Factors affecting microbial fuel cell acclimation and operation in temperate climates

ABSTRACT
For the successful scale-up of microbial fuel cell (MFC) systems, enrichment strategies are required that not only maximise reactor performance but also allow anodic biofilms to be robust to environmental change. Cluster analysis of Denaturing Gradient Gel Electrophoresis community fingerprints showed that anodic biofilms were enriched according to substrate type and temperature. Acetate produced the highest power density of 7.2 W m⁻³ and butyrate the lowest at 0.29 W m⁻³ respectively in a sucrose enriched reactor. When temperature perturbations were introduced to investigate the stability of the different substrate acclimated electrogenic biofilms, the 20°C acclimated acetate reactor was unaffected by 10°C operation but all reactors acclimated at 35°C were adversely affected. When the operating temperature was raised back to 35°C both the acetate and butyrate reactors recovered electrogenic activity but the sucrose reactor did not. It is thought that this was due to the more complex syntropic interactions that are required to occur when metabolising more complex substrate types.

Key words | acclimation, biofilm, microbial fuel cell, substrate, temperature

INTRODUCTION
An activated sludge process is typically used to treat sewage and industrial wastewaters; being an aerobic system this can involve high energy costs due to the mechanical energy used to supply the oxygen, whilst the process itself is subject to limitations associated with the efficiency of oxygen transfer to the bacteria. This is not the case in anaerobic systems where electron/hydrogen transfer can occur directly and is therefore not subject to mass transfer limitations. Aerobic systems can also generate high levels of sludge, with subsequent requirements for secondary disposal. However, the waste itself has an intrinsic energetic value; indeed it was estimated by Shizas & Bagley (2004) that the energetic content of the wastewater exceeds the energetic value to treat it by a factor of 9.3. Whilst techniques such as gasification, pyrolysis and anaerobic digestion are currently being used for renewable energy production from sewage, additional and complimentary technologies such as microbial fuel cells (MFCs) have the potential to both treat wastewaters and recover additional energy. MFCs are a fixed biofilm treatment technology which utilise microorganisms as a biocatalyst to convert chemical energy present in wastewater to electrical energy via bacterial electron transfer to a remote electrode, thus facilitating the direct recovery of electrical energy.

The observed diverse nature of anodic biofilms is matched by a diversity in the number of substrate types that electrically active bacteria can convert to electrical current, with more complex substrates reported to produce different and more complex microbial profiles (Lee et al. 2005). Substrate type has also been shown to influence MFC performance with complex substrates requiring a versatile range of microorganisms to facilitate degradation (Pant et al. 2010), whilst simple non-fermentable volatile fatty acids (VFAs) such as acetate are known to produce high current densities (Rabaey et al. 2005). It was observed by Jung & Regan (2007) that Geobacter sulphurreductans species are particularly associated with growth on acetate, whilst members of Firmicutes spp. were only found in glucose fed reactors. This is important as the former have...
been particularly associated with MFC systems that produce high power densities. The influence of substrate complexity on microbial community development is directed by how different types of microorganisms operate together as part of a functionally stable anodic community. The processing of wastewaters with anodic communities will involve the activity of at least five different trophic groups that undertake hydrolysis, fermentation, syntrophic bacterial associations, methanogens and electrochemically active bacteria (EAB); it would seem likely that a functionally robust system would require both a flexible and diverse population.

A majority of MFC system studies are run at mesophilic temperatures, within laboratory environments, even though most domestic and industrial wastewaters are released at temperatures of <18°C and can cycle annually between temperatures of 7 and 35°C (Lettinga et al. 2001; Patil et al. 2010). Although it has been recently demonstrated that MFCs can be acclimated for operation at both psychrophilic and psychrotrophic temperatures (Michie et al. 2011), non-acclimated anodic biofilms can be adversely affected by low operating temperatures (Cheng et al. 2010). As temperature can be seen to be a major regulating factor with an important influence on environmental wastewater treatment efficacy, this factor was used to introduce perturbations into different substrate acclimated biofilms.

In this study we investigated how substrate and temperature acclimation affected bacterial community development in anodic biofilms, and how this then influenced reactor performance. The effect of temperature perturbation on acclimated anodic biofilms was then investigated to assess biocatalytic robustness.

**METHODS**

**MFC reactor construction and operation**

Single chamber tubular air cathode MFCs were constructed using polypropylene tubing of 280 mm length and 40 mm diameter. The air cathode (80 × 210 mm) was manufactured with 0.5 mg/cm² platinum catalyst and was fixed with activated carbon before being attached to a cation exchange membrane (CMI-7000, Membrane International Inc., NJ, USA). The anode was constructed using carbon veil cloth (PRF Composite Materials, Dorset, UK) by winding 260 × 450 mm veil sheets around a central PVC rod (10 mm diameter). The experiments were initiated by inoculating the reactors with 24.5 mL sludge in 220.5 mL in 100 mmol phosphate buffer, sourced from Cog Moors wastewater treatment plant, Cardiff, Wales. The reactors were operated in fed-batch mode, with 5,000 ppm chemical oxygen demand (COD) L⁻¹ acetate, butyrate or sucrose in 100 mmol phosphate buffer (pH 7.0) containing vitamins and minerals (Kim et al. 2009); the reactors were maintained at 20°C or 35°C ± 1°C in heating/cooling incubators (Oxitop, WCW, Germany). The reactors were fed on a weekly basis and operated over a period of 83 weeks. During operation the MFC reactors were sited in the incubator at a fixed 45° angle, with the anode always orientated in the same position in order to maintain the anode biofilms in fixed positions for Denaturing Gradient Gel Electrophoresis (DGGE) community analysis.

Each reactor was independently connected to a 1,000 Ω external resistive load and voltages across the MFCs were recorded at 10 min intervals using LabVIEW™ software and a NI 16-Bit, Isolated M Series MIO DAQ card, (National Instrument Corporation Ltd, Berkshire UK).

**ANALYSES**

**Electrochemical and chemical analyses**

Power density plots and polarisation curves were measured using a Solartron Instruments (Farnborough, UK) 1287 electrochemical interface with an Ag/AgCl reference electrode. Analysis of the MFC was carried out using a potentiostatic method (CorrWare 2™, Scribner Associate Inc., NC, USA), where a constant potential was applied and the current was monitored as a function of time. Polarisation curves were measured after 2 hours at OCV (open circuit potential). Each cell potential was measured after an interval of 10 min to allow stabilisation of the current. Coulombic efficiencies (CE) for each batch mode operation were calculated as previously described (Logan et al. 2006; Logan 2008).

Liquid samples from each batch reactor were centrifuged at 13,000 rpm (16,060 × g) for 5 min and then tested immediately or were acidified (HCl, 12 mol/L) and then preserved frozen until measurement. Soluble COD (sCOD) measurements were undertaken using a standard method (Method 5220, HACH COD system, HACH Co., Loveland, CO, USA) and DNA concentrations were measured using a NanoDrop™ 1000 Spectrophotometer (Thermo Scientific).

**DGGE analysis**

The first layer of the anodic carbon veil was aseptically sampled with a scalpel and then trimmed by scissors to
1 cm². Test samples were taken at 8 weeks (T1), 33 weeks (T2) and 56 weeks (T3) from the time of inoculation of the reactors. DNA extraction and PCR (polymerase chain reaction) amplification (16S rDNA) were performed as previously described (Roest et al. 2005) using universal bacterial primers 0109F-T and 0515R-GC, i.e. forward primer (5'-CGC CCG GGG GGC GGC CGG GGG GCA CGG GGG G ATC GTA TTA CCG CGG CTG CTG C-3') and reverse primer (5'-ACT GCT CAG TAA CAC GT-3'). For DGGE analysis a DCode system (Bio-Rad, Hercules, CA, USA) was used with a 20–80% denaturing gradient (100% denaturant consisting of 7 mol/L urea and 40% (v/v) formamide). 8% (w/v) polyacrylamide gels (acylamide:bisacrylamide, 37.5:1, gel stock solution; Sigma, USA) in 1×TAE (40 mmol/L Tris, 20 mmol/L acetic acid, 1 mmol/L EDTA, pH 8.3), containing 0.11% (wt/v) APS (ammonium persulfate; Sigma) and 0.1% (v/v) TEMED (N,N,N,N'-tetra-methylmethylenediamine; Sigma). Gels were run at 60 C at 200 V for 5 min, then 85 V for 16 hours and were silver stained prior to imaging with a Fluor-S Multi-Imager (Bio-Rad, Hercules, CA, USA).

The DGGE gels were scanned and then analysed using GelCompar II software (Applied Maths, Belgium). Gel bands were identified and then compared in terms of intensity and position. To correct for variations in successive DGGE gels, a standard marker sample was run with each gel, this being used to normalise the banding intensities and positions. Densitometric curves were calculated for each gel track using a best-fitting Gaussian algorithm for each band.

Microbial ecological analysis of the DGGE band profiles (bacterial communities) were analysed to evaluate the diversity of bacterial communities operated at different temperatures over time. Band based nearest-neighbour correlation methods were applied using the Dice index of similarity, $C_s = 2j/(a + b)$ where $j$ is the number of common bands between samples A and B; $a$ and $b$ are the total number of bands in samples A and B, respectively; a UPGMA (average linking method) was used as the hierarchical clustering method. Community dynamics were analysed by calculating population richness and microbial clustering (Marzorati et al. 2008). The range-weighted richness ($R_k$) was calculated over time from the bacterial DGGE biofilm profiles using $R_k = N^2 \times D_p$. Here $N$ is the total number of bands in the profile (lane) and $D_p$ is the denaturing gradient between the first and the last band of the pattern.

RESULTS AND DISCUSSION

The development of voltage and anodic biofilms over time with different substrate types

The development of electrogenic activity was monitored over time for each of the substrate fed reactors (Figure 1). The lag-time to attain steady-state voltage was 2–3 weeks for the acetate fed reactor but was 12–14 weeks for both the butyrate and sucrose fed reactors. The acetate fed reactor also produced a significantly higher voltage, by a factor of 1.6, and stabilised at 0.49 (±0.02 V). The sucrose and butyrate reactors achieved steady-state voltages of ∼0.28 and 0.26 V respectively. When the influence of acetate and butyrate substrates in single chamber MFCs have been previously compared by Liu et al. (2005) it was found that power levels were up to 66% higher in acetate fed reactors; a figure comparable with the results in this study.

To quantify the growth of anodic biofilms in each substrate fed reactor over time, levels of DNA (ng L⁻¹) were measured as an indicator of biomass development (Figure 2). The results showed that sucrose produced the highest levels of biofilm with total biomass levels peaking at 840 ng L⁻¹ after 33 weeks’ operation. Acetate biomass levels continued to increase over the test period of 56 weeks but biomass levels in the butyrate reactor decreased over time showing that butyrate metabolism could not sustain high bacterial population levels. This suggests that fermentative metabolism could energetically sustain the production of high levels of bacteria/biomass, which then

Figure 1 | Voltage development during the enrichment of MFC reactors using three different substrates (butyrate, sucrose and acetate). Reactors were incubated at 35 °C and batch fed on a 1 week cycle with 5,000 ppm COD L⁻¹.
allowed this biomass to increase to a point at which cellular production was matched by biofilm losses due to cellular death and sloughing. This indicates that biomass production was a significant electron sink in the sucrose acclimated biofilm (Lee et al. 2008). In contrast, the acetate biofilm exhibited a more efficient extracellular electron transfer to the anode as demonstrated by the higher voltages produced (Figure 1).

**Community development with different substrate types and operational temperatures**

DGGE profiles were used to investigate the development of the bacterial community profiles over time (Figure 3). Early sucrose bacterial profiles (T1) clustered with butyrate biofilm communities but then further developed (T2 and T3) to cluster with the acetate fed reactor communities. It is likely that after the first sample time at 8 weeks the sucrose biofilm selected for EAB that could utilise VFA metabolites produced by fermentation, particularly acetate. Acetate has been previously found to produce high current densities in MFCs, notably through the selective enrichment of *Geobacter* spp. (Nevin et al. 2008; Chae et al. 2009) and this can explain the rapid development of electrogenic activity in the acetate reactor (Figure 1). *Geobacter* spp. are able to directly oxidise acetate to carbon dioxide, water and electrons, but electrogenic activity associated with butyrate degradation is reliant on syntrophic relationships between both bacteria and archaea (Schink & Stams 2005). This means that the capacity for the biofilm acclimation is likely to be more limited with butyrate as a substrate than is the case with acetate, which may account for the low biomass levels observed in Figure 2. It is interesting to note that incubation at 20°C, using acetate as a substrate, produced a bacterial community profile distinct from that produced by the 35°C incubation.

When range-weighted richness values were examined it was found that initial scores were low in the sucrose reactor biofilm but these then increased to high values of >200; however the butyrate scores stayed low and decreased with time (Table 1). Marzorati et al. (2008) reported that a richness score of >30 is indicative of very habitable environments, with scores of 10–30 indicative of a medium level of richness. This result shows the development of very diverse populations in both the acetate and sucrose fed biofilms, with a higher diversity in the sucrose reactor probably reflecting the diverse fermentative and VFA metabolic pathways necessary for sucrose oxidation.
The effect of substrate type on acclimated biofilm performance

Power density curves were measured using acetate, sucrose and butyrate as substrates in acetate, sucrose and butyrate acclimated MFC reactors; these were measured after 83 weeks’ operation (Figure 4).

Acetate as a substrate produced the highest power in each of the different substrate acclimated biofilms, this was higher by a factor of 7 in the acetate acclimated reactor when compared to acetate addition to sucrose and butyrate acclimated reactors (Figure 4(c)). When butyrate was added to the sucrose acclimated biofilm this produced power densities comparable to that of acetate (1 and 1.1 W⁻³ respectively), both recording higher power densities than sucrose itself (0.6 W⁻³). This indicates that the mixed bacterial population in the sucrose biofilm was able to metabolise both butyrate and acetate effectively, but the wider range of mixed VFAs produced during the fermentative metabolism were not able to be metabolised as effectively by the EAB present (Figure 4(a)). The butyrate biofilm (Figure 4(b)) using butyrate as a substrate produced the lowest maximum power measurement (0.3 W⁻³) compared to sucrose and acetate substrates added to sucrose and acetate acclimated biofilms respectively (0.6 and 7 W⁻³, respectively). This further demonstrated that

Table 1 | Richness scores at three time points using three different substrates (sucrose, butyrate and acetate)

<table>
<thead>
<tr>
<th>Time (weeks)</th>
<th>Sucrose</th>
<th>Butyrate</th>
<th>Acetate</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>25.9</td>
<td>32.9</td>
<td>267</td>
</tr>
<tr>
<td>33</td>
<td>363</td>
<td>15.6</td>
<td>267</td>
</tr>
<tr>
<td>56</td>
<td>284</td>
<td>13.8</td>
<td>230</td>
</tr>
</tbody>
</table>

Figure 4 | Power density curves using three different substrates (sucrose, butyrate and acetate) in three different substrate acclimated reactors with (a) sucrose, (b) butyrate and (c) acetate.
butyrate acclimation produced low levels of biofilm/biomass that could not energetically sustain high numbers of EAB and high rates of anaerobic respiration. It is likely that high numbers of EAB in the acetate acclimated biofilm were able to readily metabolise acetate, as shown by the high acetate substrate power measurement in Figure 4(c), with both sucrose and butyrate producing power readings of $\sim 0.5 \text{ W m}^{-3}$. A butyrate power density of $\sim 0.5 \text{ W m}^{-3}$ with the acetate acclimated biofilm was observed to be half that recorded in the sucrose acclimated biofilm ($1.0 \text{ W m}^{-3}$), suggesting that a wide range of syntrophic associations associated with sucrose electrogenic metabolism were not available within the acetate biofilm.

The differential metabolic activities of the sucrose, butyrate and acetate acclimated biofilms were further observed by measuring COD removal rates, these were calculated as the % COD loss over each 1 week batch cycle (Table 2). These results were observed to vary from power density results (Figure 4) as the values related to overall metabolic activity i.e. including electrogenic and non-electrogenic activities such as methanogenesis. CEs of the 35°C sucrose, butyrate and acetate reactors (T2) were 3.9, 8.1 and 3.12% respectively. Low CE values were caused by high substrate loading rates and the ingress of oxygen due to evaporation losses which occurred at the 35°C operational temperature. Acetate and butyrate acclimated biofilms exhibited a reduced sucrose COD removal compared to the sucrose acclimated biofilm probably due to the number of fermentative microorganisms being a limiting factor. The acetate COD removal rates for butyrate and sucrose acclimated biofilms were only 22.6 and 19.6% respectively, again showing that these biofilms lacked EAB that could directly oxidise acetate by using the carbon electrode as an external electron acceptor.

In Figure 5 sequential low temperature perturbations were used as a mechanism to test the functional

![Figure 5](https://iwaponline.com/wst/article-pdf/67/11/2568/440452/2568.pdf)

**Figure 5** Voltage development profiles of three differentially acclimatised MFC reactors using three different substrates (sucrose, butyrate and acetate), with acetate reactor acclimated at both 20 and 35°C. Reactors incubated at (a) 10°C, (b) 20°C and (c) 35°C.

**Table 2** The % COD drop over a 1 week batch cycle using three different substrates (sucrose, butyrate and acetate) in three different substrate acclimated biofilms (sucrose, butyrate and acetate).

<table>
<thead>
<tr>
<th>Acclimated reactor</th>
<th>Substrate</th>
<th>% COD drop</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate</td>
<td>Acetate</td>
<td>87.5</td>
</tr>
<tr>
<td>Butyrate</td>
<td>Acetate</td>
<td>22.6</td>
</tr>
<tr>
<td>Sucrose</td>
<td>Acetate</td>
<td>19.4</td>
</tr>
<tr>
<td>Acetate</td>
<td>Butyrate</td>
<td>41.6</td>
</tr>
<tr>
<td>Butyrate</td>
<td>Butyrate</td>
<td>41.7</td>
</tr>
<tr>
<td>Sucrose</td>
<td>Butyrate</td>
<td>58.2</td>
</tr>
<tr>
<td>Acetate</td>
<td>Sucrose</td>
<td>57.1</td>
</tr>
<tr>
<td>Butyrate</td>
<td>Sucrose</td>
<td>54.1</td>
</tr>
<tr>
<td>Sucrose</td>
<td>Sucrose</td>
<td>80.3</td>
</tr>
</tbody>
</table>
robustness of the differentially acclimated biofilms. From stable initial working voltages (as per Figure 1) each reactor was sequentially subjected to 24 hours at 10°C, 20°C and then 35°C; all metabolic responses were then monitored in terms of electrogenic activity (cell voltage). It was found that whilst all the 35°C acclimated reactors were adversely affected by low temperature operation (at 10°C), only the sucrose reactor was not able to recover electrogenic activity once the temperature was raised back to 35°C. Only the acetate reactor acclimated at 20°C was not affected by low temperature MFC operation.

It has been previously reported that microbial diversity and functional redundancy can help microbial systems withstand perturbation events (Briones & Raskin 2003). Whilst acetate had a very high bacterial diversity score (Table 1) and recovered activity quickly after the temperature was raised to 20°C (Figure 5(b)), sucrose also had a high diversity score but lost activity at 35°C, with the butyrate reactor also regaining activity despite a low diversity score. This may seem anachronistic but could be explained in the context of the levels of diversity in different levels of trophic groups that are key to overall EAB activity, measured in this case as voltage output. Where acetate biofilms have a high inherent diversity in the singular metabolism of acetate, the sucrose biofilms have a potentially greater number of functional trophic steps which individually may not have a high degree of diversity (fermentation, syntrophic breakdown of different VFA products to acetate, acetate conversion to electrons) and may also be subject to rate-limiting steps; this therefore may have had a direct impact on the electrogenic system’s capacity to deal with any perturbation event. Indeed, it has been reported that bacteria such as propionate degrading syntrophs can be affected by low temperature operation (Langenhoff & Stuckey 2000), which could then impact on overall electrogenic activity.

Whilst it may be possible to acclimate anodic biofilms for robust MFC operation over the cyclic temperature ranges typically found in temperate climates, it seems that complex bacterial interactions in complex substrate types may make these systems more vulnerable to environmental shocks (Figure 5). This may have implications for scaled-up systems using real wastewaters, indeed Cusick et al. (2011) reported that it was necessary during enrichment to add additional heat and acetate to a 1,000 L bioelectrochemical system designed to produce hydrogen from winery wastewaters.

CONCLUSION

This study demonstrates that substrate type and temperature have a dramatic effect on microbial community dynamics, the level of biofilm development and EAB activity. The trophic composition of the anodic biofilm was dictated by the substrate feed type and the capacity for microorganisms to develop syntrophic associations to degrade substrates of different complexities. The complex nature of these interactions was further demonstrated by low temperature perturbation of the acclimated biofilms, where sucrose biofilms were found to be susceptible to this type of perturbative shock. However, biofilm acclimation at sub-mesophilic temperatures may provide a strategy to extract electrical energy from mixed wastewaters in temperate climates.

ACKNOWLEDGEMENTS

This research was funded by the RCUK Energy Programme, SUPERGEN Biological Fuel Cell project (EP/D047943/1) supported by grant 68-3A75-3-150; The Low Carbon Research Institute (HEFCW). The Energy Programme is an RCUK cross-council initiative led by EPSRC and contributed to by ESRC, NERC, BBSRC and STFC.

REFERENCES


