Improvement of the anodic bioelectrocatalytic activity of mixed culture biofilms by a simple consecutive electrochemical selection procedure

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1. Introduction

During the last decade, microbial fuel cell research has developed from a scientific peculiarity seeking for development objectives and potential applications, to a serious and highly dynamic research field (Logan et al., 2006). The development of real application goals – like the use of MFC technology for wastewater treatment (Tender et al., 2004; Kim et al., 2001; Rabaey and Verstraete, 2005), the energy production from marine sediments (Angenent et al., 2004; Kim et al., 2001; Rabaey and Wilkinson, 2000) and the production of hydrogen in microbial electrolysis cells (Liu et al., 2005a; Rozendal et al., 2006; Schröder, 2008), are not only the result of this development, but also cause the further advancement of this technology. Especially the coupling of wastewater treatment and energy recovery by means of microbial fuel cells represents a major driving force in this development.

There is a long list of fundamental and technological issues to be addressed to lead microbial fuel cells from research to application (Logan et al., 2006), some major issues being an improvement of the anodic electron transfer reactions (Schröder, 2007), the search for robust and efficient cathode catalysts (Zhao et al., 2005, 2006) and the problems associated with the charge balancing ion transfer across the fuel cell separator (Harnisch et al., 2008).

The majority of modern microbial fuel cells rely on mixed bacterial cultures, usually sampled from natural environments like from soil or sewage sludge. These cultures are abundantly available in our environment, are robust and offer the access to a wide range of substrates – ranging from simple organic acids to carbohydrates like starch and cellulose, Niesßen et al., 2006; Rismani-Yazdi et al., 2007 and even to proteins (Heilmann and Logan, 2006). Fundamentally, the bacteria can be used either dispersed (planktonic) in the anodic substrate solution (Rosenbaum et al., 2006; Schröder et al., 2003), or attached to the anode surface in the form of microbial biofilms. In the recent years, these anodic biofilms and the inhabiting, electrode colonizing bacteria have attracted considerable attention. In principle, the procedure for the biofilm formation is very straightforward: An inert electrode (e.g., carbon) is immersed into an anoxic substrate solution, inoculated with, e.g., sewage, and is polarized at a slightly positive potential (e.g., at 0.2 V vs. Ag/AgCl) (Kim et al., 2004). As a result, an electroactive biofilm forms consisting of a microbial consortium that is capable of utilizing the electrode as an electron acceptor. Yet, usually this primary biofilm exhibits only a low bioelectrocatalytic activity (Rabaey et al., 2003). The reason is that in the primary inoculum the number of electroactive bacteria is low in comparison to the number of non-electroactive bacteria (Rabaey et al., 2003).

In this paper we demonstrate that the anodic, bioelectrocatalytic performance of wastewater inoculum based, mixed culture microbial biofilms can be considerably improved by using a consecutive, purely electrochemical selection and biofilm acclimatization procedure. The procedure may represent an alternative to a repetitive mechanical biofilm removal, re-suspension and electrochemically facilitated biofilm formation. By using the proposed technique, the bioelectrocatalytic current density was increased from the primary to the secondary biofilm from 250 μA cm⁻² to about 500 μA cm⁻²; and the power density of respective microbial fuel cells could be increased from 686 mW m⁻² to 1487 mW m⁻². The electrochemical characterization of the biofilms reveals a strong similarity to Geobacter sulfurreducens biofilms, which may indicate a dominating role of this bacterium in the biofilms.

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to the “normal”, electrochemically inactive, bacteria and although the electrode (and the applied potential) may especially attract the electrochemically active bacteria, the primary biofilm may still consist of mostly inactive organisms. To solve this problem, usually a tedious and time-consuming enrichment procedure is used, which consists of a repeated mechanical removal of the biofilm from the electrode, re-suspension and subsequent biofilm re-formation (Kim et al., 2005; Rabaey et al., 2003). Due to the sensibility of most electrochemically active bacteria to oxygen, all these steps have to be performed under anoxic conditions—challenging especially for non-specialists (potential future users of microbial fuel cell technology).

In this article we propose an alternative to this laborious selection procedure. We show that the selection and biofilm acclimatization procedure can be simplified by using a consecutive, purely electrochemical selection process, by which, starting from a primary, wastewater based mixed culture biofilm, an efficient electroactive biofilm with superior performance can be established within a considerably reduced period of time and with low experimental efforts. We also show that the electrochemical characteristics of the mixed culture biofilms are strongly similar to that of Geobacter sulfurreducens biofilms (Fricke et al., 2008), which may indicate a dominating role of this bacterium or related genera in the mixed culture biofilms.

2. Experimental

2.1. General conditions

All microbiological and electrochemical experiments were conducted under strictly anoxic conditions. If not stated otherwise, all potentials provided in this article refer to the Ag/AgCl reference electrode (sat. KCl, 0.195 V vs. standard hydrogen electrode, SHE).

2.2. Chemicals and electrodes

All chemicals were of analytical or biochemical grade and were purchased from Sigma–Aldrich and Merck. The working electrodes/anodes used throughout this study were graphite rods (CP-Graphite GmbH, Germany, diameter 16 mm, with a geometrical surface area of 11.5 cm²; these electrodes were paraffin impregnated, to avoid ingress of electrolyte solution into the pore structure. The electrodes were prepared following the procedures described in Scholz and Meyer (1998) and Scholz et al. (2005).

2.3. Half-cell electrochemical experiments

All half-cell electrochemical experiments were carried out under potentiostatic control, using a three-electrode arrangement consisting of the working electrode, a Ag/AgCl reference electrode (sat. KCl, Sensortechnik Meinsberg, Germany) and a counter electrode (platinum wire or a graphite rod electrode). The counter electrode was separated from the bacterial solution by a Nafion® membrane of 2.2 cm diameter was clamped between the windows separating anode and cathode compartment. Both, anode and cathode chamber, contained a reference electrode (Ag/AgCl sat. KCl) for an independent recording of the individual electrode potentials.

Current and potential measurements were carried out using a data acquisition system comprised of an Integra 2700 digital multimeter equipped with a 7700 multiplexer (Keithley Instruments, Inc., Cleveland, USA) and interfaced to a personal computer. For the recording of polarization curves and the determination of the power output a variable resistance (0–100 kΩ), used as the external load, was varied stepwise to determine the respective current and potential values. All current and potential values were recorded only after reaching steady state.

The cathode material was porous carbon fibre (NCBE microbiology, UK, 6 cm × 5 cm) with a large surface area, to avoid cathode limitations. A 50 mM K₂Fe(CN)₆ in 0.1 M phosphate buffer (pH 7) was used as cathode solution. Although such cathode is known to be not sustainable (Logan et al., 2006; Rabaey et al., 2005) it was chosen for the simplicity of its use and in order to avoid effects of oxygen crossover, as it may occur when using an air cathode.

All fuel cells were operated under non-thermostated conditions, at room temperature, around 22 °C.

2.4. Fuel cell experiments

All fuel cell experiments were carried out in batch mode (battery mode) using a self-made fuel cell model, which was composed of two 250 ml volume bottom flasks pressed together at laterally inserted windows (Rosenbaum et al., 2005). A Nafion® membrane of 2.2 cm diameter was clamped between the windows separating anode and cathode compartment. Both, anode and cathode chamber, contained a reference electrode (Ag/AgCl sat. KCl) for an independent recording of the individual electrode potentials.

Metabolic substrate consumption and non-gaseous fermentation product formation were followed applying high performance liquid chromatography (HPLC) analysis. The HPLC (Knauer, Berlin, Germany) was equipped with a Rezex™ ROA-Organic Acid column in combination with the SecurityGuard™ cartridge AJO-4490 (Phenomenex®, Aschaffenburg, Germany). The chromatograms were recorded at room temperature with 0.005 N sulphuric acid as the eluent; the detector was a differential refractometer.

The qualitative identification and characterization of the microbial biofilms was performed by Nadicom GmbH Microbiology Services, Marburg, Germany, based on polymerase chain reaction (PCR)-based methods on DNA extracted from the biofilm samples. The phylogenetic analysis of the wastewater inoculum based biofilms revealed 14 genealogical trees with in a whole about 500 bacterial species (data not shown).

2.7. Scanning electron microscopy

For the electron microscopy the biofilms were prepared as follows: after a fixation step (1 h in 1% glutaraldehyde, 2% paraformaldehyde, 0.2% picric acid, 10 mM HEPES (pH 7.4), and 50 mM NaN₃), the samples were treated with 2% tannic acid for 1 h, 1% osmium tetroxide for 2 h, 1% thiocarbohydrazide for 30 min, 1% osmium tetroxide over night, and with 2% uranyl acetate for 2 h with washing steps in between. The samples were dehydrated in a graded series of aqueous ethanol solutions (10–100%) and then critical point-dried via amylacetate and CO₂. Finally, samples were mounted on aluminium stubs, sputtered with gold and examined in a DSM 940A (Zeiss, Oberkochen, Germany).
2.8. Substrate solutions

The bacterial growth medium and electrolyte solution for the electrochemical experiments was prepared as reported by Kim et al. (2005). It contained NH₄Cl (0.31 g L⁻¹), KCl (0.13 g L⁻¹), Na₂HPO₄·H₂O (2.69 g L⁻¹), Na₂HPO₄ (4.33 g L⁻¹) and trace metal (12.5 mL) as well as vitamin (12.5 mL) solutions (Balch et al., 1979; Lovley et al., 1984). Acetate (10 mM, unless stated otherwise) served as substrate (electron donor). All solutions were sterilized in an autoclave and were adjusted to a pH 6.8. Before inoculation the sterilized substrate solutions were purged with nitrogen for 20 min to remove oxygen.

2.9. Biofilm growth and electrochemical biofilm acclimatization

The biofilm formation procedure was adapted from literature (e.g., Kim et al., 2004). Domestic wastewater, sampled from the wastewater treatment plant in Greifswald was used as inoculum for the primary biofilm enrichment procedure. The chemical oxygen demand (COD) value of the wastewater was about 1200 mg L⁻¹.

All biofilms were grown in semi-batch experiments. For the primary biofilm formation 4 mL wastewater were inoculated into the sealed electrochemical cell that contained 120 mL of the stirred substrate solution. A constant potential of 0.2 V was applied to the working electrode to facilitate the biofilm formation. The growth of the biofilm was monitored by measuring the bioelectrocatalytic oxidation current. The substrate level was monitored by HPLC analysis. Regularly the exhausted substrate solutions were replaced by fresh substrate solutions. During the first, usually four, batch cycles wastewater was inoculated (see arrows in Fig. 1) to assist the electrode colonization and biofilm formation.

For the formation of the secondary biofilms, primary biofilm modified electrodes were, together with one or more blank graphite electrodes, immersed into lightly stirred, sterile substrate solution. Depending on the used potentiostat, the electrodes were either interconnected as one working electrode or were individually addressed (when using a multi-potentiostat). All electrodes were poised at a potential of 0.2 V, and the secondary biofilm was grown under semi-batch conditions, with a regular replenishment of the substrate solution.

2.10. G. sulfurreducens biofilm electrodes

G. sulfurreducens strain ATCC 51573 was purchased from the German Collection of Microorganisms and Cell Cultures, DSMZ, Braunschweig, Germany. Growth medium contained (per L): 0.1 g of KCl, 0.15 g of NH₄Cl, 0.6 g of Na₂HPO₄, 0.82 g of Na-acetate, 2.5 g of NaHCO₃, 10 mL of trace element solution, 10 mL of vitamin solution and 1 mL of selenite–tungstate solution. The medium was adjusted to pH 6.8 and flushed with N₂–CO₂ (80:20). The pre-culturing was performed at 30 °C, and sodium fumarate (50 mM) was used as a soluble electron acceptor. For the biofilm formation, which was achieved as described above, about 0.5–1 mL of the pre-cultured bacterial suspension per 150 mL of sterile substrate solution was inoculated into the electrochemical cell.

3. Results and discussion

3.1. Primary and secondary biofilm formation

Fig. 1 illustrates the formation of a primary, wastewater bacteria-based electroactive biofilm at a graphite electrode and its bioelectrocatalytic performance. The current densities, depicted in the figure, have been extracted from the maximum current densities in the batch experiments (see inset of Fig. 1) – thus reflecting the maximum bioelectrocatalytic activity of the biofilm. They originate from the oxidation of the substrate (here 10 mM acetate) catalysed by the bacterial biofilm at the working electrode according to the following equation:

\[
\text{CH}_3\text{COO}^- + 2\text{H}_2\text{O} \rightarrow 2\text{CO}_2 + 7\text{H}^+ + 8\text{e}^- \quad (1)
\]

Due to the lack of biocatalysts at the electrode surface the oxidation current is negligible at the beginning of the experiment. About 150 h after the initial inoculation, the current rises significantly, indicating the formation of an electrochemically active biofilm. The biocatalytic current reaches its maximum of about 250 μA cm⁻² about 400 h (17 days) after inoculation, and it remains, with some fluctuations, at a stationary level of about 230 μA cm⁻² for more than 54 days (Fig. 1).

When together with the primary biofilm modified electrode, an additional blank working electrode is immersed into a sterile substrate solution, and both electrodes are poised at a constant positive potential (here, 0.2 V), one can observe that without any further wastewater inoculation a biofilm is formed at the second electrode (hereafter denoted as secondary biofilm). Such a colonization of the blank electrode may proceed via active or passive dispersal, as described in literature (see, e.g., Hall-Studley et al., 2004). As Fig. 2 illustrates, the growth of the secondary biofilm is considerably faster than that of the primary biofilm. Within 24 h – a lag time that is about 155 h shorter than that of the primary biofilm (see Fig. 1) – a noticeable biocatalytic current is observed, and the maximum biocatalytic current of the primary biofilm electrode of 250 μA cm⁻² is exceeded already after 40 h. The secondary biofilm reaches a stationary current density of about 480 μA cm⁻² after about 150 h.

Further (third, fourth) biofilm generations did not lead to a further considerable increase of the biofilm performance. Although several biofilms reached current densities of up to 560 mA cm⁻², most biofilms remained at the performance level of the secondary biofilm.

A comparison of primary and secondary biofilms revealed that not only the rate of biofilm formation is considerably faster; also
in terms of bioelectrocatalytic performance the secondary biofilms outclass the primary ones.

3.2. Fuel cell performance of primary and secondary biofilms

The promising half-cell results were confirmed by fuel cell experiments, in which the primary and secondary biofilms were compared for their performance as anode biocatalysts. As Fig. 3 shows, the secondary biofilm clearly outperforms the primary biofilm. In the depicted experiment, the performance gain of the secondary biofilm (1487 mW m$^{-2}$) over the primary biofilm (686 mW m$^{-2}$) is 801 mW m$^{-2}$, a performance increase of more than 100%.

3.3. Voltammetric characteristics of the mixed culture biofilms

Although different in their overall performance, the thermodynamics and the mechanisms of the anodic electron transfer of primary and secondary biofilm appear to be one and the same. Thus, except for the different maximum steady-state current densities, the voltammetric features of primary and secondary biofilm are virtually identical. As illustrated in Fig. 4 for the example of the secondary biofilm, the onset potential of the catalytic curve is about −400 mV. The voltammogram possesses a typical sigmoidal shape with one single inflexion point (and thus only one single maximum in the first derivative, $\Delta j/\Delta E$) at a potential of −0.321 V. Under non-catalytic conditions (i.e. in substrate exhausted medium) at the end of the batch cycles the electrochemistry of the biofilms can be studied in more detail. Here, at low scan rates, the cyclic voltammograms reveal a quite complex structure, which consists of (at least) two redox systems, with formal potentials at $E_{f,1} = −365$ mV and $E_{f,2} = −284$ mV (see Fig. 5). Since the mean value of both potentials ($E_{f,1,2} = −324$ mV) is close to the potential of the inflection point of the catalytic curve (Fig. 4) one can assume that both systems are involved in the anodic, bioelectrocatalytic electron transfer.

The voltammetric features of the wastewater bacteria based biofilms are not only similar to those obtained by other authors for wastewater based electroactive biofilms (Liu et al., 2005a); more surprisingly, they are also extremely similar to those of $G$. sulfurreducens biofilms (see, e.g., Fricke et al., 2008). Thus, the sigmoidal bioelectrocatalytic voltammograms of $G$. sulfurreducens biofilms in acetate substrate solutions possess mean inflexion points at a potential of about −335 mV, which is close to the potential observed for the mixed culture biofilm. Further, as illustrated in the inset of Fig. 5, under non-catalytic conditions, the cyclic voltammogram is also composed of at least two redox systems, at formal potentials of −376 mV and −295 mV.
Fig. 5. Cyclic voltammogram of a primary, wastewater-inoculum-based mixed culture microbial biofilm electrode. The voltammogram was recorded at the end of a batch cycle, in substrate depleted culture medium. The scan rate was 1 mV s$^{-1}$.

Inset: cyclic voltammogram of a *Geobacter sulfurreducens* biofilm modified graphite electrode, conditions as in the main figure.

The similarity of the voltammetric behaviour of the mixed culture biofilm and that of *G. sulfurreducens* may be interpreted by two different means: first of all, it is possible that different electrochemically active bacteria possess identical or at least very similar electrochemical features, i.e., possess identical outer membrane redox proteins to establish the electron transfer at the same potential and using identical mechanisms. Such similarity may especially be expected for species that belong to one genus or strongly related genera. It may also be assumed that *G. sulfurreducens*, which was in fact detected in the phylogenetic analysis of the mixed culture biofilms, is the dominating electrochemically active bacterium in the mixed culture biofilm. Certainly, in the light of 500 detected bacterial species such dominance seems astonishing, however, the phylogenetic analysis does not provide information on the quantitative composition of the biofilm, and it may well be imaginable that the proven capability of *G. sulfurreducens* to exploit an electrode as solid electron acceptor provides this bacterium with a competitive advantage, allowing it to become the dominating species at the electrode surface.

3.4. Summarizing discussion

In the light of this discussion, two important features of primary and secondary mixed culture biofilm formation shall now be discussed: (i) the time necessary for the biofilm formation and (ii) the improvement of the bioelectrocatalytic activity from the primary to the secondary biofilm. Thus, as Figs. 1 and 2 illustrate, the lag time for the formation of a fully active, electroactive primary biofilm from a wastewater inoculum amounts to more than 400 h (nearly 17 days), whereas the secondary biofilm reaches the current density level of the primary film within 48 h, attaining a final activity twice as high as that of the primary film.

Two aspects may contribute to the long lag time of the primary biofilm formation. First of all, the overwhelming majority of wastewater bacteria can be assumed to be electrochemically inactive, i.e., they are not able to utilize the anode as a final electron acceptor. Thus, the number of electrochemically active bacteria colonizing the electrode surface can be expected to be extremely

Fig. 6. Scanning electron micrographs of (A) a primary biofilm modified electrode 24 h after wastewater inoculation, magnification 3000-fold; (B) a 22-h-old secondary biofilm modified electrode, magnification 3000-fold; (C) the same biofilm as (B), but at 1000-fold magnification; (D) unmodified graphite electrode.
low at the beginning of the biofilm formation (see Fig. 6A), and only after several inoculations they start to cover the electrode surface and to prevail over the electrochemically inactive species, using their metabolic advantage of being capable of disposing the electrons from the substrate oxidation to the electrode. Once the biofilm has established, such active biofilm can emit large numbers of cells to colonize new interfaces (Flemming and Wingender, 2001), and these cells are readily available to form biofilms at a second electrode—within a short lag time (see Fig. 6B and C).

An additional reason for the different lag times of primary and secondary biofilm formation can be derived from results of the biofilm formation of G. sulfurreducens. Starting from pre-cultured bacteria (grown in a solution that contained fumarate as the terminal electron acceptor) the time necessary for the primary biofilm formation is about 150−170 h (also see Frick et al., 2008). Again, once this biofilm has evolved, it can serve as a source for further biofilm generations. Here, the lag time for the secondary biofilm formation can be as low as 24 h (data not shown). In the case of Geobacter, this finding can be interpreted by means of an acclimatization of the bacterial cells from using a soluble terminal electron acceptor (fumarate) to the presence of a solid electron acceptor (the electrode), which requires the formation of the respective periplasmic and outer membrane electron carriers (cytochromes). When these membrane features have developed, any further electrode colonization will be considerably faster than the initial one. In contrast to the mixed culture biofilms, G. sulfurreducens secondary biofilm formation did not show an increased bioelectrocatalytic performance. Both biofilms, primary and secondary, showed an average maximum current density of about 500 μA cm⁻² (Fricke et al.). The improvement of the performance of the secondary mixed culture biofilm may thus not be attributed to an increased cell performance but rather to an improved composition (e.g., increased concentration of electrochemically active bacteria in the biofilm) and structure of the secondary film. Yet, this phenomenon still has to be investigated in more detail and will be subject of a further study.

4. Conclusions

In this study we have shown that for the improvement of the bioelectrocatalytic activity of wastewater based mixed culture microbial biofilms the tedious and laborious procedure based on the repeated mechanical removal of the biofilm from the electrode, re-suspension and subsequent biofilm re-formation may be avoided, and be replaced by a comparably simple electrochemical procedure. We have demonstrated that the secondary biofilm formation is a fast process that takes place within a few days, whereas the primary biofilm formation usually requires at least 2 weeks, and a repetitive re-inoculation.

From the voltammetric characteristics of the biofilms it may also be concluded that G. sulfurreducens (or closely related genera) represents the major electrochemically active bacterial species in the mixed culture biofilms.

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