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Hybrid enzymatic and organic catalyst cascade for enhanced complete oxidation of ethanol in an electrochemical micro-reactor device

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ABSTRACT

This work combines an organic oxidation catalyst, 4-amino-TEMPO (TEMPO-NH_2), and a recombinant enzyme, oxalate decarboxylase (OxDc) to create a hybrid catalytic system able to catalyze complete ethanol electrooxidation. The catalyst system was coupled with a novel small scale electrolysis cell utilizing polycaprolactone (PCL) and carbon composite electrodes for bulk electrolysis. Electrochemical measurements and product detection by nuclear magnetic resonance spectroscopy (NMR) after 12 h of electrolysis have shown for the first time that an organic catalyst and decarboxylase enzyme can oxidize ethanol to carbon dioxide at acidic pH. The success presented here, coupling a hybrid organic catalyst/enzymatic system to simple carbon composites, has potential value for selective alcohol oxidation and is promising for a wide array of applications, including biosensors, environmental monitoring, and biofuel cells.

1. Introduction

Ethanol is a popular fuel which is obtained from sugar cane, corn, and beets, among others sources [1]. High energy density (8 kW h kg^{-1}), abundance, desirable physicochemical properties, low cost and low toxicity make ethanol an excellent renewable fuel choice to generate electricity [2]. Biological fuel cells have been extensively reported as a efficient and sustainable device to convert chemical energy to electrical energy [3,4]. Biofuel cells (BFCs) are alternative energy sources which use enzymes (enzymatic biofuel cell) or microorganisms (microbial fuel cell) as the electrocatalysts instead of the traditional noble metal catalysts [4,5].

Microbial biofuel cells have the advantage of possessing long lifetimes of up to five years [5]. Some microbial fuel cells [6,7] are capable of completely oxidizing simple sugars to carbon dioxide [8];however, these have been limited by low current and power densities owing to slow transport across cellular membranes [9]. On the other hand, enzymatic fuel cells can possess orders of magnitude higher power densities, but can only partially oxidize the fuel [10].

Enzymes are biocatalysts responsible for oxidizing fuel at the anode and reducing oxygen at the cathode to generate energy and convert it to electricity [11]. High stability and high activity at physiological pH at room temperature, in addition to fuel flexibility are some of the advantages of the enzymatic fuel cell [12,13]. Enzyme cascades for complete oxidation of ethanol have recently developed by biofuel cell researchers [14,15]. The advantage of enzymes is that they exhibit high specificity and turnover rate [15,16]; however, mimicing the natural metabolism from ethanol through electrometabolic pathways is enormously difficult [17] due to problems of stability, and a narrow optimal pH and temperature range of the enzymes used. The high number of individual enzymes required to achieve complete ethanol oxidation is also a limiting factor.

To overcome the limitations of using multiple enzymes, a small molecule organic catalyst can be employed. TEMPO (2,2,6,6-tetramethylpiperidin-1-yl) oxyl) is a small organic catalyst that does not present limited substrate specificity that enzymes face, due to its ability to oxidize oxygen, nitrogen and sulfur-containing functional groups [16]. Additionally, TEMPO exhibits favorable catalysis in biofuel oxidation processes at room temperature and under mild aqueous conditions [18,19]. However, TEMPO is not able

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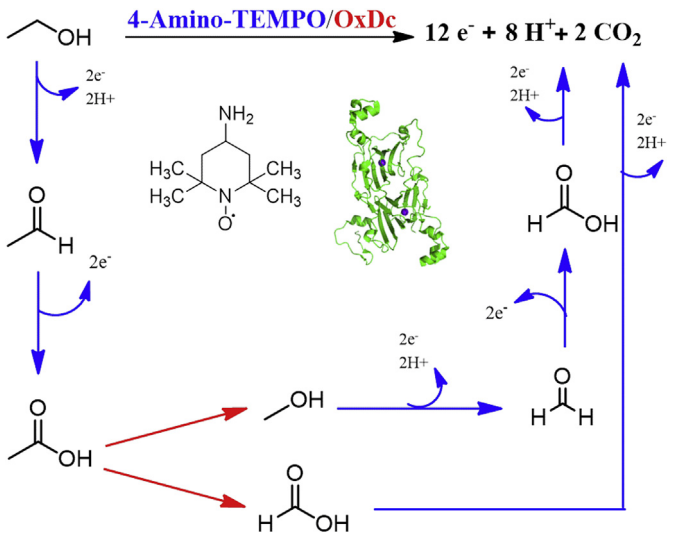
to break carbon-carbon bonds, making it impossible to achieve complete substrate oxidation with solely the use of TEMPO [20]. Despite their individual limitations, we considered the possibility of combining the advantages of a organic catalyst with enzymatic oxidation in order to obtain the benefits of each catalytic motif while minimizing the demands of complex electrooxidative cascades via hybrid systems [21,22].

Recent work reported a hybrid catalyst cascade using the organic catalyst TEMPO in the complete oxidation of fuels, such as glycerol [16,23] and ethanol [22]. Franco et al. employed TEMPO in order to oxidizes ethanol to acetic acid through 2 steps. Next, the combination of TEMPO and oxalate oxidase (OxOx) transformed acetic acid to formic acid, and finally CO_2. However, the TEMPO electrocatalyst operates best at higher pH (7–10) while the OxOx enzyme demonstrates activity only at mildly acidic pH**.** To address this issue, recent work evaluated the organic catalyst 4-amino-TEMPO, TEMPO-NH_2, and confirmed it was capable of operating under acidic conditions that are necessary for enhancing complete oxidation of fuels from hybrid systems [16].

However, the low specific activity of OxOx activity and the limited substrate range of OxOx substrates hindered the hybrid system from achieving excellent results [22,23]. To improve this cascade with OxOx, Minteer et al. demonstrated the use of a hybrid catalytic system combining TEMPO-NH_2 (an organic oxidation catalyst) with oxalate decarboxylase (OxDc) from *Bacillus subtilis*, another recombinant enzyme, for the complete electrocatalytic oxidation of glycerol [23]. OxDc and can be expressed easily with *E. coli*, while OxOx requires a eukaryote system [24,25]. Additionally, OxDc provides advantages in terms of cost, specific activity, while OxOx requires a eukaryote system [24,25].

In this work, we employed a hybrid system combining the organic oxidation catalyst, 4-amino-TEMPO (TEMPO-NH_2), and an enzyme, oxalate decarboxylase (OxDc) to complete electrochemical oxidation of ethanol to CO_2, while collecting up to 12 electrons per ethanol molecule (Scheme 1). The device used to perform the full oxidation was a bulk electrolysis micro-reactor which was an adaptation from a previous report [26].

The new electrolysis cell design is composed of simple graphite and thermoplastic materials used for commercial electrolysis/fuel flow cells. The unique fabrication method allows for miniaturization of an electrolysis cell to accommodate a small volume (≈1 mL) which is beneficial for initial fundamental studies of reaction products by nuclear magnetic resonance spectroscopy (NMR).



Scheme 1. Electrocatalytic oxidation cascade of ethanol by TEMPO-NH_2/OxDc hybrid system. The blue line represents the oxidation mediated by TEMPO-NH_2, while decarboxylation reaction catalyzed by OxDc is represented by red lines.

2. Experimental

2.1. Chemicals

4-amino-TEMPO (free radical), ethanol, acetaldehyde, acetic acid, and formic acid were purchased from Sigma Aldrich, while ethanol (2–13C, 99%) was purchased from Cambridge Isotope Laboratories, and these reagents were used as received without additional purification. 150 mM citric acid-phosphate buffers (pH = 5.2) and 200 mM phosphate buffered saline (pH = 5.7). Oxalate decarboxylase (OxDc) was also expressed and purified in the lab (*vide infra*) and stored in −80 °C until use. Water purified using a Millipore Milli-Q system was used to prepare all solutions. All chemicals were used as they were received without further purification.

2.2. Expression and purification of the enzyme oxalate decarboxylase (OxDc) from Bacillus subtilis

Oxalate decarboxylase (OxDc) expression and purification was adapted from a previously reported method [23]. The plasmid pET-9c-OxDc was used to transform the strain *E. coli* BL21(DE3). A starter culture was grown overnight at 37 °C in LB broth in the presence of 100 μg mL^{-1} kanamycin and chloramphenicol, and then was used to inoculate 6 L of LB broth. The inoculated culture was grown at 37 °C and 220 rpm until an OD600nm value of 0.5 was reached. The cells were heatshocked at 42 °C for 15 min and then induced for expression by adding 1 mM IPTG and 5 mM $MnCl_2$. The induced cells were incubated for 4 h at 30 °C and shaken at 220 rpm. The cells were then collected by centrifugation followed by resuspension in 50 mM Tris-HCl buffer (pH 7.0); these were then disrupted using a microfluidizer. After centrifugation, the soluble fraction of the cell lysate was applied to a Q-Sepharose Fast Flow column (1 × 25 cm) that had been equilibrated with 50 mM Tris-HCl (pH 7.0). Elution was performed using a 400-mL linear gradient from 0 to 1 M NaCl. The fractions that contained purified OxDc were combined and concentrated by ultracentrifugation in an Amicon centrifugal filter. The OxDc solution was then run through an FPLC desalting column (HiPrep desalting column, 15 mL, GE Healthcare) and stored at −80 °C. A protein concentration of 55.4 mg mL^{-1} was measured with Pierce™ BCA protein assay kit (Life Technologies™, Carlbad, CA).

2.3. Enzyme activity assays

OxDc activity in solution was evaluated by indirect enzymatic UV–Vis spectrophotometric assay using a 96-well plate and the Synergy HTX Multi-Mode Reader (BioTek). OxDc (60 μg) was added in 100 μL mixture containing different concentrations of oxalate (from 0 to 50 mM) in 150 mM phosphate buffer (pH 4.0). After 5 min, the reactions were quenched with 140 μL of 0.2 M K_2HPO_4 followed by the addition of 5 mM NAD^+. The levels of produced formic acid were established in a coupled assay with the addition of formate dehydrogenase and the absorbance was recorded at 340 nm ($\varepsilon = 6220\ M^{-1}\ cm^{-1}$). The formate concentration was quantified by the Michaelis-Menten equation containing K_m and V_m of the formate dehydrogenase obtained in the same condition of this essay. The obtained oxalate decarboxylase extract was determined to have a specific activity of 11.74 U/mg (μmol of substrate converted per minute per milligram of enzyme).

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2.4. Electrochemical measurements

A CH Instruments 660 E Electrochemical Workstation combined with a standard three-electrode cell was used for electrochemical experiments. Cyclic voltammetry (CV) experiments were performed using a 3 mm glassy carbon (GC) as a working electrode, a saturated calomel (SCE) reference electrode, and a Pt mesh counter electrode. CV experiments were performed at a scan rate of 10 mV s^{-1}, a step potential of 0.001 V, a potential range of 0.00−1.0 V (*vs.* SCE), and 25 °C unless otherwise noted.

Unless otherwise stated, all amperometric titrations were carried out at a fixed oxidative potential 0.8 V *vs.* SCE using constant potential amperometry in a 150-mM citric acid-phosphate buffer (pH = 5.2) with consecutive additions of substrate.

Power and current density measurements were conducted in a two-compartment cell separated by a Nafion® membrane. The cathode counterpart in direct contact with air is separated from the aqueous anodic chamber using a gas diffusion membrane (ELAT) composed of 20% platinum on Vulcan XC-72 which is hot-pressed in a Nafion® 212 membrane. The cell compartment volume of 10 mL was filled with 150 mM citric acid-phosphate buffer (pH = 5.2) containing 100 mM ethanol. The open-circuit potential of the cell was monitored for 1 h until it stabilized. Linear polarization was performed starting from OCP to 0 V at 1 mV s^{-1}. The obtained data was used to calculate power density. All results presented are based on triplicate samples. Uncertainties correspond to one standard deviation.

2.5. Ethanol oxidation cascade and CO_2 detection

Bulk electrolysis experiments were performed using a SCE reference electrode and a custom micro-reactor with integrated working and counter electrode (Fig. S1). The fabrication of the microfluidic cell and the polycaprolactone (PCL) carbon composite electrode was described by Klunder et al. [26]. The 12-h ethanol oxidation cascade was performed by bulk electrolysis Eap = 0.8 V (*vs.* SCE) using a small cell containing 30 mM ^{13}C2-ethanol, 50 mM TEMPO-NH_2, in presence of 20 U mL^{-1} OxDc with 200 mM phosphate buffered saline (pH = 5.7), at 25 °C. The oxidation cascade was run using a potential of 0.8 V (*vs.* SCE) for 12 h.

Carbon dioxide (CO_2) after bulk electrolysis was detected via NMR. ^{13}C NMR analysis was performed on a 400 MHz NMR. The NaOH was used to capture ^{13}C-enriched CO_2 through the formation of $Na_2CO_3 + H_2O$, which was detected by ^{13}C NMR (D_2O). Samples were analyzed using D_2O at 25 °C. All of the analyses were carried out in triplicate. Commercial standards were used to identify the products formed in the reaction.

3. Results and discussion

3.1. Bioelectro-oxidation of ethanol experiments at hybrid bioanodes

In order to evaluate the electrochemical properties of the hybrid system TEMPO-NH_2/OxDc, ethanol electro-oxidation was investigated. Fig. 1 shows the cyclic voltammetric (CV) profile of the

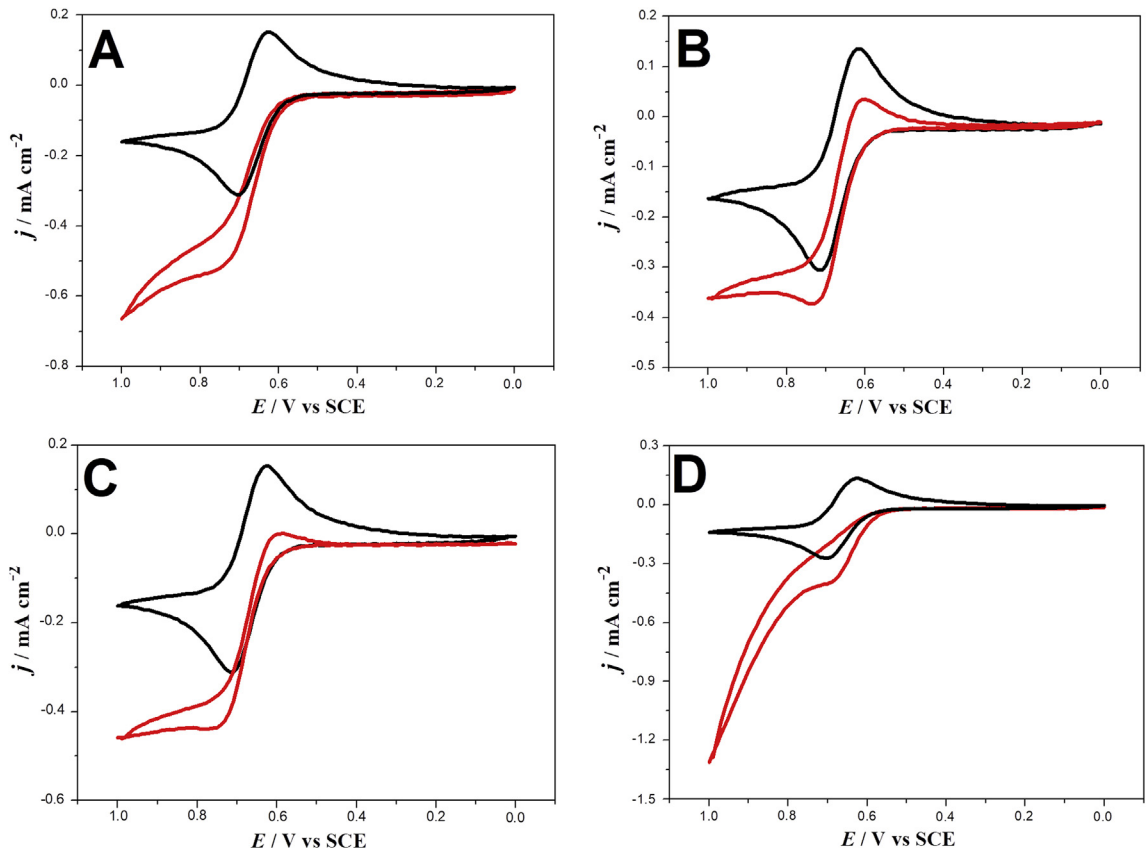

Fig. 1. Catalytic cyclic voltammetric (CV) curves of 5 mM TEMPO-NH_2 in the presence of 20 U mL^{-1} OxDc (red line). CVs were performed in the absence of any substrate (red line) and in the presence (black line) of 100 mM ethanol (A), acetaldehyde (B), acetic acid (C) and formic acid (D). Supporting electrolyte: 150-mM citric acid-phosphate buffer (pH = 5.2), potential range of 0.00−1.0 V (*vs.* SCE), ν = 10 mVs^{-1}, and 25 °C. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

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TEMPO-NH_2/OxDc electrode. The black line represents the voltammetric response in the absence of substrate (only hybrid system in buffer) and the red line represents in the presence of 50 mM of ethanol (A), acetaldehyde (B), acetic acid (C), and formic acid (D). Fig. S2 presents the CV for the bare electrode in the presence of buffer (dashed line) and with TEMPO-NH_2 (black line).

First, the electrocatalytic activity of hybrid system was tested against ethanol, acetaldehyde, acetic acid and formic acid (Fig. 1). The maximum current density generated a substantial increase in the presence of ethanol (0.56 mA cm^{-2}), acetaldehyde (0.34 mA cm^{-2}), acetic acid (0.42 mA cm^{-2}), and formic acid (0.51 mA cm^{-2}) at 0.8 V *vs*. SCE, which confirmed that TEMPO-NH_2/OxDc has the capability to readily oxidize several key intermediates in the multi-step oxidation of ethanol.

Additionally, it was observed that TEMPO-NH_2 alone is not able to react with acetic acid (Fig. S3). The use of the OxDc enzyme is required to complete this step of the cascade reaction. This finding is confirmed by Franco et al. who describe the oxidation of alcohols and aldehydes using TEMPO-mediated electrocatalysis [15,22]. The oxidative current densities by cyclic voltammetry (CV) for the system containing only OxDc did not show catalytic activity towards ethanol, acetaldehyde and formic acid (Fig. S4). These results indicate that OxDc does not catalyze the oxidation of these substrates. We demonstrated the use of recombinant OxDc with acetic acid (Fig. S4) and confirmed the capacity of OxDc enzyme to catalyze the cleavage of the carbon-carbon bond in acetic acid to produce formic acid. Thus, we conclude that the observed increase in anodic current density is the result of enzyme activity rather than the activity of the TEMPO-NH_2 catalyst.

The CV data clearly indicated that the hybrid system significantly increased the anode catalytic activity, and was able to collect more electrons from ethanol.

An oxidation potential of 0.8 V (vs. SCE) which is 50 mV above the peak oxidation potential was used in chronoamperometric studies that were undertaken with the prepared hybrid system. Concentrations of ethanol, acetaldehyde, acetic acid, and formic acid in the electrolyte solution were gradually increased. Representative amperometric titration response curves for sequential additions of ethanol, acetaldehyde, acetic acid, and formic acid (A-D, respectively) are shown in Fig. 2.

The titration curves generated maximum current densities of 0.88 mA cm^{-2} for acetaldehyde, 1.02 mA cm^{-2} for acetic acid, and 1.13 mA cm^{-2} for formic acid. The highest catalytic current density of 1.45 mA cm^{-2} was observed in the presence of ethanol for concentrations ranging between 0 and 140 mM. The high catalytic activity of TEMPO-NH_2 toward primary alcohols is demonstrated by these results.

In addition, the amperometric responses of the hybrid system with all substrates showed the current increased linearly with substrate concentration until it reached a steady state, indicating that the system has reached saturation.

Fig. S5 depicts a representative chronoamperometric assay of a TEMPO-NH_2 prepared bioanode in buffer containing acetic acid, it is observed that the organic catalyst TEMPO-NH_2 alone cannot perform acetic acid oxidation. In contrast, Fig. S6 shows the same assay performed in the presence of OxDc at 0.8 V (*vs*. SCE) with each of the four substrates/intermediates; only acetic acid demonstrates a catalytic response while ethanol, acetaldehyde, and formic acid do not.

Successive additions of different ethanol concentrations,

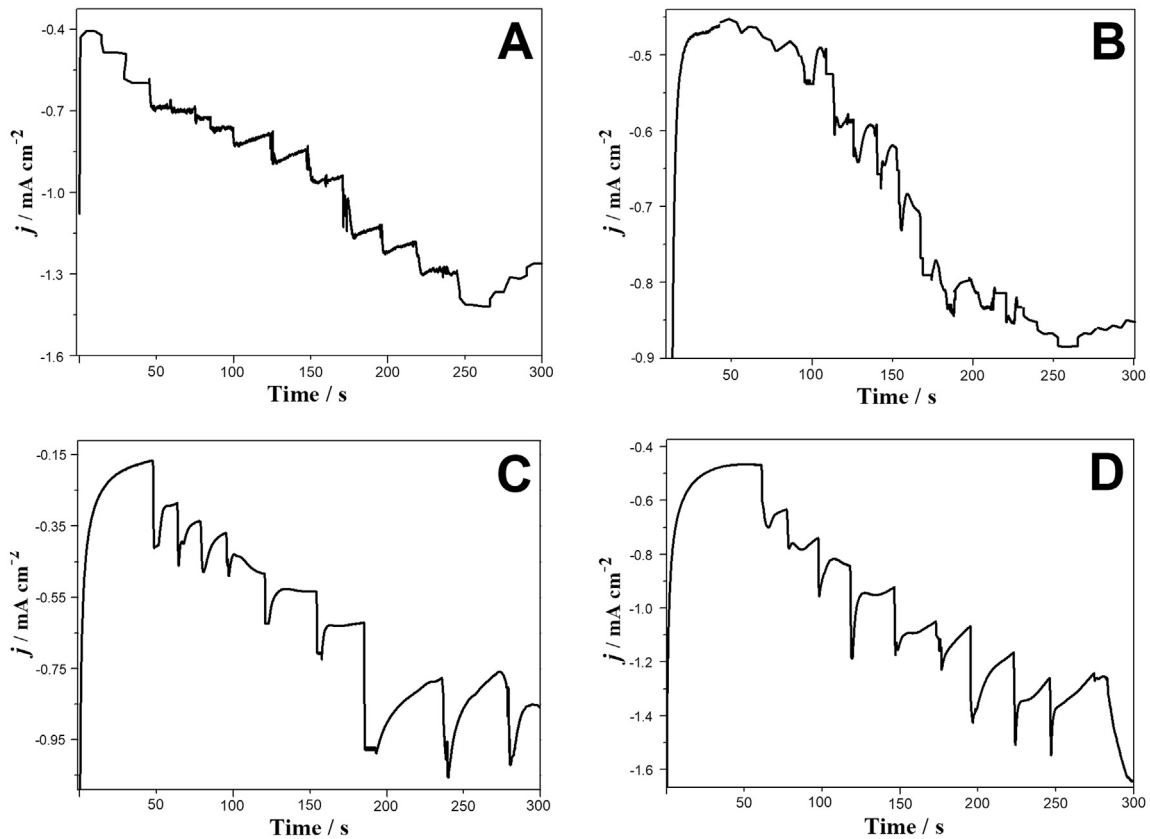

Fig. 2. Chronoamperometric assay performed with TEMPO-NH_2/OxDc with sequential addition of 10 mM ethanol, acetaldehyde, acetic acid, and formic acid (A-D, respectively). Eap = 0.8 V *vs*. SCE. 150-mM citric acid-phosphate buffer (pH = 5.2) was used as the supporting electrolyte.

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acetaldehyde and formic acid to the enzymatic system did not cause an increase in the current output. However, consecutive additions of acetic acid lead to consecutive increases in J_{max} with an observed current density maximum of 0.85 mA cm^{-2}; these results demonstrate the ability of the OxDc biocatalyst to cleave the C–C bond of acetic acid. Therefore, a noticeable increase occurs in catalytic current densities of the hybrid system in the presence of all substrates when compared to the systems containing only TEMPO or enzyme, confirming that their simultaneous presence has a synergistic interaction to oxidize these substrates and complete the total oxidation of ethanol.

Since current is a direct measurement of reaction rate then based on cyclic voltammetry and chronoamperometry data, the rate limiting oxidation step can be investigated. It can be seen from Fig. S4 that the enzyme modified electrode had the lowest current density (0.9 mA cm^{-2} at 0.8 V vs. SCE) in comparison to the other substrates. Direct electron transfer (DET) from the enzyme is limited by enzyme orientation to the electrode surface and ability to shuttle electrons to the enzyme active site. Overall, it was the main thrust of this work to demonstrate the complete oxidation of ethanol. In order to increase cell efficiencies, enzyme activity should be enhanced. Common methods to enhance active are to synergistically pair the enzyme with specialized polymers which enhance DET or increase the surface area of the electrode with porous nanocarbon [20].

Fig. 1B shows that the oxidation of acetaldehyde had the second lowest current density by cyclic voltammetry. Conversion of aldehyde to carboxylic acid moieties have been reported to have varying kinetics based on substrate structure, with simple aliphatic aldehydes having slow turnover numbers at low pH [27]. Perhaps a combination of low pH and the fact that the oxidation product, acetic acid, is a determining factor for the diminished current with acetaldehyde relative to the other substrates. Based on the current density shown in Figs. 1, 2, and S4, it is concluded that the substrates with more favorable oxidation kinetics under acidic conditions and the unique catalytic system are formic acid > acetaldehyde > acetic acid.

3.2. Bulk electrolysis and product identification by NMR

The long-term electrolysis of ethanol was performed at 0.8 V *vs.* SCE for 12 h and the products were then characterized using C13-NMR to detect the possible products formed. We chose to develop a small volume (≈ 1 mL) electrolysis cell (Fig. 3) specifically for NMR detection, which was constructed out of poly-methyl methacrylate (PMMA) with a polycaprolactone/graphite composite electrode. Polycaprolatone (PCL) is used in medical devices and is biologically compatible [28]. A traditional CO_2 laser was used to cut out the individual pieces, and the PCL/graphite composite was then hot molded into the templates [26]. The composition of the PCL/graphite composite is analogous to that of bipolar plates which are used in commercial fuel cells [29].

It is important to emphasize that the optimal ratio between 20 U mL^{-1} OxDc (55.4 mg mL^{-1}), and 5 mM 4-Amino-TEMPO (8.6 mg mL^{-1}) concentration in the reactor was 6.4, ie the enzyme concentration is 6.4 times higher than the organic catalyst concentration. The organic catalyst has a much higher catalytic activity than the enzyme, acting on the oxidation of OH group-containing compounds, while the enzyme is more specific and only acts on a specific substrate. Then, to balance the activities of both catalysts, 6 times more enzyme was added to the solution.

The profile for ethanol bioelectro-oxidation after bulk electrolysis (12 h) employing the PCL/graphite micro-reactor are described in Fig. 4. The Fig. 4 indicated that the catalytic activity of the hybrid system (red line) showed that the amount of current density

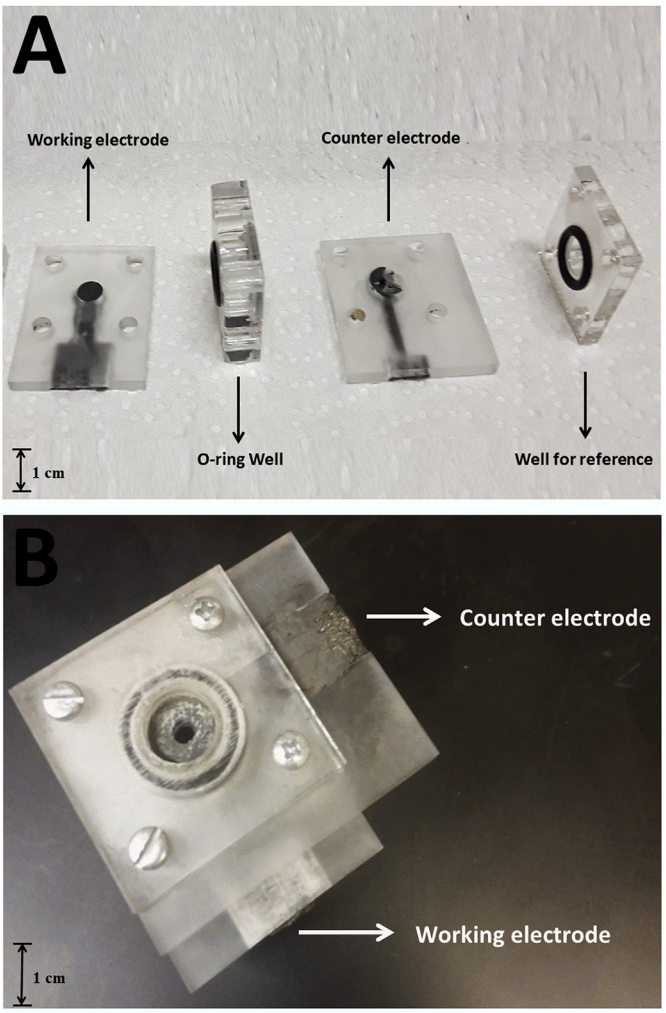

Fig. 3. (A) Pieces of the micro-reactor (B) Picture of PCL/graphite system used for electrolysis.

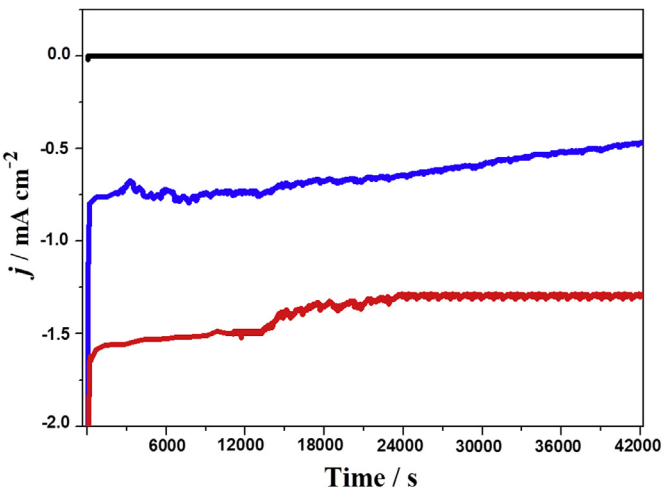

Fig. 4. Current versus time measurement for 30 mM ^{13}C2-ethanol during long-term electrolysis employing the system containing only TEMPO-NH_2 (blue line) and in the presence of a hybrid system TEMPO-NH_2/OxDc (red line). The control sample (black line) was performed, in the absence of TEMPO-NH_2 and OxDc, in the presence of buffer only. Eap = 0.8 V (*vs.* SCE). Supporting electrolyte: 200 mM phosphate buffered saline (pH = 5.7). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

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generated during the 12 h of electrolysis was two times higher compared to the system containing only TEMPO-NH_2 (blue line). Therefore, the hybrid system showed that the TEMPO-NH_2 and OxDc act together to enhance the amount of ethanol oxidized. The catalytic activity generated by the control sample - black line (absence of TEMPO-NH_2 and OxDc) was much lower when compared to the current density value of the system in the presence of organic catalyst and/or enzyme, suggesting that both are active in the ethanol oxidation.

Acetic acid electrolysis was conducted with TEMPO-NH_2 (Fig. S7) and OxDc (Fig. S8). As expected, no current density value was observed for the TEMPO-NH_2 electrode after electrolysis for 12 h with acetic acid, which indicated that organic catalyst is not able to cleave carbon-carbon bonds, acting only in oxidation of primary alcohol. We performed a long-term electrolysis for the enzymatic system (Fig. S8) in the presence of acetic acid (green line) and showed an outstanding electrocatalytic activity toward acetic acid electrooxidation, confirming the action of the oxalate decarboxylase to break the carbon-carbon bond from acetic acid.

Finally, the work here utilized PCL as a plastic binder material. While PCL has excellent biocompatibility, it is unclear if any leaching or degradation of the material may have an effect on the cell performance/stability. The choice of polymer binder as well as purity of the carbon should be a consideration when constructing devices for long-term electrolysis (days-years).

NMR experiments were performed to identify the products formed after 12 h ethanol oxidation by bulk electrolysis. To demonstrate the ability of TEMPO-NH_2 with OxDc to completely oxidize ethanol, ^{13}C-labeled ethanol was used as a substrate; the produced $^{13}CO_2$ was converted to $Na_2^{13}CO_3$ using a 0.1 M NaOH solution, and carbon-labeled substrates, intermediates, and products were observed using ^{13}C NMR. Additionally, all samples analyzed have no peaks related to the ethanol oxidation at t = 0 h.

Fig. 5A shows that the enzyme only system had no peaks

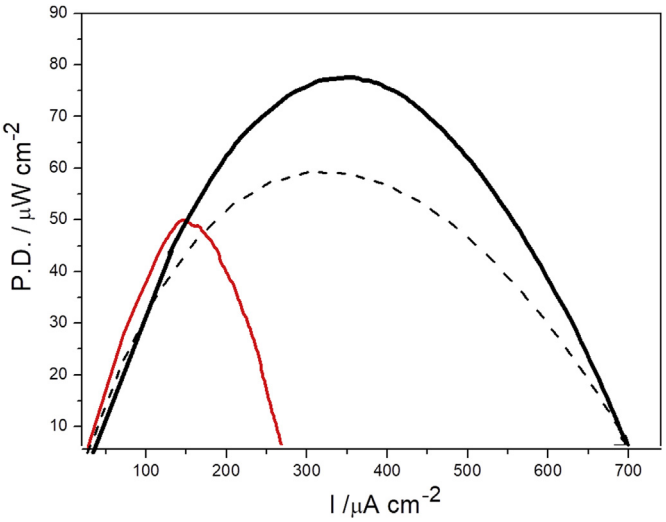

Fig. 6. Ethanol/O_2 biofuel cell power density curves obtained for different systems employed: hybrid system TEMPO-NH_2/OxDc (black line), only with organic catalyst TEMPO-NH_2 (dashed line) and only with OxDc enzyme (red line) before bulk electrolysis (12 h). Supporting electrolyte: 150-mM citric acid-phosphate buffer (pH = 5.2), and 100 mM ethanol. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

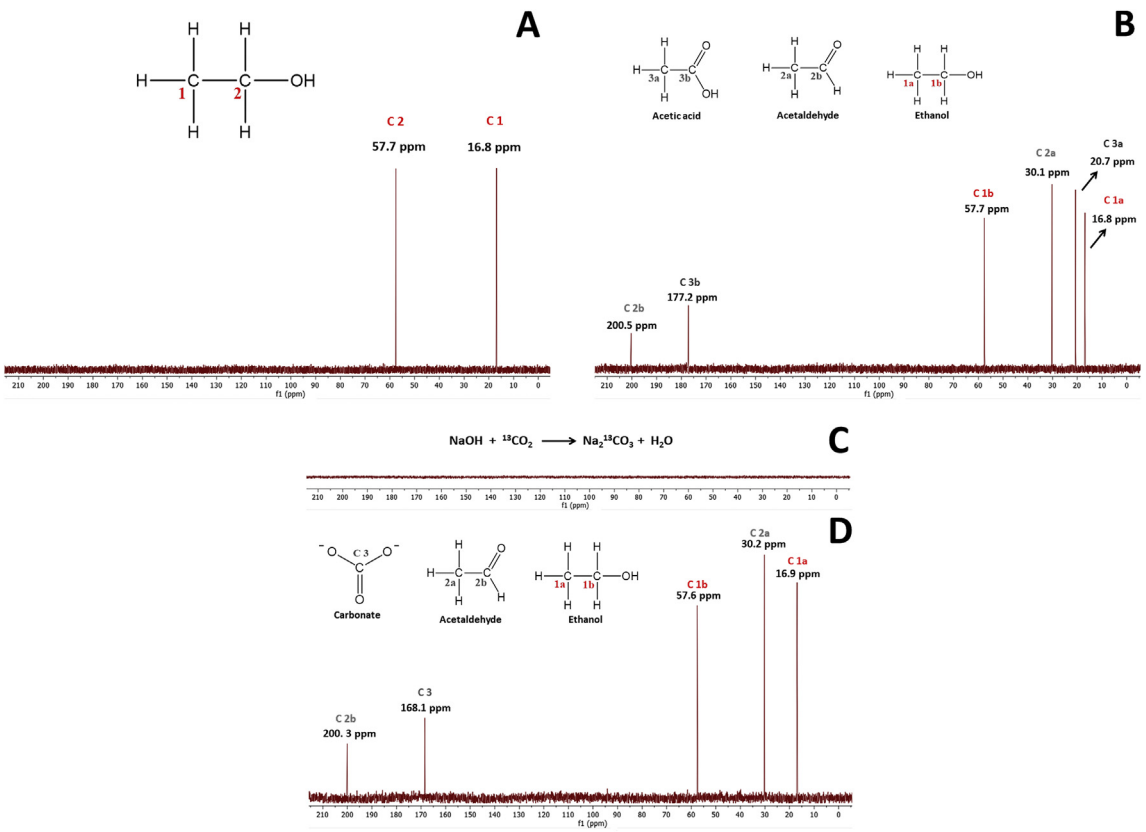

Fig. 5. ^{13}C NMR spectrum of OxDc system (A), and TEMPO-NH_2 system (B) from ^{13}C2-labeled ethanol after 12 h of electrolysis. (C) ^{13}C NMR spectrum of NaOH exposed to ambient conditions for 12 h as a control, and (D) the product of the complete oxidation of ^{13}C2-labeled ethanol by TEMPO-NH_2 and OxDc, $^{13}CO_2$, trapped in the form of $Na_2^{13}CO_3$ by reaction with NaOH.

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Table 1
Evaluation of power density values obtained for all systems analyzed before and after 12 h of electrolysis and 30 days of storage in a refrigerator. Conditions used: 100 mM ethanol; 150 mM pH 5.2 citric acid-phosphate buffer; 12 h of total electrolysis time; Eap of 0.8 V (*vs.* SCE).

	Power density ($\mu W\ cm^{-2}$) Before Electrolysis	Power density ($\mu W\ cm^{-2}$) After Electrolysis	Power density ($\mu W\ cm^{-2}$) After 15 days	Power density ($\mu W\ cm^{-2}$) After 30 days	Power density stability compared after 30 days (%)
Enzymatic (OxDc) system stability	49 ± 2	27 ± 1	21 ± 1	15 ± 1	30 ± 2
TEMPO-NH_2 system stability	59 ± 3	32 ± 28	29 ± 2	25 ± 2	43 ± 3
TEMPO-NH_2/OxDc Hybrid system stability	78 ± 5	69 ± 4	63 ± 5	60 ± 4	77 ± 6

indicating oxidation products after reaction with ethanol. In the system containing TEMPO-NH_2 only (Fig. 5B), a peak at 30.1 ppm and 200.5 ppm was attributed to acetaldehyde. The acetic acid was detected at 20.7 ppm and 177.2 ppm. These results obtained by NMR demonstrated the action of TEMPO-NH_2 to oxidize the first two catabolic steps, which involve 4 e^- in the formation of acetic acid (Scheme 1).

Control experiments were also performed with NaOH in the presence of phosphate buffered saline (pH = 5.7), which confirmed that NaOH does not oxidize the buffer, i.e., no product was formed (Fig. 5C). Finally, a significant peak at 168 ppm (Fig. 5D) indicates that the hybrid system was able to produce $^{13}CO_2$ from the cascade oxidation of ^{13}C-ethanol. The formation of acetaldehyde was observed, but not acetic acid. Probably, the acetic acid formed by the action of the TEMPO-NH_2 system was converted to CO_2 since OxDc acts on and cleaves the carbon-carbon bond in acetic acid.

A reaction scheme for the complete oxidation is proposed in Scheme 1 were TEMPO-NH_2 catalyzes the first two oxidation steps of ethanol to acetic acid (collecting up to 4 e^-), followed by OxDc-catalyzed decarboxylation of acetic acid by cleavage of a carbon-carbon bond to generate formate. Thus, due to extreme potential or TEMPO-NH_2, the formate is oxidized easily to CO_2, enabling the collection of an additional 2 e^-.

3.3. Power density tests

Following the cyclic voltammetry and the chronoamperometry assays, polarization tests for the systems investigated were performed. Fig. 6 displays representative power density curves of complete biofuel cells with freshly prepared bioanodes containing TEMPO-NH_2/OxDc (black line), only with organic catalyst TEMPO-NH_2 (dashed line) and only with OxDc enzyme (red line) before bulk electrolysis (12 h). The goal of the power density tests is to verify and compare the reproducibility before electrolysis and the stability after bulk electrolysis of the each system analyzed. A maximum current density of $147 \pm 8\ \mu A\ cm^{-2}$, power density of $49 \pm 2\ \mu W\ cm^{-2}$, and open circuit potential (OCP) of 0.394 V (*vs.* SCE) are obtained for bioanodes which were prepared with only the enzyme. Those prepared with only TEMPO-NH_2 demonstrated $316 \pm 10\ \mu A\ cm^{-2}$, $59 \pm 3\ \mu W\ cm^{-2}$, and 0.440 V for the maximum current density, maximum power density, and OCP, respectively. Finally, those with both TEMPO-NH_2 and OxDc demonstrated a maximum current density, maximum power density, and OCP of $353 \pm 15\ \mu A\ cm^{-2}$ and $78 \pm 5\ \mu W\ cm^{-2}$, and 0.468 V, respectively. Thus, the higher the power density value of a system, more energy must be generated and consequently more electrons will be collected from the ethanol molecule.

The data obtained from the biofuel cell test showed the same behavior observed by CV and chronoamperometry results, confirming once again the enhanced performance of the hybrid bioanode against the systems with only TEMPO-NH_2 or OxDc. Futhermore, the highest power density attributed to the hybrid system when compared with the others systems is explained by NMR data, which showed that this configuration was able to uptake the maximum energy possible from ethanol, e.g., 12 electrons producing CO_2.

Comparison of the power density yielded by the hybrid system with the system containing organic catalyst or enzymes only, showed significant improvement in bioanode performance. The increase in the number of electrons (12 electrons) exchanged in the complete oxidation of ethanol is likely the reason for the increased performance of the organic catalyst/enzyme system [14,30]. Notably, the developed system herein is in accordance with the literature and possesses a high enough energy density to be suitable for applications in small portable devices [31].

The evaluation and comparison of power density values obtained during storage in a refrigerator for 30 days and after bulk electrolysis for all systems analyzed, as shown in Table 1. The hybrid system presented a good stability after 12 h of electrolysis and 30 days of storage, with 89 and 77% power retention, respectively. In contrast, the stability of the individual TEMPO-NH_2 and enzymatic systems after 12 h electrolysis is much lower compared with the hybrid system. Indeed, TEMPO-NH_2 ($25 \pm 2\ \mu W\ cm^{-2}$) and OxDc ($15 \pm 1\ \mu W\ cm^{-2}$) system showed low stability after 30 days of storage, indicating 30 and 43% loss in the power density, respectively. Thus, the hybrid bioanode enables high stability when the organic catalyst and enzyme are combined in the system.

4. Conclusions

In this investigation, TEMPO-NH_2 organic catalyst and OxDc enzyme were shown to synergystically promote a compete oxidation of ethanol. The hybrid catalytic design enhances the overall steps in the cascade operating at an optimum pH for both the organic catalyst and the enzyme.To validate the reaction sequence, a novel micro-reactor device enabled low-cost product analysis and allows for high analyte sensitivity.Overall, this work demonstrated that bi-cascade of TEMPO-NH_2 and OxDc can be combined in an electrochemical cell to synergistically catalyze the oxidation of substrates that make up the steps of the ethanol oxidation cascade and enable enhanced electrochemical oxidation of ethanol to CO_2.

Declaration of competing interest

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.electacta.2019.135254.

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