First air-tolerant effective stainless steel microbial anode obtained from a natural marine biofilm

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**Abstract**

Microbial anodes were constructed with stainless steel electrodes under constant polarisation. The seawater medium was inoculated with a natural biofilm scraped from harbour equipment. This procedure led to efficient microbial anodes providing up to 4 A/m² for 10 mM acetate oxidation at −0.1 V/SCE. The whole current was due to the presence of biofilm on the electrode surface, without any significant involvement of the abiotic oxidation of sulphide or soluble metabolites. Using a natural biofilm as inoculum ensured almost optimal performance of the biofilm anode as soon as it was set up; the procedure also proved able to form biofilms in fully aerated media, which provided up to 0.7 A/m². The current density was finally raised to 8.2 A per square meter projected surface area using a stainless steel grid. The inoculating procedure used here combined with the control of the potential revealed, for the first time, stainless steel as a very competitive material for forming bioanodes with natural microbial consortia.

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**1. Introduction**

Marine sediments are rich in organic compounds that are converted by indigenous microorganisms into fermentation products, the best known being acetate. Benthic microbial fuel cells (MFCs) can supply electrical energy directly from the oxidation of these fermentation products into CO₂. Reimers and Tender were the first to present this concept with a graphite anode buried in marine sediments connected to a cathode immersed in oxygenated seawater (Reimers et al., 2001; Tender et al., 2002). Microorganisms that adhered to the anode were shown to be able to catalyse the oxidation of acetate and other compounds, transferring the electrons produced directly to the anode. These early works gave current densities of the order of 100 mA/m². Using a modified graphite anode with electronic mediators (AQDS, Mn²⁺, Ni²⁺) improved the performance of microbial anodes up to 550 mA/m² (Lowy et al., 2006). Current density has also been enhanced by addition of substrates such as cysteine, chitin or cellulose into the sediments (Logan et al., 2005; Farzaneh et al., 2007).

At the same time, several bacteria or media have been studied under constant potential chronoamperometry (CA) to focus on the anode process only (Cho and Ellington, 2007; Parot et al., 2008a,b). In this way, pure cultures of microorganisms from marine environments such as *Geobacter sulfurreducens* (Bond et al., 2002; Gregory et al., 2004; Dumas et al., 2008a,b), *Desulfuromonas acetoxidans* (Bond et al., 2002; Bond and Lovley, 2003), *Desulfovibrio propionicus* (Holmes et al., 2004) have been used to form bioanodes and have generally led to higher values of current densities, for example up to 8.0 A/m² with a biofilm of *G. sulfurreducens* developed on a graphite anode polarised at +0.2 V/Ag–AgCl (Dumas et al., 2008a).

Graphite is commonly used in different forms as an anode material (Logan et al., 2007). A few studies have been aimed at developing stainless steel anodes, because of the better expected mechanical properties of stainless steels for long-term operations and large-scale extrapolation into easy-to-handle equipment. Stainless steel anodes implemented in benthic MFCs have revealed a complex behaviour (Dumas et al., 2007) that may be partly controlled by electrochemical and semi-conductive properties of the surface oxide layers (Dumas et al., 2008c). Nevertheless, keeping stainless steel anodes in well-controlled laboratory conditions (constant potential chronoamperometry), with a *G. sulfurreducens* biofilm has led to current density of up to 2.4 A/m² for acetate oxidation (Dumas et al., 2008b).

We have recently proposed a new procedure for constructing electrochemically active (EA) biofilms, which consisted of using natural biofilms as inoculum rather than samples collected from bulk sediments as is commonly the case. Natural biofilms were scraped from a sea environment and used as inoculum to construct EA biofilms on graphite anodes under constant polarisation. Following this procedure, microbial graphite anodes led to significantly higher current densities, up to 7.9 A/m² for the oxidation of acetate at −100 mV vs. SCE (Erable et al., in press). The purpose of the present work was to check the capacity of stainless steel to form efficient microbial anodes with microbial consortia coming from a natural marine biofilm.
from marine environments. In order to use the best possible
conditions, the microbial anodes were constructed under constant
polarisation and natural marine biofilms were used as inoculum.

2. Experimental

2.1. Marine biofilm

Natural marine biofilm was collected from a plastic floating
bridge in the port of La Tremblade (Atlantic Ocean, France). The
floating bridge is situated in the low water zone and is conse-
sequently in direct contact with sediment for 6 h a day. The biofilm
was harvested using a plastic scraper and around 50 ml of biomater-
ial was stored in a glass bottle with 20 ml of fresh seawater for
days at ambient temperature. The seawater used for the experi-
ments was from the same location.

2.2. Electrochemical instrumentation and set-up

Experiments were performed at constant potential polarisation
 chronoamperometry) using a conventional three-electrode sys-
tem implemented with a multi-potentiostat (VMP2 Bio-Logic SA,
software EC-Lab v.8.3, Bio-Logic SA). Experiments were carried
out in 500 ml reactors containing 250 ml of seawater supple-
mented with acetate (10 mM final concentration) and inoculated
with 250 ml of marine biofilm samples. Reactors were closed her-
etically, with no gas flow. Stainless steel 254SMO (Fe 56.1%, Cr
19.8%, Ni 17.8%, Mo 6%, N 0.2%) plates of 25 cm² projected surface
area were used as working electrodes. The stainless steel was
cleaned before each experiment by immersion in 2% H2O/0.5 M
HNO3 solution for 20 min and was rinsed for 1 h with distilled
water. The working electrodes were embedded vertically down
to the bottom of the reactor and the electric contact was made
with a titanium wire. The auxiliary electrode was a 20 cm² pro-
jected surface area platinum mesh and was maintained near to
the surface layer of the medium. All electric potentials were re-
ported against a Saturated Calomel standard reference Electrode
(SCE, Radiometer Analytical, TR100) protected with a second por-
ous frit. The potential of the working electrode was fixed at
−100 mV/SCE during chronoamperometry. Cyclic voltammetry
was performed in situ on the active anodes and on control elec-
trodes that were in the same reactor but not polarised. The poten-
tial was scanned 3 times between −700 and 300 mV/SCE at scan
tate. Both electrodes were polarised at −100 mV and the current
was followed on each anode for a week (Fig. 1B and C). The current
density remained at almost the same value with the biofilm-anode
in the fresh medium for around 3 days and then started to increase
again. In contrast, no current was observed on the clean electrode
plunged in the environment which had been already used for
12 days. Consequently, in this case, no abiotic reaction was in-
volved in the production of current and the biofilm was respon-
sible for the whole current observed under constant potential
polarisation. In contrast to what has been observed with graphite
anodes in sediments, no compound that may have accumulated
in the medium was involved in the production of current here.

Cyclic voltammetry (CV) performed at day 12 (Fig. 2) with the
biofilm-coated anode firstly in the initial medium and then with
the same electrode immersed in fresh seawater confirmed only a
slight decrease of current in fresh seawater, as observed under
chronoamperometry. Most of the current was consequently due
to the presence of the biofilm. The slight decrease was certainly
due to the effect on biofilm activity of the modification of environ-
mental parameters (oxygen presence, pH, substrate concentration)
when the medium was changed because of the low biofilm thick-
ness (50–60 µm). At day 12 a clean electrode was plunged into
the reactor containing the initial medium. CV performed with this
clean electrode showed a weak oxidation reaction from around
−0.3 V with a plateau reaching 0.4 A/m² at −0.1 V, while no cur-
rent (less than 0.05 A/m²) was detected under chronoamperometry
(less than 0.05 A/m²). Such a discrepancy between the behaviour
observed by CV and CA has already been noted with metallic an-
odes (Dumas et al., 2008b): a significant current is detected by
CV before the chronoamperometry starts to provide current. This
difference has been attributed to the beginning of the settlement

Fig. 1. Microbial current production with a working stainless steel electrode
polarised at −100 mV vs. SCE. (A) Initial. (B) In a new fresh medium. (C) With a
clean electrode plunged into the reactor at day 12.
of EA microbial cells (Parot et al., 2008b). The first settled cells are assumed to be able to implement fast reversible electron exchange with the electrode. Only a part of the redox chain of the cells is involved in the oxidation/reduction process, which does not reach the metabolism of acetate oxidation. This process is thus similar to a redox charge/discharge of the internal electron transfer system of the cells, it can be detected by CV but cannot sustain stationary current under CA. Moreover this process was certainly responsible for the large "charge/discharge" current that enlarged the CV curves. The hysteresis phenomenon cannot be attributed here to pure capacitive current because it was not observed with the clean electrode. It was directly linked to the presence of the EA biofilm on the electrode surface. By the way, such behaviour was not reproducible, and in some experiments the hysteresis effect almost disappeared (e.g., as in Fig. 5) indicating that the settlement of the first EA microbial cells was not fully mastered.

Biofilms were observed by epifluorescence microscopy at the end of the experiments (day 18). The numerical 3-D biofilm reconstruction showed a homogeneous distribution of biofilm on the stainless steel surface (Fig. 3) with a coverage ratio of around 90% and a biofilm thickness evaluated at 80–90 μm. The biofilm seemed to be supported by some kind of pillars that anchored the biofilm and supported it a few tens of micrometres above the electrode surface. This morphology was not due to the roughness of the stainless steel surface, which was only around 0.3 μm (Dumas et al., 2008b; Erable et al., in press) and consequently could not explain the height of the pillars that formed the lower layer of the biofilm. This kind of structure can be compared to the well known mushroom configuration that has been evidenced and widely studied with pure cultures of Pseudomonas aeruginosa (Takenaka et al., 2001). Biofilms formed in close to quiescent hydrodynamic conditions are known to present a high degree of void and marked channels in their internal structure. Here the 3-D pictures revealed a similar but exacerbated structure, where anchoring to the surface seemed to be minimal. It can be supposed that the microbial cells in contact with the electrode surface, which form the “pillars” of the structure here, play a key role in the electron transfer pathway. This physical observation reinforced the model that was suggested previously from electrochemical investigations, which assumed the specific role of an "electrochemical gate" for the microbial cells that settled on the electrode surface first (Dumas et al., 2008b; Parot et al., 2008a).

The concentration of dissolved oxygen present in the aqueous phase is known to influence the structure and permeability of biofilms (Jin et al., 2006). The mechanical biofilm resistance in the presence of high concentrations of dissolved oxygen is lower to that in an anoxic environment. This has been attributed to relatively higher biofilm porosity and smaller amounts of exopolymERIC substances (EPS) in biofilms growing with oxygen (Kim et al., 2006). In MFC, the presence of oxygen in the anodic side is often a source of problems and leads to the fall-off in the power generated (Ryckelynck et al., 2005; Dulon et al., 2006; Reimers et al., 2006). Depending on the potential of the anode and its electrokinetic properties, dissolved oxygen may reduce directly on the anode and consequently lower the current provided to the external circuit. On the other hand, oxygen may be used by the microbial cells as an electron acceptor, diverting a part of the electron flow from the anode. Oxygen can also cause inactivation of the cells, as reported by Kim et al. (1999) who observed that the electroactivity of a pure culture of Shewanella putrefaciens IR-1 was inactivated reversibly by exposure to air.

The sensitivity of our biofilms to oxygen was checked by forming two EA-biofilms in parallel in two electrochemical reactors

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**Fig. 2.** Cyclic voltammetry performed on stainless steel electrodes (A) after 12 days' polarisation at −100 mV vs. SCE, (B) same electrode in a fresh medium, (C) clean electrode plunged into the bioreactor at day 12; scan rate 10 mV/s.

**Fig. 3.** 3-D structure of anodic biofilm developed on the stainless steel electrode after 18 days' polarisation at −100 mV vs. SCE. Biofilm thickness 80–90 μm, surface area 500 × 500 μm. (A) view in profile, (B) view from the electrode.

**Fig. 4.** Current density recorded with a microbial stainless steel electrode polarised at −100 mV vs. SCE. Biofilm (A) formed anaerobically (N2/CO2), (B) aerated, (C) control without inoculation (N2/CO2).
under continuous bubbling of air (aerobic conditions) for the first and continuous bubbling of N2/CO2 (80/20) (anaerobic conditions) for the second. Control experiments were carried out in the same conditions but without any microbial inoculation. These control experiments did not give any current.

In both inoculated reactors under aerobic or anaerobic conditions, current increase was observed against time but the current recorded in anaerobic conditions was 3-times higher. After 10 days of polarisation, the current produced by the anaerobic biofilm was about 2.2 A/m² while that in the presence of air was only 0.7 A/m². The biofilm was perfectly able to form in aerated conditions and provided significant current density (Fig. 4). At this potential value (−0.1 V/SCE) on stainless steel, the direct electrochemical reduction of oxygen can be neglected. The strong effect of oxygen on the current cannot be attributed to a mixed reaction on the anode, e.g. bacterial oxidation of acetate coupled to direct oxygen reduction on the same electrode. The presence of oxygen consequently acted on the microbial metabolisms and on biofilm formation in an irreversible way, as no current increase was detected when the air feed was stopped. The analysis of the biofilm structure obtained in the presence of oxygen showed a larger amount of biomass (thickness >200 µm) but no other particular difference in the structure. The capacity to implement the oxidation reaction in an aerated environment is a major advantage for the design of membrane-less MFCs or MECs. Up to now, single chamber MFCs, which have been called membrane-less, have been designed using air-breathing cathodes. Nevertheless, the anodic compartment must be in anaerobic conditions, and the air-breathing cathodes must integrate a polymeric coating (membrane, PTFE, etc.) that prevents air from penetrating into the single anodic compartment (Pham et al., 2004; Ghangrekar and Shinde, 2007; Biffinger et al., 2008; Jadhav and Ghangrekar, 2008). This coating hinders the mass transfer of protons to the cathode, and may contribute to the low performance of such cathodes by limiting proton availability. In contrast, using an anode that can work in fully aerated conditions, as was the case here, holds promise for the design of MFCs without any membrane or coating.

A reactor was implemented for 10 days following the same procedure with two stainless steel electrodes inside, one polarised at −100 mV/SCE and the second left on open circuit. The open circuit potential of this electrode was stable at around −250 mV/SCE. After 10 days, cyclic voltammetry was performed on each electrode at a scan rate of 10 mV/s. CV showed that only the polarised electrode was able to oxidise acetate, while the electrode left on open circuit generated no current in the whole range of potentials scanned (Fig. 5). The polarisation of the anode was thus absolutely necessary to the development of an electro-active biofilm capable of catalysing the oxidation of acetate. However, biofilms with the same thickness of around 60 µm were observed on both electrodes by microscopy at the end of the experiment (data not shown).

Following the same procedure, an electro-active biofilm was formed which generated a current density exceeding 1.0 A/m² after 6 days of polarisation (Fig. 6A). The biofilm was then scraped from the anode and used as the inoculum in 500 ml of fresh seawater (10 mM acetate) to construct a new electro-active biofilm. The maximum current density was again obtained after 6 days, with a relative increase of 20% with regard to the previous biofilm (Fig. 6B). Further successive scraping and inoculation did not lead to significant improvement in the electrochemical efficiency of the biofilm. In contrast, it has been reported in the literature that successive scraping/inoculation steps can lead to large successive improvements in the current supplied at each step when inocula from bulk environments are used (Liu et al., in press). It can be concluded that the procedure described here, i.e. using natural biofilm as inoculum rather than samples collected from bulk sediments, allowed the formation of efficient biofilms with stable electrocatalytic properties from the first step. Our previous work describing the biofilm-inoculum procedure on graphite anodes for the first time analysed the microbial population of the biofilms formed. It showed that the efficiency of the reconstructed biofilms was due to the presence of a few bacterial strains that were absent from the biofilm formed with the inoculum coming from bulk sediments (Erasle et al., in press). This procedure ensured the presence of the efficient strains in the biofilm from the first construction step, in contrast to the common procedure that uses samples from bulk environments as inoculum and requires further enrichment of the efficient strains by successive scraping/inoculation steps.

Finally, the same procedure was performed with 3 electrodes in the same reactor: plain stainless steel, plain graphite and a stainless steel grid, each of 25 cm² projected area. The maximal current densities reached under constant polarisation at −100 mV vs. SCE were 3.1, 5.9 and 8.2 A/m², respectively (Fig. 7). Logically, using a grid multiplied the current density by a factor of around 2.5, reaching a current density among the highest reported at such a negative potential value. The most effective acetate-fed bioanodes reported in the literature have been obtained with air-cathode MFC systems but have reached maximal values around 8.0 A/m² (Cheng and Logan, 2007; Fan et al., 2007 with smaller systems). These works were performed using large-surface-area graphite anodes but only the surface area of the cathode, which was smaller, was taken into account for the calculation of the current densities. This procedure is perfectly correct for comparing the efficiency of MFC systems but it fails when comparing anode performance levels, as the current density is multiplied by a factor equal to

![Fig. 5. Cyclic voltammograms of marine biofilms grown anaerobically for 10 days on a stainless steel electrode polarised at −100 mV vs. SCE (A) or not polarised (B); scan rate 10 mV/s.](image1)

![Fig. 6. Reconstruction of an electrochemically active marine biofilm. Current density production by initial (A) or reconstructed (B) marine biofilm on a stainless steel electrode polarised at −100 mV vs. SCE.](image2)
the anode area divided by the cathode area. So, the present results cannot really be compared with the results already published. Nevertheless, by controlling the potential of the graphite rod anode, Liu et al. (in press) improved the bioelectrocatalytic activity of a domestic wastewater mixed-cultures biofilm to reach 5.0 A/m².

Stainless steel can now be considered as a promising material for building microbial anodes from natural consortia, as has previously been observed with pure culture (Dumas et al., 2008b). The less obvious successes obtained previously with stainless steel anodes in benthic fuel cells (Dumas et al., 2007, 2008c) were certainly linked to the procedure of biofilm formation, directly in MFC devices, which did not allow the potential value to be controlled. In consequence, the free evolution of the passive layer may have been a cause of the low current densities obtained previously. The experiments performed here with and without polarisation during biofilm formation brought out the crucial role of the potential in optimising the performance of the final anode.

4. Conclusion

Reconstructing a biofilm on a polarised stainless steel electrode from a natural biofilm used as inoculum led to remarkably efficient microbial anodes. Following this procedure, stainless steel proved to be an excellent material for designing microbial anodes that could be used in MFCs, microbial electrolysis cells, or any other biotechnological applications. Thanks to the mechanical properties of stainless steel, it should be possible to increase current density still further by using electrodes with higher specific areas, superimposed grids, brushes or any other open structure. The capacity of the microbial anodes described here to form and to provide current in aerated environments is another major advantage for the design of membrane-less MFCs. The initial biofilm in its natural environment is exposed to air for several hours daily, which may partly explain the resistance to oxygen exhibited by the reconstructed biofilm. The description of the microbial populations given in a previous work indicated the presence of four specific strains in such reconstructed biofilms. The biofilm reconstructed in aerated conditions can be thought to contain aerobic anodophilic strains; we will now direct our efforts towards the identification and isolation of such strains.

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