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Alcohol Oxidase Linked Enzyme Cascade as Anodic Biofuel Cell Catalyst

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Abstract: ***This study explores the development of a biofuel cell utilizing alcohol oxidase AOx in an enzyme cascade at the anode. The aim was to increase power density by replacing NAD based dehydrogenases with AOx for the oxidation of methanol. The bioanode with AOx, coupled with formaldehyde and formate dehydrogenases, achieved enhanced power density compared to conventional systems. The electrochemical characterization of the bioelectrode demonstrated effective redox behavior and stability over time. This biofuel cell system represents a step toward more efficient energy production from alcohol substrates.***

Keywords: Alcohol oxidase, biofuel cell, enzyme cascade, methanol oxidation, NAD+ regeneration

1. Introduction

The development of EFC (enzymatic fuel cell) and their potential uses has been accelerated since the beginning of last decade and attracting worldwide attention driven by the demands for clean and renewable energy resources to a viable green technology for powering micro - scale electronic gadgets (Cracknell et al., 2008; Bullen et al., 2006; Cooney et al., 2008). In general, the redox enzymes at the anode catalyze only single step of two - electron oxidation per molecule of substrate that promote continuous accumulation of the product organic compound in the anodic compartment which ultimately may interferes the performance of the EFC due to several reasons. Effort has been made to improve the situation by using cascade of redox enzymes as anodic catalysts that facilitate the deeper oxidation of substrates through which multiple pairs of electrons could be gained from single substrate molecule resulting in an increase in power density of the EFC (Hickey et al., 2013; Sokic - Lazic and Minteer, 2008). These enzyme cascades can range from a simple two enzyme system into a cluster of enzymes belonging to complex metabolic system (Sokic - Lazic et al., 2010; Liu et al., 2013). Among the various substrates studied for EFCs, substrate alcohol have garnered wide attention. Deep oxidation of alcohol substrates (methanol) for generating power in EFC has been studied with dehydrogenase cascade consisting of the enzymes, ADH, formaldehyde dehydrogenase (FDH) and formate dehydrogenase (FtDH) and the combined action of which converts methanol to carbon dioxide (Kar et al., 2011; Akers et al., 2005). All these enzymes act in the cascade need the cofactor NAD^+ to supplement for their function that eventually escalate the cost and increase technical intricacy related to their function in the enzyme electrode interface. EFC with alcohol as fuel substrate has also been reported with other alcohol oxidizing enzymes namely, PQQ based alcohol oxidases (Ikeda and Kano, 2003). However, the magnitude of power or potential generated by using these single enzyme catalytic systems did not surpass the one yielded by using NAD based dehydrogenase enzyme cascades and one of the reasons attributed to this improved performance of the later system is the deep oxidative led high electrons yield from substrates as discussed above (Rincon et al., 2011). Dey et al., 2023 developed multienzyme cascade in carbon dioxide electroreduction fuel cell where rate of the formation of methanol is faster with co - immobilized enzymes. Co - immobilization of dehydrogenase is associated with NAD regeneration which involves (i) semiconductors or metals using solar illumination and (ii) reduction of NAD^+ under electrochemical conditions. Considering the positive traits of AOx as discussed and enzyme cascades for EFC applications, we investigated here an alcohol substrate-based EFC where the NAD based ADH present in the dehydrogenase cascade has been replaced by the AOx enzyme for initial oxidation of alcohol to formaldehyde, which is subsequently being oxidized by other dehydrogenases immobilized on the electrode surface along with the enzyme AOx. The performance of the AOx coupled FDH and FtDH bioanode has been evaluated in a half cell studied and subsequently the overall power yield of the EFC with methanol as fuel substrate was investigated in a membrane less EFC using laccase-based nanocomposite biocathode for bio - electrocatalytic reduction of molecular oxygen. A detailed account on the findings has been described in this chapter.

2. Experimental Approaches

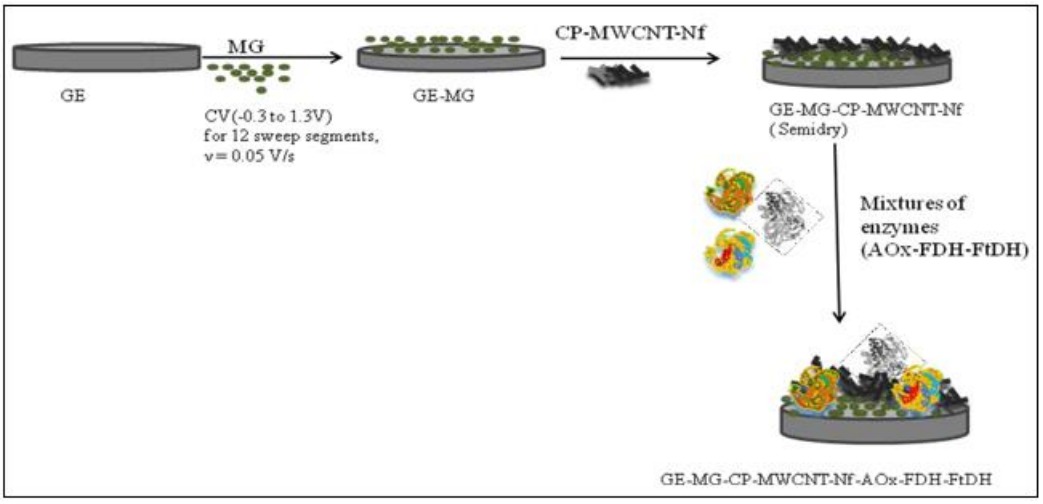
2.1 Materials and methods

AOx from *Pichia pastoris (*21 Umg^{-1} protein), ADH from *Saccharomyces cerevisiae (*300 Umg^{-1} protein), FDH from *Pseudomonas* sp. (1 - 6 Umg^{-1} solid), formate dehydrogenase (FtDH) from *Candida boidinii (*5 - 15 Umg^{-1} protein) laccase from *T. versicolor (*0.66 Umg^{-1} protein dry powder), MWCNT, (size OD 10–15 nm, ID 2–6 nm, length 0.1–10 µm), Nf (5 % w/v in isopropanol), methylene green (MG), nafion 117 membrane, OsO_4P4VP, NAD^+ were procured from Sigma - Aldrich (USA). Graphite rod electrode (GE) with disc surface area of 0.5 cm^2 was procured from Industrial carbon, India. Methanol (99.9 %), formaldehyde (35%), sodium nitrate, sodium tetraborate and ethyl benzene (99.9 %) were purchased from Merck (India). Carbon powder (CP) was purchased from Electrochem. Inc USA. All other chemicals were of analytical grade and used as received without purification. AOx (1 mg ml^{-1}), FDH (1 mg ml^{-1}), FtDH (1 mg ml^{-1}) were freshly prepared in 100 mM KPBS, pH 7.5. Laccase solution (10 mg ml^{-1}) was prepared in 0.1 M sodium citrate buffer of pH 4.8.

2.2 Fabrication of the bioelectrodes

A graphite electrode was used as a support material for

bioelectrode fabrication. At first the electrode was cleaned by sonicating in 70 % ethanol and then in distilled water for half an hour in both the steps. A thin film of MG on the electrode was prepared by performing CV using - 0.3 to 1.3 V for 12 sweep segments at a scan rate of 0.05 V/s in a solution containing 0.4 mM MG, 0.1 M sodium nitrate in 10 mM sodium tetraborate. A CP - MWCNT - Nf layer over the electrode was prepared by similar method as described in the chapter 5 section 5.2.2. A total volume of 1000 µl of CP - MWCNT - Nf was dropped into the MG modified graphite electrode. When the nanocomposite mixture in the electrode was in semi dry condition a mixture of enzyme solution containing AOx, FDH, and FtDH from the respective stock solutions was layered over the electrode surface with stepwise loading and drying to a final activities of 42 U cm - ^{2}, 10 U cm - ^{2}, and 20 U cm - ^{2}, respectively, with the total loading of six mg protein per square cm on the electrode surface. The CP included in the electrode fabrication make the layer hydrophilic for better adsoption of enzymes. The layer on the nanocomposite electrode was allowed to dry at room temperature for overnight. Using same immobilization techniques ADH based multienzyme bioanode was fabricated as control, where ADH was used instead of AOx. The final concentration of ADH, FDH, FtDH in the electrode surface was 600 U cm - ^{2}, 10 U cm - ^{2}, 20 U cm - ^{2}, respectively with the total loading of six mg protein cm $^{-2}$ on the electrode surface. For biocathode fabrication Os O_4P4VP - MWCNT - Nf is prepared by similar method as described in the work reported by (Das et al 2014) A total volume of 1000 µl of OsO_4P4VP - MWCNT - Nf was dropped above the hydrophilic microporous layer (CP - MWCNT - Nf) on graphite electrode and incubated at 140 °C overnight. In the semi dry condition, a total 1 ml of laccase stock solution was dropped by stepping method onto the OsO_4P4VP - MWCNT - Nf layer to a final concentration of 20 mg protein cm $^{-2}$ (1.32 U cm - ^{2}). The biocathode so formed was then air dried and stored at 4° C.



Scheme 2.1. Schematic depiction of the GE - MG - MWCNT - Nf - AOx - FDH - FtDH bioanode fabrication process.

2.3 Apparatus and measurements

DPV, EIS and polarization curved were performed with a potentiostat. The electrochemical measurement (DPV, EIS) for the each electrode was done in a three - electrode system containing platinum rod as counter electrode, Ag/AgCl (saturated KCl) as reference electrode, and GE or modified GE as the working electrode as describe elsewhere. All potentials were measured and reported relative to the Ag/AgCl reference electrode. EIS measurements were performed in a background solution of 5 mM K_3Fe (CN) $_6$/K_4Fe (CN) $_6$ (1: 1) and 0.1 M KCl in KPBS within the frequency range of 0.05 Hz to 10 kHz. The amplitude of the alternate voltage was 5 mV.

For the fuel cell study, all data were collected and analyzed in the test cell with a potentiostat interfaced to a PC. The bioanode with the AOx based cascade of enzyme was allowed to equilibrate in the 43 mM methanol solution with 0.5 mM NAD^+ for 1 h; whereas, the biocathode was allowed to equilibrate in the aerated buffer 100 mM, pH 7.5 for 1 h before the experiment and then open circuit potential was recorded at room temperature. Using the same fuel cell assembly and parameters, the bioanode with ADH based cascade of enzyme was allowed to equilibrate in the 0.1 M methanol fuel solution with 1 mM NAD^+ in a 100 mM KPBS, pH 7.5 for 1 h. Both bioanode and biocathode was assembled in a single chambered cell (Scheme 1). A rheostat (Stead electronics Trade, India) was used to apply load between 0 and 80 Ω. Linear polarization curves were recorded for each electrode both in the half - cell and full cell assemblies. The data discerned from the polarization curve were used to generate the power curves for both AOx and ADH based cascade bioelectrodes in the fuel cell.

For characterization of the surface morphology, the constructed bioelectrodes were scanned under a SEM (Leo 1430 vp, Germany) using 15 KeV EHT, 50 mm aperture.

3. Results and Discussion

3.1 Characterization of the bioelectrodes

The surface morphology of the fabricated bioanodes (Scheme 6.1) was investigated using SEM. GE showed a plane surface (Fig 1A). Addition of MG on the GE did not significantly alter the surface morphology on the electrode surface (Fig 1B). When MWCNT - CP - Nf was layered on

MG surface, the surface transformed to a porous morphology with a thread like structure of the adsorbed MWCNTs visible (Fig.1C). When the mixture of enzymes (AOx - FDH - FtDH) was adsorbed on the MG - MWCNT - CP - Nf layer, the surface morphology turned to globular structures indicating the immobilization of the mixtures of the enzymes on the porous CP - MWCNT - Nf film (Fig.1D).

DPV was carried out to investigate redox behavior of the fabricated AOx - FDH - FtDH bioanode (Fig 3A) in KPBS (pH 7.5) at a step potential 5 mVs^{-1}. The bioanode initiated faradic current response against substrate methanol in the range of 10 - 43 mM with a peak potential at 0.2 V (Fig 3A b, c, d). The redox reaction of NADH requires very high potentials (Blaedel and Jenkins, 1975; Karyakin et al., 2004; Cardosi and Liu, 2012). However, the MG modified electrode known to substantially reduce the over potential of NADH oxidation (Dai et al., 2008). In present case no oxidation peak higher than 0.2 V observed. Hence the potential at 0.2 V has been attribute to the oxidation of NADH mediated by MG present in the electrode interfaces. Based on the CV result following reactions at the bioanode in the presence of methanol are proposed:

$$CH_3OH + O_2 \xrightarrow{AOx} HCHO + H_2O_2 : \text{Biocatalytic oxidation}\ldots\ldots\ldots (1)$$

$$CH_3OH \xrightarrow{AOx} HCHO + 2H^+ + 2e^- : \text{Direct electrochemical oxidation}..(2)$$

$$HCHO + NAD + H_2O \xrightarrow{FDH} HCOOH + NADH + 2H^+ + 2e^- \ldots\ldots\ldots(3)$$

$$HCOOH + NAD \xrightarrow{FtDH} CO_2 + NADH + H^+ + 2e^- \ldots\ldots\ldots(4)$$

$$NADH + MG^+ \longrightarrow NAD^+ + MGH\ldots\ldots\ldots(5)$$

$$MGH \longrightarrow MG^+ + H^+ + 2e^- \ldots\ldots\ldots(6)$$

In the AOx - FDH - FtDH bioanode, the substrate methanol is initially oxidized to formaldehyde by FAD based AOx catalysis. The reaction is likely to follow both catalytically and bioelectrocatalytically [Equation 1 and 2] based on the previous work mentioned in chapter 3. Then the oxidation of the formed formaldehyde takes place by the two NAD based dehydrogenases i. e. FDH and FtDH in aqueous solution in a sequence of reaction [Equation 3 and 4]. The electron transfer in the dehydrogenase enzymes with the electrode takes place via the redox mediator, (MG) through mediated electron transfer (MET) mechanism. NADH reacts with the oxidized form of the mediator (MG^+) [Equation 5]. Then the reduced form of the mediator MGH is electrochemically reoxidized [Equation 6]. Preliminary investigation showed that the requirement of NAD in the AOx - FDH - FtDH based bioanode (apprx.0.5 mM NAD) is significantly lower than the ADH - FDH - FtDH based bioanode (1mM NAD) and previously reported ADH - FDH - FtDH based bioanode (Karyakin et al., 1994; Addo et al., 2010).

EIS was employed to investigate the electrode surface charge transfer behavior during the stepwise assembly of the bioelectrode. The spectra (Fig.3B) are presented in the form of Nyquist plots (where Z' is the real and Z" is the imaginary part of impedance) using FRA software of Autolab system and are overlaid to pinpoint the differences in electron - transfer resistance (R_{ct}) with subsequent modification of layers. The spectrum of the GE - MG (curve a) is almost straight line. When CP - MWCNT - Nf layered on the electrode surface, semicircle with resistance of 165 Ω (curve b) was found, which is due to low electronic conductivity of nafion. When mixtures of enzymes AOx/FDH/FtDH (curve d) (i. e. without MWCNT) layered on the CP - Nf electrode surface, the R_{ct} was increased to 951Ω which implies the increased interfacial resistance due to presence of poor conducting enzyme molecules and absence of electroactive MWCNT layer on the electrode. When the mixture of enzymes AOx/FDH/FtDH was layered on the CP - MWCNT - Nf electrode surface (curve c), the semi circle diameter decreased to 363 Ω due to obvious reason of highly electroactive properties of MWCNT that decreased the interfacial charge transfer resistance.

The fabricated AOx - FDH - FtDH bioanode and laccase biocathode were assembled in a fuel cell setup as shown in Scheme 6.2. The OCPs of the EFC were recorded at increasing concentrations of methanol. The OCP increased with increasing methanol concentration starting from 10 mM methanol and the potential was reached to a maximum value of 0.901 V at 43 mM methanol (Fig 4), thus showing the nernstian relation between substrate concentration and generation of potential. A methanol concentration of 43 mM was thus considered for all the electrochemical characterization of the EFC. The half cell characterization with the reference electrode (Ag/AgCl) was investigated and the anodic OCP was found to be 0.69 V. From the OCP values of the cell and anodic half cell, the OCP of the cathodic half cell has been discerned as 0.21V. Figure 5A shows the power density curve of the EFC operated with 43 mM methanol at room temperature 25 °C. Fig.6 shows the effect of external load on the power density of the EFC. Power density rose upon increasing the external load resistance and reached maximum value of 2.5 $mWcm^{-2}$ at 8 Ω. Further increase in load resistance, the cell current dropped and reached almost zero at a resistance of 80 Ω. The maximum power density generated in the EFC was 2.5 $mWcm^{-2}$ (Fig 5A). The power density of ADH - FDH - FtDH based multienzyme fuel cell (Fig 5B) was 4.6 $mWcm^{-2}$. One of reasons for lower power density may be due to the biocatalytic instead of bioelectrocatalytic function of some of the immobilized AOx. From our previous studies (Das and Goswami 2013) it has been observed that not all AOx

immobilized on the electrode surface participated DET due their inappropriate orientation on the electrode surface that likely to prevent the electron exchange between the redox centre of the enzyme and electrode. As a result majority of the AOx molecules involve in biocatalytic reactions producing the co - substrate H_2O_2 by exchanging the substrate derived electrons with the molecular oxygen bypassing the electrode. The other reason attributed to the lower power density for AOx coupled bioanode based EFC is the lower AOx enzyme loading (42 Ucm^{-2}) on the electrode than the corresponding loading (600 Ucm^{-2}) of ADH on the bioanode. Nevertheless, the power density of the present AOx couple dehydrogenase fuel cell is comparable (Sokic - Lazic and Minteer 2008; Addo et al., 2010; Hickey et al., 2013) and even higher than the previously reported methanol fuel based multienzymatic fuel cell (261± 7.6 µW cm^{-2} by Addo et al., 2010; 0.67 $mWcm^{-2}$ by Palmore et al., 1998, 2.04 $mWcm^{-2}$ by Akers et al., 2005). We also observed that the power density of the present AOx based multienzyme fuel cell is higher than our previously reported single enzyme (i. e. AOx) based fuel cell (Das et al 2014)) due to obvious reason of the involvement of additional two redox enzymes (FDH and FtDH) operated through bioelectrocatalytic mechanism which contributed higher current density in the fuel cell.

The operational stability of the bioanode was investigated from several successive measurements, each cycle of 1h duration with fresh methanol substrate of 43 mM concentration at a fixed load of 8 Ω (Fig.6). The OCP response was steady from 1st to 4th cycle. This indicates that there was no leaching and denaturation of the enzymes in the fabricated bioelectrodes till 4th cycle of operation. The functional stability implies proper nanofabrication approach followed here for immobilization of the enzymnes on the electrode surface. The MG based mediated electron exchange was facilitated by the highly conductive MWCNT present in the naocomposite matrix (Sarma et al., 2009). The nanofabrication also provides high enzyme loading on the electrode surface and tailored increase electroactive enzyme molecules on the electrode surface.

4. Conclusion

Enzymatic biofuel cell fabricated by assembling AOx coupled multienzyme based bioanode and laccase based biocathode has been developed for generating power from methanol substrate. The fuel cell generated higher power density than the single redox enzyme-based methanol fuel cell reported previously and the reason is attributed to the extraction of more electrons from the substrate methanol by its mineralization through a cascade of oxidative reaction electrocatalyzed by the NAD based dehydrogenase enzymes coupled to the FAD based AOx enzyme on the anodic electrode surface. The dehydrogenase based bioelectrocatalytic reactions on the anodic surface were mediated by MG that significantly reduce the oxidation potential of the NADH and thereby contributed to the increase potential of the NADH. Future work may explore increasing the enzyme loading and optimizing electrode orientation for further improvements in power density

The significance of this study This research provides significant advancements in biofuel cell technology by demonstrating the potential of enzyme cascades to improve the power yield from renewable alcohol substrates.

Figures

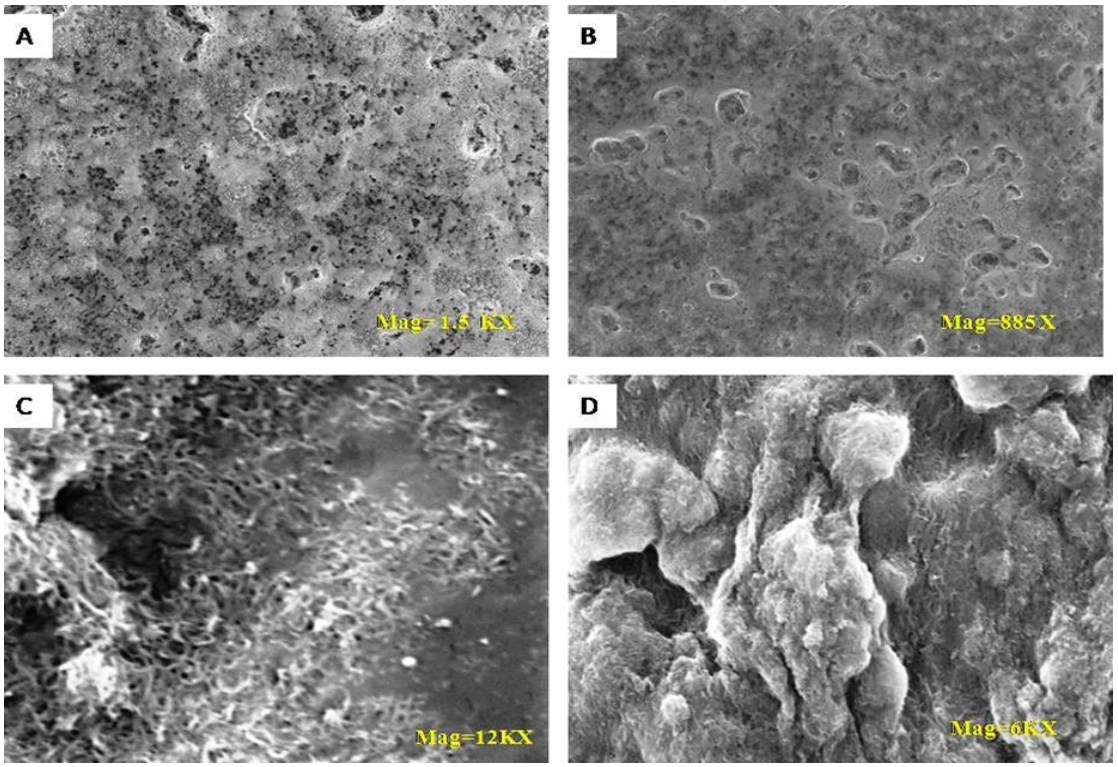
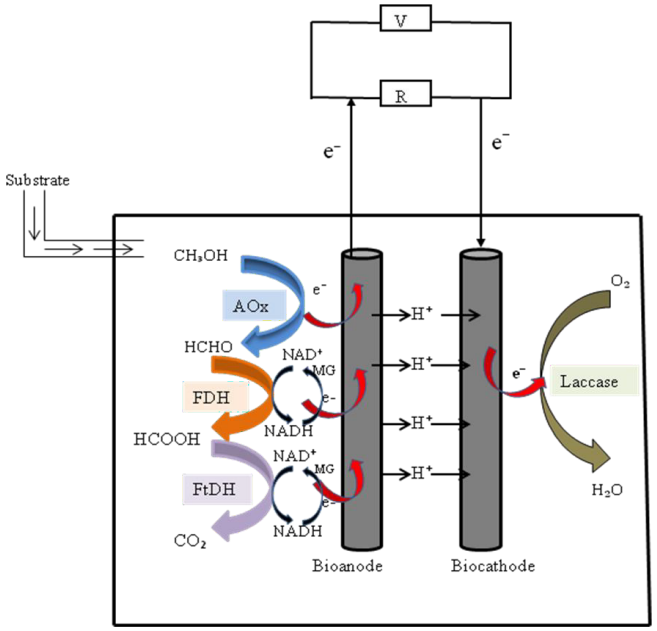


Figure 1: SEM images of the bioelectrodes at different fabrication stages: A. Bare GE, B. GE - MG. C. GE - MG - CP - Nf - MWCNT, D. GE - MG - CP - Nf - MWCNT - Mixtures of Enzymes (AOx - FDH - FtDH).



Scheme 1 Schematic diagram on the operation of AOx - FDH - FtDH and laccase based EFC using methanol as fuel substrate.

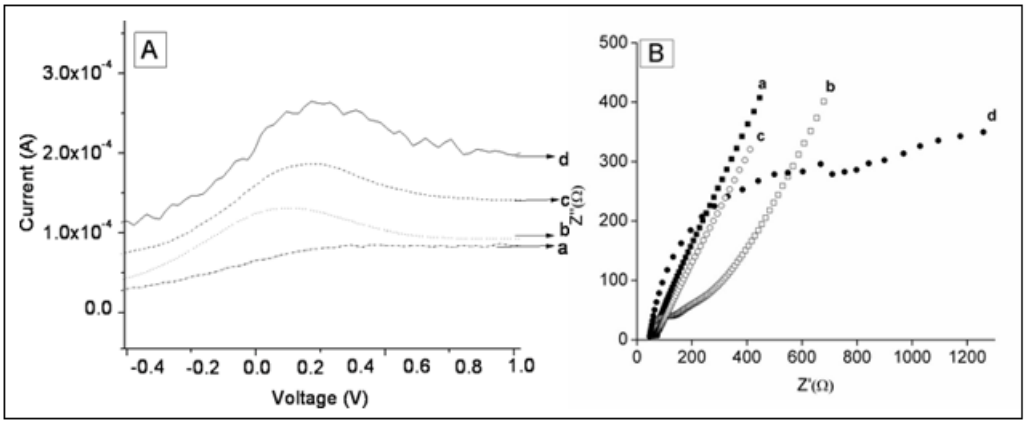


Figure 3: (A) DPV of GE - MG - CP - MWCNT - Nf - AOx/FDH/FtDH electrode at 100 mM KPBS, pH 7.5 with increasing methanol concentration (mM), (a) 0, (b) 10, (c) 21, (d) 43 at a scan rate of 50mVs $^{-1}$**.** (B) EIS of (a) GE - MG, (b) GE - MG - CP - MWCNT - Nf, (c) GE - MG - CP - MWCNT - Nf - AOx/FDH/FtDH (d) GE - MG - CP - Nf - AOx/FDH/FtDH in presence of 5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1: 1) and 0.1M KCl in KPBS within the frequency range from 0.05 Hz to 10 kHz.

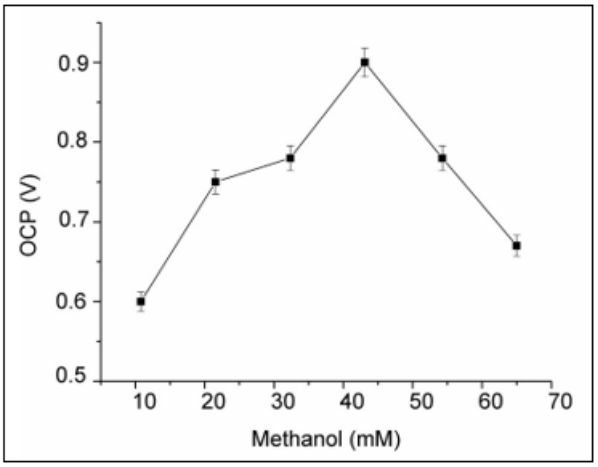


Figure 4: Effect of methanol concentration on the OCP of the EFC.

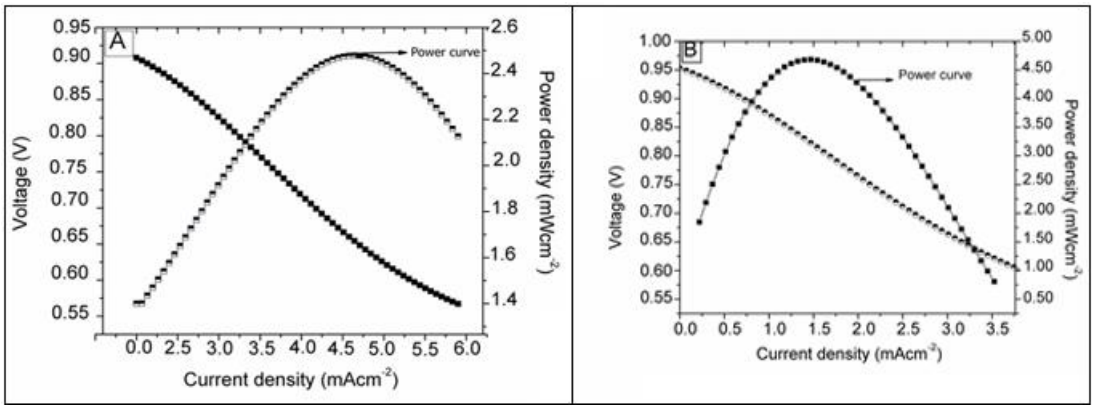


Figure 5: Power density curve of the (A) AOx - FDH - FtDH and (B) ADH - FDH - FtDH fuel cell cascade based EFC using laccase as biocathode.

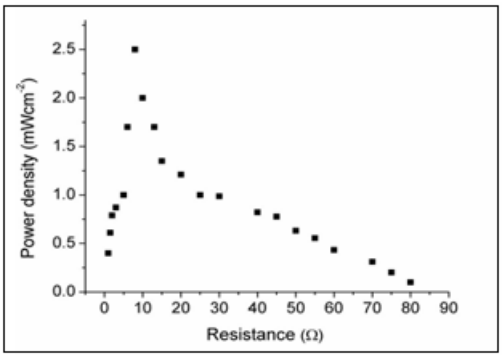


Figure 6: Effect of the resistance on the power density of the EFC fabricated by AOx - FDH FtDH bioanode and laccase based biocathode.

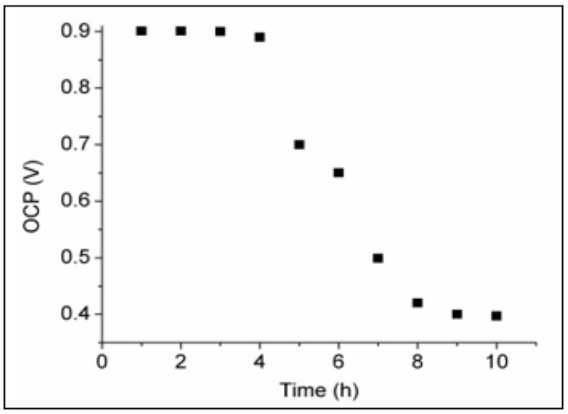


Figure 7: Stability studies of the EFC fabricated by AOx - FDH - FtDH based bioanode and laccase based biocathode in an operating condition of 43 mM methanol, 25° C temperature, 8 Ω load within 10 h duration which is maintained by charging with fresh substate (methanol 43 mM) following each 1 h runtime.

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