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Corrosion Behavior of Q235 Carbon Steel in Presence of H_2S Producing *Bacillus* sp. and a Consortium of Microbes Isolated from Inner Rust Layer

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In this work, a pure *Bacillus* sp. and a consortium of microbes were isolated from inner rust layer of carbon steel from marine environment. Corrosion behavior of Q235 carbon steel was investigated in the presence and in the absence of *Bacillus* sp. and with consortium of microbes separately. Weight loss, electrochemical impedance spectroscopy (EIS), scanning electron microscopy (SEM) and energy dispersive spectrum (EDS) tests were conducted to observe the influence on corrosion behavior of carbon steel in the presence and in the absence of bacteria. The results showed that corrosion potential (E_{corr}) decreased and the corrosion current density increased in the presence of bacteria, in contrast with the sterile medium with the same exposure time interval. The weight loss was observed and micrometer-scale pitting further confirmed by SEM on the carbon steel surface in the presence of bacteria especially with the *Bacillus* sp. Most importantly in the present work *Bacillus* sp. behaved like sulfate reducing bacteria (SRB). Further, it was confirmed that in natural environment *Bacillus* sp. could also act like sulfate-reducing bacteria and deteriorating carbon steel, especially in the form of localized pitting/corrosion.

Keywords: Carbon steel; SRB; Corrosion; Rust layer

1. INTRODUCTION

Carbon steels are widely used in oil and gas industry and in marine systems such as sea-crossing bridges, platforms and wharf because of its homogenous distribution of geometrically small

grains that has grain boundaries. Whereas, it was reported that there are many inclusions such as manganese sulfide (MnS) in carbon steel that serve as seeds for pitting corrosion [1]. Different studies found that there was closely correlation between pit initiation and microbiologically influenced corrosion (MIC) [2-5].

The existing literature on MIC is somewhat complex and confusing because bacterial activity at metal surfaces has been shown to both increase and in some cases reduce corrosion rates [6-9]. The participation of microorganisms not only induces unique corrosion features but also causes alteration in the metal-solution interface through biofilm formation [10]. It is significant part of MIC, which cause great damages to marine steel infrastructures and even induce the disasters [11]. Bacteria can form biofilm on the surface of solid in marine environment in short time (minutes or hours depending on the aqueous environment the immersed) [12]. The biofilm composed of microbial cells with extracellular polymeric substances (EPS). EPS can act as a protective barrier, which can prevent the diffusion of oxygen to cathode areas and the diffusion of aggressive anions such as chloride to anodic sites [13]. In this situation, it can protect the solid from corrosion to some extent. Simultaneously the microorganisms attaching on a metal surface, the concentration of the electrolyte constituents, pH and other factors changes where localized corrosion occurs more easily. SRB, sulfur-oxidizing bacteria (SOB), iron-reducing bacteria (IRB), iron-oxidizing bacteria (IOB), manganese-oxidizing bacteria (MOB), and methanogenic archaea are well documented to be associated with marine corrosion failures, among which SRB is thought to play the most significant role and were studied most widely [14-17]. Microorganisms cause concentration gradients of components by consuming or generating substances in biofilm e.g. H_2S and other acid in SRB biofilm. Since metabolic activity in biofilms is not uniformly distributed that leads to localized corrosion. Aged steel inner rust layer immersed in seawater because of its anaerobic condition or low oxygen concentration environments can offer SRB suitable living environment.

During the last several decades, numerous evidences has been demonstrated that biofouling is a major reason for MIC, among the SRB such as *Desulfovibrio desulfuricans* are considered to be the responsible organism which cause corrosion in anaerobic condition [15-19]. By reducing sulfur compounds such as sulfate (SO_4^{2-}), sulfite (SO_3^{2-}) or sulfur (S) to sulfide (H_2S, HS^-) using organic compounds as electron donors and gain energy for growth, in this process, metal material acts as electron donors and get corrodes. It can promote corrosion of almost all common-used materials such as iron, zinc, copper, aluminum and their alloys [20-22].

Although, many reports have investigated and revealed the relationship between microbial influenced corrosion and localized corrosion induced by bacteria [23, 24]. Studies on this mechanism can be different depending on microorganism species and environment condition. Thus, the present study aims to investigate the corrosion process and their mechanism of carbon steel in presence of *Bacillus* sp. (behaves like SRB) and microbial consortium isolated from inner rust layer of steel immersed in marine environment.

The work intends to use the weight loss, electrochemical technology, scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDS) analysis methods to gain a better understanding of the corrosion process of Q235 carbon steel in presence of pure and consortium of microorganism and to provide evidence for development anti-microbial technology. However, no data

has been found concerning the contrast of consortium, control and pure strain on the microbial corrosion behavior of Q235 CS in the presence of *Bacillus* sp.

2. MATERIALS AND METHODS

2.1 Materials

Q235 Carbon steel (wt. %) C 0.16, Mn 0.53, Si 0.3, P < 0.045, S < 0.055, balanced Fe was used in this study. For electrochemical measurement, 1 cm diameter and 1 cm thickness coupon used as working electrode. To create working electrode, an electrical contact to each steel sample was provided by length of copper wire, which connected to the back side of each specimen and then mounted in epoxy resin, leaving only 0.75 cm^2 of specimen exposed to medium solution. The surface was wet abrade with a series of silicon carbide papers up to a grit size of 2000, and then rinsed with distilled water to remove contaminations followed by sterilization with ethyl alcohol. Every side of the specimen was dried with air drier and further sterilized under UV light for 30 min to remove the entire microorganism attached to the surface.

2.2 Collection of rust layer biofilms and isolation of bacteria

Fresh inner rust layer was collected from Q235 carbon steel species after outer layer being scraped using a sterile scalpel. The coupons come from two places, some from SanYa, Hainan, China (18°15′12″N 109°30′13″E), which has been immersed in natural seawater (pH 8.3) for about 8 years with average seawater temperature 26.7 ˚C and dissolved oxygen 4.5 mg L^{-1}. Some from Qingdao, Shandong, China (35°35′ N 119°30′ E),which has been immersed in natural seawater (pH 8.3) for about half year with average seawater temperature 13.7 ˚C, dissolved oxygen 8.4 mg L^{-1} and salinity 32 marine corrosion experimental station. The collected rust layer was stored in sterile tube and transported to immediately lab in ice box (where the temperature keeps 4 ˚C). To ensure the activity of bacteria and prevent the contamination bacteria isolation was Postgate's C (PGC) to enrich the anaerobic microbes. The bottles were sealed with rubber and aluminum lid, then kept at 37 ˚C. After one week, the grown culture was diluted with sterile phosphate buffer saline (PBS) and then sub-cultured on nutrient agar plate (supplemented with ammonium ferrous sulfate to indicate SRB) to screen pure strain. Individual black colony was picked up using sterile metal loop, then inoculated in new culture medium. Subsequently cultured for five generations and further used for the experiments.

The rust layer including microorganism when grown in PGC at 37˚C, the color of culture medium change with time gradually from colorless to black, simultaneously, producing smelly gas, which is due to the bacteria release H_2S and formation of corrosion product FeS. The media contain following composition (g L^{-1}): 0.5 g KH_2PO_4, 0.5 g NH_4Cl, 0.06 g $CaCl_2{\cdot}6H_2O$, 0.06g $MgSO_4{\cdot}7H_2O$, 6 mL 70% sodium lactate, yeast extract 1 g and sodium citrate 0.3 g. The above composition dissolved in 1 L aged seawater from Qingdao offshore area, pH was adjusted to 7.2 using NaOH (1M) and autoclaved at 121 ˚C for 30 min.

2.3 Identification of the isolates

Genomic DNA extraction, 16S rRNA amplification performed as described by Karn et al. [25]. The amplified product 16S rRNA was purified and sequenced at Majorbio Shanghai, China a sequencing facility. Further sequences were compared against the available DNA sequences in Gene Bank (http://www.ncbi.nlm.nih.gov/) using the Blast-N tool. The 16S rRNA gene sequence resolved in this study was deposited in the Greenback of NCBI data library under the accession number (KY206830). Phylogenetic tree was constructed using the Mega version 5.1 software. Sequences for construction of the trees were downloaded from the NCBI Greenback site.

2.4 Weight change measurements

The coupons used for weight loss measurement are the size of 2 cm ×1.5 cm ×0.5 cm. The coupons were polished using 400 grit silicon carbide paper, degreased in alcohol, and dried in oven at 60 ˚C for 24 h until the weight wouldn't change. All the coupons used in the study were weighed, using an analytical balance with an accuracy of 0.1 mg, prior to submersion and each side of the coupons were sterilized under UV light for 30 min. Next, each coupons were immersed in PGC medium with pure strain, consortium of microbes and control separately for a period of 15 days. To determine the weight loss, the coupons were cleaned as per American Society of Testing Materials (ASTM) standard G1-03. Corrosion rate was calculated as shown follows [26-28]:

$$V_{corr} = 8.76\times10^{4}\frac{\Delta m}{St\rho}$$

Where, V_{corr}, Δm, ρ, S and t were corrosion rate (mm y^{-1}), weight loss (g), density (g cm^{-3}) and area of specimens (cm^2), culture time (h), respectively.

2.5 Electrochemical measurements

Corrosion coupons electrochemical tests were carried out in 500 ml anaerobic vials, with three-electrode system. The working electrode, made up of Q235 carbon steel, have a circular exposed area of 0.785 cm^2. Working potentials were referred to a saturated calomel electrode (SCE), Pt-plate was counter electrode. The vial was filled with 450 mL of Postgate's C medium inoculated with 5% (by volume) pure strain, consortium and control next the three-electrode system was assembled, sealed with rubber lid followed by sealing up using 704 silicon rubber. All the process was done under sterile condition or in laminar air hood chamber. The polarization measurements were performed using the Solartron Metrology (SI1287) electrochemical interface and SI1260 impedance/phase analyzer control systems.

Electrochemical impedance spectroscopy (EIS) was carried out on a steady state open-circuit potential (E_{ocp}) driven at an amplitude of 10 mV AC since wave at frequencies between 100 kHz and 10 mHz. The potentiodynamic polarization curves were obtained at a scan rate of 1 mV s^{-1}. Duplicate experiments were carried out and the differences between the two electrodes were ±1%. Further, the data was confirmed by conducting a third test. The software used in the test was CorrWare with

CorrView version 2.0 for the collection and analysis of the potentiodynamic polarization curve data and ZPlot with Princeton ZSimpWin version 3.2.1 software for the EIS data.

All the experiments were repeated three times at room temperature (~20˚C) and no renewal of any material during immersion time.

2.6 Surface analysis

Scanning electron microscopy (SEM), and energy dispersive X-ray spectroscopy (EDS) were used respectively to characterize the morphology of corrosion product and determine composition of corroded carbon steel.

For SEM and EDS observation, dehydrated using concentration gradient ethanol solutions (for 20 min each): 25%, 40%, 60%, 75%, 95% successively for fixing. Next, the coupons were dried in critical point environment, then coated with a palladium layer. Finally, the processed coupons were characterized through SEM and EDS. To observe the corrosion pit under the biofilm, the coupons were cleaned by acid solution containing hexamethylenetetramine (V_{acid}:V_{water} = 1:1, and 1 L solution including 20 g hexamethylenetetramine) to remove corrosion products and biofilm covering the coupon surface. Further, dehydrated using ethanol and dried in vacuum drier before SEM examination.

2.7 Epifluorescence measurements

To investigate the adhesion of bacteria and the biofilm formation on carbon steel surface, fluorescence micrograph was conducted at different time interval. For fluorescence micrograph, the coupons covering with biofilm were immersed in 2.5% glutaraldehyde solution for 30 min to fix the biofilm to the carbon steel surface. Further, DAPI dye was used and the green excitation light indicated the colony.

3. RESULTS AND DISCUSSION

3.1 Identification of isolates

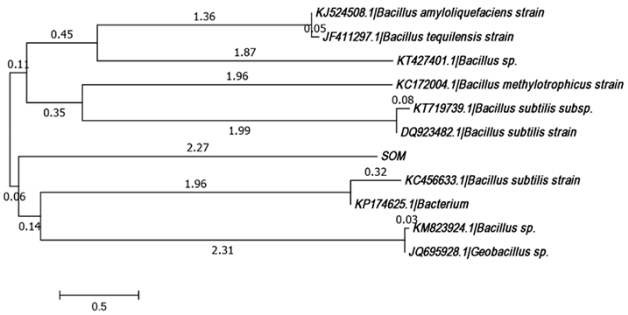

Figure 1. Neighbor-joining tree based on 16S rDNA gene sequences. Current isolate is **SOM** (KY206830) used in this study

Bacteria generally occupy in natural environment characterized by specific attributes such as pH, temperature, specific carbon or substrate availability, presence of salt and other chemical factors (solvent, inhibitors, toxicants, oxygen, etc.).

Such attributes determine the metabolic routes of microorganism inhabiting there. The BLAST analysis of the obtained sequence of pure strain showed 99% of sequence similarity with *Bacillus* sp. Based on 16S rRNA gene sequence, phylogenetic tree was constructed by N-J using MEGA software version 5.0. The phylogenetic analysis revealed that pure strain grouped with *Bacillus* sp. The constructed phylogenetic tree shown in Fig. 1.

3.2 Weight loss results

Significant amount of weight loss was observed in medium containing pure strain *Bacillus* sp. as compared to the control, which was about 7× of that in blank medium. However, the lower weight loss and low corrosion rate has been found with consortium of microbes as compared to control maybe due to the corrosion products and possibly some aerobic biofilm acting as a protective layer on carbon steel surface.

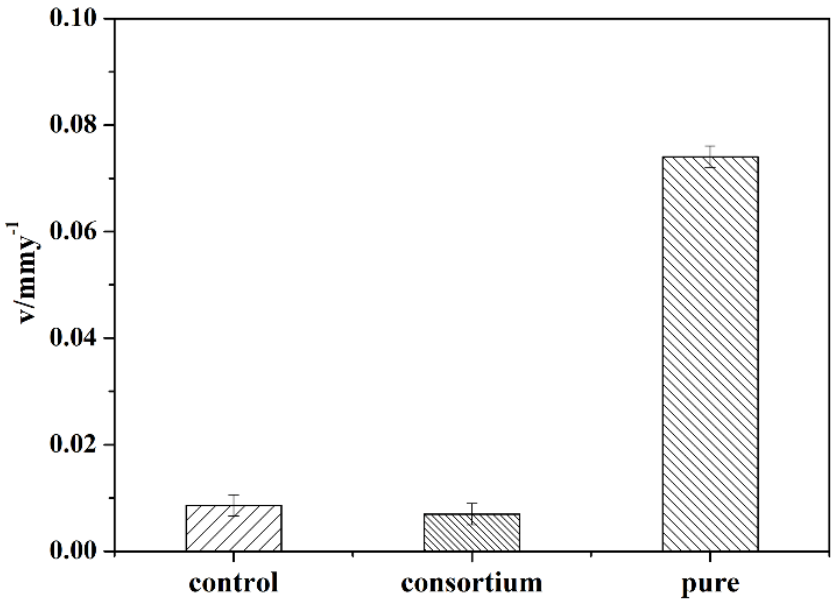

Figure 2. Corrosion rate of Q235 carbon steel calculated from the results of weight loss in the control, consortium and pure medium after immersion from 15 days

Previously it was also observed by other researcher like Moradi et al. [29] found the biofilm of aerobic microbe *Vibrio neocaledonicus* with the highest corrosion inhibition efficiency. Perhaps, in consortium different microorganism having ability to inhibit the corrosion. Fig. 2 clearly shows weight loss observed for Q235 carbon steel specimen immersed in PGC control and PGC containing pure strain and consortium of microbes separately within 15 days. The results show that Q235 carbon steel specimen in PGC with pure strain exhibit the significant weight loss, which manifests that the pure

strain has the high corrosion rate, with the average corrosion rate is 0.07407 mm y^{-1}. while the Q235 carbon steel specimen in PGC with consortium have less weight loss even as compared to control strain which suggest that the consortium has no ability to cause the corrosion and this consortium inhibit the corrosion at all, whose corrosion rate are 0.00696 mm y^{-1} and 0.00859 mm y^{-1}, respectively. These results, consistent with previous studies, which have shown similar corrosion rates in cell-free spent media and sterile (control) media [30, 31].

3.3 Electrochemical Measurements

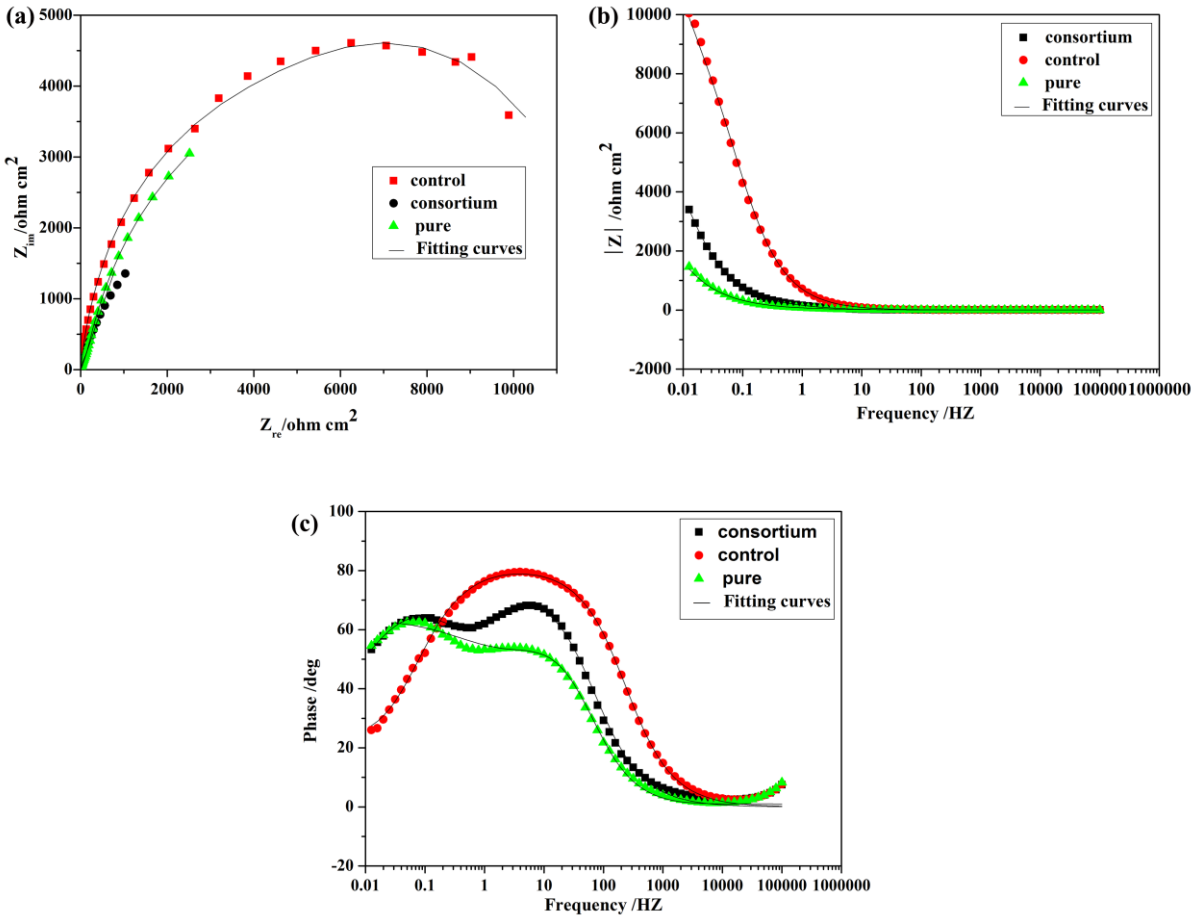

Figure 3. EIS plot of Q235 carbon steel specimen immersed in PGC and PGC containing SRB and consortium bacteria after 3 days (a) Nyquist plots (b) bode module-value plots and (c) bode phase-angle plots.

EIS has been widely recognized as a non-destructive and powerful technique for studying individual surface layers and monitoring corrosion processes [32]. EIS plot recorded by the electrode which has been immersed for 3 days in PGC control PGC containing pure *Bacillus subtilis* strain and consortium bacteria, respectively. Fig. 3 shows diameters of Nyquist semicircles for PGC including bacteria decreases as compared to diameters of Nyquist semicircles obtained from PGC control Fig. 3a

and the lower values of the total impedance magnitudes at the lowest frequency Fig. 3b. From Fig. 3c there are two time constants for Q235 carbon steel exposed to the medium with bacteria, no matter the pure one or the consortium one, the possibility of biofilms and corrosive products layer which participated in the electron transfer process will lead to the only one constant time in sterile medium [28, 33].

Fig. 4 shows the equivalent circuit for Q235 carbon steel in sterile medium where R_s is the resistance of the electrolyte, Q_{dl} describes the, usually a CPE is used in a model instead of a capacitor to compensate for non-homogeneity in the system, and the value of n can describe the content of non-homogeneity. R_{ct} describes the impedance of the corrosion product iron oxidant, (Equivalent circuit in Fig 4b is R(Q(R(QRW))) and in Fig 4c is R(QR(W))(CR),contains two time constants, and represents the formation of a corrosion product film as well as biofilm on the metal surface). Fitting electrochemical parameters obtained using ZSimDemo version 3.20 software, and summarized in table 1. The charge transfer resistance, R_{ct}, is a parameter that characterizes the corrosion rate, it clear that the R_{ct} of Q235 carbon steel exposed to sterile medium is larger than these in medium containing bacteria, and the value is the smallest in pure system, which is same with the reference [28].

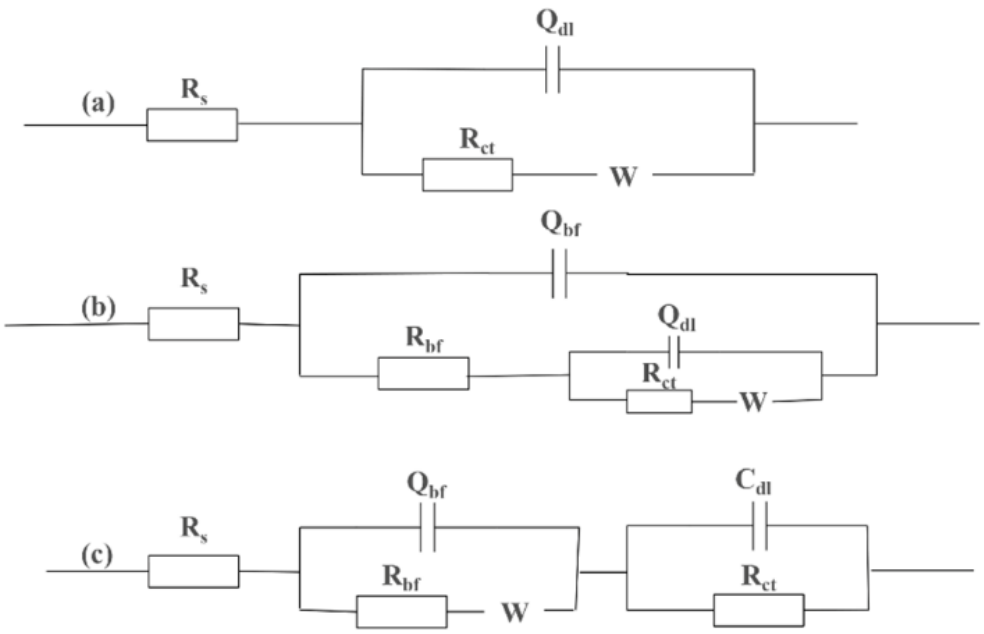

Figure 4. Equivalent circuits for Q235 Carbon steel (a) in sterile medium (b) medium containing consortium bacteria (c) medium containing pure bacteria to fit EIS data

Table 1. Parameters of EIS for Q235 Carbon steel in different medium after 3 days of immersion

Parameter / Sample	R_s/ohm cm^2	$Q_{dl}\times10^6$/ $ohm^{-1}s^n$ cm^{-2}	n	R_f/ohm cm^2	W	$Q_{dl}\times10^6$/ $ohm^{-1}s^n$ cm^{-2}	N	R_{ct}/ohm cm^2
control	5.053	264.5	0.9054		0.001083			8651
consortium	4.833	0.0009871	0.8831	428.8	0.002045	0.001146	0.8446	6507
pure	5.145	0.001746	0.8527	45.75	0.006169	0.01143	1	1816

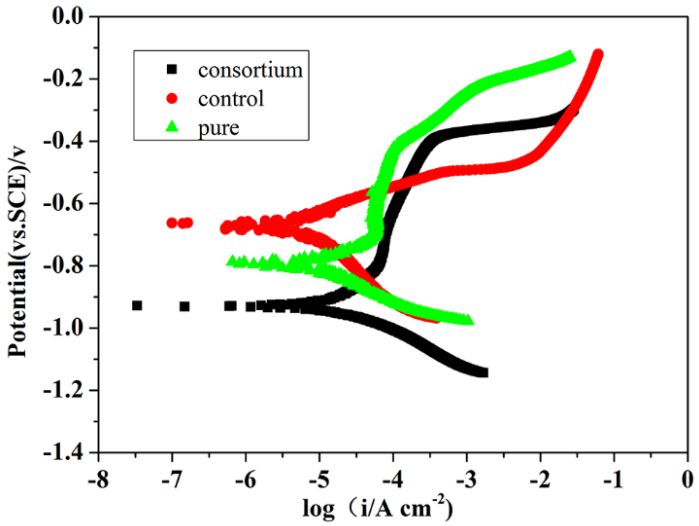

Figure 5. Potential–dynamics polarization curves of Q235 carbon steel exposed to PGC with or without bacteria after 15 days

Table 2. Tafel parameters deduced from polarization measurements

	E_{corr} (V/SCE)	i_{corr} (uA cm^{-2})	β_α (mV/dec)	β_c (mV/dec)
control	–0.67031	0.26248	47.615	65.947
consortium	–0.92823	5.843	120.41	77.616
pure	–0.79323	11.119	173.49	131.09

Fig. 5 demonstrates polarization curve for Q235 carbon steel exposed to PGC medium in the absence and presence of bacteria after 15days. Electrochemical corrosion parameters such as corrosion potential (E_{corr}), corrosion current density (i_{corr}), cathodic (β_c) and anodic (β_a) Tafel slopes were obtained from polarization curves and are given in Table 2. Fig. 5 and Table 1 show that the presence of pure strain or consortium one both decrease the corrosion potential and increase corrosion current density of carbon steel electrode dramatically. After 15 days exposure, the value of corrosion current density of electrode was 0.26 μA cm^{-2} in control medium, which increased to 11.12 μA cm^{-2} in the presence of *Bacillus* strain. The enhancement of anodic slope is affected by the amount of corrosion products and biofilms formed on the metal surface [28, 34]. According to EDS data, it was observed that products formation FeS possibly taken place with the pure strain. This finding is also supported by other researchers the products formation in SRB medium is FeS and GR (green rust) [35, 36] and iron oxides in sterile medium or consortium. Therefore, it can be concluded that the increase in anodic slope can be attributed to FeS deposition and biofilm formation on the carbon steel surface. The results also correspond to the weight loss (Figure. 2).

3.4 SEM and epifluorescence characterizations

Carbon steel coupons that were incubated under different conditions and were investigated by SEM to indicate the impact of microbial corrosion before and after the product was removed. Detail of

corrosion product developed on carbon steel surface exposed are given in Fig. 6a, 6b and 6c; Control, consortium and pure strain respectively, whereas biofilm observed under the corrosion products are given in Fig. 6e, 6f and 6g; control, consortium and pure strain respectively, under 15 days of incubation. H_2S producing *Bacillus* sp. practically the whole specimen surface immersed in PGC medium, containing bacteria was covered with black dense deposit. As shown Fig. 6a the surface of carbon steel that was incubated under sterile conditions was plain and smooth, there was little corrosion product and the surface was of metal color.

Carbon steel coupons with pure strain surfaces featured with prominent signs of corrosion exhibiting structures, mixture corrosion product and biofilms was observed. The surfaces of carbon steel with pure strain and consortium bacteria performed large and small pits respectively after removing corrosion product. So cracks and pit attacks have been formed on the carbon steel surface in presence of pure strain under biofilms. In comparison to control or sterile condition, no pit corrosion was observed when the coupons were washed with acid containing hexamethylenetetramine.

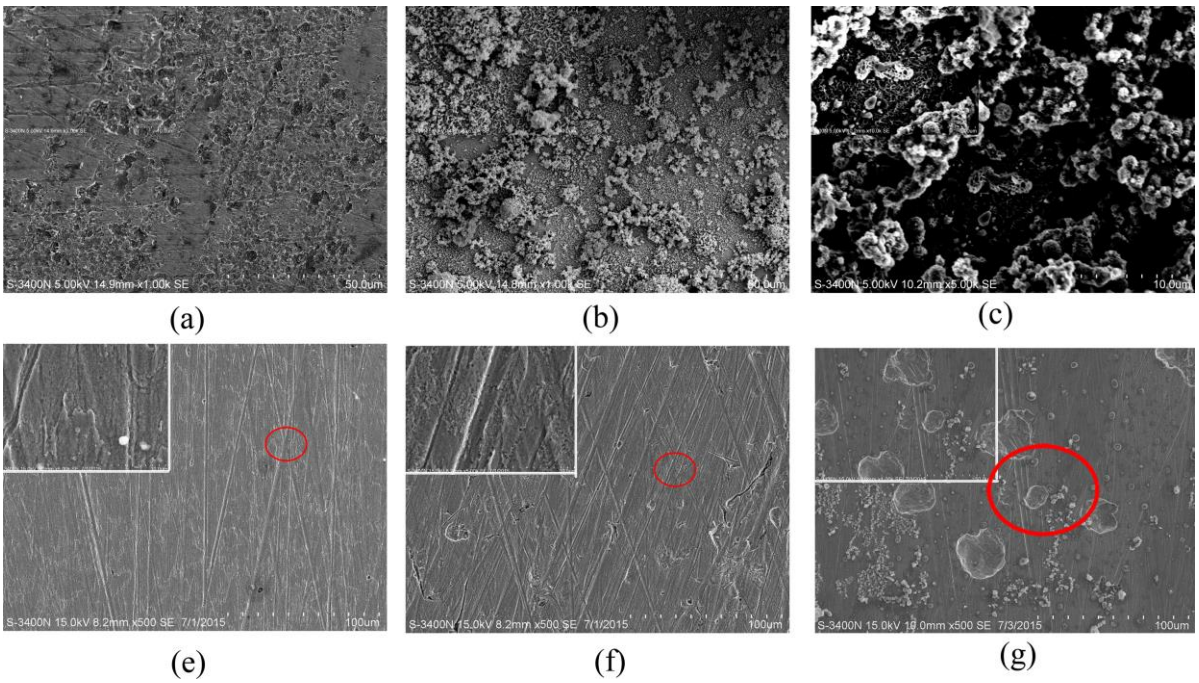

Figure 6. Corrosion morphologies of carbon steel after immersed for 15 days in three mediums: control (a) with and (e) without corrosion products; consortium microbes (b) with and (f) without corrosion products; pure strain (c) with and (g) without corrosion products.

The *Epifluorescence* analysis results show that bacteria, either pure stain or consortium can both form biofilm on carbon steel surface in short interval of time, and the biofilm can attach on the surface stably and for long time.

3.5 Energy dispersive X-ray (EDS)

EDS was performed for carbon steel surface to verify the mechanism of corrosion products formation process. In the presence of pure strain, carbon steel specimen was covered with rough and cluster black corrosion products. The EDS results of chemical composition of biofilm and corrosion products show the main elements are C, Fe, S and O, indicating that corrosion products formed are iron sulfides and some iron oxides Fig. 7a the outlook of control carbon steel is of metal color, and almost no corrosion product was observed. At the same time, EDS analysis shows that there is only a little corrosion product on the carbon steel surface subjected to control medium shown in Fig. 7, and these deposits are iron oxides, possibly this is due to the ingression of small quantities of oxygen into the anaerobic incubator [36]. In the presence of consortium, the corrosion product is also black and dense, EPS and round corrosion products was observed under SEM. EDS analysis confirms that the main composition of products are iron oxides as well as some iron sulfides. There is obvious difference between the products composition formed in three different systems. The concentration of sulfur in corrosion product and biofilm was 30 times higher for the coupons immersed in *Bacillus* strain as compared to the control system. These results support the electrochemical analysis results.

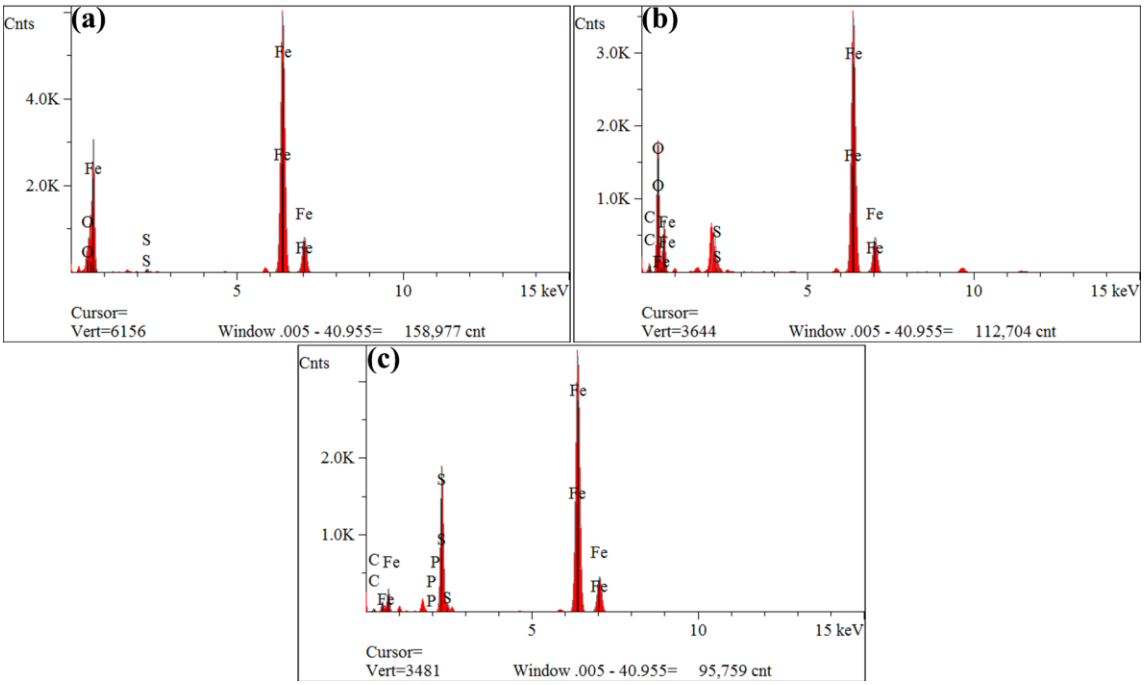

Figure 7. EDS of the carbon steel surfaces exposed for 15 days: (a) control (b) consortium of microbes (c) pure strain

Fig. 8 present the SEM image and the corresponding elements of Q235 after immersed in the consortium of microbes for 15 days. Fe was relatively uniformity distributed in the surface, Fig. 8a, while S performed almost the same of Fe Fig. 8b. In comparison, O, C and P had a relative low concentration showed less distribution see Figs. 8c, 8d and 8e, the result was corresponding with the EDS.

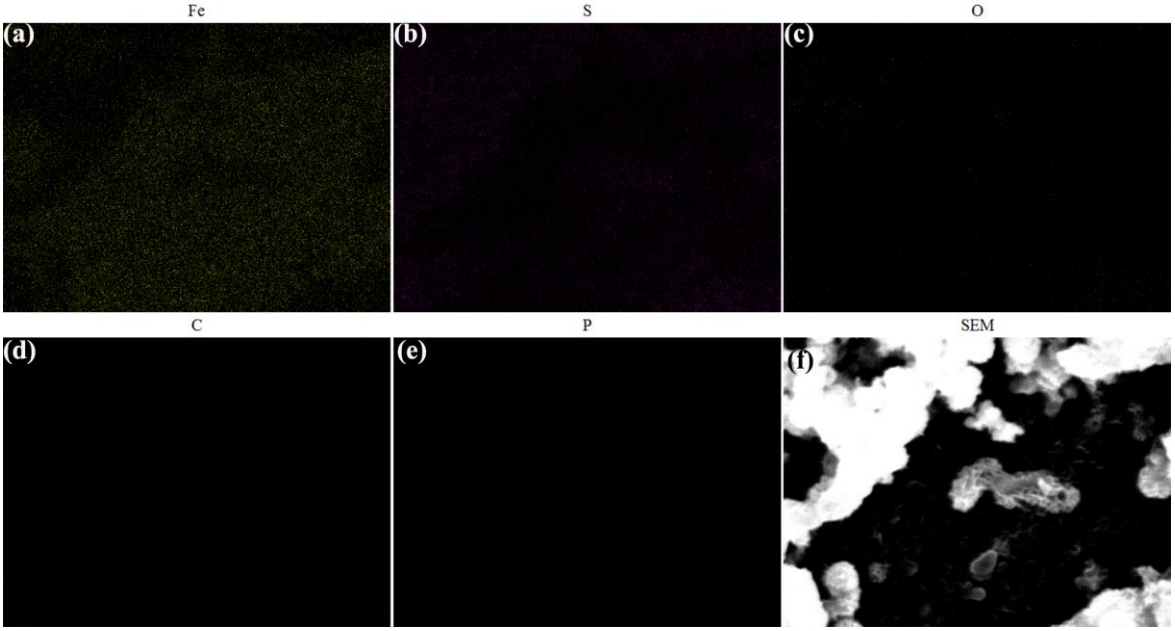

Figure 8. SEM image of Q235 after immersed in the consortium of microbes for 15 days (f) and corresponding elemental distributions of iron (a), sulfur (b), oxygen(c), carbon (d), and phosphorus (e).

EDS and surface distribution showed that the product contains a large number of S, which was produced by sulfate-reducing bacteria metabolites of sulfide iron and iron production of iron sulfide. This agreement of the of Grousset's et al. [38] results' suggested that the main product produced by SRB was iron sulphides.

4. CONCLUSIONS

Corrosion behavior of *Bacillus* sp. and consortium from rust layer on Q235 carbon steel was investigated by electrochemical technologies and weight loss. The tentative conclusions are given as follows:

（a） A corrosive strain was obtained from inner rust layer of carbon steel in marine environment, and has identified as *Bacillus* sp.

（b） Weight loss results revealed that the corrosion rate of Q235 Carbon steel in medium containing pure strain is seven times higher than in sterile medium. However, the consortium of bacteria decreased corrosion rate of Q235 carbon steel because of the function of biofilm.

（c） The EIS results support that in the presence of pure strain, the R_{ct} and the impedance module is of the lowest value which indicating the highest corrosion rate.

（d） Epifluorescence micrograph shows that a large number of bacteria as well as corrosion product attached on the surface of Q235 carbon steel. And SEM results shows that several pitting corrosions occur under the biofilm of *Bacillus* strain.

（e） EDS results confirmed that the corrosion product is FeS immersed medium with *Bacillus* sp. in mixture medium the corrosion product are irons oxides and small amount of FeS. In

sterile medium, the corrosion product was mainly ions oxides.

Bacillus sp. reporting for the first time of H_2S producing abilities and including corrosion therefore further study is required to get in detail about corrosion mechanism. Considering the fore mentioned corrosion performance caused by *Bacillus* sp., it is meaningful and further research are in underway.

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