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Short communication

Effect of initial carbon sources on the electrochemical detection of glucose by *Gluconobacter oxydans*

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Abstract

An electrochemical system consisted of *Gluconobacter oxydans* as a microorganism and 2-hydroxy-1,4-naphthoquinone (HNQ) as a mediator has been setup to examine the effect of initial carbon sources on the detection of glucose. Catalytic current due to the oxidation of glucose was observed only when both *G. oxydans* and HNQ were present. From amperometric measurements, it was found that the sensitivity strongly depended on the initial carbon sources. The sensitivity was highest for the cells cultured in a fructose-containing medium and decreased in the order, mannitol > sucrose>glucose>galactose>glycerol. The difference in sensitivity was explained by considering the current rising pattern at an initial stage of a microbial fuel cell constructed with the same components. The rising time, not the fuel cell efficiency, could directly be related to the sensitivity order. A sensor where *G. oxydans* was confined at the vicinity of the electrode by the semipermeable membrane was constructed. A linear response over a millimolar range of glucose concentration was observed with a cell grown in galactose-containing medium. This work demonstrates that the initial carbon source play an important role on glucose sensoring and should be considered in a real application.

Keywords: Glucose oxidation; Initial carbon source; *Gluconobacter oxydans*; Biosensor; Microbial fuel cell

1. Introduction

Microbial sensors utilize living microorganisms to detect substrates through the metabolic sequences of enzymes within the cell [1,2]. Despite the relatively poor selectivity compared with single enzyme-based biosensors, microbial sensors have several advantages such as physical robustness, stability, easy handling, and cheap preparation cost. Works so far have mostly been concentrated on the development of biosensors for the environmental monitoring using various detection systems, which include a Clark-type oxygen sensor [3–5] or potentiometric ammonium [6] or pH electrodes [7] as a transducer. Redox mediator-based detection systems have also been developed in which the mediator functions as an electron-shuttle between the cell and the electrode [8–10]. Particularly notable in this regard is the work by Ikeda et al. [11]. They have developed various mediated electrocatalysis systems, for example, to detect ethanol, to follow in vivo reconstruction of glucose dehydrogenase in *Escherichia coli* [12], and to measure the oxidizing activity of acetic acid bacteria [13]. It was found that the sensor performance strongly depended on the kind of mediators.

Since many enzymes are involved, the response of microbial sensors quite depends on the physiological state of living cells, and thus, it is expected that microorganisms grown in a different culture medium exhibit different sensor response even for the same substrate. However, the study of culture medium dependency is rare. Ikeda reported that *Acetobacter pasteurianus* had a higher response to ethanol and acetaldehyde when grown in ethanol-containing medium than in glucose-containing medium [13]. In our microbial fuel cell study [14], we have already demonstrated that the cell performance varied depending on the initial culture conditions. In this paper, we extended our previous work on the fuel cell to the microbial sensor to investigate the initial carbon source dependency of sensor sensitivity. Some common mono- and disaccharides as well as glycerol have been used in a culture medium. We chose glucose oxidation as a

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model system as this is one of the most extensively studied systems and has important clinical implications. An electrochemical cell made of *Gluconobacter oxydans* and 2-hydroxy-1,4-naphthoquinone (HNQ) was constructed, and voltametric and amperometric measurements were carried out to see the effect of initial culture conditions. Also, we constructed a fuel cell using the same components to compare with results from a sensor.

2. Experimental

2.1. Preparation of microorganisms

G. oxydans (ATCC 621) was obtained from the culture collection of Korean Collection for Type Cultures (KCTC) and grown aerobically at 30 °C. To test initial carbon source effects on the fuel cell efficiency, the microorganism was cultured in a medium that contained 10 g of trypton, 6.8 g of KH_2PO_4, and 10 g of a carbon source per liter. Fructose, galactose, glucose, sucrose, mannitol, and glycerol were used as an initial carbon source. Each culture containing a carbon source was daily subcultured with 5% inoculums for 3 days. Cells were harvested by centrifugation at $3000 \times g$ for 10 min at their early stationary phase and washed three times with 0.05 M of phosphate buffer of pH 7.0 at 4 °C. The washed microorganisms were resuspended in a buffer to give 20 mg (dry wt.) per ml for experiments.

2.2. Electrochemical measurements

The choice of mediator was made after testing its fuel cell performance. 2-Hydroxy-1,4-naphthoquinone (HNQ) was chosen because it gave higher coulombic output than commonly used mediators such as resazurin or thionine.

A conventional electrochemical setup with a potentiostat (Autolab PGSTAT 30, ECO CHEM) and three-electrode system ($Ag|AgCl|KCl_{(sat)}$ reference, graphite working, and Pt counter electrodes) was employed for electrochemical measurements. All the chemicals were of reagent grade and used without further purification. The solution containing a microorganism and a mediator was prepared in 0.1 M phosphate buffer at pH 7.0 after degassing with N_2. A microorganism suspension was prepared by ultrasonicating a clod of *G. oxydans* (0.24 g or 3×10^{10} cells/g) in a buffer solution (25 ml). HNQ concentration was maintained to be 0.5 mM. Amperometry of glucose oxidation was carried out at a fixed potential determined from cyclic voltammetry in the presence of HNQ and *G. oxydans*. Current was measured by adding a glucose stock solution to the system. For the rapid mixing of glucose, the electrode was rotated at 1500 rpm. A sensor was constructed according to the literature [9] where *G. oxydans* was confined at the vicinity of the electrode by the cellulose acetate membrane (pore size 0.2 μm) and current was measured as a function of glucose concentration at a fixed potential.

2.3. Fuel cell experiments

A fuel cell assembly was constructed according to our previous design [14]. The cell is composed of anode and cathode compartments separated by a cation exchange membrane (Nafion, Aldrich, USA). A reticulated vitreous carbon (RVC) plate was used as an anode. RVC has a physical structure that can allow easy access of organisms and mediators to the electrode surface through the open network and provide a high surface area for the reaction. Anolyte and catholyte were composed of 0.05 M sodium bicarbonate buffer and 0.1 M ferricyanide solutions, respectively. *G. oxydans* and HNQ were added to the anodic compartment. A platinum plate was used as a cathode. Each compartment was sealed by 1.5-mm-thick silicone rubber gaskets. During the experiments, nitrogen was flowed through the cell compartments to keep oxygen from entering the cell and to effectively mix the solution. Operation temperature was maintained constant in a water bath.

The cell discharge was done by the 560-Ω external resistor between an anode and a cathode. The discharge curve was recorded only after the open circuit voltage was stabilized with nitrogen gas flowing through the cell. The cell voltage with time was then recorded with a personal computer equipped with an analogue-to-digital board (Computer Boards, Mansfield, MA, USA). Coulombic efficiency was calculated by the equation, $\eta = Q_{dis}/Q_{th}$, where Q_{dis} and Q_{th} are charges experimentally obtained by the integration of a discharge curve and theoretically calculated for the complete oxidation of a substrate, respectively.

3. Results and discussion

Curve a in Fig. 1 shows cyclic voltammetry of HNQ in the presence of *G. oxydans*. A set of redox peaks with a formal potential at −0.36 V indicates a typical reversible

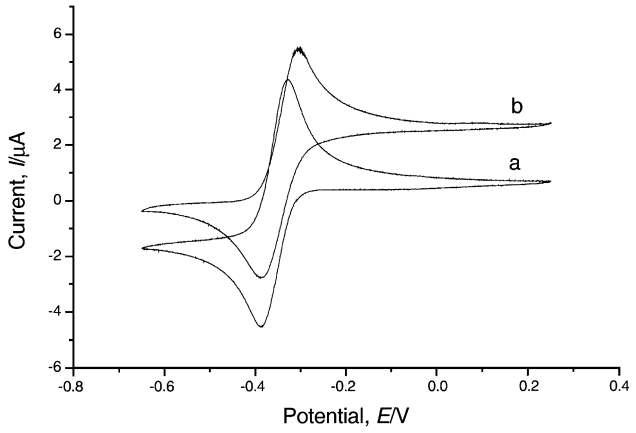


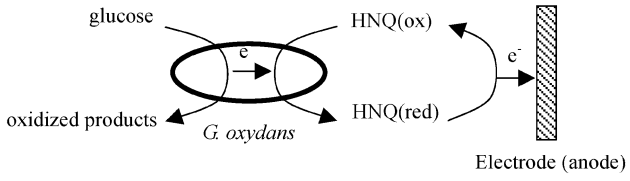
Fig. 1. Cyclic voltammetry of HNQ in pH 7.0 phosphate buffer containing *G. oxydans* in the absence (curve a) and in the presence (curve b) of glucose. $C_{HNQ} = 0.5$ mM, $C_{glucose} = 2.0$ mM. Scan rate = 5 mV s^{-1}.

electron transfer reaction of the quinone group in HNQ to hydroquinone involving two electrons.

Glucose itself does not undergo any electrochemical reactions within the potential range examined. Glucose oxidation is observed only when both *G. oxydans* and HNQ are present in the solution (curve b). Curve b is exactly the same as curve a except there is offset current. Two kinds of current contribute to the overall shape: (i) the current due to the direct redox reaction of HNQ at the electrode, and (ii) the catalytic current due to the glucose oxidation mediated by HNQ (Scheme 1). If the catalytic current prevails as in the case of enzyme-modified electrodes where a large current from the glucose oxidation is usually observed, the voltammogram should be in sigmoidal shape. In our case, however, the fact that the voltammogram is shifted upward indicates the relatively small contribution of catalytic oxidation of glucose, which in turn implies that the glucose oxidation by *G. oxydans* is slow.

G. oxydans grown at various initial carbon sources was tested by amperometric experiments. Here we denote Glu-ini, for example, to indicate the condition in which glucose was used in an initial culture medium. Likewise Gal-ini, Fru-ini, Suc-ini, Man-ini, and Gly-ini indicate that galactose, fructose, sucrose, mannitol, and glycerol were initially used as a carbon source. The working electrode was polarized at 0.1 V to detect glucose by oxidation. Fig. 2 is the current–time curve with Gal-ini condition as the concentrated glucose solution is added as indicated by arrows. *G. oxydans* was suspended in a whole electrochemical cell under 0.1 M phosphate buffer at pH 7.0.

Oxidation current increases in response to the increase in glucose concentration but it takes ca. 10 min to reach the plateau. This slow response, which has also been observed by other researchers, is typical of a sensor composed of microorganisms. The identical measurements were done with other conditions. Fig. 3 is the plot of current vs. glucose concentration for various initial culture conditions, showing the glucose oxidation is quite dependent of initial carbon sources. While somewhat large oxidation current was resulted when hexoses such as glucose and sucrose were used in a culture medium, very little oxidation current was observed with Gly-ini condition, indicating glycolytic pathway was not effectively developed in this condition. When the cell was treated glycerol initially, metabolic reactions inside the cell should be adapted to this triose. Claret et al. reported on the results that only two catabolic pathways are involved in glycerol dissimilation by this



Scheme 1. Electrochemical detection of glucose by the mediator.

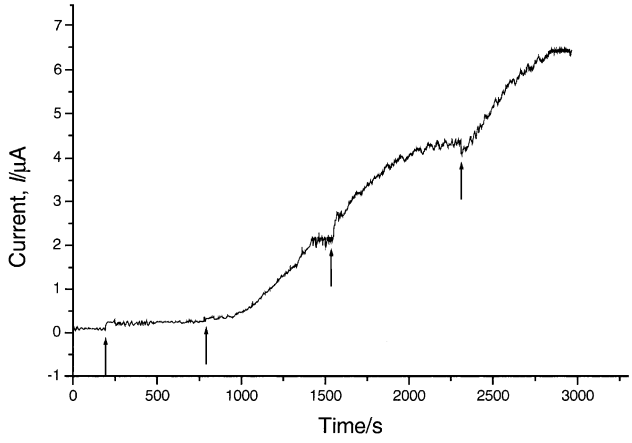


Fig. 2. Amperometric measurements of glucose oxidation by *G. oxydans* on the electrode polarized at +0.1 V vs. $Ag|AgCl|KCl_{(sat)}$. Concentrated glucose solution was injected while rotating the electrode at 1500 rpm. The injection is indicated by arrows, which correspond to 0.1, 0.5, 1.0, and 2.0 mM of glucose, respectively. Cells were cultured under Gal-ini condition and suspended in a whole electrochemical cell under 0.1 M phosphate buffer at pH 7.0 for the measurements. C_{HNQ} = 0.5 mM.

microorganism [15]. The only enzyme responsible for dihydroxyacetone (DHA) production is membrane-bound glycerol dehydrogenase, which employs O_2 as the final acceptor of reduced equivalents without NADH mediation. As the glycolytic and carboxylic acid pathways are absent, the pathway provided by the membrane-bound enzyme is indispensable for the energy requirements of *G. oxydans*. Therefore, initially glycerol-cultured *G. oxydans* cells are less preparative as well as less sensitive for the added glucose comparing with other glycolytic substrates such as galactose and glucose. The best sensitivity was achieved with Fru-ini condition although current was not linearly increased with concentration. According to recent studies of Tkachenko et al. [16], the respiratory activity of *G. oxydans* was stimulated in phosphate buffer supplemented with fructose. Stimulated respiratory activity by the fructose

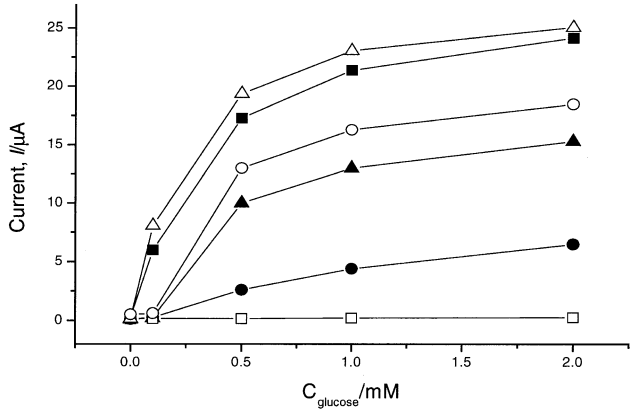


Fig. 3. Plot of oxidation current vs. glucose concentration using values obtained from amperometric measurements under Glu-ini (▲), Gal-ini (●), Man-ini (■), Fru-ini (△), Suc-ini (○), and Gly-ini (□) conditions. Other conditions are the same as those in Fig. 2.

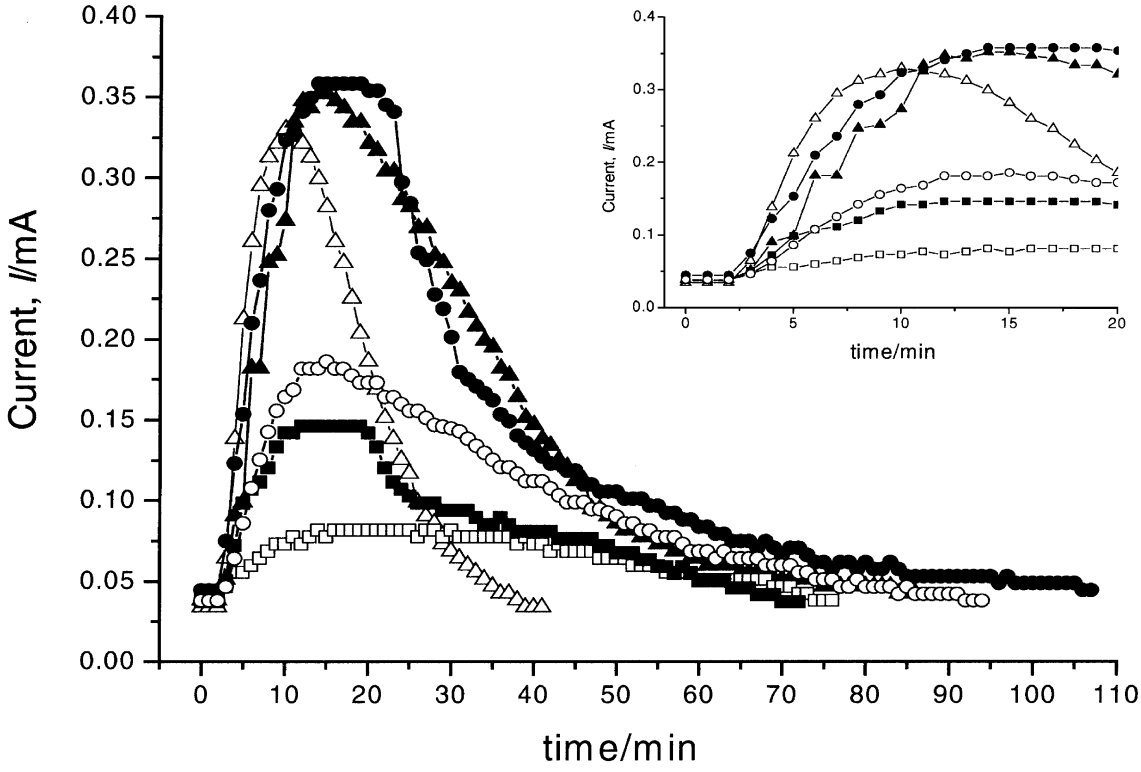


Fig. 4. Variation of current outputs of a microbial fuel cell constructed with HNQ and *G. oxydans* upon the injection of glucose as a substrate. Cells were grown under the condition where glucose (▲), galactose (●), mannose (■), fructose (△), sucrose (○), and glycerol (□) were used as an initial carbon source. Inset: The rising part of the figure.

treated cell will be responsible more efficiently to the added glucose. Another report by Elisashvili and Loitsyanskaya [17] on the levansucrase of this bacterium indicated that the highest activity of the enzyme was observed with fructose whereas the lower activity occurred with ethanol, glucose, or galactose. These previous reports might explain why the higher sensoring response to glucose could be achieved when this bacterium was initially cultured with fructose. In the meantime, Glu-ini condition gave a medium response to glucose. This result implies that the identical carbon source with the desired substrate is not necessarily used in an initial culture badge to obtain the highest sensitivity. The initial culture condition dependency has also been examined from the fuel cell point of view. We already demonstrated that the fuel cell efficiency strongly depended on the initial carbon sources [14]. Fig. 4 shows the charging–discharging curves from a microbial fuel cell consisted of the same components as those used in this electrochemical measurement as a function of initial culture conditions. Coulombic efficiencies are summarized in Table 1. The lowest efficiency (5.3%) for Gly-ini explains the lowest sensitivity for glucose. It could be explained by an inefficient metabolic adaptation by the initial treatment of glycerol as described above. However, in most cases, the

Table 1
Charging time, coulombic efficiency (η), and sensitivity order of glucose detection for different initial culture conditions

Initial culture condition	Rising time (min)	η^a (%)	Sensitivity order
Glu-ini	11.2	24.3	4
Gal-ini	12.0	25.3	5
Man-ini	8.7	9.0	2
Fru-ini	7.0	12.7	1
Suc-ini	10.2	14.3	3

[a] The standard deviation is about 5%.

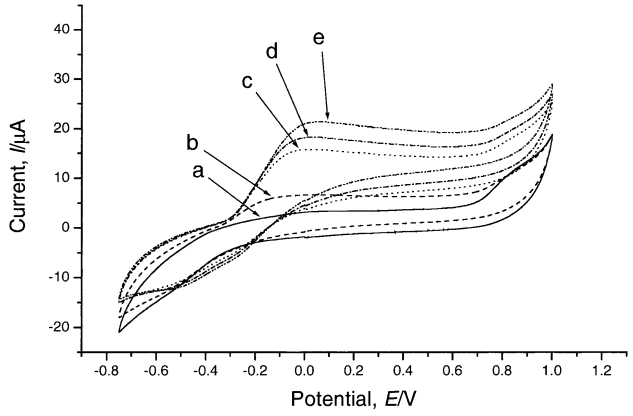


Fig. 5. Voltametric response of a sensor with *G. oxydans* confined at the vicinity of the electrode by a cellulose membrane in (a) pH 7.0 phosphate buffer only, (b) buffer + HNQ, (c) b + 5 mM glucose, (d) b + 6 mM glucose, and (e) b + 7 mM glucose solutions. *G. oxydans* was cultured in a galactose-containing medium. $C_{HNQ} = 0.5$ mM. Scan rate = 5 mV s^{-1}.

coulombic efficiencies do not follow the sensitivity order shown in Fig. 3. The highest efficiency found in Gal-ini condition (25.3%) does not mean the highest sensitivity. Actually, Gal-ini condition gave the worst glucose sensitivity except for glycerol. This seemingly contradictory result could be solved by considering the current rising pattern at the initial stage of the fuel cell operation (Fig. 4). Upon the injection of glucose, current rises to the maximum value and then decreases as glucose is being consumed. The rising pattern varies depending on the kind of initial medium in which the cell was cultured. For example, although Gal-ini condition shows the highest coulombic efficiency, the current rising takes rather long time, indicating that the cells are slowly responding to glucose. Actually, this discrepancy between fuel cell efficiency and sensor responsibility for the cells grown in the galactose-cultured medium would be explained by their unique property of galactose oxydase inside the cell. When the cell is initially treated with galactose as a carbon source, it actively induces the galatose oxidase which oxidizes galactose to DHA [18]. DHA is known to actively participate in the further metabolic pathway such as respiratory electron transport reactions. Its final effect would eventually increase the coulombic efficiency in the microbial fuel cell by way of redox mediators. However, as DHA inhibits the glycolytic pathway, the glucose-sensing ability of initially galactose-cultured cells could be decreased despite the highest fuel cell efficiency. The shortest rising time found for Fru-ini condition despite the low fuel cell efficiency (12.7%) explains why this cell gave the best glucose sensitivity. In the sensor application, the rising time should be taken into consideration more importantly than the fuel cell efficiency as measurements are usually finished in a short period of time. In this sense, the coulombic efficiency may not be a good indicator for the sensor as integration of charge over the entire discharging curve is needed to calculate the efficiency, which easily takes more than an hour. For this reason, we measured time until current reaches 90% of the maximum value, though rather arbitrarily, after the injection of glucose for each initial condition (Table 1) and compared them with the amperometric results (Fig. 2). Surprisingly, they exactly coincide with each other except for Gly-ini case. Since very low sensitivity and coulombic efficiency were resulted from Gly-ini condition, the direct comparison with other conditions is not meaningful. Based on these results, a glucose sensor has been constructed in which a microorganism was confined at the vicinity of the electrode surface by the membrane. Fig. 5 shows cyclic voltammetry of glucose oxidation with galactose-cultured *G. oxydans* in the presence of HNQ. The voltametric shape is more sigmoidal than when microorganisms are dispersed in solution (Fig. 1), indicating that the catalytic oxidation of glucose is a main contributor to the overall current. The direct reaction of HNQ is minimized because microorganisms that cover the electrode surface prevent the free access of HNQ molecules to the electrode. Oxidation current increases as the glucose concentration increases as expected.

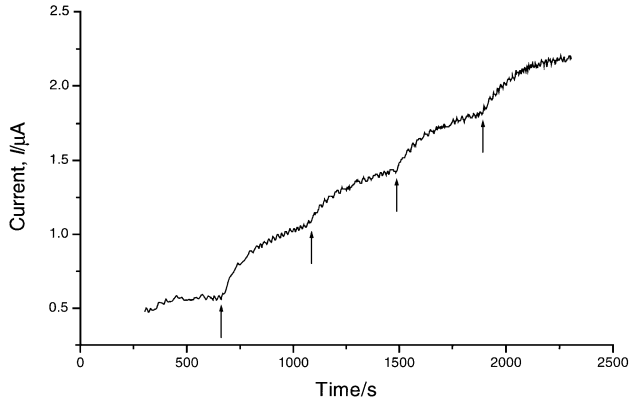


Fig. 6. Amperometric measurements of glucose oxidation by *G. oxydans* on the electrode polarized at +0.2 V vs. $Ag|AgCl|KCl_{(sat)}$. Concentrated glucose solution was injected while rotating the electrode at 1500 rpm. The injection is indicated by arrows, which correspond to 0.5, 1.0, 2.0, 3.0, and 4.0 mM of glucose, respectively. Cells were cultured under Gal-ini condition and physically confined at the electrode surface by the cellulose membrane (pore size 0.2 μm) for the measurements. $C_{HNQ} = 0.5$ mM.

Without *G. oxydans* or HNQ, no oxidation current was observed. Fig. 6 shows the amperometric measurements of glucose with the cells cultured in Gal-ini condition while rotating the electrode at 1500 rpm. *G. oxydans* was physically confined at the electrode surface by the cellulose membrane (pore size 0.2 μm). Purely based on sensitivity, a linear response over a millimolar range of glucose indicates that this type of glucose sensor may find applications in a clinical field if some means of giving selectivity is provided.

In conclusion, we have demonstrated that the changes in carbon sources in an initial culture medium induce different glycolytic pathways within a microorganism, which in turn exhibits different sensitivities toward the substrate. With an electrochemical cell consisted of *G. oxydans* and HNQ, the best sensitivity was obtained with Fru-ini condition. While the coulombic efficiency of a microbial fuel cell may not be a good indicator for the sensitivity, the current rising time at the injection of substrate could be directly related to the sensitivity. Our results show that it is possible to maximize the sensor sensitivity simply by tuning the initial carbon sources for the microorganism.

Acknowledgements

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