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Catalysis of oxygen reduction reaction by an iron-reducing bacterium isolated from marine corrosion product layers

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ABSTRACT

The impact of *Thalassospira* sp., an iron-reducing bacterium isolated from marine corrosion product layers, on oxygen reduction reaction (ORR) has been investigated in the present study. ORR catalysis has been observed on a glassy carbon (GC) electrode when it is exposed to phosphate-buffered saline containing *Thalassospira* sp., and the adhesive biofilm consisting of bacterial cells and metabolites is believed to be responsible for the catalysis. A certain time of contact between the electrode and live cells is necessary for ORR catalysis, but the metabolic activity of cells after adhesion is not vital for the catalysis, and adsorbed compounds excreted by bacteria play a more important role. The ORR catalytic activity of *Thalassospira* sp. does not depend directly on bacterial surface coverage given the results of cyclic voltammetry and scanning electron microscopy. For the ORR kinetics analysis, the 4-electron pathway with H_2O as the final product is achieved by the biofilm, which is different from the predominant 2-electron transformation on the bare GC electrode.

1. Introduction

The near-neutral pH of microbial fuel cells (MFCs) leads to sluggish kinetics of cathodic oxygen reduction reaction (ORR), which is a crucial impediment to the development of this new energy technology [1–3]. The operating conditions of MFCs permit the growth of bacteria, and in comparison with traditional chemical ORR catalysts, microbial biocathodes are inexpensive, sustainable, and resistant to poisoning [4]. Great attention has been paid to microbial biocathodes due to their advantages, and both mixed microbial communities and pure cultures have been reported to catalyze ORR. Mixed microbial communities give broad bacterial diversity, and can be derived from seawater [5,6], freshwater [7], and wasterwater treatment sludge [8–10]. Although mixed microbial communities display robust catalysis efficiency towards ORR, their complex multispecies nature brings great difficulty for mechanism investigation, which stimulates the research of ORR catalyzed by pure cultures. So far, the catalytic activity of ORR has been defined in numerous bacterial strains, such as *Pseudomonas* sp. [11], *Roseobacter* sp. [12,13], *Pseudoalteromonas* sp. [14], *Acinetobacter* sp. [2,15], *Leptothrix discophora* [16], and *Acidithiobacillus ferrooxidans* [17]. The electroactive microorganisms reported cover different genera and possess different metabolic characteristics, either aerobic, Gram-, catalase-, oxidase-positive, or facultative, Gram-, catalase-, oxidase-negative [12]. And hence it is really hard to assess the ORR catalytic activity of specific bacterial strain before determination.

Iron-reducing bacteria (IRB) have been viewed as important microorganisms involved in the anodes of MFCs [18–20], which transfer electrons to anodes without the provision of exogenous mediators under anaerobic conditions. IRB are usually facultative anaerobes, which switch from ferric ion reduction to oxygen reduction under aerobic conditions [21], and how about their catalytic performances towards ORR? To the best of our knowledge, only *Shewanella loihica* PV-4 (belonging to IRB) has been reported to catalyze ORR by the secretion of flavin compounds [22]. There is no information whether other IRB also catalyze ORR, and therefore the catalytic activity of an iron-reducing bacterium, *Thalassospira* sp., isolated from marine corrosion product layers, is investigated in the present study.

Oxygen can be transformed into H_2O_2 or H_2O as the final product in aqueous solution, depending on the nature of electrode materials and electrolytes. The lack of data on the direct comparison between cathodic current production and oxygen flux towards cathode surfaces in MFCs often leads to the speculation that oxygen is reduced to H_2O via the direct 4-electron pathway [23]. However, Babauta et al. have found that the 2-electron pathway is predominant on river-water and *Leptothrix discophora* SP-6 cathodic biofilms with the aid of microelectrode method [23], opposed to the previously accepted 4-electron pathway. Due to quite limited reports on kinetics analysis of ORR on microbial biocathodes, it is difficult to determine whether the 2-electron pathway is universal or pathways are species dependent. Consequently, herein kinetics analysis is achieved via the utilization of a rotating ring-disk

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electrode (RRDE), and it is found that oxygen is reduced to H_2O with the catalysis of *Thalassospira* sp.

2. Experimental

2.1. Microorganism cultivation and inoculation

Thalassospira sp. was isolated from Q235 carbon steel rust layers immersed in natural seawater (Haikou, China), and identified by the comparison of 16S rDNA sequences to those reference strains held in GenBank database. The culture medium for *Thalassospira* sp. was prepared by addition of 0.20 g $CaCl_2$, 0.50 g $MgSO_4$, 0.50 g $(NH_4)_2SO_4$, 0.50 g K_2HPO_4, 0.50 g $NaNO_3$, and 10.00 g ammonium ferric citrate into 1 L natural seawater, and pH was adjusted to 6.8. Conical flasks inoculated with *Thalassospira* sp. were kept in a rotary shaker with a rate of 100 rpm at 28 °C. When the color of suspension turned yellow without obvious rufous precipitates, *Thalassospira* sp. seed culture was collected, centrifuged, condensed, and washed with 10 mM phosphate-buffered saline (PBS, pH 7.4). PBS consisted of 8.00 g NaCl, 0.20 g KCl, 3.63 g $Na_2HPO_4 \cdot 12H_2O$, and 0.24 g KH_2PO_4 per liter of distilled water. The optical density at 600 nm of thus-obtained seed suspension was measured to get the bacterial concentration on the basis of a calibration curve, and the concentration was adjusted to 1.0×10^7 cfu mL^{-1}.

2.2. Electrochemical measurements

Electrochemical measurements were performed on a CHI920C station with a three-electrode system, in which Ag/AgCl (KCl-saturated) and Pt wire were used as reference and counter electrodes, respectively. Glassy carbon (GC) electrodes with a diameter of 3 mm were utilized in cyclic voltammetry characterizations. For ORR kinetics analysis, a RRDE with GC disk (diameter: 5.61 mm) and Pt ring (outer diameter: 7.92 mm, inner diameter: 6.25 mm) was adopted. All electrodes were sterilized via the exposure to ultraviolet light or immersion in 75% ethanol solution for 30 min before their introduction into measurement systems. 4 mL thus-prepared seed suspension was added to a beaker with air-saturated 36 mL PBS, and the initial bacterial concentration was calculated to be 1.0×10^6 cfu mL^{-1}. The electrolyte was stirred and kept at the temperature of 28 °C, and there was no stirring during electrochemical data recording.

Several control experiments were designed to reveal the catalysis mechanism. Cyclic voltammograms (CVs) were recorded under anaerobic conditions achieved via nitrogen bubbling for 20 min to verify the occurrence of ORR in the presence of oxygen. After exposure to PBS containing *Thalassospira* sp. for 96 h, the GC electrode was taken out and washed with PBS carefully, and its activity towards ORR was evaluated in air-saturated fresh PBS to check the role of attached biofilm. And then, the GC electrode with biofilm was immersed into PBS with 2.5% glutaraldehyde for 2 h to kill the bacteria cells, and the cyclic voltammetry characterization was carried out to assess the functions of cells and adhesive compounds. In the meanwhile, filtrate was obtained by filtering the suspension at 96 h with a 0.22 μm sterile filter, and its influence on ORR was investigated by cyclic voltammetry. Besides, 4 mL seed culture was treated with ethanol, and the dead cells were introduced into PBS to ascertain whether cell activity is necessary for ORR catalysis.

Before linear sweep voltammograms were recorded on a RRDE, pretreatment was employed on the Pt ring electrode by repetitive potential scanning until characteristic peaks of the clean Pt electrode appeared. And during recording of the voltammograms, the Pt ring electrode was kept at a constant potential of +0.8 V to get full oxidation of H_2O_2 diffused from the disk. All potentials are referred to the Ag/AgCl (KCl-saturated) electrode.

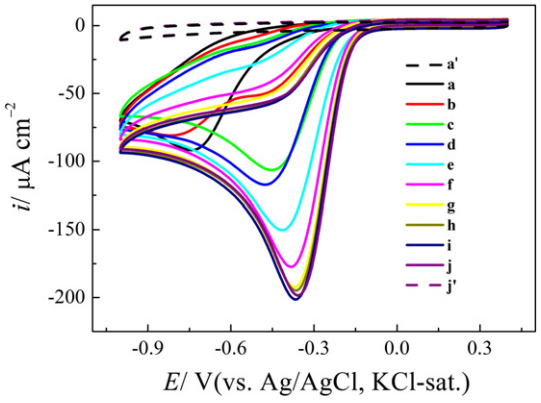

Fig. 1. CVs recorded at a GC electrode exposed to air- (a to j) and N_2-saturated (a′ and j′) PBS containing *Thalassospira* sp. for different time (a and a′: 0, b: 3, c: 17, d: 24, e: 48, f: 72, g: 96, h: 120, i: 144, j and j′: 168 h). Scan rate: 50 mV s^{-1}.

2.3. Scanning electron microscopy (SEM) characterization

SEM was applied to observe the surface morphology of GC plates after different time of exposure to *Thalassospira* sp. suspension. After the coupons were taken out from the medium and rinsed with PBS, they were immersed in PBS containing 2.5% glutaraldehyde for 2 h, and dehydrated successively with an ethanol gradient (15 min each): 30%, 50%, 70%, 90%, and 100%. Samples were then critical point dried, gold sputtered, and subjected to SEM analysis.

3. Results and discussion

3.1. ORR catalysis defined by CVs

CVs were recorded at different time points after a GC electrode with fresh surface was exposed to PBS containing *Thalassospira* sp., and the results are shown in Fig. 1. At the beginning, a cathodic peak appears around the potential of −0.75 V in air-saturated medium (curve a), and in comparison with the plot under N_2-saturated condition where peaks are absent (curve a′), this peak is ascribed to the reduction of oxygen. Due to the quite short time, few bacterial cells or metabolic compounds are attached to the electrode surface, and therefore the peak potential is close to that recorded in PBS blank [11,14]. After 3 h of immersion, there is a slight decrease in current density of the peak around −0.75 V, and a new peak is present at potentials more positive (ca. –0.45 V, curve b). The presence of two peaks demonstrates that the electrode surface is heterogeneous, and at least two kinds of sites with different ORR catalytic activity are involved. The more active sites ought to be closely associated with the adhesion of bacterial cells or metabolites. When the immersion time extends to 17 h, the previous peak at ca. –0.75 V vanishes leaving only one peak with more positive potentials (curve c), and the less active sites may be covered by bacterial cells or their metabolites. The ORR peak current density increases gradually from the time of 24 h to 96 h, and the peak potential shifts positively in the meanwhile (curves d–g), which might be related to the accumulation of adhesive bacterial cells or metabolites. Compared to the CV at 0 h, the peak current density for the plot at 96 h is two times larger and the peak potential shifts around 0.40 V in the positive direction, indicating good catalytic performance of *Thalassospira* sp. towards ORR. Further prolongation of the exposure time has limited influence on ORR activity, and the peak current density and potential are close in the range of 96 h to 168 h (curves g–j). The emergence of a steady state may be due to the full occupation of bonding sites by bacterial cells and their metabolites, or the balance between attachment and detachment of cells and metabolites. The electrochemical activity of *Thalassospira* sp. has also been observed by Rowe et al., which was capable of electrode oxidation as a surrogate for lithotrophic sulfur