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Pitting corrosion control using regenerative biofilms on aluminium 2024 in artificial seawater

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Abstract

EIS has been used to follow the pitting process of Al 2024 during exposure to sterile artificial seawater (AS) for 30 days. In the presence of a bacterial biofilm produced by *Bacillus subtilis* pitting was also observed during the first two days, however for the remainder of the exposure period the Al alloy was passive. When the biofilm was genetically engineered to secrete polyglutamate or polyaspartate, an additional small increase in corrosion inhibition occurred. Corrosion control using regenerative biofilms (CCURB) on Al 2024 in AS cannot be solely due to a reduction of the oxygen concentration at the metal surface since the experimental values of the corrosion potential E_{corr} became more noble in the presence of bacteria suggesting that production of an inhibiting species retained in the biofilm contributes to CCURB.

Keywords: Aluminium alloy; Seawater; Pitting; Inhibition; Biofilm; EIS; Corrosion control

1. Introduction

The great interest in microbiologically influenced corrosion (MIC) is documented in the proceedings of international conferences [1,2] and symposia on this subject as well as the large number of publications dealing with MIC. Little et al. have given a

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review of MIC of different materials [3]. In many cases the observation that bacteria were present in a corroded area has been taken as evidence that these bacteria have caused the observed corrosion damage. However, Little et al. have demonstrated recently that the co-location of bacteria with corrosion products did not prove that corrosion occurred due to MIC [4,5].

It is surprising that only very few publications have dealt with the possibility that bacteria can influence corrosion reactions in a beneficial way, i.e. cause MIC inhibition. One of the few exceptions is the suggestion by Eashwar et al. [6] that the often-discussed ennoblement of stainless steels in seawater is due to the production of inhibitors by bacteria which are retained in the biofilm matrix. This mechanism of ennoblement would also explain how it is possible that the corrosion potential E_{corr} observed in the presence of a biofilm can exceed the pitting potential E_{pit} determined in the same solution in the absence of a biofilm. Pederson and Hermansson [7] and Jayaraman et al. [8–10] have shown that aerobic bacteria can decrease mild steel corrosion rates. Jayaraman et al. suggested that this phenomenon is general for aerobic biofilms and that the biofilms provide corrosion protection by reducing the oxygen concentration at the metal surface [8–10]. Protective biofilms secreting antimicrobial proteins active against corrosion-causing sulfate-reducing bacteria may also reduce the corrosive attack of stainless steel by these deleterious bacteria [11], and this has led to a new approach of corrosion protection using corrosion control using regenerative biofilms (CCURB).

The CCURB concept is being evaluated at present in detail for a number of materials such as mild steel, stainless steel, copper and aluminium alloys in the presence of a large number of different bacterial communities. The results discussed in the following were obtained for aluminium 2024-T3 exposed to artificial seawater (AS) (pH 7.5) in the absence and presence of a *Bacillus subtilis* bacterial biofilm which was genetically engineered to produce the polypeptides polyaspartate and polyglutamate. Mueller et al. [12] have shown previously that polyaspartate inhibited corrosion of mild steel in seawater, while polyaspartate and some related peptides reduced corrosion rates of mild steel in fresh and brackish waters.

2. Experimental approach

2.1. Materials

Aluminium alloy 2024-T3 plates (10×10 cm^2 squares, 2 mm thick) were cut from sheet stock and polished with 240 grit paper (Buehler, Lake Bluff, IL). AS was prepared from Vätäänen nine salts solution (VNSS, pH 7.5) (Table 1) [13]. AS was used either in a sterile condition or after inoculation with one or more strains of bacteria. *B. subtilis* WB600 [14] obtained from Dr. Sui-Lam Wong of the University of Calgary is a protease-deficient strain. *Escherichia coli* XLI(Blue) was purchased from Stratagene (La Jolla, CA). Plasmid pBE92 containing the alkaline protease (*apr*) promoter, constitutive *apr* signal sequence, and the alkaline phosphatase reporter gene was obtained from E.I. du Pont de Nemours Inc. (Wilmington, DE).

Table 1
Composition of VNSS

Compound	Concentration (g/l)
NaCl	17.6
$NaHCO_3$	0.08
KBr	0.04
$CaCl_2 \cdot 2H_2O$	0.41
$SrCl_2 \cdot 6H_2O$	0.008
Na_2SO_4	1.47
KCl	0.25
$MgCl_2 \cdot 6H_2O$	1.87
H_3BO_3	0.008
$FeSO_4 \cdot 7H_2O$	0.01
Na_2HPO_4	0.01
Peptone	1.0
Starch	0.5
Glucose	0.5
Yeast extract	0.5

Polyaspartate was synthesized as a 10 amino acid polymer by Genosys Biotechnologies, Inc. (The Woodlands, TX) at 76% purity.

B. subtilis WB600 was engineered to secrete either polyaspartate or polyglutamate as 20 amino acid polymers by using recombinant DNA methods as described by Maniatis et al. [15] and Rodriguez and Tait [16]. Plasmid DNA was isolated from *Bacillus* strains according to the procedure of Bramucci and Nagarajan [17]. Plasmids pBE92-Asp and pBE92-Glu were designed to express polyaspartate and polyglutamate, respectively, as 20 amino acid peptides fused to the *apr* signal sequence (for efficient secretion of these inhibitors outside the cell) under control of the constitutive alkaline protease promoter. An *Eco* RI site was also engineered downstream of the stop codon for each construct to introduce an unique site into pBE92-Asp and pBE92-Glu. The two sets of synthetic DNA oligos for the polyaspartate gene (5′-ATAGCGAGAGCTAGCGCGGATGACGATGATGATGACGATG-ACGATGACGACGATGATGACGATGATGATGACGATTAAGAATTCAAGCTTGCTAGT, 5′-ACTAGCAAGCTTGAATTCTTAATC) and for the polyglutamate gene (ATAGACGCTAGCGCGGAAGAGGAAGAAGAGGAAGAAGAAGAGGAAGAAGAGGAAGAAGAAGAGGAAGAAGAGGAATAAGAATTCAAGCTTAGTTCC, 5′-GGAACTAAGCTTGAATTCTTATTC) were synthesized by Gibco-BRL Life Technologies (Long Island, NY) with flanking *Nhe* I and *Hind* III restriction enzyme sites (along with an internal *Eco* RI site) with an additional six bases at either end for efficient restriction digestion. For each gene, the two fully complementary oligos of each construct were annealed, and the complementary regions completed using *Taq* polymerase (1 cycle, 30 s at 94°C, followed by 30 s at 55°C, and 2 h at 72°C) with a Perkin Elmer thermal cycler N801-0150 (Perkin Elmer, Norwalk, CT). Each of the full-length genes were then digested with *Nhe* I and *Hind* III and ligated into plasmid vector pBE92 (isolated from *E. coli* XLI Blue) which was digested with *Nhe* I and *Hind* III, and *Sal* I simultaneously.

E. coli XLI(Blue) transformants containing the correct insert (pBE92-Asp or pBE92-Glu) were screened as white colonies on LB agar plates containing 100 μg/ml of ampicillin and 40 μg/ml of 5-bromo-4-chloro-3-indolyl phosphate (transformants with the correct insert produced white colonies while the reclosed vector resulted in blue colonies). The plasmids containing inserts were further characterized through restriction digests with *Nhe* I, *Hind* III, and *Eco* RI.

E. coli XLI(Blue) cells were made electrocompetent according to the method of Smith and Iglewski [18] and electroporated using a gene pulser/pulse controller (Bio-Rad Laboratories, Hercules, CA). *B. subtilis* WB600 strains were transformed with the two plasmids according to the two-step method of Cutting and Vander Horn [19] and plated on LB agar plates containing 50 μg/ml kanamycin.

Biofilms on metal surfaces were developed in glass/teflon cylindrical autoclavable, continuous reactors [8] with AS (30°C, liquid nutrient flow rate 0.2 ml/min, airflow 200 ml/min to headspace, working volume 100 ml or 150 ml, exposed surface area of test electrode 28.3 or 45.4 cm^2). A titanium counter electrode (11.34 cm^2 surface area) and autoclavable Ag/AgCl reference electrode (Ingold Silver Scavenger DPAS model 105053334, Metler-Toledo Process Analytical, Inc., Wilmington, MA) were used. The continuous reactors (sterile and inoculated) were conducted with 100 μg/ml kanamycin to ensure sterility or the presence of the engineered strain. A 1 vol% bacterial inoculum from a turbid, 16-h culture was used for all continuous experiments.

2.2. Methods

Electrochemical impedance data were obtained at the open-circuit potential in the frequency range of 20 kHz–1.3 mHz using an IM6 electrochemical impedance analyzer with a 16 channel cell multiplexer (Bioanalytical Systems-Zahner, West Lafayette, IN) running with THALES impedance measurement and equivalent circuit synthesis/simulation/fitting software interfaced to a gateway Pentium GP6 300 MHz computer (North Sioux City, SD).

2.3. Data analysis

The experimental impedance spectra were analyzed using the EC shown in Fig. 1 which has been proposed by Mansfeld and co-workers to describe the impedance behavior of Al alloys undergoing pitting corrosion [20–26]. In this pitting model R_p and C_p are the polarization resistance and capacitance, respectively, of the passive surface, while R_{pit} and C_{pit} are the corresponding parameters for actively corroding pits. $W = (K/F)(j\omega)^n$ is a transmission line element with a constant slope n $(-1 \leqslant n \leqslant 0)$ observed at low frequencies [20–26]. F $(0 \leqslant F \leqslant 1)$ is the fraction of the total surface for which pitting occurs. R_s is the solution resistance between the reference electrode and the test electrode.

Fig. 2 shows theoretical spectra calculated for the EC in Fig. 1 for a passive surface ($F = 0$) and an actively pitting surface with $F = 0.04$. For the passive surface a very simple spectrum is observed which corresponds to a parallel combination of

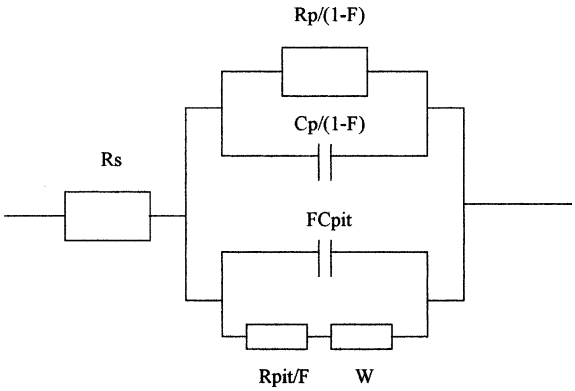

Fig. 1. EC for pitting of Al alloys.

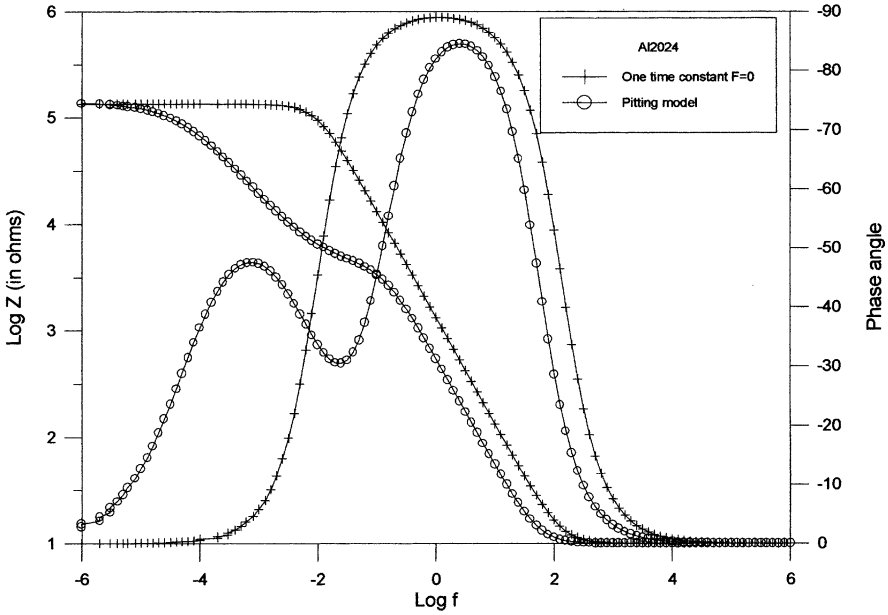

Fig. 2. Theoretical plots for the EC in Fig. 1 for a passive surface ($F = 0$) and a pitted surface ($F = 0.04$). Fit parameters were $R_s = 10\ \Omega$, $R_p = 1.35 \times 10^5\ \Omega$, $C_p = 120\ \mu F$, $R_{pit} = 200\ \Omega$, $C_{pit} = 4000\ \mu F$, $K = 25\ \Omega$ (rad/s)^n, $n = -0.7$, $A_t = 1\ cm^2$.

R_p and C_p. For the pitted surface a more complicated spectrum characterized by an increase of the experimental capacitance $C_t = C_p(1 - F) + FC_{pit}$ is obtained for frequencies f between about 100 and 0.1 Hz and a maximum and a minimum of the phase angle Φ occurring below $f = 0.1$ Hz.

The experimental values of F can be determined from the time dependence of C_t. For $t = 0$, $C_t = C_p$. Therefore if F is determined by microscopic observation at the

end of the test and a constant value of $C^0_{pit} = C_{pit,exp}/A_{pit}$, where A_{pit} is the pitted area, is calculated, F can be determined as a function of time based on the experimental values of the fit parameter $C_{pit,exp} = FC_{pit} = C_t - C_p$ assuming that F is very small. $A_{pit} = 2FA_t$, where A_t is the total exposed area and it is assumed that the pits are hemispherical [26]. The calculated values of F can then be used to determine the specific polarization resistance of the pitted area $R^0_{pit} = R_{pit,exp}A_{pit}$ ($\Omega\,cm^2$), where $R_{pit,exp} = R_{pit}/F$ is a fit parameter. The R^0_{pit} values can be used to estimate a time law for pit propagation rates [24–26].

3. Experimental results and discussion

Fig. 3 shows experimental impedance spectra obtained for Al 2024 during exposure to sterile AS for 30 days. Only four of the spectra collected during this time are plotted in the Bode plots of Fig. 3. The spectra suggest that pitting occurred during the entire test period as evidenced by the typical low-frequency minimum of Φ which is partially masked by the scatter of the data points below 0.01 Hz. Nevertheless comparison with the theoretical spectra in Fig. 2 demonstrates agreement with the pitting model [20–26]. Qualitatively it can be observed that C_t and R_{pit}, which is close to the impedance value $|Z|$ at the frequency minimum at low frequencies, increase with increasing exposure time.

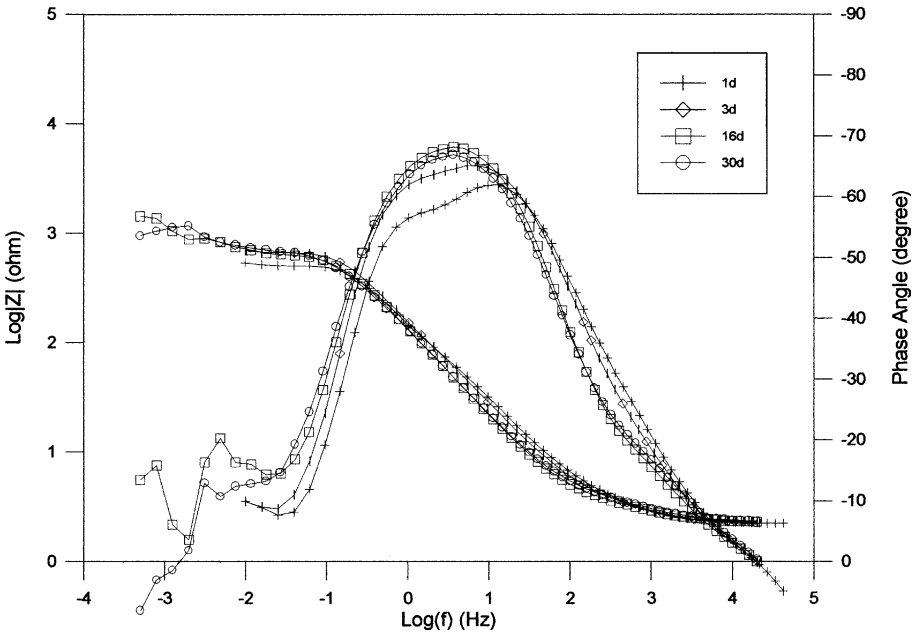

Fig. 3. Bode plots for Al 2024 exposed to VNSS (Expt. #45) ($A_t = 45.4\ cm^2$).

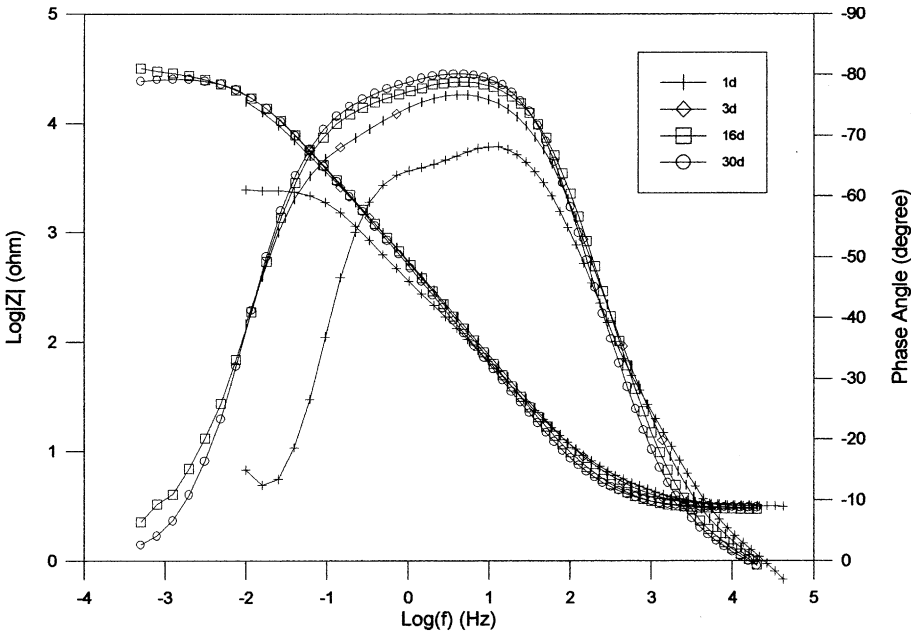

Fig. 4. Bode plots for Al 2024 exposed to VNSS containing *B. subtilis* WB600/pBE92 (Expt. #42) ($A_t = 28.3$ cm^2).

In the presence of bacteria pitting also occurred in the first two days of exposure, however after three days the spectra agreed with those calculated for a passive surface for the EC in Fig. 1. The fairly high values of R_p, which approach the M Ω cm^2 range, suggest that pits formed in the initial stages of exposure have become passivated (Fig. 4). Very similar results were obtained in the presence of bacteria which produced polyglutamate (Fig. 5) or polyaspartate (Fig. 6). The increased R_p values suggest that the inhibitors produced by the bacteria provided additional corrosion protection.

Fig. 7 illustrates the time dependence of the fit parameters R_{pit}/F for the test in the absence of bacteria (test #45) and $R_p/(1-F)$ for the tests in the presence of bacteria (tests #42–44). For the tests in the absence of bacteria the values of $R_p/(1-F)$ could not be determined due to the lack of sufficient low-frequency data (Fig. 3). For tests #42–44 the R_{pit}/F data obtained in the first two days during which active pitting occurred are also plotted in Fig. 7. The results in Fig. 7 clearly demonstrate the inhibition of pitting corrosion in the presence of bacteria and the increased corrosion resistance in the early stages of exposure due to inhibitors produced by the bacteria.

The time dependence of the experimental capacitance values is shown in Fig. 8. The increase of C_t with time is typical for pitting corrosion of Al alloys [20–25] and is mainly due to the increase of A_{pit}. The lower values of $C_p(1-F)$ for the tests in the presence of bacteria which produced inhibitors could be due to a thickening of the passive film and/or formation of adsorbed inhibitor layers (Fig. 8).

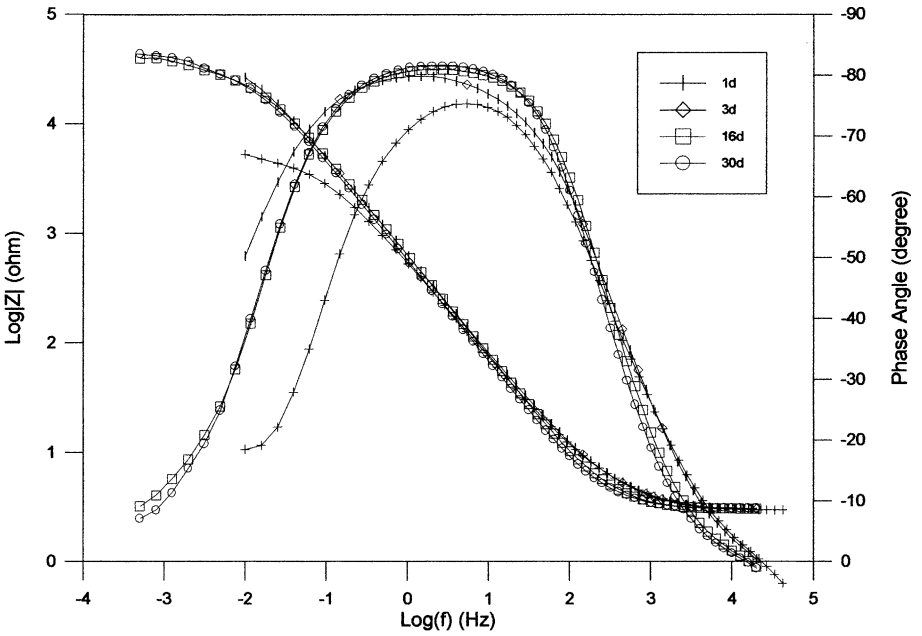

Fig. 5. Bode plots for Al 2024 exposed to VNSS containing *B. subtilis* WB600/pBE92 producing polyglutamate (Expt. #43) ($A_t = 28.3$ cm^2).

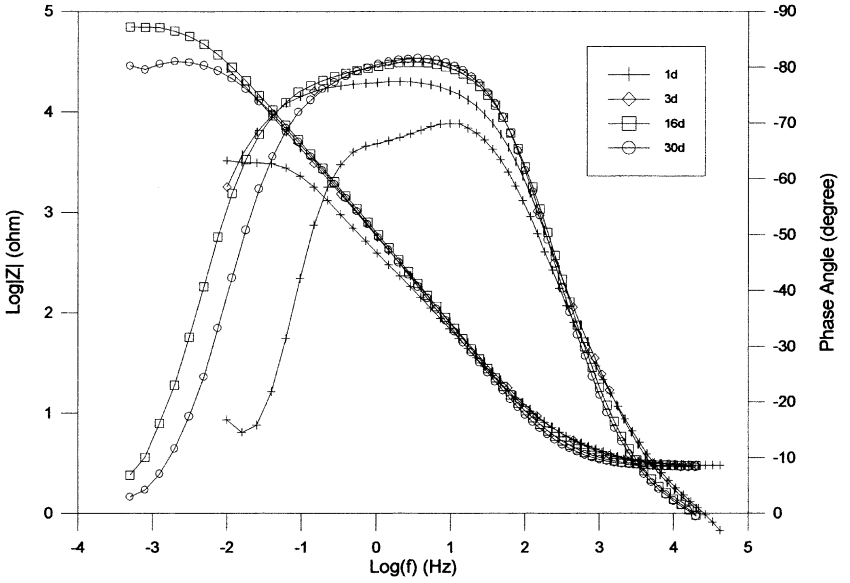

Fig. 6. Bode plots for Al 2024 exposed to VNSS containing *B. subtilis* WB600/pBE92 producing polyaspartate (Expt. #44) ($A_t = 28.3$ cm^2).

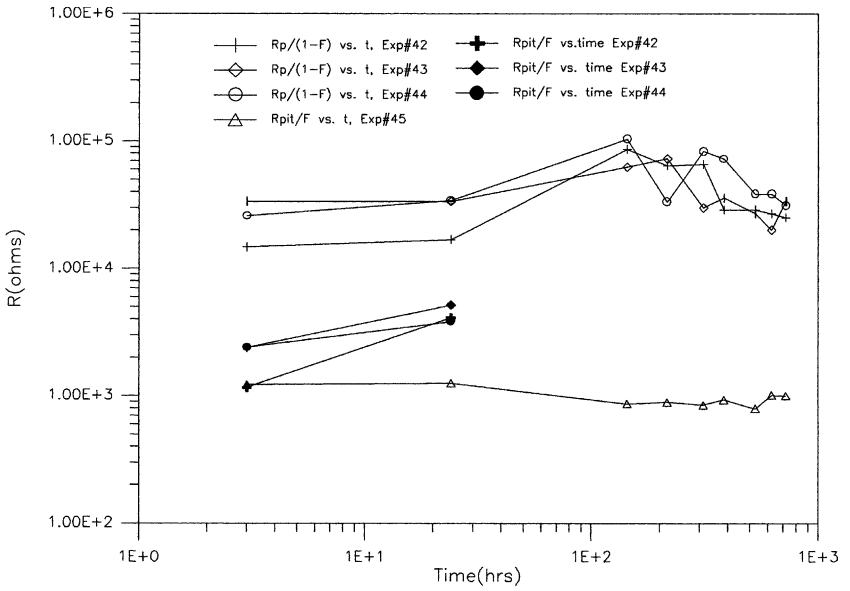

Fig. 7. Time dependence of $R_p/(1-F)$ and R_{pit}/F in abiotic solution (Expt. #45) and in the presence of *B. subtilis* WB600/pBE92 (Expt. #42–44).

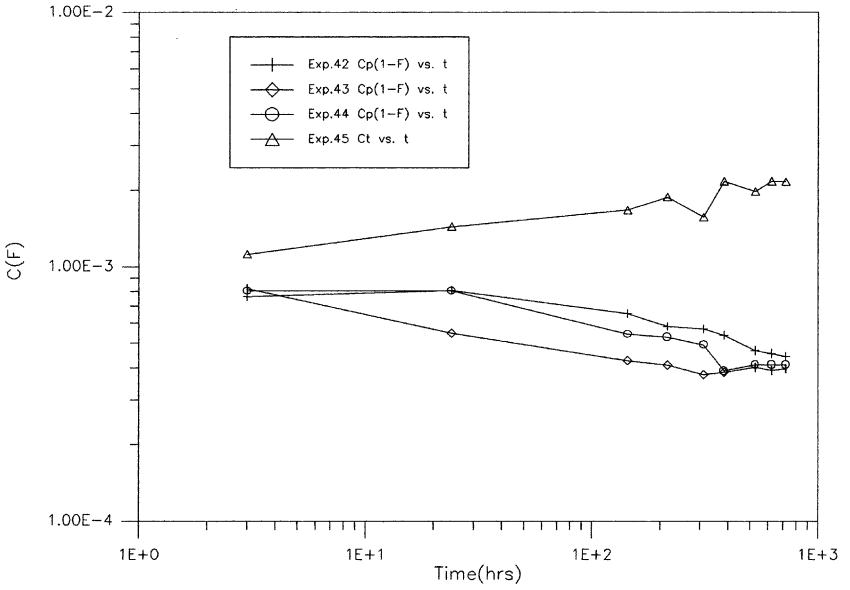

Fig. 8. Time dependence of $C_p/(1-F)$ and C_t in abiotic solution (Expt. #45) and in the presence of *B. subtilis* WB600/pBE92 (Expt. #42–44).

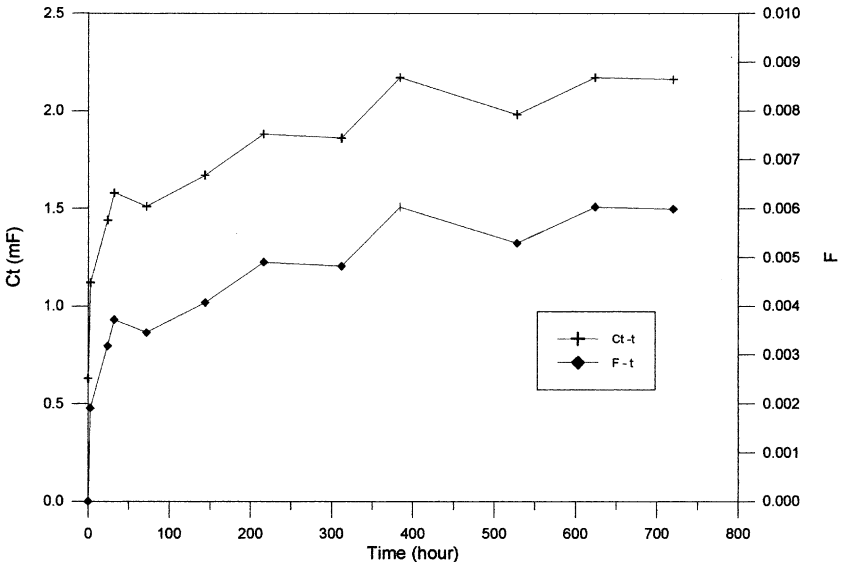

Fig. 9. Time dependence of C_t and F.

The time dependence of C_t and F is shown in Fig. 9 for the tests in the absence of bacteria. Extrapolation of the C_t–t plot to $t = 0$ results in $C_p^0 = 1.4 \times 10^{-5}$ F/cm^2. Based on the final value of $A_{pit} = 0.46$ cm^2 corresponding to $F = 1.04\%$, $C_{pit}^0 = 3.32 \times 10^{-3}$ F/cm^2 was obtained and used to determine the time dependence of F (Fig. 9). F increased from about 0.3% to about 1.0% during the 30 days exposure period.

Since R_{pit} is inversely related to corrosion rates, it is possible to estimate a time law for the propagation of pits. The time dependence of R_{pit}^0 is shown in Fig. 10 for the tests in the absence of bacteria. The decrease of R_{pit}^0 is fairly small suggesting that pit growth rates did not decrease significantly during this time. Previous experiments in 0.5 N NaCl have shown that pit growth rates were proportional to $1/t$ [24–26]. Assuming that the pits had a hemispherical shape led to the conclusion that the increase of the pit radius followed a logarithmic law. Obviously according to the data in Fig. 10 a different time law applies for exposure of Al 2024-T3 in AS.

For the tests in the presence of bacteria (tests #42–44) the values of A_{pit} determined at the end of the exposure period were much less than those determined in the absence of bacteria ($F = 1.04\%$). The final F values were 0.07%, 0.16% and 0.06% for tests #42–44, respectively.

The inhibition of pitting in the presence of bacteria could be due to exclusion of oxygen from the metal surface which would reduce the rate of the cathodic reduction resulting in a decrease of the corrosion potential E_{corr} below the pitting potential E_{pit}. However, the experimental values of E_{corr} had the lowest values in the absence of bacteria, while a certain degree of ennoblement was observed in the presence of bacteria (Fig. 11). Since the highest values of E_{corr} were observed in the presence of

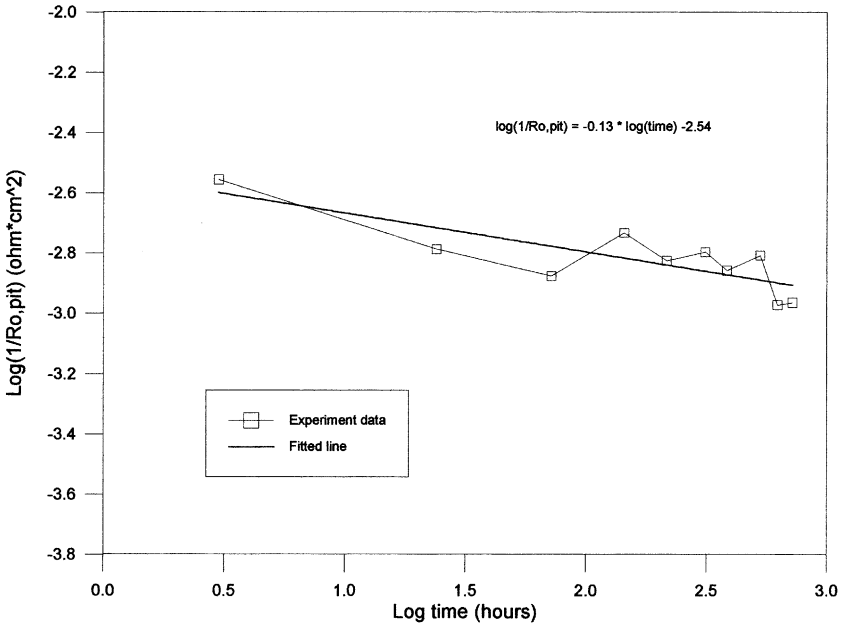

Fig. 10. Time dependence of R^0_{pit}.

inhibitors it can be concluded that the observed CCURB is due to a passivation effect which occurs in the presence of a biofilm. The beneficial effect is apparent even when the biofilm contains bacteria that were not engineered to produce inhibitors. Indeed the observation that pitting occurs in all cases in the first two days of exposure clearly suggests that formation of a stable biofilm is needed to stop growth of active pits.

4. Summary and conclusions

1. Pitting of Al 2024 was monitored by recording of impedance spectra in AS during the 30 days exposure period. Pit growth rates followed a different time law than that observed previously in 0.5 N NaCl. Pitting in AS was less severe than in NaCl most likely due to inhibiting species present in AS.
2. In the presence of bacteria (*B. subtilis* WB600/pBE92) pitting was also observed initially, however after about three days the impedance spectra demonstrated that pitting had stopped. CCURB was also achieved in the presence of the same type of bacteria which had been genetically altered to produce polyglutamate or polyaspartate as inhibitors. A small additional passivation effect was observed in these cases.
3. E_{corr} reached its most noble values after about one week of exposure to AS containing bacteria producing inhibitors. This result is similar to the ennoblement of

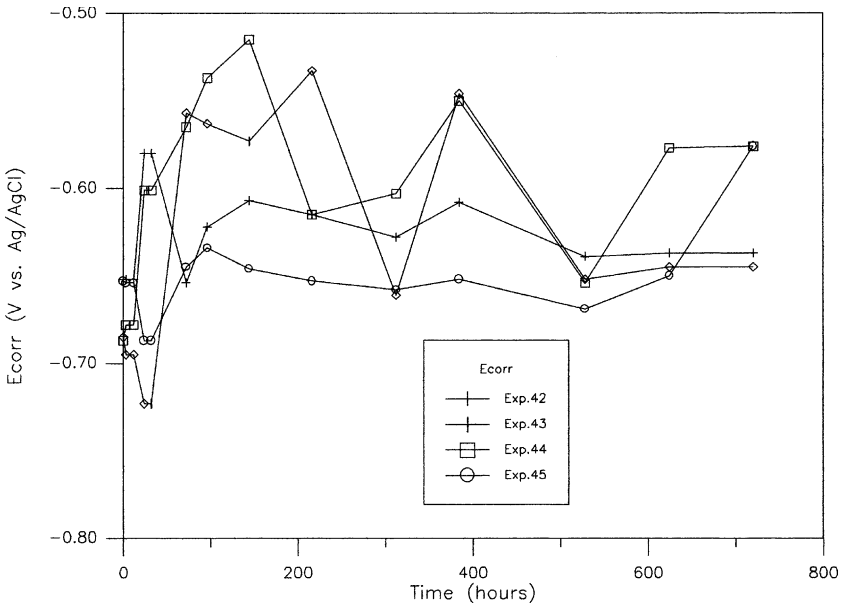

Fig. 11. Time dependence of E_{corr} in abiotic solution (Expt. #45) and in the presence of *B. subtilis* WB600/pBE92 (Expt. #42–44).

stainless steels in seawater and natural waters although the increase of E_{corr} was less dramatic. Since even in the presence of the bacteria which did not produce polyglutamate or polyaspartate E_{corr} increased beyond E_{pit} which for Al 2024 equals E_{corr} in the absence of bacteria, it is likely that the bacteria produced a chemical species which was able to stop pitting after a certain incubation period. The observed ennoblement of E_{corr} rules out the possibility that CCURB was only due to anaerobic conditions at the Al alloy surface.

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