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Extracellular electron transfer of *Bacillus cereus* biofilm and its effect on the corrosion behaviour of 316L stainless steel

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ABSTRACT

Here, a heterogeneous *Bacillus cereus* (*B. cereus*) biofilm on the surface of 316 L stainless steel (SS) was observed. With electrochemical measurement and surface analysis, it was found that *B. cereus* biofilm could inhibit SS pitting corrosion, attributing to the blocking effect of bacterial biofilm on extracellular electron transfer (EET). Differential pulse voltammetry (DPV) and cyclic voltammetry (CV) results also showed that *B. cereus* biofilm clearly impeded the EET. The proposed mechanism for the decreased corrosion rates of SS involves the interactions of extracellular polymeric substance (EPS) with SS and biofilm formation blocking electron transfer, preventing the passive layer from destroying. After biofilm formation following initial attachment of cells and EPS, electron transfer between SS and the cathodic depolarizer (oxygen) was hindered.

1. Introduction

The term microbiologically influenced corrosion (MIC) is normally interpreted to indicate an increase in corrosion rates due to the presence of microorganism that accelerate the rates of the anodic and/or cathodic corrosion reaction [1]. However, many bacteria also play a role in corrosion inhibition in water environments, such as artificial seawater and natural seawater [2–4]. Accordingly, microorganisms may cause either biologically influenced corrosion acceleration or inhibition.

Up to date, most laboratories have reported that bacteria accelerated corrosion of metallic materials as the result of the presence and activity of bacteria within biofilm [5–7]. Xu et al. [8] found that 2205 duplex SS was susceptible to an accelerated corrosion in the presence of *Pseudomonas aeruginosa* (*P. aeruginosa*) biofilms. Li et al. [9] demonstrated that 2707 hyper-duplex SS was not completely resistant to MIC due to the *P. aeruginosa* biofilm. Unlike corrosion acceleration by bacteria, corrosion inhibition by bacteria is still poorly investigated. Among the few reports in the literature, according to Duan et al. [10], mixed species of *sulfate-reducing bacteria Desulfovibrio caledoniensis* and *iron-reducing bacteria Clostridium* sp. could inhibit corrosion of steel, while single species produced iron sulfide and accelerated corrosion. Additionally, it has been reported that the pitting of cold rolled steel was obviously inhibited by *B. subtilis C2* when the compact bacterial biofilms were fully formed [1]. These reports indicated that different kinds of bacterial biofilm played different roles in corrosion of various steels.

As the main genus of the gram-positive and ubiquitous *Bacillus* species, *B. cereus* was an industrial contaminant and a public health hazard widespread in soil, air, marine environments [11,12]. Many investigations declared that *B. cereus* was readily adapted to an attached mode of growth, with forming dense and thick biofilm structure on the various solid surfaces such as glass, metal [13,14]. Nevertheless, only a few publications have dealt with MIC about *B. cereus*. Rajasekar et al. [15] revealed that *B. cereus* ACE4 was capable of converting the ferric and manganese on metal into oxides and accelerated severe pitting attack on the surface of steel API 5LX. Matsumura et al. [16] and Santana et al. [17] showed that *B. cereus* cells adhered tightly to SS specimen followed by biofilm formation, and had highly corrosive activities. However, Aïmeur et al. [18] investigated the influence of *B. cereus* on the corrosion behaviour of carbon steel in natural seawater, and found that the corrosion resistance of A60 steel was increased by the *B. cereus* biofilm. It is easy to draw a conclusion from literatures that the structure of the *B. cereus* biofilm is very important for the corrosion processes of steel in either a detrimental or beneficial way. Just like MIC of other microorganisms, the corrosion mechanisms involved in *B. cereus* biofilm are very complicated. As the process is affected by many factors, many mechanisms, such as oxygen consumption, metal-binding

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effect of the EPS have been put forward to explain the influence of biofilm on the metal corrosion, while the lack of consensus remains unresolved.

Interestingly, some researchers believed that Gram-positive bacteria biofilm presented potentially the electrochemical activity [19]. The biofilm may hamper or promote the electron transfer in the process of the MIC. Electrochemically active biofilm use a unique mode of respiration, in which terminal electrons derived from their metabolism are transferred to extracellular, insoluble electron acceptors (metallic materials and electrodes) [20–23]. The biofilm, as a conductive material, is considered to be a favorable substrate transfer to the anode in the corrosion process, which may accelerate corrosion of metal. However, the biofilm attached to the metallic material surface, with the increase of biofilm thickness, the transfer of proton might be limited. When bristle density is high, the barriers to nutrient transport are high, current density and total current will grow a little as the biofilm grows, which may play a protective role. Moreover, it is well known [23] that microorganism is surrounded by EPS which includes phospholipids and humic acids. There is a competitive relationship between the adsorption of electroactive and non-electroactive substances on the surface of a solid material. Some non-electroactive substances might be preferentially adsorbed on the metal surface, increasing EET barriers, which will inhibit the corrosion of metal. In addition, it has been confirmed that the conductive products of bacteria metabolism (riboflavin, flavin adenine dinucletide and cell-derived free enzymes) were able to accelerate corrosion [24,25]. That is, the electroactive and non-electroactive substances that affect the EET clearly may play an important role in the corrosion. However, there is still lack of direct evidence between the electron transfer of biofilm and corrosion of materials.

SS has always been a concern in inshore and offshore construction in seawater. Despite the fact that SS is being used more and more widely in offshore constructions, but there is still lack of data for SS corrosion, especially in the environment containing microorganisms. Numerous studies have shown that all kinds of steel can be corroded in this environment depending on the following variables: salinity, oxygen, availability, temperature, macro and micro-fouling [26–28]. In addition, MIC is recognized as one of the most aggressive factors present in nature. Some studies have shown that microbial activity and biofilm could accelerate or inhibit SS corrosion [29,30]. Xu et al. [31] showed that the synergies between the SS surface, abiotic corrosion products, chloride anion, and SRB, IOB, SRB + IOB and their metabolic products increased the corrosion damage degree of the passive film and accelerated pitting propagation. Xu et al. [32] indicated that the marine aerobic biofilms mainly inhibited anode action of steel. Thus, it is clear that different bacteria species play different roles in the corrosion of SS but relevant information is still scarce. To better understand the role of different bacteria species on biodegradation of metals, it is necessary to investigate the influence of other bacteria.

Therefore, the goal of this study is to investigate the effect of *B. cereus* on the corrosion behavior of SS in artificial seawater. Meanwhile, the possible mechanism of the effect of biofilm formation on SS corrosion was proposed to explain the experimental observation by CV, DPV and Fourier transform infrared spectroscopy (FTIR), *etc.*

2. Experimental methods

2.1. Preparation of the specimens, bacteria, and culture medium

The composition of AISI316L SS was shown in Table S1. For the fluorescent microscopy (FM), scanning electron microscopy (SEM) measurements, a series of SS specimens (2.0 × 2.0 × 0.1 cm) were prepared by laser cutting. For the electrochemical tests, the working electrodes were made of SS by connecting a copper wire and in PVC holder using epoxy resin with an exposed area of 1.0 × 1.0 cm^2. The SS specimens were wetly abraded sequentially using a series of silicon carbon papers with grit from 120# to 2000#. The surfaces were rinsed with distilled water to remove contamination, degreased with acetone, and dried with a warm air stream, and then the specimens were sterilized with 2.5% glutaraldehyde solution and 75% ethanol in a biosafety cabinet under the UV light for 2 h for sterilization.

B. cereus was provided by the State Key Laboratory for Conservation and Utilization of Bio-resources in Yunnan, Yunnan University (Kunming, China). It was routinely cultivated in sterile Luria-Bertani growth medium containing NaCl (10 g/L), yeast extract (5 g/L), tryptone (10 g/L) at 28 ± 1 °C.

The nutrient-rich artificial seawater was used throughout the corrosion behaviour study. The chemical compositions and preparation of artificial seawater were described in more detail elsewhere [1].

2.2. Characterization of morphology and composition

FM technique was used to investigate surface coverage of SS by *B. cereus* and EPS. SS samples were retrieved chronologically at 1 d, 3 d and 25 d, and washed twice with 1 M sterile phosphate buffered saline solution to remove bacterioplankton. Subsequently, the specimens were fixed with 2.5% glutaraldehyde solution, stained with 2-(4-amidinophenyl)-6-Indolecarbamidine dihydrochloride (DAPI) for 15 min in the dark and rinsed with sterile distilled water. Finally, coupons with immobilized bacterial cells were imaged under 20 x magnifications using a Nikon E800 fluorescence microscope, equipped for epifluorescence with a mercury lamp.

The surface morphology of SS was examined by SEM (Holland yielding XL30 ESEM-TMP) exposed to the abiotic and abiotic solution for 25 d. To immobilize bacteria, the specimens was immersed in 2.5% glutaraldehyde solution for 15 min. Afterward, the samples were respectively dehydrated successively in different proportions ethanol solutions for 10 min. 20%, 50%, 75%, 99%, 100%.

Fourier Transform Infrared Spectroscopy (FTIR) was used to measure the spectra of *B. cereus* and the corroded surface without and with *B. cereus* in artificial seawater, respectively (Thermo Fisher SCIENTIFIC Nicolet IS10, USA). The KBr disk technique was used in FTIR, which was achieved by adding 64 interferograms at a resolution of 8 cm^{-1} in the region from 500 to 4000 cm^{-1}.

2.3. Electrochemical measurements

The dynamic process of SS corrosion was conducted using potentiostat of PARSTAT 2263 electrochemical tester (Perkin Elmer™ Company, USA). A three-electrode system including a working electrode of SS, a counter electrode of 223 platinum gauze electrode, an auxiliary electrode of a saturated calomel electrode (SCE) was used for electrochemical measurements. All experiments were implemented in artificial seawater with and without *B. cereus* at 28 °C. EIS spectra were performed after open circuit potential (OCP), and carried out *via* excitation of a sinusoidal wave with the amplitude of 10 mV within the frequency range of 0.1–10^5 Hz. ZSIMPWIN (Michigan, USA) software packages was used for EIS data analysis. Tafel polarization curves was carried out at the end of EIS, at a scan rate of 1 mV/s, in the range between −0.25 and + 0.25 V with respect to the corrosion potential (E_{corr} *vs*. SCE). All electrochemical experiments were repeatedly in triplicate on different samples to ensure the reproducibility. All electrochemical data in this study represented the mean ± standard deviation of three test replicates.

Electron transfer experiment was performed using a CHI660 electrochemical workstation. Respectively, a glassy carbon disk (Ø = 3 mm), a platinum wire, and a saturated Ag/AgCl were used as the working electrode, counter electrode and reference electrode. The working electrode was polished, rinsed in distilled water, and then cleaned in acetone. The sterile Luria-Bertani was used as the electrolyte (pH = 7.5). The solutions used in the electrochemical experiments were N_2 purged to guarantee that oxygen was eliminated. EET of *B. cereus*

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