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EIS Evaluation of corrosion resistance of AISI 304 stainless steel exposed to *Pseudomonas stutzeri*

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In this work, the formation of a *Pseudomonas stutzeri* biofilm in a marine medium on AISI 304 stainless steel was observed to evaluate the effects of biofilm formation on metallic passivation and corrosion processes. Open circuit potential and electrochemical impedance measurements were carried out for 21 days on abiotic and biotic electrochemical cells. The influence of biofilm formation on corrosion potential was monitored and used to propose a mechanism based on the impedance and corrosion resistance values. In the abiotic cell, the corrosion resistance varied periodically, which was attributed to the electrolytic activity of the medium on the metal. However, corrosion resistance increased in the presence of bacteria and continued to increase as immersion time increased. Exopolymeric substances were deposited onto the electrode, suggesting that *P. stutzeri* RPh9 colonized the metallic surface, passivating the stainless steel electrode surface through continuous biofilm formation. Detachment of the biofilm on day 21 was the likely cause of decreased corrosion resistance at the end of the experiment.

Keywords: stainless steel, biofilm, EIS, OCP, *Pseudomonas stutzeri*, corrosion resistance.

1. INTRODUCTION

Corrosion as a cause for metallic deterioration is of prime importance due to enourmous hazard to environment and human life when a severe damage of a infrastructure is involved[1]. The oil industry uses carbon or stainless steels for the transfer and storage of chemical agents, but these materials are not immune to corrosion. In marine environments, microbiologically induced corrosion (MIC) can occur, wherein microorganisms initiate or accelerate corrosion through the production of corrosive metabolic products. MIC, also called biocorrosion, increases as the synergy of the bacteria with the surface increases, as in the case of biofilm formation, which might influence an aggressive site for degradation of engineering materials [2]. MIC in steel causes localized corrosion and has a considerable impact on many industries. Localized corrosion or pitting can lead to leaks or spills from metal containers, causing

production stoppages for repair and environmental damages due to pollution. The synergy between inorganic corrosion and MIC in carbon and stainless steels has not been conclusively determined. It is vitally important to know how to diagnose, prevent, and control MIC.

AISI 304 stainless steel has austenite as its primary crystalline structure [3]. AISI 304 stainless steel is currently used for numerous applications that require high corrosion-resistant materials, such as in the aeronautic, metallurgical, and medical device industries. Because it possesses a passive surface film, AISI 304 stainless steel is resistant to corrosion, although halides in the environment can induce local pitting [4-5]. Previous MIC research [6,7,8] noted the appearance of pitting in soldered zones. Specifically, biocorrosion was detected on round, welded AISI 304 stainless steel tubes submerged in stagnant water for 3 months with stainless steel tubes and welding zone with corrosion. Besides, anaerobic corrosion of 304 stainless steel by *Pseudomonas aeruginosa* biofilm has been reported with linear polarization resistance(LPR), electrochemical impedance spectroscopy (EIS) tests, involving robust biofilms of this bacterium that causes pitting corrosion.

Pseudomonas stutzeri is a member of the genus *Pseudomonas* with the ability to produce biofilms and is located widespread in soil and in water, including in sea habitats. *P. stutzeri* strains had been misidentified respect to other species before generalized use of genomic approaches to identifying bacteria. This specie differentiates from other members of the fluorescent group of *Pseudomonas spp*., due to characteristic absence of fluorescent pigments [9].

Some strains of *Pseudomonas stutzeri* have been previously associated with microbiologically influenced corrosion (MIC). An investigation of corrosion of carbon steel in seal rings at an offshore facility demonstrated the dominance of *Pseudomonas* genus in the different corroded seal rings, which supports the assumption that microorganisms participated in the corrosion phenomenon. Among the species that were found, the presence of *Pseudomonas stutzeri* CCC-IOB10 was reported [10]. Similarly, *Pseudomonas stutzeri* AP2 was isolated during a characterization of corrosive bacterial consortia inside petroleum transporting pipelines [11].

At this respect, an electrochemical investigation and biological experiments with electrochemical impedance spectroscopy (EIS) measurements, demonstrated that after 7 days of immersion, separately, *Pseudomonas flava* and *Pseudomonas stutzeri*, decreased corrosion rate of mild steel in a basal salt medium. Electrochemical results exhibited possible settlement of biofilm [12].

By other side, there are no previous studies of microbiologically influenced corrosion involving this bacterium onto stainless steel. This study emphasizes that *P. stutzeri* could be a relevant bacterial model for understanding the MIC process in marine settings, onto stainless steel surface. In this work, the *P. stutzeri* strain RPh9 was isolated from shallow marine sediments in Rosarito Port, Baja California, Mexico, and biofilm formation on AISI 304 stainless steel was monitored and characterized using electrochemical techniques. The influence of *P. stutzeri* RPh9 on the passivation and corrosion of AISI 304 stainless steel was studied to shed light on the mechanism of microbial biofilm formation on stainless steel immersed in a marine environment.

2. METHODOLOGY AND PREPARATION

2.1 Bacterial strain and culture medium preparation

Isolation of *P. stutzeri* strain RPh9 for this investigation was performed from shallow marine sediments in Rosarito Port, Baja California, Mexico. The strain was isolated by enrichment and streaking

on Bushnell Haas medium supplemented with 25 mgL^{-1} phenanthrene as the sole carbon and energy source. For this study, *P. stutzeri* was grown in Zobell broth prepared with 0.5% Bactopeptone (BD Biosciences®), 0.1% yeast extract (MCD LAB®), 75% natural seawater extracted from the coast of Baja California, and 25% distilled water. The media was sterilized at 121 °C for 30 minutes in a Quadrant® E-015R autoclave.

2.2. Preparation of working electrodes

Two AISI 304 stainless steel working electrodes were prepared: one electrode for the abiotic electrochemical system without bacteria (control), and another electrode for the biotic system seeded with *P. stutzeri*. Each electrode was mounted in Comex® Kristalizer epoxy resin with an exposed surface area of 3.75 cm^2. The surface was polished with 120-, 200-, 300-, 400-, 600-, 800-, 1200-, and 2000-grit TENAZIT® sandpaper. The polished surface was washed with distilled water and 97% ethyl alcohol (Protec®).

2.3 Characterization of AISI 304 stainless steel microstructure

The samples of stainless steel were observed using energy-dispersive spectroscopy (EDS) with an SU5000 HITACHI scanning electron microscope at variable pressures, as well as by field emission scanning electron microscopy (FESEM) as can be seen in Figure 1.

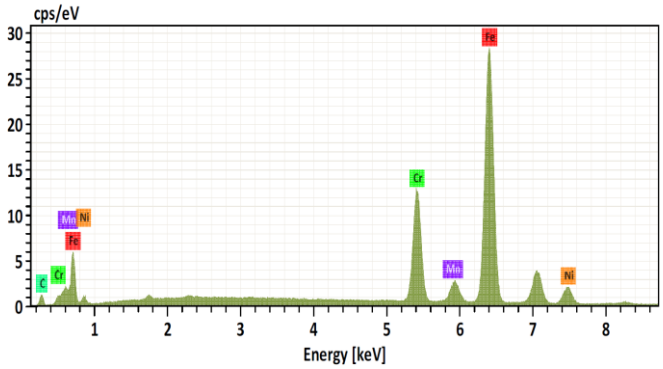

Figure 1. EDS element mapping of AISI 304 stainless steel before experimentation.

Table 1. Chemical composition of AISI 304 stainless steel.

Element	Fe	Cr	N	C	Mg
Wt. %	60.17	17.73	6.95	4.65	1.50

Table 1 shows the chemical composition of AISI 304 stainless steel. AISI 304 stainless steel is an alloy of iron and carbon and belongs to the 300 series of stainless steel [13]. Unlike carbon and low-

alloy steel, the presence of minimum chromium in AISI 304 stainless steel endows the material with corrosion resistance [14-15]. When exposed to oxygen, a chromium oxide layer forms on the surface of the material [16].

2.4 Assembly of the electrochemical cell

Electrochemical cells were sterilized in a Quadrant® E-015R autoclave. The working electrodes, saturated calomel reference (ECS) electrodes, and platinum electrodes were sterilized under UV rays in a Labtech® laminar flow hood for 35 minutes. The electrochemical cells were filled with 100 mL of sterile Zobell broth, a magnetic stirrer, the working electrodes, the ECS reference electrodes, and the platinum electrodes.

One day before electrochemical cell assembly, a liquid culture of *P. stutzeri* RPh9 was prepared. The electrochemical cell was inoculated with bacterial culture at an initial optical density (OD_{600nm}) of 0.05. Each electrochemical system was sealed both with a hermetic cap and PARAFILM "M"® paper. Every 7 days, the Zobell broth was exchanged in each cell to keep the bacterial strain in the exponential growth phase [17].

2.5. Electrochemical evaluations

After the electrochemical cells were assembled, daily electrochemical evaluations using electrochemical impedance spectroscopy (EIS) and recordings of the open circuit potential (OCP) were carried out for 21 days. Monitoring was performed with a BioLogic® VSP-300 potentiostat/galvanostat. Data were analyzed in EC-Lab® software in the following order: OCP measurement, then EIS evaluation.

2.5.1 Evaluation of the OCP

The OCP of each electrochemical cell with respect to the saturated calomel reference electrode (SCE) was monitored during 21 days.

2.5.2 Evaluation by EIS

The steel-marine medium interface in the biotic and abiotic cells was monitored with EIS. The microscope was programmed in a frequency range of 100,000 to 0.01 Hz with a voltage amplitude of 0.01 V; 10 points were taken per decade of frequency [18].

2.5.3 Analysis of the electrochemical data

The E_{OCP} *vs* SCE, as determined by OCP, was analyzed for both biotic and abiotic systems. In addition, the Nyquist and Bode plots of the EIS technique were obtained for the analysis of impedance

values and time constants, respectively. R_{ct} values were obtained from Nyquist plot fitting and were plotted with respect to time. Equivalent electrical circuits (EEC) models for both systems were obtained by the same fitting.

3. RESULTS AND DISCUSSION

Figure 2 shows the diagram of E_{OCP} over time for the abiotic and biotic systems. For the abiotic system, the E_{OCP} showed little fluctuation during the 21 days of immersion, with an average stabilization potential of -0.48 ± 0.01 V (*vs* SCE). However, there was notable variation in the potential of the bacterial system from day 1 to day 4. The E_{OCP} values tended towards the active direction of the metal, which was likely a result of the reducing nature of the medium due to bacterial metabolic activity [18]. A constant potential of -0.47 ± 0.01 V (*vs* SCE) was maintained between days 5 and 8. The potential reverted between days 8 to 19 towards the noble direction (away from the activation zone), indicating passivation on the metallic surface. This reversal was thought to be due to *P. stutzeri* RPh9 biofilm formation and the adhesion of corrosion products that passivated the metal [19]. On the last 2 days, the E_{OCP} became negative again, indicating a metallic dissolution process.

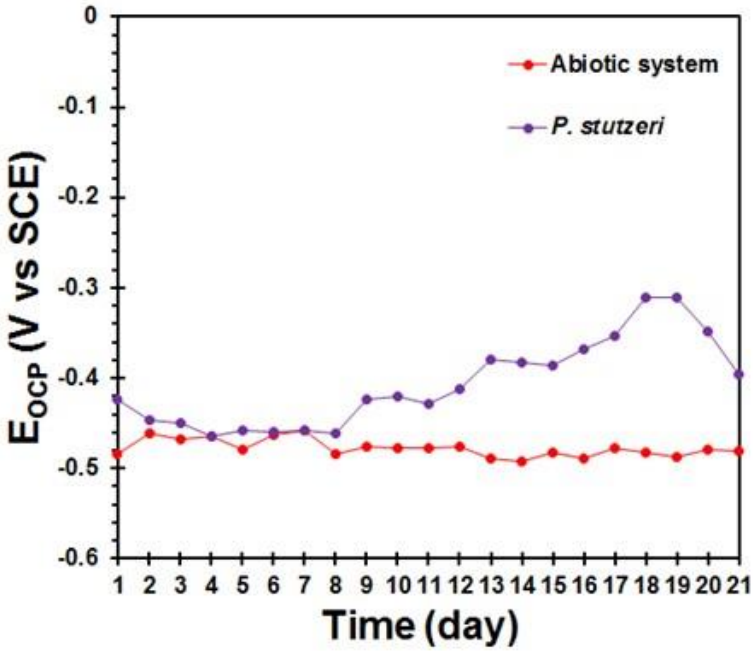

Figure 2. E_{OCP} values of the abiotic and bacterial systems immersed in a simuluated marine environment over the course of 21 days.

Figure 3 shows the EIS results obtained from the abiotic (control) cell over the course of the experiment. The fracture of the passivation layer of the steel caused a galvanic couple: the anodic zone was where superficial depassivation was occurring, and the rest of the surface was the cathodic zone [20]. Anodic zones were created inside the pits due to the increased concentration of iron cations and

other ions, deposited as hydroxides, that produced metallic dissolution and led to an accelerated destruction of the passive layer [19]. The calculated Bode parameters showed that at high frequencies, the resistance values of the solution (R_{sol}) for the immersion times were similar between abiotic and biotic systems (Figure 3b). At low frequencies, the impedance modulus (|Z|) had quite high and varied values, verifying the impedance values calculated from the Nyquist plot and the R_{ct} values (Figure 3d). At high frequencies, phase angles attributed to the passivation layer of stainless steel were observed, while at low frequencies, the process was controlled by charge transfer (Figure 3c) [21].

The variability in R_{ct} in the abiotic system was due to chloride ions that caused pitting in the metallic surface. The trends in E_{OCP} values, as well as the variable R_{ct} values (Figure 3d), correlated with the development of pitting corrosion [22]. Ions, mainly chlorides, from the marine medium caused pitting on the metal surface (decreased R_{ct}) and metallic repassivation (increased R_{ct}).

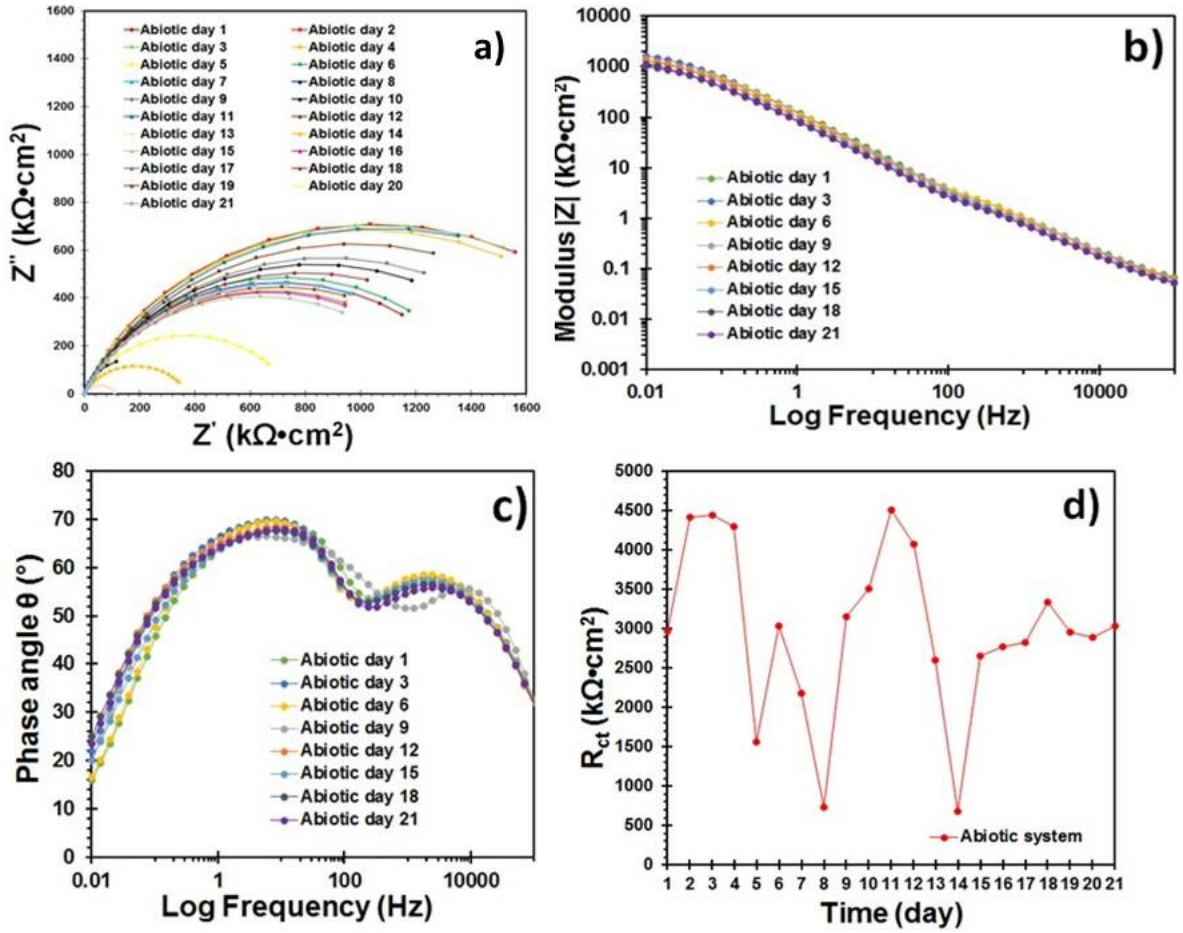

Figure 3. EIS results for the abiotic (control) cell over the course of 21 days in marine medium. a) Nyquist diagram. b) Bode diagram of the impedance modulus (|Z|). c) Bode diagram of the phase angle. d) R_{ct} variation *vs* immersion time.

Figure 4 shows the EIS results for the bacterial cell. As the immersion time increased, there was a decrease in the impedance values, except for the first 2 days and the last day. The maximum impedance (221.33 kΩ•cm^2) was on day 20.

The increase in R_{ct} (Figure 4d) was attributed to the formation of *P. stutzeri* RPh9 biofilms on the metal surface. Certain areas of the surface were covered by microbial clusters, causing the surface resistance of the metal to increase [23].

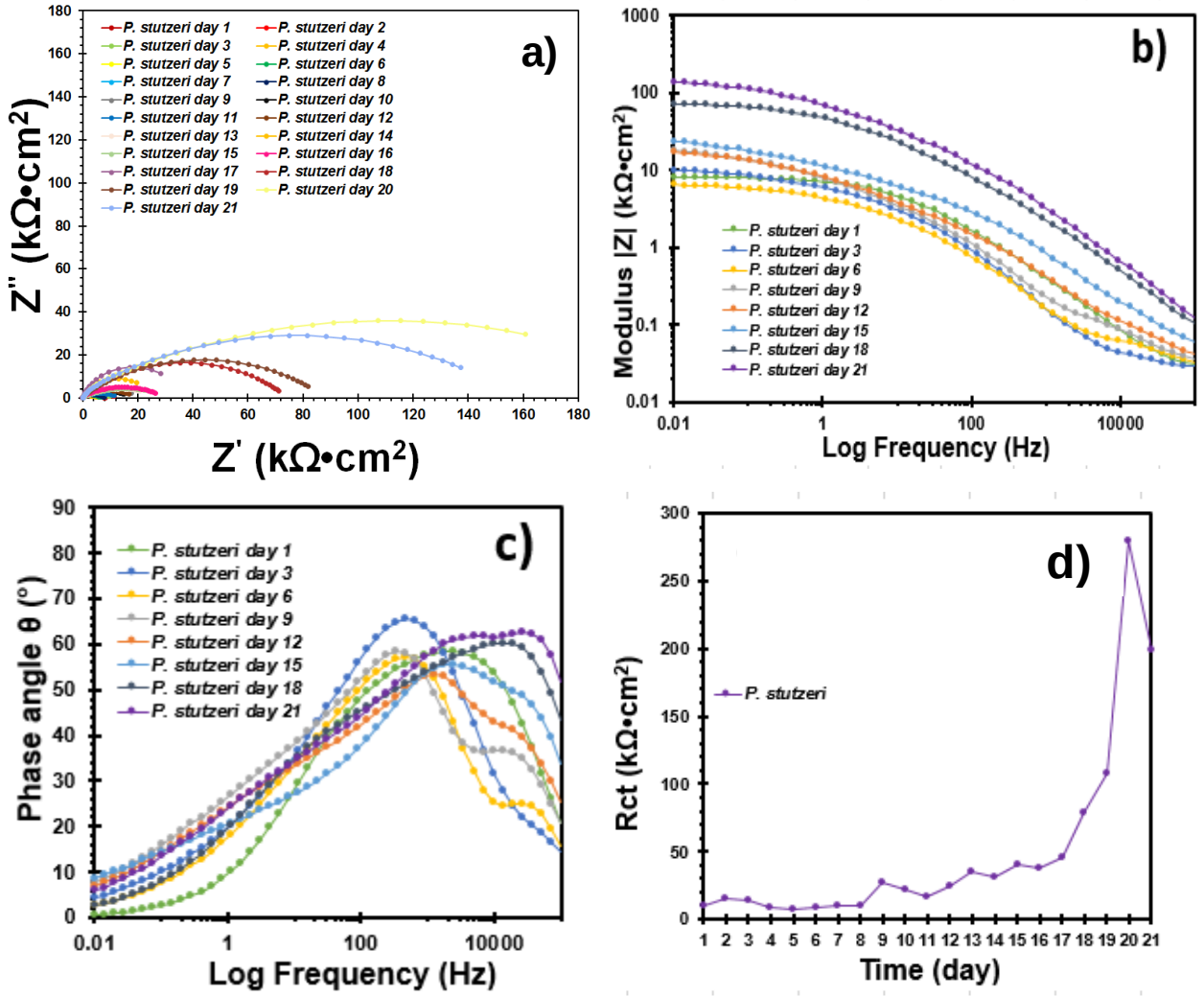

Figure 4. EIS results for the biotic cell over the course of 21 days. a) Nyquist diagram. b) Bode diagram of the impedance modulus (|Z|). c) Bode diagram of the phase angle. d) R_{ct} *vs* variation immersion time.

The biofilm lasted for 20 days, as evidenced by the increase in R_{ct} (Figure 4d). This stage of formation consisted of a mass transfer barrier for dissolved oxygen and aggressive ion deposition from the marine medium [17]. Electrons were released from the oxidation of elemental iron in anodic areas on the surface of the metal, which were consumed by the biofilm. There was no protective passivation layer due to the local shortage of dissolved oxygen, which was consumed by the upper layer of the biofilm [17, 21]. This effect manifested as a decrease in R_{ct} on day 21 (Figure 4d), suggesting that the charge transfer between the biofilm and the metal surface increased [24].

At high frequencies, R_{sol} varied with respect to immersion time. This could be attributed to modification of the ionic composition of the medium as a result of microbial activity [25, 21].

|Z| also varied at low frequencies, likely due to passivation of the metal by the bacterial biofilm (Figures 4a, d). An additional set of processes not seen in the abiotic cell occurred in the biotic cell at medium frequencies (Figure 4c): a passivation layer attributed to the formation of a *P. stutzeri* RPh9 biofilm, followed by the passivation of the chromium oxide film, and finally, the load transfer process of the steel itself [21]. Variation in the load transfer process with respect to time was also observed.

Equivalent electrical circuit (EEC) models for the abiotic cell (Figure 5a) and the biotic cell (Figure 5b) were used to model the observations.

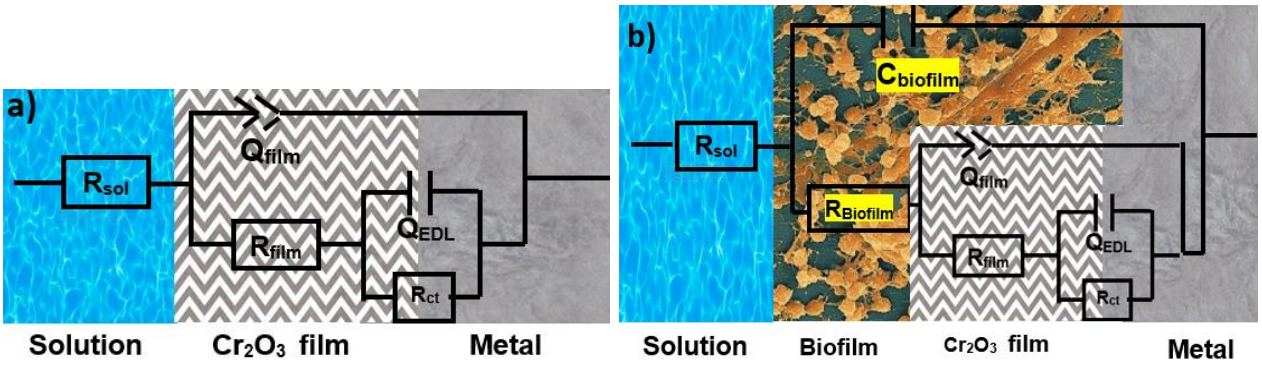

Figure 5. Equivalent electrical circuit models of a) the abiotic cell and b) the biotic cell.

The ECC model of the abiotic cell is made up of five elements: R_{sol}, resistance of the solution; Q_{pel}, passive film capacitance; R_{pel}, passive film resistance; Q_{EDL}, electrochemical double layer constant phase element; and R_{ct}, resistance to charge transfer. Two additional elements are present in the biotic cell EEC model: Q_{biopel}, an element with a constant phase in the bacterial biofilm, and R_{biopel}, which is the resistance contributed by the biofilm. Q_{biopel} was included in the biotic cell EEC because the metallic surface had increased roughness and/or fissures [26]. The mechanism of bacterial biofilm formation, coupled with the corrosion process, is illustrated in Figure 6.

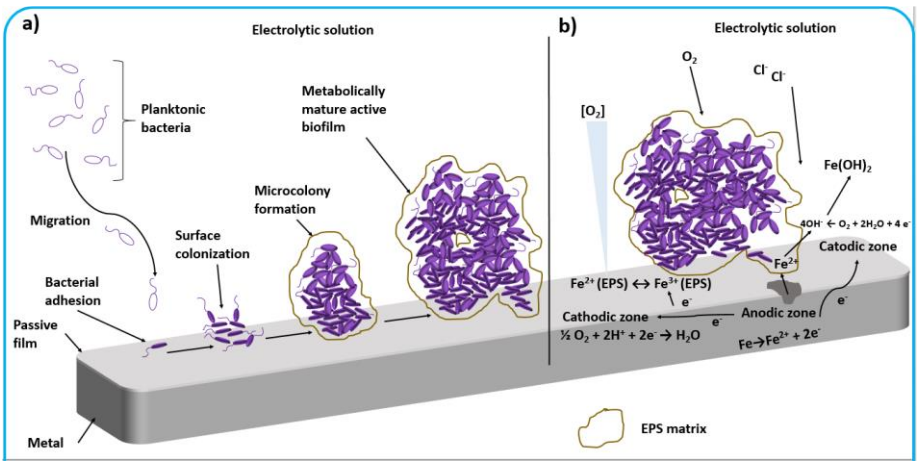

Figure 6. Proposed mechanism of a) *P. stutzeri* RPh9 biofilm formation and b) corrosion process on AISI 304 stainless steel.

Electrochemical reactions are shown in equations (2) and (3).

Anodic reaction: $Fe \rightarrow Fe^{2+} + 2e^-$ (2)
Cathodic reaction: $O_2 + 2H_2O + 4\,e^- \rightarrow 4OH^-$ (3)

For the abiotic and biotic cells, it was assumed that the cathodic reaction involved dissolved oxygen, since this gas was present in each system. *P. stutzeri* has been reported to grow well under atmospheric oxygen conditions [9]. When stainless steel is immersed in a marine medium under biotic conditions, it is subject to MIC that causes pitting [27,16]. The process includes bacterial adherence to an inert surface, such as stainless steel [28]. Flagella enable movement of bacterial cells through the medium, and fimbriae and pili allow bacterial adhesion to the metal surface. These factors may contribute to *P. stutzeri* biofilm formation in a mixed bacterial community, as *P. stutzeri* is a monotrichous flagellar Gram-negative bacterium [29].

Through cell signaling, bacteria can control the expression of the machinery necessary for the synthesis of extracellular polymeric substances (EPS), which can serve as sites of activation and facilitation of cell proliferation [30]. EPS form a surface on the biofilm with channels through which fluids, such as water and other liquids that carry nutrients, can circulate. Therefore, EPS supports and maintains the activity of the biofilm [30, 31]. It has been proposed that these water channels are circulatory systems, distributing different nutrients and removing waste materials from bacterial communities [31].

Finally, cell dispersion occurs by the shedding of new cells or by the flow-induced dispersion of aggregates. These aggregates include enzymes that cause the digestion of alginate (D-mannuronic acid and L-guluronic acid), which is responsible for the adhesion of EPS to surfaces [27]. It has been suggested that the biofilm formation process is controlled by cellular communication mechanisms including quorum sensing, in which certain molecules, called autoinducers, play an important role [28]. Quorum sensing is a mechanism of gene expression regulation that depends on cell density; the production and accumulation of autoinducers allows bacteria to establish, proliferate, and regulate the progress of biofilm formation. N-acyl homoserine lactone is a one such autoinducer found in Gram-negative bacteria such as *P. stutzeri* [31, 32].

We propose a mechanism of metallic corrosion in which bacterial biofilms, whose surfaces are metabolically active, generate corrosive metabolites that have organocatalytic activity, resulting in metallic surface deterioration [33]. At the biofilm-metal interface, the metallic iron dissolution process occurs (anodic reaction). We propose two cathodic zones: the first is the metal surface directly exposed to the marine medium, since bacterial biofilms do not cover the entire surface of the metal, and the second is the biofilm area with oxide-reductive metabolic and enzymatic activity [33,34].

The dissolved oxygen reduction reaction is found in the area directly exposed to the marine medium. The reaction generates hydroxyl ions that react with the metallic iron released from the anodic zone, generating iron hydroxides that deposit on the metallic surface. However, the presence of chloride ions in the electrolyte medium must be considered, as these also cause pitting in the passive metallic layer. We propose a synergistic effect, in which bacteria and chloride ions together promote metallic dissolution [22, 34].

In the bacterial zone, the cathodic reaction involving dissolved oxygen would occur under acidic conditions, due to the acidic microenvironment generated by metabolic byproducts. As the concentration of this gas decreases further into the biofilm, this is a secondary cathodic reaction. The corresponding enzymatic reduction process in the EPS would reduce Fe^{2+} to Fe^{3+} by means of an oxide-reductive enzyme [34, 35].

4. CONCLUSIONS

An abiotic electrochemical cell exposed to a marine environment had variable impedance values, attributable to the corrosion activity of the medium. This resulted in pitting corrosion of AISI 304 stainless steel. The corrosion resistance over time of stainless steel exposed to *P. stutzeri* RPh9 was higher than for the control cell, a phenomenon we attributed to the formation of biofilms on the metal surface. The *P. stutzeri* RPh9 biofilms created a protective environment for certain periods of time over the course of the experiment. However, a decrease in the corrosion resistance, as measured by EIS, was likely due to the organocatalytic activity of the biofilm on the metal. To our knowledge, this is the first study characterizing passivation film formation over stainless steel surfaces by the marine organism *P. stutzeri* RPh9.

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