



Electroactive mixed culture biofilms in microbial bioelectrochemical systems: The role of temperature for biofilm formation and performance

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ABSTRACT

In this paper we investigate the temperature dependence and temperature limits of waste water derived anodic microbial biofilms. We demonstrate that these biofilms are active in a temperature range between 5 °C and 45 °C. Elevated temperatures during initial biofilm growth not only accelerate the biofilm formation process, they also influence the bioelectrocatalytic performance of these biofilms when measured at identical operation temperatures. For example, the time required for biofilm formation decreases from above 40 days at 15 °C to 3.5 days at 35 °C. Biofilms grown at elevated temperatures are more electrochemically active at these temperatures than those grown at lower incubation temperature. Thus, at 30 °C current densities of 520 $\mu\text{A cm}^{-2}$ and 881 $\mu\text{A cm}^{-2}$ are achieved by biofilms grown at 22 °C and 35 °C, respectively. Vice versa, and of great practical relevance for waste water treatment plants in areas of moderate climate, at low operation temperatures, biofilms grown at lower temperatures outperform those grown at higher temperatures. We further demonstrate that all biofilms possess similar lower (0 °C) and upper (50 °C) temperature limits – defining the operational limits of a respective microbial fuel cell or microbial biosensor – as well as similar electrochemical electron transfer characteristics.

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1. Introduction

Microorganisms occupy almost every ecological niche on earth. Their outstanding physiological properties, e.g. the production of specialized chaperones, allow certain species to stand harsh environments like hydrothermal vents in the deep ocean or the arctic ice (Madigan et al., 1999). Thus, the temperature of the microbial habitats ranges from below –10 °C, occupied by cryophilic microbial strains like *Colwellia* (Bowman et al., 1997), to more than 80 °C hosting thermophilic microorganisms (see e.g. Miroshnichenko and Bonch-Osmolovskaya, 2006). In contrast to this wide temperature span of microbial life, the operational temperatures of biotechnological applications seem rather narrow, as most processes are operated between 20 °C and 45 °C (Ratledge and Kristiansen, 2006). This is not surprising, as most enzymes possess their optimum activity in this temperature range. Commonly, the indoor placement of biotechnological reactors not only results in a low variability of its environment and thus its ambient temperature, furthermore, the reactor temperature can be adjusted to the desired operation temperature using adequate cooling or process heat recovery facilities in order to realize a optimum biocatalytic performance.

In contrast, biotechnological reactors for waste water treatment are operated outdoors and without any active temperature control and thus have to face changing and less controllable temperatures. Obviously, the actual temperatures and the temperature variations on the seasonal and daily base are strongly dependent on the geographic location and thus on the climate at the location of the waste water treatment plant (WWTP). Especially in the temperature zone of the northern hemisphere (Northern Europe and Northern America) strong temperature variations between summer and winter season may lead to considerable variations in the temperature of waste water, which may severely affect the microbial degradation processes. Fig. 1 illustrates, on the example of the municipal waste water treatment plant Braunschweig Steinhof (located in the temperate climate zone at 52° 19' N, 10° 27' E and about 75 m above sea level) the annual course of the temperature of the inflowing waste water. The annual average temperature of the inflowing waste water is about 16 °C, however, as the main figure clearly shows the temperature varies between 8 °C (in February 2009) and 26 °C (in August 2009).

As the inset illustrates, additionally to the annual temperature course a daily temperature rhythm can be found. In Braunschweig this circadian temperature change was found to be usually between 5 °C and 10 °C, but up to 15 °C. In addition to these natural variations, the waste water showed rapid and extraordinary temperature extremes. Thus, temperature increases of up to 35 °C and temperature declines down to 7 °C have been observed. Obviously, as

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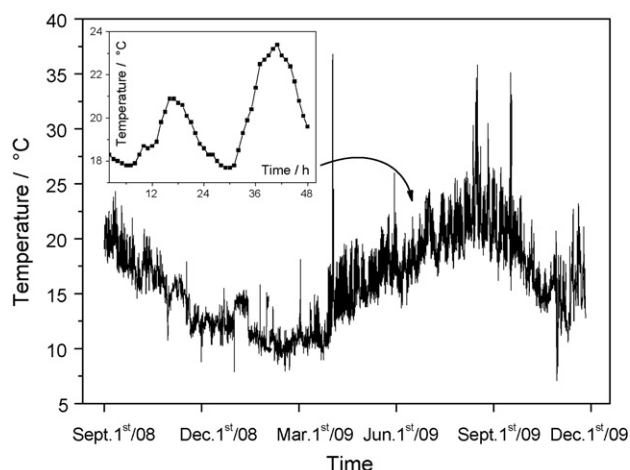


Fig. 1. Temperature of the inflowing waste water of the WWTP Braunschweig/Steinhof, Germany, measured in the influent of the primary clarifier between 2008/09/01 and 2009/11/27. Inset: zoom in for day 300–302. (Data on the courtesy of the association of sewage treatment, Braunschweig.)

Braunschweig possesses a medium sized WWTP (350,000 population equivalents and about $21 \times 10^6 \text{ m}^3$ of waste water per year), these temperature variations will be more pronounced in smaller facilities facing the same climate. From this example one can clearly deduce that the temperature variability within and between other climate conditions can be even more pronounced.

Temperature is an important parameter in microbial biotechnological processes. Thus, anaerobic digestion – representing an established technology for energy recovery from waste water – is acknowledged to operate at an optimal temperature between 25 °C and 35 °C; however, it fails below 20 °C (Cha et al., 1997; Pham et al., 2006). Despite of the obvious variability of the waste water temperature, however, the impact of temperature on many other waste water treatment processes is only scarcely reported.

Microbial bioelectrochemical systems (BES) like microbial fuel cells (MFCs) and microbial electrolysis cells (MECs) represent an upcoming technology for the exploitation and cleaning of waste water (Rabaey et al., 2009). Further, they possess a growing importance as analytical devices. Thus, MFC-type sensors have been proposed for BOD and toxicity analysis (in waste waters) as well as for the analysis of microbial activity (Kim et al., 2010; Odaci et al., 2009; Tront et al., 2008). In BESs microorganisms are exploited as biocatalysts at the anode allowing the interception of electrons released during the anaerobic oxidation of microbial substrates. For this electron transfer between microorganism and the solid electrode several mechanisms have been proposed (Schröder, 2007). The outmost majority of BES are nowadays based on anodic mixed culture biofilms derived directly from the microbial community (Kim et al., 2001a) present in the waste water by means of different selection procedures (Kim et al., 2001b, 2004; Liu et al., 2008; Rabaey et al., 2004).

So far, the great majority of BES studies have been performed at a temperature between 20 °C and 35 °C, with the actual operation temperature being chosen depending on the respective laboratory standard. A few reports deal with the effect of operation temperature in this temperature range (Di Lorenzo et al., 2009; Liu et al., 2005; Min et al., 2008; Odaci et al., 2009). Lower temperatures are only scarcely studied, and they are also based on complete microbial fuel cell devices. Thus, performance of similar MFC devices at different temperatures has been addressed with the temperatures spanning down to 10 °C (Hong et al., 2009) and 8 °C (Jadhav and Ghangrekar, 2009). In common, these studies have demonstrated that lowering the operational temperature results in a decrease

of current and power density of the BES. However, all of these reports investigated the temperature effect on entire MFC devices, which includes temperature effects of internal ohmic resistances and cathode kinetics.

In this study we illustrate the effect of temperature (in a range between 0 °C and 50 °C) on waste water derived anodic mixed culture microbial biofilms. We not only describe the performance (in terms of current densities) of waste water derived electroactive biofilms as a function of the operation temperature, but also the lower and upper temperature limits for these microbial biofilms. Furthermore, the influence of temperature on the lag time of the initial biofilm formation and the behaviour of the respective biofilms are studied in detail. All experiments reported in this paper were performed as half-cell experiments under potentiostatic control, in order to isolate the temperature dependencies of the biofilm behaviour from other fuel cell related physical properties.

2. Experimental

2.1. General conditions

All microbiological and electrochemical experiments were conducted under strictly anoxic conditions at controlled temperatures. All chemicals were of analytical or biochemical grade and were purchased from Sigma–Aldrich and Merck. If not stated otherwise, all potentials provided in this article refer to the Ag/AgCl reference electrode (sat. KCl, 0.195 V vs. SHE).

2.2. Microbial inoculum and growth medium

The source for the microbial inoculum was primary waste water, which was collected from the WWTP Braunschweig/Steinhof, Germany, and stored in sealed containers at 4 °C. The bacterial growth medium was prepared as reported by Kim et al. (2005). It contained NH_4Cl (0.31 g L^{-1}), KCl (0.13 g L^{-1}), $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (2.69 g L^{-1}), Na_2HPO_4 (4.33 g L^{-1}), trace metal (12.5 mL) and vitamin (12.5 mL) solutions (Balch et al., 1979). Acetate (10 mM) served as substrate in the growth medium that's pH was adjusted to 6.8. In order to ensure anaerobic conditions the substrate and buffer solutions were purged with nitrogen before use.

2.3. Electrochemical set-up

All 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, 0.195 V vs. SHE) and a counter electrode. The working and counter electrodes used throughout this study were graphite rods (CP-Graphite GmbH, Germany). The counter electrode was separated from the bacterial solution by a Nafion® 117 perfluorinated membrane. The experiments were conducted with a PGSTAT 30 Autolab system (Ecochemie, Netherlands) and a Solartron 1286 (Solartron Analytical, UK). Cyclic voltammetry (CV) was performed using a scan rate of 1 mV s^{-1} .

Sealed, water jacketed glass vessels, connected to thermo-/cryostats (FBC 610, Fisher Scientific, Germany) were used as electrochemical cells that allowed maintenance of strictly anoxic conditions as well as temperature control. The temperature was constantly monitored by a thermometer within the electrochemical cell.

2.4. Biofilm growth (semi-batch experiments)

As described in Liu et al. (2008) for the formation of primary biofilms 1 mL of waste water per 30 mL of the substrate solution was inoculated into the sealed electrochemical cell. In general

the electrochemical cells contained a volume of about 200 mL. A constant potential of 0.2 V was applied to the working electrode to facilitate and monitor the biofilm formation at the different temperatures. The biofilm growth was monitored by measuring the bioelectrocatalytic oxidation current. The substrate level was determined by regular sampling and HPLC analysis. The exhausted substrate solutions were replenished in intervals depending on the substrate depletion. After acclimatization of the biofilm covered electrode to a steady maximum bioelectrocatalytic current within at least three fed-batch cycles the system was switched to continuous flow mode in order to study the performance of the biofilms at different operation temperatures under continuous flow.

2.5. Continuous flow mode and temperature regime

Two plastic tanks (10 L each) served as reservoirs for the substrate and buffer solution. The flow rates of both solutions were maintained at 0.5 mL min^{-1} using a peristaltic pump (IP 65, ISMATEC Laboratoriumstechnik GmbH, Germany). After establishing a stable current generation the biofilm was set from its initial growth temperature to a step-wise temperature regime between 0°C and 55°C in 5°C intervals using a thermostat (FBC 610, Fisher Scientific, Germany). Initial experiments using a time step of 24 h per 5°C clearly showed similar current density–temperature dependencies as using time steps of 30 min per 5°C . For practical reasons the latter time interval was used throughout the study.

All reported current densities are based on at least two independent biofilm replicates and four temperature level replicates per biofilm; the standard deviation was below 5% for current densities above $140 \mu\text{A cm}^{-2}$ and below 15% for current densities below this current density value.

2.6. Metabolic analysis

Metabolic substrate consumption and non-gaseous fermentation product formation were followed by HPLC analysis. The HPLC (Spectrasystem P400, FINNIGAN Surveyor RI Plus detector, Fisher Scientific, Germany) was equipped with a Rezex HyperREZ XP Carbohydrate H+ 8 μm column. The chromatograms were recorded at room temperature with 0.005 N sulphuric acid as the eluent.

3. Results and discussion

3.1. Temperature dependence of biofilm formation from waste water

The formation of electrocatalytic microbial biofilms at an electrode surface is a time consuming process. The time span from inoculation with a natural source, like waste water or activated sludge, to the establishment of an active bioelectrocatalytic biofilm that produces a constant oxidative current flow, varies within different studies between 4 days and more than 100 days; e.g. (Aelterman et al., 2006; Freguia et al., 2008; Lee et al., 2003; Liu et al., 2008; Rabaey et al., 2004). Here we demonstrate that, when using identical inoculum, the incubation temperature substantially influences the time span needed for the formation of bioelectrocatalytic biofilms. Fig. 2 shows the lag-time, i.e. time span from inoculation with waste water as bacterial source until a reproducible maximum current density (see Section 3.2 for detailed values) was achieved, as function of the incubation temperature. One can clearly see that with increasing incubation temperature the lag time decreases considerably. Growing the biofilm at 22°C yields a lag time of 290 h (~ 12 days) which is in accordance with our previous study (Liu et al., 2008). An increase of the incubation temperature to 35°C

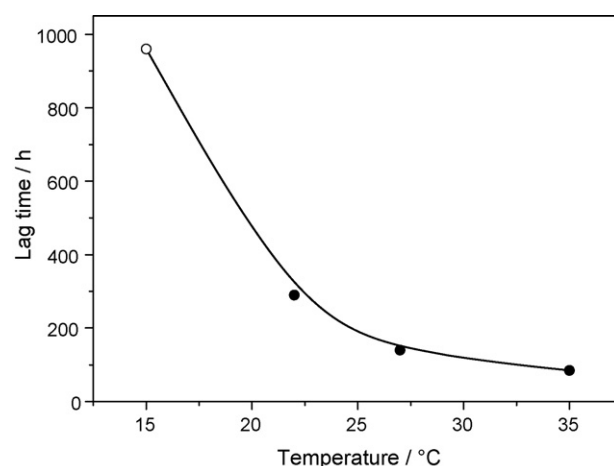


Fig. 2. Lag-time of the formation of waste water inoculum based electroactive microbial biofilms in potentiostatically controlled half-cell experiments (0.2 V vs. Ag/AgCl) at carbon rod electrodes, as a function of the applied incubation temperature. The substrate was 10 mM acetate. *The current density achieved for biofilms grown at 15°C was considerably below that of the other biofilms.

resulted in a more than threefold increase of biofilm formation rate (decrease of lag time to 85 h – 3.5 days). At 15°C , a temperature that is close to the average temperature in European WWTPs, biofilm formation took more than 40 days. Biofilm formation was not observed within more than 2 months at an incubation temperature of 5°C .

Here it has to be stressed that all biofilms within the study are formed directly from natural inoculates. Any pre-selection procedure, e.g. by using the effluent of a running BES anode (e.g. Huang and Logan, 2008), by mechanical re-suspension of electroactive biofilms (Kim et al., 2005; Rabaey et al., 2004) or by placing the fresh electrode in the vicinity of a electrode already settled with a bioelectrocatalytic active biofilm (Liu et al., 2008) will considerably decrease the lag-time of the biofilm formation.

3.2. Temperature dependence of the bioelectrocatalytic performance of waste water based biofilms grown at different temperatures

Fig. 3 shows, for the example of a biofilm grown at an incubation temperature of 35°C , the dependence of the bioelectrocatalytic current generation on the operation temperature. After adjustment of the biofilm to continuous flow conditions (~ 15 h) the biofilm was exposed to a temperature ramp to 0°C using a step wise temperature decrease of the 5°C every 30 min. One can clearly see that the current density decreases with decreasing operation temperature. The subsequent reversal of the temperature ramp clearly shows the reversibility of biological system with respect to the temperature stress. The steady state current density of the temperature plateau at 35°C between 26 h and 39 h deviates only 3% from the initial biofilm performance at this temperature. As can be clearly deduced from Fig. 3, the subsequent application of a second temperature cycle confirmed the results of the first one.

Furthermore, Fig. 3 shows that a final rise of the operation temperature to 50°C did yield stable steady state currents. Common to all biofilms exposed to these high temperatures was the continuous decrease of the bioelectrocatalytic activity to zero within 24 h. As the inset of Fig. 3 shows, an additional increase of the operation temperature from 50°C to 55°C further accelerated the performance degradation.

It is worth noticing that the short (30 min) exposure of the microbial biofilm to the freezing point did not lead to any damage to the biofilm. An exposure time of 24 h, however, resulted in

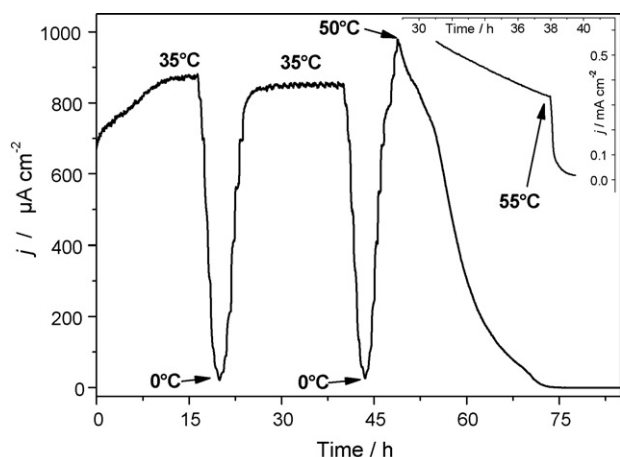


Fig. 3. Effect of temperature variation on the bioelectrocatalytic current generation of a biofilm grown at 35 °C. After adjusting the biofilm to continuous flow conditions, using 10 mM acetate as the substrate, the biofilm was exposed to two consecutive temperature ramps, each proceeding from 35 °C to 0 °C and back to 35 °C with 5 °C temperature increments in intervals of 30 min. Finally the temperature was raised up to 50 °C and remained at this till the end of the experiment; Inset: similar experiment as main figure but using a final temperature of 55 °C.

a decrease of the performance (at 35 °C) of between 42% and 50% (data not shown).

Fig. 4 summarizes the results of experiments similar to that shown in Fig. 3 by depicting the temperature dependence of the steady state current densities of waste water derived microbial biofilms that were grown at different temperatures. Regardless of the incubation temperature (biofilm formation) all biofilms possess their maximum bioelectrocatalytic activity at a temperature between 35 °C and 45 °C. Lower operation temperatures yield a decreased biofilm performance. Furthermore, common to all biofilms is the rapid decrease of the bioelectrocatalytic activity for operation temperatures exceeding 45 °C. As the bioelectrocatalytic current density can be considered as a measure of the bacterial metabolic activity, these findings are not unexpected. In mesophilic microorganisms, the most likely great majority of organism present in the studied municipal waste water, the optimum performance of the microbial metabolic machinery is between 35 °C and 45 °C. At temperatures below this optimum the metabolic rates become slower (lower enzymatic turn-over rates), above the optimum irreversible denaturation processes start to occur, which may lead to

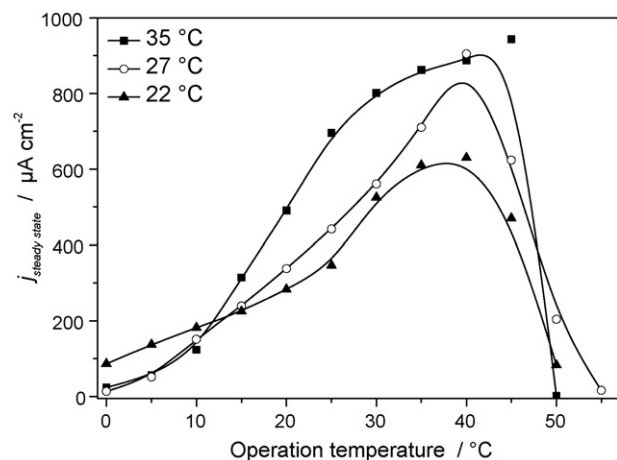


Fig. 4. Dependence of the bioelectrocatalytic steady state current densities (potentiostatic control, 0.2 V vs. Ag/AgCl) of biofilms incubated at different temperatures on the operation temperature. The data are derived from experiments analogue to those presented in Fig. 3.

full biofilm decomposition. The latter fact was supported by further experiments (data not shown) clearly demonstrating that biofilms which were exposed to temperatures above 45 °C irreversibly lost their bioelectrocatalytic activity. Even when new waste water was inoculated no bioelectrocatalytic current was re-established within more than 10 days. Only if the decomposed biofilm biomass was stripped off from the electrode surface new biofilms were successfully formed.

As it has been demonstrated before, higher operation temperatures may be achieved by utilizing thermophilic microorganisms, e.g., directly selected from a thermophilic anaerobic digester (Jong et al., 2006). In this case, a continuous operation at a temperature of 55 °C has been reported. This indicates that for specialized applications, i.e. the treatment of hot waste water, the use of adapted (thermophilic) microbial cultures is beneficial, which however may fail at lower temperatures.

As illustrated in Fig. 4, the biofilm performance at a certain operation temperature is strongly dependent on the incubation temperature during the biofilm formation. Generally, for operation temperatures between 10 °C and 40 °C a higher temperature during biofilm formation resulted in an increased bioelectrocatalytic current density. For the example of a operation temperature of 30 °C the biofilms grown at 22 °C, 27 °C and 35 °C yielded current densities of 520 $\mu\text{A cm}^{-2}$, 560 $\mu\text{A cm}^{-2}$ and 881 $\mu\text{A cm}^{-2}$, respectively. This represents a 70% increase in the bioelectrocatalytic performance for biofilms grown at 35 °C compared to biofilms formed at 22 °C. In contrast to that, at low operation temperatures (<10 °C) biofilms that were grown at low temperatures (22 °C) outperform those grown at higher temperature.

Noteworthy, the above presented results apply to young biofilms with low absolute biocatalytic activity (current density) and to mature biofilms with high activity. This means that the reported phenomena do not depend on the thickness of the electroactive biofilms.

The demonstrated impact of the temperature during biofilm formation on the bioelectrocatalytic performance at a certain operation temperature may be attributed to a differing biofilm structure, e.g. exopolymer matrix composition, and/or to a differing individual microbial performance pre-set within the initial growth phase. The latter may be considered very unlikely, as microorganisms (i.e. their protein syntheses etc.) are able to adjust rapidly to environmental changes. Therefore, it can be assumed that the temperature during the initial growth phase of the biofilm determines the abundance of the different microbial species as well as their distribution within the biofilm matrix that itself might be dependent on the temperature during biofilm formation. Once such a biofilm is formed the individual cells can adjust their metabolism to the respective operating temperature. This was clearly shown in this study by the reversible adjustment of the microbial bioelectrocatalytic activity to changing operation temperatures. Changes in the biofilm architecture, i.e. the biofilm composition and structure, might be possible on longer time scales. These biofilm reconstructions then might lead to an adjustment of the bioelectrocatalytic performance independent of the initial growth temperature. These questions, concerning the differences in the architecture and composition of the electroactive biofilms, however, require a thorough microbiological analysis that was not within the scope of this study.

3.3. Voltammetric characteristics of the waste water based mixed culture biofilms grown at different temperatures

Although their performance at certain operation temperatures differed remarkably, the voltammetric behaviour and thus the electron transfer thermodynamics of all biofilms appears to be identical. As it can be seen from the CVs recorded under turn-over

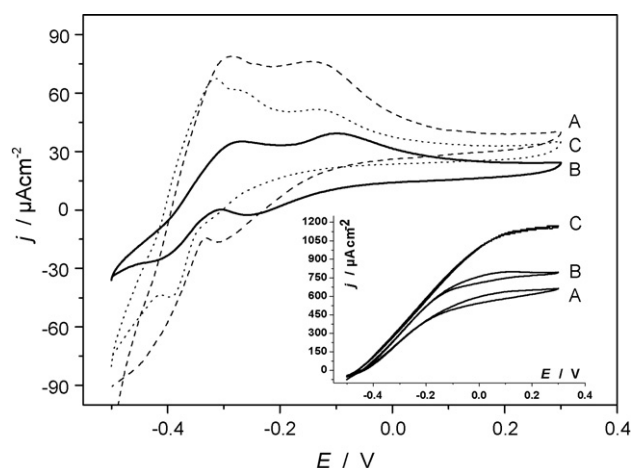


Fig. 5. Cyclic voltammograms (CV) of microbial biofilms grown at three different temperatures A) 22 °C, B) 27 °C and C) 35 °C, and recorded under non-turn-over conditions. Inset: CV curves of the same biofilms recorded under turn-over conditions at the respective growth temperature. Scan rate: 1 mV s⁻¹, the substrate was 10 mM acetate.

conditions (inset of Fig. 5) the onset potential of the bioelectrocatalysis is about -0.4 V (vs. Ag/AgCl) for all biofilms.

The voltammograms possess a typical sigmoidal shape with their main inflection points – to be derived from the maxima of the first derivatives of the respective voltammetric curves (data not shown) – between -0.4 V and -0.3 V (vs. Ag/AgCl). Under non turn-over conditions (main figure, Fig. 5) the cyclic voltammograms depict a more complex redox behaviour. This redox behaviour is not only very similar for all biofilms (the slight differences should not be overemphasized at the present, since they are typical for electroactive microbial biofilms), it is also comparable to that of our previous studies on biofilms based on primary waste water from a different treatment site (WWTP Greifswald, Germany) (Liu et al., 2008) and also to that of *Geobacter sulfurreducens* (Fricke et al., 2008; Srikanth et al., 2008).

From these results it may be concluded that the composition of the microbial biofilm with respect to the dominating, electroactive, species, was not measurably affected by changes in the studied temperature range. This means that irrespective of the studied temperature during the biofilm growth or temperature changes during biofilm operation, the biofilm characteristics remained constant.

At this point it may be emphasized that in this study acetate was used as the sole microbial carbon and electron donor. This situation will certainly lead to a preferred accumulation of species like *G. sulfurreducens* (Lovley, 2008; Richter et al., 2009), an electroactive organism that is specialized on acetate, that we have already detected in a previous study, and that shows the here reported electrochemical characteristics. In further studies it will have to be analysed if biofilms, fed on more complex substrates (e.g., real waste water), will result in similar findings.

4. Conclusions

In this study we have shown that the temperature decisively influences the lag-time of the formation and bioelectrocatalytic performance of waste water derived mixed culture biofilms. It was demonstrated that waste water derived biofilms can operate within a temperature range from above 0 °C to 45 °C and that the biofilms can adjust reversibly and rapidly to temperature fluctuations within this range.

As expected, all biofilms showed increased bioelectrocatalytic performance at elevated operation temperature. Most important, however, is the comparably good performance at temperatures

between 10 °C and 20 °C, which represents the average temperature of municipal waste water in Northern Europe and America. This performance is a great advantage against anaerobic digestion, which fails at temperatures below 20 °C.

The study further indicates that there is a relationship between the temperature used for the biofilm formation and the biofilm performance at different temperatures. Although showing the greatest overall performance, biofilms that were grown at high temperatures showed a stronger relative performance decrease upon lowered temperatures than biofilms that were grown at these temperatures.

The results of this study are of great importance to microbial fuel cells and electrolysis cells but also for electrochemical microbial biosensors.

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