Desalination and Water Treatment
www.deswater.com

doi: 10.5004/dwt.2019.23750

150 (2019) 367–373
May

Birnessite modified graphite cathode toward efficient autotrophic denitrification of *Thiobacillus denitrificans* in bioelectrochemical system

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Received 24 August 2018; Accepted 2 January 2019

ABSTRACT

Biological autotrophic denitrification by *Thiobacillus denitrificans* was investigated in double-chamber bioelectrochemical systems (BESs) and birnessite prepared by sol-gel method was used to modify graphite cathodes and investigate its effect on nitrate removal. The results showed that *T. denitrificans* could utilize the birnessite-modified graphite cathodes as the direct electron donors for autotrophic denitrification and the nitrate reduction rate increased from 65.70 ± 5.03 to 114.17 ± 5.47 mmol NO_3^--N L^{-1} m^{-2} d^{-1}. Ammonia nitrogen was not detected in the reduction products of nitrate, suggesting that dissimilatory nitrate reduction to ammonium (DNRA) did not occur in the experiment. Surface topography of the working electrode characterized by scanning electron microscopy (SEM) indicated that the electrode was colonized by biofilms of *T. denitrificans* which played an important role in facilitating electron transfer for nitrate reduction. Cyclic voltammetry (CV) tests suggested that the birnessite-modified graphite cathodes enhanced the electrochemical activity of *T. denitrificans* biofilms and effectively accelerated the nitrate reduction rate.

Keywords: Autotrophic denitrification; Bioelectrochemical systems; Birnessite; Graphite cathode; *Thiobacillus denitrificans*

1. Introduction

Nitrate pollution has become increasingly serious in natural waters which may cause serious human health damage and environmental hazards [1]. In view of the problem, many countries promulgated the standards of the nitrate concentration in drinking water. The guideline value for nitrate proposed by the World Health Organization (WHO) is 11.3 mg NO_3^--N L^{-1} in drinking water. China government sets the maximum contaminant level (MCL) of 10 mg NO_3^--N L^{-1} in drinking water, same as the United State Environmental Protection Agency (EPA).

Compared with traditional biological denitrification technology, bioelectrochemical system (BES) including microbial fuel cell (MFC) and microbial electrolysis cell (MEC) is considered to be a novel and promising approach to treat nitrate-contaminated water. So far, most studies of BES focused on the application of MFC on nitrate removal. The denitrification process in MFC is achieved with organics as electron donors in the anode chamber and nitrate as electron acceptors in the cathode chamber. However, for wastewater with a low C/N ratio, external organic carbon is generally added as the nutrients for microorganisms, leading to cost and secondary pollution. In terms of this issue, MEC may be a better choice in which microorganisms could utilize inorganic carbon sources and reduce nitrate with electrodes as the electron donors in the cathode chamber provided by a certain potential or current [2]. Gregory et al. [3] first reported that electrodes could be used as direct electron donors for biological denitrification, and the nitrate reduction rate reached 90 mmol NO_3^--N $L^{-1}d^{-1}m^{-2}$.

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After that, Park et al. [4] demonstrated that nitrate could be biologically reduced with nitrate-reducing microorganism enriched from anaerobic sludge, and nitrate could be reduced at 0.17 mg NO_3-N/(cm^2 (biofilm surface area) day). Su et al. [5] found that *Pseudomonas alcaliphila* could directly accept electrons from a carbon-based electrode and degrade nitrate by denitrification and dissimilatory nitrate reduction to ammonium (DNRA), the degradation rate reached up to 160 mmol NO_3^--N $L^{-1}d^{-1}m^{-2}$. *Thiobacillu denitrificans* showed the capability of absorbing electrons from electrodes to reduce nitrate, and nitrate was removed at the rate of 22.79 mmol NO_3^--N $L^{-1}d^{-1}m^{-2}$ [6]. However, research on the autotrophic nitrate removal by MEC with an electrode as a sole electron donor is still at an early stage, the optimization of the BES reactors and operating parameters to improve the nitrate removal efficiency is necessary.

Cathode surface modification has been a promising and feasible approach to improve the operating efficiency of BES with enhancement of biofilm development and electron transfer rate on the cathode surface. Pons et al. [7] found that the anaerobic respiration efficiency of *Geobacter metallreducens* was significantly improved by increasing the surface roughness of the cathode and the maximum of cathode current density was 1.6 times higher than that of the unmodified cathode. Nie et al. [8] developed a novel graphite electrode modified with porous nickel nanowires which efficiently increased the interfacial area and interfacial interactions between the cathode surface and the microbial biofilm and thus a 130% increase of bio-reduction rate of carbon dioxide was obtained. Also, Safari et al. [9] demonstrated that nitrate reduction efficiency in BES was increased after modifying carbon felt, carbon cloth and graphite with multi-wall carbon nanotubes.

Manganese oxides are one kind of the efficient cathodic catalysts in BES, widely used in MFC to catalyze oxygen reduction at the cathode. Liu et al. [10] found that a nano-structured MnO_x, prepared by electrodeposition, could effectively catalyze oxygen reduction and accelerate electricity production and organic removal in MFC. Zhang et al. [11] prepared α-MnO_2, β-MnO_2 and γ-MnO_2 via hydrothermal method and found that these three manganese dioxides presented good catalytic performances in oxygen reduction and β-MnO_2 was the most effective catalyst due to the highest BET surface area and average oxidation state, suggesting that the catalyst activity of MnO_x was dependent on its morphology and structures. Among manganese compounds, birnessite is a major mineral phase in soils which is mainly composed of mixed-valence manganese oxides of Mn(III) and Mn(IV) [12,13]. Birnessite presents a special layered structure that consists of the edge-shared MnO_6 octahedra, with hydrated cations in the interlayer [14]. Birnessite has the properties of large specific surface area, low isoelectric point, strong oxidizing ability, high cation exchange capacity and rich pore structure, making it present excellent performance in conductivity, magnetism, ion exchange, selective adsorption and catalysis. In addition, birnessite is easily prepared, low-cost and non-toxic to the environment.

In this paper, we modified the graphite cathode with birnessite and investigated the denitrification performance of *T. denitrificans* biofilms with the cathodes as direct electron donors in MEC of BES reactors. Electrochemical activities of *T. denitrificans* biofilms on birnessite-modified cathodes were characterized by cyclic voltammetry (CV), scanning electron microscopy (SEM) and electrochemical impedance spectroscopy (EIS) and the mechanism of enhancing autotrophic denitrification by birnessite in BES was explored.

2. Materials and methods

2.1. Preparation of birnessite-modified graphite electrodes

Birnessite was prepared by sol-gel method with $KMnO_4$ and glucose as previously described [15]. 18 mg birnessite was suspended in the mixture of 100 μL 5% Nafion and 900 μL absolute ethyl alcohol. Then the mixture was uniformly casted on one side of a graphite electrode with a load of 2 mg cm^{-2}. The other side of the graphite was coated with birnessite with the same procedure. The nafion-modified graphite electrodes coated with the dispersion liquid without birnessite were prepared in the same way.

2.2. Microorganism and cultivation

T. denitrificans (ATCC25259, a gift from Dalian University of Technology) was anaerobically cultured in the sterile PS medium at 30°C [16]. The PS medium contained the following (per liter of deionized water): 0.535 g NH_4Cl, 0.136 g KH_2PO_4, 0.0475 g $MgCl_2$, 0.420 g $NaHCO_3$, 0.0555 g $CaCl_2$, 1.7 g $NaNO_3$, 4.96 g $Na_2S_2O_3{\cdot}5H_2O$, 2.38 g Hepes, 0.5 g yeast extract, 10 mL vitamin and 10 mL mineral solution.

2.3. Bioelectrochemical system setup and operation

The experiments were conducted in double-chamber bioelectrochemical reactors. Each chamber has an effective volume of 120 mL, physically separated by a cation exchange membrane (CMI7000, MI, USA). Graphite (3 × 3 cm) was used as the cathode with the effective attachment area for microorganisms of 18 cm^2. Carbon felt (3 × 3 cm) and saturated calomel electrode (SCE) served as the anode and reference electrode, respectively. The cation exchange membranes and reference electrodes were sterilized with 75% ethanol (v/v) and ultraviolet light and other BES materials were autoclaved at 121°C for 30 min prior to use. If not otherwise stated, all electrode potentials in this study were provided vs. standard hydrogen electrode (SHE).

The bacterial culture of *T. denitrificans* at the logarithmic phase was collected and centrifuged at 8,000 rpm for 5 min. The bacterial pellets were then transferred to seed the cathode chamber containing PS medium with the addition of 2 mM NO_3^--N but $Na_2S_2O_3$ and NH_4Cl were removed. The BES reactors were connected to a multi-channel potentiostat (CHI1000C, Chenhua Co., Ltd., Shanghai, China), and maintained at 30°C for the acclimation of *T. denitrificans* biofilms for approximately 30 d. After the acclimation, the catholyte was completely replaced with fresh PS medium, and the experiments for nitrate degradation at the cathode potential of –500 mV were performed with the anolyte of 0.1 M potassium phosphate buffer solution (PBS, pH 7.0). Abiotic control experiments and open circuit potential (OCP) tests were also conducted for comparison.

2.4. Analytical methods

Small aliquots of catholyte were taken regularly to analyze nitrate, nitrite and ammonium using UV Spectrophotometry, N-(1-naphthyl)ethylenediamine dihydrochloride spectrophotometry and Nessler's reagent spectrophotometry (L5 spectrophotometer, INESA Analytical Instrument Co., Ltd., Shanghai, China), respectively.

The cathodic current was collected every 200 s by the multi-channel potentiostat. CV and EIS tests were conducted by using an electrochemical working station (CHI 760e, Chenhua). Cathodic electron recovery efficiency (CERE) was calculated as CERE (%) = $\Delta C_{exp}/\Delta C_{theor}\times 100$, where ΔC_{exp} and ΔC_{theor} represent the electrons used for the formation of denitrification products and the electrons absorbed from working electrodes by *T. denitrificans* biofilms. SEM measurements and sample preparations for SEM (JSM-6360LV, JEOL, Japan) have been described previously [17].

3. Results and discussion

3.1. Characterization of birnessite-modified graphite electrodes

The crystalline structure of birnessite was characterized by X-ray diffraction (XRD) and the result is shown in Fig. 1. The spectrum shows the strong peaks at 12.4°, 24.9° and 37.0°, corresponding to the crystal faces of 001, 002 and 110, respectively, based on Joint Committee on Powder Diffraction Standards (JCPDS) card No. 43–1456 (Table 1). The sharp and strong peaks indicate a good crystallinity of birnessite prepared by sol-gel method.

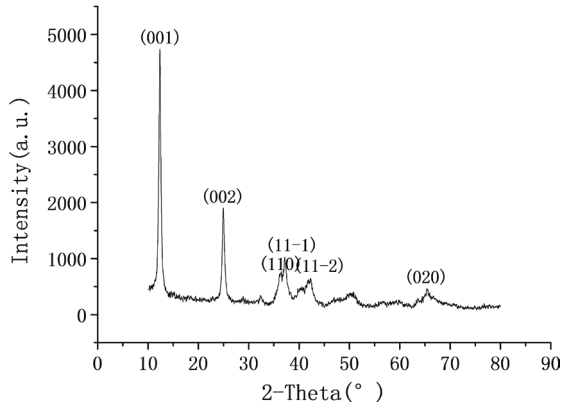

Fig. 1. X-ray diffraction pattern of the prepared birnessite powder.

SEM images show the surface topography of graphite electrodes with different treatments (Fig. 2). Compared with the graphite electrode without modification (Fig. 2(a)) and the nafion-modified one (Fig. 2(b)), the surface of the birnessite-modified electrode appeared rougher because of the formation of birnessite crystal structured layers, providing

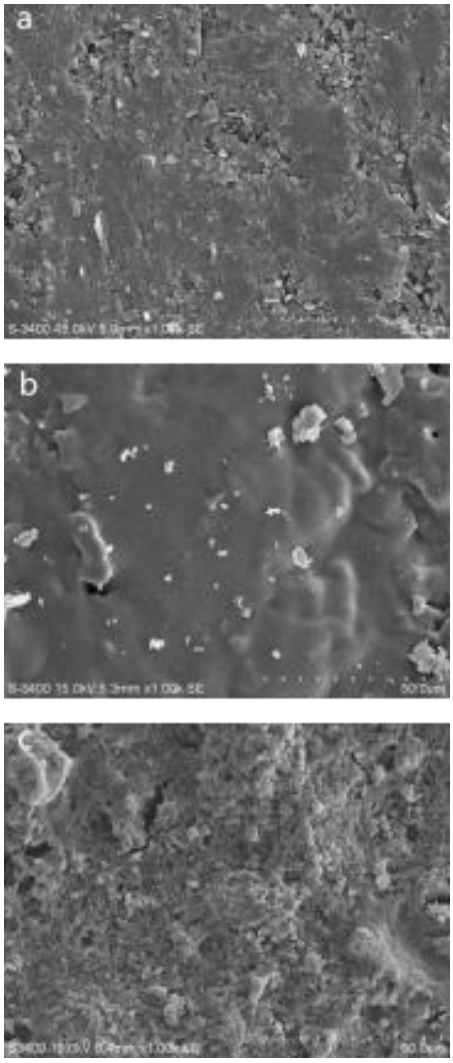

Fig. 2. SEM images of electrode surface (the magnification is 1,000). a - bare graphite electrode, b - nafion-modified graphite electrode, c - birnessite-modified graphite electrode.

Table 1
Comparison of XRD data between the prepared birnessite and JCPDS 43–1456

	XRD data of the prepared birnessite			XRD data of JCPDS 43–1456		
Crystal face	2-Theta (°)	d (A)	I (%)	2-Theta (°)	d (A)	I (%)
001	12.379	7.1446	100	12.380	7.144	100
002	24.957	3.5649	39.3	24.907	3.5720	27.0
110	36.088	2.4868	8.3	36.190	2.4800	6.0
11–1	37.184	2.4160	13.9	36.977	2.4290	13
11–2	42.332	2.1333	6.6	41.906	2.1540	7
020	65.601	1.4219	4.6	65.468	1.4245	3

a larger specific surface contact area for the attachment and growth of *T. denitrificans* biofilms.

3.2. Denitrification performance of *T. denitrificans* biofilms with electrodes as the sole electron donors

Denitrification experiments were performed in the separate BES reactors with the birnessite-modified graphite and the nafion-modified graphite as the cathodes, respectively, which were designated as sBES-bir and sBES-bare. The control abiotic reactors were designated as sAbio-bir and sAbio-bare.

After 30 d of acclimation of *T. denitrificans* biofilms, pronounced cathode currents were observed immediately in sBES-bare and sBES-bir. It was found that both sBES-bare and sBES-bir produced persistent current, and the current from sBES-bir was about two times greater than that from sBES-bare (Fig. 3(a)), while no current was observed in the abiotic BES reactors (sAbio-bare and sAbio-bir), as expected. The accumulated charge consumption by *T. denitrificans* biofilms in the sBES-bir was 46.16 C for 15 d, 50.26% higher than that in sBES-bare (30.72 C) (Fig. 3(b)). Coulombic efficiency analysis revealed that 20.45 C ± 1.43 C and 33.09 C ± 2.45 C electrons were transferred to the denitrification products in the sBES-bare and sBES-bir, and the corresponding electron recovery efficiency were 66.58% ± 3.86% and 71.69% ± 5.31%, respectively. These results indicated that *T. denitrificans* biofilms could consume electrons from the cathodes for autotrophic metabolism, and the modification of electrode with birnessite enhanced the electron absorption capacity of *T. denitrificans* biofilms, most likely due to the improvement of surface roughness. The great increment of surface roughness favored the attachment and growth of *T. denitrificans* on the electrode and thereby increasing the number of electrons consumed. The additional potential reason for enhancement of electron consumption by birnessite is that birnessite may work as a catalyst and accelerate the electron transfer rate from the cathodes to *T. denitrificans* biofilms.

Nitrate was reduced to nitrite with the continuous generation of the cathode current under the potential of –500 mV. Fig. 4 illustrates the concentrations of nitrate and nitrite in medium during a period of 15 d. Results show that 44.34% ± 3.40% and 77.06% ± 3.7% nitrate were removed in sBES-bare and sBES-bir respectively after 15 d (Fig. 4(a)) while the concentrations of nitrite reached up to 0.77 ± 0.064 mM and 1.43 ± 0.092 mM in the corresponding BES, respectively (Fig. 4(b)). The average nitrate reduction rate of sBES-bir was 114.17 ± 5.47 mmol NO_3^--N L^{-1} m^{-2} d^{-1}, which was 73.78% higher than that of sBES-bare (65.70 ± 5.03 mmol NO_3^--N L^{-1} m^{-2} d^{-1}), indicating that birnessite accelerated the nitrate reduction rate. *M*. Safari et al. [9] used multi-walled carbon nanotube to modify the graphite cathode in a bio-electrochemical reactor and the nitrate reduction efficiency was obtained 63.98%, 14.72% higher than that with virgin graphite (49.26%).

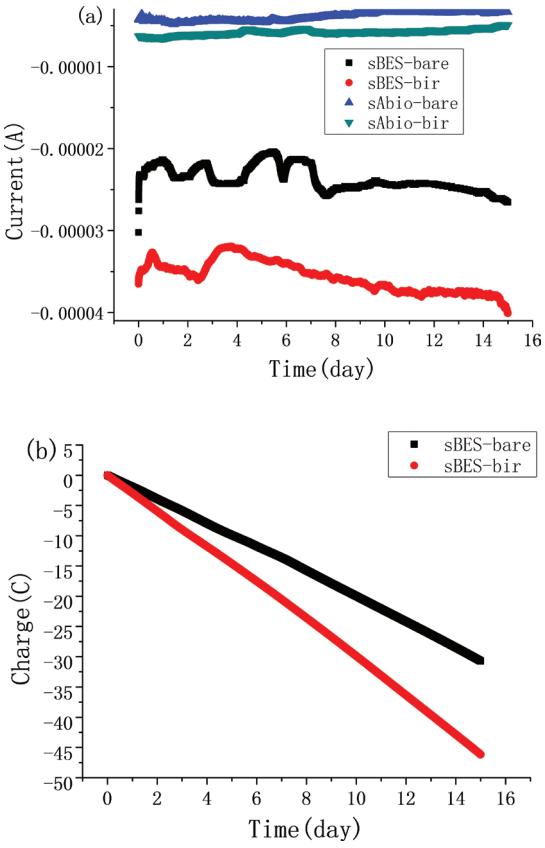

Fig. 3. Current generation by *T. denitrificans* biofilms with bare graphite electrode and birnessite- modified graphite electrode as sole electron donors (a), and the corresponding accumulated electron consumption (b).

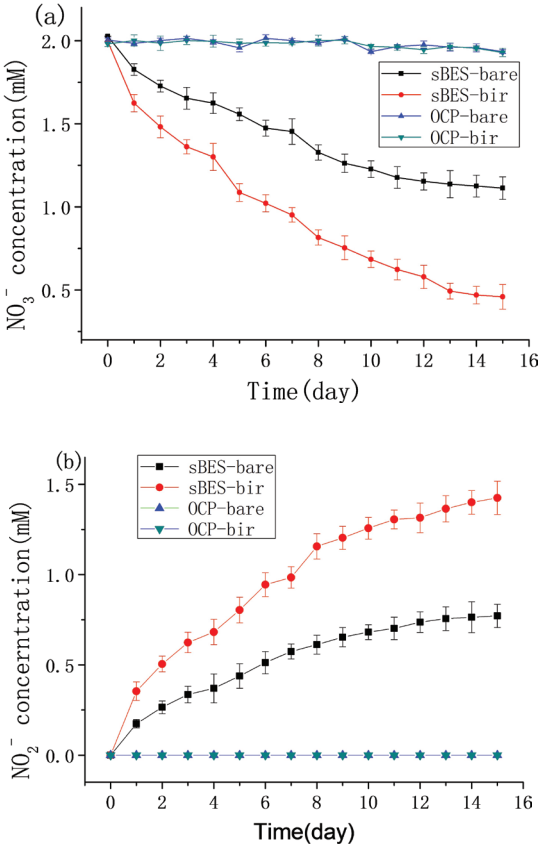

Fig. 4. Reduction of nitrate (a) and accumulation of nitrite (b) in the BES and in OCP control.

By contrast, modification by birnessite increased the nitrate reduction efficiency from 44.34% to 77.06% in our experiment and we achieved a better enhancement of efficiency. The nitrate concentration decreased slightly for the OCP control. Moreover, nitrate was not reduced and no product accumulated in the abiotic reactors (data not shown), suggesting that the graphite cathode could not directly catalyze electrochemical nitrate reduction at the potential of –500 mV. In the control experiments, no nitrite was detected. Since Yu et al. [6] demonstrated that the products of nitrate reduction by *T. denitrificans* with cathodes as the sole electron donors were mainly nitrite with very little N_2O and N_2, and N_2 originally existed in the headspace of the reactors, the gaseous products were not detected in this experiment.

Nitrate degradation has two pathways: denitrification and DNRA. In this study, no ammonia nitrogen was detected during the experiments, ruling out the possibility of the nitrate reduction through DNRA. Therefore, denitrification was the only pathway for the nitrate removal in this experiment. Denitrification process also includes the nitrate reduction and nitrite reduction. The accumulation of nitrite indicates that the rate of nitrate reduction to nitrite was higher than that of nitrite reduction. It is probably because the more negative potential is needed for nitrite reduction [18]. Alternatively, the stronger activity of nitrate reductase than nitrite reductase may also contribute to the accumulation of nitrite.

3.3. Electrochemical activity of *T. denitrificans* biofilms on electrode surface

EIS tests of the cathode were carried out in a frequency range from 100 to 10 mHz to characterize the electrochemical characteristics of *T. denitrificans* biofilms after acclimation. The appropriate equivalent circuit was used to simulate and analyze the internal resistances, potentially existing in the system in this study. The electrode potential losses in a microbial fuel cell include activation losses, ohmic losses and mass transfer losses [19]. The cathodic activation losses in this experiment existed in the two processes: absorption of electrons from the electrode by microorganisms and electron transfer from microorganisms to substrates. Therefore, we proposed the equivalent circuit model on the cathode as (*R* (*Q* (*R* (*QR*))) (*QR*)) which is illustrated in Fig. 5. R_1, R_2, R_3 and R_4 represented the ohmic resistance, the biofilm resistance, the interface resistance between the electrode and the biofilms, and the diffusion resistance, respectively. Q_2, Q_3 and Q_4 represented the biofilm capacitance, the interface capacitance between the electrode and the biofilms, and the diffusion capacitance, respectively.

Fig. 6 illustrates the Nyquist plot of the cathode with the real part Z_{re} and the imaginary part Z_{im} as X-axis and Y-axis, respectively. The diameter of the semicircle over the high-frequency range and the diameter over the low-frequency range correspond to the charge transfer resistance and the diffusion resistance. The intersection of the semicircle with X-axis over the high frequency region represented the ohmic resistance R_1. The diffusion resistance R_4 was neglected in this experiment because of the deviation for diffusion resistance in EIS tests. The chi-squared test parameter of the impedance spectra for sBES-bare and sBES-bir are 2.89×10^{-4} and 9.74×10^{-5}, respectively, suggesting that the EIS results were well fitted. The electrochemical parameters fitted based on the *R* (*Q* (*R* (*Q*))) (*QR*) model are shown in Table 2. Results showed that R_1 and R_2 were one order of

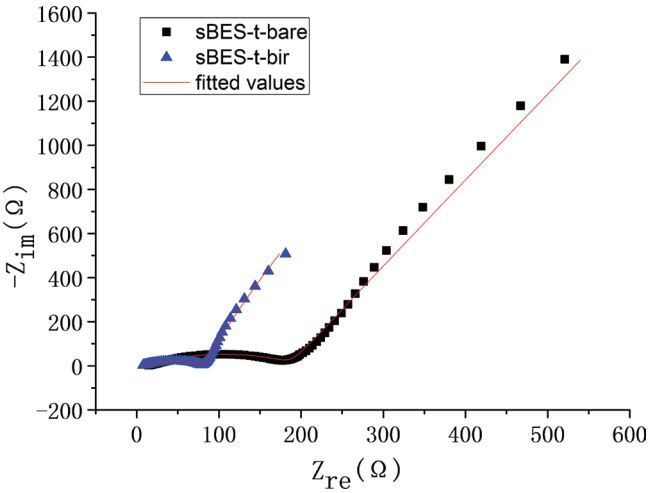

Fig. 6. Electrochemical impedance spectroscopy of BES.

Table 2
Fitting values of BES

BES	R_1 (Ω)	R_2 (Ω)	R_3 (Ω)
sBES-bare	12.13	10.57	164.8
sBES-bir	5.96	7.56	67.25

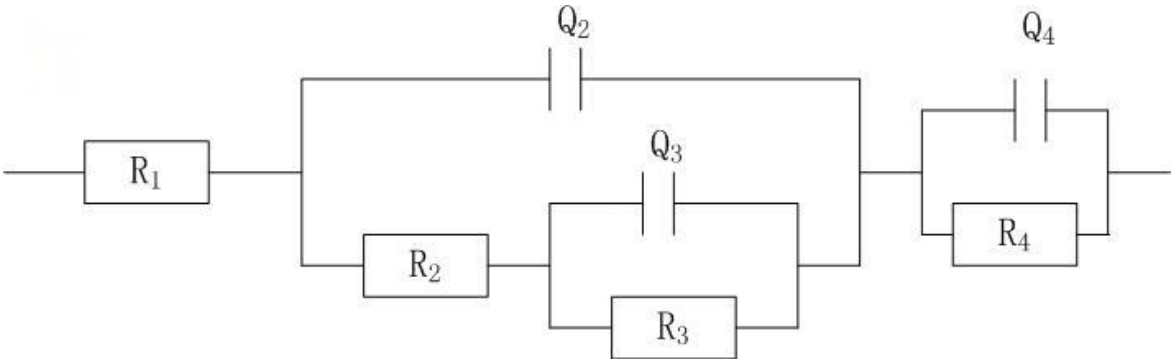

Fig. 5. Equivalent electrical circuit model: R(Q(R(QR)))(QR).

magnitude lower than the value of R_3, suggesting that interface resistance played the important role in electron transfer. The ohmic resistance (R_1) and the biofilm resistance (R_2) of sBES-bir were close to the resistances of sBES-bare, while the interface resistance (R_3) of sBES-bir was 67.25 Ω, 40% of the corresponding resistance in sBES-bare (164.8 Ω), indicating that the modification of birnessite significantly lowered the interfacial resistance between the cathode and the biofilms. This may be due to the fact that birnessite had played a role in assisting electron transfer, thereby reducing the mass transfer resistance.

CV scans were conducted at the end of the acclimation phase to investigate the electrochemical activity of the working electrode catalyzed by *T. denitrificans* biofilms. As shown in Fig. 7, the currents of the bio-cathodes in sBES-bare and sBES-bir at the potential of –500 mV were significantly higher than that of the OCP control. Furthermore, sBES-bir exhibited higher cathodic currents than sBES-bare, suggesting that the presence of birnessite enhanced the electrochemical activity of the *T. denitrificans* biofilms. It was found that a pair of redox peaks appeared in the CV curves of sBES-bare and sBES-bir, showing that at least one redox component participated in the electron transfer process. The reduction peak and the oxidation peak appeared at –640 and –415 mV in sBES-bare and at –581 and –445 mV in sBES-bir, respectively. In contrast, no obvious redox peak was observed for the OCP control.

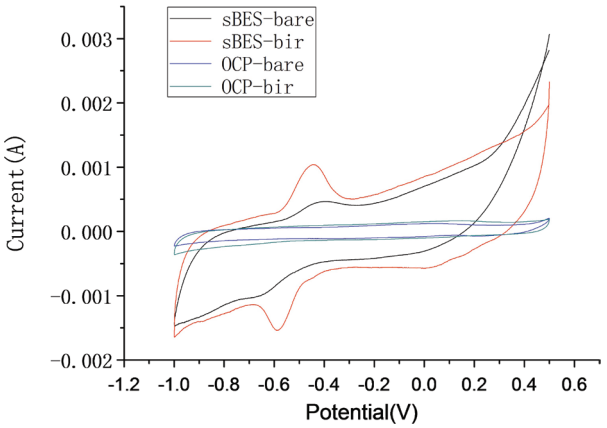

Fig. 7. CVs of the bio-cathodes and the cathodes for the OCP control at the scan rate of 5 mV s^{-1}.

The SEM images of the electrode surfaces revealed that both graphite electrodes were covered with bacteria cells of short rod shape (Fig. 8). Compared with the SEM image of nafion-modified electrode (Fig. 8(a)), the pore structure of birnessite increased the roughness of the electrode surface, which was favorable for the attachment of *T. denitrificans* to the electrodes and provided more active sites for electrochemical reactions, thereby improving the electrochemical performance of the cathode. Unlike, thick biofilms formed by anode electricigens and mixed bacteria, the biofilms in this experiment were sparsely distributed and were thinner. It was mainly because that *T. denitrificans* were acclimated with electrodes as the sole electron donor, while the anode electricigens and mixed bacteria utilize organic matter as electron donors. The electron absorption rate of *T. denitrificans* from the solid electrode may be much lower than the rate of oxidizing organics and absorbing electrons for electricigens, so the growth rate of *T. denitrificans* was relatively slower.

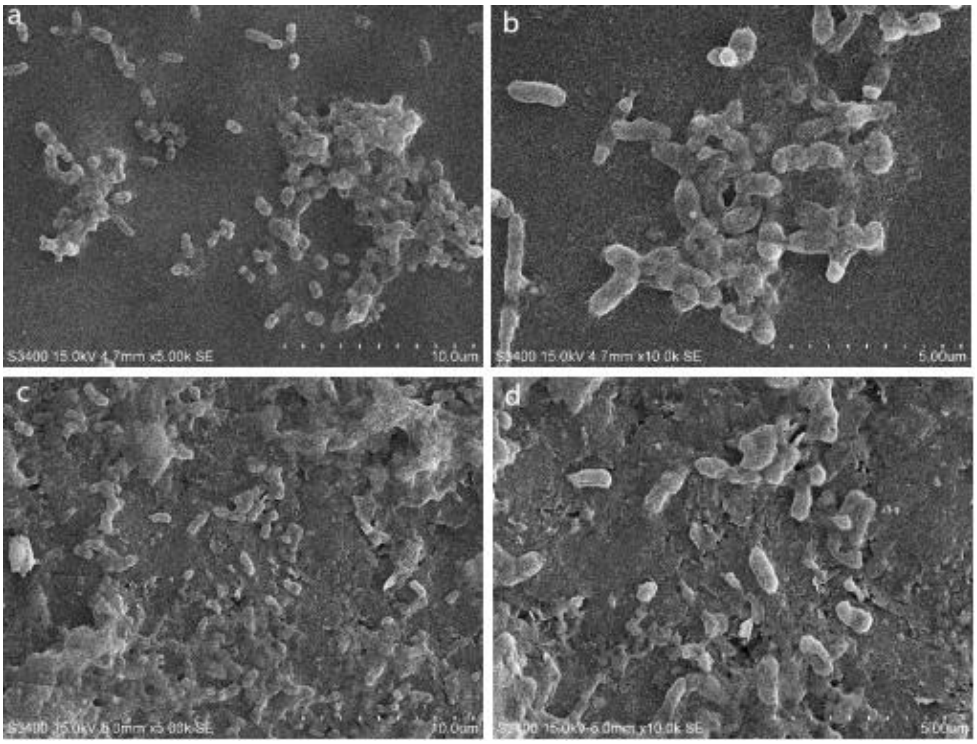

Fig. 8. SEM image of the electrode surface (a,b-nafion-modified electrode; c,d-birnessite-modified electrode; a,c-the magnification is 5,000; b,d-the magnification is 10,000).

4. Conclusions

T. denitrificans biofilms can absorb electrons from electrodes for autotrophic nitrate removal and denitrification was the only electron transfer pathway in microbial electrolysis cell. Compared with the unmodified graphite electrode, the modification of birnessite prepared by sol-gel method on the cathode enhanced the electrochemical activity of *T. denitrificans* biofilms. Meanwhile, the presence of birnessite effectively accelerated the nitrate reduction rate from 65.70 ± 5.03 to 114.17 ± 5.47 mmol NO_3^--N L^{-1} m^{-2} d^{-1}. Birnessite provides a cost-effective cathodic catalyst for autotrophic nitrate reduction in MEC.

Acknowledgment

The authors would like to thank for the help from the analysts at Center of Analysis and Test, and Laboratory for Resource and Environmental Education in East China University of Science and Technology.

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