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Morphological Evaluation of a Multispecies Biofilm on A106 GB Industry-Finished Steel Used from Heat Exchangers

Alicia Prithiraj[a*], Shepherd M. Tichapondwa[a], Jackie M. Nel[b], Evans M.N. Chirwa[a]

[a]University of Pretoria, Department of Chemical Engineering, Hatfield, Pretoria
[b]University of Pretoria, Department of Physics, Hatfield, Pretoria
alicia.prithiraj@gmail.com

Biofouling in heat exchangers is a challenge in industry leading to efficiency and maintenance losses. A 3.4 % of the global gross domestic product (GDP) in 2013, estimated the global cost of corrosion to be US$ 2.5 x 10^{12}. The purpose of the study was to evaluate the stages of multispecies biofilm formation on finished steel surfaces with emphasis on bacterial cell attachment at early stages. Although many studies have been conducted regarding bacterial attachment to metallic surfaces, little is known about the impact of the conditions and mechanisms of attachment in the early stages. It is not known which type of bacterial strains will selectively attach in this environment. Third-generation sequencing was conducted to give insight on the abundant species at the surface, and it was observed that *Firmicutes* attached to the rough and smooth surfaces and *Pseudomonas sp*. was prevalent on the rough surfaces. Scanning electron microscopy (SEM) was used to count the abundant rod-shaped bacteria in colony sites on smooth surfaces on day 3 only. However, on rougher surfaces bacteria could not be easily detected due to biofilm fully covering the sites. Atomic Force Microscopy (AFM) was used to quantify surface roughness on the bacterial biofilm. Roughness values from the AFM on the smooth surface showed a significant increase as compared to the rough surface, indicating bacterial attachment. Mature and complex structures started forming at later growth stages. Smooth finished surfaces showed reduced biofilm formation and selective attachment of *Pseudomonas sp*. on rough surfaces is known to aid in steel corrosion and subsequent failures.

1. Introduction

Initial attachment, microcolony formation, maturation, and dispersion form the biofilm process. During initial attachment bacteria get close to the metal surface and, this is caused by a combination of physical factors and chemical forces. The bacterial cell appendages further influence attachment to the metal surface. pH and temperature can also influence the bacterial attachment. Following the attachment stage, multiplication and division of the bacteria starts. Extracellular Polymeric Substance (EPS) originates as a protective layer for the bacteria (Santos et al., 2018). Once an oversaturation of microbial cells were observed during the last stages of biofilm formation, the bacteria will disperse from the biofilm once the protective layer is removed at the top of the bulb-like structure. The bacterial cells can then freely move around in a motile form (Marra et al., 2022). EPS formation in heat exchangers results in biofouling, leading to loss in heat transfer efficiency and tube failures (Mathew et al., 2021). A study conducted by Kolenbrander et al. (2006) explained that the initial attachment of the bacteria facilitated the attachment of other bacterial organisms that were not capable of binding to host surfaces and, can ultimately lead to the development of a biofilm community. This suggested that attachment of the total bacterial colony may be significantly influenced by early colonizers rather than late colonizing bacteria (Dezelic and Schmidlin 2009). Moreover, bacterial interaction and attachment play a role in biofouling, and it is hypothesized that during growth, in a multispecies system, the middle and/or late colonizers attach to the already present biofilm and not the metal surface.

The influence of surface finish using a mixed bacterial culture obtained from the petrochemical processing plant is rarely reported in literature. A few researchers have studied a mixture of 2 or 4 bacterial strains applied to modified surfaces (Jamal et al., 2018, Santos et al., 2018). This does not include the situation in the plant environment, and it is not known which type of strain bacterial will selectively attach to the steel surface.

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Traditional methods used microscope imaging to quantify bacterial attachment, mainly single strain bacteria and short time exposure to bacteria were evaluated (Duarte et al., 2009), however, in this study, this method could not be accomplished on rough surfaces. Bacteria were only visible on the smooth surface on day 3.
After multiple tube failures of an existing heat exchanger, it was reported by the petrochemical plant engineer that the sludge from a heat exchanger after 3 years of operation had contained iron-bacteria, the bacteria was not characterized, and it was not fully understood as to which bacteria started the failures. Due to accelerated equipment failure in these systems, it is imperative that early colonizers should be understood.
Understanding and visualizing multispecies attachment on different surfaces includes methods using third-generation rDNA sequencing (Park et al., 2019). AFM has proven to be a tool for characterizing, both qualitatively and quantitatively, aspects of biofilm/substratum interactions as well as surface morphology including surface roughness (Yuan et al., 2019). Biofilms may be prevented by inhibiting the initial colonization step of the biofilm lifecycle. An increase in surface roughness from a smooth surface has a significant effect on selective microbial attachment to that surface (Nouri et al., 2023). This study sheds light on this statement, with emphasis on multispecies biofilms.

2. Materials and methods

2.1 Metal preparation

The A106 GB carbon steel coupons were sourced from the petrochemical processing plant, measuring 1 cm by 1 cm, were mounted in Bakelite and automatically polished to 400 grit and 3 micron using silicon carbide waterproof paper and 3 micron polish respectively. The coupons were polished using an automatic polishing machine (Struers Tegramin-30, United States, Cleveland, Ohio) machine for 3 minutes, using a force of 35 N at 300 revolutions per minute. Under a laminar flow hood, carbon steel alloys were fastened on cable ties, marked using tape, and sterilized for 1 hour using 70 % ethanol, then removed and left to dry. The cable ties with the coupons were inserted into the batch reactor containing prepared media inoculated with bacteria and sealed with a lid and parafilm. Six coupons were used in this study which includes day 3, 6 and 13.

2.2 Bacterial cultivation and media preparation

A stainless-steel perforated mesh designed to collect bacteria, with dimensions 0.178 mm x 12.7 mm x 76.2 mm was inserted in situ at a coupon rack in a cooling tower circulation pipeline at the petrochemical plant in South Africa. The mesh was left in place for a period of 11 months. Cooling water was directly transported from the cooling tower to the coupon rack at low flow. The coupon rack was designed to hold only one biofilm mesh. The mesh was removed from the coupon rack using sterilized tweezers and inserted in a sterile sample bag containing the cooling tower water.
The bag with the mesh was transported to an on-site microbiology laboratory. Up arrival at the laboratory, the mesh was immediately transferred into a batch reactor containing autoclaved prepared media with sterilized carbon steel alloys. The batch reactor was placed in an incubator equipped with a thermostat and set at a constant temperature of 35 °C to simulate the cooling tower temperature conditions. The bacteria on the coupon surfaces was grown anaerobically. The reactor was only opened to remove the alloys, it was possible that the media was exposed to oxygen. It is to be noted that cooling towers operate as open systems. It is worth mentioning that during commissioning of a new heat exchanger and before introducing cooling water into the heat exchanger tubes, the pipelines and tubes were purged with nitrogen to ensure that there was no oxygen present when introducing the product into the heat exchanger, creating an anaerobic environment. When cooling water was supplied to the heat exchangers there were instances where the water was stagnant (such as behind the baffle plates) allowing for the growth and attachment of bacteria to these areas. Modified batch mineral medium was prepared from 0.501 g KH_2PO_4, 1.000 g NH_4Cl, 4.502 g Na_2SO_4, 0.005 g $CaCl_2.2H_2O$, 0.062 g $MgSO_4.7H_2O$, 12.012 g 50 % solution sodium lactate, 1.001 g yeast extract, 0.004 g $FeSO_4.7H_2O$, 5.002 g $Na_3C_6H_5O_7$ sodium citrate mixed in 1.00 L distilled water to simulate the mineral rich water quality of river water fed to the cooling towers with minimal carbon sources. The initial pH of 6.52 was adjusted to 7 using 5 M NaOH. The adjustment of pH was conducted for consistence with other studies on the impact of metal finish on bacterial attachment (Zhu et al., 2003, Prithiraj et al., 2019). Day 3, 6 and 13 were chosen for this study based on a previous growth model. The growth model revealed significant changes in growth on day 3 (lag phase), day 6 (exponential phase) and day 13 (death phase) (Prithiraj et al., 2023).

2.3 Microscopy

After removing alloys from the batch reactor, they were gently rinsed with sterile distilled water and placed on a paper towel, then placed on the carbon tape for observation under each microscope. The samples were studied using a Crossbeam 540 Zeiss Gemini 2 microscope (Oxford Instruments, Zeiss Gemini 2), with an accelerating voltage of 5 kV. Rough and smooth coupons were used to count bacterial cells on day 3, at a magnification of

5000x using 10 fields of vision. The samples were also studied in a Bruker Dimension Icon AFM with ScanAsyst (Germany). Samples were not coated for SEM and AFM analysis.

2.4 Culture characterization

Separate samples within the same batch reactor were used for this analysis and the middle of the samples (polished side) was swabbed using sterile swabs.
Genomic DNA (gDNA) extraction of the swabs was done by using ZymoBIOMICS DNA Mini-preparation kit (Zymo Research). The extracted gDNA was amplified in a PCR (Polymerase chain reaction) machine (Eppendorf Mastercycler Nexus Gradient), using a universal primer pair 27F and 1492R (Zhao et al., 2022). This was done in order to target the V1 and V9 region of the bacterial 16S rDNA gene. The resulting amplicons were barcoded with Pacbio M13 barcodes for multiplexing through limited PCR. The resulting barcoded amplicons were quantified and a pooled equimolar and AMPure PB bead-based purification step was then performed. The PacBio SMRTbell library was prepared from the pooled amplicons following the manufacturer's protocol. Sequencing primer annealing and polymerase binding was done following the SMRTlink software protocol to prepare the library for sequencing on the PacBio Sequel lle system.
Sample sequencing was done using the Sequel system by Pacbio software. Raw subreads were processed through the SMRTlink (v9.0) software and usearch. The taxanomic information was determined based on the Ribosomal database project's 16s database v16. Highly accurate reads were processed via Circular Consensus Sequences (CCS) and Vsearch software to produce a metagenomic report with species read count and percentage abundance.

3. Results

3.1 Culture characterization

Culture characterization was done on day 3. It was reported by Park et al. (2019) that the early colonizing bacteria attach to the metal substrate, influencing the attachment of middle and later colonizing bacteria.

Table 1: Abundant bacterial colonizers during day 3 early stages of bacterial attachment

Surface	Bacterial species	Abundance[a]	Bacterial Count
Smooth finish	*Clostridium sp.*	78 %	24.5 ± 7.9
Rough finish	*Clostridium sp. Pseudomonas sp.*	83 %	Could not be determined by SEM

[a] remaining species amounted to less than 1 % or included unknown species.

The abundant *Clostridium sp*. species attached on both surface substates, where *Pseudomonas sp*. favoured the rougher finished surface. This suggested that surface finish had an influence on the selective attachment of bacterial cells. The rough finished surface exhibited a biofilm layer which covered the surface quicker than the smooth surface and the bacterial cells could not be counted (Table 1) this was evidenced by SEM images in Figure 1 (D). The rough surface exhibited 83 % of the abundant species more than the smooth surface at 78 %. *Pseudomonas sp*. and *Clostridium sp*. bacteria being reported as slime-forming bacteria and have metal-related metabolic activates drawing them to the steel surfaces at early stages (Jeong et al., 2023). The rough finished surfaces exhibited grooves and scratches of about 1-1.5 µm (Figure 3 (C)), where the width of the bacteria generally range from about 0.5 to 0.8 µm, which further facilitated the attachment of cells to a larger surface area (Nouri et al., 2023). Future work may be conducted in quantifying the abundant bacterial cells through qPCR and use of special manufactured plasmids on day 3 initial attachment. The *Clostridium sp*. produce hydrogen-sulphide and organic acids and have been associated with corroding metals. Most hydrogen-producing *Clostridium sp*. characterized were fresh-water species. In this case the water distributed to the cooling towers was sourced from a Dam. An industrial study by Zhu et al. (2003), posited that *Pseudomonas sp*. and *Clostridium sp*. may be the main role players in steel equipment failures owing to their ability to secrete organic acids.

3.2 Topography of the alloy samples

Figure 1 below depicts the differences in bacterial attachment on the rough and smooth surfaces, indicating the first stage (day 3) to the last stage (day 13). Figure 1 (A) is a high magnification image of the smooth surface on day 3 indicating the bacterial cell which was visualized by the SEM and revealed EPS being secreted. In Figure 1 (B) is a high magnification image of the rough surface on day 13, where the bacteria was observed in its motile form depicting a smooth cell. In Figure 1 (C) smooth surface on day 3, bacteria visibly attached to the surface and could be counted in various sites, however, in Figure 1 (D) rough surface on day 3, the bacteria

produced biofilm rapidly which covered the entire surface and the bacteria could not be visualized under the microscope. This may be owing to the two dominant slime forming bacteria being present on the rough surface. It can be seen in Figure 1 (E smooth and F rough surface) which is the second stage of biofilm development, that the bacteria were no longer visible over the surface, the biofilm started to mature as compared to the first stage. Figure 1 (G smooth and H rough surface) depict the mature and complex biofilm structures which were only seen on day 13. In Figure 1 (H) the motile bacteria can be seen on top of the biofilm (indicated with a red arrow), and was not observed on the smooth surface on day 13, and may be owing to a slower biofilm maturation rate.

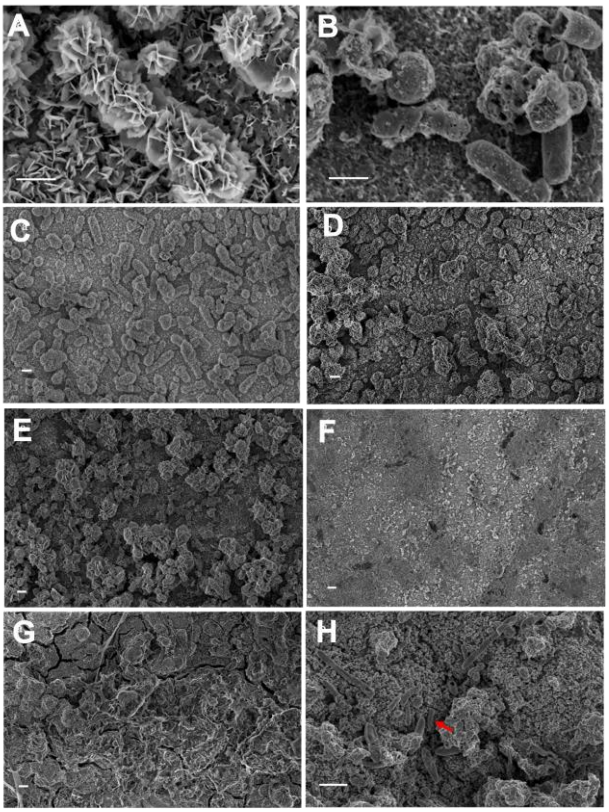

Figure 1: SEM image of day 3 smooth (A at scale 1 µm and C at 2 µm) and rough (D at scale 2 µm) surface, day 6 smooth (E at scale 2 µm) and rough (F at scale 2 µm) surface, day 13 smooth (G at scale 2 µm) and rough (B at scale 2 µm and H at 1 µm) surface.

The mature and complex structures mentioned in Figure 1 (H) rough surface on day 13 are now depicted as high magnification images in Figure 2 (A and B), which takes on a flower-like appearance and in the bulb-like structure seen in Figure 2 (A), bacteria will disperse from the opening in the structure.

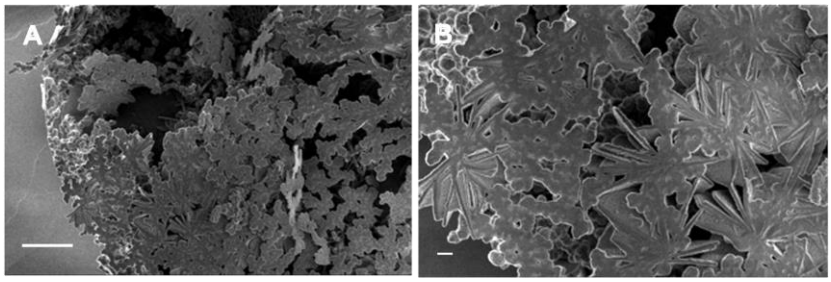

Figure 2: Day 13 SEM image of rough finish depicting the mature biofilm (A) with a bulb-like structure (scale at 20 µm) and (B) the higher magnification of part of the structure (scale at 2 µm).

3.3 Surface roughness of the biofilm

The root mean square roughness (RMS) values of the 10 µm images obtained by AFM analysis on the smooth surface on day 3, increased from 8.7 ± 1.2 nm to 114.67 ± 10.69 nm after exposure to bacteria on the smooth surface. The significant increase was due to bacterial attachment to the surface, this was evidenced in the SEM images Figure 1 (A and C). The rough surface exhibited an increase in RMS, from 39.2 ± 15.9 to 70.70 ± 2.1 nm, however, minimal bacterial cells could be observed and rather microcolonies and biofilm was observed in the SEM image in Figure 1 (D). It is then hypothesized that the biofilm roughness would increase as the biofilm matures into more complex structures. The spatial distribution and development of the biofilm (Figure 3 (D)) may influence the roughness of the sample. More understanding is needed on spatial patterns. The AFM was used to evaluate both the initial metal surfaces before exposure and the surfaces after exposure to bacteria as seen in Figure 3 (A, B and C). The matrix is composed of EPS, which provides architecture and stability to the biofilm. Nutrients needed by bacteria to grow, and other molecules move through pores and channels of the matrix (Flemming et al., 2016).

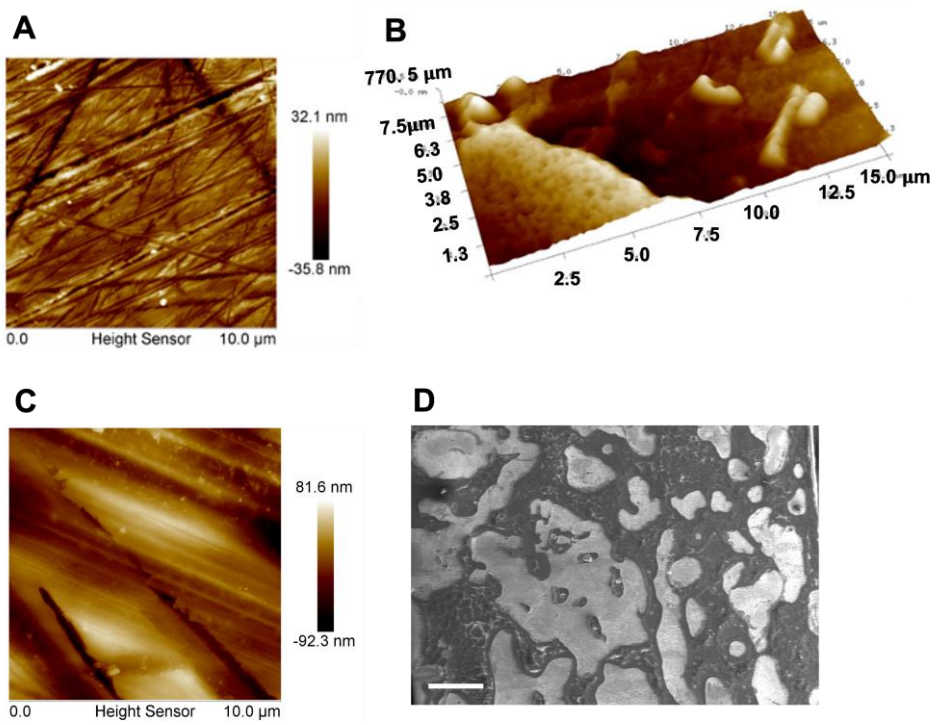

Figure 3: (A) 2D AFM image showing a smooth metal surface before exposure to bacteria. (B) 3D AFM image of the biofilm at early stages (day 3) resembling a porous exterior. Bacteria can be seen 3 µm in length attached to the surface. (C) 2D AFM image showing a rough metal surface before exposure to bacteria. (D) SEM micrograph (1 mm scale) of spatial patterns observed on the smooth surface at early stages.

In a recent multispecies study by Prithiraj et al. (2023), it was deduced that *Clostridium sp*. dominated attachment to the surfaces and produced an organic acid (acetic acid), which may influence metal corrosion. The selective attachment of *Pseudomonas sp*. with abundant *Clostridium sp*. on the rough surface may bring about a unique corrosion effect on steel. Corrosion investigations were limited in this work and will be conducted in future. *Clostridium sp*. was reported to be one of the key role players contributing to industrial failures.

4. Conclusion

This study gives insight into visualization of multispecies bacteria during initial stages of attachment to differently finished alloys. From the study it was observed that the bacterial species which selectively attached to the steel may provide more valuable information than quantifying the total bacterial surface. This information can be used in the industry to prevent initial colonizing bacteria by implementing dosing strategies targeted at the abundant species. To obtain more quantitative information about the bacterial levels on surfaces a special manufactured plasmid can be used to quantify the abundant *Clostridium sp*. on day 3, alternatively a pGEM-T plasmid can

produce data on absolute cell quantification. Further studies on time-related differences in bacterial attachment and corrosion on the rough and smooth surface is underway. Further studies can be conducted on spatial distribution on day 3 and emergent architecture of the EPS on day 13. Day 13 SEM analysis showed the opening of the bulb-like structure where cells disperse. AFM can effectively scan a 10 µm surface area and a larger scan size may be time consuming including more variation due to the heterogeneity of the biofilm. SEM analysis identified different shapes of bacteria which attached during early stages, mainly rod and coccus shaped bacteria could be seen. It was observed from this study that during initial attachment, *Pseudomonas sp*. selectively attached to the rough surface.

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