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OPTIMISATION OF THE H–TYPE MICROBIAL FUEL CELL USING WHEY AS A SUBSTRATE

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A dissertation submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science

Johannesburg, 2011

DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the Degree of Master of Science at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

_____

Josué Kassongo

7th day of June 2011

ABSTRACT

A growing interest is on the biological remediation of pollutants with the added benefit of generating electricity in microbial fuel cells (MFCs). Therefore, the analyses of suitability and potential of full-strength paper mill effluent and cheese whey were separately investigated in such devices. The most promising effluent was selected for biofilm optimization studies. In the biofilm buildup studies, anodes were enriched with microorganisms inherent to whey for a period between one and three months before their application in reactors. Independently, pre-incubated electrodes which were two-month-old were used serially in four MFCs of seven days each. In the preliminary study, the maximum power densities were 24 ± 3 mW/m^2 (0.02 % coulombic efficiency − ε_{cb}) and 16.7 ± 1.8 W/m^2 (ε_{cb} = 3.7 %) in paper mill effluent and whey, respectively. Following a three-month acclimation of whey anodophilic microbes, the power increased to 1 800 W/m^2 (ε_{cb} = 80.9 %) and 92.8 % total chemical oxygen demand (tCOD) removal after a single batch cycle in MFCs. In anode recycling experiments, the operation was characterised by power of 390 ± 21 W/m^2 (ε_{cb} = 0.25 %) in the third anode reuse; whilst the second reactor cycle had the highest tCOD removal (44.6 %). The anodophilic microbial species identified in cheese whey were from the Lactobacillus genus. This study concluded that wastes can supply fuel for power generation with simultaneous remediation; whey had greater potential than paper mill effluent; and both continual acclimation of inherent waste microbes and anode recycling improved the performance of MFCs.

This work is dedicated to my father Jean-André Kassongo wa Kassongo (“The Superior man uncovers the Truth by asking questions. Ask questions, Josh”).

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LIST OF SYMBOLS

%	percent
cm	centimetre
d	days
g/l	gram per litre
h	hour
kg	kilogram
m	metre
mg/l	milligram per litre
min	minute
ml	millilitre
mm	millimetre
mM	millimolar
mV	millivolt
mW/m^2	milliwatt per square metre
nm	nanometre
NO^-_3	nitrate ions
^oC	degree Celsius
P_d	power density
PO^{3-}_4	phosphate ions
P_Y	power yield
sec	second
SO^{2-}_4	sulphate ions
t	ton
W/m^2	watt per square metre
Δ	change
ε_{cb}	coulombic efficiency
µl	microlitre
Ω	ohm

NOMENCLATURE

BLAST	Basic Local Alignment Search Tool
CEM	cation exchange membrane
DGGE	denaturing gradient gel electrophoresis
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
I	current
MFC	microbial fuel cell
NCBI	National Center for Biotechnology Information
PBS	phosphate buffer salts
PCR	polymerase chain reaction
R	resistance
SDR	substrate degradation rate
SDV	standard deviation
SEM	scanning electron microscopy
tCOD	total chemical oxygen demand
V	voltage

LIST OF OUTPUTS

Core work in the dissertation:

Kassongo, J. and Togo, C.A. (2010). The potential of whey in driving microbial fuel cells: a dual prospect of energy recovery and remediation. *African Journal of Biotechnology*. **9**: 7885-7890.

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Related work:

Carrim, M., **Kassongo, J**., Khumalo L.E. and Togo C.A. Potential for whey as a biological catholyte: a comparative approach. **(Submitted)**

Khumalo, L.E., **Kassongo, J**., Carrim, M. and Togo, C.A. Comparison of meat and potato chips industrial effluents in driving microbial fuel cells. **(Submitted)**

1 GENERAL INTRODUCTION

1.1 Global Landscape

Humankind has always been engaged in a relentless quest for energy to drive his daily activities. With the advent of industrial revolution in the eighteenth century, fossil fuels soon became the energy currency to drive the world's economy, thus constantly keeping up with the market pressures. However, anthropogenic activities such as intensive extraction by oil drilling rigs with subsequent refining, electricity generation by coal-based power plants, and the petroleum combustion in motor vehicles have all resulted in an adverse environmental impact and the depletion of crude oil reserves (Du *et al.*, 2007; *Cheng et al.*, 2008; Samrot *et al.*, 2010). In order to mitigate the toll on the planet and at the same time meet the financial objectives, renewable, sustainable and affordable alternative strategies (solar panels, wind mills, biodiesels and anaerobic-based treatment plants) for power generation are currently under development for massive industrial exploitation.

However, there is still need to explore for more energy sources to keep the negative impacts of fossil fuels to their strict minimum. This has seen diverse efforts including the current attempts to combine effluent remediation and electricity generation. Another environmental problem is the contamination of water reserves by industrial effluents.

1.2 Potential for Anaerobic-Based Treatments

Despite the considerable feats achieved such as water recirculation and reduction of pollutants prior to disposal, conventional treatments are limited by fouling of membranes and filters, poor dissolution of oxygen in wastewater and tremendous input of electricity in aeration tanks (Thompson *et al.*, 2001; Ahn and Choi, 2004; Murray *et al*., 2008; Ahn and Logan, 2010). On the other hand, the hallmark of anaerobic treatment of effluents has been the substantial reduction of sludge accompanied by a reduction of pollutants otherwise recalcitrant to aerobic treatments (Pant *et al*., 2010).

An increasing number of studies, within the field of renewable energy, have considered anaerobic treatment for generation of volatile fatty acids, biogases (Kaksonen *et al.*, 2003; Najafpour *et al*., 2009) and direct generation of electricity in MFCs (Nimje *et al*., 2009; Cha *et al*., 2010; Kassongo and Togo, 2010). Microbial fuel cells (MFCs), still in their infancy, hold great potentials for clean and sustainable electricity generation from industrial effluents with a simultaneous decrease of pollutants.

1.3 Microbial Fuel Cells: Working Principles

In practice, regardless of their changing designs, an MFC would consist of anode and cathode compartments kept apart by a cation exchange membrane (CEM) which behaves as a sieve for the passage of protons. Electrodes are then added in both compartments and connected in series to an external resistor to complete the circuit (Figure 1.1).

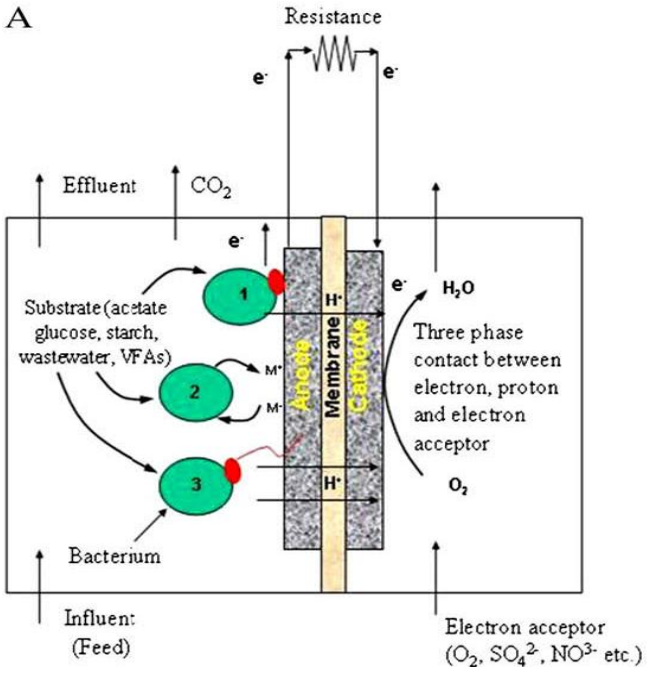


Figure 1.1 An illustration of a two-chamber MFC with possible modes of electron transfer. (1) Direct electron transfer (via outer membrane cytochromes); (2) Electron transfer through mediators; and (3) Electron transfer through nanowires (Ahn and Logan, 2010).

A liquid waste (anolyte), ordinarily municipality wastewater, is fed into the anode chamber to fulfill both carbon and electron requirements of biocatalysts (Greenman *et al*., 2009; Pant *et al*., 2010). It is imperative that the anode chamber remains anoxic because energy output would be greatly reduced in presence of a high-redox electron acceptor than the anode in the biological reactions chamber. But, the cathode chamber should be kept well aerated. For an external electron flow to occur, oxygen reduction at

the cathode will be matched by wastes oxidation at the anode. The first half-reaction in the anode chamber releases electrons, protons and various types of ions. A detectable potential difference is generated when electrons are transferred from the bacterial electron transport chain in its inner membrane to a higher redox acceptor molecule via either direct or mediated transport and conducted to the cathode externally; concurrently, protons migrate through the CEM to the anode chamber and participate in the reduction of dissolved oxygen to water (Lu *et al.*, 2009; Ahn and Logan, 2010). For this cycle to be re-generated, electrons should be constantly released upon microbial activity on substrate.

Theoretically, sugars, fats and proteins through glycolysis (anaerobic) can be converted to acetyl CoA. This molecule is then fed to the citric acid cycle for oxidation coupled to the reduction of NAD^+ and FAD which will become NADH and FADH (electron carrier molecules). These will then leave the cytoplasm to the cell membrane where intermediaries such as ATP synthase transmembrane protein will use some of the electrons to pump protons out of the cell and phosphorylate ADP to ATP (Samrot *et al*., 2010). By and large, bacteria only use a portion of their total coulombic yield toward metabolism and growth; excess electrons can thus be naturally attracted to a higher redox potential electron acceptor (mediators or anode) on the outer bacterial membrane to generate electricity. To illustrate, the fermentation of glucose follows two possible reactions:

(i) $C_6H_{12}O_6 + 2H_2O \rightarrow 4H_2 + 2CO_2 + 2C_2H_4O_2$

(ii) $C_6H_{12}O_6 \rightarrow 2H_2 + 2CO_2 + C_4H_8O_2$

As can be seen from either of the equations above, two-thirds of the initial carbon source (glucose) is transformed into a further reduced state such as acetate and butyrate and only one-third of the initial glucose can be used in electricigenesis (Rabaey and Verstraete, 2005).

A number of advantages make this nascent MFC technology ideal. In general, the simple designs of reactors which can be operated at ambient temperatures greatly reduce both the running and the maintenance cost. Despite their higher efficiency of matter-to-energy conversion, the power yield in such systems remains very low to compete with chemical fuel cells and other alternative energy sources. Therefore, various approaches have been forged to improve electricity output in line with a number of inherent biotic and abiotic factors (individually or in combination).

1.4 Intrinsic Effects of Process Parameters

1.4.1 System architecture

A single-chambered MFC, as the name suggests, has one compartment for the addition of effluents to power the device. Separators are used to keep anode and oxygen-filled cathode compartments apart; also separators accomplish the same function as a cathode. On the other hand, simpler and affordable double-chambered reactors are preferred for laboratory-scale studies (Logan and Regan, 2006). Single-chambered MFCs can perform about six-fold higher than their double-chambered equivalents (Min *et al*., 2005). However, such evidence is no cast in stone. For example, an H-type MFC was used for higher power generation in re-circulating the anolyte to reduce the pH gradient

between the two chambers and achieve greater effluent remediation while shortening the wiring of the external circuit for faster electronic flow (Freguia *et al*., 2008). Therefore, H-type MFCs have the potential of generating higher power densities than single-chambered ones. A peristaltic pump can also be added to the system in order to decrease the diffusion distance, thus favouring greater power output (Nam *et al*., 2010).

1.4.2 Separator

There are various separators employed to keep the anode and cathode chambers physically apart. Glass fibres are best because they are not biodegradable, high coulombic efficiency (ratio of electrons transferred to the anode over the theoretical maximum extractable in electricigenesis) and low oxygen diffusion into the anode chamber in order to attain an ideal potential difference across the system (Zhang *et al*., 2009). However, Nafion^{TM} 117 is used widely due to cost considerations (Li *et al.*, 2011).

1.4.3 Electrodes

Platinum and platinum-coated electrodes offer the advantage of lowering the high cathode over-potential (energy barrier on the cathode surface) for the reduction of oxygen (Freguia *et al.*, 2008). However, from a technical perspective, carbon electrodes are preferred due to their affordability, excellent conductivity and structural strength. In addition, the versatility offered by this material in coming as sheaths, rods, brushes and granules allows for its widespread applications (Logan *et al.*, 2007). Therefore, the goal has been to maximize the surface area which would support either adhesion of the

microorganisms or facilitate the reduction of oxygen. In fact, it has been established that the surface area of the cathode should be bigger than that of the anode. In that way, the cathode would offer a much larger reactive surface for the reduction of the dissolved oxygen which is a rate-limiting step of electricigenesis (Yazdi *et al.,* 2008). For long-term operation (one year and above), the cathode can be replaced for increased power (Kiely *et al*., 2010).

1.4.4 Biocatalysts

Adding supplementary pure cultures of microorganisms may positively impact on electricigenesis. There is a good degree of confidence that optimal waste oxidation by different microbes in conjunction with both intra- and inter-species secondary metabolite utilisations contribute to a better MFC performance. For example, when it comes to cellulose-rich wastes, anode chambers will generally contain a consortium of both cellulolytic and electricigenic cultures. It appears that such a binary association is relevant since cellulolytic microbes may not necessarily transfer electrons to electrodes. In the same light, cellulolytic microbes readily generate monosaccharides which can be utilized by adjacent electricigens (Logan and Regan, 2006; Du *et al.,* 2007; Ahn and Logan, 2010). Besides, high-strength effluents have an elevated mediator-to-bacteria ratio and nutrients are not limiting for microorganisms. The start-up concentration of microorganisms (biocatalysts) is of key importance in reactors with a steady state of electricity generation (Masthuriya *et al.*, 2009; Larrosa *et al.*, 2009).

1.4.5 External resistor

Changing the external resistor affects the microbial community structure on anodes and may render anode reduction of oxygen by some microbial reductases thermodynamically unfavourable, thus suppressing growth (Lydon *et al*., 2010). Ideally, the external resistor should have a value close to that of the internal resistance in order to achieve higher power output (Kiely *et al*., 2010).

1.4.6 Oxygen diffusion

Stirring the doubled-chambered reactor improves protons and dissolved oxygen transport to the cathode; also reduces substrate gradient and favours mass transport to the anode (Min *et al.,* 2005). However, excessive stirring of catholyte can lead to diffusion of oxygen into the anode compartment accompanied by low coulombic efficiency and subsequently decreased power densities. Interestingly, there is a compromise between oxygen diffusion into the anode compartment and the power output. In effect, oxygen intrusion into the anode compartment may allow for the growth of microbes which are not directly involved in electricigenesis, but which can be essential link in substrate degradation and/or electronic transfer (Kiely *et al*., 2010).

1.4.7 Buffer

Anaerobic degradation in the anode chamber is accompanied by the generation of fermentation products which may further increase the pH gradient between the compartments of an H-type reactor. Either bicarbonate or PBS can be added for stable or increased electricity output (Nam *et al*., 2010). However, elevated concentrations of

bicarbonate can encourage the growth of methanogens that are antagonistic to electricigenesis. Ideally, the appropriate buffer to use is the one with a pKa similar or close to the pH of the anolyte. At the other end of the spectrum, low buffer strength may favour the growth of methanogens and limit the activity of electricigens (Cha *et al*., 2010). A buffer like PBS in the anode chamber behaves like a carrier of protons across the CEM, thus decreasing both diffusion and electronic migration rates. This mechanism reduces the internal resistance caused by proton polarisation in the anode chamber and results in higher power densities (Fan *et al*., 2007).

1.4.8 Catholyte

Due to its high solubility, ferricyanide is the most common catholyte (aqueous solution added to the cathode chamber) to mediate oxygen reduction in the cathode chamber. However, ferricyanide successfully competes against oxygen as an active electron acceptor because of its faster reaction kinetics and comparatively greater redox potential (Yazdi *et al.*, 2008). A strategy to curb this competition would be to consistently add oxygen and remove its products (water and hydrogen peroxide) from the cathode chamber, thus minimizing the negating effect of feedback inhibition.

1.4.9 Substrate

There is a large and virtually endless base of substrate utilization (Pant *et al*., 2010): swine manure (Min *et al.*, 2005), paper mill effluent (Huang and Logan, 2008), corn stover (Wang *et al.*, 2009), dairy (Masthuriya and Sharma, 2009; Antonopoulou *et al.*, 2010), municipal wastewater (Cha *et al.*, 2010), and brewery effluent (Wen *et al.*, 2010).

It is commonly accepted that anaerobic treatments generate less sludge and offer an excellent containment strategy for noxious and fouling gases such as methane (Ward *et al*., 2008). The hallmark of MFCs, regardless of their fuel types, is that they directly convert organic matter to electrical energy without any transitory steps of purification or energy input.

1.4.10 Temperature

In the case of complex wastewaters, mesophilic temperatures can enhance the performance of MFCs when compared to ambient (room) temperatures (Ahn and Logan, 2010). However, increasingly high operational temperatures may inhibit bacterial growth which in turn results in a diminished concentration of biocatalysts with ensuing low power generation (Masthuriya and Sharma, 2009).

1.4.11 Duration

Operation in MFCs is linked to substrate depletion followed by a decrease in power output. To bypass this hurdle, fed-batch and continuous flow systems are favoured. However, there is still a point where there is decline of power despite addition of fresh medium. The reason is that long cultivation affect microbial metabolism and may result in the exhaustion of the microorganisms' exo-electrogenic capability (Nimje *et al.*, 2009).

1.4.12 Multi-level efforts

These include acclimation of microorganisms (Kim *et al*., 2007), optimisation of process parameters (Yazdi *et al.*, 2008), sedimentation of effluents (Huang and Logan, 2008), co-digestion of wastes (Ward *et al.*, 2008), regeneration of carbon dioxide in the system (Torres *et al*., 2008), bio-augmentation of catalysts (Wang *et al*., 2009), operation in fed-batch and continuous flow systems (Mohan *et al*., 2008; Huang *et al*., 2009), selection of high-performing biocatalysts (Samrot *et al*., 2010), and addition of salts to enhance conductivity (Wen *et al.*, 2010). This enumeration is by no means exhaustive as techniques are being refined and more strategies are being developed.

1.5 Advantages of MFCs over Conventional Anaerobic Treatments

There is less sludge generated in MFCs when compared to open-circuit designs (simple anaerobic modes of degradation without MFC connections and wiring), since there is an added conversion of matter to electricity. Therefore, electrogenesis enhances bioremediation (Huang and Logan, 2008). In addition, MFCs have greater substrate diversity than anaerobic bio-reactors that generate bio-hydrogen (Sun *et al*., 2009). In most cases, anaerobic treatment in bioreactors has been used to remediate lactose-rich effluents to generate bio-hydrogen. However, organic acid production causes corrosion and constant fluctuations of pH render operations difficult, not to mention additional downstream processes aimed at collecting gases (Ozmihci and Kargi, 2007). On the other hand, MFCs can be operated in glass and perspex containers which have proved effective against corrosion. Moreover, MFCs generate electricity from such corrosive wastes in one direct step.

1.6 Problem Statement

Although experiments in MFCs using pure substrates are characterized by high reproducibility (Larrosa *et al.*, 2009); nevertheless, they have been limited by the cost of artificial media and cannot be used for projections in upper-scale studies. Thus the paradigm shift in using other types of substrates such as industrial effluents to drive MFCs. There is need to continuously screen for alternative fuels, especially those that can be safely degraded and concomitantly contribute to the diminution of pollution.

1.7 Hypothesis

The hypothesis for this study was:

The whey-driven MFC performance can be enhanced by building of an electrogenic biofilm on the anode before MFC operation.

This was tested by the objective and aims below.

1.8 Objective and Aims

The main objective of the study was to use the most promising effluent (cheese whey) and enhance the MFC performance through biofilm establishment at ambient temperatures.

This was achieved through the following specific aims that were to:

(a) select effluent between whey and paper mill that generated high power density in an MFC,

(b) evaluate the effect of pre-incubation of electrodes in whey before setups, and

(c) investigate the effect of electrode reuse on MFC performance.

1.9 Research Approach and Organisation

The research started with a comparison of variability between paper mill effluent and whey using previously performed tests. Based on these calculations, the most promising effluent was then used for further studies. The latter focused on enhancing power output from waste degradation through biofilm enrichment.

The work is presented in five chapters as follows:

Chapter 1 contains the necessary background information. Chapter 2 comprises the comparative study. Chapter 3 deals with the acclimation of whey microorganisms toward reactors output. Chapter 4 examines the effect of anode recycling on MFC performance. Chapter 5 has a summative discussion of preceding chapters in a series of consolidative conclusions and culminates in potential avenues of research.

2 SELECTION OF PROMISING EFFLUENT

2.1 Introduction

Over the years, various methods have been designed and tested in the treatment and recovery of water from paper mill effluent. The paper manufacturing industry was ranked amongst the largest consumers of freshwater and polluters globally (Ali and Sreekrishnan, 2001). In addition, a value as high as 11 g/l total chemical oxygen demand (tCOD) has been reported (Thompson *et al*., 2001; Basu *et al*., 2009) which is detrimental to aquatic life. Similarly, cheese whey generated annually poses a serious environmental threat. It was estimated that 6-8 x 10^7 t of whey were produced on a global scale (Stevens and Verhé, 2004). Cheese whey discharge in the environment poses a serious threat because of its high tCOD content which leads to eutrophication of water bodies (60–80 g COD/l) and a drastic decrease of sunlight through surface water (Demirel *et al*., 2005). As a result, various chemical, physical (clarification and filters), aerobic biological treatments and/or anaerobic incubation in appropriate operation tanks have been put in place (Thompson *et al*., 2001). Therefore, it is necessary to consider these two effluents as candidates for fulfilling the fuel needs of MFCs and their concomitant remediation.

Recent works involving paper mill effluent have been focused on single-chambered MFC designs at ambient temperatures. For example, upon improving conductivity of the effluent supernatant with addition of 100 mM PBS in a 500-h fed-batch configuration, up to 672 ± 27 mW/m^2 maximum power density (P_d) and 76 ± 4 % tCOD removal were

attained (Huang and Logan, 2008). However, the maximum P_d was reduced to 210 ± 7 mW/m^2 and tCOD removal was 26 ± 2 % when effluent of the same type was fed to MFCs operated in a continuous flow system (Huang *et al*., 2009). Mathuriya and Sharma (2009) also attempted to generate bioelectricity while achieving bioremediation of different wastes, including paper industry wastewater. They found that an increase of operational parameters namely temperature, agitation, and effluent concentration directly increased the P_d within a specific range. However, little has been documented in the feasibility of electricity generation from full-strength paper mill effluent while achieving an adequate remediation in H-type MFCs.

Similarly, whey-fed MFC technology is still in its infancy with Antonopoulou *et al.* (2010) as pioneers in the field. The maximum power density generated using diluted whey in an H-type MFC was 18.4 mW/m^2 (Antonopoulou *et al.*, 2010). Hence, there is still room to explore the potential of whey as substrate in MFCs.

2.2 Objectives

The objectives of this preliminary study were to:

(a) separately examine and compare the biodegradability and suitability of live paper mill effluent and unamended cheese whey for power production in MFCs,

(b) select the better performing effluent for subsequent biofilm studies, and

(c) investigate the effect of adding an exogenous pure bacterial culture on electricigenesis.

A special attention was devoted to keeping to the strict minimum any input of nutrient(s) and/or energy to allow for practical projections in future upper-scale studies.

2.3 Materials and Methods

2.3.1 Experimental setups and analyses

MFCs of the same architecture as depicted in Figure 2.1 were used. CEM was pretreated by successively boiling discs for 1 h in each of the following solution: 3 % H_2O_2, distilled water, 0.5 M H_2SO_4, and distilled water (Nambiar *et al*., 2009; Kassongo and Togo, 2010). Paper mill effluent and cheese whey, fed to the anode compartment, were sourced from Mondi Paper (Springs, South Africa) and Greenways Deli (Kyalami, South Africa), respectively. Following collections, samples not used in setups were stored at 4 ^{o}C to delay possible changes in bio-chemical composition (Najafpour *et al*., 2009). The *E. cloacae* subspecies *dissolvens* strain 16657 (DSMZ, Germany) was obtained as a dried culture which was rehydrated, as per supplier's instructions, before inoculation into the anode chamber. In addition, *E. cloacae* is a known electricigen and was successfully used in previous electricigenic studies (Nambiar *et al.*, 2009; Rezaei *et al*., 2009; Samrot *et al*., 2010).



Figure 2.1 A prototype H-type MFC used showing the main component of the reactor.

Three separate sets of MFCs were used with each effluent: (i) Raw (untreated) waste alone to investigate the presence of electricigens in the waste and remediation ability of inherent microorganisms, (ii) raw waste with *E. cloacae* to investigate synergism between the native microorganisms and the *E. cloacae*, and (iii) sterile effluent inoculated with *E. cloacae* only to determine the amount of power generated by the pure culture alone as well as the suitability of waste as media for other microbes.

The operation of the MFCs was performed at ambient temperatures (22 ± 3 ^{o}C). Each MFC setup was run for 10 – 14 d and voltage was measured at 2-h intervals using a

TopTronic T830 digital multimeter (APelectronics, South Africa), while pollution indicators such as tCOD and solids where measured at the beginning and at the end of every MFC cycle. Ohm's law (I = V/R) was applied to calculate the current and subsequent power density (P_d), normalised to the anode surface area, was calculated using: $P_d = (VI)/A$, where; V is voltage, I is current and A is surface area (Rabaey and Verstraete, 2005). In addition, the power yield, the substrate degradation rate (SDR) and the coulombic efficiency (ε_{cb}), were calculated according to Huang and Angelidaki (2008); Mohan *et al.* (2008); and Antonopoulou *et al.* (2010), respectively. The calculations were performed in order to enable effective comparison of work by other scientists who did the same.

$$P_Y = \frac{P_t}{\Delta tCOD_t} \quad (1)$$

P_t is power at time t and $\Delta tCOD_t = tCOD_t - tCOD_o$

$$SDR = \frac{\text{tCOD removal efficiency}}{\text{Anolyte volume (ml) x Operating Time (h)}} \quad (2)$$

$$\varepsilon_{cb} = \frac{M \int_0^t I \times dt}{F \times b \times V \times \Delta(COD)} \quad (3)$$

M is the molecular weight of oxygen (32 $g.mol^{-1}$), I is current, F is Faraday's constant (96 485 $C.mol^{-1}$), b is the total number of electrons required per reduction of one mole of oxygen (4), V is the volume of effluent in the anodic chamber and Δ(COD) is the

difference in dissolved COD. The coulombic efficiency is the percentage of total charge transferred to the anode surface over the maximum charge extractable upon complete oxidation of the substrate to electricity (Antonopoulou *et al*., 2010)

2.3.2 Determination of tCOD

Total chemical oxygen demand was determined by a colorimetric method as outlined in the Hanna instruments kits (South Africa). Briefly, two millilitres of each, mixed and appropriately diluted (10^{-2} and $3x10^{-2}$ for whey and paper mill effluent, respectively), effluent were mixed with 1.5 ml digestion and 3.5 ml catalyst solutions, incubated at 150 °C for 2 h and then cooled at room temperature; absorbance was measured at 600 nm. The tCOD concentration was calculated using a potassium hydrogen phthalate standard curve from solutions processed in the same way as the whey samples (Appendix A).

2.3.3 Total solids

Total solids were determined using the drying method (SFS, 1990) both before and after the experiment. Briefly, the effluent was mixed thoroughly, pipetted 50 ml into a pre-weighed glass Petri dish, heated in an oven (100 °C) with periodic cooling (in a dessicator) and weighing until a constant weight was recorded between the heating and cooling intervals.

2.3.4 Scanning electron microscopy (SEM)

Visualisation of the electrode surfaces under SEM was performed to determine the degree of microbial colonisation. Following each MFC run, anodes (from the reactors) were prepared for SEM. Microorganisms on the electrodes were fixed by immersing the electrodes in 2.5 % (v/v) gluteraldehyde and dehydrated by successive immersion for 10 min in ethanol at the following concentrations (v,v): 30, 50, 70, 80, 90 and 100. This was then followed by critical point drying, gold palladium coating (Brunk *et al*., 1981) and visualisation using a JEOL 840 SEM.

2.4 Results

2.4.1 Effluents characteristics

At sampling, cheese whey had a greater concentration of organics than paper mill effluent (Table 2.1). In addition, cheese whey had higher conductivity.

Table 2.1 Characteristics of effluents before treatment

Parameters	**Paper mill effluent**	**Whey**
tCOD (g/l)	43.7 ± 0.3	96.5
Conductivity (mS/cm)	4.65	6.3
Glucose (g/l)	1.4 ± 0.02	59.5
pH	6.61 ± 0.1	7.85±0.1

2.4.2 Electricity generation

Without *E. cloacae*, setups with paper mill effluent did not have stable potential differences. However, introduction of *E. cloacae* in such a medium generally reduced both the magnitude and the variations of power densities, stabilising voltage readings close to 0.3 mV. The maximum power density from inherent waste microbes in presence of *E. cloacae* was 13 ± 2 mW/m^2. The highest power density, 24 ± 3 mW/m^2, was observed on setups with paper mill effluent only (Figure 2.2A). On the other hand, all the setups with whey generated higher power than paper mill effluent. The MFC setup with unamended whey and its inherent microbial flora generated the least power density (0.4 W/m^2; Figure 2.2B). Raw whey with *E. cloacae* produced power density more than double (1.1 W/m^2) that of raw whey and its native microorganisms only. Sterile whey inoculated with *E. cloacae* produced maximum power density of 16.7 ± 1.8 W/m^2 (Figure 2.2B; Appendix B).

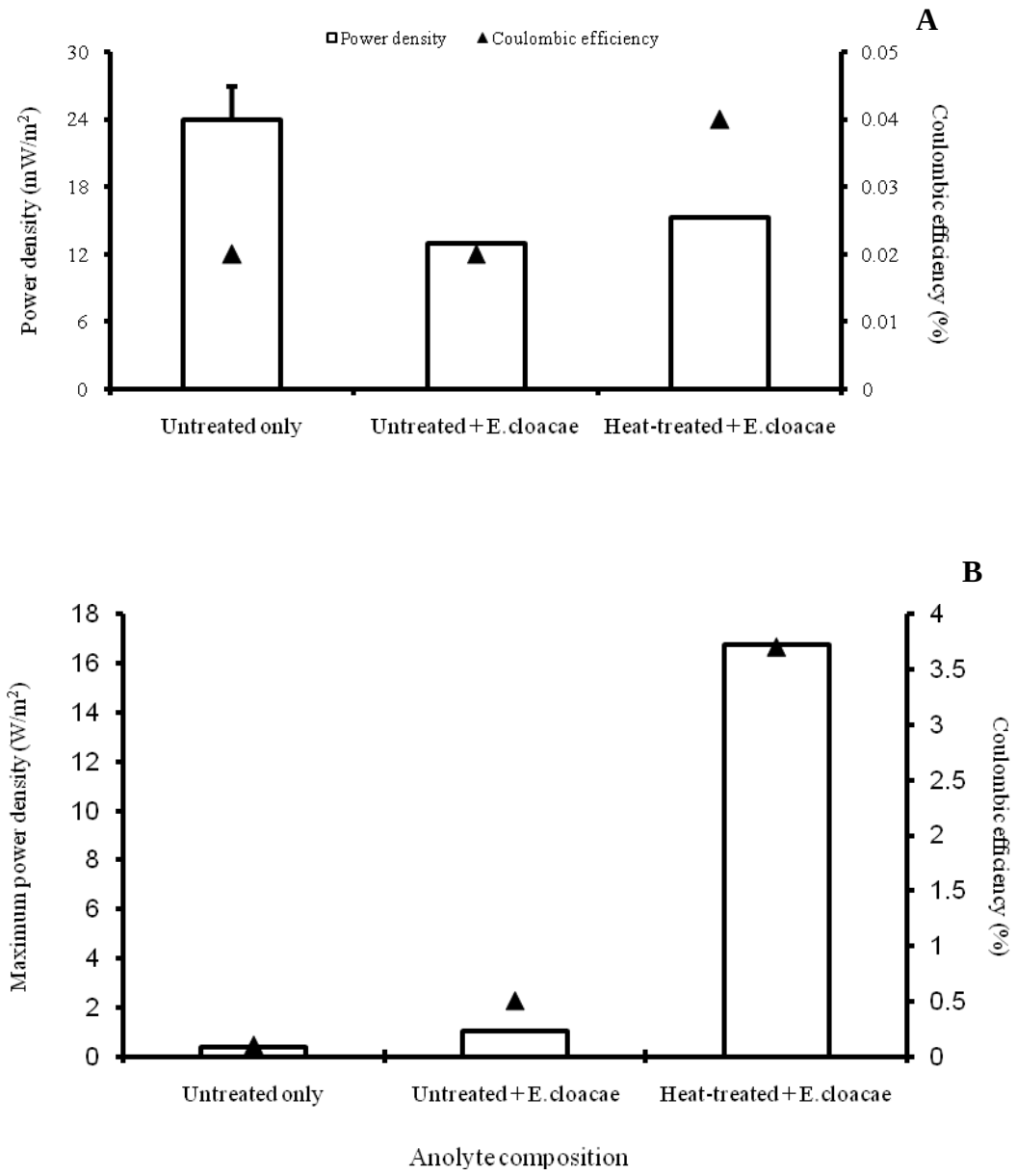


Figure 2.2 Bar graphs of maximum power densities and respective coulombic efficiencies (▲) generated from the three MFC setups with (A) paper mill effluent and (B) cheese whey.

2.4.3 Bioremediation

Pollution parameters such as tCOD and total solids were reduced in all set ups. Experiments with paper mill effluents were characterized by an inverse relationship between the maximum power density and the remediation efficiency with respect to tCOD removal and glucose utilisation (Appendix C). Interestingly, the heat treated whey with *E. cloacae* had the least tCOD and solid removals while the whey with its native microorganisms had the highest remediation efficiency (Table 2.2). Two setup types which were sterile paper mill effluent with *E. cloacae* alone and unamended paper mill effluent only had relatively close SDRs, 0.125 kg COD/m^3 day and 0.112 kg COD/m^3 day (P_Y = 0.1 μW/kg COD_R), respectively. Addition of *E. cloacae* to native paper mill effluent increased the SDR to 0.257 kg COD/m^3 day (55.4 ± 0.7 % COD removal efficiency, P_Y = 0.3 μW/kg COD_R and ε_{cb} = 0.02 %) resulting in 13 ± 2 mW/m^2, the lowest maximum power per anode surface area within this particular effluent (Figure 2.3). Although the SDR pattern when using cheese whey was observed at low levels, it shared an inverse trend to that of paper mill effluent. In MFCs, untreated whey alone and sterile whey in conjunction with *E.cloacae* had SDR values of 0.0071 kg COD/m^3 day and 0.0084 kg COD/m^3 day, respectively. The untreated whey catalyzed by *E.cloacae* metabolized at the rate of 0.0036 kg COD/m^3 day (Figure 2.3).

Table 2.2 Percentage changes in total solids and other parameters in MFC setups using whey and different microbial combinations in the anode chamber

MFC setups		COD removal	Solids removal
Pre-treatment	Microbial composition	(%)	(%)*
Autocalved	*E. cloacae* only	5.0 ± 0.7	3.4
None	Native and *E. cloacae*	22.1 ± 2.8	11.7
None	Native only	44.7 ± 0.2	19.7

*Standard deviations (SDV's) are less than 0.1 were correct to 1 decimal place.

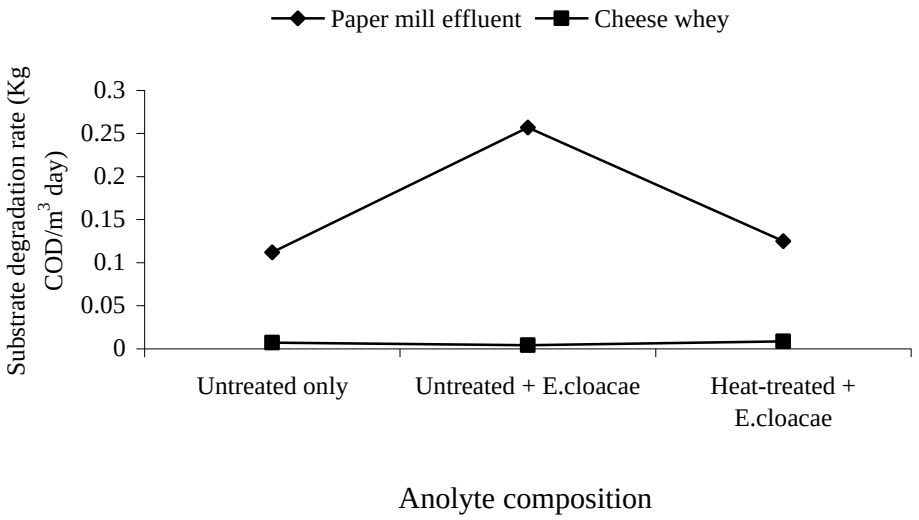


Figure 2.3 Comparison of substrate degradation rate between paper mill effluent and cheese whey.

2.5 Discussion

The present study used a concentrated paper mill effluent which contained alkalis used throughout a wood processing plant. It could be expected that such a non-pre-treated wastewater containing strong oxidizing chemicals would increase the survival pressure for microbial life and not necessarily favourable to electron generation and transport (Huang *et al.*, 2009; Hakeem and Bhatnagar, 2010). Therefore, it was noted lack of steady-state in power generation and sparse microbial growth on electrodes. Similar trends were observed by Mathuriya and Sharma (2009). The fluctuation in power densities can be attributed to the breakdown of cellulose which is a very slow process which in turn may even get intermittent in stagnant conditions or in absence of ideal mixing of bacteria within the wastewater (Huang and Logan, 2008). Nonetheless, addition of exogenous *E. cloacae* in setups reduced both the magnitude and frequency of fluctuations of electricity with a bimodal distribution of power densities in most cases.

The maximum power density from sterilised whey with *E. cloacae* achieved in this preliminary study (16.7 W/m^2) was considerably higher than the one that was reported (18.4 mW/m^2) from MFC driven by diluted whey and inherent microorganisms (Antonopoulou *et al.*, 2010). Both the elevated tCOD value (96.5 ± 4.2 g/l) and the initially high conductivity of whey had favoured higher maximum power densities. Whey tCOD has been reported to be between 61 and 80 g/l (Kalyuzhnyi *et al*., 1997; Re *et al*., 1998; Pant *et al*., 2010), which is lower than the value reported in this study. Higher conductivity reduces the resistance of the medium, thus, generally increasing the power output and shortening the onset of the maximum power density (Ramasamy *et*

al., 2008; Mohan and Das, 2009). The presence of the lag period for maximum power generation can be attributed to the complexity of whey. Rapid decrease in the power density from the maximum is not desirable because one of the objectives in MFC technology is to maintain maximum power production over a prolonged period. Under such circumstances the MFC has to be modified to feed-batch system and preserve a steady state when maximum power is generated. Alternatively, maintain a well-acclimated anodophilic culture to stabilize the power.

It could have been expected to have the setup of sterile paper mill effluent acted on by *E. cloacae* to yield the highest maximum power density due to its highest coulombic efficiency (0.04 %) coupled to a low SDR and a relatively high power yield. Surprisingly, setups with unamended effluent generated the highest power possibly due to the lowest SDR and the increase of glucose (up to 47.7 %) in the medium; whereas it was being decreased in all other setups. Two possible assumptions can be drawn. First, the slow degradation of organics together with the glucose build-up may have amounted to a long-term energy store for microorganisms toward the high electricity generated (Kassongo and Togo, 2011a). Alternatively, glucose is not the best substrate for electricity generation when compared to acetate (Ahn and Logan, 2010; Pant *et al*., 2010) which can be generated in lactic acid metabolism (Caplice and Fitzgerald, 1999). Hence maximum power can be attributed to non-utilisation of or less dependency on glucose (Kassongo and Togo, 2011c). However, the highest power yield in sterile waste metabolised by *E. cloacae* holds an indication on the adaptation of the microbial culture

and the continuation of electricity generation above power densities of both untreated waste alone and those of untreated waste with *E. cloacae* (Mohan *et al*., 2008).

The tCOD removal in MFCs with native whey only and that with native whey and *E. cloacae* show insignificant synergy in power production. One would have expected to have both tCOD removal and power density to be higher than that of sterilised whey and *E. cloacae* if there was significant synergy. This low power production could be attributed to competition for the nutrients and synthesis of intermediates that accepted electrons within the chamber (Kassongo and Togo, 2010). Thus, there is some degree of antagonism between maximum remediation and power generation (electron transfer to the anode).

In addition, heat treatment could have partly hydrolysed proteins, making them readily available to *E. cloacae* consequently producing more power than the rest of the reactors. The direct relationship between power generation and microbial density on the anode suggests a direct electron transfer to the electrode. In addition, there is a benefit on the kinetics due to reduced activation loss (Ramasamy *et al*., 2008). Thus, increased microbial competition could also have limited process or biofilm formation in the mixed culture and consequently, less power was produced. The observed decrease in glucose concentration after the experiment suggests that the microorganisms could have used it as a carbon source.

The coulombic efficiency has long been an important gauge for comparison amongst power generation studies. However, additional factors may need to be considered such as waste composition, including both biotic and abiotic factors (Kassongo and Togo, 2011a). For example, initially high tCOD content can induce bacteriostatic and bactericidal effects on certain microbial populations, resulting in reduced coulombic efficiencies (Min *et al*., 2005; Jong *et al*., 2006). Therefore, the SDR was considered alongside the coulombic efficiency in the electricity generation studies. Everything considered, the coulombic efficiencies and power yield may be low in the present investigation, but electricity generated can be significant when taking into consideration the projection of the total effluent discharge volume produced daily by a processing mill over a year, assuming that the scale-up generates electricity within the same proportion as in a pilot study (Wen *et al*., 2010).

This exploratory study has established that full-strength paper mill effluent can be metabolised and serve as both carbon and energy sources to endogenous microbes with detectable electricity generation in fuel cells. However, the constraints associated with the degradation of cellulose and the toxicity of paper mill effluent may be prominent factors in the low performance of reactors. All in all, cheese whey generated greater power densities than those of paper mill effluent, primarily due to its richness in nutrients that are readily amenable to microbial degradation.

2.6 Conclusions

Based on the results presented in this chapter, the following can be drawn:

(a) whey is better than paper mill effluent for electricigenesis in MFCs, and

(b) addition of *E. cloacae* is antagonistic to performance of reactors with native microflora.

Therefore, whey and its native microflora were studied for the subsequent biofilm studies to ascertain if power can be enhanced.

3 ACCLIMATION OF INDIGENOUS ANODOPHILES

3.1 Introduction

The booming cheese industry has fostered research toward several practical strategies to mitigate the impact of its 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; and 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 sustainable (Peters, 2005). In addition, spraying excessive volumes of whey on land farms leads to contamination of ground water and accumulation of elements such as phosphorus and potassium that disturb the mineral salt balance in crops and ultimately unsettling growth balances (Watson *et al.*, 1977).

Efforts have been directed toward 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) generated a maximum power density of 18.4 mW/m^2 using diluted whey in double-chambered MFCs operated in fed-batch mode. It is preferable to achieve maximum power generation and bioremediation without any additions of microbes or nutrients exogenous to effluents for economical and sustainable treatment (Min *et al.*, 2005; Mathuriya and Sharma, 2009; Larrosa *et al.*, 2009; Samrot

et al., 2010). Therefore, this chapter focused on utilizing unamended whey and its inherent microorganisms. The acclimation strategy was opted based on positive impact of such a stage in other effluents (Mohan *et al.*, 2008; Rodrigo *et al.*, 2009; Nimje *et al.*, 2009; Huang *et al.*, 2009).

3.2 Objectives

The objectives of the present section were to:

(a) investigate the impact of microorganism buildup on anode surfaces toward MFC performance at ambient temperature, and

(b) identify the role players in the electricity generation and remediation.

3.3 Materials and Methods

3.3.1 MFC design

Nine sets of double chambered mediator-less reactors were assembled and each filled by 200 ml of whey in the anode compartments and an equal volume of potassium ferricyanide in sodium phosphate buffer pH 6.8 (Kassongo and Togo, 2010). A Nafion^{TM} 117 membrane disc of 0.02 m in diameter was inserted halfway of the proton exchange membrane bridge with 0.06 m total length (Figure 3.1). The whey was collected from Greenways Delli (Kyalami, Johannesburg, South Africa) on the day of experimental start-ups.

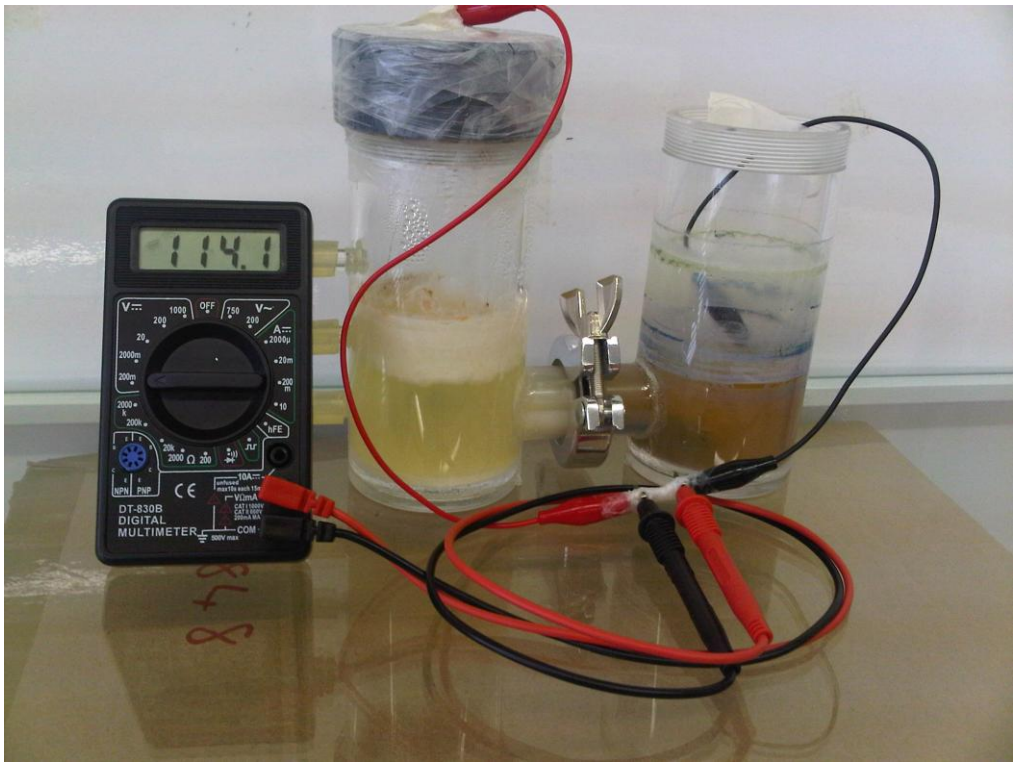


Figure 3.1 H-type microbial fuel cell used in experiments.

Anodes were stored under anoxic conditions for thirty (one-month-old) and ninety (three-month-old) days in whey replaced every three days based on previous studies which unveiled the most rapid depletion of substrate during the first three days of a batch cycle (Kassongo and Togo, 2010). This pre-incubation was conducted to:

(i) enrich i.e. promote biofilm growth on the electrodes, and

(ii) investigate the stage of optimum biofilm density for high power generation and bioremediation in MFCs.

The power densities generated, including SDR and ε_{cb}, were calculated as described in section 2.3.1.

3.3.2 Pollution analyses

The determination of tCOD was performed using Merck standard test kits. Briefly, appropriately diluted (10^{-2}) whey samples were digested with Merck COD Cell Tests in a Spectroquant® TR320 thermoreactor at 148 °C for two hours. Following cooling, the tCOD concentrations were then measured with use of Merck Spectroquant® Pharo 100 photometer. The colorimetric assay was the basis of detection of the variations of the tCOD concentration in the kits reaction cells.

Sulphates (SO^{2-}_4), nitrates (NO^-_3) and phosphates (PO^{3-}_4) concentrations were determined both before and after reactor cycles by separately mixing Hanna SO^{2-}_4, NO^-_3 and PO^{3-}_4 reagents with appropriately diluted (10^{-1}, $2x10^{-2}$, 10^{-2}, respectively) volumes of samples, as per manufacturer's instructions; and measured with use of HI83200 Multiparameter Photometer (Hanna Instruments, South Africa).

3.3.3 Biofilm confirmation

Electrodes were fixed in 2.5 % (v/v) gluteraldehyde and progressively dehydrated in ethanol in concentrations (v,v): 30, 50, 70, 80, 90 and 100 (Kassongo and Togo, 2010). Subsequent to critical point drying and coating, electrodes were viewed under FEI Quanta 400E scanning electron microscope (SEM); which was the cheapest available at the time of the study according to available resources. A number of electrodes housing microorganisms at the same developmental stage, as those prepared for SEM, were spared for molecular community studies.

3.3.4 Molecular ecology studies

Clarity that these were performed to determine diversity and identity of the electricigenic anodophiles in the biofilm. The study entailed extraction of the bacterial DNA, amplification of the 16S rDNA, denaturing gradient gel electrophoresis (DGGE), sequencing and a BLAST search.

3.3.4.1 DNA extraction

One- and three-month-old anodes and those in reactors following incubation were transferred to 20 g of 2-mm glass beads and vigorously shaken for 5 min to dislodge cells from surfaces. The resulting 50 ml- RCM medium (excluding glass beads) medium was centrifuged under refrigeration 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, as per manufacturer's instructions, to prepare ultra-pure genomic DNA templates for PCR.

3.3.4.2 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 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 sec at 94 °C), annealing (45 sec 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. An agarose gel electrophoresis was later conducted to confirm for presence, quality and purity of PCR products (Jong *et al.*, 2006).

3.3.4.3 Denaturing gradient gel electrophoresis (DGGE)

The goal with DGGE was to examine the microbial diversity (based on hydrogen production) 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 soon 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 above (without GC clamp on the forward primer) followed by another agarose gel electrophoresis were performed to confirm presence of DNA before sequencing (Inqaba Biotechnology Industries, South Africa) (Azbar *et al.*, 2009).

3.3.4.4 Sequencing and phylogenetic tree

Sequences received from Inqaba Biotechnology Industries (South Africa) were edited in 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 sequences of the isolates to construct the phylogenetic tree in the DNAMAN sequence analysis software.

3.4 Results

3.4.1 Raw effluent profile

The characteristics of the cheese whey before experimental setups are shown in Table 3.1. The parameters remained stable between samplings.

Table 3.1 Profile of the whey before reactor setups

Parameters	**Values (Mean ± SDV)**
tCOD (g/l)	93.2 ± 0.4
pH	6.46 ± 0.19
PO^{3-}_4 (g/l)	1.30 ± 0.1
NO^{-}_3 (mg/l)	72.6 ± 10.2
SO^{2-}_4 (mg/l)	50 ± 0.0
Conductivity (mS/cm)	5.96 ± 0.05

3.4.2 Electricity generation and stability

One-month-old anodes, in MFCs, produced a low maximum power density (29.1 ± 4.91 W/m^2) not significantly ($P > 0.05$) different from the control (30.9 ± 4.24 W/m^2), whereas the three-month-old anode produced the highest and most stable power (1 800 W/m^2; Figure 3.2) daily which decreased progressively for the first 25 d and was maintained above 10 % of the maximum power over a total duration of 75 d (truncated progress curve — Figure 3.3). There was low stability of power in the control and in the one-month-old anodes. The latter achieved higher power than the former over the first three days in MFCs. The maximum power density decline in the control set was linear (Figure 3.3).

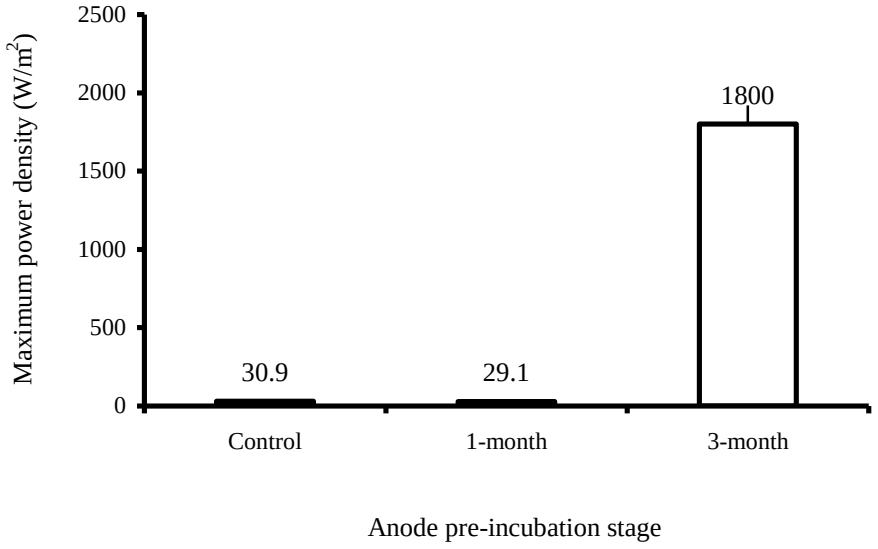


Figure 3.2 Maximum power densities (W/m^2) from reactors with the anodes subjected to different periods of biofilm build-up. In the control setup, the anode was directly used without prior immersion in the whey.

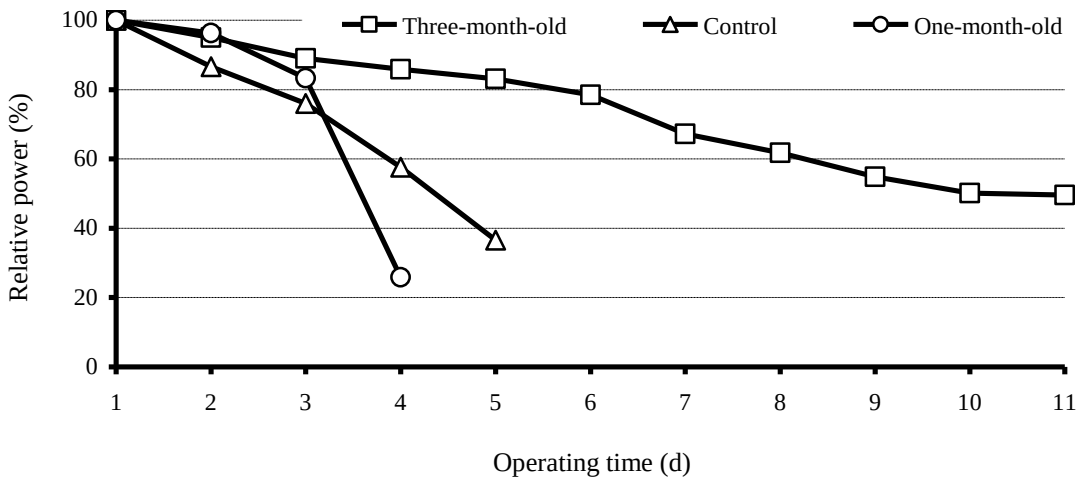


Figure 3.3 Relative power density (%) stability in microbial fuel cells with different anode treatments.

3.4.3 Membrane

There was noticeable fouling of the CEM upon dismantling of the MFC setups in all cases (Figure 3.4). This was characterized by a change from transparent to dark green.

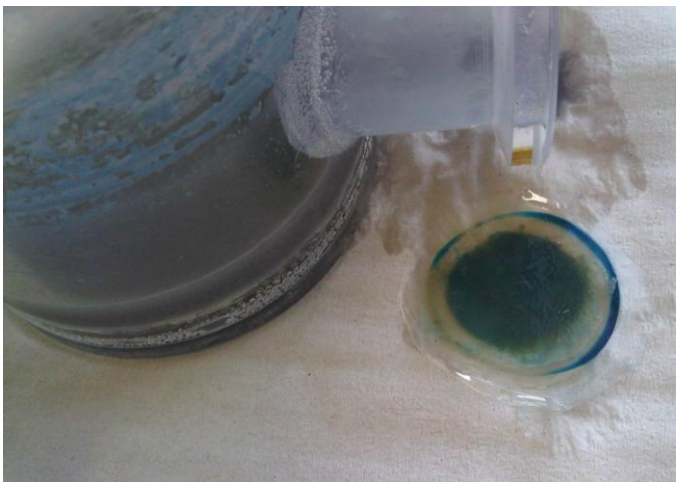


Figure 3.4 The visual of the cation exchange membrane (CEM) after operation in MFCs.

3.4.4 Substrate degradation

At termination of each reactor cycle, the final tCOD for one- and three-month-old anodes were 10.9 ± 1.9 g/l and 6.75 ± 0.15 g/l, respectively. While sulphates within the three-month-old anode setups remained unchanged, the phosphate levels doubled (2.79 ± 0.1 g/l). However, there was sulphate increase (66.7 ± 23.6 mg/l) and no significant ($P > 0.05$) phosphate change in setups using one-month-old anodes. Most notable were the increased nitrate levels across all the reactors, regardless of anode types used (Figure 3.5). 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 degradation (SDR = 2.47 kg COD/m^3 day) and the lowest coulombic efficiency (ε_{cb} = 0.13 %) when compared to 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 % (Figure 3.6). However, despite the relative similarity of maximum power densities between the one-month-old anodes and those of the controls, there were generally higher power values and greater remediation over the period of operation with the reactors using one-month-old anodes.

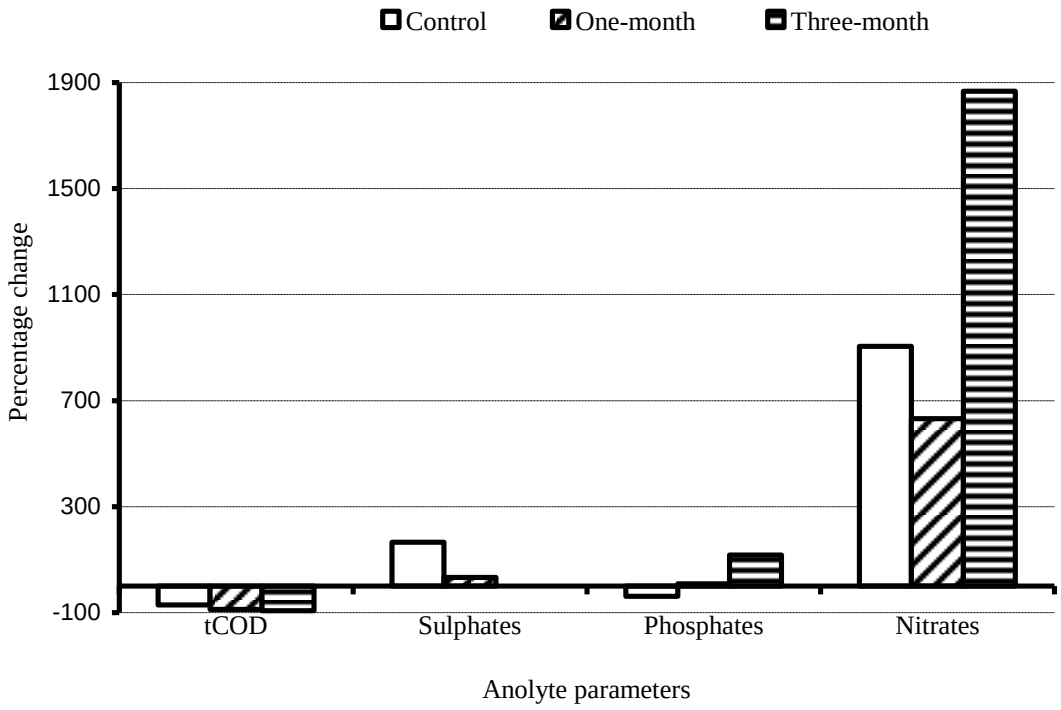


Figure 3.5 Relative changes (%) in selected parameters from anode effluent of whey-driven MFCs with different anode treatments.

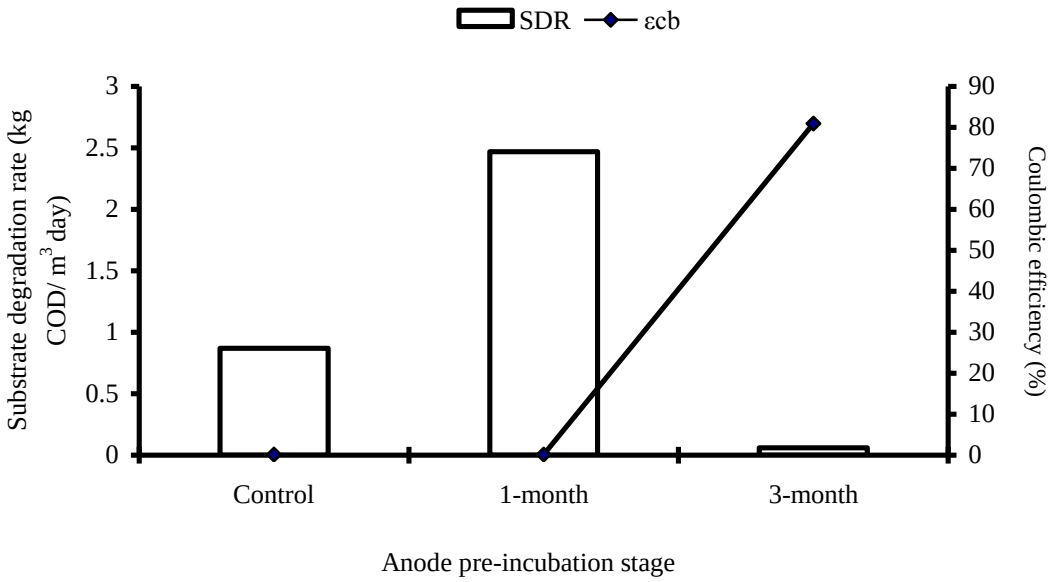


Figure 3.6 Comparison of substrate degradation rates and respective coulombic efficiencies of anodes at different pre-incubation stages.

3.4.5 Scanning electron microscopy

Incubation of electrodes in reactors increased bacterial biofilm density on anodes and extra polymeric substances (EPSs) were seen on the one-month-old anodes (Figure 3.7B). While there was a predominance of rod-shaped cells and less cocci on all the one-month anodes after residence in MFCs, the three-month-enriched anode was exclusively made of filament-like structures resembling rod-shaped cells put end to end under higher magnification (Figure 3.7D insert). 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 3.7E & F).

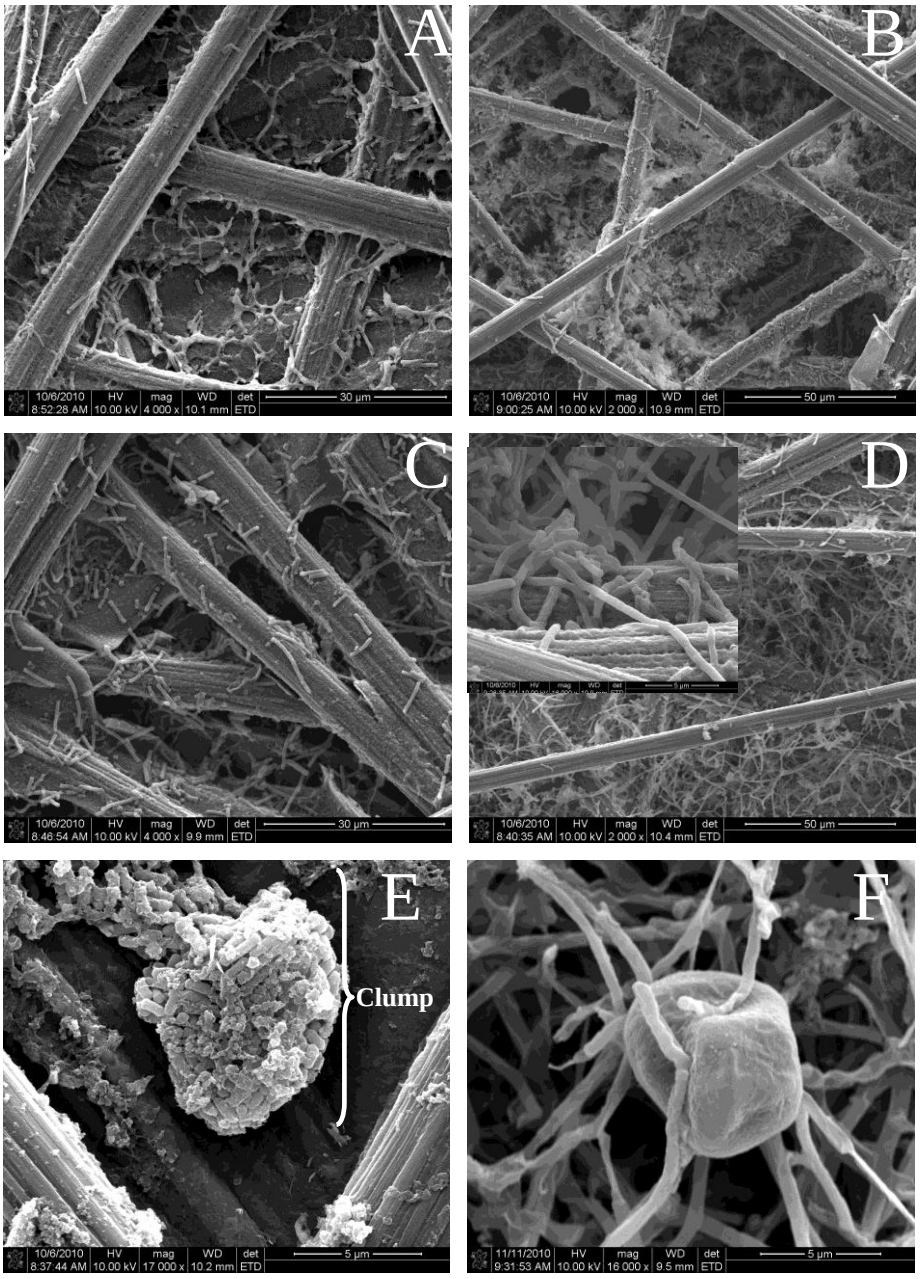


Figure 3.7 Scanning electron micrographs of anode surfaces for one (A) and three (C) months of pre-incubation before MFC setup, and respectively for same treatments (B) and (D) after use in the MFCs. E and F further respectively illustrate the morphologies of microorganisms on one- and three-month-old anodes subsequent to incubations in MFCs.

3.4.6 Molecular ecology

A DGGE analysis revealed similar patterns of band brightness on all the lanes that were loaded with the PCR products from the one- and three-month-old anodes before and after residence in MFCs (Figure 3.8). Although bands were lacking in sharpness, two bands (somewhat defined regions) 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 (Figure 3.9).

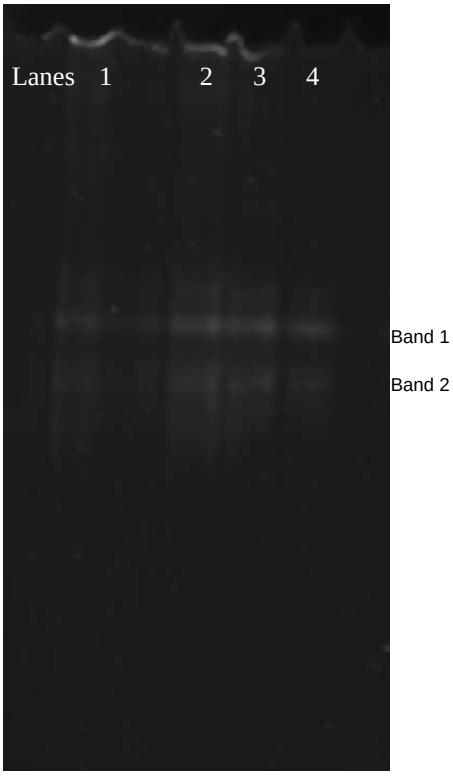


Figure 3.8 A DGGE profile of one- and three-month-old anodes recovered from the reactors. Left to right, lane 1 (3) and 2 (4) represent PCR amplicons from one- and three-month-old anodes, respectively, before (after) residence in reactors.

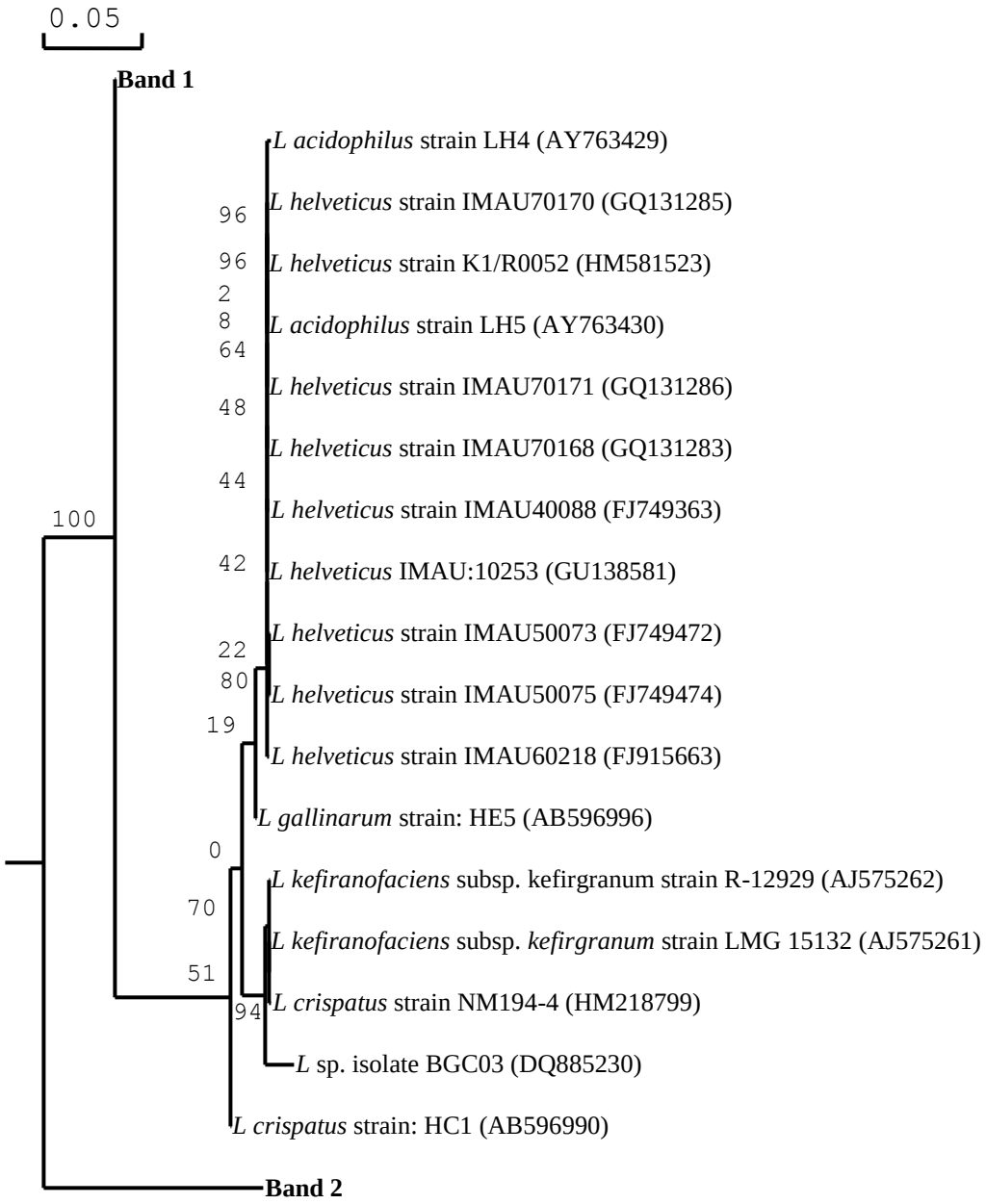


Figure 3.9 A phylogenetic tree showing the relationship between the sequences obtained from excised bands and those deposited in GenBank (accession numbers are indicated in brackets).

3.5 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 (1 800 W/m^2) when compared to those of anodes pre-incubated for one month (29.1 ± 4.91 W/m^2). This technique built up the density of biofilm for stable electricity generation (Lee *et al.*, 2003). The lack of significance in maximum power densities achieved by the one-month-old anodes when compared to those of the control anodes (not acclimated for enrichment) suggests that there should be a threshold for acclimation to yield desirable power outputs (Kassongo and Togo, 2011b). However, differences in power stabilities between the control and one-month electrode (Figure 3.3) shows the importance of microbial colonization of the electrode if high power densities are to be sustained over a prolonged period.

Interestingly, the related electrical performance of one-month-old and control anodes may hold an indication that there may be an abundance of electron shuttles (mediators) in whey. Electron-mediated transport generally faster than direct electron transfer, in some cases, may have compensated for the little or complete absence of acclimation in the control reactors (Samrot *et al.*, 2010).

The linear decrease of power density between the one- and three-month old anodes may be due to factors (either individually or in combination) such as substrate depletion in batch mode operations, declined ionic transport due to fouling of the CEM and

establishment of a nutrient gradient which further diminished mass transport (Nambiar *et al*., 2009, Kassongo and Togo, 2010).

It can be argued 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. In addition, lactic acid bacteria contribute to this low pH through production of organic acids. Contamination of anodes by non exo-electrogenic microorganisms can also occur inside reactors, not only during pre-incubation (Antonopoulou *et al*., 2010). Yet, a refining of the microbial anodophilic composition occurs depending on operating conditions (such as pH and MFC configuration) in favour for electricity generation instead of methanogenesis (Ishii *et al.*, 2008; Kassongo and Togo, 2011c) and microbial consortia themselves evolve over time (Kiely *et al*., 2010a).

The elevated 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 elevated 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 efficiency due to an inversely proportional relationship (Antonopoulou *et al*., 2010;

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 tCOD was achieved only because of anode and whey residence time in the MFC (Kassongo and Togo, 2011b). Therefore, an even higher tCOD 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^{2-}_4, NO^-_3 and PO^{3-}_4) generation and/or removal were observed in MFCs. It could be that the lowering of phosphate and sulphate levels by one- and three-month-old anodes, respectively, was due to reduction of compounds in accepting electrons. Alternatively, the interspecies distance between microbe-producing and microbe-consuming was either short or long with respect to their spatial distribution and their population sizes, thus affecting removal of the metabolites (Trzcinski *et al.*, 2010). The increased nitrate concentration across reactors may have partly been due to proteolysis. It is noteworthy 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 densities between the two setup types. In the same light, the supreme coulombic efficiency of the three-month-old anode had the best explanation for its towering maximum power density generated in spite of the phosphates and nitrates after an MFC run.

The oxygen gradient in the anode chamber had most likely dictated the distribution of bacteria on the anode with the strict and facultative anaerobic microbes on the innermost carbon layers (Kiely *et al.*, 2010b). The micrographs revealed two distinct patterns of polymeric substances production on anode surfaces depending on enrichment time prior to reactor setups. This observation might suggest presence of polymer-producing microbes on the one-month-anodes not found on the three-month-anode counterpart. Alternatively, the tremendous degradation rate (SDR = 2.47 kg COD/m^3 day) achieved by microbial populations on the one-month-old anodes was expressed in form of an external polymers production, instead of microbial growth (Kassongo and Togo, 2011b).

Microorganisms aggregated and formed specialized structures for the degradation of complex compounds (Lee *et al*., 2003); however, the bacteria on the one- and three-month-old anodes, after residence in MFCs, appeared to be morphologically different. Such a qualitative study would further aim the quantification of the microbial density per surface area. As illustrated in the DGGE profile (although not core to the work), there were same microbial populations across reactors. Therefore, it could be that the period of batch operation in MFCs was not long enough for the one-month-old microorganisms on anodes to considerably differentiate from those in pre-incubation and form clumps with a well-defined margin (Kiely *et al*., 2010a). To gain a fairly precise representation of the diversity of anodophilic cells, there was no cloning or sub-culturing of anodes to improve microbial density. A phylogenetic analysis of the microbial ecology on anode surfaces was conducted and had up to 92 % identity to strains of lactic acid bacteria (including during the pre-incubation stage). Nevertheless,

factors such as the formation of heteroduplexes during PCR, co-migration of phylogenetically heterogeneous bands and contamination of bands during excision of the acrylamide gel (DGGE) may have generated biased sequences (Riemann *et al*., 1999; Sekiguchi *et al.*, 2001).

3.6 Conclusions

Based on the results presented in this chapter, the following can be drawn:

(a) continual acclimation and enrichment significantly improved the overall performance of whey-fed reactors, and
(b) species of Lactobacillus were involved in power generation.

Further studies were thus conducted to investigate the impact of pre-incubating anodes followed by the serial reuse of electrodes across reactors.

4 RECYCLING OF THE BIO-CATALYTIC INTERFACE

4.1 Introduction

Large volumes of whey produced annually may prove to be a renewable substrate to fuel MFCs (Ozmihc and Kargi, 2007; Souza *et al*., 2010). Therefore, cheese whey which is a high-organic-content watery by-product of the cheese manufacturing has recently been investigated in electricity generation studies (Antonopoulou *et al*., 2010; Kassongo and Togo, 2010). Previously, it was established that the enrichment time of electrodes in whey during acclimation in advance of an MFC operation improved the performance of the reactors in terms of levels of electricity generated and bioremediation achieved (Chapter 3).

4.2 Objectives

The objectives of the present section were to:

(a) explore the impact of a pre-incubation stage of electrodes in whey before reactor setups followed by a series of multiple incubations in mediator-less H-type MFCs, and

(b) identify the role players in electricity generation.

4.3 Materials and Methods

Anodes were stored under an anoxic environment in whey replenished every three days for enrichment of anodophilic microorganisms on surfaces during a period of two months (pre-incubation). Then, electrodes were transferred to whey-fed MFC setups for

seven days before being reused in new setups. This electrode recycling was performed four times in reactors and lasted thirty days for a continual cumulative enrichment period of ninety days which started at pre-incubation. Experiments in fuel cells started off with six reactors and one reactor's anode was "sacrificed" to microscopy and molecular identifications at the termination of each round of MFCs. Fresh cheese whey was sourced from Greenways Delli (Kyalami, Johannesburg, South Africa) on the day of reactor setups. All the operational parameters, power density calculations, molecular work and equipments used were as described previously (Kassongo and Togo, 2010; Chapter 3).

4.4 Results

4.4.1 Whey composition

Chemical analyses revealed that the whey samples contained phosphates, nitrates, solids and glucose before reactor setups (Table 4.1). The measured parameters remained relatively stable between repetitive effluent samplings from the factory. Both phosphates and nitrates had similar patterns of concentration changes across batch cycles with anodes reuse. The lowest concentrations were found in the first batch cycles whereas the fourth cycles experienced an increase of concentrations in the reactors (Figure 4.1).

Table 4.1 Profile of the whey before reactor setups

Parameters	**Values (Mean ±SDV)**
tCOD (g/l)	76 ± 19
Solids (g/l)	3.4 ± 0.12
PO^{3-}_4 (mg/l)	1410 ± 14
NO^{-}_3 (mg/l)	90 ± 8.5
Glucose (mg/l)	261.1 ± 33
Conductivity (mS/cm)	6.74 ± 0.51

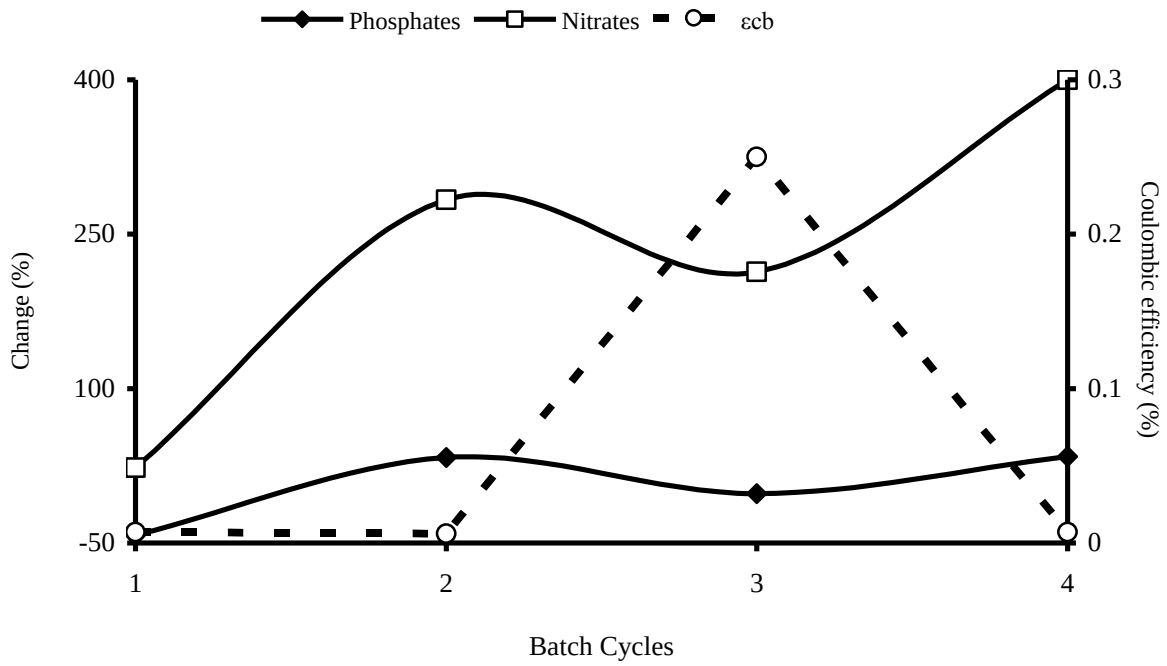


Figure 4.1 Relative changes (%) in nitrates, phosphates and coulombic efficiency during each cycle of anode reuse.

4.4.2 Electricity output

A recurrent pattern of electricity generation was observed with the two-month-old anodes. The first setups generated 42.5 ± 10 W/m^2 which was twice the power of same anodes when reused in the second batch cycles (20.7 ± 9 W/m^2). However, the third anodes re-cycling raised the power to 390 ± 21 W/m^2 accompanied by a decline to levels of 26.5 ± 12 W/m^2 in the fourth round of setups (Figure 4.2).

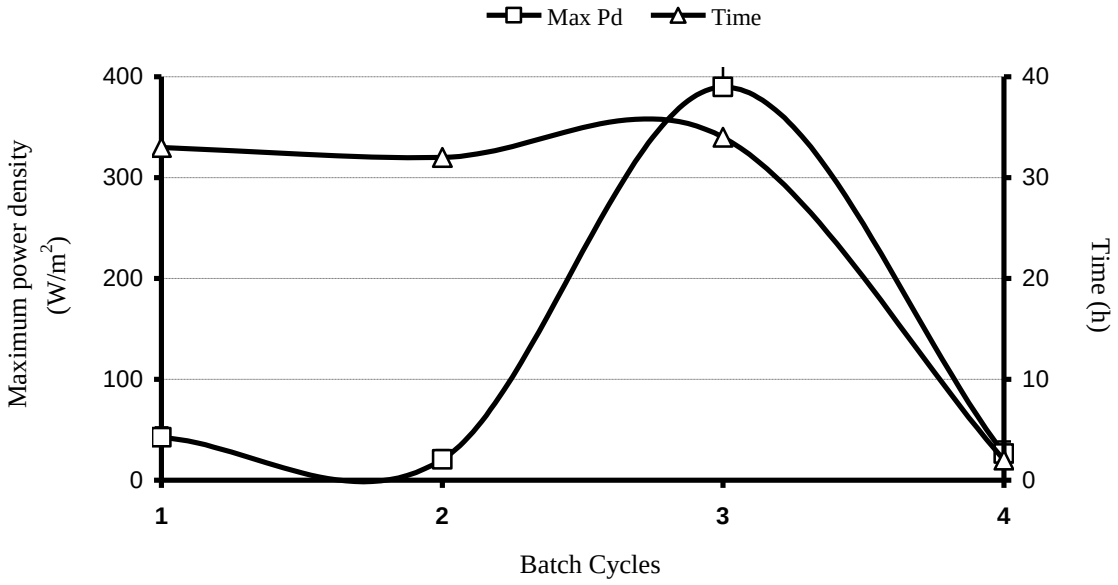


Figure 4.2 Maximum power density and time taken to reach each peak density in different stages of anode reuse.

4.4.3 Bioremediation

The solids removal efficiencies were not significantly different ($P > 0.05$) between the first and second cycles, 66.5 % and 66.2 %, respectively; whereas there was no significant ($P > 0.05$) solids removal in the last two cycles (Figure 4.3A). There was variable tCOD removal through successive anodes use (Figure 4.3B). Interestingly, the substrate degradation rate (SDR) curve was directly proportional to that of the tCOD removal efficiency (Figure 4.3). The tCOD removal increased from 19.7 % (0.493 Kg COD/m^3 day), in the first cycles, to 44.6 % (0.776 Kg COD/m^3 day) in the following batch. For same anodes, the tCOD removal went up to 7.4 % (0.185 Kg COD/m^3 day) and 6.6 % (0.115 Kg COD/m^3 day) in the third and forth cycles, respectively. As depicted in Figure 4.3, the glucose concentrations were increased throughout the whey treatment and were clearly correlated to both the tCOD removal and the change of SDR.

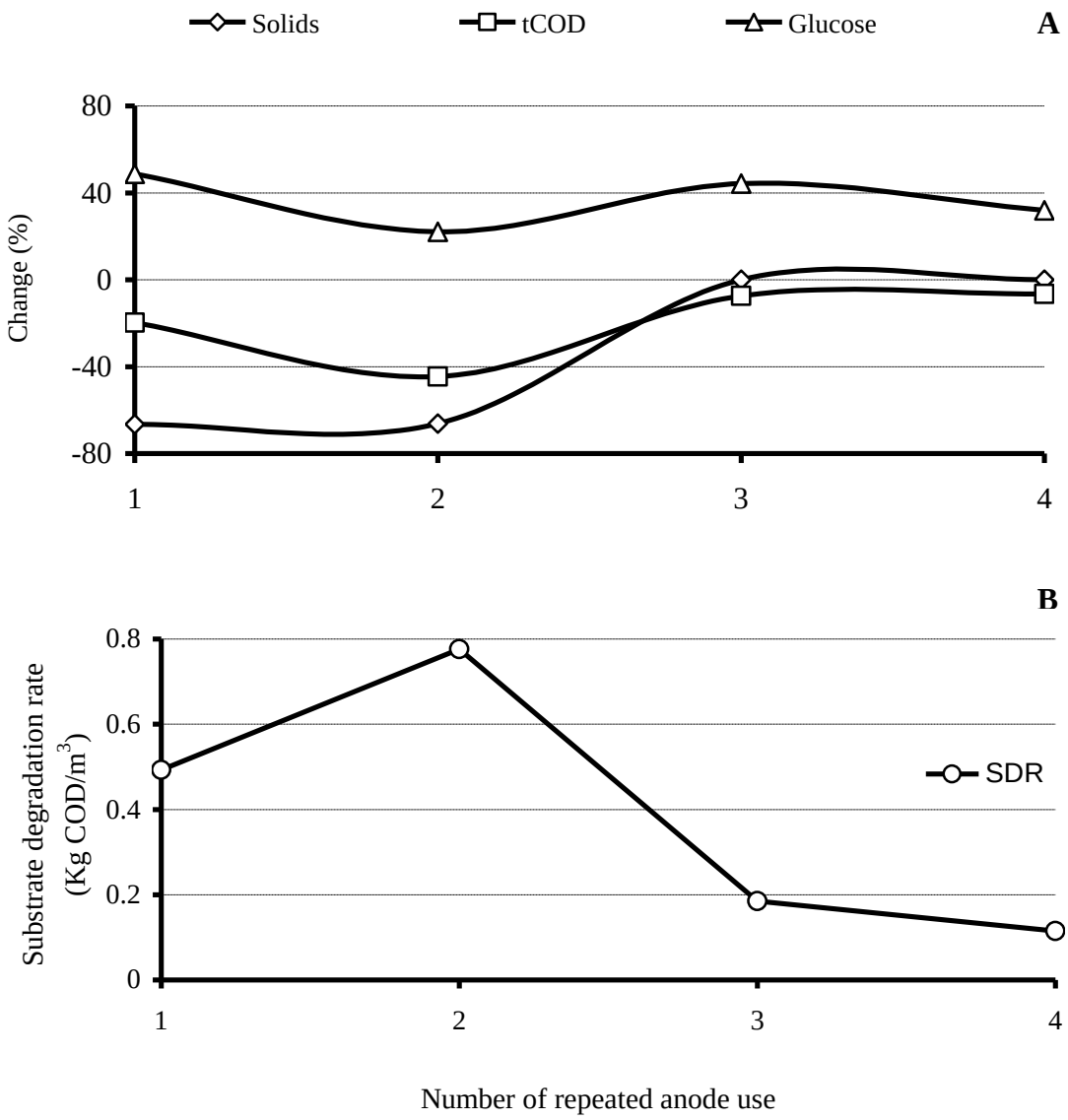


Figure 4.3 Changes (%) in whey parameters (A) and the substrate degradation rate (B) for each anode reuse setup.

4.4.4 Scanning electron microscopy

After the two-month period of anodes acclimation ahead of reactor setups, electrodes were enriched with a biofilm of rod-shaped cells. The recycling of anodes in MFCs continued the enrichment phase which in turn increased the microbial density over time. However, there was a differential distribution of cells on same anodes, irrespective of their batch cycles. Also, the outermost layers of carbon tubes on the anode surfaces were sparsely colonized (Figure 4.4). The presence of clumps (aggregates of cells) was noted on electrodes before the first MFC batch cycles and increased with the progression of the reuse cycles. At the termination of the third batch cycles, the clumps were a dominant characteristic of anodes' topography.

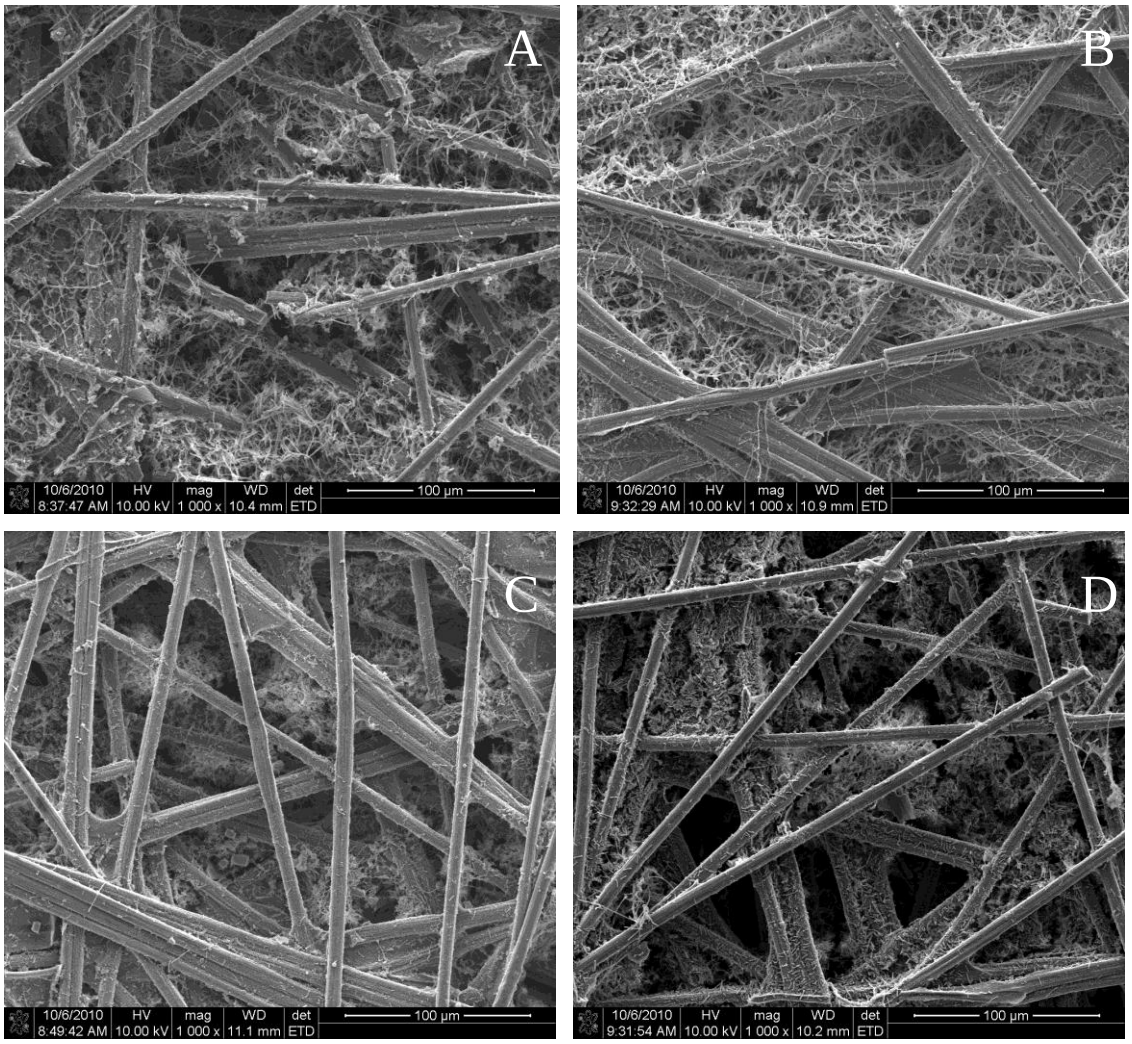


Figure 4.4 Scanning electron micrographs of anodes at the termination of the first (A), second (B), third (C) and fourth (D) MFC reactor sets.

4.4.5 Molecular analysis and phylogeny

In the case of the DGGE, there was an increasing brightness of bands characterised by progressive refinement and resolution with anode reuses across reactors (Figure 4.5). As illustrated, the DNA extracted from anodophiles on the first MFC cycles appeared as a single and faint band (lane 1; Figure 4.5) which became well defined in the third (lane 3; Figure 4.5) and fourth batch cycles (lane 4; Figure 4.5). Also, certain bands not visible initially became noticeable in the last two MFC runs. Band 1 had 85 % identity to strains of *Lactobacillus helveticus* while bands 2 and 3 had 96 % identity to *Proteus mirabilis* and *Escherichia coli*, respectively (Figure 4.6).

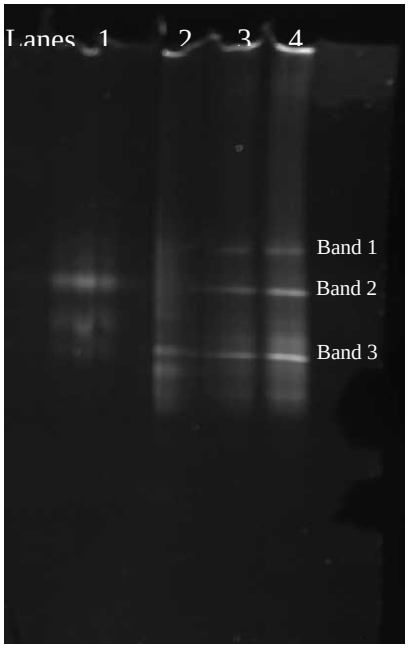


Figure 4.5 Profiles of the denaturing gradient gel electrophoretic analyses.

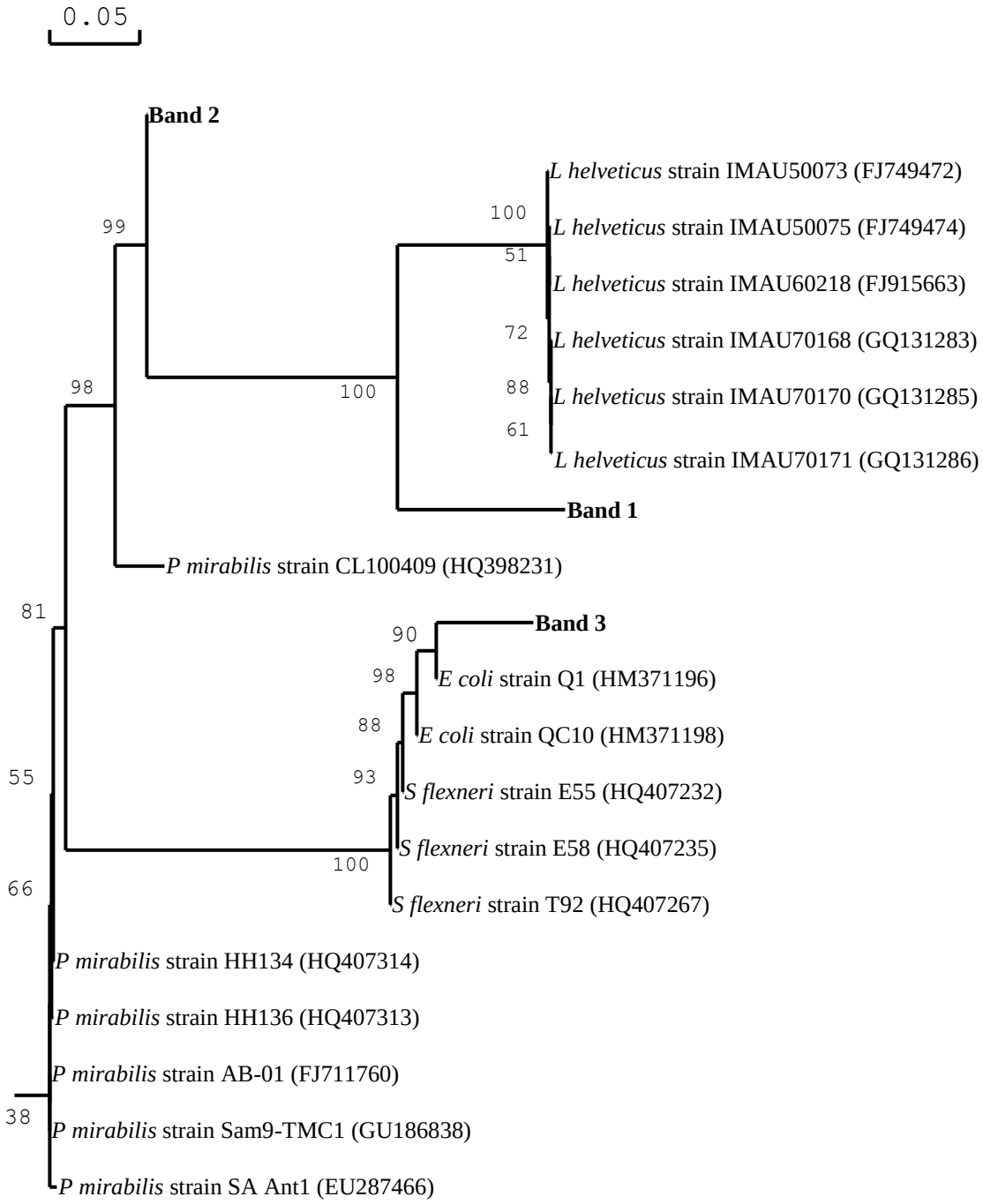


Figure 4.6 A phylogenetic tree based on the 16S rDNA from anodophiles. Numbers at internodes indicate bootstrap values (percentages of 1 000 replications).

4.5 Discussion

It stands to reason that the pre-incubation period of two months allowed anodophiles to attach, colonise electrodes and progressively increase the concentration of signalling molecules which resulted in a harmonious collective behaviour on surfaces (quorum sensing). The continual exposure of anodes to fresh whey provided the substrate needed to the growing microbial populations to reach the critical density for a global cell-to-cell communication (Kassongo and Togo, 2011c). There appears to be a global and precise control of timing for onset and magnitude of both power density and bioremediation at the interface between the anode and its exo-electrogenic biofilm. Regardless of the incubation stage, once triggered by a crucial amount of signalling molecules within the biofilm, the anode behaved as a single respiring entity at the centre for exchange between microorganisms donating and those receiving electrons (Tinsley *et al*., 2010). Anode reuse in MFCs contributed to the build-up of cell density as confirmed by microscopy imaging. It can be postulated that the biofilm may be providing an efficient conduit for electron flow, but this requires to be verified by further studies (Kassongo and Togo, 2011c). Despite the peak of metabolism (highest SDR) in the second batch cycle, there was a drop of electricity which can be attributed to the remarkable increase of electron acceptors; followed by decreasing levels of metabolism (low SDRs in 3rd and 4th cycles) in spite of substrate availability.

Increase in power density with electrode reuse up to the third cycle was expected partly because of increase in the population of electricigenic microorganisms and improved efficiency that comes with acclimation (Rodrigo *et al*., 2009, Kassongo and Togo,

2011c). The clock for maximum power density had set at 33 ± 1 h for the first three cycles with an initial twitch (peak) of maximum power density at the 6^{th} hour in the third cycle which subsequently increased to correspond to the early cycles. Interestingly, this occurrence may have reset the threshold for maximum electricity generation to a lower onset as depicted in the fourth cycle. It is also likely that the reset may be attributed to microbes suffering exhaustion that saw failure of the microbial community to sustain their systems, in the fourth cycle, for the same period as that observed in previous cycles (Nimje *et al.*, 2009; Kassongo and Togo, 2011c). Such a mechanism of communal microbial behaviour is frequent in bacteria and has recently been documented in bioelectrochemical systems (Venkataraman *et al*., 2010). The quorum sensing cascade of anode-respiring bacteria may encode for redox-shuttles which can behave as signalling molecules to planktonic microorganisms in whey, thus providing the general consistency observed between the patterns of electricity (P_d and ε_{cb}) and those of bioremediation (tCOD, glucose and solids) despite frequent replacement of the whey in reactors. Therefore, both electricigenic and non-electricigenic microorganisms work in symphony to dictate the overall performance of fuel cells.

The differential distribution of cells on same anode or across MFC cycles can be the result of absence of mixing which created both a substrate gradient and lack of transport to anode for non-motile microorganisms (Mohan *et al*., 2008). Microbial aggregates on electrode topography are viewed as hotspots for degradation of complex substrates (Lee *et al*., 2003). In the context of quorum sensing, clumps may be command centres for both local and global microbial activities. The prevalence of such structures in the third

batch cycle holds an indication that the enrichment period plays a key role in their development.

Decrease in coulombic efficiency can be partly attributed to the existence of alternative pathways that do not generate electricity. For example *L. helveticus* is known for its lactic acid-producing abilities and production of lactate alone does not guarantee improved electricity generation. A DGGE analysis revealed appearance of new bands with increasing brightness, thus confirming a common feature of electrogenesis which is the refinement of microbial composition on anode surfaces over time (Kiely *et al*., 2010). The conditions increasingly favoured growth of electricigenic microorganisms. It is noteworthy that *L. helveticus* is common to cheese whey (Gatti *et al*., 2004), while *E. coli* and *P. mirabilis* could have been present at undetectable levels or were fortuitous contaminants (Kassongo and Togo, 2011c). The latter is unlikely considering that MFCs select for electricigenic microorganisms (Rabaey *et al*., 2004; Kiely *et al*., 2010) and the repeated use of the anodes proved a selective advantage of the electricigenic , *E.coli* and *P. mirabilis* (Du *et al*., 2007).

4.6 Conclusions

Based on the results presented in this chapter, the following can be drawn:

(a) the recycling of anodes improved performance of reactors with respect to substrate utilisation and electronic shuffling in whey,

(b) acclimation initiated by a pre-incubation phase of anodes before setups lead to the establishment of a collective microbial behaviour, and

(c) species of *L. helveticus, E.coli* and *P. mirabilis* were involved in power generation.

5 OVERALL DISCUSSION, CONCLUSIONS AND FUTURE PERSPECTIVES

5.1 Overall Discussion

The road to successful electricity generation in MFCs using biocatalysts to unlock energy enclosed in substrates into detectable and viable power needs to take into account all the necessary parameters involved in such dynamics. A large extent of the existing development in fuel cells have aimed at improving the design of reactors and the engineering of diverse electrode materials, concurrently exploring a vast array of enhanced operational parameters (Chapter 1). To complement such progress, extensive studies have been conducted to fully appreciate the complexity of the mechanism involved in electricigenesis as well as the biology of microorganisms in relation to their substrates of preference. In addition, an on-going screening is needed in the search for affordable substrates to drive efficiently MFCs. Parameters such as microbial ecology; organic composition and chemical concentration of effluents all influence the power output.

The preliminary work on H-type MFCs permitted the initial selection of promising waste, subsequent design and construction of reactors with improved performance. Cheese whey and paper mill effluent were thus used for electrogenesis with their simultaneous remediation in reactors. Addition of an exogenous microbial culture to improve the concentration of biocatalysts in wastewaters was antagonistic to MFC performance owing to possible lack of synergism between catalysts inherent to wastes

and intruders (Chapter 2). As a result, there were shifts in the coulombic efficiencies. However, cheese whey performed better than paper mill effluent after treatment in reactors. In view of the foregoing, supplementary work was performed on cheese whey which revealed that continual acclimation and enrichment (using same effluent as in later setups) of anodophilic populations prior to MFC operations enhanced bioremediation and electricity generation (Chapter 3). Molecular analyses of anode biofilm revealed the presence of various species from the Lactobacillus genus. Furthermore, the recycling of anodes in reactors following a pre-incubation stage uncovered the likely existence of a motor command centre housed on the anode surface which in turn signals (quorum sensing) to other microorganisms within the anode biofilm and those in suspension in the whey, thus dictating the overall performance of a whey-powered MFC (Chapter 4).

In order to expand the current wealth of knowledge, studies in whey will need to construct polarization curves for the determination of the internal resistance and the choice of an optimum external resistor. In addition, cyclic voltammograms will be required to measure the specific contribution of mediators (signalling molecules). Until then, the human control of the bacterial clock for electricity in whey treatment may remain a mystery that we have only started to grasp.

5.2 Overall Conclusions

Based on the results presented in this dissertation, the following can be drawn:

(a) a high-strength effluent such as cheese whey is better than paper mill effluent and a promising candidate for affordable electricity generation and bioremediation,

(b) addition of an exogenous microbial culture to increase the concentration of biocatalysts may impact adversely on electricigenesis, and

(c) both acclimation of the biocatalyst population on electrodes ahead of MFC setups and anode recycling are necessary when using whey so as to improve bacterial communication and subsequent performance of reactors.

5.3 Future Perspectives

As a whole, the field of electricigenesis in MFCs has attained respectable feats in laboratory-based implementations. In the relatively novel whey-based research, more requires to be performed so as to refine the duration of the acclimation of anodes and enhance the volumetric power per anode surface area while using an unamended effluent at ambient temperature. Further investigations to look at various enrichment periods spanning one and three months in order to determine the critical duration required for an onset of a significant potential difference may need to be considered in future work. Also, pre-incubations longer than three months should be considered to determine the optimal biomass density needed in acclimation.

In addition, operational parameters such as the ones listed below may need to be developed.

5.3.1 Reactor design

Parallel studies aimed at improving the architecture of the H-type by exploring the upper limit of the CEM diameter for maximum power density. Also, the length of the wiring of the system would progressively be shortened to the optimum. Investigate the impact of operating multiple reactors in series and in parallel.

5.3.2 Electrode

Experimentations with platinum-coated electrodes to minimize over-potentials and nanotechnology to increase the reactive surface area should be conducted. Alternatively, increase the size of electrode sheets in order to compensate for the nutrient gradient created by the sludge blanket of effluents in reactors.

5.3.3 Resistor

Constructions of polarization curves to provide insight on areas where there are over-potentials and thus assist in the choice of an appropriate external resistor. Also, the connection of a potentiostat to the MFC in order to provide an interrupted flow of electro-analytical data.

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APPENDICES

APPENDIX A

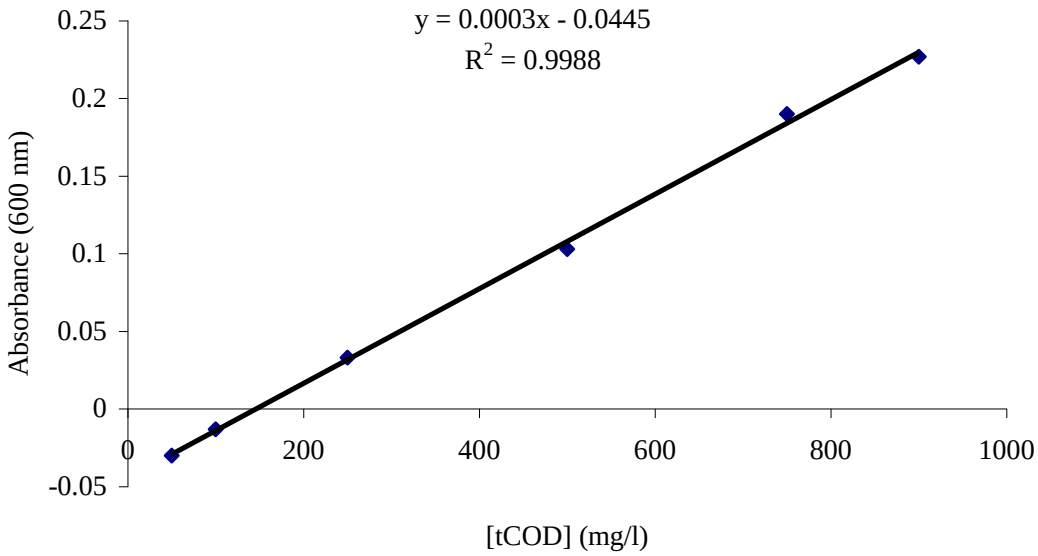


Figure A1 Standard curve of potassium hydrogen phthalate

APPENDIX B

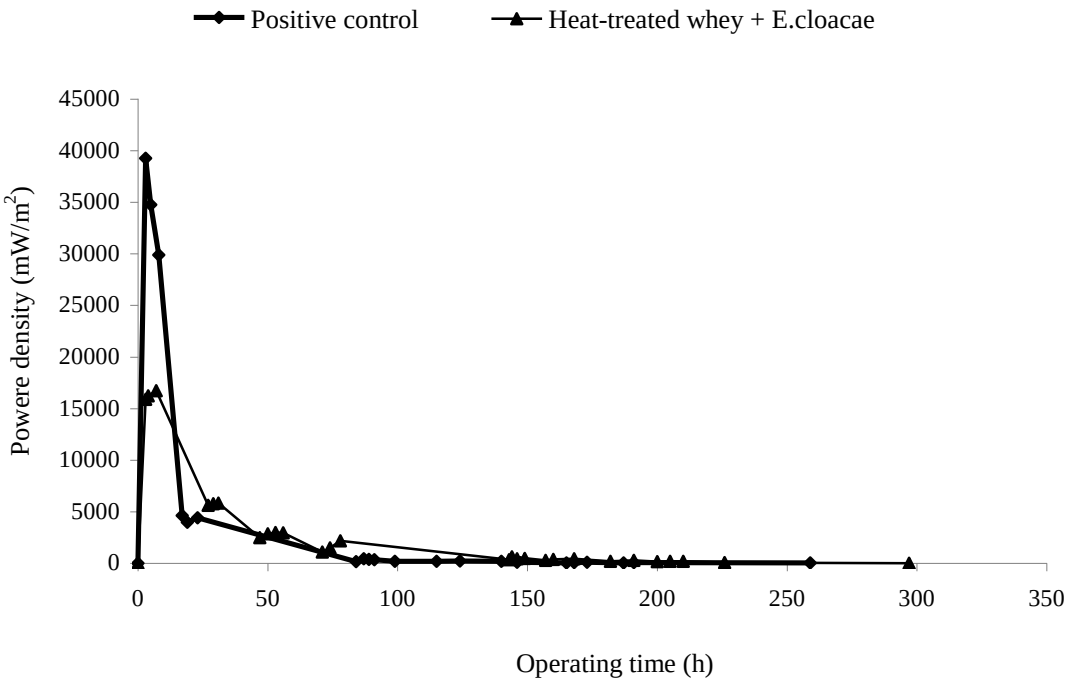


Figure B1 A typical MFC performance when fed with heat-treated whey and using *E. cloacae* inoculum. Maximum power density: 16.7 ± 1.75 (W/m^2); the positive control (RCM inoculated with *E. cloacae*) is also shown.

APPENDIX C

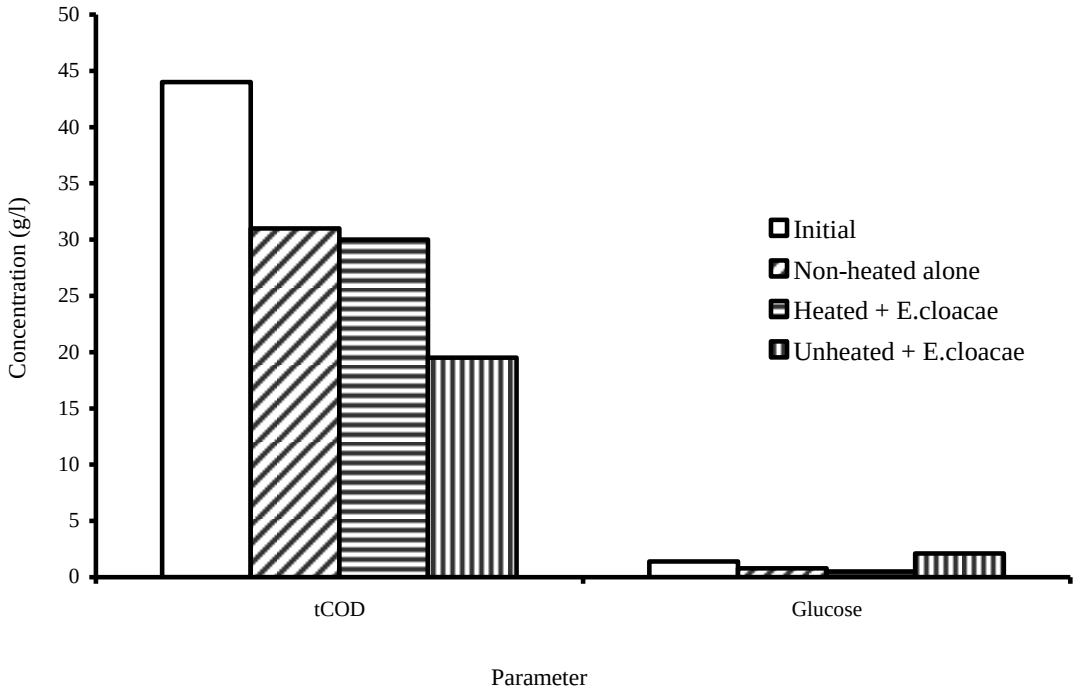


Figure C1 Bioremediation profile of paper mill effluent in different setups after MFC cycles in relation to initial concentrations.