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Ethanol generation, oxidation and energy production in a cooperative bioelectrochemical system

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ABSTRACT

Integrating in situ biofuel production and energy conversion into a single system ensures the production of more robust networks as well as more renewable technologies. For this purpose, identifying and developing new biocatalysts is crucial. Herein, is reported a bioelectrochemical system consisting of alcohol dehydrogenase (ADH) and *Saccharomyces cerevisiae*, wherein both function cooperatively for ethanol production and its bioelectrochemical oxidation. Here, it is shown that it is possible to produce ethanol and use it as a biofuel in a tandem manner. The strategy is to employ flexible carbon fibres (FCF) electrode that could adsorb both the enzyme and the yeast cells. Glucose is used as a substrate for the yeast for the production of ethanol, while the enzyme is used to catalyse the oxidation of ethanol to acetaldehyde. Regarding the generation of reliable electricity based on electrochemical systems, the biosystem proposed in this study operates at a low temperature and ethanol production is proportional to the generated current. With further optimisation of electrode design, we envision the use of the cooperative biofuel cell for energy conversion and management of organic compounds.

1. Introduction

The development of electrochemical devices based on biomolecules for use as more environmentally favourable alternative energy resources has been extensively studied [1–3]. These systems have many potential applications as amperometric biosensors [4], implantable biodevices for power generation and biofuel cells (BFCs) [5–8]. The first study in the literature on energy production using microorganisms was reported by Potter [9], in which the author quoted, 'the disintegration of organic compounds by microorganisms is accompanied by the liberation of electrical energy'. This study provided a simple example of energy production by microbial fermentation of glucose media and later, it resulted in the development of a new class of biodevices known as BFCs. BFCs are electrochemical biodevices that utilise biological entities, such as enzymes and/or microorganisms for producing electrical energy. These devices are considered one of the most promising energy converters because they can use fuels produced from biomass [1,10–14].

Yeasts, bacteria, and other microorganisms can be potentially used as bioelectrocatalysts. However, the problems concerned with the use of BFCs that utilise the chemical pathways of living cells include low volumetric catalytic activity of the microorganisms and low power densities due to slow mass transport of fuel across the cell wall [15]. Despite the promising features of BFCs, it is still necessary to establish systems that produce and electrochemically regenerate the materials used as fuel, in order to develop an efficient bioelectrode. Thus, the development of an economically viable and thermodynamically favourable device with high power density, which integrates the complete microbial oxidation of fuel and the formation of product by enzymatic catalysis into a single system consisting of enzymes and microorganisms, is expected.

The high catalytic activity and selectivity of enzymes make them attractive for use in BFCs [15]. Dehydrogenases are responsible for the oxidation of specific substrates, such as alcohols and aldehydes and are used as biological catalysts at bioanodes of BFCs [2,16]. Alcohol dehydrogenase (ADH) (EC 1.1.1.1) is a member of a large family of zinc-containing dehydrogenases. ADH of *Saccharomyces cerevisiae* is most active with ethanol than other substrates and it was one of the first enzymes to be isolated and purified [17,18]. It is a homotetramer with a molecular weight of 150 kDa and is composed of four identical subunits and 347 amino acid residues [19]. ADH catalyses the oxidation of ethanol to acetaldehyde with the reduction of nicotinamide adenine dinucleotide (NAD^+) to NADH, which are the key electron carriers in living cells [20]. Also, NAD^+ is an important coenzyme that participates in the reactions catalysed by many dehydrogenases [20,21].

A hybrid system that integrates enzymes and microorganisms has been proposed [22], for instance, the lactate/air BFC with a microbial anode containing *Shewanella* MR1 and the laccase air-breathing cathode. The reactions occurred by direct electron transfer between the two compartments of the enzymatic-microbial fuel cell. The flow-through microbial anode consumed lactate that was generated during

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metabolism in *Shewanella* and it was compatible with the cathode, where both can successfully function in the 'power generation' mode for several days with no apparent performance decay. The critical advantage of the combination of enzymes and microorganisms is the flexibility that it offers to the design of BFCs. Another report [23] described a BFC containing *S. cerevisiae* cells with glucose oxidase (GOx), which was immobilised on their surfaces, used as the biocatalyst for the oxidation of glucose. In this case, GOx catalysed the redox reactions with high efficiency when it was immobilised on the yeast-cell surfaces and hence, the power densities of the BFCs with GOx immobilised on the surfaces of the yeast cells were much higher than those that used purified GOx or unmodified yeast. Furthermore, enzymatic BFCs suffer a major disadvantage with respect to long-term use, which is the loss of enzyme activity. Therefore, a system in which the enzyme can be regenerated would be beneficial.

Herein, we introduce the concept of cooperative BFCs, wherein *S. cerevisiae* is employed as a cooperative biocatalyst for the oxidation of glucose to ethanol and the further oxidation of ethanol to acetaldehyde via the loss of 2 electrons is catalysed by ADH in a cascade of reactions. The cooperation between the two biocatalysts was evaluated in order to demonstrate the catalytic activity from the oxidation of ethanol, which is produced upon yeast fermentation under anaerobic conditions using glucose as the substrate. The main pathway involved in the energy metabolism of yeast is anaerobic glycolysis, whereby glucose is oxidised to produce ethanol, which is the major final glycolytic product and the substrate for ADH, concurrently. The oxidation of ethanol to acetaldehyde occurs with the concomitant reduction of NAD^+ to NADH. Also, the use of *S. cerevisiae* as a biocatalyst is advantageous, since it is a microorganism whose metabolism is well understood with good biological condition at room temperature and it has the ability to survive in aerobic and anaerobic environments. Furthermore, *S. cerevisiae* has been one of the most popular microorganisms traditionally employed in industrial fermentation, mainly for the production of alcoholic beverages [24]. It is also used for metabolic engineering studies and metabolomics of biological systems [25,26].

The catalysis of the oxygen reduction reaction (ORR) by bilirubin oxidase (BOD) via the four-electron reduction pathway from oxygen to water at the biocathode was considered [27,28]. BOD is an attractive enzyme, since it catalyses the ORR with high efficiency at neutral pH without the need for redox mediators, it is not inhibited by chloride ions and it has high thermal tolerance [29]. For developing a cooperative BFC with *S. cerevisiae* and ADH at the bioanode, we chose to employ BOD at the biocathode, not only because of the reasons mentioned previously, but also since the optimum pH of this enzyme for ORR is compatible with that of ADH and the optimum conditions for yeast growth.

The proposed system has some advantages, which include that fact that ethanol shows great promise for use as a renewable energy source in BFCs due to its low toxicity and desirable physicochemical properties, as a high energy density [30,31]. Also, ethanol is attractive as an alternative fuel, since it is widely available and it can be produced in large quantities through microbial fermentation of agricultural products [31]. This study allows that the ethanol generated from the fermentation process by yeast could be used as a fuel in an enzymatic reaction. Thus, the ethanol is utilised as substrate to produce electrical energy in the cooperative BFC employing just one enzyme as biocatalyst on the anode. Furthermore, the anodic reactions of this device generate products with wide industrial application. The acetaldehyde produced from the reaction of ethanol oxidation catalysed by ADH, for example, is an important intermediate in the synthesis of other compounds in chemical industries and it is used especially in the manufacture of acetic acid and its derivatives.

2. Methods and materials

2.1. Materials

S. cerevisiae was obtained commercially from Fleischmann® (Pederneiras, São Paulo, Brazil) and used without pretreatment. ADH from *S. cerevisiae* (EC 1.1.1.1) was used without further purification; absolute ethanol (extra pure), nafion® solution (5 wt%) and sodium hydroxide (microbeads) were purchased from Sigma-Aldrich® (St. Louis, Missouri, USA). BOD from *Myrothecium* sp. (EC 1.3.3.5) was obtained from Amano Enzyme Inc. (Elgin, Illinois, USA) and used without further purification. Carbon cloth (PWB-3) was obtained from Stackpole Electronics, Inc. (Raleigh, North Carolina, USA), a polytetrafluoroethylene (PTFE) 60 wt% dispersion (Teflon® T-30) was purchased from DuPont™ (Barueri, São Paulo, Brazil) and carbon powder (Vulcan® XC-72) was obtained from Cabot (Boston, Massachusetts, USA). Flexible carbon fibres (FCF) electrode was extracted from a carbon cloth (CCS200), as described in our previous work [32]. NaH_2PO_4 and Na_2HPO_4 salts were used for preparing the buffer solution, anhydrous D-glucose P. A., sulphuric acid P. A., hydrochloric acid P. A. and potassium permanganate were obtained from Synth® (Diadema, São Paulo, Brazil). Nicotinamide adenine dinucleotide free acid was purchased from Merck (Darmstadt, Hessen, Germany).

2.2. FCF-ADH bioanode

Enzyme immobilisation is important since the electrical contact between the enzyme and the electrode is essential for optimal bioelectrocatalytic activity of the electrode. Before immobilising the enzyme by adsorption, the FCF electrode was treated according to the procedure described in our previous work [32]. The FCF-ADH bioanode was prepared by the physical adsorption [2,33] of the enzyme and the electrode was sealed using epoxy resin. After the resin has dried, the FCF electrode was placed in an ADH solution (8 mg mL^{-1} in sodium phosphate buffer, pH 7.5) for 24 h at 4 °C. Then, nafion® solution (20 μL, 2.5%) was dropped onto the FCF surface on which ADH was adsorbed. Finally, the bioelectrode was vacuum-dried for 10 min.

2.3. BOD-based biocathode

Firstly, the FCF electrodes were washed twice in isopropanol and twice in deionised water. After drying under vacuum at room temperature, the fibres were pulverised to a powder consisting of carbon fibre particles with a mean length of 75 μm and diameter of 6.5 μm. The gas-diffusion biocathode (see Fig. S1 in the Supplementary Information) was formed of two layers: the diffusion layer and the catalytic layer, both of which were supported on the carbon cloth. The diffusion layer was prepared using 3.0 mg cm^{-2} of a mixture constituted by 70 wt% of Vulcan® XC-72 and 30 wt% of PTFE and it was deposited on one face of the carbon cloth, as described previously by Paganin and coworkers [34]. For preparing the catalytic layer, 2.3 mg cm^{-2} of FCF powder was suspended in isopropanol and applied over the diffusion layer. After the evaporation of isopropanol at room temperature, BOD was immobilised by physical adsorption onto the FCF electrode. For this, 36.3 μL of a mixture containing 1.7 mg of BOD, 0.8% (v/v) of nafion® solution and 0.8% (v/v) of glutaraldehyde in phosphate buffer (0.10 mol L^{-1}) of pH 7.5 were dropped onto an area of 1.18 cm^2 of the electrode. After this, the electrode was kept at 4 °C overnight. The final amount of BOD on the electrode surface was 1.4 mg cm^{-2}. The electrical contact with an array of FCF electrodes, which were fixed on the carbon cloth, was made using epoxy resin.

2.4. Enzyme kinetics

For evaluating the enzyme kinetics of ADH, the reaction was followed at room temperature in air and the absorbance at 340 nm, which corresponds to the formation of NADH, was monitored. The reaction proceeded until the saturation of the enzyme was reached, i.e. when the absorbance did not increase anymore. In a cuvette, 0.20 mL of 0.10 mg mL^{-1} ADH solution, 1.0 mL of 1.5 mmol L^{-1} NAD^+ and 0.30 mL of 0.10 mol L^{-1} sodium phosphate buffer (pH 8.2) were added. The absorbance was measured during the reaction and 0.05 mL