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CELLOBIOSE DEHYDROGENASE/ EPOXY-GRAPHITE COMPOSITE WITH ARYL DIAZONIUM REDUCTION FOR LACTOSE DETECTION

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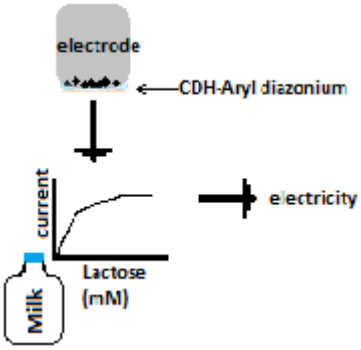
Graphical abstract



Abstract

Milk is an important ingredient in our day to day diet bacause of the high quality nutrients in it. In the dairy industry including cheese fermentation processes, there is a need to control the release of lactose into wastewater streams. There are methods adopted to recover the lactose and to transform the lactose into energy through renewable energy (RE) pathways. These methods however are expensive and require certain skill to operate them. In this study, in-house electrode, which is simple and can be applied after one day of fabrication were investigated. The method was by using graphite-epoxy composite electrode, surface modified with cellobiose dehydrogenase (CDH) enzyme using aryl diazonium. These designed composite electrodes were tested on its capability as biosensor for sensitivity on detecting the lactose as well as its capability as an anode in enzymatic fuel cell (EFC) on long term electrochemical stability in generating electricity from lactose oxidation. The results showed that the CDH-Aryl diazonium modified on surface of fabricated graphite-epoxy electrodes had Michaelis Menten constant K_m for CDH (0.65 – 0.75 mM) comparable to available commercial electrodes reported in the literature (0.7 mM). They are also conductively sensitive with the current intensity 86% more with the above mentioned electrodes when modified with embedded multi-walled carbon nanotube (MWCNT) and gave a high reproducibility signal (63% more than fabricated electrodes without MWCNT). In addition to the above, its performance stability in continuous mode operation for 25 days, recorded almost consistent in current detection (19.2 ± 3.8 μA/ cm^2). Hence, these fabricated electrodes give alternative for a sensitive lactose detector which is cheap and simple to fabricate.

Keywords: Biosensor, cellobiose dehydrogenase/aryl diazonium, enzymatic fuel cell, graphite-epoxy composite, lactose

1.0 INTRODUCTION

Milk is an important constituent of our daily life because of its inherent nutrients, such as carbohydrates, fat, protein, vitamins and minerals and enzymes. In the dairy industry, there is a need to control the lactose effluent polluting the wastewater streams. Raw milk has a BOD_5 of about 100 g L^{-1} [1] while the soluble carbonaceous tolerance adopted by the New Zealand government is less than 0.002 g L^{-1} daily [2]. This standard was set to reduce water pollution by bacteria while ensuring the accessibility of aquatic ecosystem to soluble oxygen in waterways. For this reason, there have been reports published on

methods to remove lactose from the waterways including recovering lactose from dairy waste streams using filtration techniques, ultra and nano and reverse osmosis [3, 4], and converting lactose and other organic matters to biogas by using the up flow anaerobic sludge-fixed film (UASFF) [5]. The latter technique is a branch of renewable energy where it uses the internal energy from unwanted biomass and transforms it into a useful source of energy. Another branch in renewable energy, which also is an extension from biosensor technology that directly converts energy through electrochemical pathway from biomass into electricity is the enzymatic fuel cell (EFC). Enzyme applied in EFC would have specific activity and highly selective in choices of substrates. For instance, lactose can be determined using biosensors employing a single cellobiose dehydrogenase (CDH) enzyme. CDH is an extracellular enzyme with narrow substrate specificity: active on cellobiose and lactose while showing distinct discrimination over glucose and maltose [6]. The enzyme has two prosthetic groups, the dehydrogenase (FAD domain) and a cytochrome (heme domain). During lactose oxidation to lactobionic acid in an external mediator- free environment, the FAD domain with the maximum capacity of accepting two electrons, will oxidized the lactose while reducing itself to $FADH_2$. The electrons on the other hand will be channelled one at a time via a flexible linker through the heme domain, which lies about 15 Å away from the FAD domain and get transferred to the electron acceptors, an electrode [7]. Various studies were done on the CDH- modified electrodes for biosensor and EFC applications, targeting lactose as the substrate with trend to investigate and improve the electron transfer from the CDH to the electrodes (Table 1). The CDH capability in performing DET and high substrate specificity without need to compete with oxygen makes the enzyme a preferred choice [8] when involving lactose / and cellobiose as substrate.

Graphite material is a common electrical conductor due to its π bonding between C atoms creating layered, planar structure that allow electrons to move freely [9]. At the same time, graphite gives good electrochemical reversibility to electrode reactions [10]. CNT on the other hand is well known for its ability to mediate fast electron transfer kinetics on a wide range of electroactive species [11-13]. So far, amalgamation of these two electrical conductive materials; graphite and CNT, has shown good conductivity improvement in electrodes activity [10, 14, 15]. In recent advances, graphite-epoxy composites are being studied for improvement in mechanical and electrical features. In view of utilization, research works were done particularly in biosensor [16-19] and bipolar plate [20, 21] sectors. Since the epoxy resins have good electrical insulation properties, suitable conductive fillers and curing agents need to be applied to make the resins conductive or semiconductive [22]. The advantages of a conductive graphite-epoxy composites, lie on the versatility in fabricating custom made electrodes, i.e. having various sizes and shapes, simple to prepare and easy adaptation to wide electrode configurations [17]. From previous research, we were able to quick fabricate conductive graphite-epoxy composites consist of more than 70% (w/w) of graphite content within a day, using cheap and simple technique. Aryl diazonium salts on the other hand is simple to prepare, rapid in electroreduction, large choice of reactive functional groups and provides strong covalent bonding between the aryl and the surface: polymers, biomacromolecules and nanoparticles [23]. Tasca *et al*. observations on covalent binding of CDH to glassy carbon electrode, found that it is better to make the electrode's surface positively charged using diazonium salts from amine group, since it will create a better interaction between the modified surface and the negatively charged heme domain [24].

To our knowledge, there are no reports on the capability and reliability of graphite-epoxy composite for use as an electrode for lactose detection. By combining the graphite-epoxy composite with CDH using aryl diazonium bonding, new insights could be obtained for better and more sensitive lactose detection.

In this study, graphite-epoxy composites were fabricated with MWCNT embedded in the matrix surfaces. Safranin/ aryl diazonium was electrochemically grafted to the composites' surfaces to create strong covalent bonding and induced preferred orientation between the negatively charged CDH and positively charged electrodes' surfaces. The sensitivity of the composite electrodes in detecting lactose was compared to the capability of other published papers on lactose biosensor while the electrochemical stability of the electrodes in continuous operation was monitored through EFC system.

The aim of this study was to investigate performance of immobilized CDH bonded using aryl on graphite-epoxy electrodes with and without embedded MWCNT, for sensitivity in lactose detection and continuous detection stability. Emphasis was on performance of the electrode in increasing the maximum detection of lactose analysis, shorter response time, and sensitivity of the sensor in boosting the amount of electron transfer from the heme domain of the CDH to the electrode and long term operational stability of the electrode performance in continuous flow environment.

Table 1 Some publications on interaction between lactose and CDH-modified electrodes for biosensor and EFC applications

Electron acceptor	Interest	Source	Year
Graphite rod (Ø 3.05 mm)	Investigate the performance of DET between whole CDH / FAD fragment adsorbed directly on electrode surface and whole CDH / FAD fragment cross-linked in a mediator on electrode surface for biosensor application (CDH source: *Phanerochaete chrysosporium*)	[15]	1996
Graphite rod (Ø 3.05 mm)	Effect of pH and ionic strength on the bioelectrocatalysis of cellobiose and lactose at CDH-modified graphite electrodes for biosensor application (CDH source: *Phanerochaete chrysosporium*)	[25]	2000
Graphite rod (Ø 3.05 mm)	Evaluating detection limit, linear range, sensitivity of sensor and long term stability of CDH-modified graphite electrodes for lactose and cellobiose in flow injection mode: in the presence and absence of 1,4-benzoquinone for biosensor application (CDH source: *Myriococcum thermophilum*)	[26]	2007
Graphite rod (Ø3.05 mm)	Investigate the DET performance of CDH from different sources of fungi in the presence and absence of SWCNT modified to anode surface for EFC application (CDH source: *Phanerochaete sordida, Sclerotium rolfsii*, *Myriococcum thermophilum, Trametes Villosa and Phanerochaete chrysosporium*)	[27]	2008
Graphite (3 mm x 3 mm)	Comparing DET and MET between CDH and anode in a membrane-less EFC (CDH source: *Phanerochaete sordida*)	[6]	2009
Graphite rod (Ø3.05 mm)	Investigate the performance of integrating CDH with specifically developed polymer mediator with respect to conversion of lactose for EFC application.	[28]	2009
Graphite based SPE (Ø1.80 mm)	Improve efficiency of direct bioelectrocatalysis by CDH- electrosynthesised PANI –graphite based SPE (CDH source: *Myriococcum thermophilum*)	[29]	2009
Carbon based SPE (Ø na)	Improve a lactose biosensor designed by Stoica *et al*. (2006) using graphite rod as based with cross-linked CDH-MWCNT modified carbon based SPE to make it small, more sensitive and suitable for on-line mode (CDH source: *Phanerochaete sordida*)	[30]	2010
Glassy carbon electrode (Ø3.00 mm)	Investigate the effect of negatively/ positively charged SWCNTs on DET interaction between heme and electrode with respect to current density and stability of the produced electrodes for EFC application (CDH source: *Phanerochaete sordida*)	[24]	2011
Graphite (Ø na)	Investigate the influence of different concentrations of sodium chloride on the performance of CDH from various sources in solution and immobilised on electrode area for EFC application (CDH source: *Phanerochaete chrysosporium, Myriococcum thermophilum, Pichia pastoris, Humicola insolens and Aspergillus oryzae*)	[31]	2012
Graphite rod (Ø na)	Combined thermometric/amperometric biosensor, which is separated from immobilised CDH on pore glass in a flow system assisted by benzoquinone (BQ) as mediator (CDH source: *Phanerochaete chrysosporium*)	[32]	2012
Carbon based SPE (Ø 4.00 mm)	Developed an automated at-line lactose biosensor for monitoring dairy wastewater streams using SPE modified with MWCNT using a prototype design, Lactosenz TM	[33]	2013

Note: DET = direct electron transfer, MET= mediatored electron transfer, SPE = screen printed electrode and MWCNT = multi-walled carbon nanotube, CNT = carbon nanotube

2.0 METHODOLOGY

2.1 Chemicals

Chemicals were of analytical grade. Potassium ferrocyanide $K_4Fe(CN)_6$ was purchased from AnalaR®, BDH Laboratory Supplies (Poole, England). Potassium ferricyanide $K_3Fe(CN)_6$ was purchased from UNIVAR, Ajax Finechem (Wellington, NZ). N,N-Dimethylformamide (DMF), sodium nitrate $NaNO_2$ and aryl diazonium were purchased from Sigma (Auckland, NZ). Lactose $C_{12}H_{22}O_{11}.H_2O$ was purchased from Fisher Scientific (New Hampshire, US). Citric acid monohydrate $C_6H_8O_7.H_2O$ powder, hydrochloric acid (11.7 M HCl) and sodium hydroxide NaOH pellets were purchased from LabServ™, Thermo Fisher Scientific, NZ Ltd. (North Shore City, NZ).). Tri-sodium citrate $C_6H_5Na_3O_7.2H_2O$ and safranin were purchased from BDH Laboratory Supplies (Poole, England). The 5% functionalized-COOH Multi walled carbon nanotubes (MWCNT) was purchased from DropSense (Spain). Graphite powder with ultra 'F' purity was purchased from Ultra Carbon Corp. (Michigan, US). Resin–norSKi® part A and hardener norSKi® part B were purchased from norSKi® (Wellington, NZ).

All analytical solutions were made using Milli-Q water from EASYpure UV (Barnstead, New Hampshire) unless otherwise stated. The buffer used for lactose analysis was a citrate buffer (CB) (0.1 M $C_6H_8O_7.H_2O$/ 0.1 M $C_6H_5Na_3O_7.2H_2O$, 1.0 M KCl) with pH adjusted to 4.5 using 1.0 M NaOH [6, 30].

The stock reagent of lactose (0.2 M $C_{12}H_{22}O_{11}.H_2O$) was made into several range of concentrations by diluting lactose solution with CB solution using the following equation: $V_1 = (M_2 x V_2)/ M_1$. The V_1 is the volume to be added in mL from the original lactose source, M_1 is the concentration of the original lactose source, M_2 is the end concentration of lactose while V_2 is the end volume required for testing. All of the prepared lactose with different concentrations were stored in different bottles and stored at 4 °C when not in use.

2.2 Enzyme Strain

Solution of CDH from *Phanerochaete sordida* was provided by Dr. Roland Ludwig, BOKU, Austria. The CDH has a volumetric activity of 291 U mL^{-1} and a specific activity of 23.9 U mg^{-1} protein. The enzyme unit (U) is a unit for the amount of a particular enzyme. One U is defined as the amount of the enzyme that produces a certain amount of enzymatic activity, that is, the amount that catalyzes the conversion of 1 micro mole of substrate per minute.

2.3 Preparation of Working Electrodes

The base for the anodes were made from graphite powder mixed with epoxy resin in % (w/w) ratio of 78:22. The total filler loading was more than 50 wt% to produce a fast drying conductive electrode with low inherent resistance. Ratio between the resin (norSKi® part A) and its hardener (norSKi® part B) followed Pumera *et al*. at (w/w) of 20:3 [18]. The combined material was manually blend using spatula until the texture turned flaky and packed into the PCR tubes (ø 0.4 cm) at the length of 1.5 cm. For the embedding of MWCNT into the matrix of graphite epoxy composite, MWCNT was prepared through mixing of 1 mg of MWCNT with a 0.4 mL N,N-Dimethylformamide (DMF) in a vibrator for 1 min. The mixture was later topped up with 0.6 mL of 70% ethanol and sonicated in water bath for five minute. Loading of the MWCNT ink into the base of graphite-epoxy composite was done at % (w/w) ratio of 0.04:1. The MWCNT mixtures were manually blend using spatula and dried under room temperature until the texture became muddy dry. The mixture paste was topped up about 0.3 cm high on the packed graphite- epoxy resin mixture in the PCR tube, to become the electrode's surface. Prior to the surface modification methods, each of the packed PCR tubes was centrifuged at 14, 000 g for 1.5 min to compress and remove remaining air in the paste. A copper wire was inserted at the bottom of the tube for electrical contact. The filled PCR tubes were then cured at 80 °C for 12 h. They were then allowed to cool at ambient temperature for 30 min. Later, the excess PCR tubes wall were cut until the wall was at the same level with the surface of the fabricated graphite-epoxy composite electrodes. The electrode surface area were polished first on a wet fine emery paper (Norton, P400), rinsed with Milli-Q water and dried on paper towel before polished on white paper until mirror like surface appeared. Modification with aryl diazonium adapted steps from Picot *et al*. and Commault *et al*. was done on the fabricated electrodes [34, 35]. Here, 0.1 M HCl was mixed with 0.01 M safranin and dissolved in Milli-Q water for total volume of 5 mL. The mixture was placed in ice bath and under nitrogen flow while kept in complete darkness. About 0.02 M of $NaNO_2$ was added into the mixture. Surface of the electrode was then modified through electropolymerization in a three-electrode electrochemical cell consisting of the anode as working electrode, a Pt auxiliary for counter electrode and an Ag/ AgCl for reference electrode. A potentiostat (EC epsilon, BASi, IN, USA) was used to control the constant potential of -0.161 V (vs. Ag/ AgCl) applied to the anode until total coulombic charge consumed is about 300 mC cm^{-2} (Initial P= 0 mV, t_{Quiet}= 2s, 1st step = -161 mV; 1,200 s, 2nd step = 0 mV; t= 0s). The freshly prepared modified electrode was rinsed several times in Milli-Q water and absorbed dry on paper towel. The modified electrodes were rinsed with large volumes of water and allowed to air dry. CDH- modified electrodes were prepared by pipetted 10 µL of CDH solution onto each aryl/ graphite-epoxy electrode's surface to adsorb by the surface at 4 °C under controlled humidity, overnight.

2.4 Enzymatic Fuel Cell Air-Cathode System Construction

Stability of the fabricated anodes during continuous lactose detection were analysed using a miniature air-cathode enzymatic fuel cell (EFC) (Figure 1). The EFCs consist of a thin drum shaped polyethylene reactor (ø 4 cm) with one wall made from hard polyethylene and the other from Ultrex membrane (BASF Fuel Cell Inc, Somerset, NJ, USA). The air-cathode made from 10% Pt-carbon cloth (Fuel Cell Earth LLC, Stoneham, MA) (12.6 cm^2) was fastened to the exterior wall of the Ultrex membrane with a nickel plate (4 cm x 1 cm) and an elastic band. The working electrode had the modified area facing the Ultrex membrane interior wall at a constant distance of about 1-2 mm and was inserted through a small hole made by butynol Dunlop sheet (2 cm x 1 cm) that was fixed onto an opening on the hard polyethylene wall. This is to ensure snug fitting between the electrode and the electrode insertion port at the EFC wall to avoid electrolyte leaking. To enable application of reference electrode (Ag/ AgCl) during the amperomety analysis, a small insertion hole was made at the top of the polyethylene wall, almost parallel to the Ultrex membrane and perpendicular to the inserted graphite rod. The maximum volume of the EFC was 5 mL and the system was equipped with self-drained ability (cut 1 mL polyethylene pipette tip) to naturally maintain the volume of electrolyte and avoid overflow from occurring.

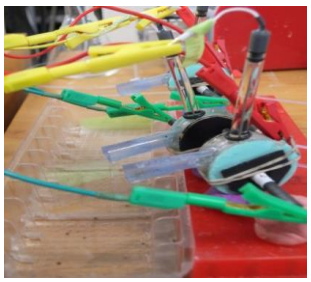


Figure 1 The in-house EFC made in this study equipped with self-drained system

2.5 Electrochemical Measurements

Electrochemical analyses were conducted using direct current potential amperometry (DCPA) with a four- channel potentiostat (QuadStat 164, eDAQ Pty Ltd, New South Wales, Australia) and data acquisition system (e-corder 1621, eDAQ Pty Ltd, New South Wales, Australia) connected to a computer. A three-configuration electrodes were applied: bioanodes for working electrodes, auxiliary electrodes and Ag/ AgCl (3 M KCl) for reference electrodes. Analyses done in batch mode applied Pt while in continuous mode applied the air-cathode itself for the auxiliary electrodes. Sensitivity in lactose detection was done in both batch and continuous mode. The anode was set at potential +100 mV (vs. Ag/ AgCl), which was adapted from Safina *et al*. where she found that *Phanerochaete sordida* worked well when the electrodes were poised at +100 mV (vs. Ag/ AgCl) [30]. Here, 12 different lactose concentrations were tested: 0.04 mM, 0.08 mM, 0.3 mM, 0.5 mM, 1 mM, 2 mM, 3 mM, 4 mM, 5 mM and 10 mM, 15 mM and 25 mM. Analysis in continuous mode introduced flushing with 0.1 M CB in between the lactose detection with the flow rate of 0.5 mL min^{-1}. The anode performance stability analysis was done using a fixed lactose concentration of 5 mM, following Tasca *et al*. based on the same K_m value obtained in this study [24].

3.0 RESULTS AND DISCUSSION

3.1 Sensitivity in Lactose Detection

The study of maximum detectable lactose concentration could show the highest lactose concentration capable to be oxidized by the enzyme before the enzyme becomes saturated and no longer produce significant current even though there is an availability of substrate.

From Figure 2, both mode of analyses, batch or continuous, regardless whether the surface modified graphite-epoxy electrodes are with or without embedded MWCNT, shows having similar capability to detect a wide range of lactose concentrations, up to 70 mM. This shows that the maximum lactose concentration the anode can oxidized under potential poised of +100 mV, would be around 70 mM. Higher than that, there will be no increase in current intensity. In a study done by Stoica *et al*. on lactose detection by CDH-osmium/spectroscopic graphite rod, under anode potential poising of +205 mV (vs. Ag/ AgCl), the maximum lactose concentration detected was until 34 mM generating about 7.86 µA cm^{-2} [28]. When they applied it as anode in an air-cathode EFC, with 34 mM lactose as anolyte, the maximum current density obtained was 1.7 fold higher than the current at the saturated point. In this study, more than 30 µA cm^{-2} could be generated at 34 mM lactose concentration (Figure 2). This gives the possibility of the anodes in this study to generate higher maximum in current and power density when operated in EFC. The Michaelis- Menten constants (K_m) obtained from the fabricated electrodes were within the range of 0.65 – 0.78 mM, which is not much different from the K_m obtained for *Ps*CDH in Tasca *et al*. [24] (Table 2). K_m focusses on enzyme activity of CDH, measured via the generated current produced by the liberated electrons from the oxidation of lactose concentrations through the external circuit. Study on the K_m when analysed in electrochemical cell upon different concentrations, helps selecting bioelectrodes with a larger K_m value for application in tough condition of continuous lactose detection in an EFC system. A high K_m value will show the capability of the system to operate at higher concentration of

lactose before the enzyme became saturated. This would offer the possibility to tap into more electrons out from the oxidation of high lactose concentration to generate current with greater intensity. For the studied bioelectrodes, applying surface modification

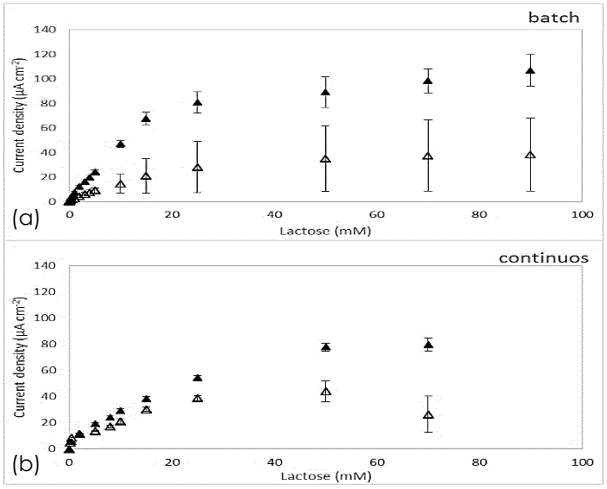


Figure 2. Lactose detection capability in different lactose concentrations via batch mode (a) and continuous mode (b). The (△) represents the modified electrodes without embedded MWCNT, while (▲) represents modified electrodes with embedded MWCNT. Data were obtained using DCPA analysis with working electrode poised at +100 mV s^{-1}. The electrolyte was 200 mM lactose diluted in 0.1 M CB to make into different lactose concentrations. Error bars represent standard error of the mean (n=3)

with embedded MWCNT did not significantly increase the K_m values. This indicates that the high current intensity achieved was due to the contact between CDH and MWCNT located on the electrode's surface [36]. To obtain a high electron transfer rate, the heme domain of the CDH must be in the correct orientation to the electrode surface, which is less than 20 Å away [24]. This was proven by Tasca *et al*. in their experiment on glucose biosensor via *Corynascus thermophiles* (*Ct*CDH) [37]. By comparing between the presence and non-presence of SWCNT, they found the performance of *Ct*CDH through application of SWCNT on the surface of the spectrographic graphite electrode, had increased the K_m of glucose concentration higher by 1.2-fold and improved the current intensity by almost two-fold at 300 mM glucose (the maximum glucose concentration analysed in the study) while still having the same maximum linear detection range as the SWCNT-free biosensor. Effect of continuous lactose flow with intermittent buffer flushing had given a slight increase in K_m values. K_m is affected by several factors, such as pH, temperature, ionic strengths and the substrate concentration. The slight increase in K_m could be due to the continuous flow mode maintaining consistent environment for the enzyme by supplying fresh feed every time into the system.

The only difference in observation is the current intensity generated from this study, where the current intensity is greater in batch rather than in the continuous mode (Table 2). Significant statistical differences (t-test, $p<0.05$) however, were seen only in the batch mode at lactose concentrations of 0.5 and 5 mM, where current intensity from the surface modified graphite-epoxy electrodes were higher than the electrodes without embedded MWCNT.

At the same time, on average for each electrode types, the current reproducibility is higher (smaller RSD %) on the electrodes with embedded MWCNT, especially in the continuous mode (t-test, $p<0.05$). This shows that the existence of embedded MWCNT had improved the conductivity of the electrodes.

Although graphite-epoxy composite electrodes have rather high inherent resistance (Kirgoz *et al*., 2006), the adding of MWCNT into the system however was able to reduce it (Spitalsky *et al*., 2010). The MWCNT may have dampen the effect of the non-conductive epoxy resin and eventually increased the reproducibility of results, compared to the graphite-epoxy electrodes without embedded MWCNT that had rather low reproducibility.

A similar effect due to surface treatment with CNT yet with a different approach from this study, was also observed by Safina *et al*. (Safina *et al*., 2010). Their study on biosensor on the effect of CDH from *Phanerochaete sordida*, when cross- linked directly onto MWCNT on carbon screen printed electrode (cSPE) for lactose detection, showed that the existence of MWCNT on the cSPE showed an improvement in current density by 1.5 to 2.5 times higher than the cSPE without MWCNT. In fact, other studies that had CDH directly linked to SWCNT showed high current intensity (Table 2).

In this study, MWCNT was not exposed on the surface of the base electrode, instead the MWCNT was mixed into the matrix graphite composite. The embedded MWCNT in the fabricated graphite composites might create continuous electrical wires to allow easy DET flowing from cytochrome that bonded covalently onto the aryl diazonium compound to the fabricated electrode and all the way to the copper wire, which acts as current collector. Synthesis of aryl diazonium salt from safranin was chosen to obtain protonated state from phenazine-NH2 when in acidic environment, pH 4.5.

The negatively charged cytochrome from the CDH will be attracted to the dangling positively charged - NH_3^+ with its other end covalently bonded to the graphite surface (Figure 3). Hence, these functional charged groups should increase both the interaction forces and orientation of the enzyme on the surface of the electrode [24] and improve the DET.

The fast response time, less than a minute per sample is slightly faster than 2 minutes per sample obtained by Yakovleva *et al*. for their lactose biosensor [32]. This fast sampling obtained through fabricated graphite electrode might be due to the wide electrode active surface area and correct orientation of CDH and - NH_3^+ that favours easy flow of electron transfer from CDH to electrode.

Table 2 Anode reproducibility test at several lactose concentrations

		Lactose (mM)			
		0.5		5	
Anode sample	K_m (mM)	Current Density (Mean) (μA/ cm²)	RSD (%)	Current Density (Mean) (μA/ cm²)	RSD (%)
MWCNT added					
CDHAryl diazonium/ MWCNT epoxy graphite composite (*batch*)	~0.65	4.6 ± 0.5	20	24.0 ± 1.5	11
CDHAryl diazonium/ MWCNT epoxy graphite composite (*continuous*)	~0.75	5.2 ± 2.3	78	19.2 ± 3.8	35
No MWCNT (Control)					
CDHAryl diazonium/ epoxy graphite composite (*batch*)	~0.75	1.7 ± 0.1	10	9.3 ± 2.0	30
CDHAryl diazonium/ epoxy graphite composite (*continuous*)	~0.78	8.5 ± 8.3	138	13.6 ± 12.7	132
[a]CDHAryl diazonium/SWCNT/ Glassy carbon	0.70	na	na	500	na
[b]PEGDGE-CDH/SWCNT/graphite rod	2.3	na	na	80	na

[a][24] & [b][27]. PEGDGE = poly(ethylene glycol) diglycidyl ether

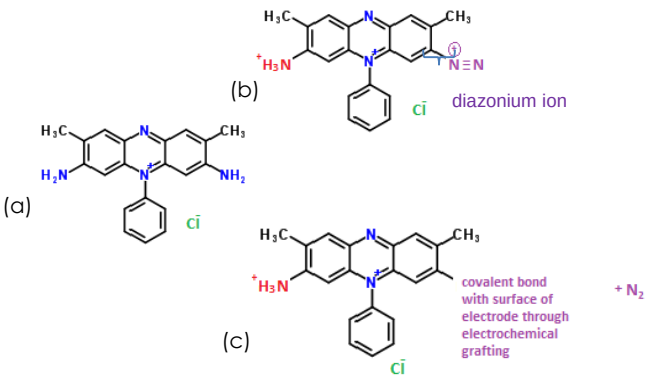


Figure 3 Molecular representation of safranin (a), the formed diazonium ion getting ready to make bonding (b) and the attachment of the aryl diazonium salt through covalent bond onto a surface (c) Safranin = 3,7-Diamino-2,8-dimethyl-5-phenylphenazin-5-ium chloride. Having molecular formula of $C_{20}H_{19}ClN_4$

3.2 Prepared Electrodes Provide Continuous Current Signal

The duration stability test was done in air-cathode MFCs with 5 mM of lactose continuously flowing into the system. The analysis was carried out onto the fabricated graphite composite electrodes for a length of 25 days (Figure 4). Results showed that anodes with MWCNT embedded into the surface matrix gave almost consistent and higher in averaged current density, about 86% more than anodes without embedded MWCNT at the fixed lactose concentration. The average current intensity during the stability test is however 2.4 fold lower than obtained by anodes with MWCNT embedded into the surface matrix during continuous operation (Table 2). This could be the result of sufficient rinsing time washing away the excess safranin before used for stability analysis. The safranin that was left behind has strong bonding created between itself and the graphite showed from the stability of the current.

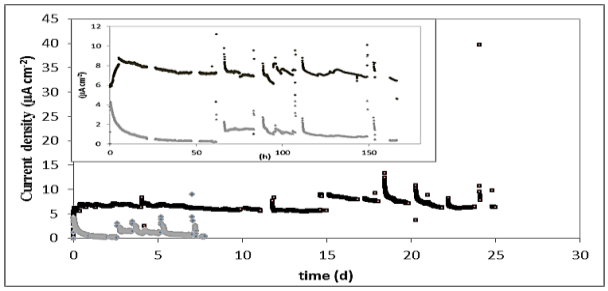


Figure 4 Stability test done in an air-cathode EFC for fabricated graphite-epoxy anodes with CDH on aryl diazonium surface modification. (■) represents anodes with embedded MWCNT while (□) represents anodes without embedded MWCNT. Data were obtained using DCPA analysis with working electrode poised at +100 mV s^{-1}. The electrolyte was 5 mM lactose in 0.1 M CB at 0.5 mL min^{-1} (n=3)

Due to technical problems, analysis done to anodes without embedded MWCNT was discontinued on the 7th day of EFC operation. Set aside the technical problems, this shows that the fabricated anodes are capable in working within long duration and still sensitive without any significant decrease in analytical response. To our knowledge, continuous stability analysis on lactose detection conducted to a CDH modified electrodes for more than a day [30, 32, 33] have been reported on commercial electrodes such as SPEs and graphite rods, however none on graphite-epoxy composite electrodes. Although using the commercial SPE modified with MWCNT could give better performance in RSD% and smaller concentration detection as in Glithero *et al*. [33] (0.002 mM to 29 mM), the in-house fabricated graphite epoxy composite electrode in this study offers the flexibility and freedom in designing the electrode shape based on the desired reactor, while maintaining equally fast detection time as the SPEs. In addition, this study targets on high generation of current from the lactose oxidation besides concentration detection for lactose monitoring. Hence, electrode stability analysis on the graphite-epoxy composite while functioning as anode under potential poising for lactose continuous detection and generating high currents look promising for further development in real EFC condition without any anode poising.

4.0 CONCLUSION

It is very important and crucial to detect a wide concentration range of substrates and at the same time use them as a stable anode in EFC to generate high current intensity for a long duration (more than 30 days). It serves the purpose of both biosensor and EFC. This study was able to show that the simple and fast drying graphite-epoxy composite electrodes were capable of producing K_m of CDH similar to commercial electrode materials. In addition to that, it was able to detect a higher maximum lactose concentration than achieved by commercial electrode materials and can withstand strenuous long term operation. The embedded MWCNT did not give intense current compared to the MWCNT on the surface due to the non-existence of a direct link between the CNT and the CDH-aryl diazonium. Embedding the MWCNT within the graphite-epoxy matrix however shows that it was able to significantly increase the reproducibility of the signal and intensify the current up to 86% compared to an electrode without embedded MWCNT. In this study, the ability to fabricate anodes gives flexibility to cater with system design. Further studies can be directed to examine the effect of half embedded and half protruded MWCNT on electrode surface for electricity generation.

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