EVALUATION OF BIOFILMS FORMATION AND CORROSION OF STEEL BY MICROSCOPIC TECHNIQUES AND ELECTROCHEMICAL IMPEDANCE SPECTROSCOPY

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ABSTRACT

A clean metal surface which contacts natural or industrial waters undergoes a series of processes that lead to the formation of inorganic deposits and biofilms. In these structures, microorganisms adhere irreversibly to the substrate, embedded in a matrix of extracellular polymeric substances (EPS). The problems arising from biofilm formation, such as microbiologically influenced corrosion (MIC), loss of equipment performance, product damages, generate economic costs and may lead to structural failures with consequences for operators and/or users. The aim of this study was to evaluate the corrosion associated with the formation of bacterial biofilms on carbon steel surfaces. Bacterial cultures used in the experiments were isolated from different systems that presented MIC. SAE 1010 carbon steel coupons were placed in cultures for biofilm development. After 48 h coupons were extracted and bacterial adherence was measured by viable bacteria counts, epifluorescence microscopy, crystal violet assay and EPS quantification. The biofilm morphology was analyzed by scanning electron microscopy (SEM) and epifuorescence microscopy. Surface deterioration was monitored using electrochemical impedance spectroscopy and open circuit potential measurements. Studies carried out allowed

correlating the adherence of the tested strains with the degree of attack suffered by the SAE 1010 carbon steel coupons.

Key words: SAE 1010 carbon steel, Microbiologically influenced corrosion, Extracellular polymeric substances, Microscopic techniques, Electrochemical impedance spectroscopy.

INTRODUCTION

Biofilms are adherent microbial populations enclosed in a matrix of extracellular polymeric substances (EPS) [1]. Biofilms can develop on metal surfaces in natural environments and increase the rate of corrosion [2-4]. The presence of the biofilm could modify locally the chemical parameters at the biofilm/metal interface. These modifications could affect the kinetics of reactions at the interface leading to electrochemical variations [5-7]. In the case of carbon steel, an increase of the corrosion rate has been observed with different measurement methods [8].
Depending on the chemical composition of the EPS, these substances can exhibit high ability for complex-binding metal ions and thus are discussed to promote corrosion [9]. However, inhibition or a decrease of corrosion rate in the presence of EPS has also been reported [10]. Carbon steel is one of the most widely used material in the transport of water, petroleum products and chemicals [11], and external corrosion of buried pipes is a major problem for these pipeline systems [12]. The consequences of pipe failure are borne by a wide range of industries and utilities and it can include the costly loss of production, contamination of the environment, expensive and difficult repairs, suspension of critical services such as water supplies and serious safety hazards including public health risks due to contamination of water that can occur during a failure event.
Typically, microscopic techniques provide a qualitative assessment, while the surface chemical techniques provide qualitative and quantitative estimations of the characteristics of the biofilm and the corrosion processes [13]. Microscopic images of the biofilms, combined with the data from the chemical analysis of surfaces [14], and electrochemical measurements [15-17] provide information about the chemical composition of the corrosion products and microbiological deposits, and can be used to estimate the level of corrosion.
The aim of this study was to evaluate the corrosion associated with the formation of bacterial biofilms on the carbon steel surfaces with different microscopic, analytical and electrochemical techniques.

EXPERIMENTAL PROCEDURE

Metal substrate

Carbon steel SAE 1010 coupons were used. For all the experiments, the coupons were degreased with acetone and sterilized by UV light during 30 min on each side before exposure to the experimental media.

Biofilm formation and characterization

Three bacterial strains isolated from corroded metallic systems and identified through 16S rDNA sequence analysis were selected for these experiments (Table 1). Besides, a strain of *Pseudomonas* sp. was used.

Table1: Bacterial strains isolated from corroded metallic systems used in this work.

Strains	Taxa	GeneBank Accession Number
165	*Bacillus* sp.	KM349191
175	*Acinetobacter* sp.	KM349193
178	*Paenibacillus* sp	KM349194

For biofilm formation, flasks containing 80 mL of nutrient broth were inoculated with strains (initial OD ≈ 0.1) in which up to 6 coupons were placed. The flasks were incubated at 28ºC during 48h.
For bacterial counts, 2 coupons were taken, rinsed with distillated water, scraped into 1mL of sterile physiologic solution, serial diluted and 0.2mL were seeded in nutrient agar plates (in duplicate) and incubated at 28ºC, 24h.
For evaluation of the biofilm formation capacity, 2 coupons were rinsed with distillated water, covered with crystal violet 0.1% (w/v) during 10 min. at room temperature. The excess was washed out with water, and the coupons were air-dried. The dye was solubilized with acetic acid (30% v/v) and the color was measured in a spectrophotometer at 590 nm.
Other 2 coupons were scraped in 1 mL of saline and sonicated with its scraping products: 3 pulses of 1 min. each one for exopolysaccharide extraction. The carbohydrates content of the extract was determined by the phenol-sulfuric acid method, described by Dubois (1956) [18]. For epifluorescence microscopic observation of biofilms, 2 coupons were extracted, rinsed with distillated water stained with DAPI 5 min. at room temperature, then rinsed with ethanol 80%. Stained coupons were kept in dark at -20°C until observation using an Olympus BX51 microscope.
For scanning electron microscopic (SEM) observations of biofilms, 2 coupons were used. They were rinsed with distillated water, fixed with glutaraldehyde 2.5% in phosphate-buffered saline (PBS), dehydrated with ethanol 20 to 100%, then critical point drying and surface-conductive ultra-thin coating were applied on all samples. ESEM observations were carried out using a FEI Quanta Microscope.

Electrochemical measurements

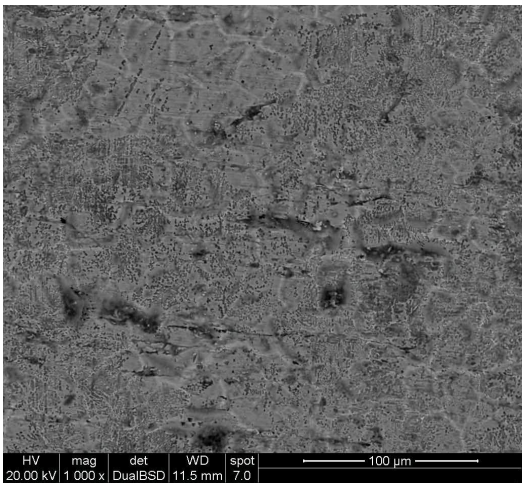
The open circuit potential of the carbon steel coupons was measured periodically during biofilm formation. Coupons were connected to a high impedance digital multimeter using a saturated calomel electrode as reference.
Electrochemical Impedance Spectroscopy (EIS) measurements on coupons with bacterial biofilms after 48 h of formation were performed. The cell contained the working electrode, a platinum counter electrode and a saturated calomel reference electrode and sterile nutrient broth as electrolyte. EIS data were recorded using a Solartron 1286 and a frequency analyzer Solartron 1250, *ac* 10 mV at OCP between 0.01 Hz and 20000 kHz was applied via Zplot software. The ZView software (Version 2.6b) was used for graphs drawing, equivalent circuit fitting and calculation of the parameters.
The same electrochemical measurements were done using SAE 1010 carbon steel coupons immersed in sterile nutrient broth as controls.

RESULTS

Biofilm formation and characterization

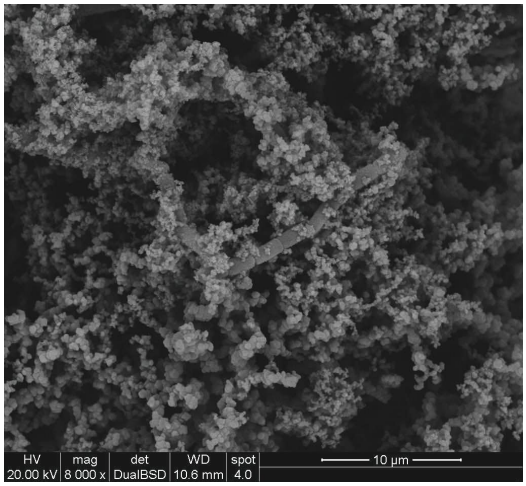
The micrographs obtained for SAE 1010 carbon steel exposed in the sterile medium showed the attack suffered by the material, associated with the active surface of the same (Fig. 1 a). In the presence of bacteria, a more complex film could be observed (Fig.1 b-e). Different layers with corrosion products, bacteria, and EPS can be seen. The characteristics of these films varied according to tested strain. EDX microanalysis show the presence of oxygen associated not only with the formation of oxides and heterogeneity of corrosion products of SAE 1010 carbon steel but also indicate the bioorganic presence. Epifluorescence microscopic images allowed to observe bacterial adherence and the EPS matrix formed for each strain (Fig. 2 a-d). The production of EPS resulted more noticeable in the case of strains 178 and *Pseudomonas sp.*

a)



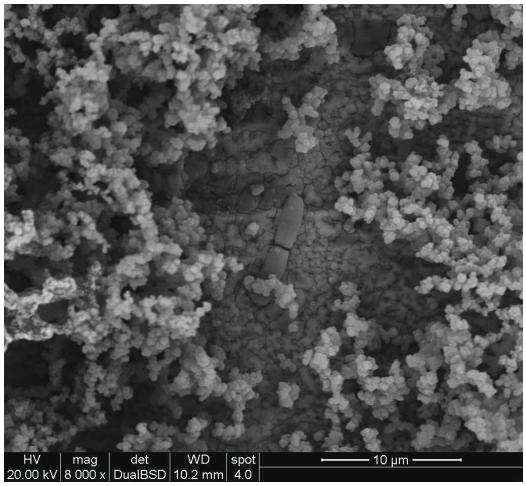
C 2.81, O 4.38, P 0.92, Fe 91.89

b)



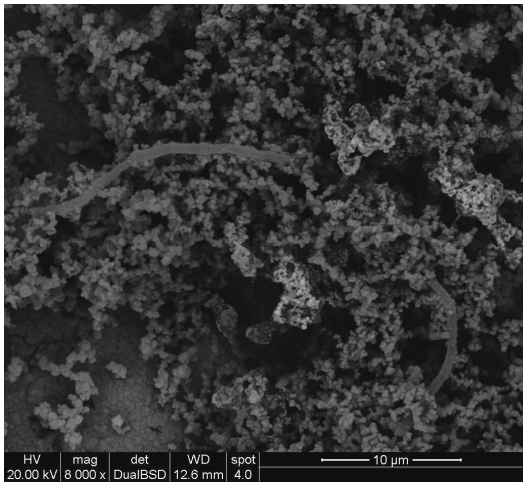
C 2.30, O 3.17, Na 1.42, Fe 93.10

c)



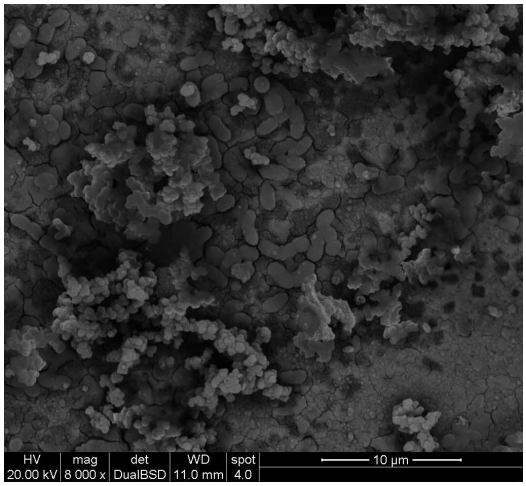
C 3.30, O 10.28, Na 2.88, P 7.28, K 0.95, Ca 0.51, Fe 74.80

d)



C 3.35, O 22.81, Na 3.58, P 15.19, K 2.73 Ca 1.35, Fe 51.00

e)



C 4.12, O 12.95, P 5.44, Fe 77.48

Figure 1: SEM micrographs (x8000) of the carbon steel coupons after 48 h. of exposure a) in the sterile culture and b-e) in the bacterial cultures: b) strain 165; c) strain 175, d) strain 178; e) *Pseudomonas* sp.

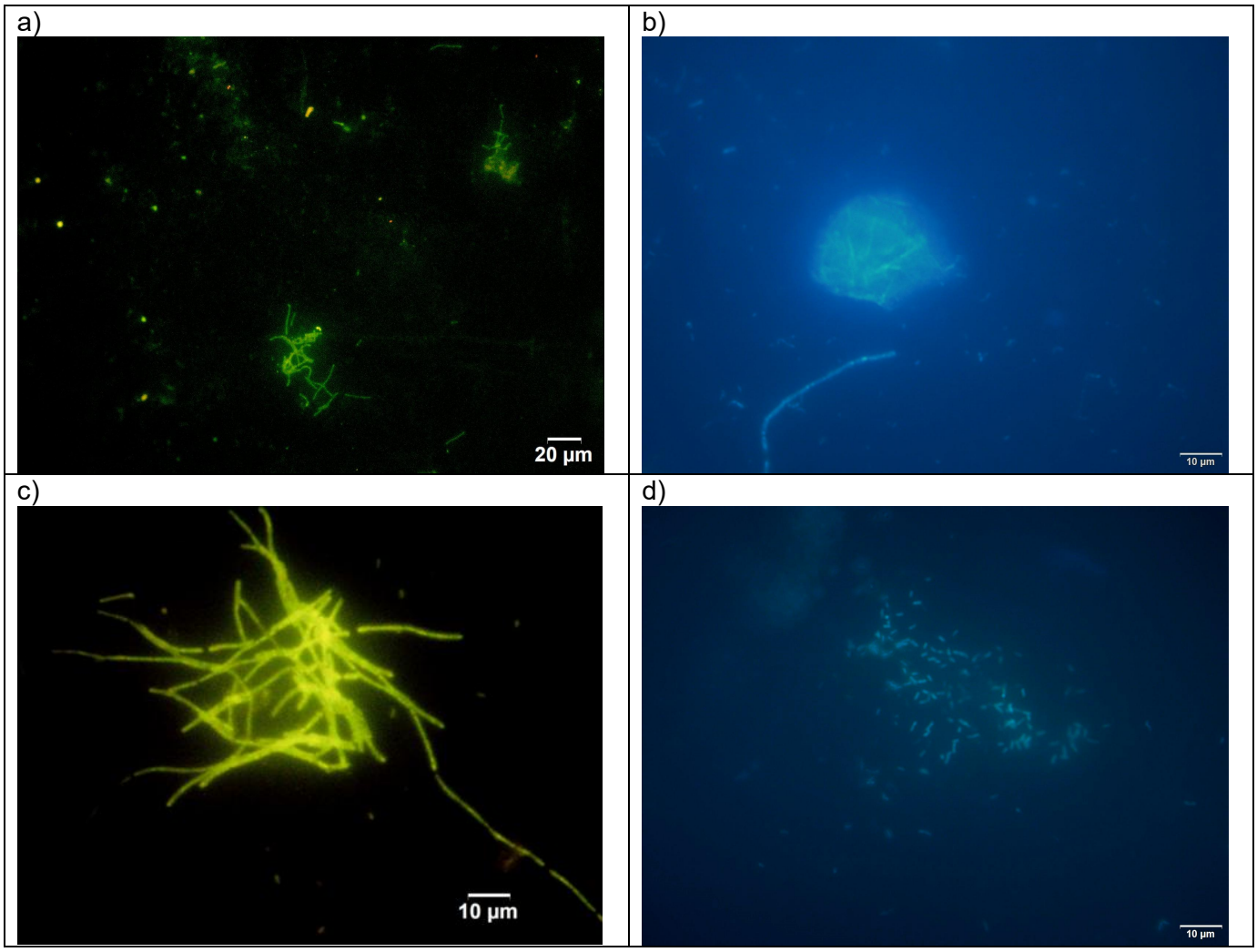


Figure 2: Epifluorescence micrographs of the carbon steel coupons after 48 h. of exposure in the bacterial cultures: a) strain 165 (x40); b) strain 175 (x100); c) strain 178 (x100); d) *Pseudomonas* sp (x100).

Figure 3 shows the number of sessile bacteria (in UFC.cm^{-2}) in biofilms formed on SAE 1010 carbon steel coupons. Biofilms of *Pseudomonas* sp. showed the highest number of microorganisms and the highest production of EPS. The other three strains used did not show significant differences in the number of attached bacteria, however, the amount of EPS presented differences; strain 178 produced a significantly higher amount of EPS than the other two strains. The results obtained with the CV assay did not allowed to see differences in the attachment of these bacteria. EPS production may be related to the strain tested and not the ability to form biofilms [10].

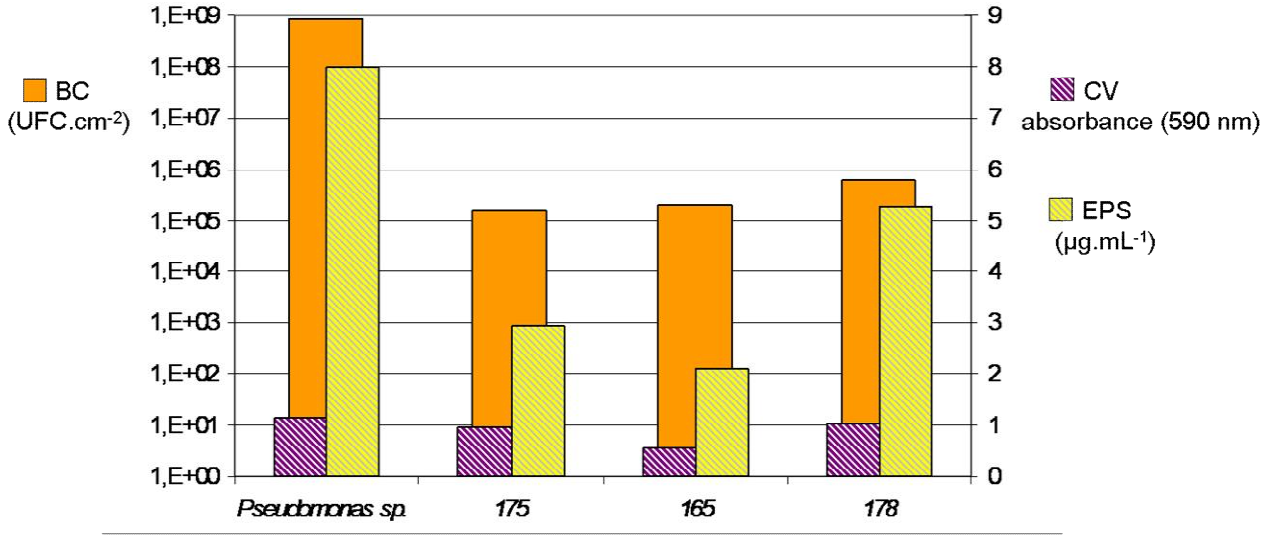


Figure 3: The graphic shows the values obtained for bacterial count (BC), exopolysaccharides (EPS) and crystal violet (CV) of the assayed strains.

Electrochemical measurements

The evolution of the OCP during biofilm formation can be seen in Figure 4. In the absence of bacteria OCP values showed small fluctuations around 0.660 V (vs SCE) were observed over time. In the presence of the different bacterial strains assayed, the OCP values were more anodic and showed more important variations over the time than in the case of the sterile medium. The decreased in the OCP values is related with the presence of active sites located on the metal surface and therefore the occurrence of localized corrosion. These would be associated with the metabolic activity of the different bacterial strains, which influenced the corrosion behavior of the carbon steel [19]. The most active OCP values found in the presence of *Pseudomonas* sp. indicating that this strain was more aggressive for SAE 1010 carbon steel than the other strains assayed.

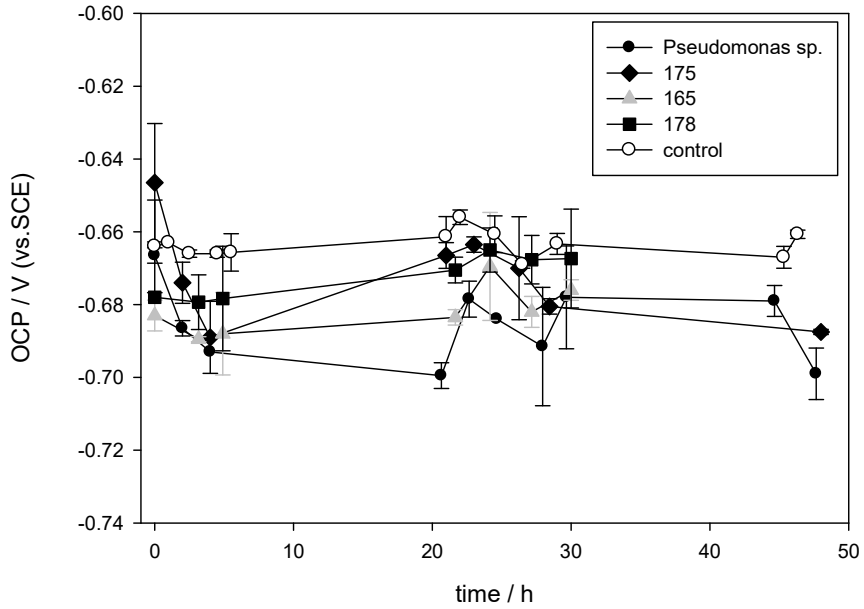


Figure 4: Open circuit potential vs. time plot for SAE 1010 carbon steel in the presence of different strains.

EIS results obtained for the biotic system is observed in Figure 5. The diagram shows a defined semicircle corresponding to a charge transfer process. An increase in the diameter of the Nyquist semicircle was obtained in the presence of bacteria, indicating that the bacteria influenced the corrosion rate. The asymmetry observed in the semicircles, can be associated with inhomogeneous surfaces which can result in dispersion in the time constant [20]. This dispersion would be associated with the presence of heterogeneous bacterial biofilms of different strains.

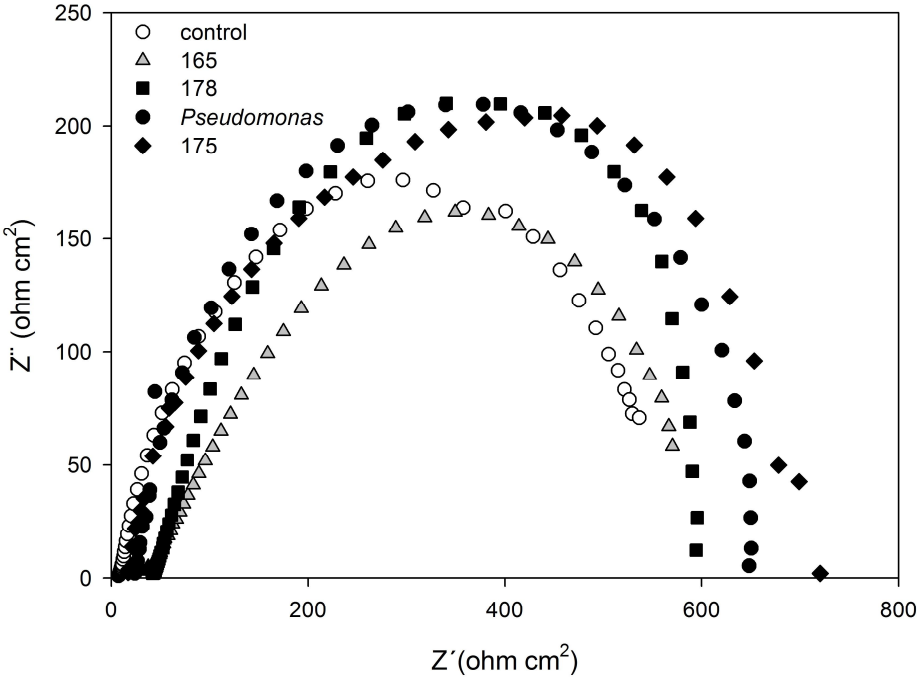


Figure 5: Nyquist diagram of the electrochemical impedance spectra of SAE 1010 carbon steel with bacterial biofilms.

CONCLUSIONS

- The results obtained with the CV showed a similar biofilm forming capacity in all the bacterial strains assayed.
- The amount of EPS excreted varied with the different strains tested. The production of EPS resulted more noticeable in the case of strains 178 and *Pseudomonas sp.* EPS production may be related to the strain tested and not the ability to form biofilms.
- The most active OCP values obtained in the presence of *Pseudomonas* sp. indicated that this strain was more aggressive for SAE 1010 carbon steel than the other strains assayed. The dispersion in the time constant observed in the Nyquist diagrams would be associated with the presence of heterogeneous bacterial biofilms.

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