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Full Length Research Paper

Performance improvement of whey-driven microbial fuel cells by acclimation of indigenous anodophilic microbes

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Various methods are available for the recycling and treatment of cheese whey with the objective of enhancing sustainable manufacturing. Currently, an increasing interest is on the anaerobic bioremediation of whey with the added benefit of generating electricity in microbial fuel cells (MFCs). Since microorganisms are the biocatalysts in MFCs, their initial density plays a paramount role both towards electricity generation and bioremediation. Hence, this study was aimed at evaluating the effects of anode enrichment with microorganisms on power generation. Anodes were enriched with microorganisms inherent to whey for periods of 30 and 90 days before their application in whey-powered MFCs. At the termination of reactor cycles, the one-month-old pre-incubated anodes had 0.13% coulombic efficiency (ε_{cb}), 88.3% total chemical oxygen demand (tCOD) removal efficiency and maximum power density (P_d) was 29.1 ± 4.9 W/m^2, whereas the three-month-old pre-incubated anode had ε_{cb} = 80.9 and 92.8%, tCOD removal and maximum P_d was 1800 ± 120 W/m^2. Two non-acclimated anodes used as control in separate setups exhibited 0.17% coulombic efficiency, 71.6% tCOD removal and maximum P_d of 30.9 ± 4.2 W//m^2. Microscopy analyses revealed different morphologies on anode surfaces depending on the length of the enrichment periods and further molecular analyses of electrode communities indicated up to 92% identity to various species from the *Lactobacillus* genus. This study established that, an initial acclimation step ahead of MFC setups significantly improved the performance of reactors utilising live cheese whey as fuel.

Key words: Cheese whey, microbial fuel cell, enrichment, alternative energy, bioelectricity, bioremediation.

INTRODUCTION

Large volumes of cheese whey generated annually pose a serious environmental threat due to its high total chemical oxygen demand (tCOD). It was estimated that, 6-8 x 10^7 t of whey were produced on a global scale (Stevens and Verhé, 2004; Peters, 2005). The booming cheese industry has fostered research toward several practical strategies to mitigate the impact of whey discharge into the environment. These include the manufacturing of whey cheese (whey to cheese); the direct application of liquid whey on farms to enhance the crop yield; feed for livestock; fungicide along with herbicide formulations (Lambou et al., 1973; Philippopoulos and Papadakis, 2001; Saddoud et al., 2007). In addition, whey can be filtered, condensed and dried to extract its proteins, solids and fats for food supplements (Arvanitoyannis and Kassaveti, 2008; Souza et al., 2010). However, the cost of recovery of whey constituents may not necessarily be economically profitable (Peters, 2005). Furthermore, excessive application of whey to farm lands leads to accumulation of elements such as phosphorus and potassium that cause serious pollution both to ground and surface water. Additionally, excess minerals in the soil change the delicate salt balance for crops leading to growth abnormalities (Watson et al., 1977).

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Abbreviations: MFCs, Microbial fuel cells; **tCOD,** total chemical oxygen demand; **SEM,** scanning electron microscopy; **DGGE,** denaturing gradient gel electrophoresis.

Recently, efforts have been directed towards the anaerobic treatment of whey in MFCs with the advantage of generating electricity (Antonopoulou et al., 2010; Kassongo and Togo, 2010). Antonopoulou et al. (2010) managed to generate a maximum power density of 18.4 mW/m^2 using diluted whey in double-chambered MFCs operated in fed-batch mode. In our previous work (Kassongo and Togo, 2010), we demonstrated that the introduction of *Enterobacter cloacae* subspecies *dissolvens* in sterile raw whey increased the maximum power density by 15-fold (from 1.1 W/m^2) corresponding to a partial and variable COD removal of 5%. It is however, worthwhile to maximise power from whey using its native microflora, while achieving sustainable waste-water treatment. Therefore, the purpose of this work was to improve power generated from whey and its native microflora through enrichment of the microorganisms on the anode before operation of the microbial fuel cells (MFCs).

MATERIALS AND METHODS

MFC design

The H-type MFCs were used as described by Nambiar et al. (2009). Nine sets of double chambered mediator-less MFCs were assembled and each filled to 200 ml with whey in the anode compartments. A Nafion™ 117 membrane of 2 cm in diameter was inserted halfway of the proton exchange membrane bridge (6 cm total length). The catholyte and the operational parameters remained as previously described (Kassongo and Togo, 2010). Whey was collected from Greenways Delli (Kyalami, Johannesburg, South Africa) on the day of experimental start-ups.

Anodes were stored under anoxic conditions for thirty (30) (one-month-old) and ninety (three-month-old) (90) days in whey replaced every three days based on our previous studies which unveiled the most rapid depletion of substrate during the first three days of a batch cycle. This pre-incubation was conducted to (1) enrich that is, promote microorganisms' adhesion on electrodes; (2) investigate the stage of optimum biofilm density for high power generation and bioremediation in MFCs. The power density generated by the devices was calculated by $P_d=VI/A$; where, V is the voltage; I is the current and A is the anode surface area (Huang and Angelidaki, 2008a). Both efficiency of electron transfer to the anode surface (coulombic efficiency (ε_{cb})) and substrate degradation rate (SDR) were calculated as explained by Huang and Angelidaki (2008b), Mohan et al. (2008) and Antonopoulou et al. (2010).

Pollutant analyses

The determination of tCOD was performed by incubating appropriately diluted whey samples with standard Merck COD Cell Tests in a Spectroquant® TR320 thermoreactor (Merck, Johan-nesburg, South Africa) at 148°C for two hours. Following cooling, the tCOD concentrations were then measured using the Merck Pharo 100 spectrophotometer.

Sulphates (SO_4^{2-}), nitrates (NO^-_3) and phosphates (PO_4^{3-}) concentrations were determined both before and after reactor cycles by separately mixing appropriately diluted volumes of samples with Hanna SO_4^{2-}, NO_3^- and PO_4^{3-} reagents, as per manufacturer's instructions and measured with use of HI83200 Multi-parameter Photometer (Hanna Instruments, Johannesburg, South Africa).

Biofilm confirmation

Electrodes were fixed with 2.5% (v/v) gluteraldehyde and progressively dehydrated in ethanol as previously described by Kassongo and Togo (2010). Subsequent to critical point drying and coating, electrodes were viewed under FEI Quanta 400E scanning electron microscope (SEM). A number of electrodes housing microorganisms at the same developmental stage, as those prepared for SEM, were spared for molecular microbial community studies.

DNA extraction

One- and three-month-old anodes and those in reactors following incubation were transferred to 2 mm glass beads and vigorously shaken for 5 min to dislodge cells from surfaces. The medium was centrifuged at 10 000 rpm for 1 min, the supernatant was discarded and the pellet was re-suspended then mixed with Zymo Research ZR Fungal/Bacterial DNA MiniPrep™ kit (Inqaba Biotechnical Industries, South Africa) as per manufacturer's instructions to prepare ultra-pure genomic DNA templates for PCR.

PCR-amplification

For amplification, one tube contained: 25 µl of 2 X PCR Master mix (Taq DNA polymerase in reaction buffer, $MgCl_2$ and dNTPs - 0.4 mM of each), 1 µl each for reverse and forward primer 16S rDNA (UNIV1392R: 5'-ACG GGC GGT GTG TRC-3', EUB968F AAC GCG AAG AAC CTT AC with GC clamp), 22 µl DNase and RNase-free water (Fermentas, USA) and 1 µl of the extracted DNA aliquot. A second tube was incubated with all the components for PCR, but without the whey-isolated DNA in order to check for any possible contaminations which may have occurred in the course of experiments. Applied Biosytems 2720 Thermal Cycler was used for PCR and set at the following parameters: initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation (30 s at 94°C), annealing (45 s at 60°C and extension, 90 min at 72°C) and a final extension at 72°C for 7 min before storage at -4°C. The products were electrophoresed on an agarose gel to confirm their presence, quality and purity (Jong et al., 2006).

Denaturing gradient gel electrophoresis (DGGE)

The goal with DGGE was to examine the microbial diversity on the anode biofilms of whey by running a mix of amplified 10 µl PCR products and 5 µl of the DGGE loading dye in 6% PAG at 130 V, 60°C for 4 h. After that the gel was stained in 250 ml 0.5 X TAE buffer (10 $mg.ml^{-1}$ ethidium bromide) for 15 min followed by a distaining for 15 min in 0.5 X TAE buffer without ethidium bromide (Muyzer et al., 1993). A Gel Doc system was used to view the gel under UV light and help in the cutting out of dominant bands which were seen after immersed in 50 µl TE buffer at 4°C overnight to remove the DNA template from the DGGE gel (Lee et al., 2003). A second re-amplification as specified earlier (without GC clamp on the forward primer) followed by another agarose gel electrophoresis were performed to confirm presence of DNA before sequencing (Inqaba Biotechnical Industries, South Africa) (Azbar et al., 2009).

Sequencing

Sequences received from Inqaba Biotechnical Industries (South Africa) were edited with FinchTV and a nucleotide BLAST search for highly similar sequences was performed in the NCBI database. The strongest matches obtained were used together with the

sequence of the isolate to construct the phylogenetic tree (not sown) in the DNAMAN sequence analysis software.

RESULTS

Raw effluent profile

The parameters measured initially were: 93.2 ± 0.4 g/l tCOD, 1.30 ± 0.1 g/l PO_4^{3-}, 50 ± 0.0 mg/l SO_4^{2-}, 72.6 ± 10.2 mg/l NO_3^-, 6.13 mS conductivity and pH 6.46 ± 0.19.

Electricity generation

One-month-old anodes produced insignificantly different ($P > 0.05$) maximum power density (29.1 ± 4.9 W/m^2) from that of the control (30.9 ± 4.2 W//m^2), as shown in Figure 1. However, the power from the former anode was more stable than in the control during the first three days (Figure 2). The three-month-old anode produced the highest and most stable power (1800 ± 120 W/m^2) (Figures 1 and 2). When compared at day 3, power densities decreased by 42.4, 74 and 14.2% in the control, one-month and three-month pre-incubated electrodes, respectively. To compensate for the volume lost to evaporation in the cathode compartment, this long operation time necessitated the addition of fresh catholyte every two weeks, resulting in a slight surge of power which soon returned to previous measured levels.

Substrate degradation

At the termination of reactor cycles, the final tCOD for one- and three-month-old anodes were 10.9 ± 1.9 and 6.8 ± 0.2 g/l, respectively while sulphates within the three-month-old anode setups remained unchanged, the phosphates levels doubled (2.9 ± 0.1 g/l). However, there was a significant ($P < 0.05$) increase in sulphate levels and no significant phosphates change in setups using one-month-old anodes (Figure 3). Most notable were the high nitrates levels across all the reactors, regardless of anode types used (Figure 3). Assuming that, ionic transport and oxygen diffusion across the Nafion^{TM} stayed constant, the SDR was calculated to compensate for the variable operation time of reactors. The reactors with the one-month-old anodes had the fastest rate of whey decomposition (SDR = 2.47 kg COD/m^3 day) and the lowest coulombic efficiency (ε_{cb} = 0.13%) when compared with the three-month-old anode (SDR = 0.06 kg COD/m^3 day; ε_{cb} = 80.9%). The control reactor had an SDR of 0.87 kg COD/m^3 day and ε_{cb} = 0.17%.

Scanning electron microscopy

There was a direct relationship between electrode exposure period and biofilm density on all electrodes (Figure 4a). Extra polymeric substances (EPSs) was observed (Figure 4b), pointing out to biofilm formation. While there were essentially separate rod-shaped cells and scattered cocci on all the one-month anodes after residence in MFCs, the three-month-enriched anode was exclusively made of filament-like structures resembling a few rod-shaped cells put end to end (Figure 4d). Irrespective of their acclimation periods, all the anodes had developed visible aggregates of microorganisms (clumps) after incubation in MFCs. In the case of the one-month-old anodes, the envelope of such clumps had not yet reached maturation and was open at many regions, thus, revealing that, extra-polymeric substances held bacteria together. Conversely, clumps characterized by a well-defined ovoid margin were seen on the three-month old anode (Figure 4e, f).

Molecular ecology

A DGGE analysis revealed similar patterns of brightness on all the lanes that were loaded with the PCR amplicons from the one- and three-month-old anodes before and after residence in MFCs (Figure 5). Two bands were visible in all the lanes. Both sequenced bands shared a variable identity (92 and 84%, bands 1 and 2, respectively) with same species within the *Lactobacillus* genus.

DISCUSSION

Residence of anodes in cheese whey for a period of three months before MFC cycles allowed for an enrichment of microorganisms, including exo-electrogenic microbes (capable of an electron transfer to anode surfaces) which then resulted in an elevated maximum power density (1800 W/m^2) when compared with those of anodes pre-incubated for one month (29.1 W/m^2). This technique built up the density of biofilm for a stable electricity generation (Lee et al., 2003). The lack of significance in maximum power densities achieved by the one-month-old anodes when compared with the control anodes (not acclimated for enrichment) suggests that there should be a threshold for acclimation to yield desirable power outputs. However, differences in power stabilities between the control and one-month electrode (Figure 2) shows the importance of microbial colonisation of the electrode if high power densities are to be sustained over a prolonged period.

The power generated in MFCs with one-month and control anodes indicated the presence of electron shuttles (mediators) in whey because electron-mediated transport is generally fast and can compensate for the little or lack of acclimation period (Samrot et al., 2010). Despite the relative similarity of maximum power densities

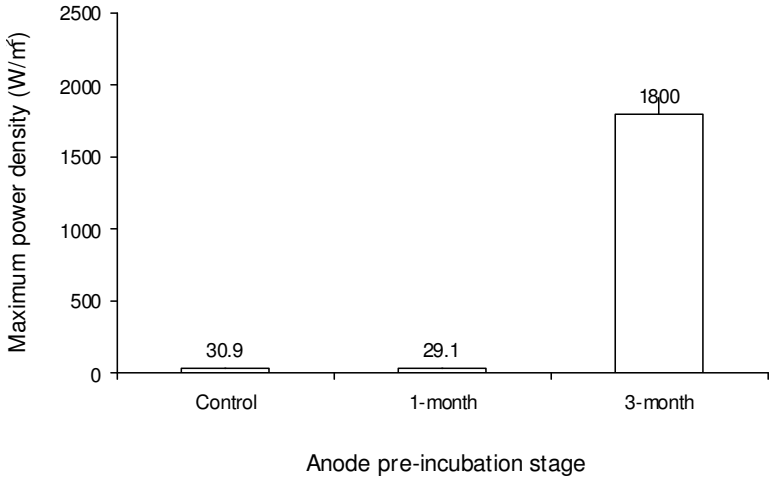

Figure 1. Maximum power densities (W/m^2) from reactors with the anodes subjected to different periods of biofilm build-up. In the control setup, the anode was directly used without prior immersion in the whey.

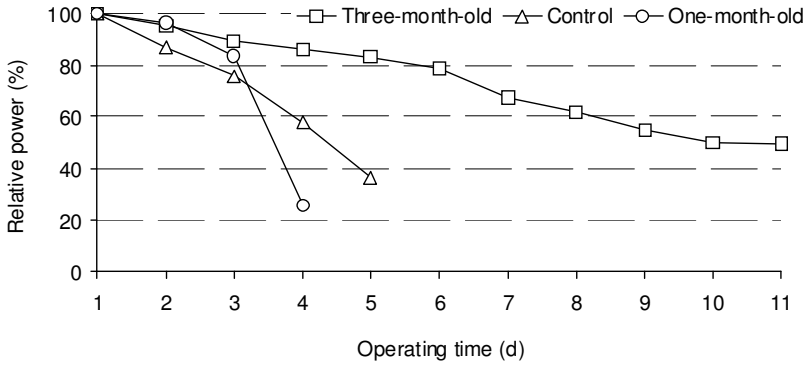

Figure 2. Relative power density (%) stability in microbial fuel cells with different anode treatments. The 100% power densities for MFCs with the control, one-month and three-month anodes were 30.9 ± 4.2, 29.1 ± 4.9, and 1 800 ± 120 W/m^2, respectively.

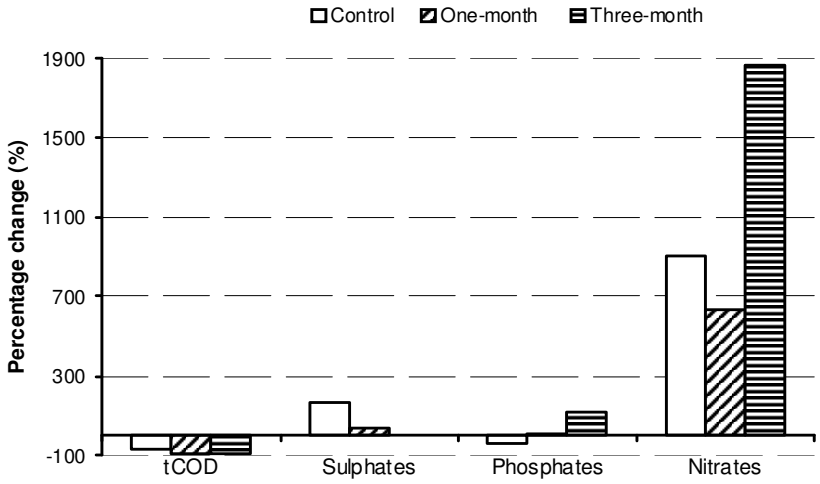

Figure 3. Relative changes (%) in selected parameters from anode effluent of whey-driven MFCs with different anode treatments.

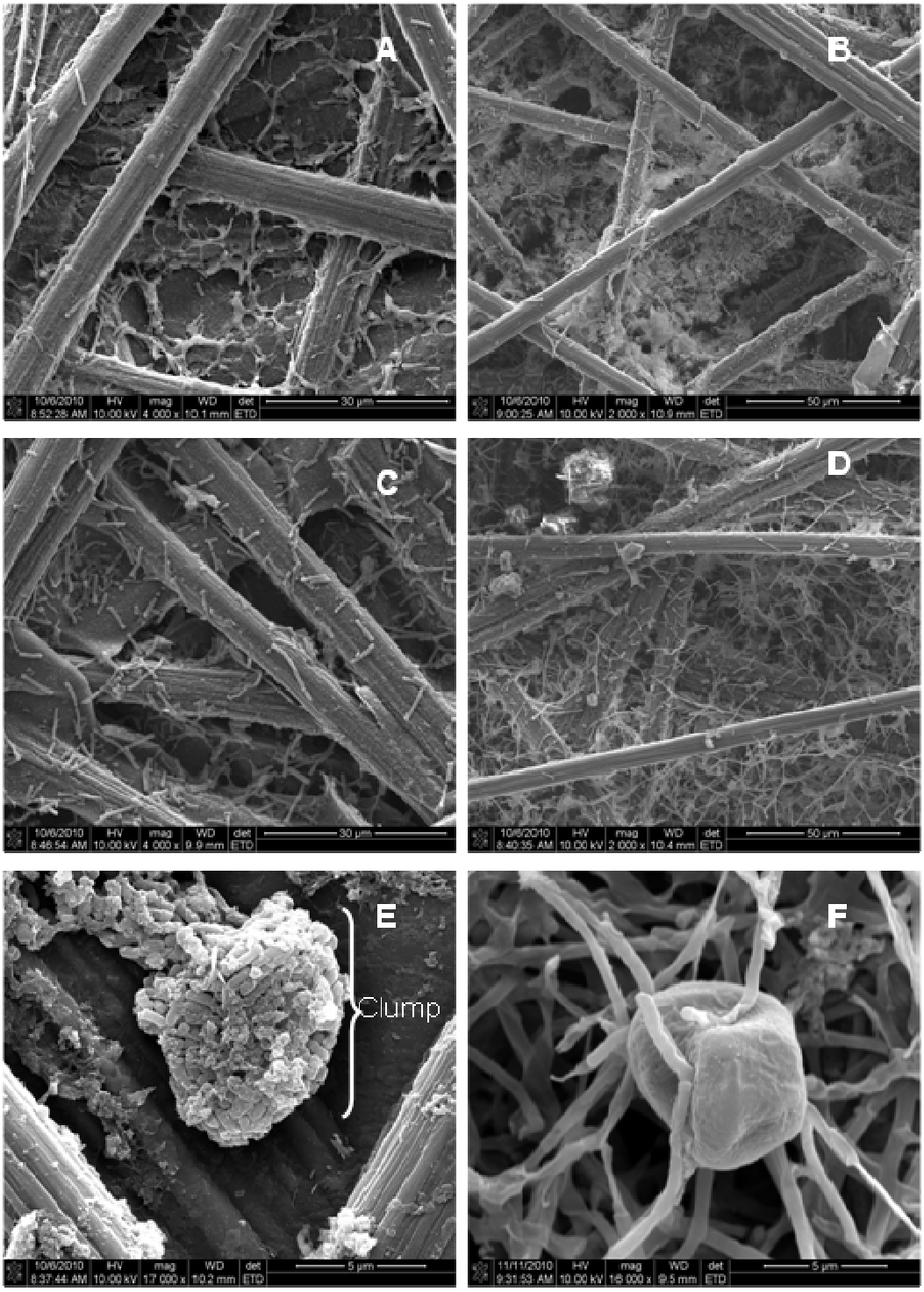

Figure 4. Scanning electron micrographs of anode surfaces for one (A) and three (C) months pre-incubation before MFC set up and respectively for same treatments (B) and (D) after use in the MFCs. E and F further respectively illustrate the morphologies of microorganisms on one- and three-month old anodes subsequent to incubations in MFCs.

between the one-month-old anodes and those of the controls, there were generally higher power values and a greater remediation over the period of operation with the reactors using one-month-old anodes. A linear and steady decrease of power density which corresponded to substrate depletion was achieved by the three-month-old

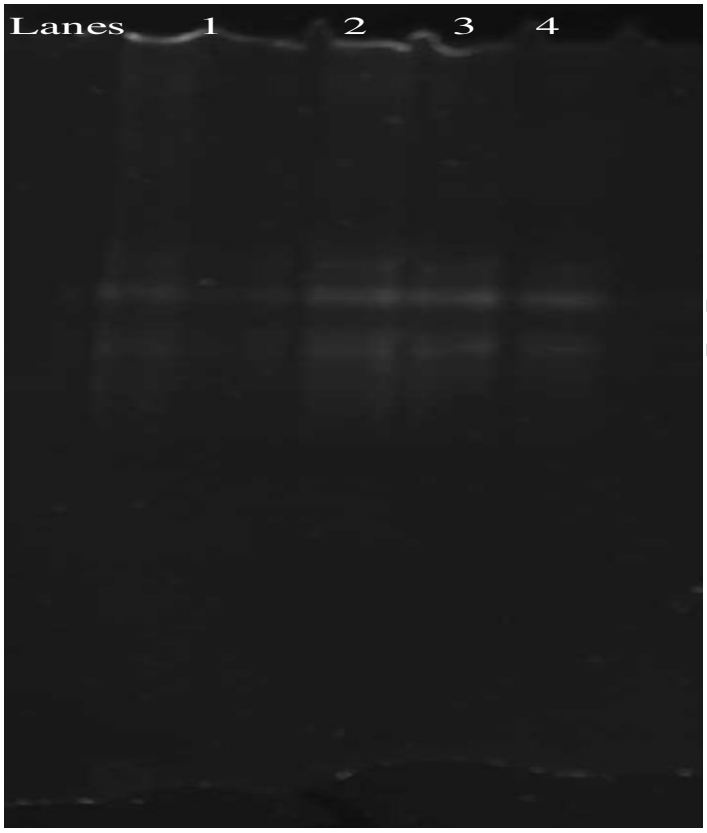

Figure 5. A DGGE profiles of one- and three-month-old anodes before and after MFCs cycles. Lanes 1 and 3 have samples from one-month anode, while lanes 2 and 4 have samples from the three-month anode, both respectively before and after reactor setup

anode in a MFC. Such a pattern seen for the initial 24 h corresponds to the adaptation phase of the catalytic interface of the anode when transferred to a fresh medium. In comparison, this behaviour is much like the incubation of a non-acclimated anode which is characterized by a lag phase followed by an exponential increase of power generation in a MFC operated in batch mode (Nambiar et al., 2009).

One can argue that, the acclimation setup used in this study may promote methanogenesis and give a biofilm community structure antagonistic to electrogenesis (Ishii et al., 2008). However, the growth rate of methanogens is greatly reduced below pH 6.6 (Ward et al., 2008). The collected fresh whey for experiment was pH 6.46 ± 0.19, thus, positive for electrogenesis. Lactic acid bacteria contribute to this low pH through production of the acid. Furthermore, refining of the microbial anodophilic composition occurs based on operating conditions (such as pH and MFC configuration) in favour for electricity generation instead of methanogenesis (Ishii et al., 2008) and microbial consortia themselves evolve over time (Kiely et al., 2011).

High tCOD (93.2 ± 0.4 g/l) at the outset provided microorganisms with the organics needed and thus, substrate availability would not be limiting factor (Samrot et al., 2010). The high coulombic efficiency (80.9%) from the reactor with the three-month-old anode indicates that both electronic extraction and transfer to the electron acceptor surface were close to an ideal reactor performance. It could have been expected that this high tCOD removal efficiency (92.8%) would result in a very low coulombic due to an inversely proportional relationship (Kassongo and Togo, 2010). However, the substrate degradation rate (SDR) was very low (0.06 kg COD/m^3 day) indicating that the cumulative removal of the COD was achieved only because of anode and whey residence time in the MFC. Therefore, an even higher COD removal could have been achieved if the reactors ran for a longer period.

Depending on the duration of enrichment in acclimation, different patterns of ionic compounds (SO_4^{2-}, NO_3^- and PO_4^{3-}) generation and/or removal were observed in MFCs. It appears that, stable specialised degradation systems formed over time. The three-month-old anode may have housed adequate microbial colonies for utilisation of excess sulphate, whereas the one-month-old anodes were excellent to buffer variations of phosphates in reactors. Alternatively, it could be that the interspecies

distance between microbe-producing and microbe-consuming was either short or long with respect to their spatial distribution and their populations, thus, affecting removal of the metabolites (Trzcinski et al., 2010). It is interesting to note that the control reactors have higher sulphates and nitrates than those of the one-month-old anodes. Despite the possibility of such compounds acting as electron acceptors to decrease the maximum power density of the control, the sulphate levels were reduced and the coulombic efficiency of the control (ε_{cb} = 0.17%) was higher than that of the one-month-old anode (ε_{cb} = 0.13%); thus, maintaining the relative similarity of maximum power density between the two setup types.

Microorganisms aggregate and form specialised structures for the degradation of complex compounds (Lee et al., 2003). This explains the differences in densities and aggregate forms between films on the one- and three-month anodes. The DGGE results (although not core to the work) highlighted that, lactic acid bacteria were responsible for power generation and in-depth studies in this aspect is on going in our group. These will also answer the question of presence of spherical micro-organisms on the electrode is a result of environmentally induced morphological change of the electricigenic lactic acid bacteria or presence of other species. In general, the duration of acclimation and enrichment was of significance to the performance of MFCs. Other future studies will entail investigation of serial re-use of anodes across reactors, varying the external resistors of H-type MFCs.

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