stainless steel coupon was determined via cell viability on the coupons. Briefly each of the stainless steel coupon was aseptically suspended in 10 mL sterile PBS

and vigorously vortexed for about 3 min to remove the biofilms and ensure homogenous dispersion of cells (Schlisselberg and Yaron, 2013; Soleimani *et al.*, 2013). Serial 10-fold dilutions of the dislodged biofilms in PBS were prepared in order to obtain cell concentration of approximately 10^4–10^5 cells/ mL. 10 µL of each serially diluted sample was spread onto LB agar plates and incubated for 24 h at 37 °C. The resulting colonies were enumerated with the aid of colony counter

Scanning Electron Microscopy and Field Emission Electron Microscopy of the Biofilm

The biofilms were grown in a continuous flow system following the same procedure described earlier for three days; however, after rinsing with PBS, the coupons were suspended in chemical fixative containing 2.5% glutaraldehyde, 4% formaldehyde and 0.1 M potassium buffer and left at room temperature for 4 h to fix the biofilms on the surface of the coupons. The coupons were subsequently rinsed in potassium buffer 3 times for 15 min each and further dehydrated in serial dilutions of 20, 30, 50, 70, 95 % ethanol for 15 min each and 100% Absolute ethanol for 30 min. the coupons were further treated with acetone for 20 min and freeze dried for 30 min. The freeze dried coupons were finally mounted on Aluminium stub using double sided sellotape and viewed with Table top Scanning electron microscope (TM 3000) at an acceleration voltage of 1500 V and working distance of 6000 µm. Five images were obtained randomly from each coupon. In the case of FESEM, the freeze dried samples were coated with gold using Auto fine coater (JFC-1600). The coupons were subsequently mounted into the exchange chamber of the FESEM (JOEL JSM8701F) for imaging. The images were acquired at accelerating voltage of 5.0 KV, working distance of 0.8 mm and magnification of 5000×. The measurement of single *E.coli DH5α* cell was also obtained with the FESEM

Results

Biofilm Formation on the Stainless-Steel Coupons

.

Figure 3 shows the bacterial attachment to the three substrate profiles tested. The bacterial attachment to stainless steel coupons 2 and 3 with Ra of 1.5 ± 0.18 µm and 2.0 ± 0.09 µm were both higher as compared to stainless coupon 1 with Ra of 0.38 ± 15 µm.

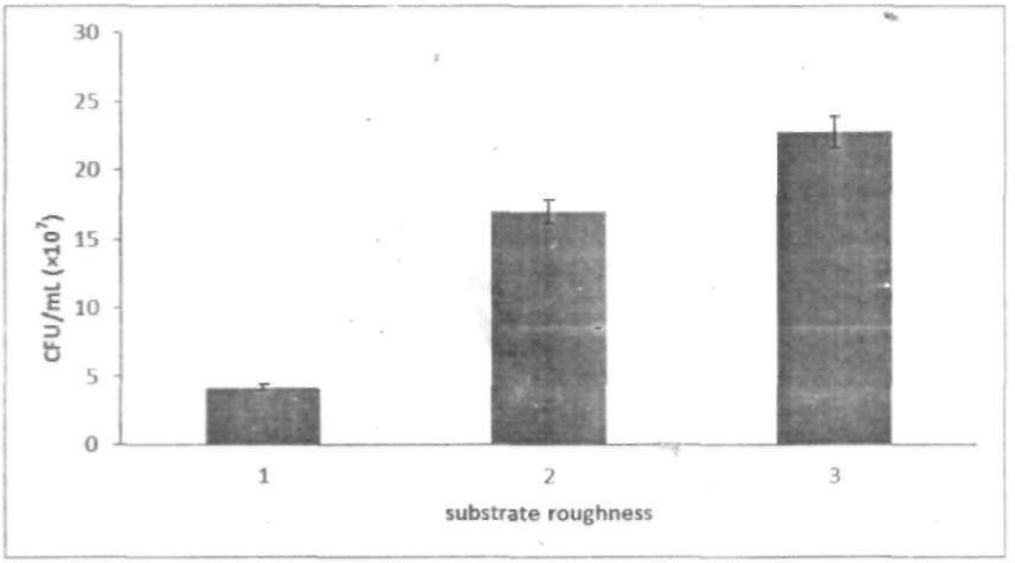


Figure 3: The bacterial attachment on three substrate roughness. The cells were enumerated using CFU count. The experiments were conducted using three coupons in each case and the error bars represent the standard deviations of the mean

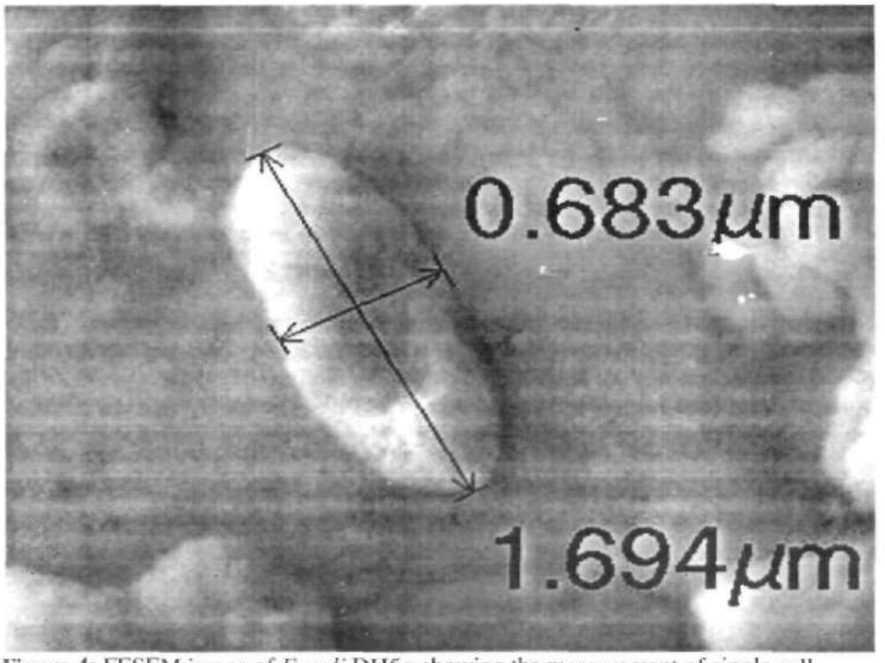
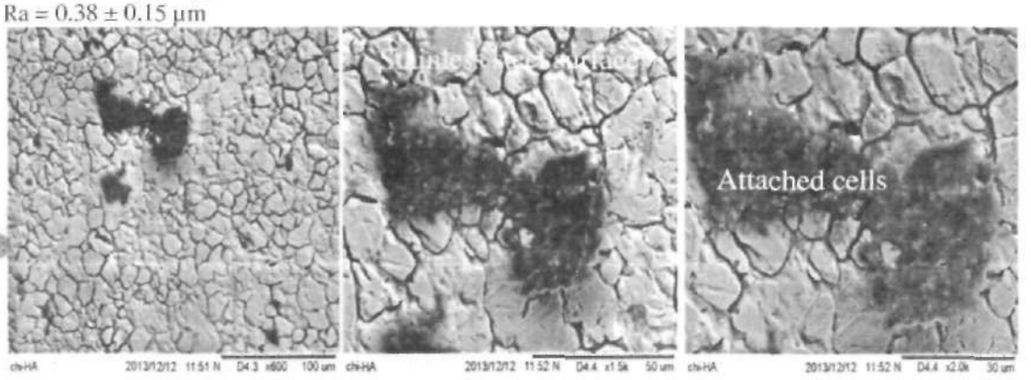


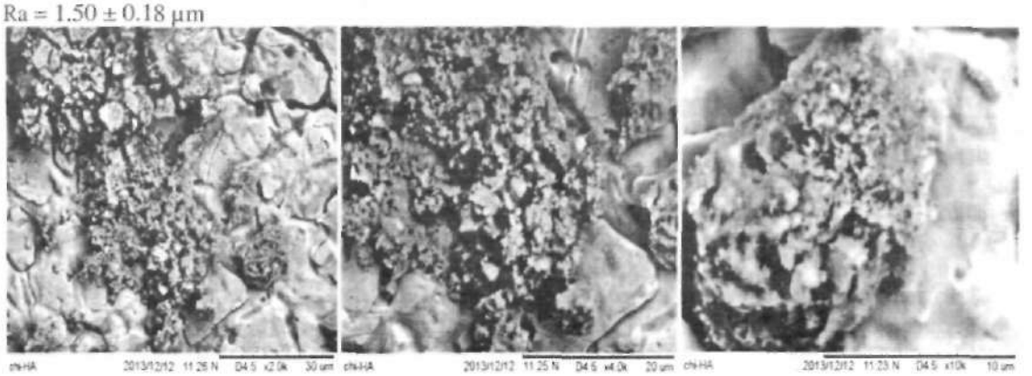
Figure 4: FESEM image of *E. coli* DH5α showing the measurement of single cell

Biofilm Morphology

Ra = 0.38 ± 0.15 μm



Ra = 1.50 ± 0.18 μm



Ra = 2.00 ± 0.90 μm

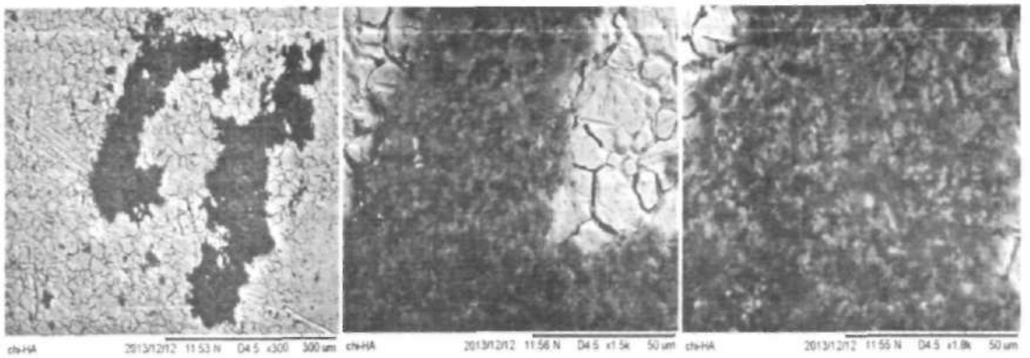
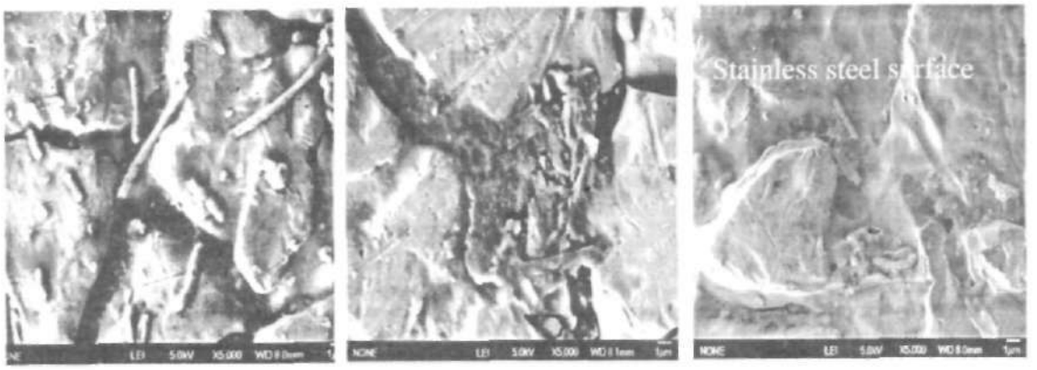
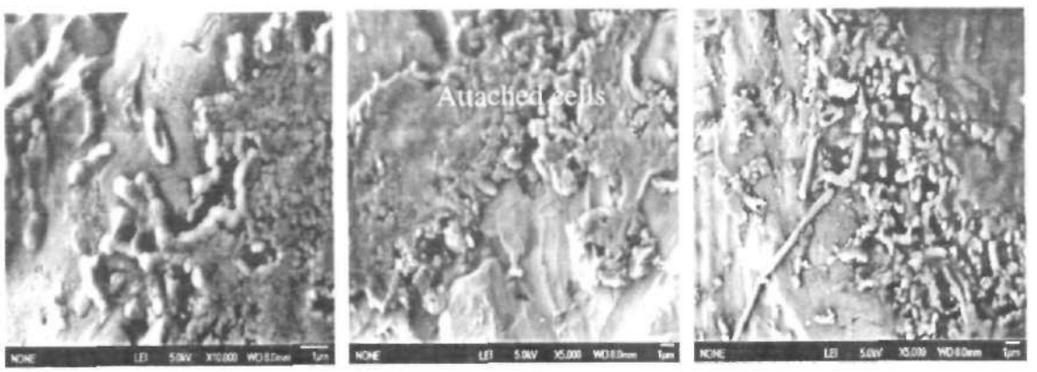


Figure 5: SEM micrographs of stainless steel coupons covered with *E. coli* DH5α biofilm. The images are in increasing order of magnification.

Ra = 0.38 ± 0.15 µm



Ra = 1.50 ± 0.18 µm



Ra = 2.00 ± 0.90 µm

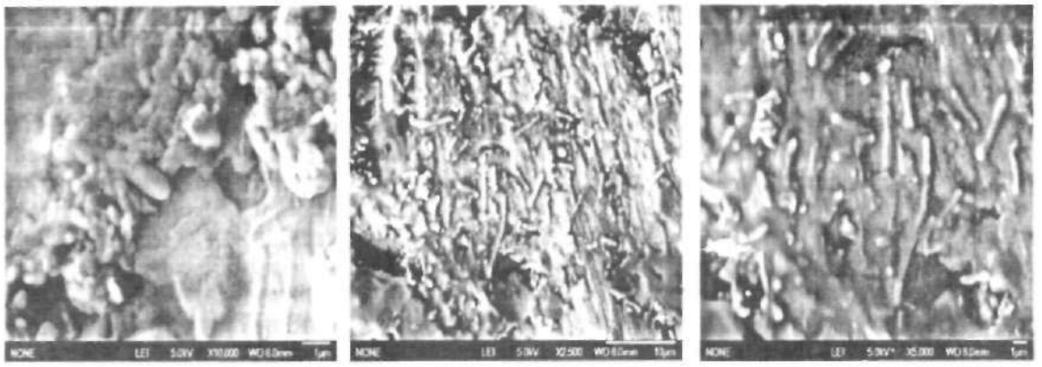


Figure 6: Representative FESEM micrographs of *E. coliDH5a* biofilms formed on the surface of stainless steel coupons. The images were taken at random from different positions at magnification of x 5000 and working distance of 8.0 mm.

Figure 7: FESEM image of *E. coli DH5a* biofilm showing dehydrated EPS.

Discussion

Adherence of bacteria to surfaces and subsequent biofilm formation are influenced by a number of factors as reviewed earlier in this study. This study laid emphasis on surface topography as one of the major factors influencing bacterial attachment. The initial adherence of bacteria to a substratum involves a two-step process; the initial attachment mediated by vdW forces, hydrophobicity and electrostatic attractions. At this phase, the reversibly adhered cells can be easily washed away by the shear force generated in the process of rinsing. The irreversible adhesion is brought about by the accumulation of exo-polysacharides and the actions of bacterial surface appendages such as fimbriae and pili (Hori and Matsumoto, 2010; Mohammed *et al.*, 2013; Muri, 2013). The ability of the *E. coli* cells to remain adhered to the coupons despite rinsing with PBS at high flow rate indicates irreversible attachment of the cells. The reversibly adhered cells can be washed away at relatively high flow rate of about 30 mL/min (32 rpm). The experiment was run for three days during which the *E. coli DH5a* that grow very fast have formed a matured biofilm.

Based on the results of CFU/ mL count obtained from the experiments using the coupons with three different roughness profiles, it can be inferred that bacterial attachments may increase with increase in

surface roughness to a certain threshold. The high bacterial attachment to coupons 2 and 3 can be attributed to the ability of deep crevices and fissures found in the both coupons to hold the bacteria and offer them resistance against the shear forces. Although, the cell surface characteristics, i.e. the presence/absence of fimbriae, flagella (Hori and Matsumoto, 2010), surface charge (Shi and Zhu, 2009) hydrophobicity (Chmielewski and Frank, 2006) and the spreading pressure of bacteria polar force and vdW forces (Frank, 2001; Gall *et al.*, 2013, Mohammed and Dagang, 2017) have all been reported to contribute to bacterial attachment to different surfaces, the size and shape of the bacterial cells play an important role on the surface roughness at which the attachment would be optimal. *E. coli DH5α*, a rod shape bacterium with about 1.67 by 0.68 µm (Figure 4) can easily attached to the stainless steel coupon 2 and 3 with average surface roughness of 1.5 ± 0.18 µm and 2.0 ± 0.09 µm than the stainless steel coupon 1 with roughness of 0.38 ± 0.26 µm

In a related research, (Flint *et al.*, 2000) observed higher *Streptococcus* adherence to stainless steel when the roughness profile was close to bacterial length (0.9 mm). Based on this observation, given that the surface irregularity of stainless steel coupon 1 is far smaller than the *E. coli* cell, only little if any bacterial entrapment is expected on this surface. The geometry of the cells have also been reported to play a leading role in transferring the cells to the substrate surfaces (Raya, 2009).

Meanwhile, there are contradictions in literatures on the effect of surface roughness on bacterial adhesion and inconsistence observations between the theoretical forecast and the experimental results of bacterial attachment to surfaces (Araújo *et al.*, 2010). These conflicting observations may be attributed to the degree of roughness used, the bacterial *spp* tested and the experimental procedures employed by the various researches.

In another development, the biofilm images were obtained with Table top SEM (TM3000). The coupons were carefully and aseptically removed from the flow cell after rinsing with PBS and prepared for imaging. SEM observation illustrated that the *E. coli DH5α* firmly attached to the crevices and fissures found on the surface of the coupons and developed to mature biofilms as shown in Figure to the extents that the biofilms could not be washed away despite rinsing with PBS at high flow rate. This further reinforced the irreversible attachment of the cells and demonstrates their resistance to the shear force generated by the rinsing medium. SEM observation also shows that less bacteria adhered to the coupon with the lower Ra of 0.38 ± 0.15 µm as compared to the other two coupons which has Ra of 1.50 ± 0.18 µm and 2.00 ± 0.90 µm respectively thereby complementing the results of the CFU/ mL count discussed above.

It is however important to note that the extensive sample preparation steps (fixation, dehydration and freezing drying) might alter the structure of the biofilm and make the EPS clearly invisible (McKinlay *et al.*, 2004) and as such microscopic examination with CLSM and ESEM that requires no or less sample preparation may reveal better structure and morphology of the biofilm.

Meanwhile, the FESEM images (Figure 6) show more internal details of the biofilms than the SEM images as the individual cells can be seen at the edge of the biofilms. The dehydrated EPS can also be partially seen (Figure 7). The ability of the FESEM to reveal more internal structures of the biofilm is due to the better resolution and sputter coating of the coupons prior to imaging. Similar to the SEM images, there seems to be less bacterial attachment to the coupon with the least Ra (0.38 ± 0.15 µm) while coupon 2 (1.50 ± 0.18 µm) and coupon 3 (2.00 ± 0.90 µm) were seen to be covered with much biofilm (Figure 7) with some part of coupon 3 entirely covered. This observation is expected considering the fact that the 3D laser micrographs of coupon 2 and 3 show them to be completely rougher than coupon 1 thereby supporting the hypothesis that the *E. coli DH5α* with estimated measurement of 1.67 by 0.68 µm can easily get entrapped to the substrate with Ra of 1.50 ± 0.18 and 2.00 ± 0.90 µm.

Conclusion

A continuous flow system was successfully designed and represented a very useful tool to study biofilm formation on stainless steel coupons with different roughness. The system was able to minimized bubble formation and interference with growing biofilm architecture. Both CFU/ mL and microscopic evaluation of the coupons after three days of continuous flow revealed that coupons 2 and 3 with higher surface roughness, respectively support bacterial adherence and biofilm formation than coupon 1 with lower Ra. Results of this study showed that surface profile exert influence on bacterial adherence and subsequent biofilm development on stainless steel (Type 304 No 4 finish). However, this does not always show a mutual relationship. The substrate elemental composition may also play a vital role in addition to surface profile since the surface topography may differ in other factors that enhance biofilm formation. We recommend a further study of continuous flow system along with detail study of elemental analysis of the substratum on which biofilms grow

References

Araújo, E.A., de Andrade, N.J., da Silva, L.H.M., de Carvalho, A.F., de Sá Silva, C.A., and Ramos, A.M. 2010. Control of microbial adhesion as a strategy for food and bioprocess technology. *Food and Bioprocess Tech.* 3, 321-332.

Beloin, C., Roux, A., and Ghigo, J.-M. (2008) Escherichia coli biofilms. In *Bacterial Biofilms*. Springer, pp. 249-289.

Chmielewski, R., and Frank, J. 2006. Biofilm formation and control in food processing facilities. *Comprehensive reviews in food science and food safety* 2, 22-32.

Flint, S., Brooks, J., and Bremer, P. 2000. Properties of the stainless steel substrate, influencing the adhesion of thermo-resistant streptococci. *J. Food Eng.* 43, 235-242.

Frank, J.F. 2001. Microbial attachment to food and food contact surfaces. *Advances in food and nutrition research* 43, 319-370.

Gall, I., Herzberg, M., and Oren, Y. 2013. The effect of electric fields on bacterial attachment to conductive surfaces. *Soft Matter.*

Hori, K., and Matsumoto, S. 2010. Bacterial adhesion: from mechanism to control. *Biochemical Engineering Journal* 48, 424-434.

Huang, C.T., Peretti, S.W., and Bryers, J.D. 1994. Effects of medium carbon-to-nitrogen ratio on biofilm formation and plasmid stability. *Biotechnology and Bioengineering* 44, 329-336.

McKinlay, K.J., Allison, F.J., Scotchford, C.A., Grant, D.M., Oliver, J.M., King, J.R., Wood, J.V., and Brown, P.D. 2004. Comparison of environmental scanning electron microscopy with high vacuum scanning electron microscopy as applied to the assessment of cell morphology. *Journal of Biomedical Materials Research Part A* 69, 359-366.

Mohammed, J.N., Abubakar, B.M., Yusuf, H., d Sulaiman, M., Saidu, H., Idris, A., and Tijani, H. 2013. Bacterial biofilm: a major challenge of catheterization. *J. of Microbiology Res* 3, 213-223.

Mohammed, J.N. and Dagang, W.R.Z.W., 2017. Effect of surface roughness on susceptibility of Escherichia coli biofilm to benzalkonium chloride. *Malaysian Journal of Fundamental and Applied Sciences, 13*, (1) 14-18

Muri, I.D. (2013) Atomic force microscopy measurements of the surface and the interaction characterization to optimize the surface patterning for bacterial micro arrays. Norwegian University of Science and Technology.

Nielsen, M.W., Sternberg, C., Molin, S., and Regenberg, B. 2011. Pseudomonas aeruginosa and Saccharomyces cerevisiae biofilm in flow cells. *Journal of visualized experiments: JoVE.*

O'Sullivan, C., Burrell, P., Pasmore, M., Clarke, W., and Blackall, L. 2009. Application of flowcell technology for monitoring biofilm development and cellulose degradation in leachate and rumen systems. *Bioresource Technology* 100, 492-496.

Pamp, S.J., Sternberg, C., and Tolker-Nielsen, T. 2008. Insight into the microbial multicellular lifestyle via flow-cell technology and confocal microscopy. *Cytometry Part A* 75, 90-103.

Raya, A. (2009) Fundamental Study of the Initial Bacterial Attachment of Pseudomonas aeruginosa, Pseudomonas putida and Escherichia coli. The University of Akron.

Schlisselberg, D.B., and Yaron, S. 2013. The effects of stainless steel finish on< i> Salmonella</i> Typhimurium attachment, biofilm formation and sensitivity to chlorine. *Food microbiology.*

Sezonov, G., Joseleau-Petit, D., and D'Ari, R. 2007. Escherichia coli physiology in Luria-Bertani broth. *Journal of bacteriology* 189, 8746-8749.

Shi, X., and Zhu, X. 2009. Biofilm formation and food safety in food industries. *Trends in Food Science & Technology* 20, 407-413.

Soleimani, S., Ormeci, B., and Isgor, O.B. 2013. Growth and characterization of Escherichia coli DH5α biofilm on concrete surfaces as a protective layer against microbiologically influenced concrete deterioration (MICD). *Applied Microbiology and Biotechnology* 97, 1093-1102.

Watnick, P., and Kolter, R. 2000. Biofilm, city of microbes. *Journal of bacteriology* 182, 2675-2679.

White-Ziegler, C.A., Um, S., Pérez, N.M., Berns, A.L., Malhowski, A.J., and Young, S. 2008. Low temperature (23 C) increases expression of biofilm-, cold-shock-and RpoS-dependent genes in Escherichia coli K-12. *Microbiology* 154, 148-166.