

Biocorrosion Behaviour of Carbon Steels by Tropical Microbes in the Presence of Corrosion-Inhibiting Bacterium

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ABSTRACT

A set of microbiologically induced corrosion was carried out on different types of carbon steels: AISI 1006 and API 5L X52, iron-oxidizing bacterium and sulfur-oxidizing bacterium, a mixed culture of *Alicyclobacillus ferrooxydans* SKC/SAA-2 and *Aspergillus niger* and *Comamonas thiooxydans* SKC/SAA-1, in the presence of the corrosion-inhibiting bacterium, *Pseudomonas plecoglossicida*. According to the immersion test experiments, weight loss of API 5L X52 was lower (0.06-0.27%) than AISI 1006 (0.14-0.32%). AISI 1006 showed more detrimental localized pitting corrosion than API 5L X52. During the longer incubation time, the corrosion-inhibiting bacterium was more homogenous and compact, which affected the specimen surface more protective characteristics. The 2-week-old biofilm effectively protected the API 5L X52, as indicated by the low amount and more negligible pitting corrosion. This study will be the first report on the biocorrosion behaviour of carbon steels using different corrosion-causing microbes in the presence of the corrosion-inhibiting bacterium.

1. Introduction

Microbiologically influenced corrosion (MIC) or biocorrosion, by definition, refers to an electrochemical process whereby microorganisms may be able to initiate, facilitate or accelerate corrosion reactions through the interaction of the three components that make up this system: metal, solution, and microorganisms (de Romero *et al.* 2004). In recent decades, MIC research has been extensively investigated due to the significant damage of materials caused by this process, leading to a tremendous treatment cost (Hays 2010; Summer *et al.* 2009). Several microorganisms accelerate corrosion: sulfate-reducing bacteria, sulfur-oxidizing bacteria, iron/manganese-oxidizing bacteria, slime-forming bacteria, organic acid-producing bacteria, and acid-producing fungi (Javaherdashti 2017; Lane 2005). These microorganisms can be found in pipelines/storage tanks, cooling systems,

underwater structures, vehicle fuel tanks, power generation plants, and fire sprinkler systems (Scott 2004). The metals used in the application include mild steel, stainless steel, copper alloys, nickel alloys, and titanium alloys. Primarily, uniform corrosion to environmentally-assisted cracking can be performed in mild steels, while localized forms were exhibited in the remaining alloys (Lane 2005).

Some researchers have reported many MIC studies on carbon steels caused which were used as pipelines, storage tanks, or cooling systems by iron-oxidizing and sulfate-reducing bacteria (Dong *et al.* 2011; Herrera and Videla 2009; Liu *et al.* 2015; Miranda *et al.* 2006; Rao *et al.* 2000; Starosvetsky *et al.* 2001). Corrosion study of AISI was conducted at various types, including AISI 304 (Dagbert *et al.* 2006; Damborenea *et al.* 2007; Nivens *et al.* 1986), AISI 316 (Dexter and Gao 1988; Sheng *et al.* 2007), AISI 420 (Dan *et al.* 2005; Ni *et al.* 2012), AISI 904 (Dagur *et al.* 2017), and AISI 1006 (Hartomo *et al.* 2010; Widyanto *et al.* 2020). Other types of carbon steel, e.g., API 5L X52, has been comprehensively studied (AlAbbas *et al.* 2013a, 2013b; Angeles-Chávez *et al.* 2001;

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Elshawesh *et al.* 2008; Javidi and Bahalaou Horeh 2014). As reported, most of the resultant corrosion types were pitting and localized corrosion (AlAbbas *et al.* 2013b; Hartomo *et al.* 2010; Lane 2005).

In parallel, to establish MIC prevention, an environmentally friendly and low-cost method has been developed, excluding biocides, such as the utilization of biofilm-forming bacteria. Corrosion inhibition mechanisms include: 1) a diffusion barrier to corrosion formed by the biofilm, 2) diminishing the concentration of oxygen to the metal surface, 3) metabolic products generated by microbes that act as corrosion inhibitors (e.g., siderophores), 4) antibiotics produced by microbes which prevent the reproduction of corrosion-causing organisms (Little *et al.* 2007). Few *Pseudomonas* spp. have been reported in reducing the metal corrosion rate due to its biofilm-forming ability. Ismail *et al.* described *P. fragi* has successfully diminished the mild steel corrosion by 20 times (Ismail *et al.* 2002). Likewise, *Pseudomonas* sp. S9 demonstrated a protective effect on carbon steel (ASTM A619) at 18–20°C (Pedersen and Hermansson 1989, 1991). However, some references stated that *Pseudomonas* sp. has increased and reduced the corrosion rate (Little and Ray 2002). A contradiction emerged when some biofilm microbes could induce localized corrosion while others hinder corrosion. For example, *Pseudomonas* sp. was confirmed in enhancing iron and nickel corrosion rate than sterile control (Pedersen *et al.* 1988). It was believed that metal-binding by extracellular polymeric substances was involved as a mechanism for both biocorrosion (Geesey *et al.* 1988) and corrosion prevention (Ford and Mitchell 1990). Thus, using an indigenous bacterium *Pseudomonas* sp. was potentially applicable as a corrosion-inhibiting bacterium. Considering several aspects influencing MIC, this study concentrated on comparing the factors affecting biocorrosion of different carbon steels: The American Iron and Steel Institute (AISI 1006 type) and The American Petroleum Institute (API 5L X52 type), using indigenous microbial isolates of iron- and sulfur-oxidizing bacteria and fungus: a mixed culture of *Alicyclobacillus ferrooxydans* SKC/SAA-2 and *Aspergillus niger*, *Comamonas thiooxydans* SKC/SAA-1, and the presence of a locally isolated biofilm-forming bacterium, *Pseudomonas plecoglossicida*, which has the potential as a corrosion-inhibiting bacterium. This will be the first report on biocorrosion comparative study between carbon steels, especially AISI 1006, using the new locally isolated corrosion-inhibiting bacterium. Several parameters were investigated, including weight loss, the incubation time of corrosion-inhibiting biofilm,

corrosion product characterization, and surface and cross-section morphology of the specimen.

2. Materials and Methods

2.1. Specimen Preparation and Characterization

The American Iron and Steel Institute (AISI 1006 type) and The American Petroleum Institute (API 5L X52 type) were cut into coupons with a length of 2 cm, a width of 1 cm, and a thickness of 1 cm. These coupons were polished using polycrystalline oil-based diamond paste 6, 3, and 0.5 µm in succession and degreased using isopropyl alcohol to obtain a mirror surface finish. Finally, the coupons were cleaned using isopropyl alcohol, ethanol, and deionized water, dried in a nitrogen flow, and kept in desiccators for immersion experiments. A string was tied to each specimen for hanging the coupon at the immersion test. In parallel, the coupon was characterized using optical emission spectroscopy (OES).

2.2. Cultivation of Microorganisms

The consortium of *Alicyclobacillus ferrooxydans* SKC/SAA-2 (an iron- and sulfur-oxidizing bacterium) and *Aspergillus niger* (a sulfur-degrading fungus), *Comamonas thiooxydans* SKC/SAA-1 (a sulfur-oxidizing bacterium), and *Pseudomonas plecoglossicida* (a corrosion-inhibiting/biofilm-forming bacterium) were used in this study. Each microorganism was cultivated separately using different growth media. For a consortium of *A. ferrooxydans* SKC/SAA-2 and *A. niger* and *C. thiooxydans* SKC/SAA-1, Erlenmeyer flasks (500 ml) containing 100 ml of Norris broth medium ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g L⁻¹, $(\text{NH}_4)_2\text{SO}_4$ 0.4 g L⁻¹, K_2HPO_4 0.2 g L⁻¹, KCl 0.1 g L⁻¹, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 13 g L⁻¹; diluted to 1 L of distilled water; sterilized at 121°C for 15 minutes) were inoculated with the consortium of *A. ferrooxydans* SKC/SAA-2 and *A. niger*, or *C. thiooxydans* SKC/SAA-1 at 10% v/v, and then incubated at room temperature (28°C). $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ was filter-sterilized instead of autoclaving. The pH of the media was adjusted to 4.5 using 1 M of sulfuric acid for its optimal growth condition. Flasks were then incubated at room temperature (28°C) and agitated at 150 rpm.

The corrosion-inhibiting bacterium, *Pseudomonas plecoglossicida*, was pre-grown using modified Luria Bertani (LB) medium (tryptone 10 g L⁻¹, yeast extract 5 g L⁻¹, NaCl 10 g L⁻¹; diluted to 1 L of distilled water) under a similar sterilization procedure. Flasks were then incubated at room temperature and left unshaken to observe the formation of a layer on the medium surface. The growth of microorganisms was monitored by optical density (OD) measurement

using a spectrophotometer at the wavelength of 550-600 nm.

2.3. Corrosion-Inhibiting Biofilm Formation on the Specimen Surface

Before biofilm formation, a string-tied specimen was sterilized by alcohol to remove any microorganisms attached to the specimen. Subsequently, the biofilm of *P. plecoglossicida* was grown on the specimen surface by immersing the specimen in the microbial culture for 1 and 2 weeks to form an even surface with different thicknesses of the biofilm layer. The biofilm formation was indicated by a white sticky layer covering the specimen surface. After incubation time, the specimen was retrieved for the corrosion immersion test experiment. In parallel, a fresh medium for the corrosion immersion test experiment was prepared.

2.4. Corrosion Immersion Test

Corrosion immersion test experiments were performed in a 300 ml Erlenmeyer flask containing a 100 ml liquid medium inoculated with each microorganism. Each bacterium was inoculated to a fresh medium to give a final OD₆₀₀ = 0.5. The experimental matrix for both tested specimens is described in Table 1. Biofilm-formed string-tied coupons were hung in an Erlenmeyer flask, immersed in the test media. The flasks were well-sealed and incubated at 30°C for 1 and 3 weeks. Flasks were put inside an alcohol-sterilized glass chamber to avoid contamination during the immersion test and kept at room temperature (±25-30°C). At the end of the immersion test experiment, the coupons were withdrawn and dried up for corrosion product and morphology analysis. Immersion test experiments were carried out in duplicate. Error bars are shown

based on the data presented as the arithmetic mean ± standard deviation of the mean. The diameter and depth of pits were calculated based on the average of at least three pits.

2.5. Characterization of Corrosion Products

After the corrosion immersion test had been completed, the specimens were withdrawn from the flask and dried up at 55°C in an oven overnight to eliminate any excessive moisture. The corrosion product layer covering the specimens was peeled off and collected for mineral composition characterization by X-ray diffraction (XRD) and scanning electron microscopy-energy dispersive x-ray (SEM-EDX). In parallel, to confirm the presence of Fe(III) ions as the products of steel corrosion, an inorganic analysis was carried out by adding KSCN to the dissolved corrosion product by HCl (Charlot 1954).

2.6. Deterioration Morphology of the Specimens

The peeled off specimens were prepared and cleaned by following the standard method to remove the remaining corrosion products and other organic substances excreted by bacteria (ASTM 2003). Subsequently, specimens were weighed to observe the weight loss after the corrosion experiment in the presence of biofilm-forming bacteria following ASTM D2688-05 (Standard 1983) after cleaning procedures in the ASTM G1-03 standard (ASTM 2003). To observe the cross-sectional view of deterioration morphology using SEM (JEOL JSM-6610), coupons were wire-cut into the size of 0.5 x 0.5 x 0.5 cm for length, width, and thickness, respectively.

Table 1. Experimental methodology for the corrosion study consisted of 16 different treatments, including carbon steel types (AISI 1006 and API 5L X520, the incubation time for immersion experiments (1 and 3 weeks); iron- and sulfur-oxidizing microbes (mixed culture of *Alicyclobacillus ferrooxydans* SKC/SAA-2 and *Aspergillus niger*, and *Comamonas thiooxydans* SKC/SAA-1); and incubation time of corrosion-inhibiting biofilm *Pseudomonas plecoglossicida* (1-and 2-week-old)

AISI 1006		1-week-old biofilm		2-week-old biofilm	
Incubation time (week)	Mixed culture of <i>A. ferrooxydans</i> and <i>A. niger</i>	<i>C. thiooxydans</i>	Mixed culture of <i>A. ferrooxydans</i> and <i>A. niger</i>	<i>C. thiooxydans</i>	
1	AISI A1.1	AISI C1.1	AISI A2.1	AISI C2.1	
3	AISI A1.3	AISI C1.3	AISI A2.3	AISI C2.3	
API 5L X52		1-week-old biofilm		2-week-old biofilm	
Incubation time (week)	Mixed culture of <i>A. ferrooxydans</i> and <i>A. niger</i>	<i>C. thiooxydans</i>	Mixed culture of <i>A. ferrooxydans</i> and <i>A. niger</i>	<i>C. thiooxydans</i>	
1	API A1.1	API C1.1	API A2.1	API C2.1	
3	API A1.3	API C1.3	API A2.3	API C2.3	

3. Results

3.1. Specimen Characterization

The specimen characterization using optical emission spectroscopy (OES) following ASTM E 212-66 is shown in Table 2. The results were within the standard range of AISI 1006 and API 5L X52.

3.2. Microbial Growth and Biofilm Formation on the Specimen Surface

The growth of microorganisms was visually indicated by the colour change of the growth medium, from clear to turbid, which was subsequently quantified by a UV-Vis spectrophotometer at 600 nm to measure the optical density. Furthermore, the optical density of the bacterial cultures was set to 0.5, equal to approximately 10^7 CFU/ml for iron-oxidizing bacteria. The optical density of the consortium of *A. ferrooxydans* SKC/SAA-2 and *A. niger*, and *C. thiooxydans* SKC/SAA-1 was set to $OD_{600} = 0.5$ (equal to 3.5×10^6 CFU ml⁻¹) at initial week (day 0). The growth of each iron-oxidizing and sulfur-oxidizing bacterium is shown in Table 3. Since the isolation stage, strain *A. ferrooxydans* SKC/SAA-2 was associated with *A. niger* but was separated with *C. thiooxydans* SKC/SAA-1.

Owing to the biofilm-forming characteristics leading to metal corrosion inhibition, *P. plecoglossicida* was utilized in this study. This

bacterium was isolated from a local Indonesian mining site. In this study, the biofilm incubation time was varied, 1-week-old and 2-week-old, to generate different thicknesses of biofilm layer grown on the specimen surface to cover the specimen surface. It was expected that the older biofilm, the more it can inhibit the specimens' corrosion process. According to SEM results in Figures 1A and B, it was observed that 2-week-old biofilm had a more homogenous, rod-shaped, and thicker cells layer compared to 1-week-old biofilm, while there was still some hollow in 1-week-old biofilm. The biofilm layer may be deficient after two weeks due to the bacterial cells' stationary to death phase, resulting in the non-homogeneity coverage of the materials' surface.

3.3. Characterization of Corrosion Products

Corrosion products of AISI and API were characterized qualitatively by XRD. The dried-up corrosion product from AISI specimens was selected and analyzed further for its mineral composition. API corrosion product was unable to proceed for analysis due to insufficient sample. According to Figure 2, it was observed that corrosion products of AISI specimens for both a mixed culture of *A. ferrooxydans* and *A. niger* and *C. thiooxydans* were amorphous. Due to the amorphous characteristics of ferric hydroxide deposits, SEM-EDX analysis was

Table 2. Elemental composition (wt.%) of carbon steel types: AISI 1006 and API 5L X520 measured by OES

Elements	This experiment		ASM vol. 1, 10 th ed	
	AISI 1006 (%)	API 5L X52 (%)	AISI 1006 (%)	API 5L X52 (%)
Fe	99.6	97.9	NM*	NM*
C	0.046	0.159	0.08	0.22
Si	0.015	0.289	0.1-0.4	NM
Mn	0.311	1.18	0.3-0.5	1.4
P	0.09	0.023	0.05	0.025
S	0.014	0.006	0.05	0.015
Ni	0.018	0.083	NM*	NM*
Mo	0.008	0.023	NM*	NM*
Cu	0.033	0.143	NM*	NM*
Al	0.04	0.028	NM*	NM*
Zn	0.002	0.001	NM*	NM*

*not mentioned

Table 3. Viable bacterial counts in the media inoculated with a mixed culture of *A. ferrooxydans* and *A. niger* and *C. thiooxydans* during immersion test

CFU \pm SE (10 ⁷ cell ml ⁻¹)	AISI 1006			API 5L X52		
	0-week	1-week	3-week	0-week	1-week	3-week
Mixed culture of <i>A. ferrooxydans</i> and <i>A. niger</i>	0.35 \pm 0.02	1.16 \pm 0.01	1.68 \pm 0.04	0.35 \pm 0.02	1.05 \pm 0.05	1.65 \pm 0.05
<i>C. thiooxydans</i>	0.35 \pm 0.03	0.91 \pm 0.03	1.47 \pm 0.05	0.35 \pm 0.02	0.98 \pm 0.04	1.54 \pm 0.04

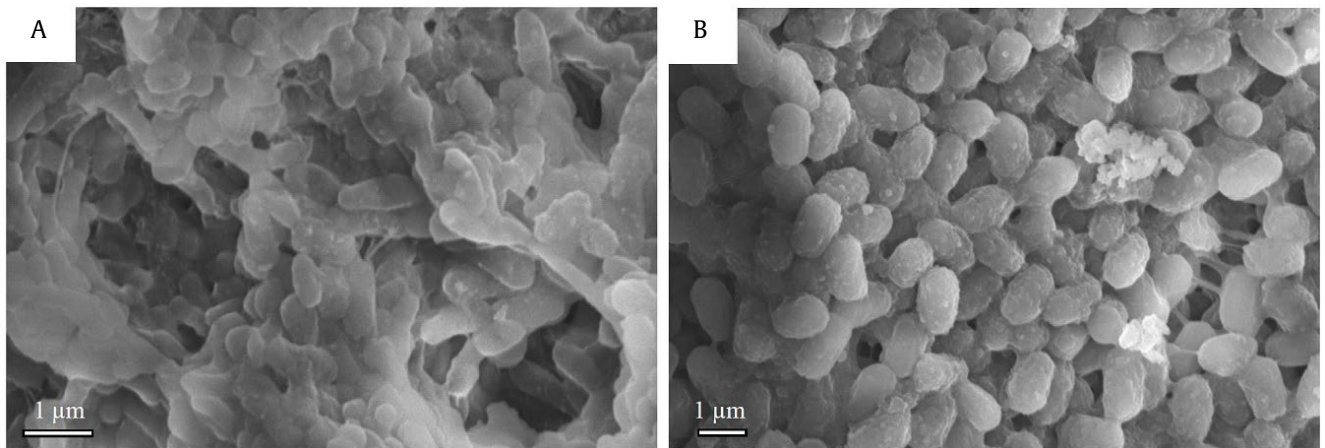


Figure 1. (A) SEM images of the corrosion-inhibiting bacterium *Pseudomonas plecoglossicida* for the 1-week-old biofilm and (B) the 2-week-old biofilm

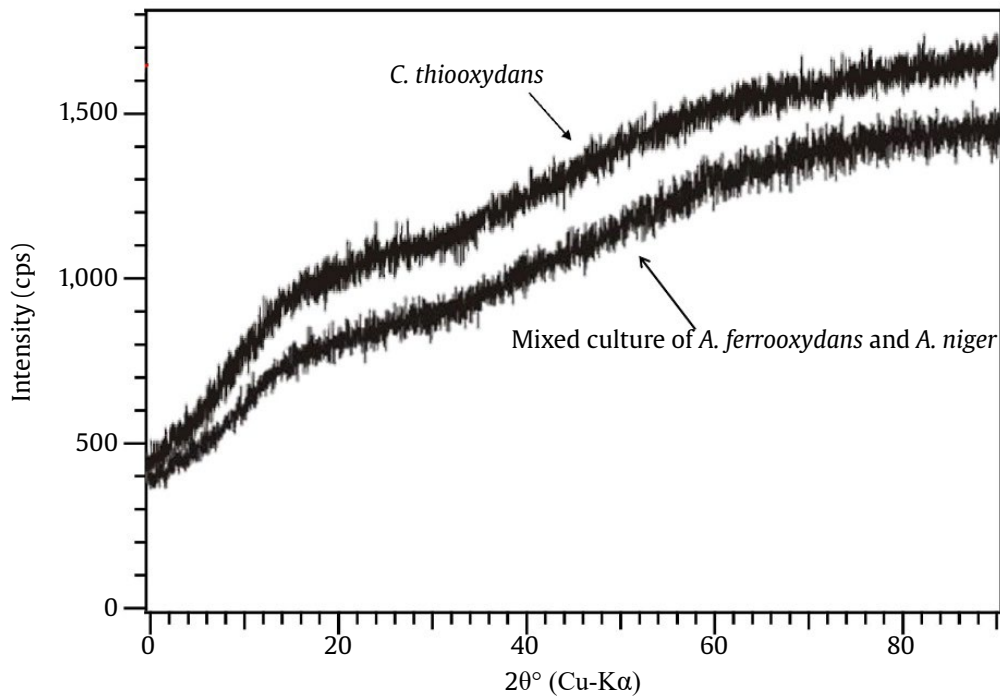


Figure 2. Diffractogram of the corrosion products by a mixed culture of *Alicyclobacillus ferrooxydans* SKC/SAA-2 and *Aspergillus niger*, and *Comamonas thiooxydans* SKC/SAA-1

carried out to analyze the mineral composition. As shown in Figures 3A and B, it was quantified that the elements were dominated by FeO, approximately 60% and 74.56% for mixed culture of *A. ferrooxydans* and *A. niger* and *C. thiooxydans*, respectively. In parallel, to confirm the presence of Fe(III) ions as the product of carbon steel corrosion, a qualitative inorganic analysis was conducted. The result has confirmed the formation of red colour as iron(III) oxide.

3.4. Comparison of Specimen Weight after Immersion Test

All specimens were weighed before and after the immersion test in a dehydrated form to investigate the effect of microbiologically influenced corrosion. The initial and final weight was obtained before the immersion test and after the specimens peeled for their corrosion product and all remaining organic matters attached. This procedure has to

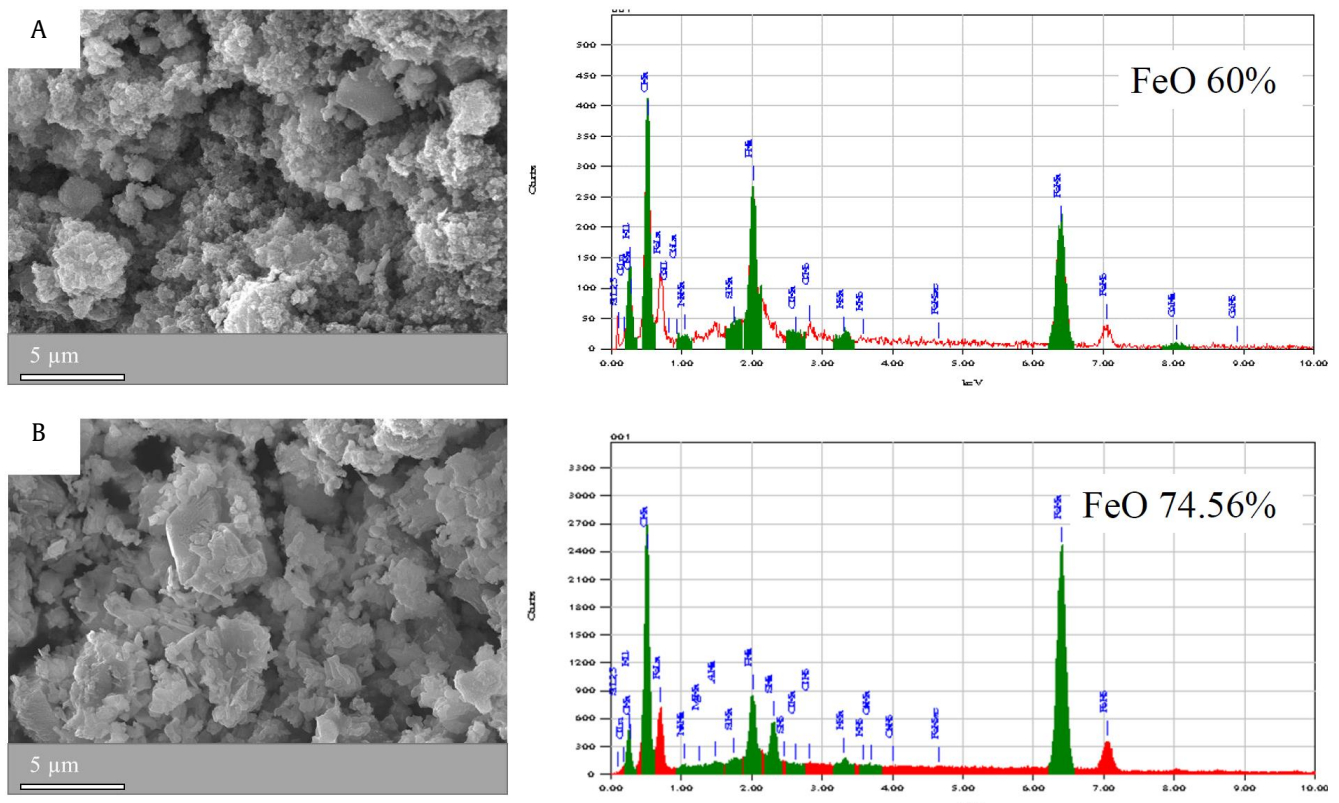


Figure 3. SEM-EDX images of corrosion products resulted from: (A) a mixed culture of *Alicyclobacillus ferrooxydans* SKC/SAA-2 and *Aspergillus niger*, and (B) *Comamonas thiooxydans* SKC/SAA-1. The contents of FeO are shown

be performed to control the data validity at clear specimens condition. The initial and final weights of each specimen are shown in Figure 4A. The weight for all AISI and API specimens was in the range of 15 to 25 g. The weight difference of each specimen was due to the specimen preparation, including manual polishing started with #60 to #400 to achieve a mirror-like specimen with no surface impurities. The weight change after the immersion test was indiscernible since the change was less than 10%. According to Figure 4B, by focusing on weight loss, it was observed that the weight loss was in the range of 0.08–0.32%. The weight loss gradually increased for A2.3, C2.3, C1.1, C1.3, and A1.3 by 0.08%, 0.14%, 0.18%, 0.27%, and 0.32%, respectively. This suggested that the 2-week-old *P. plecoglossicida* biofilm had prevented the weight loss by decreasing the corrosion rate due to its more homogenous and compact layer covering the specimen surface. In contrast, a 1-week-old biofilm layer was insufficient to prevent corrosion, resulting in a 2-fold increased weight loss. It was observed from Figures 4B and C that API 5L X52 showed less weight loss than AISI 1006. In contrast to AISI 1006, the weight of API 5L X52 specimens was constantly reduced under all conditions.

Nonetheless, as shown in Figure 4C, the reduction of weight occurred in both treatments with a mixed culture of *A. ferrooxydans* SKC/SAA-2 and *A. niger*, and *C. thiooxydans* SKC/SAA-1, which were also protected by 1- and 2-week-old biofilms of *P. plecoglossicida*, showing less weight loss. From Figure 4C, it was revealed that the 3-week incubation period resulted in almost doubled weight loss compared to the 1-week incubation period in all cases and the presence of the 2-week-old *P. plecoglossicida* biofilm. In the case of weight loss caused by a mixed culture of *A. ferrooxydans* SKC/SAA-2 and *A. niger*, the weight loss was slightly higher than *C. thiooxydans* SKC/SAA-1, indicating that the mixed culture of *A. ferrooxydans* SKC/SAA-2 and *A. niger* had a more remarkable iron-oxidizing ability to deteriorate the specimens.

3.5. Comparison of Deterioration Morphology Using SEM

The scale bars shown on the SEM images determined the pit depth and diameter. The resultant corrosion damage was elaborated according to the carbon steel type and the incubation period of the corrosion-inhibiting bacteria as follows:

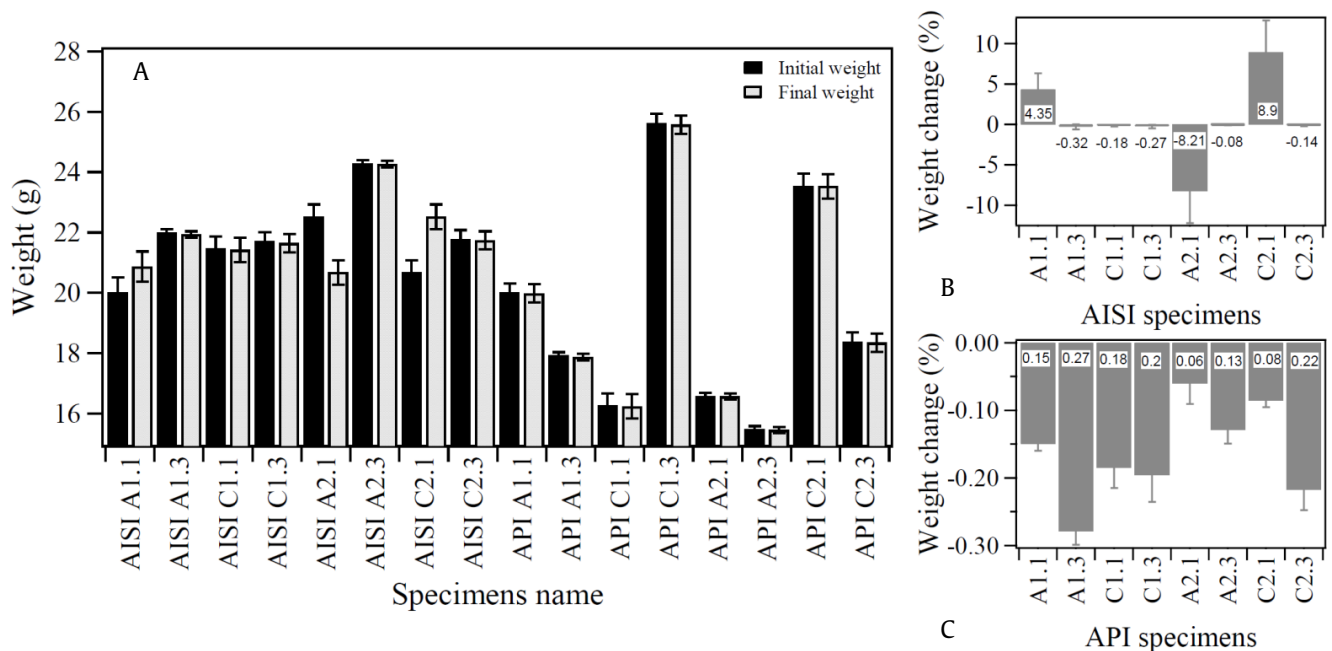


Figure 4. (A) The actual weight of AISI 1006 and API 5L X52 specimens before and after immersion test, the weight change of (B) AISI 1006 and (C) API 5L X52 in percent. Positive or negative weight change indicated weight gain or weight loss, respectively

3.5.1. AISI 1006 Protected with a 1-week-old *P. plecglossicida* Biofilm

As previously stated in section 3.3, the 1-week-old *P. plecglossicida* biofilm evaluated on a 1-week immersion did not protect the specimen surface due to the biofilm's uneven layer structure and thickness, which was caused by the rough layer structure and thickness of the specimen. This was confirmed by Figure 5A (1) that some steep localized pitting corrosion, with the various depths of 5-10 μm and diameter of approximately 5 μm , was detected on the specimen surface as well as on the cross-section view for both immersed with a mixed culture of *A. ferrooxydans* SKC/SAA-2 and *A. niger*. Pitting corrosion will only develop in the existence of aggressive anionic species, in that chloride ions are usually, but not always, the cause of it. In the 3 weeks of immersion test, as shown in Figure 5A (2), it can be predicted that the mixed culture of *A. ferrooxydans* SKC/SAA-2 and *A. niger* caused more severe damage with the pit diameter of 10 μm while similar pit depth to 1-week immersion. As previously mentioned that *A. niger* produced organic acid, it was evident that *A. niger* caused several types of damages but not significantly correlated with the weight loss. A few shallow and round micro pits (a depth of $\sim 2 \mu\text{m}$) were detected at the front surface. Moreover, from the cross-section and surface view, worse surface topography and surface crevice of the specimen were observed along the surface, respectively, indicating

some release of ionic Fe from the specimen due to microbial iron-oxidizing activity. In the case of 1-week immersion with *C. thiooxydans* SKC/SAA-1, the resultant deterioration was similar to the mixed culture of *A. ferrooxydans* SKC/SAA-2 and *A. niger*. A few pitting corruptions were detected, as shown in Figure 5A (3), with less localization, suggesting that the iron-oxidizing ability of *C. thiooxydans* SKC/SAA-1 was lower than that of the mixed culture of *A. ferrooxydans* SKC/SAA-2 and *A. niger*. Either the pit depth or diameter was approximately 5 μm . Compared with the 3 weeks of immersion test using the mixed culture of *A. ferrooxydans* SKC/SAA-2 and *A. niger*, *C. thiooxydans* SKC/SAA-1 resulted in more numbers of pitting corrosion as observed in surface view (Figure 5A (4)). The resultant pitting corrosion was about 3-5 μm in diameter and 5 μm in depth with evenly pitting distribution along the surface.

3.5.2. AISI 1006 Protected with a 2-week-old *P. plecglossicida* Biofilm

It was expected that using a 2-week-old *P. plecglossicida* biofilm should lower the corrosion effect caused by iron-oxidizing bacteria. Despite protecting the specimen surface better, Figure 5B (1) showed similar behaviour to a 1-week-old biofilm, about 5 μm and 10 μm of pit depth and diameter observed by immersing in the mixed culture of *A. ferrooxydans* SKC/SAA-2 and *A. niger*. In contrast to 1-week-old biofilm, 2-week-old biofilm notably

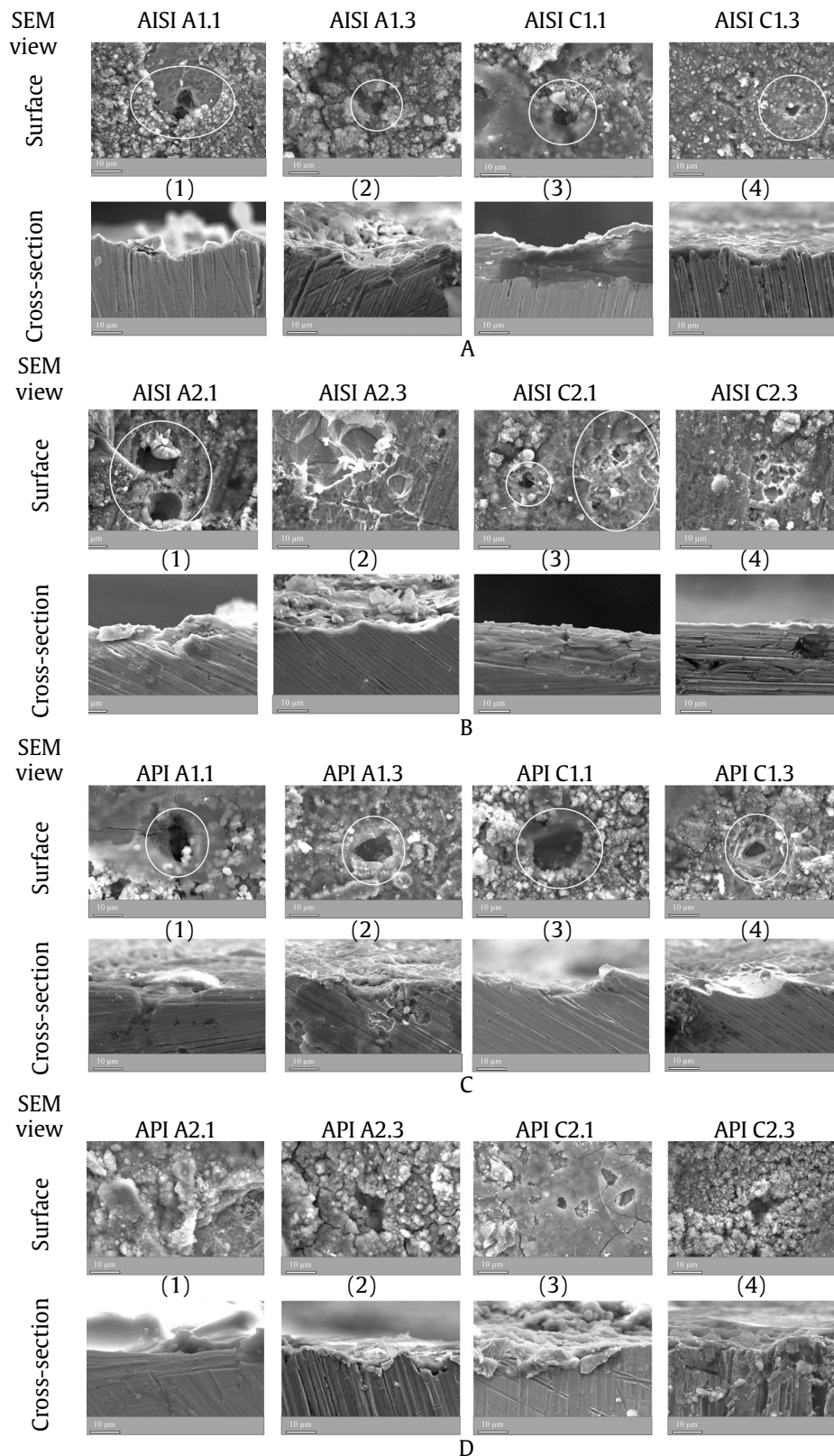


Figure 5. SEM images of surface and cross-section view for (A) AISI 1006 using the 1-week-old corrosion-inhibiting bacterial biofilm, (B) AISI 1006 using the 2-week-old corrosion-inhibiting bacterial biofilm, (C) API 5L X52 using the 1-week-old corrosion-inhibiting bacterial biofilm, and (D) API 5L X52 using the 2-week-old corrosion-inhibiting bacterial biofilm. Scale bars are shown in each image

caused more uniform corrosion along the specimen surface, probably due to the even layer of a 2-week-old *P. plecoglossicida* biofilm, which also added the deterioration effect on the surface, leading to the uniform corrosion. Meanwhile, the corrosion effect in a 3-week immersion with the mixed culture of *A. ferrooxydans* SKC/SAA-2 and *A. niger* was lower in the number of pitting corrosion (Figure 5B (2)). The pitting was shallow but large in diameter, with a diameter of approximately 2 μm and 10 μm , respectively. Some rod-shaped cells of *P. plecoglossicida* were also observed from a cross-section view. In the case of a 1-week immersion with *C. thiooxydans* SKC/SAA-1 using the 2-week-old *P. plecoglossicida* biofilm, some pitting corrosion was noticed in Figure 5B (3). However, the 2-week-old *P. plecoglossicida* biofilm could protect the specimen according to the cross-section view since a subtle crevice was detected. Regardless of the few pitting produced as recognized on the surface, the pit depth and diameter were only about 2 μm and 5 μm , respectively. In the 3-week immersion with *C. thiooxydans* SKC/SAA-1 using 2-week-old *P. plecoglossicida* biofilm (Figure 5B (4)), minor damage was produced as spotted on the surface. Even though few pitting corrosion were generated, the most oversized diameter was about 5 μm with uniform corrosion along the specimen surface.

3.5.3. API 5L X52 Protected with a 1-week-old *P. plecoglossicida* Biofilm

In API 5L X52 immersed with a mixed culture of *A. ferrooxydans* SKC/SAA-2 and *A. niger* for a week, only a few pitting corrosion was encountered, as shown in Figure 5C (1). The pit's diameter was varied, at the biggest about 5 μm , while the cross-section view suggested the diverse pit depth of 3-10 μm . In the 3-week immersion with a mixed culture of *A. ferrooxydans* SKC/SAA-2 and *A. niger*, the number of pitting was higher than the 1-week immersion (Figure 5C (2)). The uneven biofilm layer was obvious to cause a higher number of pitting corrosion. The depth and diameter of the pit were similar to a week of immersion; 3 μm , and 5 μm , respectively. In the case of the 1-week immersion with *C. thiooxydans*, SKC/SAA-1 using a 1-week-old *P. plecoglossicida* biofilm, uniform corrosion was dominantly generated with a limited amount of pitting corrosion (Figure 5C (3)). Despite the low pitting corrosion, the pit diameter was comparable to AISI 1006, about 10 μm , with a depth of 3 μm . In the 3-week immersion with *C. thiooxydans* SKC/SAA-1 using a 1-week-old *P. plecoglossicida* biofilm (Figure 5C (4)), some spots appeared like their

original surface before the immersion, but pitting corrosion was observed as well.

3.5.4. API 5L X52 Protected with a 2-week-old *P. plecoglossicida* Biofilm

API 5L X52 was better protected against corrosion when immersed in a mixed culture of *A. ferrooxydans* SKC/SAA-2 and *A. niger* for one week employing a 2-week-old *P. plecoglossicida* biofilm (Figure 5D (1)). Although there was no pitting corrosion on the specimen surface, there was some little crevice or damage visible on the surface of the specimen. Surface degradation was less severe and occurred more uniformly. In comparison to the 1-week immersion, less pitting corrosion was developed in the 3-week immersion (Figure 5D (2)), but in reality, most of the surfaces were well covered by the biofilm during the whole immersion period (Figure not shown). The most significant corrosion was found in minor crevices along the surface, as seen in the surface image. The trench measured 3 μm in depth and 5 μm in diameter. In a 1-week immersion with *C. thiooxydans* SKC/SAA-1 using a 2-week-old *P. plecoglossicida* biofilm (Figure 5D (3)), subtle crevice was noticeable on the surface with minor pitting corrosion (depth and diameter of 2 μm and 3 μm). The 2-week-old *P. plecoglossicida* biofilm efficiently shielded the specimen, confirmed by the smooth surface view. The 3-week immersion responded similarly to the 1-week immersion, with a slightly larger diameter (5 m) than the 1-week immersion.

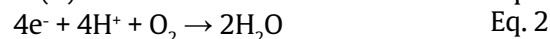
4. Discussion

From the microbiological standpoints, in the current study, the optical density of the bacterial cultures was set to 0.5, equal to approximately 10^7 CFU/ml for iron-oxidizing bacteria, which was sufficient for the corrosion test (Sutton 2006). This value is believed to be in the exponential phase of bacterial growth, where the bacteria are doubled in total number and metabolically active (Madigan *et al.* 2010). It was found from this study that strain *A. ferrooxydans* SKC/SAA-2 was associated with *A. niger* since the isolation stage but was separated with *C. thiooxydans* SKC/SAA-1. Jiang *et al.* (2008) have reported that the bacterium *A. ferrooxydans* is Gram-positive, strictly aerobic, rod-shaped, optimally grows at room temperature for 7 days and oxidizes ferrous iron to gain energy and support its growth. The bacterium *C. thiooxydans* is known as iron- and sulfur-degrading bacterium (Gerritse *et al.* 1992) and is involved in the corrosion process (Cheng *et al.* 2005; Morrow 2012). Moreover, *P. plecoglossicida* is

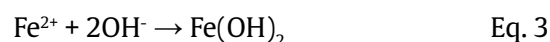
Gram-negative, aerobic, rod-shaped (Nishimori *et al.* 2000; Park and Nakai 2003), and the biofilm-forming bacterium (Li *et al.* 2009), which was previously demonstrated as the PAHs-degrading bacterium (Parellada 2011, 2013). For a few decades, the genus of *Pseudomonas* sp. has become strategies for metal corrosion inhibition (Chongdar *et al.* 2005; Jayaraman *et al.* 1997; San *et al.* 2014; Zuo 2007). Based on the thickness of biofilm formation on the specimen surface, it was expected that the older biofilm, the more its ability to inhibit the corrosion process of the specimens. This hypothesis was based on one possible mechanism of biocorrosion inhibition: the formation of a protective layer of EPS (extracellular polymeric substances) by non-damaging microbes (Videla and Herrera 2009; Zuo 2007). The influence of EPS in inhibiting carbon steel corrosion has been reported by Dong *et al.* (2011), stating that a sufficient amount of EPS successfully inhibited carbon steel corrosion. In general, the genus of *Pseudomonas* sp. has been reported to produce a high molecular weight of EPS when attached to a steel surface, as demonstrated by FTIR and electron microscopy (Zinkevich *et al.* 1996). Moreover, despite the living biofilm, EPS likely acts as protective properties investigated to be similar to paint or coating layer in conventional corrosion protection (Zuo *et al.* 2005). As shown by SEM images (Figures 1A and B), the biofilm formation suggested that it was crucial to produce a more homogenous and thick biofilm layer by extending the incubation time, which led to better material protection. This behaviour was in agreement with the work of Bakke and Olsson (1986), showing the biofilm layer thickness of ~0.627 mm and 1.025 mm for the 1-week-old and 2-week-old biofilms, respectively, for material protection. In general, the growth of biofilm is mainly affected by two factors, nutrient availability and the amount of cells populations (Kokare *et al.* 2009). Sufficient nutrients from the environment will lead to robust cell growth. To produce bacterial biofilm in the substrate, it is necessary to achieve a minimum amount of bacterial cells to establish every colony where they can communicate intracellularly between cells, known as quorum sensing (Jia *et al.* 2019). This process demonstrated cell to cell signalling to play a role in cell attachment. It has been revealed from the genetic studies that multiple steps are required to produce biofilms, including intracellular signalling and transcribing different sets of genes different from planktonic cells. In other words, a developmental process occurs in biofilm formation (Watnick and Kolter 2000).

Based on the characterization of corrosion products of AISI specimens (Figure 2), the amorphous

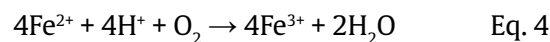
characteristics of the corrosion product indicated the freshly formed iron(III) hydroxide, which would be transformed into a crystalline form of iron oxyhydroxide (Jegdi *et al.* 2011). The following reactions are that the primary reaction couples the iron oxidation (anodic reaction) to the oxygen reduction (cathodic reaction) at the surface of the metal (Sharma and Eddy 2011):



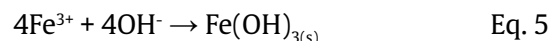
Ferrous ion reacts with hydroxide (OH^-) in the water to form rust:



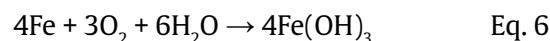
Ferric ion is generated by the combination of oxygen and hydrogen in the water:



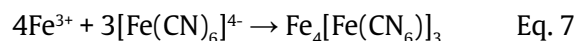
Ferric ions play an important role in the generation of amorphous reddish deposits, known as iron(III) hydroxide:



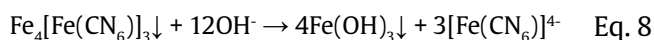
In general, the corrosion process can be simplified as follows:



Moreover, a qualitative inorganic analysis was conducted (Charlot 1954; Svehla and Vogel 1987) to confirm the presence of Fe(III) ions as the product of carbon steel corrosion, exhibiting the formation of red colour as the presence of iron(III) oxide. This method utilized the following reaction to occur:



The precipitate was insoluble in dilute acids but decomposed in concentrated HCl. A considerable excess of the reagent dissolved it partly or entirely when an intense blue solution was obtained. Sodium hydroxide turned the precipitate red as iron(III) oxide and hexacyanoferrate(II) ions were formed:



Furthermore, a comparison of specimen weight after immersion test suggested that API 5L X52 had better physical properties than AISI 1006 due to its elemental composition, particularly, higher carbon

content (0.22% vs 0.08%) (Committee 1990). This property affected the deterioration development by bacteria (Kokare *et al.* 2009), as shown in Figures 4B and C that API 5L X52 showed less weight loss than AISI 1006. Some metallurgical factors are recognized to play a role in MIC: chemical composition, surface roughness, microstructure, grain boundaries, and residual stresses (Noël 2003). Those factors were well-reported on the MIC initiation, propagation, and resultant in stainless steel alloys. Unlike stainless steel, metallurgical features studied in carbon steel MIC were limited. Mara and Williams (1972) reported that the MIC rate increased with the carbon content of the steel, but the cause was still a mystery. Another study described that *E. coli* enhanced the corrosion rates of different iron-carbon alloys; however, the correlation between carbon content and corrosion remained unclear (Ashton *et al.* 1973). Recently, the influence of the composition and microstructure of different carbon steel grades on the bacterial attachment during the MIC process was investigated (Javed *et al.* 2016). It was reported that corrosion rates of different grades of carbon steels were enhanced with the pearlite content, consistent with the study by Mara and Williams (1972). Given this behaviour, more research is needed to understand the relationship between the complexity of metallurgical characteristics and the MIC of carbon steels. Furthermore, the ability of a mixed culture of *A. ferrooxydans* SKC/SAA-2 and *A. niger* to deteriorate the specimens more effectively than *C. thiooxydans* SKC/SAA-1 could be attributed to the production of organic acids by the fungus *A. niger*. Some studies have reported that *A. niger* has been determined to produce some organic acids, i.e., oxalic acid, citric acid, lactic acid (Chaerun *et al.* 2017), which enhance metal dissolution from solid substrates, thus enhancing corrosion rate as well as metal recovery (Castro *et al.* 2000; Dai 2016; Mulligan 2004; Sayer and Gadd 1997; Zhang *et al.* 2015). A 1-week-old *P. plecoglossicida* biofilm caused higher weight loss, implying that the biofilm layer was insufficient to protect the specimen surface evenly. Therefore, a 2-week-old biofilm was required to produce an even layer for specimen protection against iron- and sulfur-oxidizing bacteria.

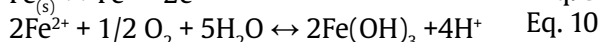
Comparing the deterioration morphology by SEM observation among the specimens, particularly those protected by the 1-week-old biofilm, exhibited that both corrosion-related microbes yielded pitting corrosion (Figure 5). This pitting corrosion will only

occur in the presence of aggressive anionic species, with chloride ions being the most common, but not always, source of the corrosion. The presence of oxidizing agents in a chloride-containing environment is immensely destructive and will further enhance localized corrosion (Newman 2002). Even a low concentration of chloride ions (60 ppm) has been reported to generate corrosion (Anuradha *et al.* 2007). Moreover, Dai (2016) reported that the presence of chloride ions was obligatory for pitting corrosion. Heterogeneous corrosion pits in the presence of NaCl were detected on several surfaces, ranging from ~8 µm to 130 µm in either depth or diameter, with the average pit depth of ~22 µm, and the more bottomless pits and pit clusters (a depth of 15 µm) were observed at the edge. Also, the logarithm of the bulk chloride concentration led to various severities of pitting corrosion (Leckie and Uhlig 1966). Additionally, using a 1-week-old *P. plecoglossicida* biofilm to protect API 5L X52 specimens against the corrosion bacterium *C. thiooxydans* SKC/SAA-1 during a 3-week immersion revealed that some spots retained their original surface appearance before the immersion, but pitting corrosion was also observed (Figure 5C (4)). Two alternatives emerged as a result of this occurrence: (1) The corrosion damage caused by *C. thiooxydans* SKC/SAA-1 was minor when compared to the corrosion damage caused by a mixed culture of *A. ferrooxydans* SKC/SAA-2 and *A. niger*, or (2) *C. thiooxydans* SKC/SAA-1 had reached its stationary phase to almost dead phase, which affected its iron-oxidizing ability. A small amount of pitting corrosion was discovered at depths and diameters of 3 m and 5 m, respectively. The corrosion process in the presence of biofilm has been reported by (Zuo 2007). The formation of corrosion-inhibiting biofilms/bacterial biofilms on the metal surface may accelerate or delay the corrosion process. When bacteria colonize on metallic substratum, they will form non-uniform patch which, in the presence of aerobic respiration, results in the differential aeration of cells formation, with the areas below thicker colonies (lower oxygen concentration, more respiration activity) into anodic and areas below thinner colonies (higher oxygen concentration, less respiration activity) into cathodic, hence accelerating corrosion. In contrast, biofilm matrix establishes a transport barrier, which may hinder the diffusion of corrosive agents (e.g., oxygen, chloride, etc.) and diminish their contact with the

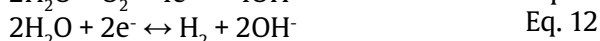
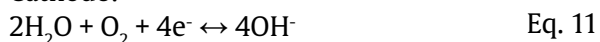
metal surface, resulting in corrosion reduction (Zuo 2007).

Comparing the corrosion damage between AISI 1006 and API 5L X52 specimens, this corrosion research for AISI 1006 in the presence of corrosion-inhibiting bacteria will be the first report to our knowledge, regardless of the MIC process of AISI carbon steels, which has been extensively investigated. Previously, MIC for AISI 1006 has been tested using different iron-oxidizing bacteria, *Acidithiobacillus ferrooxidans*, and sulfate-reducing bacterium, *Desulfovibrio piger* (Hartomo *et al.* 2010). The results revealed that *At. ferrooxidans* was found to have higher weight loss than *D. piger* by creating uniform corrosion damage rather than pitting corrosion. This result suggested that oxygen and inorganic compounds were beneficial for iron-oxidizing bacteria to gain energy (Pronk *et al.* 1990). Compared to this case, both a mixed culture of *A. ferrooxydans* SKC/SAA-2 and *A. niger* and *C. thiooxydans* SKC/SAA-1 produced pitting corrosion, either localized in many spots or limited on the surface, with varied depth and diameter within 2-10 μm . MIC studies of API 5L X52 have been well documented, primarily using sulfate-reducing bacteria (AlAbbas *et al.* 2013a, 2013b; Angeles-Chávez *et al.* 2001; Elshawesh *et al.* 2008; Wu *et al.* 2014). Localized pitting corrosion with some subtle crevice was reported in this study. However, the pit depth and diameter were unknown. Several causative agents contribute to the corrosion process, especially pitting and crevice corrosion. The collapse of the passive film leads to pitting and crevice corrosion. The presence of ferric ions in the solution is beneficial and thus initiate localized corrosion. The passivating oxidizer (ferric ions are reduced to ferrous ions) and chloride are the pitting agents. Salt solution hydrolysis from either the medium or the specimen generates an acid pH of 1.2. The coalescence between a strong oxidizer, an acid solution, and chloride results in a threatening environment toward pitting and crevice corrosion (Jones 1996). The following equations are anodic and cathodic reactions for corrosion.

Anode:



Cathode:



Classical pit morphologies include shallow pits (e.g., AISI A2.3), deep pits (AISI A1.1, API A1.1), and deep closely pits bordering on an irregular type of uniform corrosion. It is suspected that a crevice might be an onset for pitting (Asphahani *et al.* 1987). Based on the results of this study, which compared the corrosion damage caused by different iron-oxidizing and sulfur-degrading/oxidizing bacteria on AISI 1006 and API 5L X52 steels, it can be concluded that the corrosion damage caused by a mixed culture of *A. ferrooxydans* SKC/SAA-2 and *A. niger* was more detrimental and localized than the corrosion damage caused by *C. thiooxydans* SKC/SAA-1. Figure 6 provides an excellent illustration of the comparison of the resulting corrosion. As a result of the decreased carbon content of API 5L X52 compared to AISI 1006, the number of pits and crevices in the resulting pit corrosion was less numerous, with a mild crevice in some specimens. Hence, corrosion-inhibiting bacteria in biofilm was more effective in protecting the specimen surface against corrosion-causing bacteria.

In conclusion, different types of carbon steel were tested for biocorrosion employing different iron- and sulfur-oxidizing bacteria in the presence of corrosion-inhibiting bacteria in a comparative biocorrosion study. The mixed culture of *A. ferrooxydans* SKC/SAA-2 and *A. niger* caused localized corrosion that was more deleterious than *C. thiooxydans* SKC/SAA-1, resulting in distinct corrosion tendencies. The weight loss of API 5L X52 was more minor (0.06-0.27 percent) than that of AISI 1006 during the immersion testing (0.14-0.32 percent). It was determined that the corrosion caused by the immersion duration became more severe as the time spent in the water increased. During the extended incubation period, the corrosion-inhibiting bacteria became more homogeneous and compact, which resulted in the specimen surface exhibiting more robust protective qualities than previously observed. The API 5L X52 was effectively protected by the 2-week-old biofilm, as evidenced by the low amount of pitting corrosion. We believe that this will be the first report on comparative research between carbon steels utilizing a variety of corrosion-causing microbes in the presence of corrosion-inhibiting bacteria, to the best of our knowledge.

Conflict of Interest

The authors declare no conflict of interest.

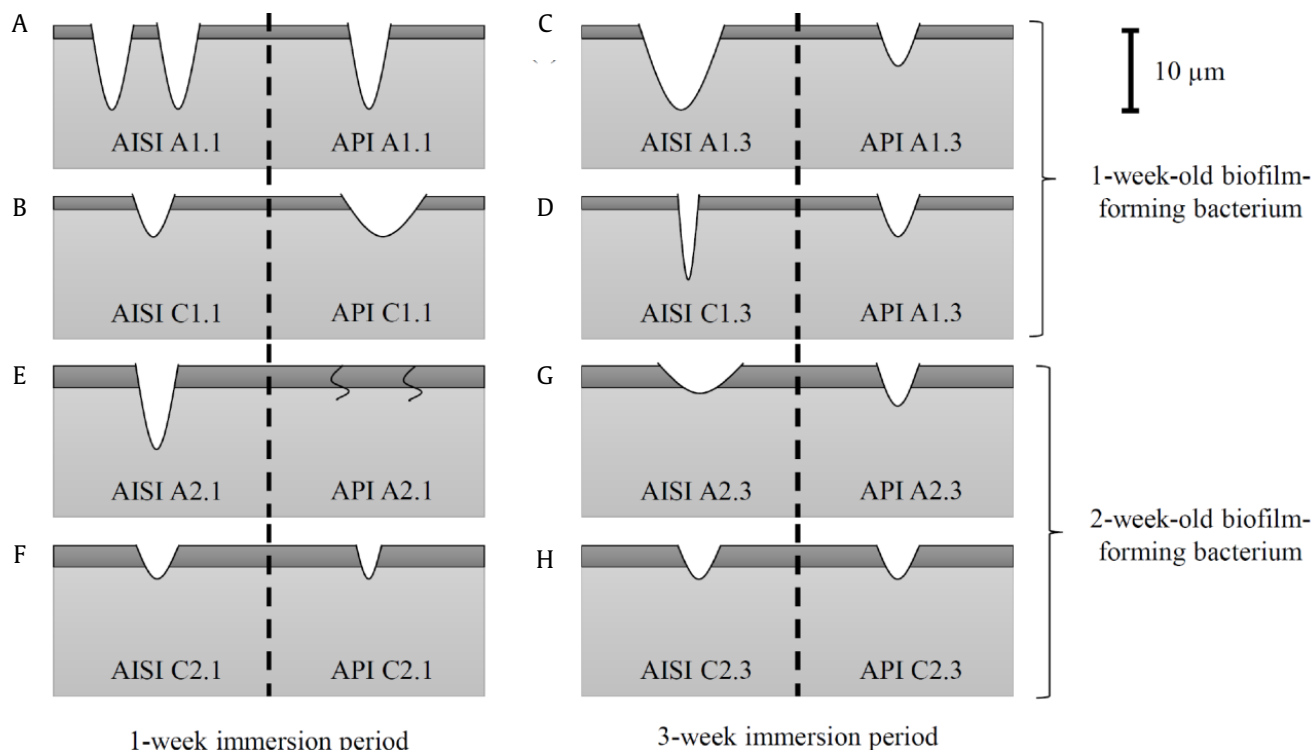


Figure 6. Schematic illustration of a cross-section view of MIC: (A-D) in the presence of the 1-week-old biofilm-forming bacterium, (E-F) in the presence of the 2-week-old biofilm-forming bacterium. Scale bar is shown for measuring the pit depth and diameter. It is shown that a 2-week-old biofilm-forming bacterium is effective for protecting the specimen surface against corrosion than a 1-week-old biofilm-forming bacterium. The corrosion damage shown in API 5L X52 is less than AISI 1006

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