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How to advance the frontiers of current biofuel cells: design of a H<sub>2</sub>/O<sub>2</sub> biofuel cell based on thermostable enzymes

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## **Abstract**

A new generation of mediatorless H<sub>2</sub>/O<sub>2</sub> biofuel cells was designed based on a hyperthermophilic O<sub>2</sub>-tolerant hydrogenase and a thermostable bilirubin oxidase both immobilized on carbon nanofibers. A power density up to  $1.5 \pm 0.2 \text{ mW.cm}^{-2}$  at 60°C was reached. This first demonstration of a H<sub>2</sub>/O<sub>2</sub> biofuel cell able to deliver electricity over a wide range of temperatures, from 30°C up to 80°C, and over a large pH window, allows considering this device as an alternative power supply for small portable applications in various environments, including extreme ones.

*Keywords:* Enzymatic H<sub>2</sub>/O<sub>2</sub> biofuel cell; Hydrogenase; Bilirubin oxidase; Thermostable enzymes; Direct electron transfer; Carbon nanofiber.

## 1. Introduction

H<sub>2</sub> is an alternative to fossil fuels and is the subject of intensive research in the field of fuel cells. Its production from biomass is an increasing and promising domain which may improve its sustainability [1, 2]. Its use has also been envisioned for the elaboration of H<sub>2</sub>/O<sub>2</sub> enzymatic biofuel cells (BFC) which may power small *ex-vivo* electronic devices in a sustainable manner.

Hydrogenase (Hase) is the key enzyme for hydrogen oxidation in many microorganisms. Many fundamental researches on mechanistic aspects of hydrogen oxidation by Hase have been done for more than 20 years [3-5]. However, H<sub>2</sub>/O<sub>2</sub> BFCs are only emerging, essentially because of the high sensitivity to O<sub>2</sub> of many Hases. This limitation was recently overcome by the identification of O<sub>2</sub>-tolerant Hases [6-10]. Armstrong's group established the proof of concept of H<sub>2</sub>/O<sub>2</sub> BFCs using a mesophilic O<sub>2</sub>-tolerant Hase and multicopper enzymes (laccase or bilirubin oxidase (BOD)) to reduce oxygen [11-13]. The mW.cm<sup>-2</sup> power density landmark was reached last year for H<sub>2</sub>/O<sub>2</sub> BFC by entrapping the enzymes in compacted carbon powder electrodes [14]. Therefore, H<sub>2</sub>/O<sub>2</sub> BFC equaled the highest performances of the commonly developed glucose/O<sub>2</sub> BFCs [15-17]. This BFC operated in mild conditions, *i.e.* at pH 6 and room temperature. We also proved two years ago that power densities up to 300 μW.cm<sup>-2</sup> could be reached with another O<sub>2</sub>- and CO-tolerant Hase purified from the hyperthermophilic bacterium *Aquifex aeolicus* (MbH1) and a commercially available BOD, both covalently bound on carboxyl functionalized carbon nanotubes [18]. Due to the ability of MbH1 to oxidize H<sub>2</sub> at high temperatures [7], we envisioned the development of a H<sub>2</sub>/O<sub>2</sub> biofuel cell delivering electricity in unconventional environments, such as hot ones. However the performances of the H<sub>2</sub>/O<sub>2</sub> BFC were limited at temperatures higher than 40°C by the strong decrease in enzymatic activity of the biocathode, linked to the BOD denaturation.

In the present work we use a thermostable BOD from *Bacillus pumilus* (*Bp* BOD) [19, 20] for the direct reduction of oxygen at the biocathode in tandem with the hyperthermophilic MbH1 at the anode. This is the first attempt to address the problem of operating BFCs even at temperatures above those tolerated by enzymes from conventional microorganisms.

## 2. Experimental

### 2.1. Chemicals and materials

*Bacillus pumilus* BOD (*Bp* BOD) was produced and purified according to Durand and co-workers [19] and MbH1 was purified as described in Luo et al. [7]. BOD from *Myrothecium verrucaria* (*Mv* BOD) was a gift from Amano Enzyme Inc. (Nagoya, Japan). Hydrophobic and hydrophilic carbon nanofibers (CNF) were synthesized and functionalized as reported in de Poulpiquet et al. [21].

### 2.2. Bioinformatics

Swissmodel automated mode was used to construct the homology model of *Bp* BOD. The *Bp* BOD protein sequence (Pubmed entry AFL56752.1) was used as a query and the crystallographic structure of *Bacillus subtilis* (1GSK chain: A) was used as a template.

### 2.3. Instrumentation and measurement procedures

Electrochemical experiments were performed using a potentiostat from Bio-Logic. The Ag/AgCl (NaCl sat.) reference electrode was separated from the electrolyte using a side junction maintained at room temperature. A pyrolytic graphite (PG) electrode from Bio-Logic was the working electrode. All current densities are calculated using the projected area of the PG electrode ( $A=0.07 \text{ cm}^2$ ). Current densities were measured at -0.25 V or +0.3 V for MbH1 and BOD, respectively. The electrodes were placed at 6 cm (this distance was not optimized but imposed by the geometry of the cell) from the Nafion<sup>®</sup> membrane (Nafion<sup>®</sup> 117 from DUPONT-USA) separating the compartments. The biofuel cell performances were examined

under 100 % H<sub>2</sub> and 100 % O<sub>2</sub> for anode and cathode respectively. Gas bubbling was maintained into the electrolyte solution to limit substrate depletion. The cell current and voltage were measured by polarization curves, after stabilization of the system. Scan rate was 3 mV.s<sup>-1</sup>.

#### 2.4. Electrode preparation

MbH1 and BOD were immobilized respectively on hydrophobic and hydrophilic fishbone carbon nanofibers (CNF) [21]. In-depth characterization of the biohybrid electrode led to the following optimized procedure. CNFs were diluted in a mix of Milli-Q water and N-N-dimethylformamide and sonicated before use. 5 μL of the 4 mg/mL CNF suspension was deposited on the PG electrode and left to dry at 60°C. This operation was repeated 3 times. Then 5 μL of 5 μM MbH1, or 5 μL of 10 μM *Bp* BOD, or 10 μL of 20 μM *Mv* BOD were deposited and left to dry at 4°C for 2h, 15h and 1h, respectively.

### 3. Results and Discussion

#### 3.1. Direct electron transfer for H<sub>2</sub> oxidation and O<sub>2</sub> reduction is allowed by the CNF network on a large range of temperature (30 to 70°C)

Direct H<sub>2</sub> oxidation by MbH1 at a CNF-bioelectrode has been recently detailed at 60°C [21]. Thanks to a hierarchical porosity and a large number of anchoring sites available for MbH1, hydrophobic fishbone nanofibers allowed stable and high current densities for H<sub>2</sub> oxidation [21, 22]. The evolution of the catalytic current as a function of temperature is reported in Figure 1A. Current densities increase with temperatures reaching values around 4 mA.cm<sup>-2</sup> at 70°C. It must be noted also that H<sub>2</sub> oxidation is still quite efficient for lower temperatures ( $j_{\max}$  at 30°C is about 45% of  $j_{\max}$  at 70°C).

Although O<sub>2</sub> reduction by *Bp* BOD in redox osmium hydrogels has been reported [20, 23], direct electron transfer (DET) has never been investigated. Actually, bacterial *Bp* BOD

shares less than 33% sequence homology with the fungal BOD usually studied by electrochemistry such as *Mv* BOD [19]. A consistent structural model of *Bp* BOD has been constructed by homology modeling based on the crystallographic structure of CotA from *B. subtilis* which displays 67% sequence identity (Figure 1B). The *Bp* BOD exhibits a strong dipole moment of 2073 Debye pointing toward a positive patch at the surface of the protein opposite to the T1 copper center (for a review on multicopper oxidases see [24]). Besides, hydrophobic amino acids form only isolated entities at the surface. Consequently, hydrophilic CNFs were chosen and simple adsorption of the enzyme led to efficient DET for O<sub>2</sub> reduction with an onset potential of + 0.56 V vs Ag/AgCl at pH 4 (Figure 1A). Hydrophobic CNFs could also be used but led to a less important and stable O<sub>2</sub> reduction.

The biocathode proved to be efficient over a wide range of pH and temperature. The enzyme was electroactive at acidic and neutral pH, with an optimum at pH 4 (data not shown), which is close to the optimum activity reported for oxidation of the natural substrate bilirubin [19]. As similar observations were reported for *Bp* BOD wired in osmium polymer [20], this suggests that direct electrical connection of the enzyme does not alter its enzymatic properties. The current densities for direct enzymatic O<sub>2</sub> reduction increase with increasing temperatures up to 60°C, depicting a two-fold increase between 30°C and 60°C (Figure 1A). A decrease in the maximum current density was recorded at higher temperatures than 60°C. Based on spectrophotometric assays, Mano's group reported a four-time higher activity at 60°C than at 30°C with an optimum temperature of 75°C [20]. A three-time enhancement of the catalytic current was also previously recorded for O<sub>2</sub> reduction with *Bp* BOD wired in osmium polymer when temperature was increased from 37°C up to 70°C [20]. The differences between homogeneous and heterogeneous experiments might be explained by the increasing difficulty to overcome the mass transport limitation through the redox polymer or the CNF network because O<sub>2</sub> solubility in water decreases with increasing temperature, and/or

by the orientation of the enzyme on the electrode surface. A change in the CV profiles at the highest temperatures was sometimes noticed, which is also observed for H<sub>2</sub> oxidation (Figure 1A). This behaviour might be related to a distribution of electron transfer rates inside the CNF film because of a distribution of enzyme orientation [25]. The intensity for direct O<sub>2</sub> reduction by *Bp* BOD immobilized onto CNFs is 90%, 70% and 50% of the initial current after 30 min of continuous cycling at 50°C, 60°C and 70°C, respectively. Again these results are close to those reported before for homogeneous catalysis at 80°C [19] and when the BOD was wired in Os polymer at 70°C [20]. A different result is obtained when immobilizing *Mv* BOD at the CNF-modified electrode: 100% loss of current for O<sub>2</sub> reduction at 50°C in 30 minutes is observed.

### 3.2. Performances of the biofuel cell

Individual experiments were performed either with HEPES at the anode or citrate-phosphate at the cathode. In the BFC configuration, phosphate buffer was determined to be the best compromise.

The thermostability of both cathodic and anodic enzymes permits to apply a unique temperature in the two BFC compartments allowing to overcome the previous limitation reported using MbH1 and *Mv* BOD [18]. At 50°C, the optimal power density  $1.2 \pm 0.2 \text{ mW} \cdot \text{cm}^{-2}$  was reached at pH 6 with a cell voltage of 0.75 V. In the 4 to 7 pH range, the BFC power densities were comprised between  $0.6 \pm 0.07$  and  $1.2 \pm 0.2 \text{ mW} \cdot \text{cm}^{-2}$  for cell voltages varying from 0.6 to 0.75 V (Figure 2A). In contrast a maximal power density of  $10 \mu\text{W} \cdot \text{cm}^{-2}$  was reached for electrodes modified with CNFs in absence of enzymes. 40% of the initial power density is retained after 24h of continuous operation at  $E_{\text{cell}} = 0.5 \text{ V}$ . During this period (24h), the anodic and cathodic currents have decreased by the same ratio (Inset in Figure 2A). The loss of power density is most probably a combination of enzyme leaching and denaturation.



As seen in Figure 2B, the H<sub>2</sub>/O<sub>2</sub> BFC delivers high power densities over a broad range of temperature comprised between 30 and 80°C. At 30°C, a power density of 650 μW.cm<sup>-2</sup> was reached while it was 1.5 ± 0.2 mW.cm<sup>-2</sup> at 60°C. These results contrast with the previous BFC performances using *Mv* BOD where the power density strongly decreased upon increasing the temperature above 40°C in the cathodic compartment [18]. The power density of this new BFC is 5 times higher than our previous BFC [18]. Measurements with CNF-modified electrodes were realized with *Mv* BOD instead of *Bp* BOD while maintaining the anode and cathode compartments respectively at 60 and 25 °C as previously published [18]. A maximal power density of 495 μW.cm<sup>-2</sup> was obtained, *i.e.* a 1.5-fold increase which shows the importance of optimizing the electrode material, but which essentially underlines the key role of the thermostability of *Bp* BOD.

#### **4. Conclusion**

We demonstrate in this work that associating together two thermostable enzymes allows the development of a H<sub>2</sub>/O<sub>2</sub> BFC that can deliver high power densities over a wide range of temperatures. A power density of 1.5 mW.cm<sup>-2</sup> with a fill factor of 0.63 is reached which constitutes one of the highest power density ever obtained for BFCs. This new generation of H<sub>2</sub>/O<sub>2</sub> BFCs whose functioning is not limited by temperature can reasonably be considered as an alternative energy for future applications in environments in which enzymes from conventional microorganisms are denaturated. Preliminary studies on the stability of this BFC reveal that only 40% of the power density remains after 24 h of operating. In the next step of this research, long term stability as well as the scale-up of the bioelectrodes need to be envisioned for future applications. To this end, enzyme engineering and material design are underway.

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## Captions to Figures

**Figure 1:** Direct H<sub>2</sub> oxidation and O<sub>2</sub> reduction at CNF-modified bioelectrodes. (A) Temperature dependence of the voltametric signals (0 rpm, 5 mV.s<sup>-1</sup>) for the bioanode in 50 mM Hepes buffer pH 7 and for the biocathode in 100 mM phosphate – 200 mM citrate buffer pH 4 (H<sub>2</sub> and O<sub>2</sub> flow rates were 3 cm<sup>3</sup>.s<sup>-1</sup>): 30°C (violet), 40°C (blue), 50°C (red), 60°C (green), 70°C (orange). CV at 60°C for CNF electrodes with no enzyme is superimposed in grey. (B) Model structure of *Bp* BOD based on *Bacillus subtilis* CotA crystallographic structure (pdb ID: 1GSK). The hydrophobic amino acids are colored white, the positive and negative ones in blue and red, respectively. Copper atoms appear as black spheres.

**Figure 2:** High temperature H<sub>2</sub>/O<sub>2</sub> BFC performances in 100 mM phosphate buffer. (A) Influence of pH at 50°C, inset: performances of the biofuel cell before (blue) and after (red) 24h of continuous operation at E<sub>cell</sub> = 0.5 V and pH 6; (B) Temperature dependence at pH 6.







