Bioelectrochemistry 112 (2016) 53–60

Contents lists available at ScienceDirect

Bioelectrochemistry

journal homepage: www.elsevier.com/locate/bioelechem

Yeast surface display of dehydrogenases in microbial fuel-cells

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ARTICLE INFO

Article history:
Received 15 March 2016
Received in revised form 17 July 2016
Accepted 17 July 2016
Available online 19 July 2016

Keywords:
Cellobiose dehydrogenase
Pyranose dehydrogenase
Yeast surface display
Microbial fuel cells
Enzymatic fuel cells

ABSTRACT

Two dehydrogenases, cellobiose dehydrogenase from *Corynascus thermophilus* and pyranose dehydrogenase from *Agaricus meleagris*, were displayed for the first time on the surface of *Saccharomyces cerevisiae* using the yeast surface display system. Surface displayed dehydrogenases were used in a microbial fuel cell and generated high power outputs. Surface displayed cellobiose dehydrogenase has demonstrated a midpoint potential of −28 mV (vs. Ag/AgCl) at pH = 6.5 and was used in a mediator-less anode compartment of a microbial fuel cell producing a power output of 3.3 μW cm^{-2} using lactose as fuel. Surface-displayed pyranose dehydrogenase was used in a microbial fuel cell and generated high power outputs using different substrates, the highest power output that was achieved was 3.9 μW cm^{-2} using D-xylose. These results demonstrate that surface displayed cellobiose dehydrogenase and pyranose dehydrogenase may successfully be used in microbial bioelectrochemical systems.

1. Introduction

Biofuel cells are electrochemical devices that spontaneously convert chemical energy into electrical energy using biochemical pathways and redox enzymes as the biocatalysts. Biofuel cells can be classified into enzymatic fuel cells (EFCs) and microbial fuel cells (MFCs) that use either isolated redox enzymes or whole living microorganisms as their respective biocatalysts [1,2]. EFCs can only partially oxidize the fuel and have limited lifetime owing to relatively short term enzyme stability, while MFCs are typically stable for longer periods and can catalyze full oxidation of different fuels by using the microorganism's entire metabolism for power production [3,4]. Nevertheless, the major factor influencing power production in MFCs relies on the biocatalyst ability to communicate efficiently with the electrode [5]. Hence, it is important to optimize the microorganism itself, using genetic engineering tools, for energy production in order to improve electrobiocatalysis in MFCs [6].

Electrons can flow between the biocatalyst active site and the electrode either by mediated electron transfer (MET) using small redox active molecules, redox mediators, or by direct electron transfer (DET), where the biocatalyst is able to communicate directly with the electrode. DET possesses some important advantages over MET. First, mediators are often toxic and their use leads to potential losses arising from the potential difference between the redox potential of the enzyme active site and that of the mediator. Second, a mediator-less system is less prone to interfering reactions. Third, DET allows the possibility of modulating the desired properties of the system by protein modification using genetic and chemical engineering tools [7,8].

In our recent studies, an anode containing glucose oxidase (GOx) from *Aspergillus niger* displayed on the surface of *Saccharomyces cerevisiae* (*S. cerevisiae*), using D-glucose as a fuel, was described [9–11]. The surface-displayed GOx allows bypassing of the cellular membrane [12], while offering the possibility of in situ regeneration of the active enzyme by the living organism [13]. Although GOx has been widely used in biofuel cells, it still has a number of caveats in bio-fuel cells (BFCs) applications. First, GOx belongs to a group of redox enzymes that harbor a catalytic center buried deep inside the protein matrix [14]. This prevents DET between the enzyme and the electrode. Second, GOx is limited to the oxidation of only one possible anomeric form of glucose (β). That means that it may utilize only up to 64% of the total soluble glucose content in aqueous solutions [15]. Third, GOx oxidizes glucose only at its C-1 position, releasing only two electrons per one molecule of glucose, resulting in low Coulombic efficiency. Fourth, GOx produces hydrogen peroxide in the presence of oxygen, allowing oxygen to compete continuously with mediators in the solution and eventually leading to a decrease in Coulombic efficiency.

Cellobiose dehydrogenase (CDH, EC 1.1.99.18) is an extracellular sugar oxidoreductase produced by various wood degrading fungi [16]. CDH is a monomeric N-glycosylated peptide which has a distinguishable structure as the only known extracellular flavocytochrome. CDH is composed of two domains, the small cytochrome domain (CYT), carrying a heme b redox cofactor, which is located in the N-terminus and connected by a long flexible linker to the flavodehydrogenase domain (DH), where a flavin adenine dinucleotide (FAD) is used as the redox cofactor [17]. CDH can oxidize the β anomers of mono-, di- and oligosaccharides such as cellobiose, lactose, maltose and glucose on their C-1 position, with a preference towards cellobiose and lactose [18,19]. In

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http://dx.doi.org/10.1016/j.bioelechem.2016.07.006
1567-5394/

the saccharide oxidative reaction, two electrons are obtained in the DH domain and can be transferred to a two electron acceptor only by the DH domain or to a one electron acceptor by either the DH or the CYT domain after an internal electron transfer (IET) from the DH domain to the CYT domain [20]. In addition, CDH can transfer the electrons directly to an anode through DET using the CYT domain, which acts as a built-in mediator and eliminates the need for soluble redox mediator in bioelectrochemical systems [21,22]. CDH from *Corynascus thermophilus* (*Ct*CDH) is a very attractive dehydrogenase, which can operate under a wide pH range, with pH optima of the DH domain between 5.0 and 6.0 and pH optima of CYT domain at 7.5 with a broad activity peak over the range of pH 6.0–9.0. The latter indicating that IET between DH and CYT domains is considerably favored at neutral pH, which is an important trait for future applications such as implantable devices [20,23].

Pyranose dehydrogenase (PDH, EC 1.1.99.29) is an extracellular sugar oxidoreductase that can be found in "litter-decomposing" fungi which grow mostly on lignocellulose-rich forest litter [24]. The native enzyme is a monomeric glycosylated polypeptide with one covalently bound FAD molecule acting as the redox cofactor. PDH has a broad substrate tolerance due to its excellent ability to oxidize different aldopyranoses including mono-, di- and oligosaccharides [15]. PDH can oxidize its substrate at the C-1, C-2 or C-3, as well as a double oxidation at C-1,2, C-2,3 and C-3,4 by acting on the CH–OH groups of non-phosphorylated sugars, converting them to the corresponding aldonolactones (C-1), 2/3-dehydro sugars or di-dehydro sugars [15,25]. PDH ability for double oxidation increases current densities by gaining four electrons per one molecule of substrate [15] thus has a potential to increase MFCs power outputs. In addition, PDH lacks an anomeric specificity which can increase the number of oxidized substrate molecules in a solution, improving oxidation reaction efficiency. The preferred saccharides of PDH from *Agaricus meleagris* (*Am*PDH), with the highest catalytic efficiencies, are D-glucose (double oxidation), D-galactose (C2 oxidation), L-arabinose (C2 oxidation), D-xylose (double oxidation) and cellobiose [26–28]. *Am*PDH has a broad pH stability between 4.0 and 10.0, with maximum stability at pH 7.0 [26]. It is an important trait for applications such as biofuel cells proposed for power generation in remote locations.

Herein, we wanted to characterize the activity of two novel surface-displayed dehydrogenases in MFCs. *Ct*CDH and *Am*PDH displayed on the surface of *S. cerevisiae*, using the a-agglutinin yeast surface display (YSD) system [29,30]. Schematic 1 describes the two different surface displayed enzymes used in our system. *S. cerevisiae* displaying *Ct*CDH were used in a mediatorless anode compartment of an MFC. *S. cerevisiae* displaying *Am*PDH were used in an MFC, fed with different sugars as fuels while methylene blue (MB) was used as the redox mediator. The ability of *Am*PDH to oxidize various fuels while still performing double oxidation, enables an improved MFC performance while rendering the MFC more suitable for wastewater treatment applications.

2. Materials and methods

2.1. Materials and reagents

*Ct*CDH and *Am*PDH genes originated from *C. thermophilus* and *A. meleagris*, respectively. Both genes were cloned into a YSD vector and transformed to *S. cerevisiae* (Strain EBY100) yielding *Ct*CDH modified *S. cerevisiae* and *Am*PDH modified *S. cerevisiae* strains. 2,6 dichlorophenol-indophenol (DCIP), Cyt c, ferricenium hexafluorophosphate ($Fc^+PF_6^-$), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), methylene blue (MB), D-xylose, L-arabinose and lactose were purchased from Sigma-Aldrich (Rehovot, Israel). D-glucose and D-cellobiose were purchased from Chem-Impex international Inc. (Wood dale, USA) and D-galactose was purchased from Acros Organics (Geel, Belgium). Dionized water (DW) were purified using a milli-Q water system (18.2 MΩ cm, Millipore, Bedford, MA) to result in DDW.

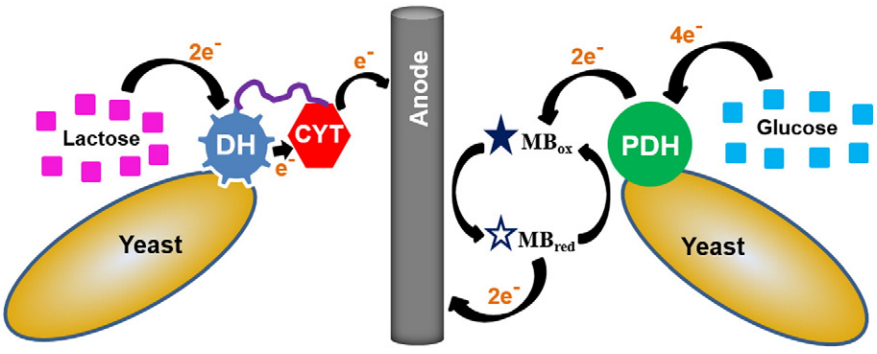
2.2. Biochemical activity assays

2.2.1. CtCDH separation from S. cerevisiae

*Ct*CDH transformed *S. cerevisiae* were induced by standard induction protocols (SI section), concentrated to an $OD_{600} = 2.0$ and re-suspended in 0.1 M acetate buffer, pH 5.5. 20.0 mL of *S. cerevisiae* expressing *Ct*CDH were treated with 200 mM β-Mercaptoethanol (Biorad) and protease inhibitor set IV-for yeast (Calbiochem) at 40 °C for 2.5 min. Samples were precipitated right away at 4 °C for 20 min and the supernatant was filtered using a 0.45 μm PVDF filter (Millex-HV) followed by 4 centrifuge cycles with a 50 kDa cutoff VIVASPIN 20 filter (Sartorius Stedim Biotech GmbH, Goettingen, Germany) at 4 °C for 15–20 min. After each cycle samples were re-suspended in acetate buffer 0.1 M pH 5.5. Afterwards, 3.5 kDa cutoff cellulose tubular dialysis membranes (Membrane Filtration Products, Seguin, TX, USA) were filled with samples and placed in a stirred 1 L beaker, containing acetate buffer 0.1 M pH 5.5, at 4 °C for 24–48 h. The beaker buffer was replaced, and incubated again for 24–48 h, after which samples were collected for *Ct*CDH activity assay. This procedure was based on previously reported protocols [31–34].

2.2.2. CtCDH activity assay

*Ct*CDH activity was determined using a modified procedure by spectrophotometrically following the lactose-dependent reduction of DCIP to $DCIPH_2$ at 520 nm ($\varepsilon 520 = 6.8\ mM^{-1}\ cm^{-1}$) for 1 h at 37 °C [20,23,35]. 100 μL of each sample were transferred to a flat-bottom 96-well plate (Nunclon Delta Surface, Thermo Scientific, Denmark) in triplicates. To each sample, 100 μL of 0.6 mM DCIP and 60 mM Lactose (Sigma-Aldrich) in 0.1 M acetate buffer (pH 5.0) were added. Absorbance measurements at 520 nm were taken every 1 min using a microplate reader (BioTek Instruments, Winoosky, VT, USA), and a plot of the



Schematic 1. Schematic presentation of *Ct*CDH and *Am*PDH MFCs anodes. Left: mediator-less MFC anode containing *S. cerevisiae* surface displaying *Ct*CDH. Right: MFC anode containing *S. cerevisiae* surface displaying *Am*PDH.