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The effect of *Pseudoxanthomonas* sp. as manganese oxidizing bacteruim on the corrosion of carbon steel

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Abstract

The present study investigated the role of manganese oxidizing bacterium (MOB), namely *Pseudoxanthomonas sp.* on the corrosion of carbon steel. This bacterium was isolated from sewage treatment plants and identified by biochemical and molecular methods. The microbially influenced corrosion (MIC) of the carbon steel in the presence of this bacterium was studied. The electrochemical techniques such as open circuit potentiometry, anodic polarization measurement and electrochemical impedance microscopy (EIS) were used to measure the corrosion rate and observe the corrosion mechanism. Also, SEM and XRD studies were applied to surface analysis. This study revealed strong adhesion of the biofilm on the metal surface. It indicates that the corrosion of carbon steel accelerated in the presence of this bacterium. X-ray identified MnO_2 within these biofilms. This is the first report that discloses the involvement of *Pseudoxanthomonas* sp. as MOB on the corrosion of carbon steel.

Keywords: Manganese Oxidizing Bacteria (MOB); *Pseudoxanthomonas* sp. bacterium; microbially influenced corrosion (MIC); carbon steel; Electrochemical methods.

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1. Introduction

Biocorrosion is the damage caused or accelerated by the presence of bacteria and other microorganisms and their metabolic activities [1]. One of the main types of these bacteria is manganese oxidizing bacteria. These bacteria are the phylogenetically diverse assemblage, which is characterized by the ability to catalyze the oxidation of divalent, soluble Mn (II) to insoluble manganese oxides [2]. The manganese oxides deposited on the surface elevate the potential, creating an environment where the pits will be initiated by microbes [3]. In fact, the deposits on the metal surfaces, such as corrosion byproducts, scale, silt, and sludge or biofouling materials may also have an effect on modifying the environment where accelerated corrosion related to microbiological growth occurs [4]. Some researches have noticed the effect of MOB on the corrosion of metal surface. The first report was related to Molica and Trevis who showed the ennoblement of stainless steel exposed to sea water [5]. Then Linhardt [6], Dickinson et al [7], Maruthamuthu [8], Oleoson [9] and Lewandowski [3, 10] observed appreciable increase in corrosion potential and deposition of MnO_2 on stainless steel by MOB, namely *Leptothrix discophora*. It has been found [2] that these bacteria deposit MnO_2 by their actively metabolizing, thus creating an anaerobic zone. This anaerobic zone can later be used by other anaerobic bacteria such as sulphate reducing bacteria (SRB). This shifting between aerobic and anaerobic bacteria that will eventually result in more enhanced corrosion has been well documented in systems as diverse as buried (metallic) pipelines, wharfs and jetties as well as concrete sewage pipes [10]. Hence the role of bacteria such as MOB on the corrosion process is important, in this research the impact of a novel manganese oxidizing bacteria namely *Pseudoxanthomonas* sp. on the corrosion of carbon steel has been studied. The changes

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occurring on the metal surface and the formation of biofilms were analyzed with electrochemical, microscopic and spectroscopic methods.

2. Material and methods

2.1. Isolation of bacteria, microbial cultivation and incubation

The corrosion products were collected from pipelines of sewage treatment plants using a sterile metal loops. For isolation of the bacteria, 1g of corrosion product was transferred to 250ml of Erlenmeyer flask containing 50ml of Bushnell Hass medium (BH); consisting of (g/L), MgSO4.7H_2O 0.2, $CaCl_2$ 0.02, NH_4NO_3 1, K_2HPO_4 1, KH_2PO_4 1, $FeCl_3$ 0.05 at pH=7 under aerobic chamber and incubated at 30°C in a rotator shaker at 100 rpm for 48hr . Before using this solution, it was autoclaved at 121°C for 15min. Therefore, the grown cultures were subcultured on nutrient agar for selective isolation. Individual colonies were purified by repeated streaking on nutrient agar plates. The pure cultures were streaked repeatedly with an inoculation loop on pre-prepared sterile Mn agar (MPSV) [12] plates separately, the composition of which is given in Table 1. Plates were wrapped in plastic bags to prevent desiccation after few days and incubated at 37 °C for 12 days. Brown color colonies were observed in the petriplates and scored as ‘‘manganese oxidizer’’ by noticing the appearance of brown coloration in the colonies. The colonies were picked up and smeared on a filter paper using a sterile inoculation loop. Then 0.04% of Leukoberbelin blue (LBB) was added on the filter paper, with the subsequent scrapping of the smear with the inoculation loop. Blue color was formed after a few minutes on bacterial

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colonies, which indicated the accumulation and conversion of manganese to manganese-dioxide by manganese oxidizers [13].

2.2. Taxonomic studies

Morphological and biochemical characteristics of the isolated bacteria were studied according to the Bergey's Manual of Systematic Bacteriology [14]. The strains were characterized based on their morphological and biochemical characteristics. The main strains identification were confirmed according to observation of 16s rRNA gene. Hence, genomic DNA of the strains were extracted and subjected to polymerase chain reaction (PCR) to amplify a partial 16srRNA gene. Amplification reactions were accomplished by the eubacterial 16sr DNA primers in an optimized counteraction conditions and the PCR products were confirmed by gel electrophoresis. After the purification of the implications by gel extraction kits, they were sent to sequencing. The similarity of the obtained sequences was examined by a Blest search at National Center for Biotechnology Information NCBI.

2.3. Material

The CK45 steel used in the study has the following composition: C≤0.45, Si≤0.4, and Mn≤1.4, balance Fe. The cylindrical metal specimens (diameter 3cm) were used in this study. To create working electrodes, an electrical contact to each sample was provided by a length of copper wire connected to the back side of each specimen mounted in an epoxy resin, and then,

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the specimens were abraded through silicon carbide metallurgical paper up to grit size of 1000. After polishing, they were rinsed in distilled water followed by chloroform and acetone.

2.4. Electrochemical studies

In the present study, the BH medium with 0.02g/lit $MnSO_4$ as manganese source was used as the control system and 1ml inoculums MOB was added it and used as the experimental system. The solutions were stirred vigorously for the periods of 7 and 30 days. After that, the coupons were removed and electrochemical measurements were carried out. All electrochemical tests were carried out in a corrosion cell with three-electrode system consisting of a saturated Ag/AgCl electrode as reference, a platinum sheet as counter electrode and a carbon steel sample as working electrode. The measurements were concluded using an AUTOLAB Potentiostat-Galvanostat (PGSTAT30). Anodic polarization curves were acquired potentiodynamically with a scan rate of 0.005V/s. EIS measurements were made at the open circuit potential using a 5 mV amplitude sinusoidal signal over the frequencies ranging from 10 mHz to 10 kHz.

2.5. Surface analysis

After exposing for 30 days under freely corroding conditions in medium containing bacteria, the samples were examined for their surface characteristics and corrosion features by scanning electron microscopy (SEM) after sputter coating with gold. To immobilize the bacterial cells in order to confirm the adhesion of bacteria, the samples were immersed for 4hr in a 2%

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glutaraldehyde solution. Then, the coupons were dehydrated using four ethanol solutions (15 min each): 25, 50, 75and 100% successively.

A computer controlled X-ray diffraction technique (XRD), JEOL Model JDX-8030 was used to scan the corrosion products between 10° and 85° – 2θ with copper Kα radiation (Ni filter) at a rating of 40 kV, 20 mA. The carbon steel specimens were used directly for XRD analysis to determine the nature of oxides present in the corrosion products.

3. Results and discussion

3.1. Isolation and identification of the microorganism

Isolation was made by two media. The first was nutrient agar that various types of microbial colonies were obtained in this media. The second stage of identification performed by the MPSV agar medium which is specific medium for manganese oxidizing bacteria. In this medium, three microorganisms were isolated as MOB. One of them exhibited higher corrosion affected on the metal surface, and then it was used for further studies. The physiological and biochemical characteristics of this strain are shown in Table 2. On the basis of the nucleotide sequence of the partial 16S rRNA gene, this bacterium was classified as species of genus *Xanthomonas*. A pairwise alignment (BLASTn) analysis of 16S rDNA sequence of the bacteria indicated that the DNA sequence had the highest similarity with *Pseudoxanthomonas* (more than 99% similarity at the level of DNA). From the above data, the isolated bacterium was identified as *Psuedoxanthomonas* sp.

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3.2. Surface analysis

Fig. 1 shows the SEM images of carbon steel specimens after 30days exposure to the corrosion media. It can be seen that a heterogeneous surface biofilm was formed showing a high concentration of cells and EPS which interact with bacteria, while in control media, the carbon steel shows thick layer of oxide all over its surface. This may suggest that the metabolic byproducts produced by the microorganisms created an aggressive microenvironment with the colonization structure adhering to the surface of base metal. Lewandowski [15] suggests that accelerated corrosion of metals involving microbiological growth is related to the modifications of the environment near the metal surfaces. The growth of biofilm is considered to be a result of complex processes involving transport of organic and inorganic molecules and microbial cells to the surface, adsorption of molecules to the surface and initial attachment of microbial cells that followed by their irreversible adhesion facilitated by intracellular polymeric substances (EPS) [16]. The forms of deterioration which can be stimulated by the interaction of biofilms with metals are numerous.

The X-ray diffraction pattern (Fig. 2) of the carbon steel samples with and without bacteria gives qualitative information about the possible phases present. The phase identification carried out using a search and match fit of the XRD data to the patterns in the ICDD database. Four major phases were recognized, namely FeOOH, Fe_2O_3 and Fe_3O_4 as well as MnO_2. As said earlier, *Psuedoxanthomonas* sp. converts Mn (II) to MnO_2. Also the ferrous ions get oxidized into ferric which in turn are combined with oxygen to form ferric oxide. These oxides were found in steel samples however with different intensities at the surface. In the presence of the bacterium where there was biofilm growth MnO_2 constituted the major peaks whereas under

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control medium, it was the peaks for Fe_2O_3 and FeOOH. Consequently, relative compound percentage was affected by the biofilm growth.

3.3. Open circuit potential versus time

Fig. 3 shows the variation of an open circuit potential (E_{corr}) with the exposure time for carbon steel in sterile medium and MOB solutions in BH medium at 30°C. In inoculated medium containing bacterium, E_{corr} began to increase, exceeded -0.201V after 30 days. In contrast, little change in the E_{corr} was observed for sample exposed to control medium. This small change is usually attributed to steel passivation. Molica and Trevis [5] discovered that biofilm formation on stainless steel changed their electrochemical behavior and OCP was shifted in positive direction from initial values which are believed that it is due to microbial colonization and production of a thin film on the metal surface. Then it can be said that the OCP shift towards the positive direction may endanger the material as, the potential may approach the critical pitting potential and thereby increases the risk of localized corrosion [11].

3.4. Anodic polarization measurements

Fig. 4 demonstrates anodic polarization curves for carbon steel in BH medium in the absence and the presence of the *Pseudoxanthomonas* sp. Electrochemical corrosion parameters such as corrosion potential (E_{corr}), cathodic and anodic Tafel slopes, corrosion current density (i_{corr}) and corrosion rate were all derived from the polarization curves (Table. 3). As can be seen, the presence of this bacterium increased the corrosion current density of sample.

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It is important to note that the effect of MnO_2 on the corrosion rate of the active metals, such as carbon steel, is controlled by the rate and amount of MnO_2 deposition. For these metals, the corrosion current is limited by the anodic process so that increased cathodic efficiency has little impact on the metals corrosion. Instead, the cathodic reaction anodically polarized the metal at localized sites, shifting the OCP to the positive or nobler direction [4]. Therefore, the anodic slopes of the control samples were lower than the samples exposed to media containing the bacterium. Also, during the exposure time, the i_{corr} changes in the control media and this is usually attributed to changes in passivation current that occurs as the passive film ages [7]. As a result, it is understandable that microorganisms growing on the metal surfaces and forming the biofilm can affect this process. These biofilms acts also as the diffusion barrier and can lead to concentration cells for metabolic and corrosion products [17]. On the other hand, biofilms are complex microbial population composed by microbial clusters separated by channels and void spaces, which develop on virtually all immersed surfaces. Due to this ubiquity, biofilms are associated with many natural and industrial processes and play a key role in the way how microorganisms influence corrosion process [18]. In each case, the change in electrochemical parameters could be attributed to the action of the bacteria changing the localized chemistry at the metal surface [19]. *Pseudoxanthomonas* sp. is heterotrophic bacterium which requires an organic energy source. This strain catalyzes Mn(II) oxidation at the cell wall or other extracellular surfaces and frequently deposits MnO_2 in a slime layer of biofilm biomass. Deposition can also occur directly on to the metal surface as a tightly adhering film [4].

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3.5. Electrochemical impedance spectroscopy (EIS)

Fig. 5 gives a comparison of impedance spectra observed in BH medium in the absence and presence of *Psuedoxanthomonas* sp. at 30°C after 30 days. In the sterile medium, a semicircle obtained at immersion time showed an irregular response that can hardly be associated with the presence of a single time constant. This type of Nyquist plot can be modeled by a simple Randels circuit. But in the culture medium, the Nyuist plot has two time constant because of the formation of a layer of the biofilm on the metal surface. The first time constant, which appears at high frequencies, relates to the response of the corrosion products layer. The second time constant appears at low frequencies could be related to the growth and stabilization of the biofilm. Indeed, when the biofilm like passive layer is formed, an EIS diagram with two semi-circles is distinguished. In bacteria medium, a diffusion tail appears in Nyquist plot, and thus the electric equivalent circuit involving a Warburg component becomes necessary. The presence of Warburg components has been related with the diffusion of oxygen vacancies [20] and manganese ion from the metal toward solution through the biofilm [21].

To describe the impedance response of the corrosion phenomenon in the presence of the bacterium, equivalent circuit of Fig. 6 has been proposed. Table 4 shows the values of electric parameters obtained on simulating experimental impedance diagrams using equivalent circuit. As it can be seen in the Table 4, R_s values commonly associated with solution resistance, vary within exposure time, which may be indicated the existence of a small change in the composition of the medium. The film capacitance value is seen decreased in the presence of this bacterium which suggests changes in porosity and structure of the corrosion products layer and biofilm formed at the metal surfaces. The roughness of surfaces is confirmed by n parameter values and

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this interface exhibits non-ideal behavior of the capacitor. Charge transfer resistance (R_{ct}) exhibits decreased values in the sterile medium which suggest accelerated character of the steel oxidation process, while R_{diff} corresponding to manganese ion diffusion from the metal toward solution through the biofilm, is favored since the values of this resistance increased with respect to the immersion time function. Indeed, the metabolism of the microorganisms enclosed in the biofilm includes changes in the local physico- chemistry at the material/biofilm interface, which may influence the corrosion processes [22].

Conclusions

This study concluded that:

(1) The isolated bacteria are indeed *Pseudoxanthomonas* sp. as the manganese oxidizing bacteria which identified by the biochemical and molecular methods.

(2) SEM morphology showed that the biofilm was formed on the surface of carbon steel in the presence of this bacterium.

(3) The XRD studies ascertained that MnO_2 compound were developed during the biofilm formation.

(4) In the presence of this bacterium, the open circuit potential and corrosion rate were higher than the sterile medium.

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(5) A different shape of EIS diagram is distinguished in the presence of the biofilm and a diffusion tail appears in the Nyquist plot because of the diffusion of oxygen vacancies and manganese ions from the metal toward solution.

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Figure Caption:

Fig.1. SEM images of the specimens exposed for 30 days in BH medium in absence (a, c) and presence of *Psuedoxanthomonas* sp. (b, d)

Fig.2. XRD analysis of corrosion product samples after 30 days (a) blank (b) *Psuedoxanthomonas* sp.

Fig.3. Open circuit potential (E_{corr}) during exposure time for carbon steel in sterile BH medium and MOB solutions at 30°C

Fig.4. Anodic polarization curves for carbon steel in BH medium in the absence and presence of *Pseudoxanthomonas* sp. after 30 days

Fig.5. Nyquist plots for carbon steel in BH medium in the absence and presence of *Psuedoxanthomonas* sp. at 30°C after 30 days

Fig.6. Equivalent circuit proposed to simulate experimental impedance diagrams in the evaluation of carbon steel in BH medium in absence (a) and presence of *Psuedoxanthomonas* sp. (b).

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Tables:

composition	g/L
$(NH_4)_2SO_4$	0.24
$MgSO_4.7H_2O$	0.06
$CaCl_2 \cdot 2H_2O$	0.06
KH_2PO_4	0.02
$Na_2HPO_4 \cdot 7H_2O$	0.05
HEPES buffer	1.15
$FeSO_4$ 10mM	1ml

Table. 1: Composition of MSPV medium

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characters	results
Morphology	Cells are ro shape, Gram- negative and aerobic
Motility	Positive
Catalase	Positive
Growth at 10°C	Positive
Growth at 42°C	Positive
Hydrolysis of Urea	negative
Hydrolysis of Tyrosine	Positive
Hydrolysis of Starch	negative
Hydrolysis of Gelatine	Positive
Assimilation of D-Glucose	Positive
Assimilation of L-Arabinose	negative
Assimilation of D-Mannose	negative
Assimilation of D-Manitol	negative
Assimilation of D-Maltose	negative
Assimilation of D-Lactose	Positive
Utilization of Citrate	negative
Oxidase	Positive
Methyl red	negative
Voges-Proskauer test	negative
Growth in NaCl	Up to 3%
Nitrate reduction	Positive
Nitrite reduction	Positive

Table 2: Morphological, phisiological and biochemical characteristics of strains *Pseudoxanthomonas* sp.

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	sample	E_{corr} (V vs Ag/AgCl)	I_{corr} ($\mu A.cm^{-2}$)	β_c (mV/dec)	β_a (mV/dec)	*Rate* (mm/year)
After 7 days	Blank	-0.501	1.41	96	64	0.017
	Psuedoxanthomonas sp.	-0.327	3.6	190	309	0.042
After 30 days	Blank	-0.517	6.08	131	292	0.071
	Psuedoxanthomonas sp.	-0.217	10.11	100	491	0.118

Table 3: Polarization parameters for carbon steel in BH medium in the absence and presence of *Pseudoxanthomonas* sp. at 30°C.

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	sample	R_s (Ω)	R_{ct} (Ω)	R_{diff} (Ω)	C_{dl1} (mF)	W_R (mF)	n1	W_T
After 7 days	Blank	37.1	4097	------	2.6	------	0.61	-------
	Psuedoxanthomonas sp.	-29.2	2055	200.2	4.96×10^{-3}	0.34	0.64	1.21
After 30 days	Blank	131.5	163.5	------	9.1	------	0.76	-------
	Psuedoxanthomonas sp.	80.65	459.8	3382	2.66×10^{-3}	0.64	0.76	112.8

Table 4: Electrical elements obtained from the best fitting of experimental impedance diagrams of the carbon steel/ electrolyte interface, using Zview 2 program.

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Figures:

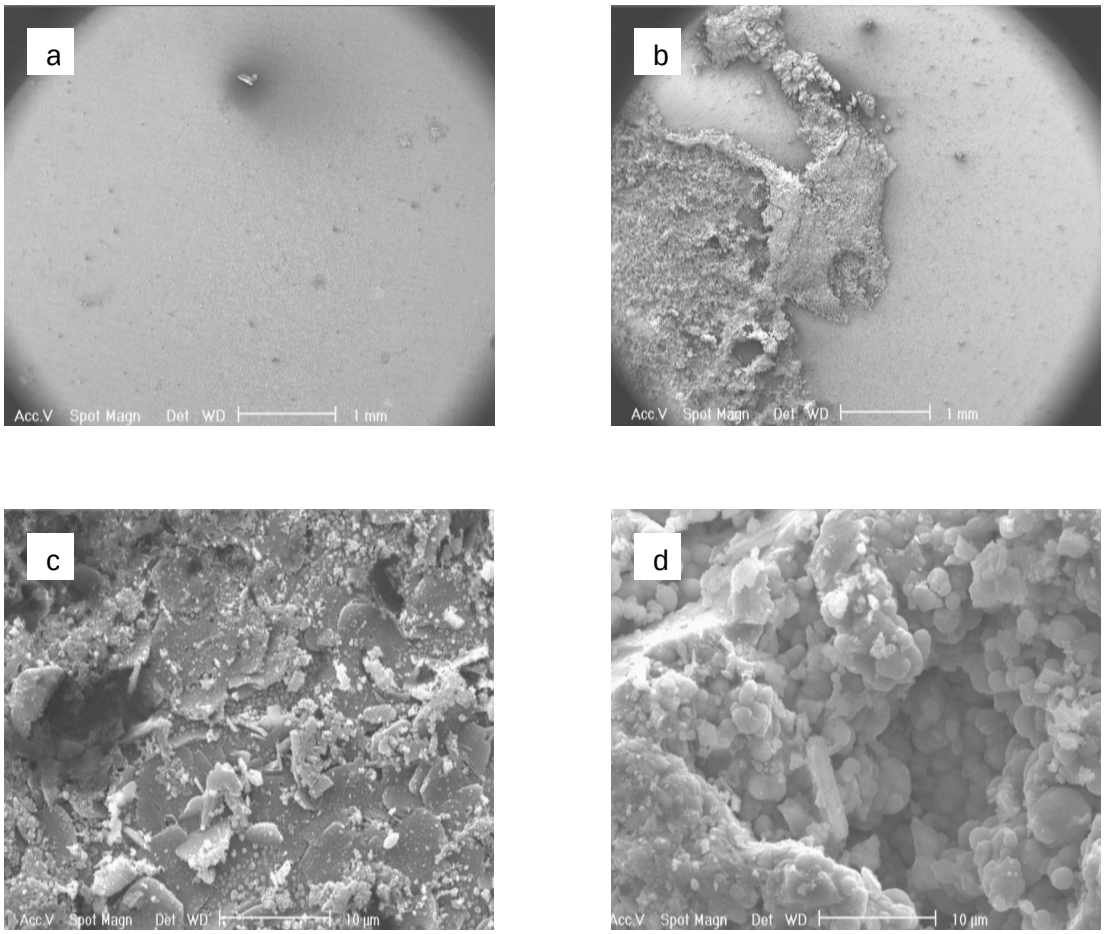

Fig.1. SEM images of the specimens exposed for 30 days in BH medium in absence (a, c) and presence of *Psuedoxanthomonas* sp. (b, d)

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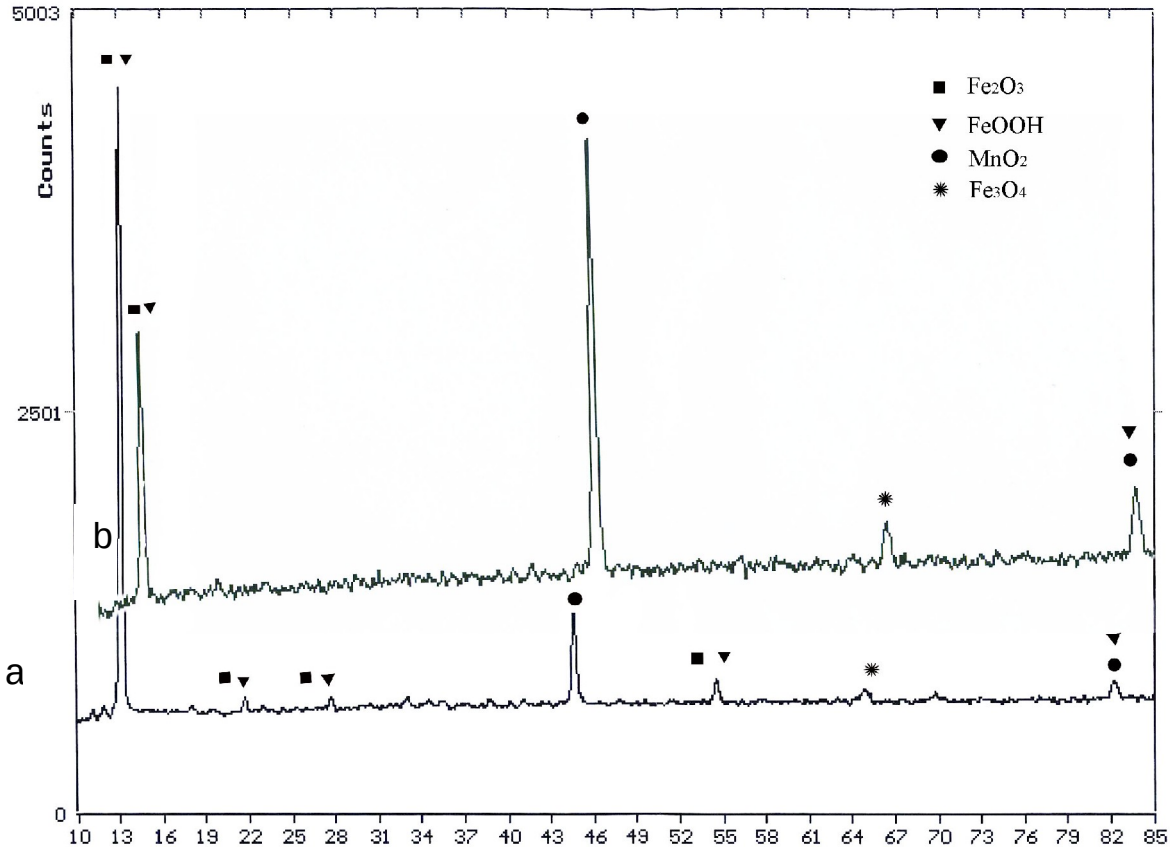

Fig.2. XRD analysis of corrosion product samples after 30 days (a) blank (b) *Psuedoxanthomonas* sp.

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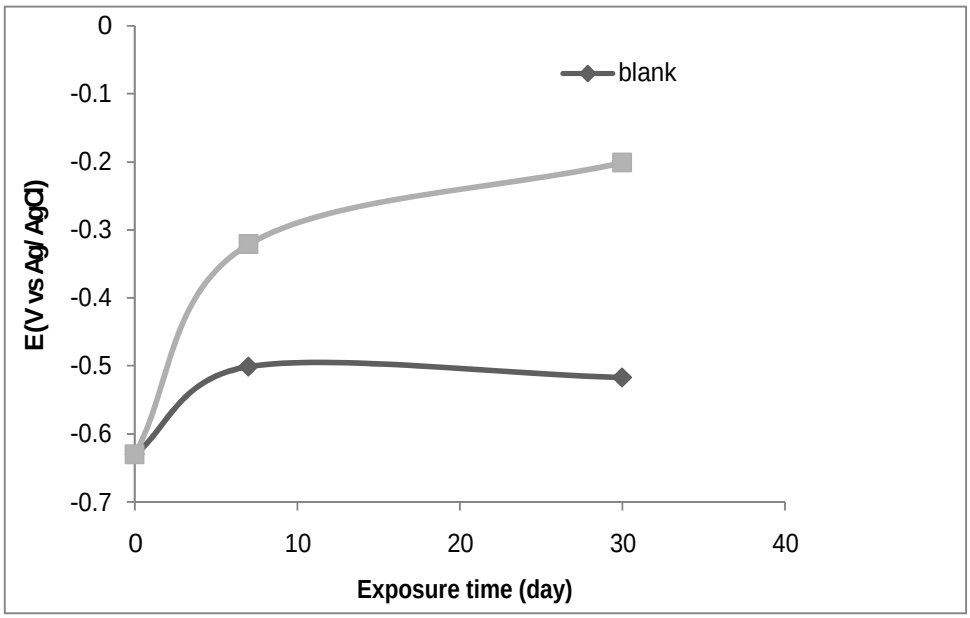

Fig.3. Open circuit potential (E_{corr}) during exposure time for carbon steel in sterile BH medium and MOB solutions at 30°C

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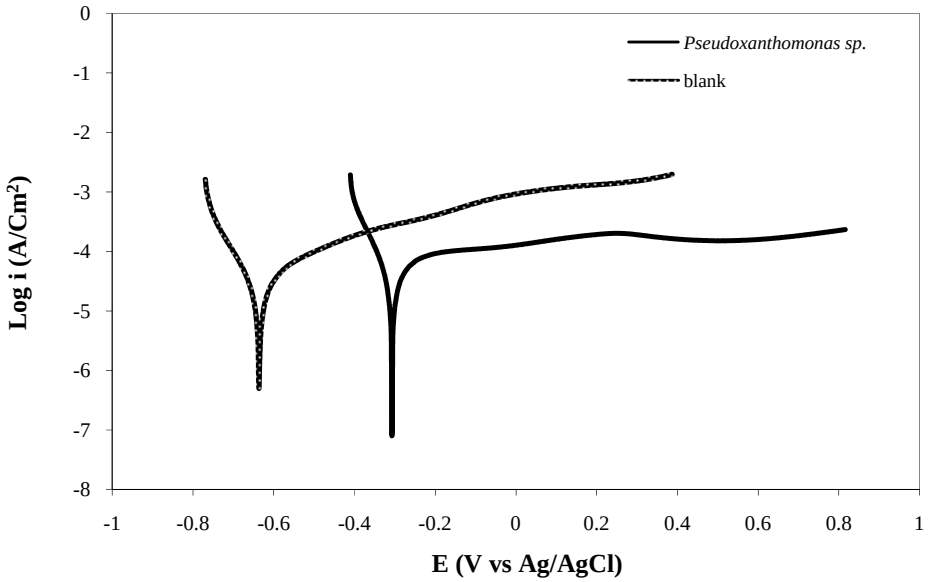

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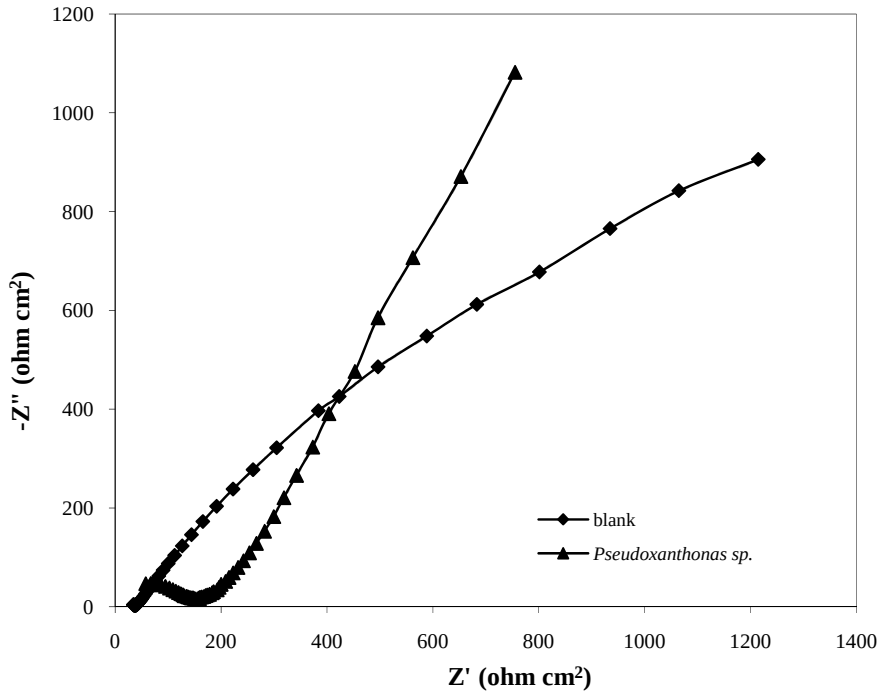

Fig.5. Nyquist plots for carbon steel in BH medium in the absence and presence of *Psuedoxanthomonas* sp. at 30°C after 30 days

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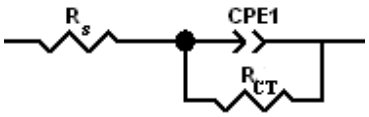
a:



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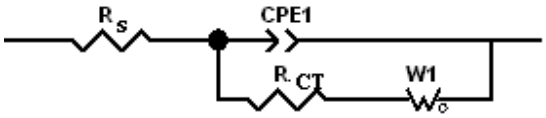

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