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Article

A Hybrid Microbial-Enzymatic Fuel Cell Cathode Overcomes Enzyme Inactivation Limits in Biological Fuel Cells

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Abstract: The construction of optimized biological fuel cells requires a cathode which combines the longevity of a microbial catalyst with the power density of an enzymatic catalyst. Laccase secreting fungi were grown directly on the cathode of a biological fuel cell to facilitate the exchange of inactive enzymes with active enzymes with the goal of extending the lifetime of laccase cathodes. Additionally, a functionally graded coating was developed to increase enzyme loading at the cathode. Directly incorporating the laccase producing fungus at the cathode extends the operational lifetime of laccase cathodes while eliminating the need for frequent replenishment of the electrolyte. Additionally, the hybrid microbial-enzymatic cathode addresses the issue of enzyme inactivation by using the natural ability of fungi to exchange inactive laccases at the cathode with active laccases. Finally, enzyme adsorption was increased through the use of a functionally graded coating containing an optimized ratio of titanium dioxide nanoparticles and single walled carbon nanotubes. The hybrid microbial-enzymatic fuel cell combines the higher power density of enzymatic fuel cells with the longevity of microbial fuel cells and demonstrates the feasibility of a self-regenerating fuel cell in which inactive laccases are continuously exchanged with active laccases.

Keywords: Oxygen reduction reaction; Multi-functional catalysts for ORR; Bioelectrocalysis; Biocathodic microbial communities; Optimization of catalyst layers and electrode design

1. Introduction

Biological fuel cells (BFCs) use bioderived fuels and catalysts to produce electricity. Most BFCs utilize the oxygen reduction reaction at the cathode, thus BFCs can be categorized by the type of catalyst used at the cathode: (I) metal catalysts, (II) enzymatic catalysts, and (III) microbial catalysts[1–5]. Platinum-group metal catalysts are most common due to their superb catalytic properties, but the high price of the catalysts prevent large-scale adoption of biological fuel cells. Enzymatic catalysts hold promise to replace metal catalysts but suffer from short lifetimes and mass transport limitations[1,3,4,6–8]. Microbial fuel cells rectify the short lifetimes of enzymatic catalysts at the expense of additional mass transport limitations and lower power densities[2,9–11]. The construction of optimized biological fuel cells requires a cathode which combines the longevity of a microbial catalyst with the power density of an enzymatic catalyst.

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Laccases are commonly used enzymatic catalysts in biological fuel cell cathodes because they demonstrate reversible adsorption to carbon electrodes and an ability to directly transfer electrons to carbon nanotubes[12,12–15]. Laccases catalyze the one-electron oxidation of diverse chemical substrates at a single-copper containing site near the surface of the protein[16]. Concomitantly, laccases catalyze the four-electron reduction of dioxygen to water at a tri-copper site in the interior of the protein without the production of superoxides or peroxides[17]. Despite their promise, laccase cathodes are plagued by short enzyme lifetimes, suboptimal enzymatic reaction velocities, enzyme inactivation, and low enzyme adsorption[6,15,18–22]. Many attempts have been made to engineer laccases with faster reaction velocities, but additional work is required to address losses from these other factors[23,24]. Filamentous fungi overcome short laccase lifetimes and laccase inactivation in nature by secreting laccases to catalyze the breakdown of lignin[16,25,26]. Fungi address the problem of laccase inactivation via specialized endocytic pathways which facilitate the absorption of inactive enzymes for breakdown and reuse[18–20,25].

This work presents a hybrid microbial-enzymatic cathode leveraging fungal metabolism to overcome the short enzyme lifetimes associated with pure laccase cathodes (Figure 1). Five different filamentous fungi were screened for their ability to produce extracellular laccases. Three inducers were analyzed to maximize laccase secretion while maintaining the medicinal and/or nutritional value of the resulting fungal biomass. To increase enzyme loading at the cathode, a functionally graded coating was applied to the cathode prior to introducing the fungus. The selected fungus was grown directly on the cathode to secrete extracellular laccases and facilitate the exchange of inactive enzymes with active enzymes to extend power production.

Figure 1

2. Results

2.1. Assessment of Laccase Secretion

The ability of five different fungi to secrete enzymatically active laccases were compared with and without induction (Figure 2). In the absence of a laccase inducing substance all tested fungi produced less than 10 U*mL-1 of laccase. When copper sulfate, a common laccase inducer, was added all fungi increased the amount of extracellular laccase. In 200 mL culture bottles the *Pleurotus ostreatus* cultures demonstrated the highest extracellular laccase activity. The genetic differences between the subspecies of *P. ostreatus* had a notable effect on extracellular laccase production with the commercial strain *P. ostreatus* N001 producing the least active extra cellular laccases on day 4 and the in-house cultured *P. ostreatus* white producing the highest.

Figure 2

When smaller reaction vessels (20 mL) were compared to larger reaction vessels (200 mL) *Fusarium oxysporum* and *Trametes versicolor* produced extracellular laccases with the highest activity (Figure 3). The high activities of extracellular laccases produced by *F. oxysporum* are likely due to *F. oxysporum*'s role as a plant pathogen while the high activity of cellular laccases produced by the *T. versicolor* and the *P. ostreatus* strains can be attributed to their roles as a lignin degrading saprotrophs. Despite the ecological relevance of laccases to all tested species it remains unclear why smaller culturing vessels significantly improved the activity of secreted laccases in *F. oxysporum* and *T. versicolor* cultures while having less of an influence over *P. ostreatus* cultures. The 20 mL setup was used for all subsequent studies as it provided higher laccase activity, enough biomass for analysis, and was more manageable for subsequent trials.

Figure 3

2.1.1. Inducing Laccase Secretion with a Biocompatible Inducer

Trametes versicolor has medicinal properties that make the biomass a useful byproduct of this biological fuel cell [27]. The use of copper sulfate as an inducer leads to concerns of heavy metal

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sequestration by *T. versicolor* precluding its use for medicinal purposes. We explored Tween 20 as an alternative laccase inducer as the detergent causes oxidative stress to the fungus and is non-toxic to humans and animals.

Figure 4

When copper sulfate, Tween 20, and a mixture of the two were compared, it was discovered that the extracellular laccase activity of all tested fungi was significantly higher in copper sulfate induced cultures than in Tween 20 induced cultures (Figure 4). However, the small reduction in electrical production could be offset by the benefits of a valuable byproduct in edible or medicinal biomass that is not contaminated with heavy metal inducers.. No significant differences between biomass were observed between any of the treatments. Copper sulfate was chosen as the inducer for the proteomics study as the higher activity of extracellular laccases was desirable for electrochemical studies.

2.2. Trametes versicolor Secretome Analysis

The secretome of *T. versicolor* was analyzed for protein composition under copper sulfate induction and compared to an uninduced culture. Of particular interest are laccases, peroxidases and proteases. Laccases facilitate the four-electron reduction of oxygen to water while peroxidases facilitate the two-electron reduction of oxygen to peroxide which poisons the cell and can kill the fungus and inactivate functional laccases. Proteases hydrolyze the peptide bonds between amino acids in a protein rendering the protein inactive and the amino acids useable for metabolism or protein synthesis.

Copper sulfate induction raised the laccase composition of the secretome 21%, from 33% to 54% (Figure 5). Additionally, the peroxidase composition of the secretome was decreased by 8%, to 14% of total protein. Proteases were not found in abundance in the uninduced culture but comprised 1% of the total protein in the copper sulfate induced culture. The small number of proteases supports our hypothesis that *T. versicolor* is able to break down and recycle inactive laccases in situ, without disturbing active laccases at the electrode surface. In addition to the proteins of interest, glucose oxidase and glycosidase enzymes were detected in the *T. versicolor* secretome. These metabolic enzymes do not prohibit the laccase function at the cathode but can occupy sites otherwise usable by laccases for oxygen reduction.

Figure 5

2.3. Comparison of Electrode Coatings in Terms of Electrode Surface Area

The carbon cathodes used in this study do not provide a large enough surface area to demonstrate an increase in power density due to the recycling of laccase at the cathode. We therefore developed a functionally graded cathode coating with the goal of improving enzyme loading on the cathodes while facilitating the time-dependent change of active laccases adsorbed to the electrode. An optimized ratio of 0.2% titanium dioxide nanoparticles to 0.7% carbon nanotubes (w:w) was found to increase the laccase loading, open circuit potential, and power density of the tested cathodes (Table 1).

Table 1

At lower carbon nanotube loading the number of direct electron transfer sites accessible to laccases is insufficient to promote efficient oxygen reduction resulting in a lower open circuit potential and low power density (Figure 6). However, at higher CNT loading the nanotubes form aggregates significantly diminishing the effective surface area of the coating. A scanning electron micrograph of the optimized coasting is depicted in Figure 7.

Figure 6

2.4. Comparison of Electrochemical Properties with and Without Enzyme Regeneration

Figure 7

The power density of pure laccase in sterile electrolyte was 20% higher than that of the crude laccase from the *T. versicolor* culture (Figure 8a). Incorporating the fungus at the cathode lowered the open circuit potential of the pure laccase to 244 mV, but raised the power density and open circuit

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potential of the crude laccase treatment (Figure 8b). The higher performance of crude filtrate over pure laccase is in agreement with the findings of Sane et al. (2013) [20]

Figure 8

Forty-eight hours after introducing electrolyte to the fuel cells linear sweep voltammetry was performed again. In cultures without *T. versicolor* (Figure 8c) open circuit potential and power density were significantly diminished. However, in cultures with *T. versicolor* growing on the cathode, current density and open circuit potential were aligned (Figure 8d). Interestingly, the performance of the media control, containing no laccase at initiation, was "rescued" by the presence of the fungus. The higher bioelectrocatalytic activity of the electrodes indicates that active laccases produced by the fungus are successfully exchanged with inactive laccases previously adsorbed to the cathode.

3. Discussion

Biological fuel cells continue to gain recognition as a solution to global energy challenges due to their multifunctionality [2]. However, neither enzymatic nor microbial cells have achieved commercial viability due to the shortcomings of brief lifetimes, poor power density, limited enzymatic lifetimes, and poor catalyst loading. The hybrid microbial-enzymatic fuel cell combines the higher power density of enzymatic fuel cells with the longevity of microbial fuel cells. Additionally, the hybrid microbial-enzymatic fuel cell demonstrates the feasibility of a self-regenerating fuel cell in which an enzyme-producing microbe is incorporated in the electrode by continuously recycling inactive laccases to active laccases.

In previous work, laccase cathode lifetimes were extended by periodic exchange of laccase-containing electrolyte without the enzyme-producing microbe in the electrode [5,19,20] . This proof-in-concept work demonstrated the feasibility of using unpurified fungal culture broth as an electrolyte in a laccase-based cathode, but the manual replenishment of the laccase-containing electrolyte limited the device's application space. Devices which had grown a fungus in the cathode chamber utilized yeast mediators to facilitate electron transfer between laccase and the cathode which is not viable for commercial applications [6,15,21,22,28**?** ,29]. Directly incorporating the laccase producing filamentous fungus at the cathode overcomes the limitations imposed by the short lifetime of laccases while eliminating the need for frequent replenishment of the electrolyte or mediator. Additionally, the hybrid microbial-enzymatic cathode addresses the issue of enzyme inactivation by using the natural ability of filamentous fungi to exchange inactive laccases with active laccases. Finally, enzyme adsorption was increased through the use of a functionally graded coating containing titanium dioxide nanoparticles and single walled carbon nanotubes.

The production of edible and medicinal biomass was used as the metric to analyze secondary benefits of this system. *T. versicolor* has shown promise in boosting the immune response in cancer patients, increasing lymphocyte counts, natural killer cell functional activity, CD8+ T cell counts, and CD19+ B cell counts [30]. These benefits have come from taking capsule of freeze-dried fungal biomass produced for the sole purpose of consumption. *Pleurotus ostreatus* is a gourmet mushroom with highly sought-after taste and high mineral content [27]. The increasing adoption of plant-based proteins provides a growing market for *P. ostreatus* biomass produced in the hybrid microbial-enzymatic cathode. The harvesting of fungal biomass for edible and medicinal purposes represents a new area of high-profit secondary benefits that should be explored as an avenue to amend the functionality of microbial fuel cells. Fungi are also adept at heavy metal sequestration and waste remediation presenting additional opportunities to customize and combine secondary benefits based on application [31,32].

The various ecological roles played by filamentous fungi in nature allow the hybrid microbial-enzymatic fuel cell to be tuned to provide a variety of secondary and tertiary benefits during operation. Future research will explore the use of additional fungi and the genetic and culture conditions that can increase the laccase composition of the secretome in fungi of interest and reduce

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the number of detrimental (peroxidase) and metabolic (glucose oxidase and glycosidase) enzymes in the extracellular environment.

4. Materials and Methods

Unless otherwise stated, all chemicals were of reagent grade and purchased from Sigma Aldrich (St. Louis, MO). Bacto yeast extract was obtained from Fisher Scientific (San Diego, CA); *T. versicolor* laccase standard (13.6 U/mg) was purchased from MyBioSource (Cerritos, CA). *P. ostreatus* var. pearl, *P. ostreatus* var. blue, and *T. versicolor* were purchased from Fungi Perfecti (Olympia, WA); *P. ostreatus* var. N001 was purchased from American Type Culture Collection (http://www.atcc.org/ /ps/32783.ashx); *F. oxysporum* f.s. *lycopersici* was obtained from Dr. Hans VanEtten. Five hundred mL media bottles and 50 mL miniature bioreactors were purchased from Dow Corning (Midland, MI); Single wall carbon nanotubes (95 wt%, OD: 10-20 nm, ID: 5-10 nm, L: 10-30 µm) were provided by Dr. Mike Foley. Spectroscopic carbon rods were purchased from Ted Pella Inc (Redding, CA). PTFE heat shrink tubing was purchased from McMaster Carr Inc. (Elmhurst, IL). Difco yeast extract was purchased from Fisher Scientific (Waltham, MA).

4.1. Fungal Strain Maintenance

All fungi were maintained on 100 mm X 15 mm petri dishes containing glucose yeast extract agar (GYEA) composed of 10 g/L glucose, 10 g/L yeast extract, 15 g/L agar. Ten mL of Acidified Glucose Yeast Extract Broth at pH 4.5 (10 g/L glucose, 10 g/L yeast extract, 1 g/L succinic acid) was dispensed onto a 7-day-old culture to create pre-inoculum. The mycelium was dislodged from the plate using a 10mL wide-bore pipette and the resulting slurry was added to 190 mL of AGYEB in a sterile 500mL reagent bottle. The reagent bottles were kept shaking at 250 rpm and 27 C for 7 days. The resulting inoculum was used at 10% volume-to-volume ratio to inoculate all experimental cultures. Experimental culture conditions were as follows: 500 mL media bottles or 50 mL bioreactors were filled were with 200 mL or 20 mL AGYEB, respectively. Cultures were either kept shaking at 250 rpm or 0 rpm. All fungi were grown at 27 C for the predetermined time course. After 72 hours of growth one of three inducers was added aseptically to promote laccase secretion. After the appropriate time course, cultures were filtered through pre-weighed cheesecloth, then cell free filtrate was collected using 0.45 um low-protein binding cellulose acetate filters.

4.2. Laccase Activity

Laccase activity was determined spectrophotometrically using a Bio Tek Synergy II microplate reader with microinjector [33]. 100 uL of sample, standard, or blank was pipetted into the corresponding well, then 100 uL of 2 mM ABTS (2,20-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) was injected into each well of the plate and absorbance was read at 410 nm continuously after injection. The reaction was allowed to proceed for 10 minutes at 27 C under the medium shaking condition. Data reduction was performed according to the standard fluorometric protein quantification parameters in the Gen5 software provided with the reader.

4.3. Dry Biomass

After straining, the fungal biomass and the cheesecloth were placed into beakers, frozen at -80 C, and lyophilized under a 200 militorr vacuum at -60 C. Dry biomass was determined by weighing the biomass-cheesecloth combination and subtracting the weight of the cheesecloth.

4.4. Total Protein

Bradford assay was performed for total protein quantification [34]. Briefly, one part cell-free filtrate was used to one part Bradford reagent, and absorbance was monitored at 595 nm after 10 minutes.

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4.5. Proteomics

Cell-free filtrates for proteomics were prepared as before, then passed through 0.22 um low-protein binding syringe filters. The resulting solution was ultrafiltered using an Amicon stirred cell concentrator [35] . After ultrafiltration, the concentrated protein solution was dialyzed against 50mM ammonium bicarbonate pH7.5. Finally the dialysates were frozen until they were sent to the University of Arizona's proteomics core facility. Tandem mass spec analysis was performed using a LTQ Orbitrap Velos mass spectrometer (Fisher Scientific, Waltham, MA) equipped with a nano electrospray ion source; the instrument was operated in data-dependent acquisition (DDA) mode. Raw data was processed using Scaffold Proteome Software.

4.6. Culture Conditions

T. versicolor was, obtained from Fungi Perfecti, Olympia, WA. Cultures were maintained on 100 mm X 15 mm petri dishes containing glucose yeast extract agar (GYEA) composed of 10 g/L glucose, 10 g/L yeast extract, 15 g/L agar. Inoculum was created by dispensing 10 mL of pH 4.5, acidified glucose yeas extract broth (AGYEB), composed of 10 g/L glucose, 10 g/L yeast extract, and 20 mM succinic acid, was dispensed onto a 7-day-old culture to create pre-inoculum. The mycelium was dislodged from the plate using a 10 mL wide-bore pipette and the resulting slurry was added to 190 mL of AGYEB in a sterile 500 mL reagent bottle. The reagent bottles were kept shaking at 250 rpm and 27 C for 7 days. The resulting inoculum was used at 1% volume-to-volume ratio to inoculate all experimental cultures. Experimental cultures were shaken at 250 rpm and 27 C for 72 hours when 1 mM copper sulfate was added aseptically to induce laccase secretion. After 96 hours of growth cultures were prefiltered through cheesecloth, and cell free filtrate was collected using 0.45 um milipore filters.

4.7. Titanium Dioxide Nanoparticle Synthesis

Tetramethylammonium hydroxide was added to titanium isopropoxide in a 1:1 ratio, then 3 parts milipure water was added, and the mixture was stirred at 95 C for 2 hours. After two hours the solution was peptized at 70 C for 48 hours. The resulting solution was passed through a 0.2 um filter and 1 mL aliquots were placed in a roto evaporator to determine the final concentration of nanoparticles. The stock solution was diluted to 20 weight-percent using milipure water and the resulting working solution stored at 2 C.

4.8. Dip Coating

The tops of the working electrodes were passivated using PTFE heat shrink tubing. Surface area was normalized to 10 cm2 after modification with PTFE. Dip coating solutions of 0.9 weight percent were created with varying amounts of titanium dioxide and CNT and cured at 120 C for three minutes. This process was repeated three times; a constant pull rate of 2 mm/s was used for all coatings. The electrodes were then dip coated in 0.1% Nafion under sterile conditions at the same pull rate. For microbe-containing coatings, functionalized electrodes were placed on 1 mm X 3 mm round carbon supports in the 20 mL bioreactors and fungi were allowed to colonize for two days. The electrodes were then dip coated in 0.1% Nafion under sterile conditions at 2 mm/s pull rate.

4.9. Linear Sweep Voltammetry

All electrochemical measurements were carried out on a biologic VSP potentiostat with a saturated calomel reference electrode and a 10 cm X 0.5 cm carbon counter electrode. Functionalized 0.32 cm X 10 cm spectroscopic carbon rods were used as working electrodes. The catholyte was stirred for 10 minutes under air before open circuit potential was determined and linear sweep voltammetry began. Catholytes were either sterile AGYEB (media), crude T. versiolor filtrate (crude), or sterile AGYEB spiked with purified *T. versicolor* laccase (pure).

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