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Direct electron transfer with yeast cells and construction of a mediatorless microbial fuel cell

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Abstract

The direct electron transfer exhibited by the yeast cells, *Hansenula anomala* has been demonstrated using the electrochemical technique cyclic voltammetry by immobilizing the microorganisms by two different methods viz., physical adsorption and covalent linkage. The analysis of redox enzymes present in the outer membrane of the microorganisms has been carried out in this work. This paper demonstrates that yeast cells with redox enzymes present in their outer membrane are capable of communicating directly with the electrode surface and contribute to current generation in a mediatorless biofuel cells. The efficiency of current generation has been evaluated using three anode materials.

Keywords: Direct electron transfer; *Hansenula anomala*; Mediatorless biofuel cells

1. Introduction

Microbial fuel cells (MFCs) are bioelectrochemical transducers that convert microbial reducing power (generated by the metabolism of organic substrates) into electrical energy. The recent interest in microbial fuel cells is attributed to the possibility of combining waste degradation with energy generation (Shukla et al., 2004; Rabaey and Vestraete, 2005; Heydorn and Gee, 2004). Microorganisms can transfer electrons to the anode electrode in three ways; viz., (i) using exogenous mediators such as potassium ferricyanide, thionine or neutral red; (ii) using mediators produced by the bacteria themselves; (iii) direct transfer of electrons from inside of the bacterial cells (Leropoulos et al., 2005). There are several drawbacks of using exogenous mediators such as their high cost, short lifetime and toxicity to the microorganisms. However when the bacteria produce their own mediators or they transfer electrons directly to the electrode, the system can operate at a high-sustained level of activity.

Organisms such as *Shewanella putrefaciens*, *Geobacter sulfuurreducens*, *Geobacter metalli reducens* and *Rhodoferax ferrireducens* have been shown to generate electricity in mediatorless MFC systems. Some of the species that belong to the genera *Geobacter*, *Geovibrio*, *Shewanella*, reduce Fe(III) through their respiratory, fermentative or photosynthetic metabolism (Holmes et al., 2004a). Some of them are able to conserve the energy for growth by coupling the oxidation of organic acids, aromatic hydrocarbons and H_2 to Fe(III) reduction or Mn(IV) reduction (Lovely and Philips, 1988; Lee et al., 1999). The Fe(III) reducing bacterium, *S. putrefaciens* is known to localize the majority of its membrane-bound cytochromes on its outer membrane (Myers and Myers, 1992; Pham et al., 2003). The cytochromes on the outer membrane are believed to be involved in the reduction of water soluble Fe(III). Intact cells of anaerobically grown *S. putrefaciens* were electrochemically active and the bacterium could grow in a fuel cell-type electrochemical cell in the absence of electron acceptors. Similar studies were made using another Fe(III) reducing bacterium *G. sulfurreducens*. *Desufobulbus propionicus* was found to grow with Fe(III) or humic acid analogue or a graphite electrode as electron acceptor (Holmes et al., 2004b). In these cases the electroactive enzymes present in the outer membrane of the cell is responsible for direct electron transfer between microorganisms and electrode. Dissimilatory ferric reduction is proposed to be an early form of microbial respiration. It has been shown (Varges et al., 1998) that Archaea and Bacteria, which are

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very closely related to the last common ancestor, can reduce Fe(III) to Fe(II) and generate energy from the respiration to support growth. Dissimilatory iron reducing microorganisms designated as GS-15, have been recently discovered that they reduce extra-cellular amorphous Fe(III)-oxyhydroxide as the terminal electron acceptor for the oxidation of organic matter to magnetic iron oxides under anaerobic conditions (Lovely et al., 1987; Pierre et al., 2002). This is an interesting finding since this microbial metabolism might have played an important role in the magnetization of anaerobic sediments and could account for the accumulation of magnetite in ancient iron formation. Magnetite accumulation has also been used as an indication that Fe(III) reduction was an important respiratory process on early Earth (Walker, 1987) and is significant in modern hot deep terrestrial biospheres, allowing a life in the absence of solar energy (Liu et al., 1997). So far only two classes of *Ferricyanide reductases* are known, the soluble prokaryotic *Flavin reductase* and the membrane cytochrome *b* like reductases found in prokaryotes. *Ferricyanide reductase of Saccharomyces cerevisiae* has been analysed in detail and it is characteristic of a b-type cytochrome and very similar to *flavocytochrome b*558 of human neurophils. This enzyme takes electrons from NADPH in the cytoplasm and passes them across the membrane via FAD and heme to molecular oxygen generating superoxide that is expelled into the lumen of the vacuole (Shatwell et al., 1996).

Electrochemical techniques have been extensively used to characterize redox proteins including cytochromes. In general, bacterial cells containing electrochemically active proteins tend to be electrochemically inactive as their cell wall structures consist of non-conducting material such as lipid and peptidoglycan (Park and Zeikus, 2002). Hence mediators were used to facilitate electron transfer between an electrode and electrochemically inactive microbial cells. Alternatively, the bacterial cells can be modified with hydrophobic conducting polymers to render them electrochemically active (Park et al., 1997). In this work we have explored the possibility of direct electrical communication between the yeast cells and electrode surface and demonstrated current generation in aerobic microorganisms for the first time.

The mediatorles systems described so far make use of Ferric ions as terminal electron acceptors instead of oxygen. During electron generation the reduction of ferric ions is found to be the intermediate step before electron collection on electrode surfaces. In the case of *Geobacter sulfurreducens* and *D. propionicus* the dissimilatory iron reduction is the intermediate step before electron generation at the electrode surface. In the case of *S. putrefaciens*, the cytochromes present in the outermembrane are involved in the reduction of water soluble ferric salts and this is the intermediate step before electron generation. In the present work, the redox proteins present in the outer membrane of the yeast cells facilitate direct electron transport between bacterial steps without any external mediators and without the intervention of reduction of ferric ions as the intermediate step. It is likely that the redox potentials of the proteins ferricyanide reductase and Lactate dehyrogenase and that of the electrode material (present in the membrane fraction of *Hansenula anomala*) are favorably arranged for the electron transport relay from the cells directly to the electrode surface. The efficiency of current generation has been evaluated using three anode materials.

2. Experimental

2.1. Sub-culturing of H. anomala

H. anomala was sub-cultured using the medium comprising D-glucose: 1 g; peptone: 0.5 g; malt extract: 0.3 g; yeast extract: 0.3 g; phosphate buffer: 100 ml.

The buffer solution was prepared by dissolving 1 g of KCl; 6 g of NaH_2PO_4; 2.9 g of NaCl and 2 g of Na_2CO_3 in 1 l.

2.2. Construction of the biofuel cell

A two-compartment cell made of Perspex with Nafion (961) membrane as the separator was used. Three different anode materials were used for the investigations—graphite, graphite felt and polyaniline (PANI)–Pt composite coated graphite electrodes. Graphite was used as the cathode material. The electrodes were suspended from the top cover and it is tightly sealed. Cathode compartment contained 0.1 M ferricyanide. The anode compartment was filled with deaerated suspension of nutrient broth in phosphate buffer.

2.3. Pretreatment of graphite felt

Carbon felt was kept immersed in distilled water for 12 h and later in methanol for another 12 h. Felt was heated to 120 °C for 1 h.

2.4. Preparation of PANI–Pt composite graphite electrode

Polyaniline (synthesized in our laboratory) was dissolved in a solution containing *N*-methyl-2-pyrrolidone and polyvinylidine fluoride. Graphite was coated with a thin layer of this PANI solution and dried for 2 h. The electrode was refluxed in a 10 mM K_2PtCl_4 solution containing 1% $NH_2OH.HCl$ at 60 °C for 5 h. Platinum(II) is reduced by NH_2OH and deposited on the PANI-coated electrode.

2.5. Immobilization of microorganisms

The nutrient broth containing the microorganisms was subjected to refrigerated centrifuge and the residual pellet is collected after several washings with water. The cells were suspended in phosphate buffer and a few microlitres of the suspension were immobilized by physical adsorption on to the pores of a membrane filter fitted inside a crucible connected to a vacuum pump. The filter is then removed and fixed around the gold electrode of a three-electrode assembly (Type-I). A silver reference electrode and a Pt foil counter electrode were assembled encircling the gold electrode. The bacterial leaching was prevented by tying a dialysis membrane on the bottom of the assembly. Similar electrode assembly has been used by our group earlier as a biosensor for BOD (Sumathi et al., 1992;

Sangeetha et al., 1996). However, unlike the earlier designs, the present construction does not use a gas permeable Teflon membrane. Phosphate buffer was used as the internal filling solution (see Supplementary files).

Immobilization has also been carried out using a different procedure described as follows: a gold disk electrode is modified with a self-assembled monolayer of cystamine by immersing the electrode in a solution of cystamine (50 mM) overnight. Then it is treated with 1% glutaraldehyde and then with the cells suspended in phosphate buffer. Then the modified electrode is washed with distilled water and the electrochemical activity of the immobilized microorganisms was tested using a three-electrode assembly (Type-II) (modified electrode as the working electrode, Pt as the counter electrode and calomel electrode as reference electrode) in phosphate buffer (see Supplementary files).

2.6. Preparation of specimens for scanning electron microscope (SEM) analysis

The anode material used in the fuel cell was removed at the end of the experiment and washed with phosphate buffer followed by distilled water. Chips of 1 cm × 1 cm were cut and used for SEM analysis.

2.7. Analysis of end products by HPLC

The end products in the anode compartment were analyzed by HPLC (Shimadzu, Japan). Methanol was used as the mobile phase and silica gel was used as the stationary phase. The experimental parameters used for the analysis is as follows:

- Main column analytical–Shim-Pack CLC–octadecyl silane(ODS-C1*) (4.6 mm i.d. × 35 cm). Guard column–Shim-Pack g-ODS (4 mm i.d. × 1 cm); detector-UV spec trophotometer; flow rate: 1 ml/min; column head pressure −125 kg f/cm^2; injection/sample: 20 μl; wavelength: 254 nm.

2.8. Analysis of enzymes

2.8.1. Extraction of membrane fraction from yeast sells

Yeast membrane fraction was prepared by the methods briefly described below (Christensen and Cirillo, 1972; Zinser et al., 1991). In brief, washed yeast cells were mixed with 2–3 ml of glass beads per gram of yeast cells which dissolved in homogenizing buffer (10 mM Tris–HCl pH 7.6, 0.15 M NaCl, 5 mM EDTA, 10 mM dithiothreitol) and the mixture was ground for 5 min with a mortar and pestle. After grinding, the mixture was allowed to stand in the mortar until the beads settled and the overlying liquid was decanted in to centrifuge tubes. Unbroken cells and debris were removed by centrifugation at 250 × *g* for 3 min. Pellet was discarded and supernatant liquid was centrifuged at 12,000 × *g* for 15 min. The resulting pellet was re-suspended/mixed well with homogenizing buffer and re-centrifuged at 12,000 × *g* for 15 min. The resulting pellet was dissolved in homogenizing buffer and stored at −20 °C for further enzyme analysis.

2.8.2. Enzyme assay

All the enzymes were assayed using UV–visible spectrophotometer. Cytochrome *b*2 was assayed by the method of Appleby and Morton (1959). The rate of reduction of potassium ferricyanide is determined spectrophotometrically at 420 nm. One unit reduces one micromole of ferricyanide per minute. Cytochrome *b*5 was determined by the method of (Omura and Sato, 1964). NADPH ferricyanide reductase activities were measured by decreasing absorbance of ferricyanide at 420 nm using the method described in the reference quoted here (Smith et al., 1963).

Polarization experiments were carried out using Electrochemical Analyzer PARSTAT 2263. SEM analysis was carried out with Hitachi Model-S-3000H. Enzyme analysis was carried out using the UV–visible spectrophotometer.

3. Results and discussion

3.1. Analysis of membrane fraction of H. anomala

The membrane fraction of the species *H. anomala* has been separated and analyzed. The presence of some redox enzymes could be demonstrated by our analysis. The isolated membrane fraction was found to contain *Lactate dehydrogenase* (cytochrome *b*2), NADH-*Ferricyanide reductase*, NADPH-*Ferricyanide reductase*, and cytochrome *b*5 (see Supplementary files for bar graph). The total protein content of the membrane fraction was found to be 52.35 μg protein/10 μl of the sample.

In earlier reports, the presence of *cytochrome b*2 on the outer membrane has been advantageously used for the sensing of lactate in presence of the exogenous mediator, potassium ferricyanide (Kulys et al., 1992). The presence of *Ferricyanide reductase* in yeast cells has also been observed earlier (Carlos et al., 1998). The presence of electro-active enzymes in the membrane suggests that they can mediate the transfer of electrons generated during metabolism to the electrode directly without external mediators. Oxygen, which is the terminal electron acceptor in the aerobic respiration is completely avoided in the anode compartment.

3.2. Evaluation of direct electron transfer

Fig. 1 (curve 1) represents the cyclic voltammogram (CV) taken using the Type-I electrode assembly. The three-electrode assembly is immersed in 5 ml of phosphate buffer in the electrochemical cell and it is completely de-aerated. The CV reveals the presence of two pairs of redox peaks A and B. The anodic peaks corresponding to A and B occur at 0.09 and 0.38 V. Based on the E^0 values, the peaks are assigned to *Ferricyanide reductase* and *Lactate dehydrogenase*. The equilibrium redox potential of *cytochrome b*2 is 0.12 V versus NHE. The redox potential at which the neutrophilic iron reducers release their electrons is in the same range as that of the fumarate–succinate couple (+30 mV versus NHE) (Seeliger et al., 1998). These two enzymes are responsible for the electrochemical activity of the organism. *Ferricyanide reductase* is an externally directed redox enzyme located in the plasma membrane and its activity is traditionally

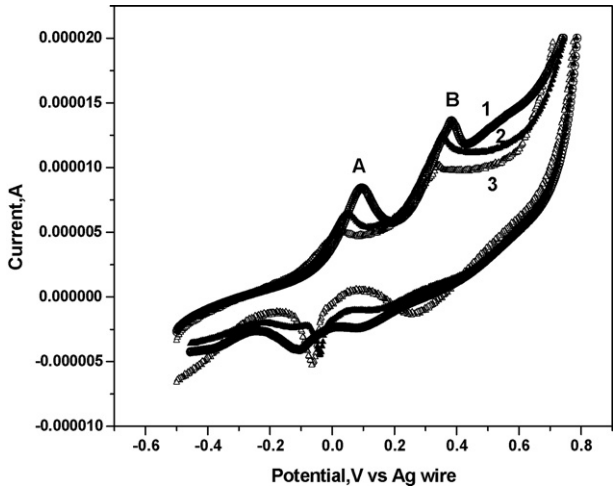


Fig. 1. Cyclic voltammograms demonstrating the electrochemical activity of *H. anomala* in phosphate buffer using electrode assembly I; scan rate 50 mV/s. (1) Blank CV; (2 and 3) after successive additions of 0.5 ml each of lactate stock solution (0.5 M).

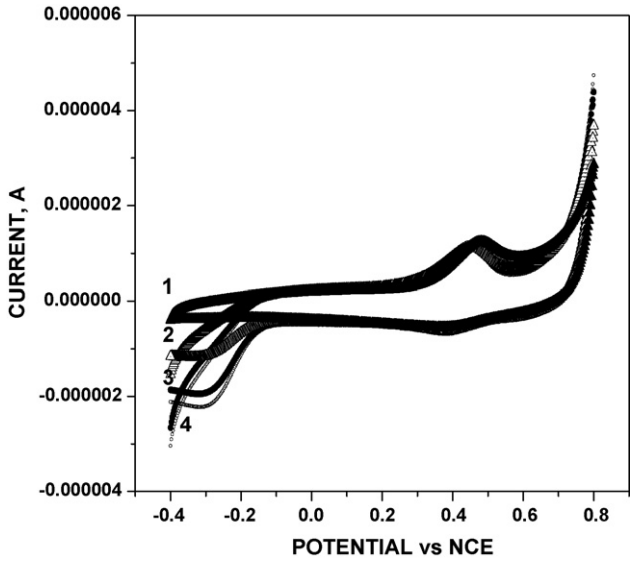


Fig. 2. Cyclicvoltammograms demonstrating the electrochemical activity of *H. anomala* using electrode assembly II; scan rate 50 mV/s. (1) Blank CV in phosphate buffer; (2–4) after successive additions of 0.5 ml each of lactate stock solution (0.5 M).

measured through the ferricyanide anion which is also impermeant within the cells. *Ferricyanide reductase* is involved in the iron uptake system of yeasts. The electrochemical activity of the two enzymes could be demonstrated by immobilizing the microorganisms on a filter membrane. The degassing of the electrolyte with an inert gas removes the terminal electron acceptor, oxygen from the medium and consequently the electrochemical activity of the microorganisms could be demonstrated. When nutrients like lactate or glucose are added, the peak currents decrease indicating that the enzymes directly communicate with the electrode. The electroactive enzymes in the outer membrane accept the electrons generated during oxidation of glucose or lactate and get themselves reduced and transfer the electrons to the electrode. Hence its concentration keeps on decreasing. The Fig. 1, curves 2 and 3 represent additions of lactate to the phosphate buffer solution. Both the peak currents decrease on the addition of lactate. This shows that these enzymes act as gates for direct movement of electrons towards the electrode.

Fig. 2 depicts the CV obtained for the Type-II electrode assembly, which shows the electrochemical activity of microorganisms immobilized on gold electrode surface. Curve 1 represents the blank CV for the modified electrode and curves 2–4 represent different lactate additions. Curve 1 shows the presence of one peak, which is quasi reversible. The position of the peak suggests that it is due to *Lactate dehdrogenase*. The peak corresponding to *Ferricyanide reductase* does not appear in this case. This indicates that this method of immobilization does not favour proper orientation of enzymes present in the outer membrane for direct electron transfer. Hence the immobilization procedure used in the Type-I electrode assembly is more efficient in revealing the electrochemical activity of the microorganisms.

Fig. 3 presents the open circuit potential variation with time for the biofuel cells constructed with the three anode materials, graphite, graphite felt and PANI–Pt-graphite electrode. In the

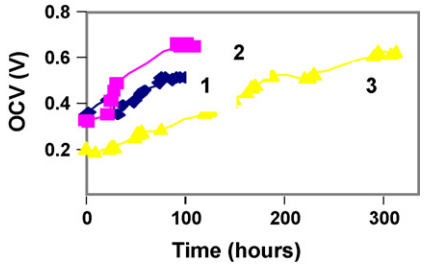


Fig. 3. Open circuit voltage variations for different types of electrodes. (1) Graphite; (2) graphite + felt; (3) graphite + polyaniline + platinum.

case of the graphite electrode maximum cell voltage of 0.52 V was reached over a period of 74 h and in the case of graphite felt, maximum cell voltage of 0.658 V was reached over a period of 96 h and in the case of PANI–Pt-graphite electrode, maximum cell voltage was attained after 285 h. The voltage remained constant after this period. The values remained constant after the time period mentioned here.

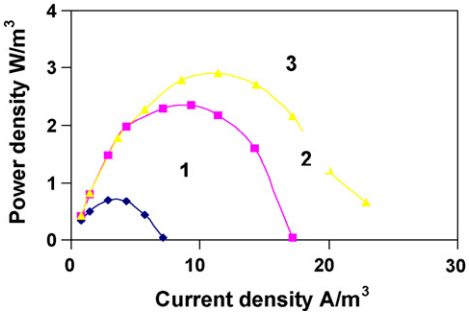


Fig. 4. Polarisation curves for different electrode materials. (1) Graphite; (2) graphite felt; (3) graphite + polyaniline + platinum.

3.3. Evaluation of different anode materials in mediatorless biofuel cells

Polarization experiments were carried out after the attainment of steady, maximum voltage. The anode was polarized at different current densities and the resulting steady state cell voltage is noted. Power density is calculated by multiplying the current density with the corresponding steady state cell voltage. The results are expressed in W/m^3 as per the current trend (Rabaey and Vestraete, 2005). Each biocatalyst will have specific requirements and occupy a certain volume in the reactor thus decreasing free space and pore size. Each bacterial cell acts as a tiny electron generator and each cell has its own energy requirements and occupies a finite volume in space. The observed current is due to the electrons generated by all the active cells in combination. Every study refers to a specific combination of reactor volume, membrane separator catholyte, anode surface and organic loading. Hence it will be useful to express the power density in W/m^3. The results are given in Fig. 4. It can be seen from the figure that maximum power density was obtained from PANI-

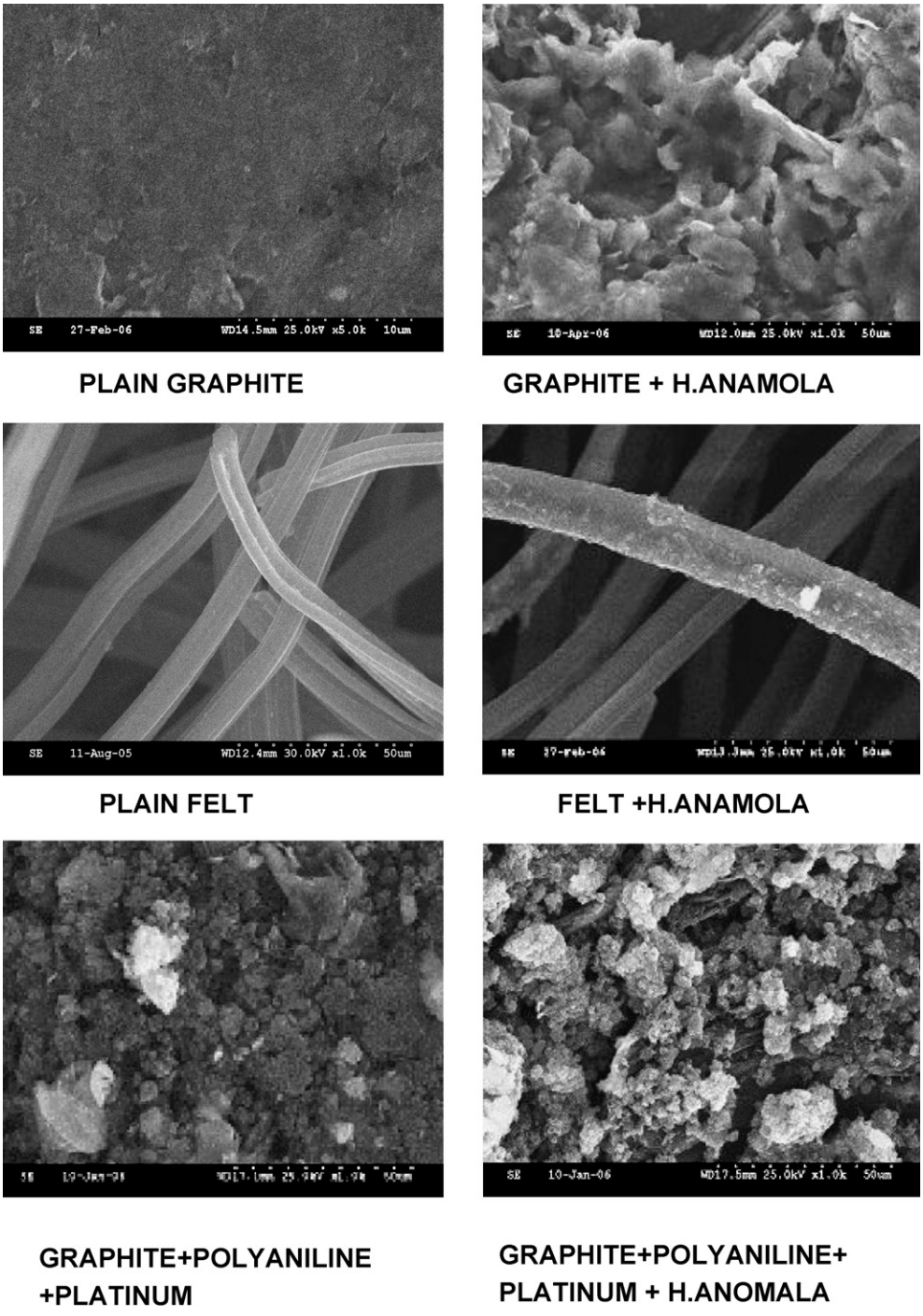
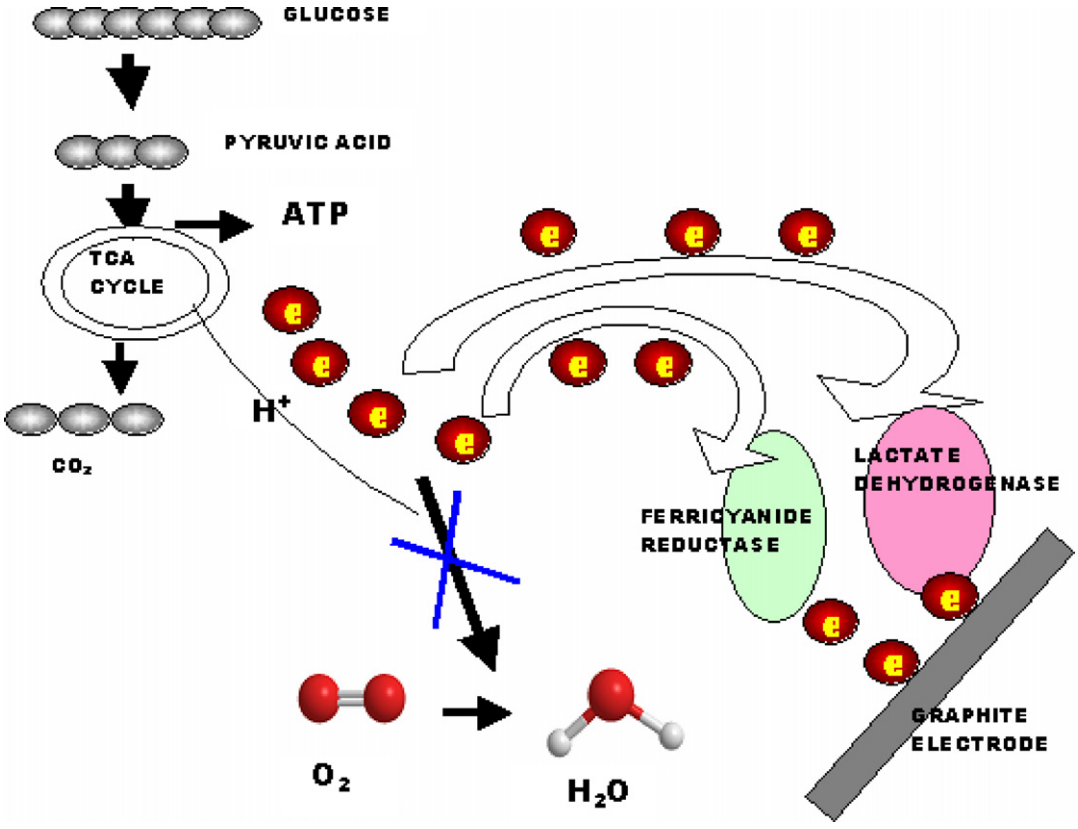


Fig. 5. SEM images showing the microbial growth on electrode surfaces.



Scheme 1. Scheme depicting the transfer of electrons to electrode surface.

Pt electrode, which was found to be 2.9 W/m^3. In the case of graphite-felt electrode, a maximum power density of 2.34 W/m^3 was obtained. And in the case of plain graphite, 0.69 W/m^3 was obtained.

3.4. Analysis of morphology of anode surfaces

Fig. 5 shows the SEM images showing the growth of microorganisms on the anode materials. The SEM images of the bare surfaces are given for comparison. The growth of microorganisms on electrode surfaces is clearly seen in the pictures.

3.5. Mechanism of direct electron transfer

In the literature, the papers related to direct electron transfer as mentioned earlier in the introduction, talk about iron uptake associated with direct electron transfer. For example, *Shewanella* and *Geobacter* are Fe(III)-reducing in nature. They can grow in a medium with organic compound as the donor and Fe(III) as the electron acceptor. Since *H. anomala* is aerobic in nature, oxygen can be an electron acceptor. Presence of *Ferricyanide reductase* in the cell membrane suggests, that Fe(III) can also act as electron acceptor. In the absence of terminal electron acceptors like oxygen and Fe(III), the electrons produced during the metabolism of organic compound are accepted by electrode surface. The electron transfer is facilitated by the redox enzymes present in the outer membrane, since they act as electron transfer shuttles or gates for electron transfer release. They do not show any specificity for lactate or glucose. Lactate and glucose are found to behave as electron donors and the electrode acts as acceptor (Scheme 1). The influence of electrode material in improving the electron transfer kinetics is shown in Fig. 4. Compared to bare graphite (0.69 W/m^3), the power density has increased in the case of graphite felt (2.34 W/m^3) and graphite modified with PANI and Pt (2.9 W/m^3). This shows that all the three materials employed in the study are good electron acceptors. The equilibrium potentials of these electrodes are sufficiently positive to accept electrons released from the metabolic reactions of the microorganisms. The electron transfer kinetics is increased due to the high surface area of the graphite material in one case. In the case of graphite electrode modified with PANI and Pt, the electron transfer kinetics is improved due to the presence of catalytically active Pt.

HPLC analysis showed that the end products formed contained gluconic acid, succinic acid and pyruvic acid. These products are generally observed in the metabolic cyle of yeasts and confirm that glucose can be utilized as a fuel for current generation by the species *H. anomala*.

4. Conclusions

In this work, *H. anomala*, which belongs to the family of *yeast*, is demonstrated to exhibit direct electron transfer without the aid of mediators. The electro-chemical activity of the

microorganisms could be demonstrated by two different immobilization methods. The redox enzymes present in the cell membrane, viz., ferricyanide reductase and lactate dehydrogenase which are responsible for its electroactivity, have been analyzed by extracting the membrane fraction of the yeast cells. It has also been demonstrated that *H. anomala* can transfer electrons directly to electrode surfaces in presence of glucose as carbon source. Direct electron transfer between microbial cells and electrodes was shown to occur with low efficiencies. Hence, the direct electron transfer kinetics exhibited by *H. anomola* has been studied as a function of anode material. Biofuel cells have been constructed with three different anode materials (graphite, graphite felt and polyaniline–Pt composite coated graphite) and the performance of the biocatalyst for direct electron transfer is compared. This work illustrates how the redox potentials present in the membrane fraction of *H. anomola* are favorably arranged in comparison with that of electrode material for direct electron transport without any external mediator and without any intermediate redox reaction like ferric ion reduction. This work demonstrates that microorganisms with suitable redox potential arising due to relay of electrochemically active proteins in the membrane, can be suitably tuned for dye reduction which will be useful for waste water treatment and in the preparation of bioassisted preparation of metal nanoparticles which we will be addressing in our future work.

Acknowledgements

The authors wish to acknowledge Director, CECRI for his keen interest in this work Supplementary files available for schematic diagram representing the Type-I and Type-II electrode assemblies and bar graph representing the results of enzyme analysis in the membrane fraction.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2006.10.028.

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