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An air-breathing enzymatic cathode with extended lifetime by continuous laccase supply

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ABSTRACT

We present a novel concept of an air-breathing enzymatic biofuel cell cathode combined with continuous supply of unpurified laccase-containing supernatant of the white-rot fungus *Trametes versicolor* for extended lifetime. The air-breathing cathode design obviates the need for energy-intensive active aeration. In a corresponding long-term experiment at a constant current density of 50 μA cm^{-2}, we demonstrated an increased lifetime of 33 days (cathode potential above 0.430 V vs. SCE), independent of enzyme degradation. The obtained data suggest that theoretically a longer lifetime is feasible. However, further engineering efforts are required to prevent clogging and fouling of the supply tubes. These results represent an important step towards the realization of enzymatic biofuel cell cathodes with extended lifetime and enhanced performance.

1. Introduction

Microbial fuel cells bear great potential for the generation of electricity from renewable resources such as wastewater or other organic residues (Logan, 2005). However, the overall fuel cell performance is often limited by the sluggish kinetics of the oxygen reduction reaction at the cathode (Harnisch and Schröder, 2010). On this account, enzymatic biocatalysts such as laccase are an inexpensive alternative to expensive and rare noble metal catalysts such as platinum. The multi-copper oxidase laccase exhibits high electrocatalytic activity for oxygen reduction, that compares favorably to platinum (Cracknell et al., 2008). In nature, laccase is for instance secreted by the white rot fungus *Trametes versicolor*. It catalyzes the oxidation of phenolic compounds in timber with the simultaneous four electron reduction of dioxygen to water (Ivnitski and Atanassov, 2007; Piontek et al., 2002). In addition, it is capable of direct electron transfer for instance to carbon-based electrodes, which obviates the need for artificial electron mediators (Christenson et al., 2004). However, the long-term application of enzymes is significantly impaired by the intrinsic protein instability and the associated loss of biocatalytic activity. The lifetime of enzymes is typically in the range of days. For instance, in case of laccase dissolved in citrate buffer (pH 5), a half-life time in the range of only 9 days has been reported (Rubenwolf et al., 2012). In contrast, microbial fuel cells can typically be operated over periods of several years (Kim et al., 2003), and probably beyond. Therefore, to be combined with microbial anodes (hybrid microbial-enzymatic fuel cell) the lifetime of enzymatic cathodes has to be significantly increased.

In literature, different approaches to extend the enzyme lifetime are reported, which include stabilization by genetic engineering of the enzyme's amino acid sequence, by immobilization of the enzymes by binding to an external matrix, or by reduced interactions with the solvent (Minteer et al., 2007; Rasmussen et al., 2016; Rubenwolf et al., 2011). Although considerable improvements have been achieved, these methods typically only decrease the enzyme's denaturation rate but do not fully prevent degradation and the associated loss of bioelectrocatalytic activity over extended periods of time. To decouple enzyme lifetime from the lifetime of the electrode, Rubenwolf et al. (2012) previously introduced the periodical renewal of purified laccase enzyme reversibly adsorbed to a carbon-based cathode. This approach was further developed by Sané et al. (2013) who showed that the periodical re-supply of crude laccase-containing supernatant of *T. versicolor* (without purification) to a cathode leads to a more than 5 times extended electrode lifetime of at least 120 days. Furthermore, they demonstrated that it is not necessary to perform time-consuming and expensive enzyme purification processes to apply laccase as oxygen-reduction catalyst in biofuel cells. However, a limitation of these

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previous studies is the use of cathodes submerged in the enzyme containing catholyte, which has to be actively aerated to supply oxygen (Sané et al., 2013). A further drawback of immersed cathodes is the low oxygen solubility in aqueous electrolytes. Thus, the focus of recent research lies on air-breathing cathodes to maximize the oxygen concentration at enzymatic cathodes, however only few studies on this topic have been published so far (Leech et al., 2012; Rasmussen et al., 2016; Santoro et al., 2016).

In the present work, we demonstrate that the promising concept of enzyme renewal can also be applied in an air-breathing gas-diffusion cathode design. This work significantly exceeds the partial results already communicated in earlier conference proceedings (Kipf et al., 2014, 2015), and to the best of our knowledge, this combination has never been shown before. Relying purely on diffusive O_2 supply, this concept enables passive operation of the cathode without the need for energy-intensive aeration – which is of high relevance for practical application in a biofuel cell. The novel cathode design features an integrated flow-through system to continuously supply laccase-containing supernatant of a *T. versicolor* culture, and a silicone rubber membrane as diffusion layer, which enables passive oxygen diffusion. Silicone rubber is an alternative to PTFE which is used in conventional air-breathing cathodes, since it has a higher permeability for oxygen compared to PTFE (Wetser et al., 2017). Furthermore, applied as a dense layer it overcomes the typical problems of conventional microporous gas diffusion layers such as electrolyte leakage and salt precipitation (Kipf et al., 2013b; Logan et al., 2006) which often lead to a performance loss due to clogged micropores in the catalyst layer (An et al., 2017).

2. Materials and methods

2.1. Culture conditions

To supply laccase the strain of the white rot fungus *T. versicolor* ATCC 32745 was used in this study. It was cultivated according to the procedure described elsewhere (Sané et al., 2013). In short, every week a new liquid culture of *T. versicolor* was prepared by transferring three pieces (0.5 × 0.5 cm^2 each) of mycelium grown on a YDP agar plate, onto the surface of 150 mL sterile synthetic complete laccase medium (SCL, pH 5) in a 250 mL Erlenmeyer flask. This culture was cultivated for two weeks in the dark under non-shaking conditions at 30 °C. In the next step, after this cultivation time the supernatant was harvested, centrifuged for 10 min at 9511*g*, and then sterile filtered using a PES filter with 0.2 µm pore size (514–0030, VWR, Bruchsal, Germany). Afterwards the pH value and the enzyme activity were analyzed as described elsewhere (Sané et al., 2013). One unit of enzyme activity (1 U) is defined as the amount of enzyme required to oxidize 1 µmol of ABTS per minute at pH 5 and a temperature of 30 °C.

In the long-term experiment, different supernatant batches with differing enzyme activities were used to supply the cathode throughout the experiment, as indicated in Fig. 3. To this end, every week a new flask with SCL medium was inoculated with *T. versicolor* as described above (described as batches A-G). The cultivation time until supernatant was harvested is listed in Table 1. After harvesting approx. 30 mL of supernatant from a flask, this flask was further cultivated until the next time point when supernatant was harvested (batches 1, 2, …).

As comparison to the culture supernatant of *T. versicolor*, commercial laccase from *T. versicolor* (Sigma-Aldrich, Germany) dissolved in 0.1 M sodium acetate buffer (pH 5) (approx. 3.6 U mL^{-1} towards ABTS) was used as catholyte.

2.2. Electrode and reactor set-up

To construct the air-breathing cathode, at first a buckypaper electrode was prepared from dispersed carbon nanotubes (Baytubes C150 HP, Bayer Material Science AG, Germany) as described elsewhere (Hussein et al., 2011a; Hussein et al., 2011b). For the detailed procedure of the buckypaper preparation the reader is referred to the supplementary data of our previous report (Kipf et al., 2013a). The buckypaper electrode was then equipped with a 0.5 mm thin silicone rubber membrane (Ketterer + Liebherr, Freiburg, Germany) as gas diffusion layer, which enables sufficient passive oxygen supply and prevents electrolyte leakage and salt precipitation. A schematic of the flat-plate construction of the air-breathing cathode is shown in Fig. 1. The buckypaper side with the carbon nanotubes faced towards the silicone rubber membrane and the nylon filter support faced the catholyte chamber. The overall exposed area of the air-breathing cathode was 1 cm^2.

The cathode was operated in a half-cell setup against a platinum mesh counter electrode (Goodfellow, Bad Nauheim, Germany), separated by a Nafion membrane (Nafion 115, Quintech, Göppingen, Germany). As reactor an octagonal polycarbonate vessel with a liquid volume of 300 mL was used. To its outside eight air-breathing cathodes were attached using different polycarbonate frames and silicone gaskets. The cavity between the porous filter backside of the buckypaper cathode and the Nafion membrane (see Fig. 1) amounted to only 0.2 mL volume. The reactor was filled with 300 mL of 0.1 M sodium acetate buffer (pH 5), and its interior volume was continuously purged with

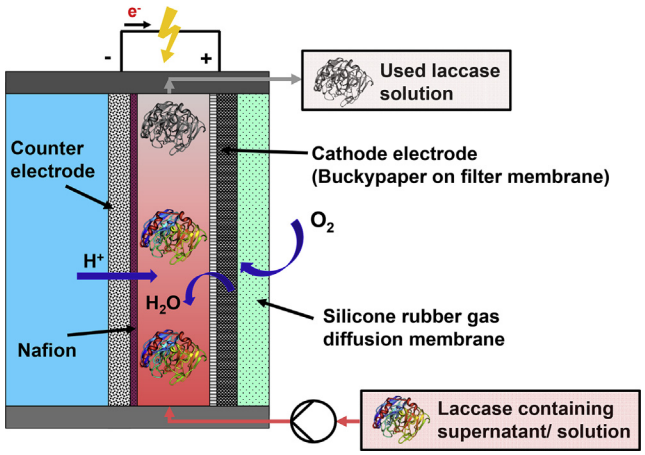

Fig. 1. Schematic of the flat-plate construction of the air-breathing laccase cathode with continuous flow-through. The image of the laccase enzyme was created using the protein data bank (PDB) and the software Protein Workshop.

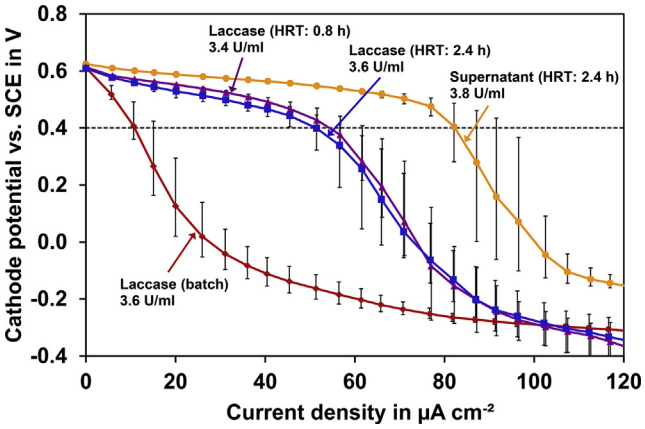

Fig. 2. Cathode polarization curves of air-breathing cathodes, recorded either in batch-mode, or with continuous supply of commercial laccase solution in sodium acetate buffer or cultivated supernatant of *T. versicolor*. Different hydraulic retention times (HRTs) were tested, as indicated. In all cases similar enzyme activities were used (approx. 3.6 U mL^{-1} towards ABTS). Data points depict the mean values and bars correspond to the maximum and minimum values of at least triplicate experiments.

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