Comparison of Biofilms Morphology for Two Strains of Marine Sulfate-Reducing Bacteria using Atomic Force Microscopy
(Perbandingan Morfologi Oleh Dua Strain Bakteria Penurun Sulfat Marin Menggunakan Force Microscopy Atom)

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ABSTRACT

AISI 304 stainless steel coupons were immersed in VMNI medium containing two strains of Sulfate-reducing bacteria which were isolated from Port Dickson, Negeri Sembilan. The immersion period were set for twenty-four hour, seven days, nine days and fifteen days. At the end of each period, the biofilm were formed on the surface will be maps using Atomic Force Microscopy. The image were taken using contact mode in the 70 µm x 70 µm and 10 µm x 10 µm resolution. Bacteria counts were done using the dilution and plate count technique. Image showed that SRB2 contain more bacteria cell attached to the metal surface by the 7th day of immersion compare to SRB1. But SRB1 produce more condensed and slime like biofilm with some crystallization precipitation while SRB2 bacteria cell were loosely attached. SRB1 bacteria cell were engulf in the slime like matrix while SRB2 bacteria cell is densely packed together. SRB2 showed a slightly higher rate growth in the medium compared to SRB1 but produce low amount of biofilm matrix while SRB1 produce a lot of bio-matrix precipitation and slime formation.

Keywords: Sulphate-reducing bacteria, Extra-Polymeric Substances, biofilm, Atomic Force Microscopy

ABSTRAK

AISI 304 kupon keluli kalis karat telah direndam dalam medium VMNI yang mengandungi dua strain bakteria bakteria penurun sulfat marin yang telah disampel daripada Port Dickson, Negeri Sembilan. Tempoh rendaman telah ditetapkan untuk dua puluh empat jam , tujuh hari , sembilan hari dan lima belas hari. Pada akhir setiap tempoh, biofilm yang terbentuk di permukaan akan diukur dengan menggunakan Atomic Force Microscopy. Imej diambil menggunakan mod kenalan beresolusi70 µm x 70 µm dan resolusi 10 µm x 10 µm . Pengiraan bakteria telah dilakukan dengan menggunakan teknik pencairan dan kiraan plat . Imej menunjukkan SRB2 mengandungi lebih banyak bakteria sel yang melekat pada permukaan logam pada hari ke-7 rendaman berbanding SRB1 . Tetapi hasil SRB1 lebih pekat dan lendir seperti biofilm dengan beberapa penghabluran termendap sementara sel bakteria SRB2 adalah kurang termendap. SRB1 sel bakteria telah melanda dalam lendir seperti matriks tetapi sel bakteria SRB2 dibungkus padat. SRB2 menunjukkan pertumbuhan pada kadar sedikit lebih tinggi di dalam medium berbanding SRB1 tetapi menghasilkan jumlah matriks biofilm yang rendah manakala SRB1 menghasilkan banyak mendapan bio -matriks dan pembentukan lendir .

Katakunci: Bakteria penurun sulfat, bahan tambahan polimer, biofilm, Atomic Force Microscopy

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INTRODUCTION

In natural environments, bacteria can live independently, as planktonic cells, but often form complex communities that survive attached to surfaces (Oh et al. 2007). These can be formed by one single or by multiple species, with different metabolic mechanisms, which in many cases function as cooperative consortia (Cordas et al 2008). Bacterial adhesion to biological surfaces is the first step in the formation of a biofilm that initiate biofouling, i.e. the undesirable colonisation of a surface by microorganisms that immobilise particulate matter and heavy metals and influence corrosion processes (Me'ndes-Vilas et al 2004; Bachman & Edyvean 2006).

The biofilm can be defined as microbial communities, containing large different numbers of microorganisms which produce a wide range of biopolymers and adhere to solid surface (Kreft & Wimpenny 2001; Kujundzic et al. 2007; Mahmoud et al. 2008; Pradhan et al 2008). They are typically of 30 to 40 mm in thickness and have channels for transport of water, nutrients and waste by molecular diffusion. Bacteria in biofilms use some combination of pili, fimbriae, and secreted exopolymeric substances (EPS) to adhere to an interface (Pradhan et al 2008). Biofilms are of particular interest because of their involvement in infections and biofouling of industrial surfaces (Kujundzic et al. 2007; Pradhan et al 2008; Houdt & Michiels 2010). It also caused various problems such as medical infections, fouling of water cooling system, product contamination, and microbiologically influenced corrosion (MIC) (Wen et al. 2009).

Extracellular polymeric substances (EPSs) are composed of a mixture of macromolecules, such as proteins, polysaccharides, nucleic acids and lipids, and are often referred to as glycocalyx or slime, which facilitate bacterial attachment to the substratum. The cellular component can be as little as 15% in the biofilm volume and all others are organic components of EPS, including proteins, polysaccharides, nucleic acids and water (Pradhan et al 2008; Cordas et al 2008; Flemming & Wingender 2010). They are responsible for the increase of friction resistance changes of surface properties such as hydrophobicity, roughness, and color. Rapid succession of species within the community seems to be mainly Gram Negative rods that produce copious quantities of extracellular binding materials (Mahmoud et al. 2008). Once thought to be an anomaly, in fact most bacteria can and do form biofilms. Because they are formed at interfaces and particularly well-suited to study by AFM (Volle et al. 2008).

AFM have been used to observe the development of bacterial biofilms on stainless steel, to determine the degree of steel deterioration owing to the occurrence of extensive pitting corrosion beneath the heterogeneous biofilms (Yuan & Pehkonen 2007; Volle et al. 2008). The combination of high resolution and non-damaging preparation has made AFM especially valuable for studying a wide variety of bacteria in both air and liquid environments (N'unez et al. 2005). It also were used to investigated the MIC rates on mild steel in the presence of sulfate-reducing bacteria (SRB), and further profiled the changes in the width and the depth of pits with time using AFM (Yuan & Pehkonen 2007).

SRB are anaerobic microorganisms that are widespread in anoxic habitats, where they use sulfate as a terminal electron acceptor for the degradation of organic compounds, resulting in the production of sulfide (Muzyer & Stams 2009). SRB are among the anarobic bacteria to blamed for causing MIC by forming forming biofilm where pitting happen. With further development of the biofilms, metabolic products may lower the local pH, thus deepening the pits (Wen et al. 2009).

This research was conducted in order to investigate the morphology of biofilm constructed from two different strain of SRB, designated as SRB1 and SRB2. To determine the adhesion of bacteria cell on the metal surface and captured the development of biofilm on each stage along the 15 days immersion. Also to relate the growth curve of SRB bacteria with the formation of the biofilm on the metal surface.

MATERIALS AND METHODS

Bacteria Culture

Bacteria strain SRB1 and SRB2 were cultured in 300 ml VMNI broth. In this experiment, 4 cultures were prepared for each strain. Stainless steel AISI 304 coupon with a diameter 1cm x 1cm x 1cm was grind with 400, 600, 800, 1000 and 1200 grit of sand paper before polished using 1μm diamond spray until it creates a mirror image on the surface. The coupons then were immersed in SRB1 and SRB2 cultured respectively. Each culture was incubating at 37°C for a different length of time; 24 hours, 7 days, 9 days and 15 day. Each day, lactate solution will be provided into the broth as the carbon source. After the end of the day, the surface of the metals were maps using Atomic Force Microscopy technique.

Atomic Force Microscopy

The metal is removed from the immersion and dried by wicking the liquid off of the edge with a paper towel or lightly blowing the liquid off with a stream of nitrogen and allowing it to sit in a covered clean petri dish for a few minutes until any remaining water had dried. Bacteria were imaged immediately without any further treatment (N'unez et al. 2005).

Biofilms were imaged in contact mode using silicon nitride cantilever with resonance frequencies of 6–21 kHz and a spring constant of 0.07–0.4 N/m in air. Contact mode is to provide morphological data in conjunction with the tip and the materials interested. Fresh cantilevers were used for each experiment to prevent sample cross contamination (Oh et al. 2007). Images were taken first with the width area of 70 μm x 70 μm. And in that area, a 10 μm x 10μm image were taken to view in the surface. Image than were analyze using Image Analysis (IA) 2007 NT-MD to get a better view of the AFM image.

Bacteria Growth

During 15 days of immersion, the number of bacteria growth were observed and recorded using dilution and plate counting technique then the number of growth were calculated using the below equation (Sahrani et al. 2008) :

$$CFU = \frac{No.\,of\,colony\ \times dilution\,factor\ \times\ 1ml}{0.1\,ml}$$

Where:

    CFU = Colony form unit

RESULTS AND DISCUSSION

Atomic Force Microscopy Image

Figure 1 showed the image of biofilm formation for SRB1 and SRB2 for day 1, day 7, day 9 to day 15 in the area width of 70 x 70. Figure 2 showed the image of biofilm formation, in pairs, for SRB1 and SRB2 for day 1, day 7, day 9 to day 15 in the area width of 10 x 10.

A small trace of bacteria and extra polymeric substances can be seen in both figure 1 and 2 for day 1 for SRB1 and SRB2. But the coverage of biofilm in this stage is still low. Crystallization substances can be seen on both of the metal surface SRB1 and SRB2 immersion. SRB2 showed more bacteria cell present compare to SRB1.

Day seven for both figure 1 and figure2 showed a lot of differences between SRB1 and SRB2. The metal surface is almost covered either by the bacteria or EPS. SRB1 have a lot of crystallization substance on the surface of the metal. A close up of the surface in figure 2 showed a bacteria covered by crystal like substances on it exterior. There is a lot of patches of this substances scattered all over the surface. SRB2 consist of more packed bacteria cell that covered its metal surface. Figure 2 for SRB2 on day seven showed that the bacteria were still loosely attached to each other even though they are densely packed together.

There was an increase in the number of bacteria cell for SRB1 on day nine. Bacteria cell were visibly seen but the present of a crystallization substance can still be detected on its surface. In Figure 2b, for SRB1 during day nine, a bacteria form under slime like EPS layer that covered the bacteria cell plus the formation of crystallization substance on top of the layer. SRB2 increase in cell density and surface coverage percentage, as more cells were observed in figure 1. Figure 2 showed that SRB2 still contain loosely attached cell where the form of the bacteria cell can be trace. But there is a formation of a solid body layer that was form from stack of cell together.

Day 15 for both SRB1 and SRB2 showed the formation of a very thick layer of biofilm on the surface. SRB1 still have the crystallize substances forming on the metal surface, but most of the bacteria were under a thick formation of EPS substances that densely foiled together forming a hard body shaped. SRB2 form a thick, but porous form of EPS on the surface. In figure 1, day 15 for SRB2, it showed that the biofilm is very thick at the center but become loose at the edge. This is confirmed when the shape of the EPS can be trace as it does not compactly build towards each other.

Atomic force microscopy (AFM) is a powerful tool that allows for imaging of biological samples down to nanometer scale resolution and under their physiological conditions (Pradhan et al 2008), which will be difficult or otherwise impossible using electron or light microscopy. Another advantage of AFM is that it requires minimum sample preparation and can be operated under wet or dry conditions. Consequently, AFM has been frequently used in interpreting corrosion phenomena induced by biofilms formed on metal surfaces (Yuan & Pekhonen 2007).

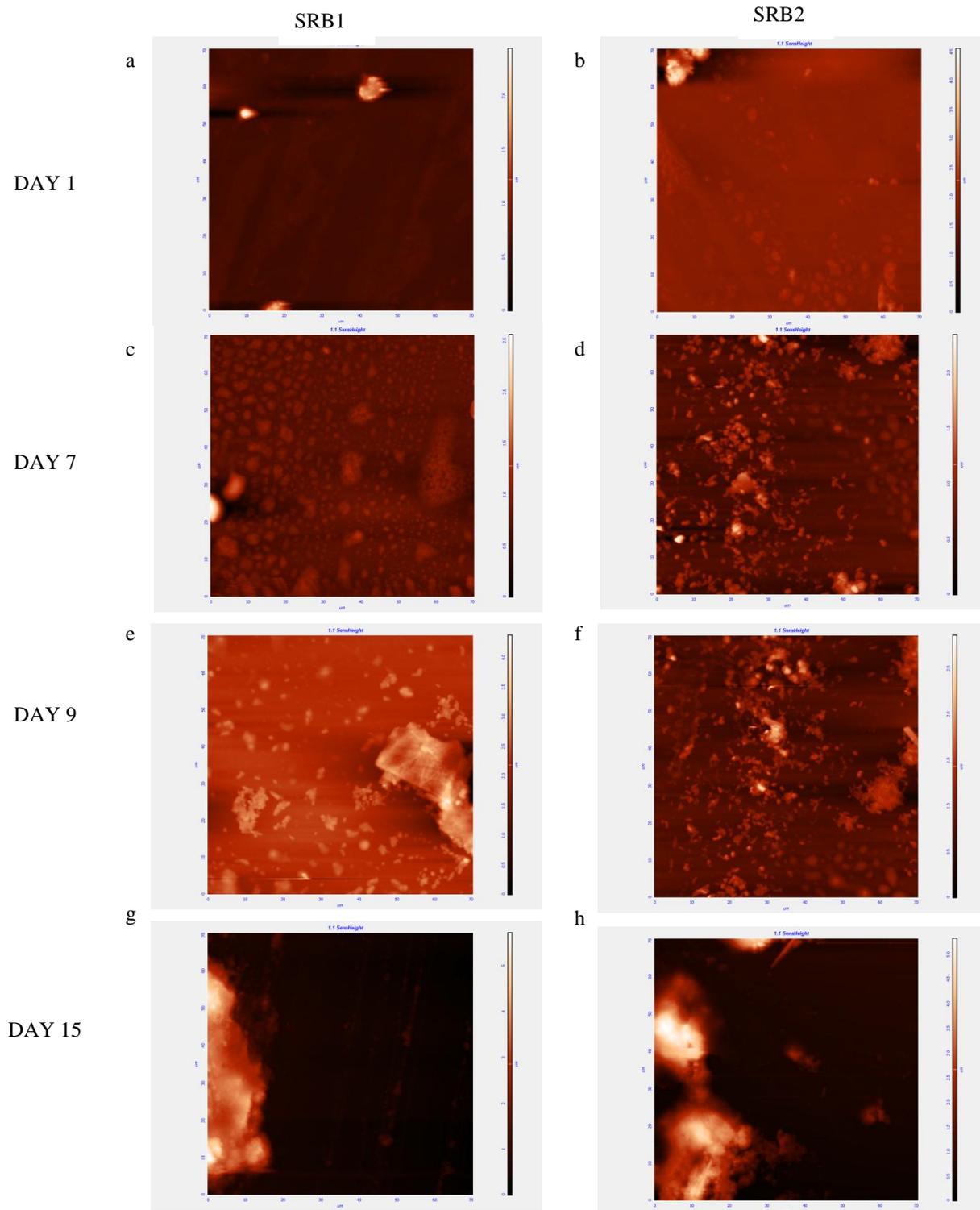SRB1   SRB2

Figure 1: AFM image of biofilm formation on AISI 304 stainless steel for SRB1 (a, c, e & g) & SRB2 (b, d, f & h) for day 1 ; (a) & (b), day 7 ; (c) & (d), day 9 ; (e) & (f) and day 15 (g) & (h). Magnification 70 µm x 70 µm.
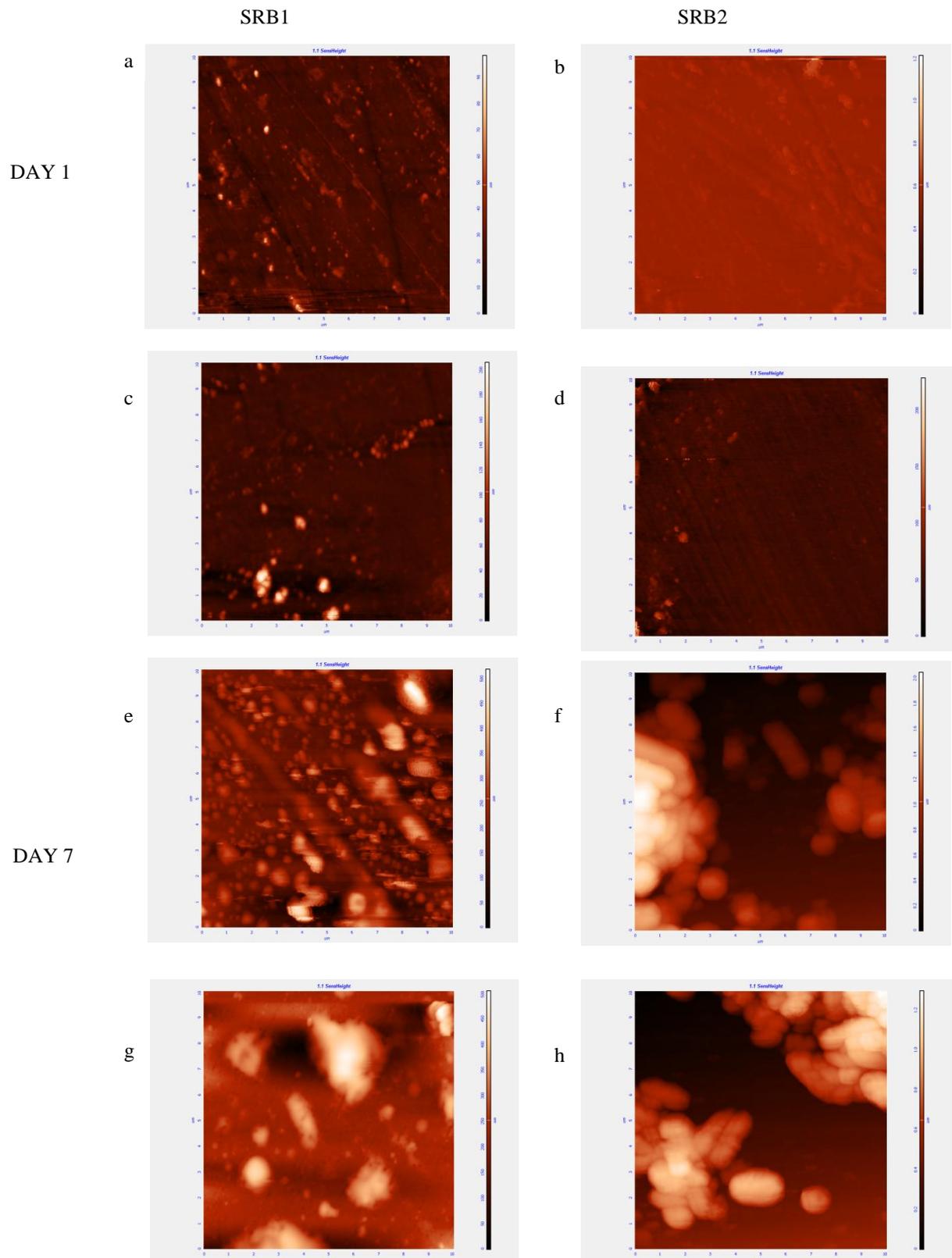
Figure 2a: AFM image of biofilm formation on AISI 304 stainless steel for SRB1 (a, c, e & g) & SRB2 (b, d, f & h) for day 1 ; (a), (b), (c) & (d) day 7 ; (e), (f), (g) & (h). Magnification 10 µm x 10 µm.

SRB1   SRB2

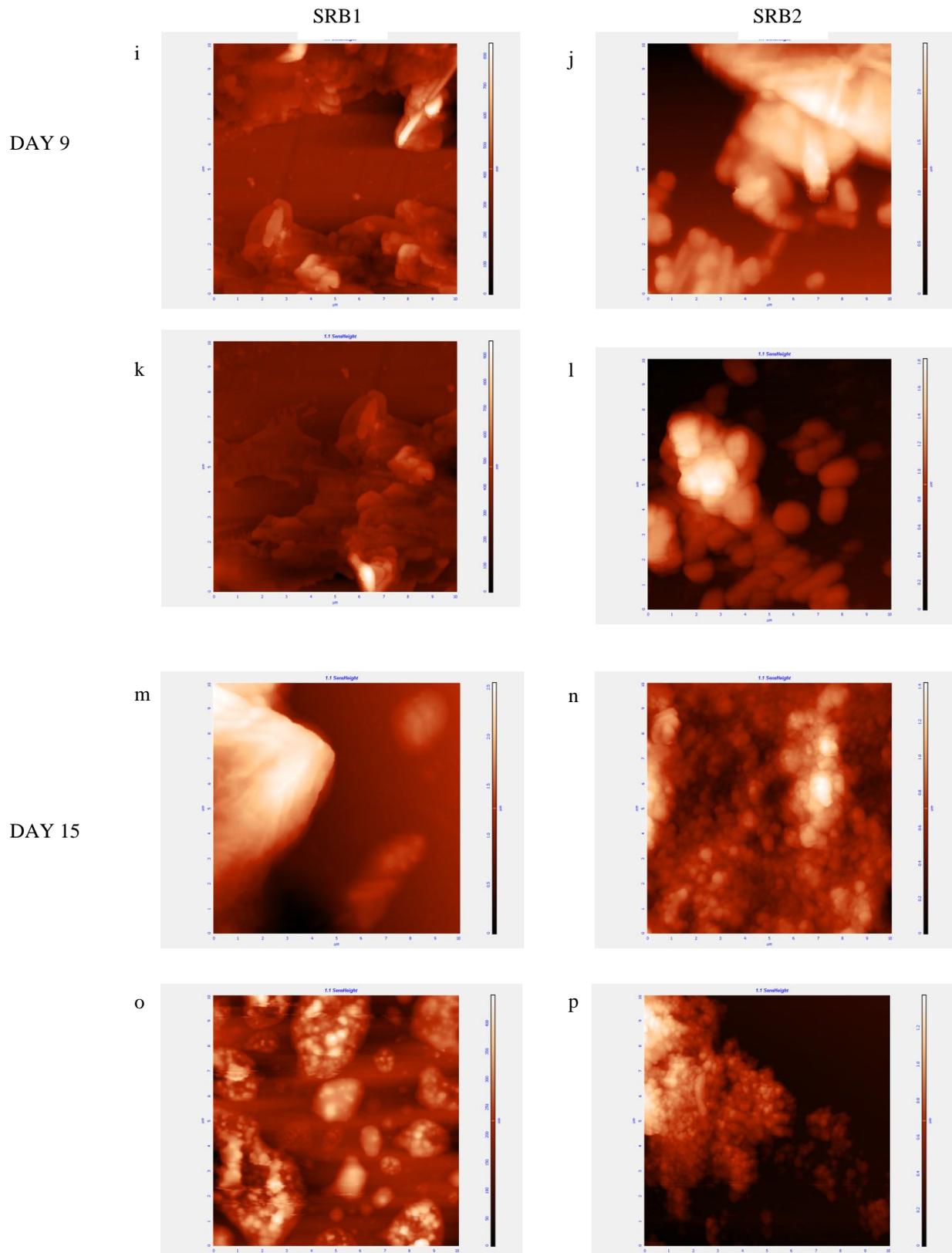i   j

DAY 9

k   l

m   n

DAY 15

o   p

Figure 2b: AFM image of biofilm formation on AISI 304 stainless steel for SRB1 (i, k, m & o) & SRB2 (j, l, n &p) for day 1 ; (i), (j), (k) & (l) day 7 ; (m), (n), (o) & (p). Magnification 10 µm x 10 µm.

Transition from planktonic cells in liquid to biofilms in surfaces is a complex process that can be divided into four steps: initial attachment to abiotic surfaces, microcolony formation, macrocolony formation, and maintenance of a mature biofilm (Oh et al. 2007). Some divide it into five stages of attachement that is, initial reversible attachment, irreversible attachment by formation of EPS, formaton of biofilm architechture, development of microcollonies to mature biofilm and dispersion of cells from the biofilm into the surrounding environment and return to the planktonic state (Houdt & Michiels 2005). The conditioning of the abiotic substrates should be taken into consideration as the attachment of bacteria towards a surface does account the type of nutrient available and in the case of SRB, the solubility of the metal itself (Mahmoud et al. 2008; Cordas et al. 2008).

A preliminary phase can take place where organic matter, glycoproteins and polysaccharides are adsorbed to the surface to a depth of 10 - 80 μm, this is called surface conditioning that leads to adhesion to surfaces, within hours of immersion (Mahmoud et al. 2008). The trace of this substance can be seen in figure 1 day 1 for both SRB strain. A few bacteria cell can be detected on the metal immerse in SRB2. Crystallize substances that were detected on SRB1 metal surface may come from salt residue from the seawater that were used for the medium or metabolite substances that were secreted from the bacteria itself. There are some bacteria that can generate ammonia and elevating the pH of the biofilm. Under these conditions, crystals of calcium and magnesium phosphate will be precipitated (Stickler & Morgan 2006).

The second phase of fouling development is the attachment of bacteria to the conditioned surface. This occurs within few days of immersion (Mahmoud et al. 2008; Diaz et al 2011). Organisms multiply during the next few days of exposure and forms of microcolonies enclosed in a slime of their extracellular polymer (Mahmoud et al. 2008). This is the early stage of the biofilm development (Houdt & Michiels 2005). This is shown in figure 1 of SRB2 where a huge number of bacteria cell can be detected on the surface. But a closer view (figure 2) on the surface of SRB2 for day 7 indicated that the formation of EPS substances is still low as the cell is loosely condensed together. SRB1 bacteria were covered with crystallize substances showed that the formation of its EPS is closer to the cell as so to protect itself from the environment like a case (Flemming & Wingender 2010). Patches of this EPS substances can be seen scattered along the surface.

The positioning of bacteria and the biofilm construction is possible due to cell aggregation and attachment to surfaces (Cordas et al 2008). One important aspect in the microbe–surfaces interaction is the influence of the extra cellular metals, since its availability (solubility) play a key role on biofilm stabilization. The participation of metals, either intra or extra cellular, for the microbial metabolism is dependent on the metal capacity to act as electron donor or acceptor. The most relevant redox couples in this context are Fe(II)/Fe(III) and Mn(II)/Mn(IV), not only for its abundance but also due to its formal redox potentials, compatible with biological activities (Cordas et al. 2008). Bacteria want to be attached or stay as close as possible to sulfide mineral surfaces to maximize their efficiency of substrate utilization. The close adherence reduces the time needed for diffusion of iron between the mineral and the bacterium (Pradhan et al 2008).

The formation of EPS substance in day 9 is where the stage of architectural of the biofilm started to begins. It is the intermediate stage between biofilm development and maturity of the microcolonies. The formation of a biofilm takes place due to the presence of electrostatic and adhesion conditions that allows one microorganism to bind irreversibly to a steel surface further followed by exopolymeric substances (EPS) production by

microorganisms (Pe'rez et al. 2007). The structure of the biofilm was dramatically influenced by EPS production or capsule formation (Kreft & Wimpenny 2001). Bacteria that have formed adherent biofilms exist not as a tightly packed unit but rather as columns of loosely associated cells, some fixed, others motile such that were showed in SRB2 (Mireles et al. 2001). SRB1 showed higher rate of EPS production rates due to the death of those cells that have spent more energy on EPS synthesis than they can gain (Kreft & Wimpenny 2001).

EPS is implicated in biocorrosion processes due to its selective metallic ion binding properties (Pradhan et al 2008). These bacteria including SRB are embedded into slimy polysaccharide capsule can cause pitting of stainless steel. Expolysaccharides released by biofilm population had a higher content of uronic acids compared to expolysaccharides released into the bulk phase (Mahmoud, Abdel-Samie & El-Mokadem 2008).

The architecture of biofilms is influenced by many factors, including hydrodynamic conditions, concentration of nutrients, properties of abiotic, cell surfaces bacterial motility and intercellular communication as well as exopolysaccharides and proteins may all be involved in initial adhesion to abiotic surfaces (Houdt & Michiels 2005; Oh et al. 2007; Cordas et al. 2008; Flemmig & Wingender 2010). Upon adhesion, bacteria induce the production of extracellular polymeric substances (EPS), which contribute to the architectural and functional characteristics of biofilms. As a result, cells embedded in EPS become resistant against potentially detrimental conditions including nutrient deprivation. Details of biofilm development vary, depending on bacterial species and environmental conditions (Oh et al. 2007).

Figure 1 for day 15 show the stage of development of microcolonies into a mature biofilm. During this stage, extracellular polymeric substances that serve as an adhesive matrix and trap nutrients from the environment continue to be produced. Complex architectures with pedestal-like structures, water channels and pores are formed, in which bacteria develop specific patterns of growth and a different physiology and metabolism from planktonic cells (Houdt & Michiels 2005). SRB2 produce a porous type of biofilm where it traps water in between the bodies. SRB1 produce a more solid body where slime filled the gap between the bacteria cell and the formation of crystallize substances covered the surface of the biofilm. This type of biofilm consists of densely packed polysaccharides and precipitation of metabolite substrate.

Bacteria Growth Curve

Figure 3 showed that SRB2 had a faster growing rate compare to SRB1. In day 1 to day 7 the increments is a little bit slow as it goes from 500 CFU/ml to 900 CFU/ml for SRB1 and 450 CFU/ml to 1000 CFU/ml for SRB2. A sudden increase happens on day 7th and 11th where the highest number was recorded, 33000 CFU/ml for SRB1 and 36000 CFU/ml for SRB2. The numbers keep on declining after passing the 11th day until the 15th. SRB1 showed a decline to 29000 CFU/ml on day 13 and become almost constant until day 15 at 28500 CFU/ml. SRB2 still showed a decline in number as it goes from 30000 CFU/ml to 27000 CFU/ml for day 13 and 15.
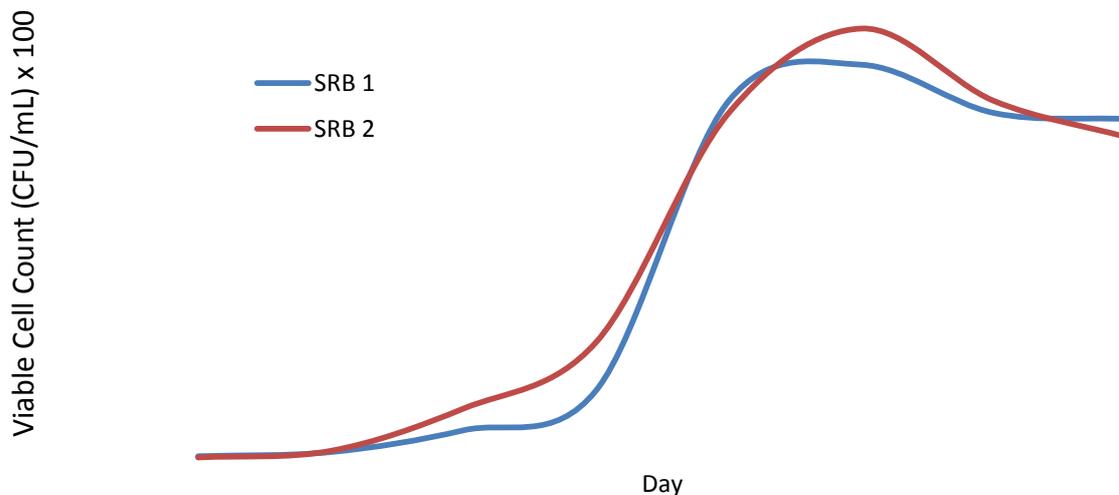
Figure 3: Bacteria growth curve for SRB1 and SRB2 for 15 days of culturation

The growth period contain four stage of growth that is the latent period, the rapid growth period, the stable growth period and the declining period (Liu et al. 2007). Both SRB1 and SRB2 give the same pattern of growth curve only difference in number. The number of bacteria corresponded with the thickness of biofilm recorded from the AFM. In the latent period, number of bacteria is low thus the biofilm formation is thin as in the initial stage, adaptation is still occurring. The rapid growth period started from day seven to day nine as the increasing number of bacteria occurs, so thus the thickness of biofilm for both SRB1 and SRB2. SRB1 showed a low formation of biofilm and swarming of bacteria compare to SRB2 thus related to the higher number of bacteria in SRB2.

Day 9 is steady growth period where the number of bacteria is stable, where the number of growth is equal to the number of bacteria death. Production of slime creates a sessile environment on the surface. Rate of biofilm production is now dependable with the nutrient supplies rather than number of bacteria. Day 15 marked the declining period where there is no more growth due to nutrient and space limitation. The rate of bacteria die is greater than bacteria produce. The production of material will occur under the biofilm as it creates its own environment. Eventually the production become low due to the lack of nutrient from outside and the growth of bacteria in the biofilm matrix will be suppressed.

CONCLUSION

Biofilm formation on metal surface is one of the key roles of SRB biocorrosion method of metal. Understanding the growth phase of the bacteria is important in preventing biofouling from happening. Different strain of SRB may produce different type of biofilm in a different period of time. SRB1 produce a slime like biofilm on the metal surface, along with crystallize precipitation that cover the bacteria cell. SRB2 biofilm consist of bacteria cell densely packed, creating a porous biofilm at the maturity level. SRB2 showed a slightly more high

rate growth in the medium compared to SRB1 but produce low amount of biofilm matrix while SRB1 produce a lot of bio-matrix precipitation and slime formation.
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