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Enhanced Corrosion of 7075 Alloy by the Presence of *Bacillus megaterium*

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A *Bacillus megaterium* strain with corrosive properties was isolated from a bacterial community collected from the drain valve of a C130 aircraft principal fuel tank. This strain and the consortium present in the valve were studied microbiologically and electrochemically to evaluate the risk of corrosion. Aluminum alloy 7075 was used as a substrate and the corrosion behavior was studied in sterile minimal salt medium (MSM) as a function of immersion time. The isolation and characterization of the consortium by DNA sequencing showed that the bacterium with corrosive properties is closely related to *B. megaterium*. Electrochemical results revealed that the corrosion rate was not influenced in sterile and inoculated media with the consortium. However, a corrosive behavior was determined in presence of the isolated *B. megaterium* strain after 14 days immersion time. The effect might be related to interfacial local pH changes by oxygen reduction to OH^- ions. Further, SEM examinations and EDX analysis of metallic samples exposure in inoculated media showed localized attacks.

Keywords: microbiologically influenced corrosion, biocorrosion, aluminum alloy, 7075, *Bacillus megaterium*

1. INTRODUCTION

Microbiologically influenced corrosion (MIC) is mediated by microorganisms attached to the

metal surface and/or embedded in a biofilm, which may produce changes in terms of pH, dissolved oxygen concentration, and organic and inorganic compounds in the biofilm/metal interface from that of the bulk solution. Therefore, microorganisms can accelerate corrosion rates [1-10]. In particular, in the aeronautic industry, microbial contamination of jet fuels line system has been reported as a problem for at least 40 years, which have included a diverse group of bacteria and fungi, such as anaerobic sulfuric-reducing, iron-oxidizing, manganese-oxidizing, and acid-producing bacteria [11-20].

Aluminum alloys (AA) are widely used in aircraft construction due to their good corrosion resistance, high strength and low density. However, those alloys are susceptible to localized corrosion, such as MIC. For example, aerobic and anaerobic heterotrophic bacteria, sulfate reducing bacteria, fungi and yeasts have been found in corroded sites of different airplanes [21]. Hagenauer et al. [22] in 1994 investigated the biocorrosion behavior of different aircrafts. The authors concluded that some of the isolates might cause corrosion of AA7075. In addition, Ayllon [23] and Palermo [24] published that the presence of fungus *Hormoconis resinae* caused damage to AA7075 surface. On the other hand, corrosion of AA2024 in the presence of *Bacillus cereus* ACE4 exposed to a simulated fuel tank environment showed pitting corrosion damage, which was associated with the higher bacterial adhesion of *B. cereus* ACE4 [25]. Additionally, AA2024 exposed to *Bacillus subtilis* in minimal salt medium (MSM) showed also evidence of corrosion by using electrochemical measurements [26].

In this work we studied the corrosion of AA7075 in sterile and inoculated media. Samples of corrosion products were collected from a drain valve of a military aircraft, which carried JP-8 fuel. It should be noted that most of the time the drain valve was exposed to the environment. The corrosion products were analyzed by electrochemical, microbiological and surface analysis. The microbiological techniques revealed the presence of a *B. megaterium* strain in the consortium. *B. megaterium* is a spore-forming and gram-positive bacteria, named for its large size “megat(h)erium” (Greek for big animal) of 1.5 to 4 μm, even this microorganism is the largest of all bacilli. Porwal et al. [27] showed that *B. megaterium* is only distantly related to the *B. cereus* and *B. subtilis* groups and that it is more deeply rooted in the phylogenetic tree than previously thought. Even though no information may be found on the direct relationship between *B. megaterium* and aluminum alloys, might be postulated that the magnesium present in the AA7075 (close to 2.7wt. %) would be crucial to achieve its maximal growth [28]. Magnesium favors the generation of extracellular products [29] and the porosity of the cell wall and membrane [30]. Therefore, the magnesium can be used by the bacterium as part of its metabolic cycle, but the physical interaction between them is not understood yet. In addition, local pH changes at the biofilm/metallic surface interface have been related to the metabolic products of microorganisms. In this case, the local pH changes might influence the stability of the aluminum oxide film formation [31].

2. EXPERIMENTAL

2.1. Substrates

Samples correspond to 7075-T6 aluminum alloys (AA7075-T6) currently used in the aeronautic industry with nominal composition (wt. %): 5.51 Zn, 2.69 Mg, 0.291 Fe, 0.22 Cr, 1.58 Cu,

0.091 Si, 0.025Mn, 0.016 Ti and Al-remainder, which were provided by the Empresa Nacional de Aeronautica (ENAER). The specimens consisted of 1 mm x 10 mm ×10 mm cm^2plates machined from rolled plate. The specimens were mechanically ground sequentially with SiC paper of 800, 1200 and 2400 grit, then washed with distilled water and degreased with acetone.

2.2. Sample Collection and Bacterial Strains

Samples of corrosion products were obtained from a drain valve of a C130 aircraft. The corrosion products were placed in sterile plastic receptacles (Zelle, Germany) containing Lysogeny Broth medium (LB), which was composed of (per L): 10 g tryptone, 5 g yeast extract, and 10 g NaCl. Samples were transported in a cooler packed with ice to the laboratory. Bacteria were isolated with serial dilutions and conventional plating from the general enrichment conditions. The consortium and the isolated strains were stored at −18 °C to ensure the purity and consistency of the bacterial material in all subsequent experiments.

2.3. Bacterial identification

To identify the microorganisms present in the consortium was used a method for molecular identification based on 16s rRNA gene sequencing. The 16S rRNA (ribosomal ribonucleic acid) gene was amplified from genomic DNA (deoxyribonucleic acid) by polymerase chain reaction (PCR) using bacterial specific primers 515F [31] and the universal primer 1492R [32]. The reaction mix was performed using 0.25 μL of Taq DNA polymerase (500 U/mL), 5 μL of each deoxy (d) nucleotide (2mM) (dATP, dCTP, dGTP and dTTP; deoxyadenosine triphosphate, deoxycytidine triphosphate, deoxyguanosine triphosphate and deoxythymidine triphosphate), 5 μL of buffer, 4 μL of $MgCl_2$ (0.75 mM) and 0.5 μL of each primer (100 mM). The following thermal conditions were applied: 95 ºC for 45 s, 55 ºC for 45 s, 72 ºC for 45 s. Each cycle was repeated thirty times and a final elongation step of 72ºC for 10 min was added. Amplification reactions were carried out using a Palm Gradient Cycler (PCR) (Corbett). Verification of PCR amplifications were carried out by running the sample on a 1.5% agarose gel stained with SYBR gold (Invitrogen). Sequences obtained were assembled, analyzed, and manually edited using ChromasPro software (Technelysium Pty Ltd.) for a final sequence extension of (~450 bp). Strains were taxonomically classified using the Naïve Bayesian rRNA Classifier function from the RDP-Ribosome Database Project [33]. To determine the phylogenetic distance between the bacteria isolated and other bacterial strains with 16s rRNA gene sequences available on databases, a neighbor joining tree based on 16S rRNA gene sequences was generated using the software available at the NCBI web page.

2.4. Development of biofilms and corrosion in an aerated inoculated medium

Specimens were exposed to UV light (15 min per side) in a laminar flow hood to sterilize them. To simulate a corrosive environment, the sterile culture medium was MSM at pH 6.4, which contained

(per L): 0.22 g of $(NH_4)_2SO_4$, 1.20 g of KH_2PO_4, 0.23 g of $MgSO_4 \cdot 7H_2O$, 0.25 g of $CaCl_2$, and 0.024 g of yeast extract [24]. In addition, 0.3 mL of the microbial strain and the consortium, previously grown in LB media, were cultured in MSM at 25°C (1/100 mL) over night. The culture was inoculated ($\sim 10^8$ CFU/mL) and maintained for different times on an electrochemical cell containing AA7075-T6 plate. To maintain bacterial density near the steady state growth phase throughout the experiment, a semi-continuous mode of culture growth was employed, i.e. 75% of medium was drained and replaced with an equal amount of fresh sterile medium every 96 h.

2.5. Electrochemical Measurements

The electrochemical measurements were registered with a SP 200 Bipotentiostat (Biologic, USA), using triplicate coupons at 23 ± 2°C. All experiments were performed in a three-electrode electrochemical cell with a large platinum grid as the counter electrode, and all of the potentials were referenced to a KCl saturated calomel electrode (SCE). The working electrode area was 21 cm^2. The control electrolyte consisted of 100 mL of MSM, and a 1 mL of the respective bacterial strain was added to 100 mL of MSM for each sample. It should be noted that the specimens were sterilized with alcohol, washed with distilled water and were exposed to UV light for 15 min before to measure the electrochemical test. Electrochemical techniques were obtained as a function of immersion time. The I - E curves were carried out at a scan rate of 0.1 mV/s and the electrochemical impedance spectroscopy (EIS) diagrams were obtained at E_{corr} using a sinusoidal signal with 10 mV as amplitude over frequencies ranging from 3 mHz to 50 kHz.

2.6. Morphological characterization

A scanning electron microscope (JEOL, model JSM-6010LA) with a beam voltage of 14-15 kV was used to analyze the morphology of the bacteria, the consortium and the AA7075-T6 surface. Specimens were exposed to sterile and inoculated (~10^8 CFU/mL) media for 14 days as immersion time. The bacteria on the coupons were then fixed for 45 min in a 2.5% glutaraldehyde solution at 4 °C. Later, the specimens were dehydrated using solution with increasing ethanol concentrations (25, 50 and 75%) for 15 min [34]. The topography and elemental composition of the AA7075-T6 surface were examined after the removal of biofilms by using ethanol and ultrasonic (Elma, model S3) method for 15 min, before subjecting the coupons to SEM-EDX analyses.

3. RESULTS AND DISCUSSION

3.1. Bacteriological analysis

The consortium was composed of seven cultivable strains, which were isolated through several serial dilutions and plating rounds in LB media. The 16S rDNA sequence analysis was used to identify the most corrosive strain-which was determined by electrochemical techniques and will be described

later- that showed a close affiliation to *B. megaterium* strain NBRC 15308 (99 % identity) by BLASTN search database [35]. Fig. 1 shows the neighbor joining tree based on 16S rRNA gene sequences, revealing phylogenetic relationships between sequences of the bacterial phylum *B. megaterium* and the phylum Firmicutes (*Bacillus* related species).

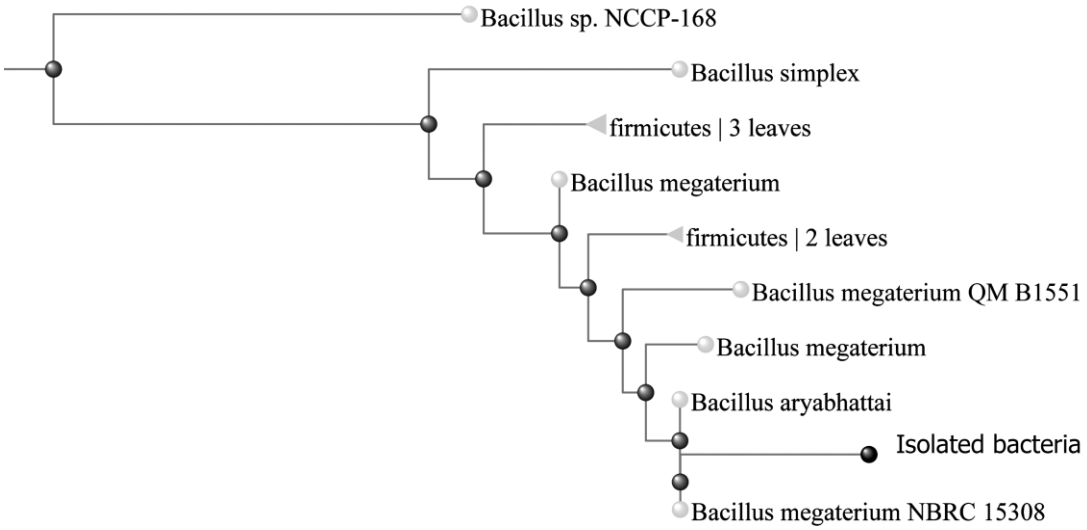

Figure 1. Neighbor-joining tree based on the 16S rRNA gene sequence of the highly corrosive bacterial isolate.

3.2. Corrosion behavior

3.2.1. Electrochemical responses after 14 days as immersion time

Fig. 2 shows the electrochemical response of AA7075-T6 in sterile and inoculated media. In particular, Fig. 2a shows the variation of the open circuit potential (E_{OC}) as a function of immersion time. For sterile media, E_{OC} value was shifted to more negative values, from -0.4 to -0.6 V, as the immersion time increased. For inoculated media with consortium, E_{OC} value remains relatively constant even up to 14 days of immersion time, with values near -0.8 V. For inoculated media with *B. megaterium*, E_{OC} value was shifted to more positive values, from -0.8 to -0.55 V, after 14 days as immersion time. In addition, Fig. 2b shows $I - E$ curves of AA7075-T6 in sterile and inoculated media, which reveals that the corrosion potential values (E_{corr}) tended to be similar in comparison to the previously mentioned after 14 days, as shown in Table 1. The corrosion current density (I_{corr}) value was much higher in the inoculated media with *B. megaterium* than in the inoculated media with consortium and sterile media, as also shown in Table 1.Tafel slopes were obtained from Fig. 2bin sterile and inoculated media. In sterile media, anodic Tafel slopes (β_a) as cathodic Tafel slopes (β_b) were close to 106 and 178 mV/dec, respectively. On the one hand, in inoculated media with *B. megaterium* the β_a was not significantly modified, however, β_c was decreased drastically to 96 mV/dec. On the other hand, in inoculated media with consortium the β_a decreased considerably to 67 mV/dec and the β_c was not influenced (see Table 1). Let us know, I_{corr}, Tafel slopes and the Stern-Geary

constant *B* were employed to obtain corrosion rate values (V_{corr}). A complete description was reported in reference [25,36]. V_{corr} values were close to 8.27, 160.00 and 0.013 μm/year in sterile, inoculated media with *B. megaterium* and consortium, respectively. From these results, the ability of *B. megaterium* to corrode the metallic surface might be related to the stability of the alumina oxide layer formation, which might influence local pH changes [35]. These pH changes might be related to enzymatic catalysis effect of oxygen reduction promoted by catalase.

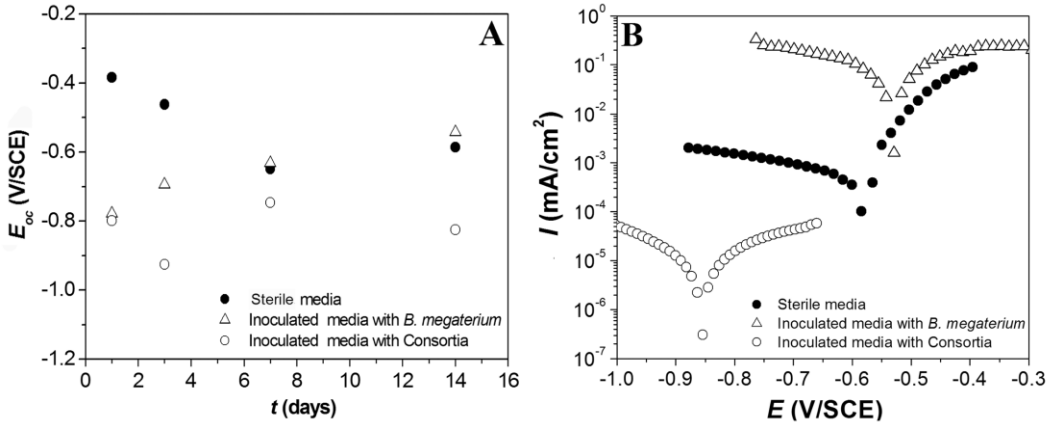

Figure 2. Electrochemical responses of AA7075-T6 in sterile and inoculated media. (a) E_{OC} curves obtained as a function of immersion time and (b) $I - E$ curves obtained after 14 days at 0.1 mV/s. Electrode surface Area = 21 cm^2

Table 1. Corrosion parameters obtained from $I - E$ curves of AA7075-T6 in MSM media

Media	*t* (days)	β_a ($mV \cdot dec^{-1}$)	β_c($mV \cdot dec^{-1}$)	E_{corr} (V vs SCE)	I_{corr} ($\mu A \cdot cm^{-2}$)	V_{corr} ($\mu m \cdot year^{-1}$)
Sterile (MSM)	14	106	171	-0.55	0.075	0.827
MSM/*B. megaterium*	14	118	96	-0.52	14.500	160.000
MSM/Consortium	14	67	112	-0.85	0.001	0.013

Fig. 3 shows the evolution of Bode diagrams corrected by electrolyte resistance (R_e) of AA7075-T6 as a function of immersion time in sterile and inoculated media. In all these cases a capacitive behavior was observed, which was characterized by a single time constant that can be attributed to the aluminum oxide film formation, except in the case of inoculated medium with *B. megaterium* after 14 days of immersion time, where the dissolution rate of the oxide layer could have been higher than its formation rate. On the one hand, no significant differences were observed in the modulus of the impedance ($|Z|$) at high and medium frequency range, except in inoculated media with *B. megaterium* after these 14 days of immersion. The V_{corr} was determined by using the $|Z|$ at low frequency range, as described in reference [25], that revealed V_{corr} values close to 0.147 and 680 μm/year, in sterile and inoculated medium with *B. megaterium*, respectively, after 14 days of immersion time, as shown in Table 2. On the other hand, the phase of the impedance (ϕ) for all

systems were close to 70° at high and medium frequency range, which may be also related to the aluminum oxide film formation. However, for inoculated media with *B. Megaterium* after 14 days of immersion, the ϕ values were close to 70° at very high frequency range and showed a significant deviation at medium and low frequency range that might be also associated with a higher dissolution rate of the aluminum oxide film than the formation rate.

In order to determine if the pH changes were associated with local or bulk behavior of the media, pH measurements of the bulk media were obtained, as shown in Fig. 4. The impedance results revealed that the alkaline media was similar to the impedance responses obtained in inoculated media with *B. megaterium* after 14 days of immersion time. Therefore, the behavior of *B. megaterium* might be related to a local increase of the OH^- ions concentration due to chemical and biological reactions resulting from the adherence of microorganisms, which might increase the dissolution rate of the aluminum oxide film or further this microorganism does not allow the oxide film formation [41-42].

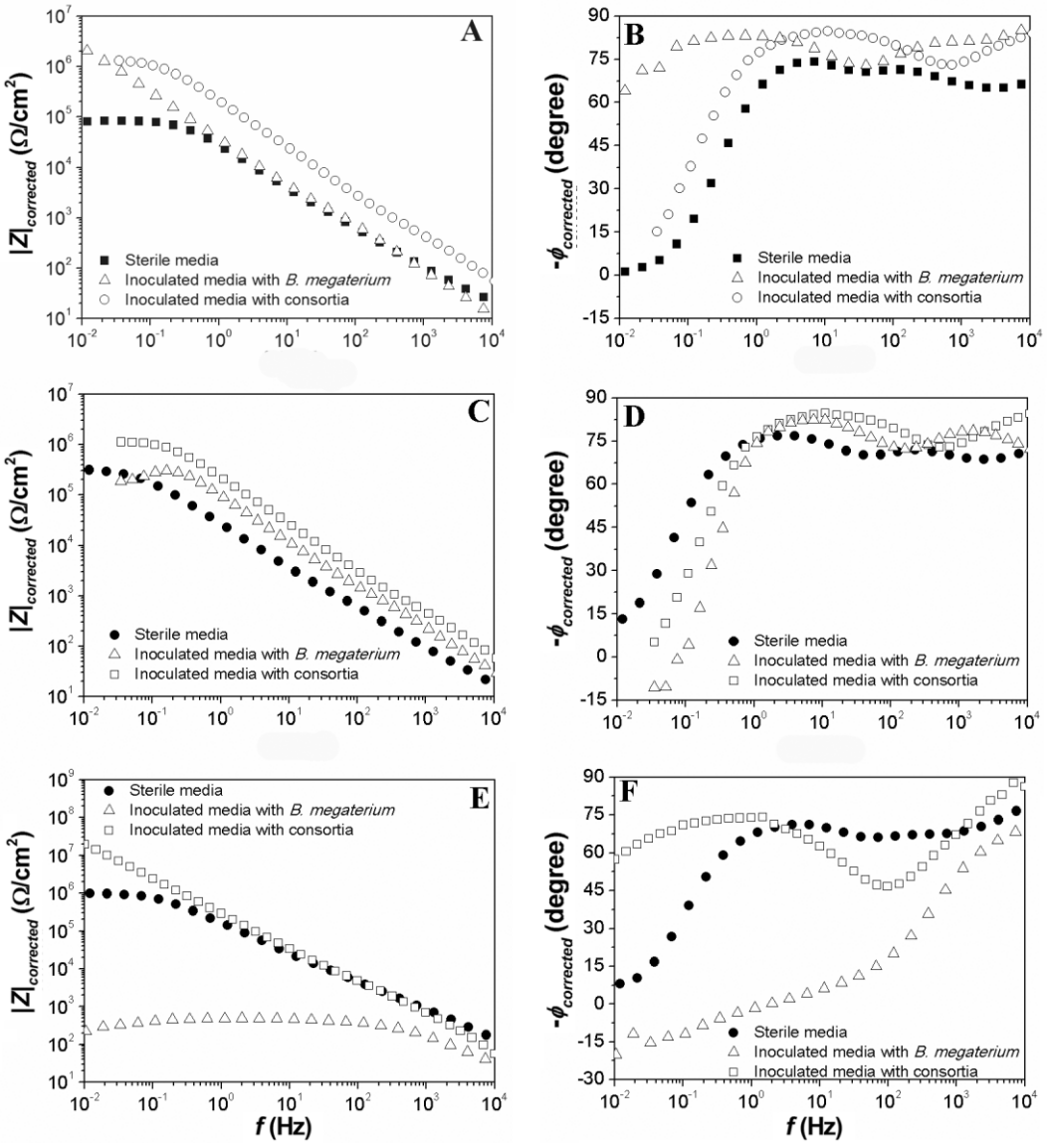

Figure 3. Bode diagrams of AA7075-T6 in MSM as a function of immersion time. Electrode surface Area = 21 cm^2

Table 2. Corrosion parameters obtained from EIS measurements of AA7075-T6 in MSM media

MSM	t (days)	$\|Z\|_{30\ mHz}(\Omega/cm^2)$	I_{corr} ($\mu A/cm^2$)	V_{corr} (μm/year)
Sterile	14	9.68×10^5	1.33×10^{-2}	1.47×10^{-1}
B. megaterium	14	3.71×10^2	6.17×10^1	6.80×10^2
Consortia	14	8.14×10^6	2.23×10^{-3}	2.46×10^{-2}

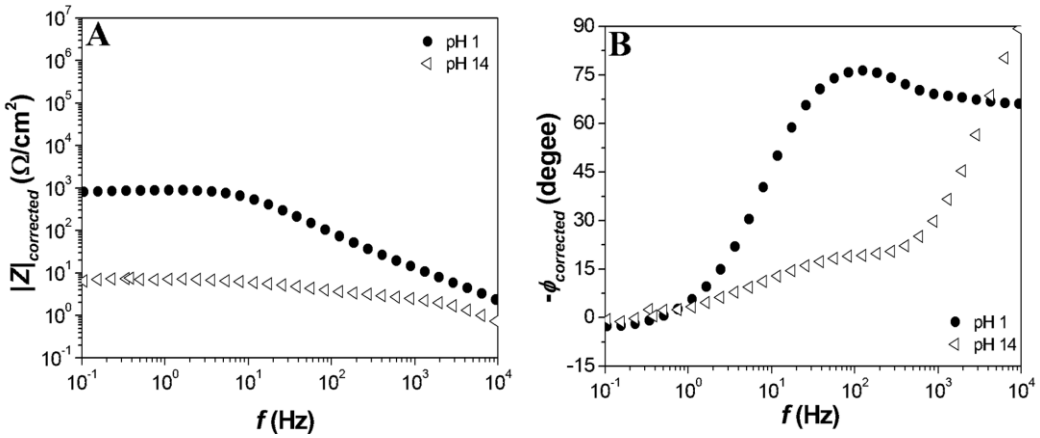

Figure 4. Bode diagrams of AA7075-T6 in sterile MSM media after 1 day as immersion time at pH (a) 1 and (b) 14.Electrode surface Area = 21 cm^2

It should be mentioned that the pH values of the bulk media were also determined after every impedance measurements and they remained close to neutral pH values (see Fig. 5).

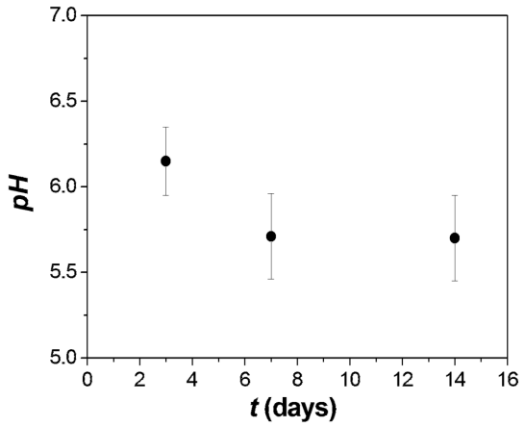

Figure 5. variations of pH values of inoculated MSM media with *B. megaterium*

3.3. Morphological characterization of strain

Fig.6 shows SEM micrographs of AA7075-T6 surface after 14 days of immersion time in inoculated media with *B. megaterium*. The SEM micrographs revealed that the *B. megaterium* were close to 4 µm long and 1.5 µm in diameter. The plan view shows cells organized in pairs and in chains.

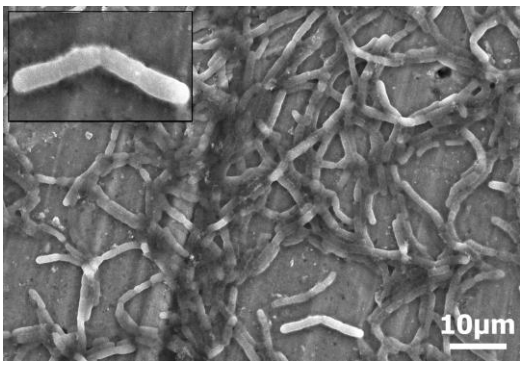

Figure 6. SEM micrographs of AA7075-T6 sample after exposure to MSM media with *B. megaterium*.

Fig. 7a and c show SEM micrographs of the surface morphology of AA7075-T6 samples after 14 days of exposure to sterile and inoculated media with *B. megaterium*, after that the corrosion products were removed. Localized corrosion zones were evident (pitting) and the corrosion products were distributed discontinuously on the sample surface. The pit sizes on the surfaces exposed to the sterile were smaller than in inoculated media with *B. megaterium*, which is in agreement with the results obtained from the electrochemical experiments. The EDX analyses suggest corrosion products were contained of Al, Mg, Cu, Zn and O, as shown in Fig. 7b and d. Moreover, local zinc content in the surface varies among the differently exposed samples. For instance, the sample exposed to the sterile media had a zinc content of 4 wt.%, while zinc content in the samples exposed to inoculate media with *B. megaterium* was close to 11 wt.%.

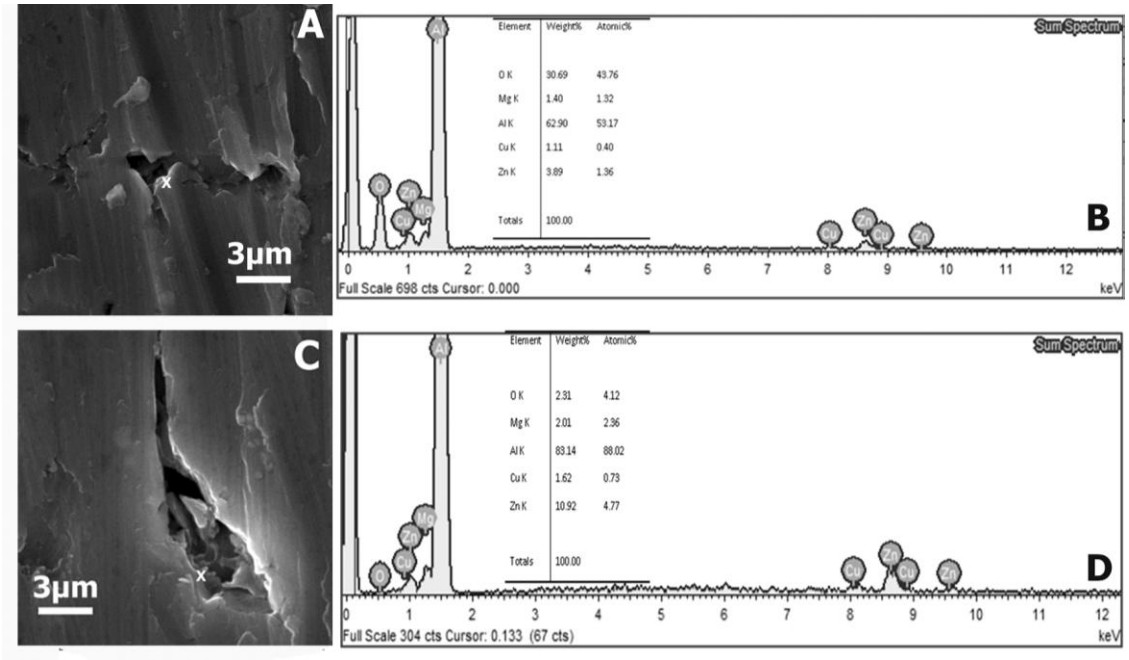

Figure 7. SEM micrographs of AA7075-T6 sample after exposure to (a, b) sterile and (c, d) inoculated MSM media with *B. megaterium* (corrosion products were removed).

The high level of local zinc enrichment on surface regions suggests that corrosion might be related to intermetallic zones. In general, the microstructure of the AA7075-T6 consists of primary α-Al, η-phase ($MgZn_2$), Al_7Cu_2Fe and $(Al,Cu)_6(Fe,Cu)$ second phases [43]. Therefore, corrosion events may occur preferentially at $MgZn_2$intermetallic particles.

4. CONCLUSIONS

We have found evidence of the presence of a consortium in the fuel line of an aircraft manufactured with AA7075-T6. The consortium was characterized by microbiological methods. 16S rRNA analysis revealed that one of the microorganisms of the consortium was a strain closely related to *B. megaterium*. The corrosion rate of AA7075-T6 was significantly higher in inoculated media with *B. megaterium* than in sterile media. In particular, the impedance results showed a decrease of $|Z|$ at low frequency range after 14 days, which may be attributed to the dissolution of the aluminum oxide layer by the action of this strain. While no information was found on the direct relationship between *B. megaterium* and the metallic surface, SEM/EDX and electrochemical results revealed a relationship with the local pH changes and the stability of the aluminum oxide layer and the dissolution of second phases.

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