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Electrochemical growth of *Acidithiobacillus ferrooxidans* on a graphite electrode for obtaining a biocathode for direct electrocatalytic reduction of oxygen

Sofía Carbajosa[a,b], Moustafá Malki[a,b], Renaud Caillard[c], María F. Lopez[c], F. Javier Palomares[c], José A. Martín-Gago[c,d], Nuria Rodríguez[d], Ricardo Amils[b,d], Victor M. Fernández[a], Antonio L. De Lacey[a,]*

[a]*Instituto de Catálisis, CSIC, c/ Marie Curie 2, 28049 Madrid, Spain*

[b]*Centro de Biología Molecular Severo Ochoa, Universidad Autónoma de Madrid, 28049 Madrid, Spain*

[c]*Instituto de Ciencia de Materiales de Madrid, CSIC, 28049 Madrid, Spain*

[d]*Centro de Astrobiología, INTA-CSIC, 28850 Torrejón de Ardoz , Spain.*

* Corresponding author. Tel: +34-915854802; fax: +34-915854760. *E-mail address*: alopez@icp.csic.es

Abstract

An aspect in microbial fuel cell research that is currently of great interest is the development of bacterial cathodes. Bacterial cathodes that catalyze oxygen reduction to water at low pH have the advantage of overcoming the kinetic limitations due to the requirement of 4 protons per molecule reduced. In this work we have studied the performance of a biocathode using as electrocatalyst an acidophile microorganism: *Acidithiobacillus ferrooxidans*. Growth of the microorganism directly on the electrode took place using an applied voltage of 0 V *vs.* SCE as the only energy source and without adding redox mediators to the solution. Current densities of up to 5 A/m^2 were measured for O_2 reduction in the *At. ferrooxidans* cathode at pH 2.0 and the electrocatalytic wave was shifted 300 mV to higher potential compared to the control graphite electrodes without the bacterium.

Keywords: microbial fuel cell; biocathode; acidophile; oxygen reduction, extracellular electron transfer

1. Introduction

In a Microbial Fuel Cell (MFC) the oxidation of the fuel at the anode is catalyzed by microorganisms. Up to date MFCs are not economically feasible because they have very low efficiency. They produce power very slowly and with low density (Pham et al., 2009). There are several bottlenecks that need to be eliminated in MFC development, one of these being the performance of the cathode. Various catholytes such as ferricyanide or acidic permanganate have been used in MFCs. However, these electron acceptors are impractical and unsustainable because they need to be regenerated. Alternatively, the use of O_2 as final electron acceptor in

the cathode greatly improves the sustainability of MFCs (Oh et al., 2004; Zhao et al., 2006). The reduction of O_2 at a carbon cathode is kinetically hampered, thus a catalyst, normally platinum, is required to accelerate the reaction. The problem is that platinum is a very scarce and expensive noble metal, which contributes in making the MFCs economically unfeasible.

An interesting alternative in MFCs is the use of microorganisms as biocatalysts not only in the anode but also in the cathode. The study of biocathodes for MFC applications is very recent (Bergel et al., 2005; Rabaey et al., 2008). Special interest has the study of microbial cathodes that may operate at low pH because the electrocatalytic reduction of O_2 to H_2O is kinetically limited by the availability of protons (Zhao et al., 2006; Erable et al., 2009). Therefore, acidophilic microorganisms that may be reduced directly at an electrode and use O_2 as final electron acceptor are of great interest.

Acidithiobacillus ferrooxidans is an autotrophic, aerobic and strictly chemolithotrophic bacterium. This microorganism uses CO_2 as a carbon source and obtains its energy from the oxidation of ferrous iron, elemental sulphur or reduced sulphur compounds at low pH, using O_2 as final electron acceptor (Malki et al., 2006). It has been shown that *At. ferrooxidans* cells are able to grow using electricity as sole energy source by using soluble iron as an electron-transfer mediator (Nakasono et al., 1997; Thrash and Coates, 2008). In the present work we have studied the performance of an *At. ferrooxidans* biocathode without using redox mediators. The goal has been to grow the microorganism directly on the electrode using applied voltage as the only energy source and to study the electrocatalytic properties of the bacterial cathode for O_2 reduction at low pH.

2. Materials and methods

2.1 Microbial growth and culture manipulations

At. ferrooxidans cells were grown at pH 2.0 in Mackintosh liquid medium composed by three solutions: Sol A, which contained basalt salts; Sol B, which contained $FeSO_4$ as

energy source; and Sol C, which contained $MnCl_2$, $ZnCl_2$, $CoCl_2$, $CuCl_2$, H_3BO_3 and $NaMoO_4$ as trace elements. These solutions were prepared as reported (Gonzalez-Toril et al., 2006). The culture preparation before the bacterium inoculation into the electrochemical cell involved the following steps: a) 150 ml of exponential phase growth culture was filtered through a filter paper in sterile conditions to take out the Fe^{2+} traces from the medium; b) the filtered culture was centrifuged at 15,000 × g during 6 minutes to sediment the bacteria, c) all the pellet was suspended in 1 ml of Sol A from Mackintosh medium.

2.2 Electrochemical measurements

A sterilized glass electrochemical cell from Radiometer thermostatized at 30ºC was used. The working electrodes were graphite felt (9 μm fibre diameter; 3,500 cm^2 g^{-1} surface area, RVG 4000, Le Carbon Lorraine) prisms of 11.8 cm^2 projected area. Before use, the prisms were cleaned in 1 M H_2SO_4 and then sterilized at 120ºC and 1 atm for 30 min. A standard calomel electrode (SCE) was used as the reference electrode and a platinum electrode as the counter electrode; both of them were supplied by Radiometer. Redox potential values mentioned in the text are versus SCE if not specified. *At. ferrooxidans* was inoculated into the electrochemical cell that contained 90 ml of Sol A and the trace elements of Sol C at a final concentration of 60 μg l^{-1}. A constant voltage of 0 V was applied on the working electrode with an Autolab PGSTAT 30 potentiostat (Eco-Chemie) while the solution was stirred with a magnetic bar. Cyclic voltammetry experiments were performed at different scan rates with stationary solution. Polarization curves were performed applying the linear voltammetry pGES Autolab procedure at 1 mV s^{-1} scan rate with magnetic stirring of the solution. In some experiments O_2 or N_2 (99.999% purity, Air Liquide) were bubbled into the electrochemical solution for at least 10 minutes.

2.3 Scanning electron microscopy

Scanning electron microscopy (SEM) of modified electrodes was done according to a protocol described previously (Alphenaar et al., 1994) with a JEOL-5600LV Scanning Electron Microscope.

2.4 X-ray photoelectron spectroscopy

X-Ray photoelectron spectroscopy (XPS) spectra were recorded with a SPECS Phoibos 150 electron spectrometer and a nine segments delay line detector, using Al Kα radiation (1486.61 eV) in an ultrahigh vacuum chamber with a base pressure of 10^{-9} mbar. Prior to the XPS measurements, the samples were dried out at room temperature by pumping down at a vacuum pressure of 10^{-3} mbar. All samples were analyzed by XPS no later than 24h after removal from solution. The overall surface composition was determined from survey scans. Additionally, high resolution XPS spectra were recorded for the C 1s, Fe 2p, N 1s and O 1s core-level peaks at normal emission.

3. Results and discussion

3.1 Electrochemical growth of At. ferrooxidans cells

At. ferrooxidans was grown in an electrochemical cell at a constant applied potential of 0 V, pH 2.0 and in absence of redox mediators. Negative currents were registered that increased slowly during 20 days after bacterial inoculation, and then the current oscillated around a mean value of -2.3 ± 0.3 mA (Supplementary Fig. S1). The slow increase of the current is typical of electrodes that are being colonized by electrochemically-active bacteria (Malki et al., 2008; Erable et al., 2009). In the control experiments without bacterial cells inoculated into the solution the measured currents were negligible during the same time period (Supplementary Fig. S1).

The SEM image of Fig. 1A shows that bacterial cells had colonized the graphite felt electrode after 28 days of 0 V applied potential. A biofilm formation can be observed with cells embedded in extracellular polymeric substances (EPS).

Fig. 1 here

Although it has been demonstrated that *At. ferrooxidans* can grow oxidizing Fe^{2+} regenerated at an electrode as the only energy source of the system (Nakasono et al., 1997), to our knowledge it has not been reported before that this bacterium could accept electrons directly from the electrode without the need of adding iron to solution.

3.2 Electrocatalytic reduction of oxygen at the bacterial cathode

The electrocatalytic properties of the bacterial cathode were studied by cyclic voltammetry at low scan rate. In this way, the background capacitive current and the kinetic limitations due to sluggish interfacial electron transfer between the bacterial cells and the electrode are minimized (Marsili et al., 2008). Fig. 2A shows the cyclic voltammograms (CV) recorded before and after purging the electrochemical solution with N_2. Under anaerobic conditions three pairs of redox waves are observed with formal redox potentials (E') of 0.405 V, 0.045 V and -0.210 V. Under aerobic conditions there is a clear catalytic effect in the redox process centred at 0.045 V, as the oxidation wave decreases considerably, whereas the reduction wave has a great increase in peak current. On the contrary, the redox process at 0.4 V is independent of O_2 concentration in the electrochemical cell. Therefore, electrocatalytic reduction of O_2 by the bacterial cathode is associated to a redox species with E'= 0.045 V. It has been suggested that anodophilic microbial consortia adapt their electroactivity such that their operative terminal reductases have redox potentials 35-55 mV more negative than the anode potential, in order to balance their oxidation kinetics at the electrode with microbial metabolism (Finkelstein et al., 2006). In agreement with this, our study of the cathodophilic bacterium *At. ferrooxidans* shows that the redox species that is associated to microbial-catalyzed O_2 reduction has a formal redox potential growth that is 45 mV more positive than the cathode potential applied for microbial growth.

A moderate catalytic effect in presence of O_2 is also appreciated for the redox process at -0.210 V. However, the control CV performed with an electrode non-modified by the bacterial cells show that direct reduction of O_2 at the graphite electrode takes place at such negative potentials (Supplementary Fig. S2). Therefore, the redox process at -0.210 V has seldom relevance for application of the bacterial electrode as a biocathode for O_2 reduction.

Fig. 2 here

Fig. 2B shows the polarization curves measured for the bacterial and control cathodes during which pH 2.0 solution was bubbled with pure O_2 and stirred, in order to decrease mass transfer limitations and to maximize the current of oxygen reduction (Oh et al., 2004; Zhao et al., 2006). It can be observed that for the bacterial electrode the electrocatalytic reduction of O_2 is shifted almost 300 mV to higher potentials compared to the graphite electrode non-modified with bacterial cells. A plateau current density of almost 6 mA was achieved at 0 V for the bacterial cathode, which corresponds to a current density of 5 A/m^2 taking into account the projected surface area of the electrode. This result suggests that an *At. ferrooxidans* cathode could be used for MFCs operating at acidic pH. *At. ferrooxidans* cells immobilized on a solid support have been used for regenerating Fe^{3+} in the cathodic compartment of a MFC (Ter Heijne et al., 2007), but to our knowledge the use of this organism as electrocatalyst in a O_2-reducing cathode has not been reported before.

A question that may be raised is about the nature of the redox species detected in the CV of the *At. ferrooxidans* electrode. One possibility is that they are redox active compounds excreted by bacteria, some of which could react with oxygen and give an electrocatalytic effect on the CV (Freguia et al., 2010). Nevertheless, the redox species at 0.045 V must be linked to the metabolic activity of the bacterium, otherwise the cells would not had been able to grow on the electrode surface at 0 V and using oxygen as final electron acceptor. Another possibility is that the redox species are redox proteins located in the cells' outer membranes.

There are evidences of outer membrane cytochromes of some electricigenic bacteria participating in direct transfer with electrodes (Busalmen et al., 2008; Richter et al., 2009). It has been proposed that an outer membrane bound cytochrome *c* (Cyc2) is the first electron acceptor in the protein complex for Fe^{2+} oxidation coupled to O_2 reduction in *At. ferrooxidans* (Castelle et al., 2008). However, the redox potential reported for Cyc2 (+ 0.560 V vs. SHE) is too positive to be involved in the electrocatalytic O_2-reduction measured with our biocathode.

3.3 XPS study of the bacterial cathode surface

It is possible that the redox signals detected are not due to redox proteins but to metallic ions bound to the bacterial biofilm on the electrode, acting as redox relays between electrode and the redox proteins of the cell. XPS characterization of the bacterial cathode was performed to detect the presence of metallic ions on the electrode surface.

Fig. 3 shows the Fe 2p XPS spectrum of the bacterial cathode poised at 0 V and measured following the procedure described in the experimental section. This figure also displays the spectrum of a control graphite electrode in which all the steps of the experimental protocol were followed but the bacterium was not included in the solution. In the case of the graphite electrode modified with *At. ferrooxidans*, the XPS spectrum exhibits a clear emission from Fe, whereas the negative control electrode does not. In the first case, the analysis of the Fe 2p line shape reveals the presence of two main peaks, which can be assigned to the doublet corresponding to the Fe 2p spin-orbit splitting, i.e. the Fe $2p_{3/2}$ and Fe $2p_{1/2}$ emissions. For each contribution, both Fe^{2+} and Fe^{3+} oxidation states can be distinguished. The Fe^{2+} and Fe^{3+} states for the Fe $2p_{3/2}$ emission are located at binding energies of approximately 710 eV and 712 eV, respectively and they are indicated in Fig. 3 by dashed lines (Wagner et al.,1979). The relative intensity of both Fe components indicates the predominant presence of Fe^{3+} species. In addition, the XPS survey scans for the two samples (not shown) revealed the presence of other atomic elements on the electrode surface, which are representative of the

chemical species present in the solution. However, their contributions with respect to the Fe peak were insignificant.

Fig. 3 here

It has been shown that the EPS excreted by *At. ferrooxidans* cells attached to pyrite contain exopolymer-complexed Fe^{3+} ions that allow electron transfer between the cells and the mineral (Dziurla et al., 1998; Gehrke et al., 1998). In the present work, *At. ferrooxidans* was grown electrochemically on the electrode in absence of Fe ions in the medium, although the cells inoculated into the electrochemically were from a pure culture grown using Fe^{2+} as energy source. Thus, it is expected that these cells contain plenty of Fe ions, as has been previously observed by Transmission Electron Microscopy (Malki, 2003). Furthermore, it is possible that the Fe species excreted by the cells into the biofilm could be mediating electron transfer between the graphite electrode and the cell.

5. Conclusions

Growth of the acidophile bacterium *At. ferrooxidans* directly on a graphite electrode is possible using an applied voltage of 0 V as the only energy source, without adding redox mediators to the solution, and oxygen as the ultimate electron acceptor. Current densities of up to 5 A/m^2 were measured for O_2 reduction in the *At. ferrooxidans* cathode at pH 2.0 and the electrocatalytic wave was shifted 300 mV to higher potential compared to the control graphite electrodes without the bacterium. The electrocatalytic reduction of oxygen at the biocathode is mediated by a redox compound with a formal redox potential of 45 mV that is related to bacterial growth. XPS characterization of the biocathode suggests that a Fe species excreted by the cells into the biofilm could be mediating electron transfer between electrode and the cell.

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Appendix A. Supplementary data

Supplementary data associated with this article.

References

Alphenaar, P.A., Groeneveld, N., van Aelst, A.C., 1994. Micron 25, 129-133.

Bergel, A., Feron, D., Mollica, A., 2005. Electrochem. Commun. 7, 900-904.

Busalmen, J.P., Esteve-Nuñez, A., Berna, A., Feliu, J.M., 2008. Angew. Chem. Int. Ed. 47, 4874-4877.

Castelle, C., Guiral, M., Malarte, G., Ledgham, F., Leroy, G., Brugna, M., Giudici-Orticoni, M.T., 2008. J. Biol. Chem. 283, 25803-25811.

Dziurla, M.A., Achouak, W., Lam, B.T., Heulin, T., Berthelin, J., 1998. Appl. Environ. Microbiol. 64, 2937-2942.

Erable, B., Etcheverry, L., Bergel, A., 2009. Electrochem. Commun. 11, 619-622.

Finkelstein, D.A., Tender, L.M., Zeikus, J.G., 2006. Environ. Sci. Technol. 40, 6990-6995.

Freguia, S., Tsujimura, S., Kano, K., 2010. Electrochim. Acta 55, 813-818.

Gehrke, T., Telegdi, J., Thierry, D., Sand, W., 1998. Appl. Environ. Microbiol. 64, 2743-2747.

González-Toril, E., Gómez, F., Malki, M., Amils, R., 2006. Methods Microbiol. 35, 436-502.

Malki, M., 2003. *Acidithiobacillus ferrooxidans* y su papel en la geomicrobiología del ciclo del hierro en el Río Tinto, Ph. D. Thesis, Universidad Autónoma de Madrid.

Malki, M., González-Toril, E., Sanz, J.L., Gómez, F., Rodríguez, N., Amils, R., 2006. Hydrometallurgy 83, 223-228

Malki, M., De Lacey, A.L., Rodriguez, N., Amils, R., Fernandez, V. M., 2008. Appl. Environ. Microbiol. 74, 4472-4476.

Marsili, E., Rollefson, J.B., Baron, D.B., Hozalski, R.M., Bond, D.R, 2008. Appl. Environ. Microbiol. 74, 7329-7337.

Nakasono, S., Matsumoto N., Saiki, H., 1997. Bioelectrochem. Bioenerg. 43, 61-66.

Oh, S., Min, B., Logan, B.E., 2004. Environ. Sci. Technol. 38, 4900-4904.

Pham, T.H., Aelterman, P., Verstraete,W., 2009. Trends Biotechnol. 27, 168-178.

Rabaey, K., Read, S.T., Clauwaert, P., Freguia, S., Bond, P.L., Blackall, L.L., Keller, J., 2008. ISME J. 2, 519-527.

Richter, H., Nevin, K.P., Jia, H., Lowy, D.A., Lovley, D.R., Tender, L.M., 2009. Energy Environ. Sci. 2, 506-516.

Ter Heijne, A., Hamelers, H.V.M., Buisman, C.J.N., 2007. Environ. Sci. Technol. 41, 4130-4134.

Thrash, J.C, Coates, J. D., 2008. Environ. Sci. Technol. 42, 3921-3931.

Wagner, C.D., Riggs, W.M., Davis, L.E., 1979, in: Mulenberg, G.E. (Ed.), Handbook of X-ray Photoelectron Spectroscopy, Perkin-Elmer, Minnesota, pp. 76-77.

Zhao, F., Harnisch, F., Schröder, U., Scholz, F., Bogdanoff, P., Herrmann, I., 2006. Environ. Sci. Technol. 40, 5193-5199.

Figure Captions

Fig. 1. Bacterial electrode characterization by SEM**.** A) Image of a fiber of a carbon felt electrode 28 days after inoculation of the electrochemical cell with *At. ferroxidans* and a applied redox potential of 0 V. B) Control image of a carbon felt electrode fiber before inoculation with *At. ferroxidans*.

Fig. 2. A) CV of the bacterial electrode after 22 days of 0 V applied potential. The dashed line corresponds to the CV measured under aerobic conditions, whereas the solid line corresponds to the CV measured after purging the electrochemical cell with N_2. Scan rate was 1 mV/s. B) Polarization curves of bacterial (dashed line) and control (dotted line) electrodes measured by lineal voltammetry at 1 mV/s scan rate with magnetic stirring and bubbling with pure O_2. The control electrode had been poised previously at 0 V but in absence of bacterium. The polarization curve with pure N_2 bubbling is also shown for the bacterial electrode (solid line).

Fig. 3. Fe 2p XPS spectrum of the bacterial cathode poised at 0 V (a), and for the negative control in absence of bacteria (b). The contributions of Fe^{2+} and Fe^{3+} are shown by dashed lines.

Fig. 1

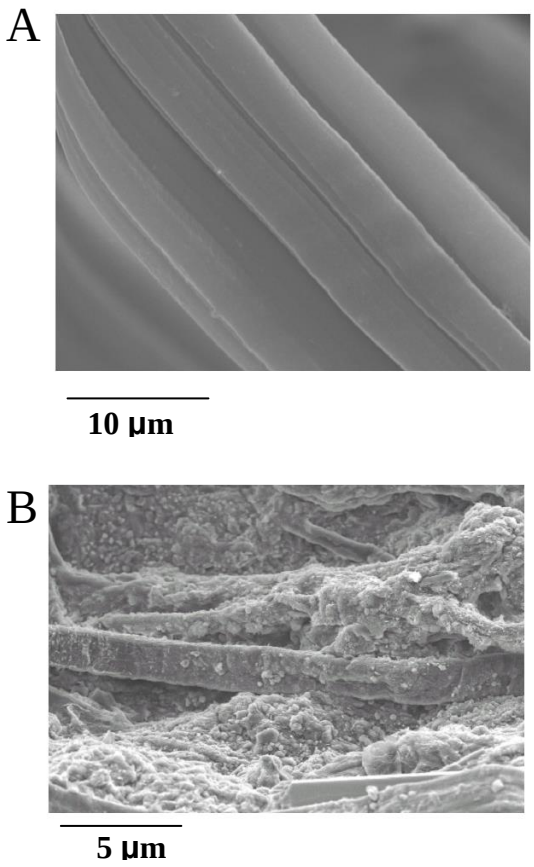

A
10 µm
B
5 µm

Fig. 2

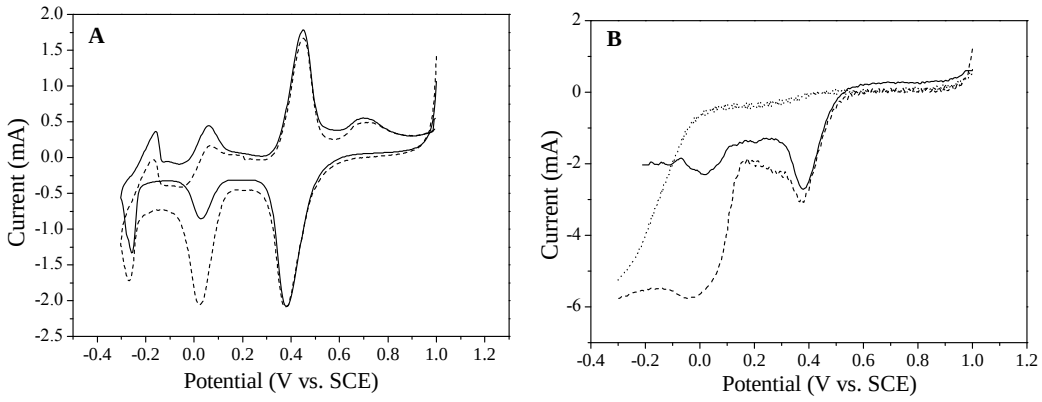
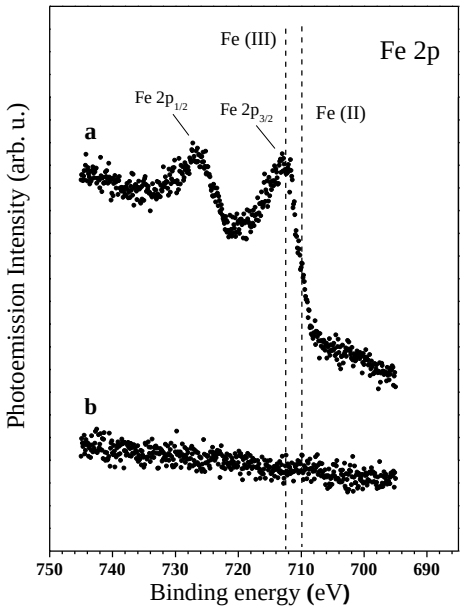

A
Current (mA)
Potential (V vs. SCE)
B
Current (mA)
Potential (V vs. SCE)

Fig 3


Fe (III)
Fe 2p
Fe 2p1/2
Fe 2p3/2
Fe (II)
a
b
Photoemission Intensity (arb. u.)
750
740
730
720
710
700
690
Binding energy (eV)

Appendix A. Supplementary data.

Fig. S1. Growth of *At. ferroxidans* cells on a graphite electrode constantly poised at 0 V under aerobic conditions. The increase of cathodic current during time for the bacterial electrode (filled squares) is compared with those of control electrodes in absence of bacteria (open circles) and in absence of both bacteria and trace metals (filled triangles). The current intensity was monitored continuously, although only one value is plotted per day for the sake of simplicity. The current intensity oscillated approximately ± 5% for each point represented.

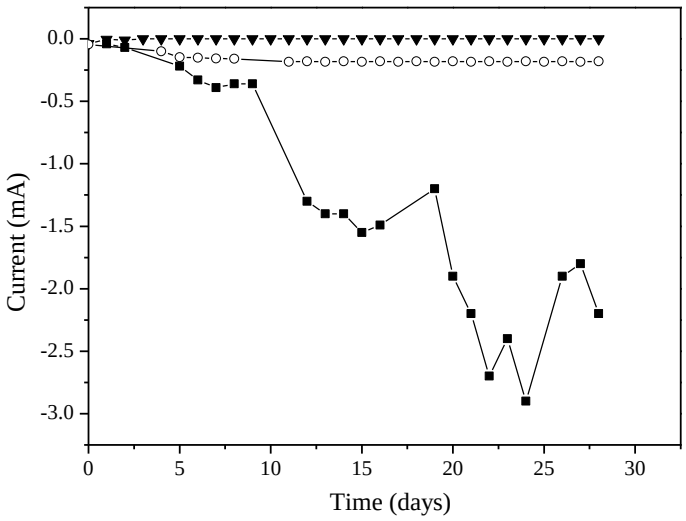


Fig. S2. Comparison of the CV of the bacterial electrode under aerobic conditions (dashed line) with CVs of the control electrode in absence of bacteria under aerobic conditions (dotted line) and after purging with N_2 (solid line). Scan rate was 1 mV/s. Both electrodes had been poised at 0 V during more than 20 days.