Oxygen Reduction Reaction Affected by Sulfate-Reducing Bacteria: Different Roles of Bacterial Cells and Metabolites

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Abstract Sulfate-reducing bacteria (SRB) were found to be capable of tolerating a certain amount of oxygen (O_2), but how they affect oxygen reduction reaction (ORR) has not been clear. The present work investigated the impact of SRB on ORR in 3.5 wt% sodium chloride solution with the cyclic voltammetry method. The addition of SRB culture solution hampered both the reduction of O_2 to superoxide (O_2^-) and hydrogen peroxide (H_2O_2) to water (H_2O), and the influence of SRB metabolites was much larger than that of bacterial cells. Sulfide and extracellular polymeric substances (EPS), typical inorganic and organic metabolic products, had great impact on ORR. Sulfide played an important role in the decrease of cathodic current for H_2O_2 reduction due to its hydrolysis and chemical reaction activity with H_2O_2 . EPS were sticky, easy to adsorb on the electrode surface and abundant in functional groups, which hindered the transformation of O_2 into O_2^- and favored the reduction of H_2O_2 to H_2O .

Keywords Sulfate-reducing bacteria · Oxygen reduction reaction · Sulfide · Extracellular polymeric substances · Cyclic voltammetry

Introduction

Microbial biocathodes for oxygen reduction reaction (ORR), commonly utilized in microbial fuel cells, take advantages of cost efficiency, sustainability, and resistance to poisoning in comparison with conventional chemical catalysts [1]. Both mixed microbial communities and pure cultures have been reported to catalyze ORR, and the former holding broad bacterial diversity has been derived from seawater [2], freshwater [3], and wastewater treatment sludge [4]. Although mixed microbial communities displayed robust catalysis efficiency towards ORR, their complex multispecies nature brought great difficulty for mechanism investigation, which stimulated the research of ORR catalyzed by pure cultures. So far, the catalytic activity of ORR has been defined in numerous bacterial strains, such as *Roseobacter* sp. [5], *Pseudoalteromonas* sp. [6], *Acinetobacter* sp. [7], *Yersinia enterocolitica* [8], and *Thalassospira* sp. [9]. These reported strains were either aerobic or facultative bacteria, and it is unknown whether ORR could be influenced by anaerobes.

Sulfate-reducing bacteria (SRB) are a diverse group of prokaryotes gaining energy via the dissimilatory sulfate reduction, and a complete absence of oxygen (O_2) has for long been viewed to be vital for SRB. Recently, some SRB strains were reported to be able to tolerate a certain amount of O_2 [10, 11], motivating researchers to study possible O_2 defense strategies. These adaptation strategies could be divided into two groups: behavioral strategies and molecular strategies [12]. The former mainly referred to the formation of cell aggregates while the latter allowed SRB to generate relevant enzymes to consume O_2 or eliminate reactive oxygen species [13, 14]. However, these reports were concentrated on the impact of O_2 on SRB, and conversely how SRB affected ORR was neglected.

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ORR is sensitive to the characteristics of electrode surface and electrolyte [15, 16]. The attachment of bacterial cells and adsorption of metabolites would impact the electrode surface condition, and the accumulation of metabolites in surroundings led to changes in the electrolyte, which were expected to affect ORR. Consequently, the impacts of SRB and their typical metabolites on ORR were concerned in the present study. For the influence of metabolites, sulfide and extracellular polymeric substances (EPS) were involved, which were believed to play quite an important role in the alterations of electrode surface and electrolyte characteristics.

Materials and Methods

Chemicals and Solutions

All chemicals purchased from Sinopharm Chemical Reagent Co. Ltd, China, were of analytical grade and utilized without further purification. Sodium chloride (NaCl) and sodium sulfide nonahydrate ($\text{Na}_2\text{S}\cdot 9\text{H}_2\text{O}$) were used for the preparation of 3.5 wt% NaCl electrolyte and solutions containing sulfide with different concentrations. The concentration of sulfide was determined by iodometric titration, in which sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3$), iodine (I_2), potassium iodide (KI), hydrochloric acid (HCl), and starch were utilized. A series of reagents, including calcium chloride (CaCl_2), sodium sulfate (Na_2SO_4), dipotassium hydrogen phosphate (K_2HPO_4), ammonium chloride (NH_4Cl), yeast extract, magnesium sulfate (MgSO_4), and sodium lactate ($\text{C}_3\text{H}_5\text{NaO}_3$), were applied to prepare the modified Postgate's culture medium. Ultra-high purity nitrogen (N_2) and O_2 for the preparation of N_2 - and O_2 -saturated solutions were provided by Qingdao Heli Gas Co. Ltd, China.

Cultivation, Phylogenetic Analysis, and EPS Extraction of SRB

The SRB strain was isolated from the marine sludge of Huiquan Bay, Bohai Sea, China via the enrichment and isolation procedures similar to those reported by Duan et al. [17]. A modified Postgate's medium was utilized for the cultivation of SRB, with the composition per liter of natural seawater: 0.1 g CaCl_2 , 0.5 g Na_2SO_4 , 0.5 g K_2HPO_4 , 1.0 g NH_4Cl , 1.0 g yeast extract, 2.0 g MgSO_4 , and 4 mL $\text{C}_3\text{H}_5\text{NaO}_3$.

DNA was extracted with the TIANamp Bacteria DNA kit (Tiangen, Beijing, China), and the 16S rRNA gene sequences were amplified with the universal primer set 27F/1492R (27F: AGAGTTTGATCCTGGCTCAG, 1492R: CTACGGCTACCTTGTACGA). Sequencing

was carried out by Sunnybio (Shanghai, China). Sequences were submitted to the GenBank database, and compared with those of reference strains held in the database by BLAST search. The closest 16S rRNA gene sequences were retrieved from GenBank, and the finalized alignment by ClustalW was analyzed by the MEGA program to construct the phylogenetic tree.

After kept in a thermostatic incubator at 30 °C for 4 d, a measured amount of SRB culture solution was added into 3.5 wt% NaCl electrolyte for the influence of SRB and their metabolites on ORR. When the role of SRB was focused, the bacterial cells separated from bulk solution by centrifugation (6000 rpm, 20 min) were rinsed three times with phosphate buffer solution and introduced into the electrolyte. The supernatant was then used for the effect of metabolites on ORR. EPS were extracted from culture solution according to the method reported by Chan et al. [18], consisting of centrifugation, dialysis, and lyophilization. And the final dried product yielded 330 mg L^{-1} of SRB culture.

Electrochemical Measurements

Cyclic voltammetry experiments were performed on a CHI760C station (CH Instruments, Inc.) in N_2 - and O_2 -saturated 3.5 wt% NaCl electrolyte with different concentrations of SRB culture solution, SRB bacterial cells, SRB metabolites, sulfide and EPS. A conventional three-electrode system was adopted, in which a glassy carbon (GC) electrode, a platinum wire, and an Ag/AgCl (KCl-saturated) electrode were used as working, counter, and reference electrodes, respectively. Prior to experiments, the GC electrodes were polished with 1.00 and 0.05 μm alumina slurries, and cleaned with Milli-Q water in an ultrasonic bath for 10 min. All potentials were reported versus the Ag/AgCl (KCl-saturated) electrode and all experiments were carried out at room temperature.

Results and Discussion

Phylogenetic Analysis of SRB

The 16S rRNA gene sequences of the present SRB strain were available in the GenBank database with the accession number of MF461625, and 27 reference strains were found to display high sequence similarity (97–99%), which were utilized to construct the phylogenetic dendrogram shown in Fig. 1. Among these 27 strain, 15 exhibited the similarity of 99% with the accession numbers of AB546251.1, AB546252.1, AB546253.1, CP003220.1, EU137840.1, HE797785.1, KJ576624.1, KJ576625.1, KJ576626.1, KJ576627.1, KJ576632.1, KJ576634.1, KJ576635.1, KM494507.1, and

Fig. 1 Phylogenetic 16S rRNA tree reflecting the relationship of the present SRB strain with reference microorganisms

KU892724.1, 6 showed the similarity of 98% (HQ878123.1, KP682306.1, KT750867.2, NR025078.1, U53465.1, and U85475.1), and the others gave 97% similarity. Figure 1 demonstrates that the target SRB strain is a member of the *Desulfovibrio* group, and is closely related to *Desulfovibrio dechloracetivorans* SRB1 (99% similarity).

Effect of SRB and Their Metabolites on ORR

Cyclic voltammograms (CVs) for ORR obtained at a GC electrode in N_2 - and O_2 -saturated 3.5 wt% NaCl electrolyte with different concentrations of SRB culture solution are shown in Fig. 2A. For N_2 -saturated solutions, hydrogen evolution reaction (HER) occurred at potentials more negative than -1.20 V and the introduction of SRB culture solution increased the HER current slightly. While in O_2 -saturated 3.5 wt% NaCl electrolyte, three cathodic peaks were present at potentials more positive than those for HER, corresponding to three steps of ORR. The first two peaks at ca. -0.32 and -0.71 V were both attributed to the 2-electron electrochemical reduction of O_2 to hydrogen peroxide (H_2O_2), in which the former involved the transformation of superoxide anion ($O_2^{\cdot-}$) with the catalysis of

active surface quinone-like groups (reactions I and II) and the latter was a direct reduction (reaction III) at the GC surface [19]. Subsequently, H_2O_2 was reduced to water (H_2O) at the third stage (reaction IV). The addition of SRB culture solution in 3.5 wt% NaCl electrolyte brought a strong impact on ORR processes (curves b and c), resulting in an obvious decrease of the current at the first and third steps. When the content reached 3% (in volume, curve d), these two peaks vanished. To find out the origin of these changes, the influence of SRB bacterial cells and their metabolites was investigated separately.

As shown in Fig. 2B, C, bacterial cells and metabolites gave a similar effect trend to that of the culture solution, but differed in degree. According to the growth curve of SRB in the modified Postgate's culture medium [20], the bacterial number on the 4th day was ca. 1.0×10^6 colony-

Fig. 2 CVs recorded on a GC electrode in different media. **A** CVs in N_2 - (a' and b') and O_2 -saturated (a – d) 3.5 wt% NaCl containing SRB culture solution with different contents (a and a' : 0; b : 0.6%; c : 1.0%; d and b' : 3.0%). **B** CVs in O_2 -saturated 3.5 wt% NaCl containing different numbers of SRB bacterial cells (a : 0; b : 6.0×10^3 ; c : 6.0×10^4 ; d : 2.0×10^5 cfu mL $^{-1}$). **C** CVs in O_2 -saturated 3.5 wt% NaCl containing SRB metabolites with different concentrations (a : 0; b : 0.3%; c : 6.0%; d : 10.0%). **D** CVs in N_2 - (a' and b') and O_2 -

saturated (a – d) 3.5 wt% NaCl with different contents of sulfide (a and a' : 0; b : 0.43; c : 0.87; d and b' : 4.34 mM). **E** CVs in N_2 -saturated 3.5 wt% NaCl containing 1 mM H_2O_2 and different concentrations of sulfide (a : 0; b : 0.08; c : 0.38; d : 0.75 mM). **F** CVs in N_2 - (a' and b') and O_2 -saturated (a – d) 3.5 wt% NaCl electrolyte with different contents of polysaccharides (a and a' : 0; b : 1.61; c : 4.02; d and b' : 4.82 g L $^{-1}$). Scan rate: 100 mV s $^{-1}$

forming units per milliliter (cfu mL⁻¹). Therefore, when the bacterial count in 3.5 wt% NaCl electrolyte was 6.0×10^4 cfu mL⁻¹, SRB culture solution with a volume ratio of 6% to NaCl ought to be harvested, centrifugated, rinsed, and introduced. Even the population of SRB increased to 2.0×10^5 cfu mL⁻¹ in the electrolyte (curve d, Fig. 2B), current density of the first cathodic peak was close to 0.4 mA cm^{-2} . However, the first cathodic peak disappeared in 3.5 wt% NaCl electrolyte with 6% (in volume) of SRB metabolites. Consequently, the impact of metabolites on ORR was much larger than that of bacterial cells, which motivated us to understand the role of typical metabolic products separately.

Effect of Sulfide on ORR

Figure 2D exhibits the impact of sulfide on CVs in N₂- and O₂-saturated 3.5 wt% NaCl solution. The hydrolysis of sulfide led to an increase in pH, and the pH values for the solutions with 0, 0.43, 0.87, and 4.34 mM sulfide were 6.69, 10.87, 11.31, and 12.22, respectively. However, there was no obvious difference in the HER current between the solution free of sulfide and that containing 4.34 mM sulfide (curves a' and b', Fig. 2D), although HER was pH-dependent. Similarly, the current at peaks around -0.32 and -0.71 V changed slightly with the introduction of sulfide, suggesting that sulfide had little influence on the electrochemical reduction of O₂ into O₂⁻ and H₂O₂. The indirect and direct electrochemical reduction of O₂ to H₂O₂ was dependent on electrolyte pH (reactions II and III), and such slight variations demonstrated that they were not sensitive in the pH range of 6.69–12.22. In the meanwhile, the reaction of sulfide with O₂ was favorable thermodynamically, which seemed to be contradictory to our results. Actually, the reaction of sulfide with O₂ could be divided into two stages, induction and acceleratory periods, and sulfide scarcely reacted with O₂ in the former step [21, 22]. Under the present condition, the induction period lasted for several hours, which was far longer than the time required for electrochemical experiments. As a result, there was no significant decrease in the cathodic current of the first two peaks with the addition of sulfide.

At potentials around -1.46 V, the introduction of sulfide resulted in a sharp decrease of current. The impact of sulfide on cathodic reduction of H₂O₂ was further investigated and the result is illustrated in Fig. 2E. As shown, in N₂-saturated 3.5 wt% NaCl solution containing 1 mM H₂O₂, the current of cathodic peak at around -1.46 V was decreased remarkably by the introduction of sulfide, and the influence of sulfide on H₂O₂ reduction was verified. Two possible factors were responsible for that current decrease: negative shift of the potential for H₂O₂ reduction from the increase in pH and the decrease in H₂O₂

concentration from the chemical reaction of sulfide with H₂O₂. It was normal to obtain two cathodic peaks for the indirect and direct reduction of O₂ to H₂O₂ on CVs in alkaline media, and the further reduction of H₂O₂ occurred at more negative potentials than that of HER [19, 23]. Meanwhile, sulfide was expected to react with H₂O₂ quickly due to the facile reaction kinetics [24, 25].

It can be concluded that sulfide resulted in the decrease or even disappearance of cathodic current for H₂O₂ reduction, which was closely associated with the electrolyte pH increase and chemical reaction between sulfide and H₂O₂. Since H₂O₂ was detrimental to SRB, the function of sulfide was also favorable to the survival of SRB in aerobic environments.

Effect of EPS on ORR

It has been reported in our previous study that EPS isolated from the SRB strain were mainly composed of polysaccharides with plenty of hydroxyl and carboxyl groups [26], and their impact on CVs is shown in Fig. 2F. It can be seen that the influence of EPS was quite different from that of sulfide. EPS affected both the reduction of O₂ to H₂O₂ and H₂O₂ produced to H₂O. The current of the first reductive peak at ca. -0.32 V decreased with the introduction of EPS, and finally vanished when the concentration was more than 4.02 g L^{-1} , which was relevant to the viscosity and adsorption of EPS [27]. The adsorption of EPS on the GC electrode covered surface active quinone-like groups, and consequently hampered the reduction of O₂ to O₂⁻. In comparison with the first peak, the current of the second one increased slightly, resulting from less sensitivity of active sites for the direct 2-electron reduction of O₂ on GC surface to EPS. For the third step, the peak current was increased greatly and the peak potential was shifted in the positive direction by the addition of EPS, demonstrating good catalytic performance of EPS towards H₂O₂ reduction. This behavior was relevant to the abundance in functional groups of EPS [28, 29].

To recap briefly, EPS had a great effect on the first and third steps of ORR, and their adsorption on GC surface hindered the transformation of O₂ into O₂⁻ but favored the reduction of H₂O₂ to H₂O.

Conclusions

The present work demonstrated that SRB culture solution had a great impact on ORR, and the influence of SRB metabolites was much larger than that of bacterial cells. The impact of typical inorganic and organic metabolic products of SRB (sulfide and EPS) was focused. Sulfide heavily affected the third reduction step of ORR, due to its

hydrolysis and chemical reaction activity with H_2O_2 . EPS not only hampered the reduction of O_2 to O_2^- , but catalyzed the reduction of H_2O_2 to H_2O . The role of other metabolites of SRB in ORR and the cooperative action of these products will be concerned in the future, which is significant for deeper comprehension on the relations between O_2 and SRB.

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