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# Immobilizing Enzymes on a Commercial Polymer: Performance Analysis of a GOx-Laccase Based Enzymatic Biofuel Cell Assembly

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**Abstract:** Enzymatic Biofuel Cell (EBC) represents a promising green source since it is capable of harvesting electricity from renewable and abundantly available biofuels using enzymes as catalysts. Nevertheless, nowadays long-term stability and low power output are currently the main concerns. To this end, several research studies focus on using complex tridimensional and highly expensive nanostructures as electrode support for enzymes. This increases cell performance whilst drastically reducing the economic feasibility needed for industrial viability. Thus, this paper analyzes a novel flow-based EBC consisting of covalent immobilized GOx (bioanode) and Laccase (biocathode) on a commercial flat conductive polymer. A suitable immobilization technique based on covalent ligands is carried out to enhance EBC durability. The experimental characterization demonstrates that the cell generates power over three weeks, reaching 590 mV and 2.41 μW $cm^{-2}$ as maximum open circuit voltage and power density, respectively. The most significant contributions of this configuration are definitely ease of implementation, low cost, high scalability, and reproducibility. Therefore, such a design can be considered a step forward in the viable EBC industrialization process for a wide range of applications.

**Keywords:** covalent immobilization; direct electron transfer; enzymatic biofuel cell; enzymes; laccase; glucose oxidase; electrochemical stability; green power; low-cost polymer; power generation

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## 1. Introduction

With the increasing energy demand and the need of pollutants and greenhouse gases reduction, governments and research are focusing on exploiting green renewable energies. Several ways to produce green energy are investigated in the literature, moving from wind, wave, and solar generation to biomass and fuel cells. Among the potential green energy device, Enzymatic Biofuel Cell (EBC) is considered one of the most promising since it is capable of harvesting electricity from renewable and abundantly available biofuels using enzymes as catalysts [1].

EBC shows unique advantages in the conversion of chemical energy into electric energy because it can work in mild operating conditions (i.e., ambient temperature and neutral pH) [2]. Moreover, employing enzymes as catalysts makes this technology

sustainable and renewable since no scarce noble metal is required to enhance reaction kinetics. Properties such as great specificity toward substrates (e.g., sucrose, fructose, glucose, etc.), high catalytic activity with low overvoltage for substrate conversion, low cost, renewability, and biodegradability represent the main advantages of enzymes [3].

Several possible applications for EBCs are emerging, moving from power generation and waste treatments to biomedical devices, such as implantable artificial organisms, self-powered biosensors, cardiac pacemakers, and drug delivery, as indicated in [4–13].

EBC consists of a bioanode and a biocathode where the fuel is oxidized, and oxidant is reduced by means of catalysts (i.e., enzymes) immobilized on the surfaces of electrodes [14]. The electron transfer mechanism can be distinguished in direct electron transfers (DET), in which no mediator takes part in the electron transferring between the enzyme and the electrode, and mediated electron transfers (MET), in which an external redox mediator shuttles electrons from the protein to the electrode surface [15]. Although MET allows higher current densities than DET, the reaction overpotential introduced by mediator reduces the cell potential. Moreover, the possible toxicity of the mediator limits the development of MET EBCs for a wide range of applications, such as implantable biosensors and biodevices. Therefore, EBCs based on DET are currently the most investigated in the literature, allowing open circuit voltages (OCVs) nearly to 1 V and subsequently higher power output, as indicated in [2].

Nowadays, amongst the several enzymes suitable for EBCs, glucose-oxidase (GOx) and Laccase are the most investigated for bioanode and biocathode, respectively. GOx is widely used as enzyme for bioanodes because of its ability to oxidize glucose to gluconolactone, since glucose is a widely available substrate characterized by high-energy intensity, renewability, environmental friendliness, low cost, and simplicity of transportation [16]. Furthermore, glucose is one of the most important energy sources of many living organisms [17]. On the other hand, Laccase reduces oxygen at the cathode, thanks to its high redox potential which determines low-overpotential in oxygen reduction electrocatalysis.

Nevertheless, low power output and long-term stability of EBCs represent the main current issues that have to be faced for further development of this technology. Concerning such limitations, research efforts have been addressed to optimize immobilization techniques and to employ nanomaterials as electrodes with the purpose of enhancing DET and stability between enzyme and electrode surface. Several immobilization techniques, such as physical adsorption and entrapment or chemical covalent bonds and cross-ligands, have been developed [18–21].

In the field of electrodes engineering, 3D porous structures, based on nanomaterials, such as carbon nanotubes (CNTs), multi-walled carbon nanotubes (MWCNTs), graphene, and gold nanoparticles, have been widely explored since their dimension is very close to the protein structure, allowing a deep interaction between electrode surface and enzyme [2,4,9,14,22–28]. Such 3D electrodes often have displayed higher current than flat electrodes, when the current is normalized by the projected surface area, as analyzed in [29].

Furthermore, scientists are investigating fluidic and microfluidic design configurations with the purpose of obtaining miniaturized EBCs with higher mass transfer and reaction rates, higher surface to volume ratio, lower reagent volume and power consumption, automated fluid delivery, faster response, and reduced operational costs [3,30].

As example, Kim et al. [31] analyzed micro-scale Y-shaped and cross-shaped paper-based mediator-less microfluidic EBCs, in which GOx and Laccase were immobilized on MWCNT electrodes, at varying the glucose concentration. Specifically, the highest performances of power density (104.2 ± 3.35 μW $cm^{-2}$) and current density (615.6 ± 3.14 μA $cm^{-2}$) were obtained with the Y-shaped channel configuration at a glucose concentration of 100 mM.

Jayapiriya et al. [14] analyzed the performance of carbon nanotubes functionalized laser-induced graphene (C-LIG) electrodes incorporated into a microfluidic device. These C-LIG electrodes were able to generate 2.2 μW $cm^{-2}$ power density without using any metal catalyst, at an optimized 200 mL $min^{-1}$ flow rate.

Hui et al. [32] presented an EBC with spider nest shaped multi-scale 3D substrate of reduced graphene oxide and nickel foam as structure for enzymatic electrodes, measuring a maximum power output of 7.05 ± 0.05 mW $cm^{-2}$.

Di Lorenzo and du Toit [33] demonstrated continuous power generation by flow through miniature EBCs fed with an aerated solution of glucose and no redox mediators. Non-toxic highly porous gold was used as the electrode material and the immobilization of the enzymes onto the electrode surface was performed via cost-effective and easy-to-reproduce methodologies. Two different designs (i.e., single channel and parallel channel EBCs) were compared, registering the highest power density (1.6 μW $cm^{-2}$) for the parallel configuration.

However, in the case of 3D porous electrodes, normalization by total surface area is generally calculated by dividing the output current by the electrode surface area that has been determined by either mercury intrusion or nitrogen sorption porosimetry [29]. Therefore, when current normalized by these values is compared to current density values on flat electrodes, 3D porous electrodes usually underperform. Furthermore, 3D electrodes result in a very high cost with respect to flat electrodes and the complex production process is limited at laboratory scale to date.

Hence, taking into account the current issues in the EBC development, our research activity is addressed to show a DET flow-based design EBC consisting of covalent immobilized GOx (bioanode) and Laccase (biocathode) on a commercial conductive polymer (PV15, SGL carbon, GmbH), usually employed in fuel cell and flow battery applications.

To the best of our knowledge, no similar commercial polymer has been studied for EBCs in the literature. Moreover, this particular EBC configuration, using widely abundant substrates such as glucose and air and enzymes immobilizing on the commercial polymer PV15, could toughly affect the development of EBC allowing greater reproducibility while lowering the complexity and related costs.

Thus, such a design can be considered a step forward in the viable EBC industrialization process for a wide range of applications.

The paper is organized as follows: Section 2 concerns the description of the immobilization technique for bioanode and biocathode and the experimental test rig for the characterization of the single cell. The main obtained results are analyzed in Section 3, and the conclusions are treated in Section 4.

## 2. Materials and Methods

### *2.1. Materials*

All enzymes, salts, and reagents were purchased from Sigma-Aldrich (Saint Louis, MO, USA): Laccase from Trametes versicolor, Glucose Oxidase (GOx) from Aspergillus niger, Peroxidase from horseradish, Sodium acetate (NaOAc), Potassium phosphate, 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), o-Dianisidine, D-Glucose, N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), N-Hydroxysuccinimide (NHS), Diethylenetriamine, and 25% Glutaraldehyde stock solution.

A commercial conductive fluoropolymer, Sigracell® PV15, was purchased from SGL Carbon (Wiesbaden, Germany) and used as electrode material support for the immobilization of the enzymes.

### *2.2. Methods*

The immobilization procedures for the bioanode and biocathode, as well as the enzymatic activity assays, are described in the following sections. The immobilization was carried out on PV15 supports, each featuring a surface area of about 30 $cm^2$ to be tested in the developed EBC prototype. In parallel, 30 $cm^2$-PV15 sheets were cut in four pieces (surface area ≅ 7.5 $cm^2$) to evaluate the enzymatic activity of the immobilized enzymes over time.

#### 2.2.1. Bioanode Immobilization Technique

The protocol for the immobilization of GOx on the PV15 support has already been described before [19]. It consists of a functionalization phase using diethylenetriamine, followed by an activation step with glutaraldehyde.

The GOx enzyme was then immobilized on the activated material placing 15 mL of the enzymatic solutions (1 mg/mL in a 50 mM NaOAc pH 5.0 solution) in an overnight incubation at 4 °C on a laboratory shaker (Bibby Stuart Platform Rocker STR6) set at 20 rpm.

The quantity of GOx covalently immobilized on PV15 was determined by the difference between the GOx enzymatic activity, assessed by the Glucose Oxidase assay, of the enzyme stock solution and that of the solution re-covered after the immobilization [34]. The results are the mean of four GOx-PV15 preparations.

#### 2.2.2. Biocathode Immobilization Technique

A method based on the use of a solution consisting of EDC/NHS was employed for the immobilization of Laccase [18]. The surface of the PV15 material was activated with 15 mL of a mixture containing 0.5 mM EDC and 0.1 mM NHS in deionized $H_2O$. The solution also contained a 400 μg/mL concentration of Laccase to be immobilized and was left over the PV15 for at least 24 h at 4 °C on a laboratory shaker (Bibby Stuart Platform Rocker STR6) set at 20 rpm.

The quantity of Laccase covalently immobilized on PV15 was determined by the difference between the Laccase enzymatic activity, assessed by the Laccase assay based on the oxidation of ABTS [35], of the enzyme stock solution, and that of the solution re-covered after the immobilization. The results are the mean of four Laccase-PV15 preparations.

#### 2.2.3. Determination of GOx Enzymatic Activity

The activity of GOx, both in its free and immobilized forms, was performed by a colorimetric assay that has been widely described elsewhere [34]. The reaction velocity was determined by an increase in absorbance at 460 nm resulting from the oxidation of o-Dianisidine through a peroxidase coupled system.

The reaction was set up by mixing 2.5 mL of o-Dianisidine (prepared by diluting 0.1 mL of 1% o-Dianisidine in 12 mL of 0.1 M potassium phosphate buffer pH 6.0) with 0.30 mL of 15% (*w*/*v*) glucose in 50 mM NaOAc pH 5.0 and 0.10 mL of peroxidase at a concentration of 200 μg/mL. A volume of 0.10 mL of GOx enzyme solution (1 mg/mL) was then added to the solution for a final volume of 3 mL.

For the immobilized GOx, the reaction was carried out in a petri dish (35 mm × 10 mm). A volume of 0.1 mL of 50 mM NaOAc pH 5.0 was added to the solution, in order to keep the final volume of 3 mL, and then placed over the enzyme-loaded PV15 films. The reaction mixture was incubated at room temperature for 5 min on a Stuart vortex mixer SA8 (Staffordshire, United Kingdom) set at 1000 rpm.

The incubation time was chosen based on preliminary tests which ensured us that there is a linear relation between product release and time of up to 10 min. During the incubation, a brownish-red color develops which indicates the progress of the reaction.

The oxidation of o-Dianisidine was followed by recording the absorbance increase at 460 nm ($\varepsilon_{460}$ = 11.3 × $10^4$ $M^{-1}$ $cm^{-1}$). Enzyme activity is expressed in Units (where 1 U = amount of enzyme that oxidizes 1 μmol of o-Dianisidine per minute).

#### 2.2.4. Determination of Laccase Enzymatic Activity

The enzyme activity of Laccase was determined by the ABTS oxidation method as described elsewhere [35]. Briefly, the ABTS dye is oxidized by Laccase to its radical cation which shows an intense blue-green color.

The concentration of the radicals, derived from the absorbance read at 420 nm, can be correlated to the enzyme activity [36,37]. The assay mixture contained 1.0 mL of 0.5 mM ABTS dissolved in 50 mM NaOAc pH 5.0, 0.9 mL of the same buffer, and 0.1 mL of the Laccase solution (400 μg/mL).

As is the case with GOx, also immobilized Laccase was tested in a petri dish (35 mm × 10 mm). A volume of 0.1 mL of 50 mM NaOAc buffer pH 5.0 was added to the solution, aiming at keeping the final volume of 2 mL, and then placed over the enzyme-loaded PV15 films. The reaction mixture was incubated at room temperature for 15 min on a Stuart vortex mixer SA8 (Staffordshire, United Kingdom) set at 1000 rpm.

The incubation time was chosen based on preliminary tests which ensured us that there is a linear relation between product release and time up to 20 min. Oxidation of ABTS was followed by the absorbance increase at 420 nm ($\varepsilon_{420}$ = 3.6 × $10^4$ $M^{-1}$ $cm^{-1}$). Enzyme activity was expressed in Units (1 U = amount of enzyme that produces 1 μmol of oxidized ABTS per minute).

### *2.3. Experimental Test Rig Description*

The EBC experimental setup is depicted in Figure 1. A peristaltic pump (Gilson) was used to flush the fluids from the tanks to the cell. The flow rate was fixed at 50 mL $min^{-1}$ for both the cathode and anode sides. The anolyte tank contained a NaOAc buffer solution with 15% (*w*/*v*) of glucose at pH 5.0. Catholyte consisted of NaOAc buffer solution at pH 5.0. In order to increase Laccase reaction kinetics, occurring at the cathode, the cathodic tank was continuously purged with air. BioLogic galvanostat/potentiostat SP-240 was used to perform experiments. The bioanode (negative electrode) and biocathode (positive electrode) are respectively connected to the instruments by means of blue and red cable. Tests were conducted at ambient temperature (i.e., 20 ± 1 °C).

The single cell, whose active area is equal to 12 $cm^2$, is composed of the following:

- Aluminum end plates;
- Copper plates as current collectors;
- Bioelectrodes (PV15 Sigracell, SGL Carbon GmbH, Meitingen, Germany);
- Teflon frames;
- Nafion™ 211 (Ion Power GmbH, München, Germany) membrane as half-cells separator.

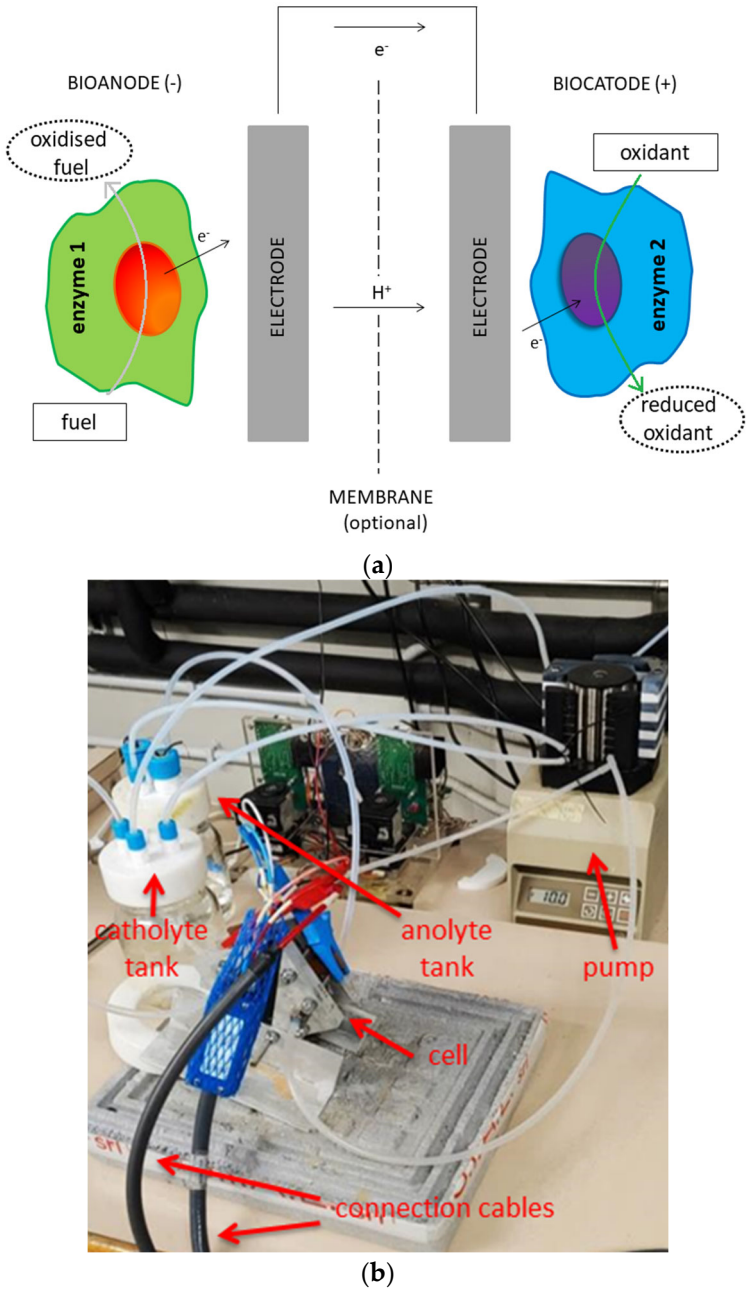


**Figure 1.** (**a**) Schematic view of a typical EBC (reproduced from [3]) and (**b**) experimental test rig for the developed EBC.

The experimental campaign was based on the measurement of the following:

- Cyclic Voltammetry (CV) performed for bioanode and biocathode, in presence of glucose at the bioanode and both in absence and presence of air in the NaOAc buffer solution at the biocathode side. CV tests were carried out with the galvanostat/potentiostat BioLogic SP-240 in a three electrodes setup, using the investigated bioelectrode of 2 $cm^2$ as working electrode, an Ag/AgCl KCl saturated as reference electrode, and a platinum wire as counter electrode.
- Open circuit voltage, in order to evaluate the stability of the enzymes over time;
- Polarization curve, aiming to determine the maximum power output. A voltage cut-off of 0.1 V was set for this experiment.

Such techniques are widely used in the field of electrochemistry with the purpose of evaluating the electric performance of the EBCs.

OCV measurements were performed continuously for more than 120 h starting from the first operating day of the cell (day 0). Then OCV was measured over a few hours after three weeks before the acquisition of the polarization curve.

Polarization curves were obtained at both day 0 and after three weeks in order to assess EBC maximum power output and its decay over time.

## 3. Results and Discussion

In this section, the main results relating both to the evaluation of the enzymatic activity and the entire EBC electrochemical performance are discussed.

### *3.1. Cyclic Voltammetry Preliminary Tests*

CV is a widely used and powerful technique in electroanalytical chemistry. CV provides direct insights concerning the kinetics of electrode reactions [38]. CV experimental tests were conducted to assess the GOx and Laccase electrocatalytic activity on the chosen commercial support (PV15). Specifically, Figure 2 depicts the CV carried out on the developed GOx bioanode. Two different current peaks (cathodic peak at −310 mV vs. Ag/AgCl and anodic peak at −270 mV vs. Ag/AgCl) can be distinguished. Being the catalytic processes involving the bioelectrodes are very slow, clear catalytic curves are formed at very low rates of potential change, as described in [24]. Thus, the CV tests were conducted at a relatively low scan rate (20 mV $s^{-1}$).

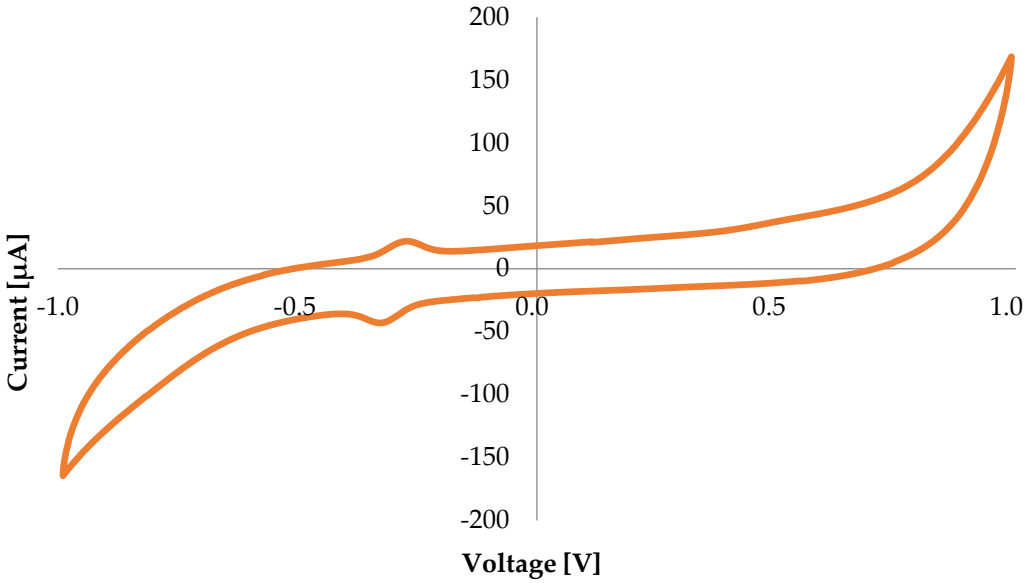


**Figure 2.** Cyclic voltammetry of the GOx bioanode in a solution containing 15% *w*/*v* of glucose (pH 5) at 20 mV $s^{-1}$ scan rate.

On the other hand, Figure 3 shows the measured CV for the biocathode in a phosphate buffer solution saturated and not saturated with air, respectively.

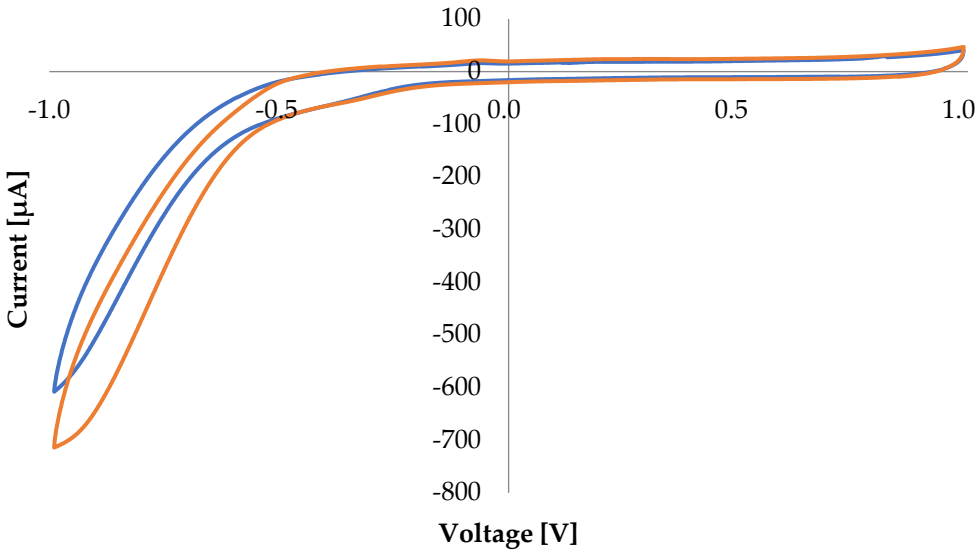


**Figure 3.** Cyclic voltammetry performed for Laccase biocathode in absence (blue line) and presence (red line) of air in NaOAc buffer solution (pH 5.0) at 20 mV $s^{-1}$ scan rate.

### *3.2. Enzymatic Activity of Bioelectrodes*

The immobilization of the enzymes was evaluated in terms of immobilization yield, expressed as the percentage of immobilized molecules over the total enzyme that had been administered to the polymer film and enzymatic activity for units of area (in U/cm²) (Figure 4). The stability of the enzyme-loaded PV15 films over time was tested every second day for a total time of 3 weeks. In the case of GOx, the immobilization yield was found to be 63%, meaning that the enzyme concentration on the PV15 support was 315 µg/cm². Right after the immobilization, at day 0, the enzymatic activity for units of area was about 3.18 U/cm². After an initial drop of about 30% in activity, immobilized GOx showed a rather constant functionality at least up to the 20th day.

Analogously, the immobilization of Laccase was evaluated as for the percentage of bound molecules and an immobilization yield of about 83% was assessed. The concentration of Laccase on the PV15 films was thus 170 µg/cm². The Laccase-PV15 system was found to be stable over time with only a slight drop after the first revelation at day 0, with an average activity of about 20 mU/cm².

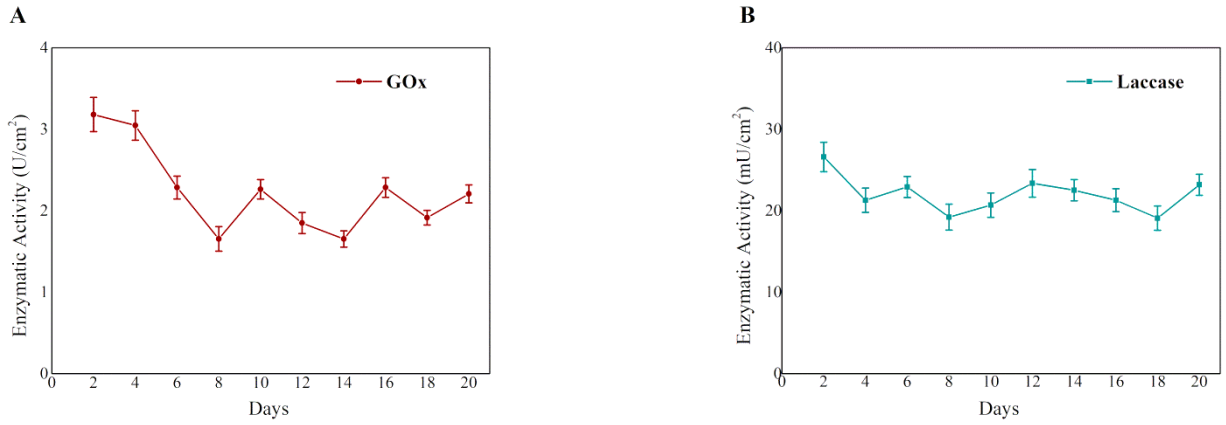


**Figure 4.** Trend of enzyme activity of GOx (**A**) and Laccase (**B**) immobilized on PV15 films as a function of time.

### *3.3. EBC Electrochemical Characterization*

Electrochemical characterization carried out on the studied EBC was performed through BioLogic galvanostat/potentiostat SP-240 and the main EBC parameters, i.e., open circuit voltage and polarization curve were collected.

Specifically, at the first operating day of the cell (day 0), the polarization curve was acquired after leaving the cell at OCV for a few hours for stabilization. In particular, before performing experimental tests, the electrolytes were flowed into the cell for a few hours with the aim being to allow enzymes to start the substrate consumption increasing EBC voltage potential.

During the working period, the catholyte and anolyte tanks were refueled in order to avoid emptying.

Figure 5 depicts the measured polarization curve and the power output of the considered EBC at day 0. The trend of power output is typical of these applications, as illustrated in [39–42].

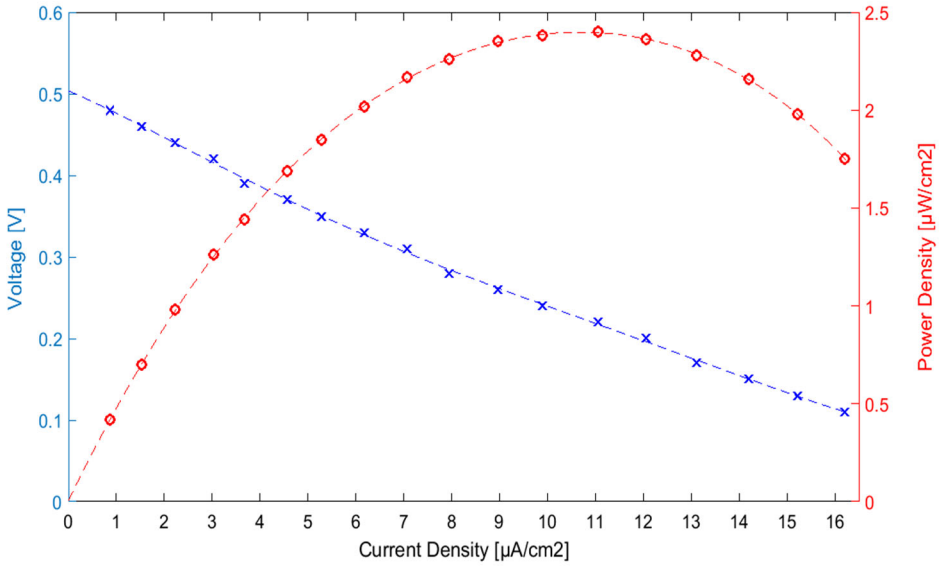


**Figure 5.** Polarization curve (blue crosses: measured data; blue dashed line: fitted data with a 5th order degree polynomial) and power density curve (red circles: measured data; red dashed line: fitted data with a 5th order degree polynomial) registered at day 0 for the studied EBC.

A maximum power output of 2.41 µW $cm^{-2}$ at 11.3 µA $cm^{-2}$ (i.e., 28.94 µW for the studied cell) was registered at day 0.

After the polarization curve was measured, the OCV of the cell was continuously gathered for more than 120 h, as illustrated in Figure 6. It is noteworthy that the considered EBC showed high and stable OCVs of more than 500 mV for 60 h of operation, with only a decrease of −7.6% from the initially registered voltage of 590 mV. Then, a rapid decrease of the OCV occurred until the cell reached 150 mV after 120 h (−74% with respect to the initial voltage).

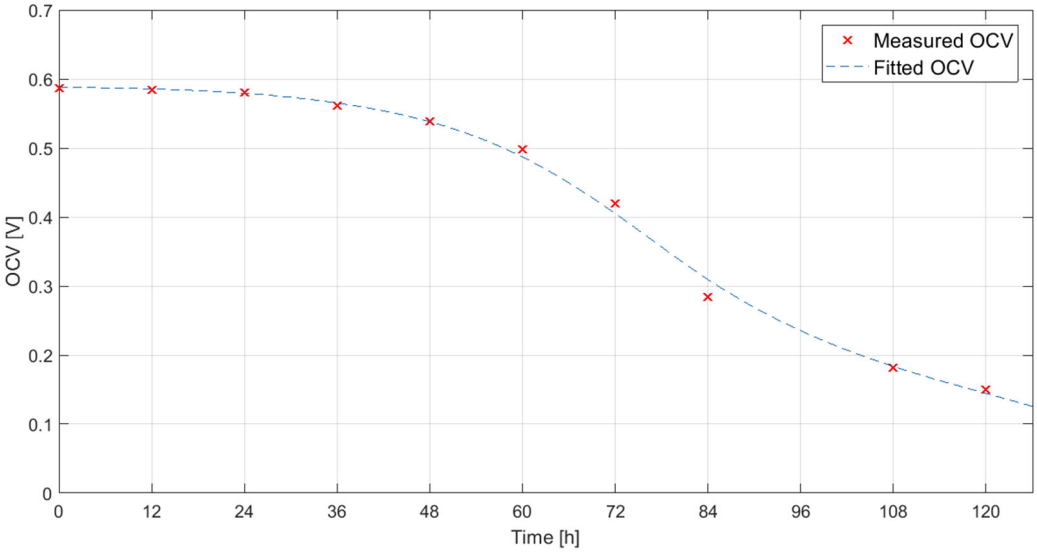


**Figure 6.** Open circuit voltage measurement of the studied EBC. Gathered data are represented in red crosses, fitted OCV evolution in blue dashed line.

After three weeks, both the OCV and polarization curve were gathered in order to assess cell performance (OCV, EBC maximum power output) and its decay over time.

It is highlighted since, in order to evaluate the long-term power stability of the studied EBC, both catholyte and anolyte were replaced with fresh solutions at the 3rd week before the OCV and polarization curve were collected.

Concerning the solutions replacement, the same procedure described in Section 2.3 was followed. The catholyte solution was purged with air in order to completely saturate the solution with oxygen. Consequently, an increase in the cell OCV was registered of up to 430 mV.

After the stabilization was achieved, OCV was measured for almost 23 h showing a stable value of 430 mV ± 10 mV. Such OCV value corresponds to 72.9% of the one registered at day 0 (590 mV). This can be interpreted as a clear sign of enzymes reactivation due to the use of fresh substrates.

Figure 7 depicts the measured polarization curve and the power output of the considered EBC at the 3rd week.

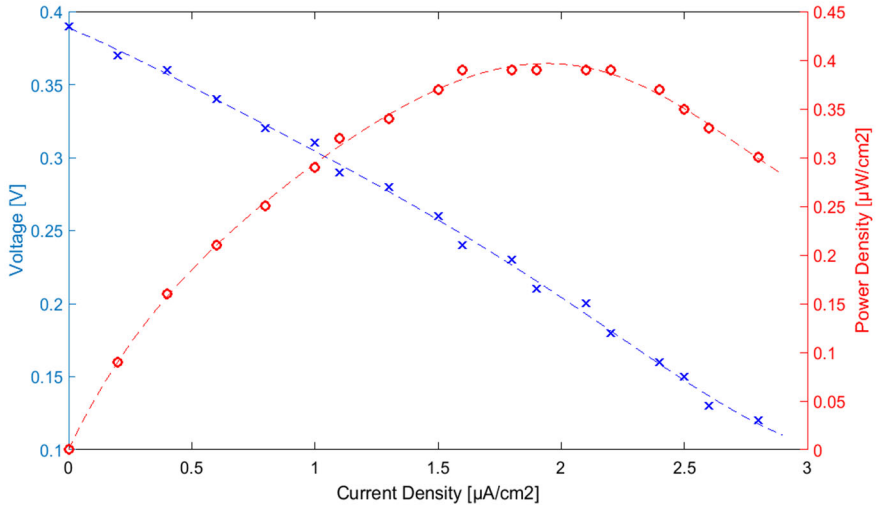


**Figure 7.** Polarization curve (blue crosses: measured data; blue dashed line: fitted data with a 5th order degree polynomial) and power density curve (red circles: measured data; red dashed line: fitted data with a 5th order degree polynomial) registered after three weeks for the studied EBC.

As evident from Figures 5 and 7, current density moved from 16 μA/cm² recorded at time 0 to less than 3 μA/cm² after three weeks.

This strong current and power density drop, respectively, corresponding to −82.7% and −83.4% after three weeks, can be ascribed to the degradation of the linkers used in the covalent immobilization, rather than the decay of GOx and Laccase enzymatic activity. Indeed, as shown in Section 3.2, tests conducted on GOx and Laccase immobilized on two PV15 samples demonstrated good enzymatic activities over time, although a rapid performance decrease, particularly for GOx, was registered only during the first days.

With reference to the literature, the performance of the considered EBC in terms of power output is in line with similar designs including flat electrodes, as stated in [30,33,43]. As analyzed in the Introduction, despite 3D porous structures often reaching very high power densities, no consistent comparison can be carried out per unit of area with respect to flat electrodes. As a matter of fact, when the power output is normalized by dividing the output current by total surface area, 3D porous electrodes tend to underperform flat electrodes. Furthermore, it is noteworthy that such particular cell configuration based on commercial and low-cost polymer immobilizing enzymes can strongly enhance the EBC evolution, especially in reference to green power production from waste and wastewater. Indeed, the high scalability and industrial viability of the studied EBC could play a key role for example in the food industry, generating electricity from widely available substrates for disposal.

## 4. Conclusions

Although EBCs are still a technology in development, the many advantages of using enzymes, such as great specificity toward substrates, high catalytic activity, and renewability, make this technology one of the most promising, increasing their number of prospective application fields.

Notwithstanding several research works reporting high power densities up to hundreds mW cm$^{-2}$ by means of 3D electrodes structures based on conventional materials such as CNTs, graphene, and nanoparticles, the great costs of such materials and the difficulties related to fabrication have to be faced in order to make industrialization feasible.

The studied EBC, based on GOx and Laccase enzymes immobilized on a conductive commercial polymer used as electrode, shows a maximum power output (2.41 μW cm$^{-2}$), which is, according to available literature, in line with applications of flat electrodes based on conventional materials. Moreover, also the proven stability over time overcomes the results reported in the literature, in that a good enzymatic activity was maintained over a period of three weeks, as demonstrated by the enzymatic activity assays carried out on enzyme-loaded PV15 film samples.

Therefore, the most significant key points concerning the EBC here presented are definitely its ease of implementation, high scalability, low cost, and use of widely available substrates (glucose and air) and enzymes. These technological aspects can be considered of paramount importance in the industrial viability of EBCs and their wide range of applications, moving from biosensors and biodevices to waste treatments and green power production. In reference to the specific EBC here presented, a possible application concerns the conversion of organic substrates contained in wastewater produced, for example, by oil mills and other applications in the food industry. After enzymatic distillation processes potentially performed to extract polyphenols and other compounds for the pharmaceutical, cosmetic, and feed sectors, organic substrates are reduced to glucose being exploited for power generation through EBC.

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