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Using planktonic microorganisms to supply the unpurified multi-copper oxidases laccase and copper efflux oxidases at a biofuel cell cathode

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HIGHLIGHTS

- Supply of unpurified laccase and copper efflux oxidase to a biofuel cell cathode.
- Using crude culture supernatant from planktonic microorganisms.
- Live cells at a cathode provide copper efflux oxidase.
- Easy to operate and cost effective enzymatic biofuel cells.

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ABSTRACT

The feasibility to apply crude culture supernatants that contain the multicopper oxidases laccase or copper efflux oxidase (CueO) as oxygen reducing catalysts in a biofuel cell cathode is shown. As enzyme-secreting recombinant planktonic microorganisms, the yeast *Yarrowia lipolytica* and the bacterium *Escherichia coli* were investigated. The cultivation and operation conditions (choice of medium, pH) had distinct effects on the electro-catalytic performance. The highest current density of 119 ± 23 μA cm^{-2} at 0.400 V vs. NHE was obtained with the crude culture supernatant of *E. coli* cells overexpressing CueO and tested at pH 5.0. In comparison, at pH 7.4 the electrode potential at 100 μA cm^{-2} is 0.25 V lower. Laccase-containing supernatants of *Y. lipolytica* yielded a maximum current density of 6.7 ± 0.4 μA cm^{-2} at 0.644 V vs. NHE. These results open future possibilities to circumvent elaborate enzyme purification procedures and realize cost effective and easy-to-operate enzymatic biofuel cells.

1. Introduction

Biofuel cells (BFC) have great potential for the eco-friendly direct conversion of biochemically stored energy into electricity (Bullen et al., 2006; Osman et al., 2010, 2011). A prominent application is to combine the treatment of wastewater with the generation of electricity (Logan, 2005), hydrogen, or fine chemicals (Rosenbaum et al., 2011). Furthermore, implantable, glucose powered fuel cells which supply medical implants (Cinquin et al., 2010; Kerzenmacher et al., 2008), or miniature biofuel cells that digest organic matter to power energy-autonomous robots (Ieropoulos et al., 2012) are currently under development.

Usually, enzymatic catalysts or the biochemical pathways of complete microorganisms are employed to catalyse the electrode reactions in biofuel cells. In this way, the use of expensive and unsustainable noble metal catalysts is circumvented and renewable electrode materials such as carbon can be utilized as electrodes (Bullen et al., 2006; Lapinsonnière et al., 2012; Osman et al., 2010, 2011).

However, to bring biofuel cells from the lab-scale into practical application, in particular the cathode performance needs to be improved (Harnisch and Schröder, 2010; Schaetzle et al., 2009).

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A preferred oxidizer for biofuel cell cathodes is oxygen, because of its abundance and comparably high redox potential of 0.918 V vs. NHE at pH 5 and 30 °C (calculated according to (Logan, 2008)). Furthermore, the reaction product of the 4-electron reduction of oxygen is harmless H_2O (Ivnitski and Atanassov, 2007; Schaetzle et al., 2009). In this context, enzymatic catalysts such as bilirubin oxidases and laccases enable the reduction of O_2 at a lower overpotential compared to microorganisms (Rosenbaum et al., 2011) or even expensive noble metal catalysts such as platinum (Lapinsonniére et al., 2012; Mano et al., 2003; Soukharev et al., 2004), and therefore promise higher power densities. However, the drawback of using enzymatic electrodes is their short lifetime in the range of several weeks at best due to gradual deactivation and loss of catalytic activity (Rubenwolf et al., 2011). Furthermore, the prevailing use of purified enzymes is associated with technical efforts, and therefore additional costs (Lapinsonniére et al., 2012; Liu et al., 2014; Schaetzle et al., 2009).

In a previous publication, the use of crude culture supernatant of the white-rot fungus *Trametes versicolor* to supply the unpurified enzyme laccase to a biofuel cell cathode was successfully demonstrated (Sané et al., 2013). This operational strategy decreases time and costs needed for purifying enzymes. In addition, it was shown that by regular exchange of the crude culture supernatant it is possible to extend the lifetime of the cathode by at least 5-fold.

However, a disadvantage of using fungi as an enzyme source is that these organisms grow in cell agglomerates and therefore are comparably difficult to cultivate in a continuous manner. To continually provide enzymes to a biofuel cell cathode it is preferable to cultivate planktonic microorganisms such as yeasts and bacteria.

The aim of the present work is thus to explore, under which conditions planktonic enzyme-secreting microorganisms can be used to supply multicopper-oxidases to an oxygen reduction cathode via untreated culture supernatant. These investigations are an important step towards the realization of extended lifetime enzymatic fuel cells, in which enzyme-secreting microorganisms grow in an electrode-integrated micro-bioreactor to continually supply fresh enzymes. To supply multi-copper oxidases to a biofuel cell cathode the recombinant strains of the yeast *Yarrowia lipolytica YL4* and the bacterium *Escherichia coli*$_{CueO}$ constructed in the present work are compared.

The recombinant yeast *Y. lipolytica YL4* secretes laccase IIIb from the fungus *T. versicolor* (Jolivalt et al., 2005). The oxygen reduction potential of laccase from *T. versicolor* can be as high as 0.856 V vs. NHE at pH 5 and 30 °C (Sané et al., 2013) which makes laccase an attractive candidate as a cathode catalyst in biofuel cells (Lapinsonniére et al., 2012). When expressed by *Y. lipolytica*, laccase IIIb exhibits higher activity towards the redox indicator ABTS (2,2 Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) at acidic pH, and shows little to no activity at a pH of 6 and above (Madzak et al., 2006). Some electrochemical activity has been achieved under physiological conditions (pH 7.4) with the help of osmium-based redox polymers as mediators (Barrière et al., 2006).

The recombinant bacterium *E. coli*$_{CueO}$ was constructed to secrete the laccase-like enzyme copper efflux oxidase (CueO). Compared to laccase, CueO shows a higher overpotential towards oxygen reduction with an onset potential of oxygen reduction of around 0.649 V vs. NHE (0.450 V vs. Ag/AgCl (Miura et al., 2009)). However, in experiments with rotating disc electrodes it was found that CueO exhibits an about 9 times higher limiting catalytic current compared to laccase (Miura et al., 2007). Similar to laccase, CueO shows no activity towards ABTS at pH 7 (Supporting information S1), but reportedly exhibits electrochemical activity in the broad range from pH 2–8 (Tsujimura et al., 2008).

Both, laccase and CueO have the advantage of performing direct electron transfer. They do not require a mediator which shuttles electrons between the enzyme and the electrode (Christenson et al., 2004; Miura et al., 2009). In general, mediators can increase the overpotential (Harnisch and Schröder, 2010) and at the same time become an additional cost factor (Lapinsonniére et al., 2012). For both microorganisms, the influence of cultivation parameters on the enzyme activity in crude culture supernatant was investigated. Subsequently, the electro-catalytic activity towards the cathodic oxygen reduction was assessed by means of recording polarization curves for culture supernatant as well as complete cultures with live *E. coli* cells at pH 5.0 and pH 7.4.

2. Methods

2.1. Expression of laccase IIIb by the yeast Y. lipolytica

For the expression of laccase IIIb from *T. versicolor* the yeast strain *Y. lipolytica YL4* (Jolivalt et al., 2005) was used. The non-laccase-producing strain *Y. lipolytica Po1g* (Madzak et al., 2004), which was also used as the host for the heterologous laccase expression of *Y. lipolytica YL4*, served as control. Both strains were maintained on YPD (Yeast Extract Peptone Dextrose) agar plates (10 g/l yeast extract, 20 g/l vegetable peptones, 20 g/l agar, 0.1 M glucose monohydrate, Sigma–Aldrich, Taufkirchen, Germany).

A pre-culture (15 ml) was inoculated with *Y. lipolytica* from a YPD plate and cultivated overnight. For enzyme production, 50 µl of the pre-culture were transferred to 50 ml of liquid medium in a 100 ml baffled Erlenmeyer flask. The cultures of *Y. lipolytica* were shaken at 180 rpm and 30 °C.

For cultivation, YNB medium (yeast nitrogen based medium), containing 6.7 g/l yeast nitrogen base (Carl Roth, Karlsruhe, Germany), 50.5 mM glucose monohydrate, and 20 mM 2,2 dimethylsuccinic acid (both Sigma–Aldrich, Taufkirchen, Germany) was used. Furthermore, modified PPB medium (peptone phosphate buffered medium) according to (Jolivalt et al., 2005) and SCL medium (synthetic complete laccase medium) according to (Sané et al., 2013) were investigated. All media were supplemented with 1 mM $CuSO_4$ (Merck KGaA, Darmstadt, Germany) and the pH adjusted to a value of 5.0 with 10% acetic acid (Carl Roth, Karlsruhe, Germany).

Aliquots of the supernatants were collected daily to measure the enzyme activity towards ABTS (Sigma–Aldrich), as described in Section 2.3.

2.2. Cloning and expression of copper efflux oxidase by the bacterium E. coli

The multi copper efflux oxidase *CueO* is a periplasmic enzyme exported via the twin-arginine-transport machinery. In order to export the protein into the medium, the *CueO* gene was fused to the first 430 nucleotides of the *pulA* gene. *PulA* encodes for the pullulanase protein of *Klebisiella oxytoca*, an exoprotein that degrades the polysaccharide polymer pullulan. With the help of the Pul-secreton expressed from a second plasmid (pCHAP710) the fusion construct can be secreted into the medium (Francetic and Pugsley, 2005). Therefore, *CueO* was amplified using primer pBad_CueO_for (TCCA TACCCGTTTTTTTGGGCTAGAAATAATTTT-GTTTAACTTTAAGAAGGA-GATATACATACCATGCAACGTCGTGATTTCTT) and CueO_rev (GAA GAGGATCCGTTATCCATTACCGTAAACCCTAACATCA). The primers contain homologous regions to the pBAD plasmid and the 5′ region of the *pulA* gene, respectively. The first 430 nucleotides of *pulA* were amplified using primer *pulA*_for (ATGGATAACGGATCCTCTTC) and pBAD_*pulA*_rev (CAGGCTGAAAATCTTCTCTCATC-CGCCAAAACAGC CAAGCTGGAGACCGTTTTTAAAACGGTCTGGTCCCAGG). The amplified fragment contained sequences complementary to the pBAD plasmid. After digesting the plasmid vector using the restrictions enzymes PmeI and NcoI, the three fragments were combined in