Available online at www.sciencedirect.com
ScienceDirect
Bioelectrochemistry 71 (2007) 38–45
Bioelectrochemistry
www.elsevier.com/locate/bioelechem

Electrical wiring of *Pseudomonas putida* and *Pseudomonas fluorescens* with osmium redox polymers

Suna Timur [a,b], Behzad Haghighi [a,c], Jan Tkac [a], Nurdan Pazarlıoğlu [b], Azmi Telefoncu [b], Lo Gorton [a,*]

[a] *Department of Analytical Chemistry, Lund University, P.O. Box 124, SE-221 00, Lund, Sweden*
[b] *Ege University, Faculty of Science, Biochemistry Department, 35100-Bornova, Izmir, Turkey*
[c] *Department of Chemistry Institute for Advanced Studies in Basic Sciences Gava Zang, Zanjan, P.O. Box 45195-1159, Iran*

Received 9 March 2006; received in revised form 14 August 2006; accepted 16 August 2006
Available online 18 August 2006

Abstract

Two different flexible osmium redox polymers; poly(1-vinylimidazole)$_{12}$-[Os-(4,4′-dimethyl-2,2′-di'pyridyl)$_2$Cl$_2$]$^{2+/+}$ (osmium redox polymer I) and poly(vinylpyridine)-[Os-(*N*,*N*′-methylated-2,2′-biimidazole)$_3$]$^{2+/3+}$ (osmium redox polymer II) were investigated for their ability to efficiently "wire" *Pseudomonas putida* ATCC 126633 and *Pseudomonas fluorescens* (*P. putida* DSM 6521), which are well-known phenol degrading organisms, when entrapped onto cysteamine modified gold electrodes. The two Os-polymers differ in redox potential and the length of the side chains, where the Os$^{2+/3+}$-functionalities are located. The bacterial cells were adapted to grow in the presence of phenol as the sole source of organic carbon. The performance of the redox polymers as mediators was investigated for making microbial sensors. The analytical characteristics of the microbial sensors were evaluated for determination of catechol, phenol and glucose as substrates in both batch analysis and flow analysis mode.

Keywords: Osmium redox polymers; Microbial biosensor

1. Introduction

Microbial cells have a number of advantages as biological sensing materials in the fabrication of biosensors. They are present ubiquitously, are able to metabolise a wide range of chemical compounds and have a great capacity to adapt with unfavourable conditions and to develop the ability to metabolise new chemicals. Microbes are also susceptible for genetic modifications through mutation or through recombinant DNA technology and serve as an economical source of intracellular enzymes. Purified enzymes, biological elements with high specific activities and high analytical specificity, are expensive and unstable and in this regard, the utilisation of whole cells as a source of intracellular enzymes avoids the lengthy and expensive operations of enzyme purification, preserves the enzyme in its natural environment and protects it from inactivation by external toxicants. Whole cells also provide a multipurpose catalyst especially when the process requires the participation of a number of enzymes in sequence [1–4]. The major limitation of microbial biosensors as compared to enzyme sensors is the slow response, which has been attributed to diffusional problems associated with the cell membranes. It is well known that microbial cells are able to reduce small redox compounds such as ferricyanide, dichlorophenolindophenol, and other organic dyes in the presence of organic compounds such as glucose and ethanol [5]. This indicates that microbial cells are able to catalyse the oxidation of the mentioned substrates using redox compounds as electron acceptors [6]. To follow electrochemically such processes through mediated electron transfer from microbial systems to electrodes represents a promising alternative to the use of a Clark electrode [7,8]. Perturbations in microbial respiration due to changes in substrate or microbial concentration have previously been detected via the interaction of redox mediators at electrochemical transducers and found the basis for a number of devices. Establishing a method for quantitative evaluation of intact cells as biocatalysts is beneficial for developing more advanced biofuel cell systems, whole cell-based

* Corresponding author. Tel.: +46 46 222 7582.
E-mail address: Lo.Gorton@analykem.lu.se (L. Gorton).

1567-5394/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.bioelechem.2006.08.001

biosensors and bioreactors [5] and also provides useful information concerning the enzymatic reactions proceeding within the intact cells under physiological conditions. Rapid detection of the concentration of bacteria was achieved using redox-mediated amperometry [9], which is free from influence by the turbidity of the bacterial cell suspension and allows the measurements of the consumption rates of artificial dyes by bacterial catalysis [10]. Mediated whole-cell biosensors have also been developed for on-line pesticide screening [11]. The reduction of a range of redox mediators by bacteria [12], including the reduction of ferricyanide by *E. coli* [13], have been studied to identify the most effective mediator-microorganism combinations for utilising substrates in microbial fuel cells [5,14,15]. Electron mediators perform a special function in biosensors; their role is to replace the natural electron acceptor usually oxygen, thus preventing the process from the problem of having a low oxygen concentration. An advantage of applying mediators is that the amperometric measurement can be performed at a less drastic potential, which reduces the possibility of interfering reactions to contribute to the response signal and thus enhancing selectivity. A notable number of mediator type biosensors based on either enzyme or whole microbial cells have been developed. Aqueous freely soluble mediators such as ferricyanide and *p*-benzoquinone, as well as less aqueous soluble mediators including ferrocenes have successfully been used in these systems [16,17].

Since the first applications of osmium redox polymers for reagentless mediated biosensing were described [18–20], polymeric mediators still attract attention due to the efficient electron shuttling properties combined with the polymeric structure promoting a stable adsorption as well as a possibility for multiple layers of immobilised enzymes as well as microbial cells on the electrode surface [21]. In developing biosensors, polymers containing dispersed redox centres are promising because of their synthetic flexibility and the ability to control the formal potential ($E^{\circ\prime}$), and hence the electron transfer properties [19,21].

In this work, *Pseudomonas fluorescens* (*Pseudomonas putida* DSM 6521) and *P. putida* ATCC 126633, which are well-known phenol degrading organisms, were wired with two different Os-polymers, one polymer with a high $E^{\circ\prime}$-value but with a restricted length of the side chains (poly(1-vinylimidazole)$_{12}$-[Os(4,4-dimethyl-2,2-di'pyridyl)$_2$Cl$_2$]$^{2+/+}$, osmium redox polymer I) [19], and one with a low $E^{\circ\prime}$-value but with long-side chains and with a much higher flexibility (poly (vinylpyridine)-[Os-(*N*,*N*-methylated-2,2-biimidazole)$_3$]$^{2+/3+}$, osmium redox polymer II), [22,23]. The positive effect of increasing the chain length of the side chain containing the mediator by the end of the side chain, was shown by Mao et al. in a recent publication [23], where the efficiency of wiring glucose oxidase by two different Os-polymers having the same $E^{\circ\prime}$-value was compared. One of the Os-polymers contained the Os-functionality at the end of a short length side chain and the other at the end of a long side chain. The two types of osmium redox polymers (osmium redox polymer I and II) were used recently, for electrical wiring of pyranose oxidase to graphite [22]. Osmium redox polymer I was recently shown also to wire whole living gram-negative bacteria *Gluconobacter oxydans* cells [24].

In this work the bacterial cells were entrapped together with either of these two redox polymers behind a dialysis membrane onto the surface of cysteamine modified gold electrode to form microbial biosensors. The response characteristics for catechol, phenol and glucose of the biosensors were investigated in both batch and flow mode.

2. Material and methods

2.1. Reagents

Poly(1-vinylimidazole)$_{12}$-[Os(4,4′-dimethyl-2,2′-di'pyridyl)$_2$ Cl$_2$]$^{2+/+}$(osmium redox polymer I) and poly(vinylpyridine)-[Os-(*N*,*N*′-methylated-2,2′-biimidazole)$_3$]$^{2+/3+}$(osmium redox polymer II) were generously provided as gifts from TheraSense (Alameda, CA, USA). Phenol, catechol, glucose, and cysteamine were purchased from Merck AG (Darmstadt, Germany). A 5 mM cysteamine solution was prepared by dissolving the appropriate amount in ethanol. All other chemicals were of analytical grade and used without further purification. Solutions used for immobilisation were prepared in ultrapure distilled water (Millipore, Milford, CT, USA) and the others used as substrate were in working buffer. Dialysis membranes with a cut-off of 6000–8000 Da were used.

Mineral standard medium (MSM) with the following compositions were used as growth media for *P. putida* (A) and *P. fluorescens* (B), respectively;

A. 0.1% NH_4NO_3, 0.05% $(NH_4)_2SO_4$, 0.05% NaCl, 0.05% $MgSO_4{\cdot}7H_2O$, 0.15% K_2HPO_4, 0.05% KH_2PO_4, 0.0014% $CaCl_2{\cdot}2H_2O$, 0.001% $FeSO_4{\cdot}7H_2O$ and trace element solution (1 ml/L), [25,26].
B. 0.244% Na_2HPO_4, 0.152% KH_2PO_4, 0.050% $(NH_4)_2SO_4$, 0.02% $MgSO_4{\cdot}7\ H_2O$, 0.005% $CaCl_2{\cdot}2H_2O$ and trace element solution (10 ml/L), [27]. The pH of the growth media was adjusted to 6.9.

2.2. Biological materials

P. fluorescens (*P. putida* DSM 6521) and *P. putida* ATCC 126633 were obtained from DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) and CCM (Czech Collection of Microorganisms, Masaryk University, Brno, Czech Republic), respectively, and were sub-cultured in nutrient agar. Adaptation of the cells to phenol (250 mg/L) was performed by gradually increasing the phenol and decreasing the glucose (250 mg/L) concentrations by daily inoculations until the medium contained 250 mg/L phenol. When the cells were grown, the biomass was harvested by centrifugation at 10,000 *g*, suspended in MSM and then re-centrifuged. The cellular paste was used for making the biosensor [25].

Cell growth was followed spectrophotometrically by measuring the optical density at 560 nm and the relationship between optical density and the living cells was also investigated [25]. In all experiments, log-phase bacterial cells were used. Daily prepared enzyme electrodes including fresh cells were used in all experimental steps.

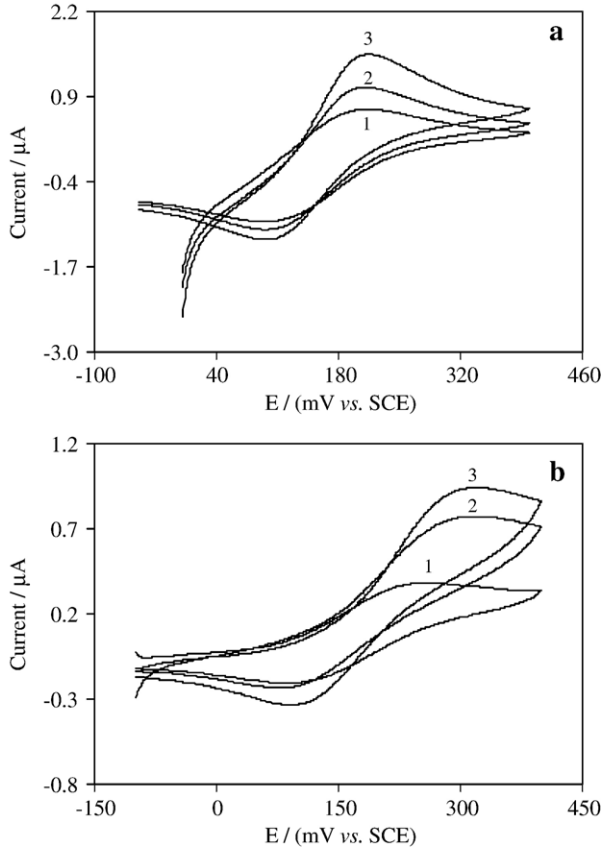

Fig. 1. Cyclic voltammograms of (a) *P. putida* and (b) *P. fluorescens* cells mediated with osmium redox polymer I on cysteamine modified gold electrodes in the absence and presence of substrates at a scan rate of 5 mV s^{-1}. [(1) Blank, (2) Glucose; 1 mM, (3) Catechol; 0.1 mM].

2.3. Equipment

Cyclic voltammetric studies were performed using a CV-100W voltammetric analyser (BAS, West Lafayette, IN, USA) with a conventional three-electrode set-up. The microbial sensors were used as the working electrodes, a saturated calomel electrode (SCE, Radiometer, Copenhagen Model K-401) as the reference electrode and a platinum rod served as the auxiliary electrode.

Amperometric measurements were done in both batch and flow injection modes. Batch mode of analysis was performed using the same electrodes as for cyclic voltammetry. Successive portions of sample solution containing substrate were added into the electrochemical cell containing 25 ml of phosphate buffer solution (0.1 M at the various pHs) using a sampler. During the course of experiment the phosphate buffer solution was stirred using a magnetic stirrer.

Flow injection mode of analysis was performed using a single line flow injection manifold with a three-electrode electrochemical flow through cell of the wall-jet type [28]. A Gilson peristaltic pump model Minipuls 2 (Villier-le-Bel, France) equipped with silicon tubing (0.89 mm i.d.) propelled the phosphate buffer (0.1 M at the various pHs) as the carrier into the flow line with a flow rate of 0.5 ml min^{-1}, if not stated otherwise. The flow line was made from Teflon tubing (0.5 mm i.d.). A microbial sensor, an Ag|AgCl (0.1 M KCl) electrode and a platinum wire were used as the working, reference and auxiliary electrodes, respectively. A 50 μl sample solution containing substrate was injected into the carrier stream via a LabPRO six-port injection valve (PR700-100-01, Rheodyne, Cotati, CA, USA).

The working potential for both modes of analysis was applied by a three-electrode potentiostat (Zäta Electronics, Lund, Sweden) and the output signal was recorded by a strip chart recorder (Kipp and Zonen, type BD111, Delft, The Netherlands). All measurements were performed at room temperature.

2.4. Preparation of the electrode modified with bacterial whole cells

Gold disk electrodes (A = 0.031 cm^2, BAS, West Lafayette, IN, USA) were polished on Microcloth (Buehler, Germany) in aqueous alumina suspension (0.1 μm, Stuers, Copenhagen, Denmark), rinsed with Milli-Q water, and then, electrochemically cleaned by cycling in 0.1 M H_2SO_4 between −0.3 and +1.7 V vs. SCE, washed thoroughly with water and immediately used for surface modification.

The electrode surface was modified with cysteamine to form a self-assembled monolayer by immersion of the electrochemically activated gold electrode into a 5 mM solution of cysteamine in ethanol for 45 min. Poly(1-vinylimidazole)$_{12}$-

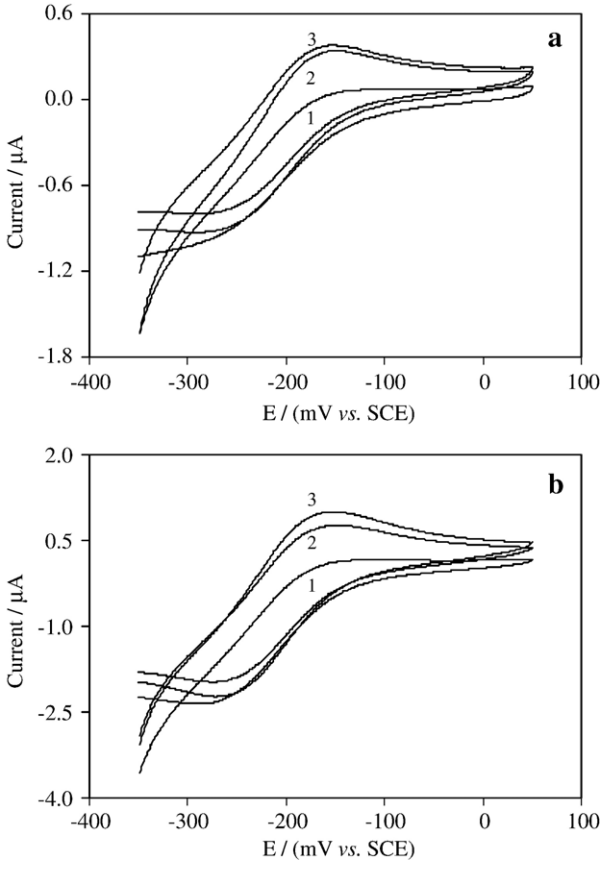

Fig. 2. Cyclic voltammograms of (a) *P. putida* and (b) *P. fluorescens* cells mediated with osmium redox polymer II on cysteamine modified gold electrodes in the absence and presence of substrates at a scan rate of 5 mV s^{-1}. [(1) Blank, (2) Glucose; 1 mM, (3) Phenol; 0.2 mM].

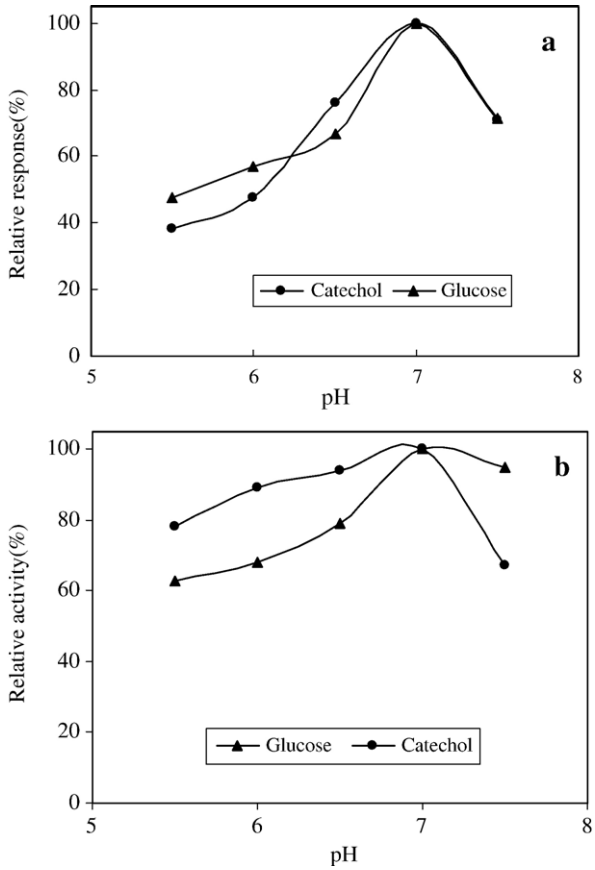

Fig. 3. Variation of response versus pH for (a) *P. putida* and (b) *P. fluorescens* based systems with osmium redox polymer I, [pH 5.5–7.5; potassium phosphate buffer (50 mM), (For *P. putida*; catechol concentration; 0.02 mM and glucose concentration; 0.4 mM), (For *P. fluorescens*; catechol concentration; 0.01 mM, glucose concentration; 0.2 mM), applied potential; 300 mV].

$[Os(4,4'\text{-dimethyl-}2,2'\text{-di'pyridyl})_2Cl_2]^{2+/+}$(osmium redox polymer I) and poly(vinylpyridine)-$[Os\text{-}(N,N'\text{-methylated-}2,2'\text{-biimidazole})_3]^{2+/3+}$(osmium redox polymer II) were used, respectively for the preparation of the biosensors. A 5 μl portion of a solution of the osmium redox polymer (10 mg/mL in water) in Milli-Q water was spread over the surface of the modified gold electrode and water was allowed to evaporate at 25 °C (20 min). In the following step, 5 μL of the cellular paste (from 0.3 g/L of bacterial mass, in 0.1 M phosphate buffer, pH 7.0), was evenly spread on top of the modified electrode and gently dried-up for 1 h at room temperature. Finally the surface of the bacterial modified electrode was covered with a permselective dialysis membrane (MWCO < 6000–8000) pre-soaked in water. The membrane was fixed tightly with a silicone rubber O-ring [24]. The microbial sensors were initially equilibrated in MSM (growth medium) solution and phosphate buffer, respectively. After 30 min, substrates were added individually to the reaction cell. Nitrogen was passed from all solutions before use.

3. Results and discussion

A variety of redox-active substances can serve as electron acceptors and can thus be reduced by certain microorganisms. They can also serve as electron shuttling molecules between microbial cells and electrodes. Such 'mediators' have been applied for making microbial fuel cells and for microbial detection [4,5]. It has been suggested that reduction of the redox mediator, rather than molecular oxygen, is due to the metabolic reactions of microorganisms. Hence, instead of oxygen as an indicator of respiratory metabolic activity, various mediators, which could be used either in solution or polymerised on the electrode surface, have been used as the electroactive compound for the development of amperometric microbial sensors [29]. Gram-negative bacterial cells have respiratory redox proteins located in the cell membrane and accessible from the periplasm via porins, which make the outer membrane permeable for a wide variety of low molecular-weight mediators [30]. In this work, two different flexible osmium redox polymers; poly(1-vinylimidazole)$_{12}$-$[Os\text{-}(4,4'\text{-dimethyl-}2,2'\text{-di'pyridyl})_2Cl_2]^{2+/+}$ and poly(vinylpyridine)-$[Os\text{-}(N,N'\text{-methylated-}2,2'\text{-biimidazole})_3]^{2+/3}$ were used for electrical wiring of the bacterial cells to the cysteamine modified gold electrode. The microbial cells grown initially on glucose but slowly adapted to grow on phenol as the major organic carbon source were used as the biological component. After the adaptation step, both bacterial cells are able to degrade phenolic compounds because of their

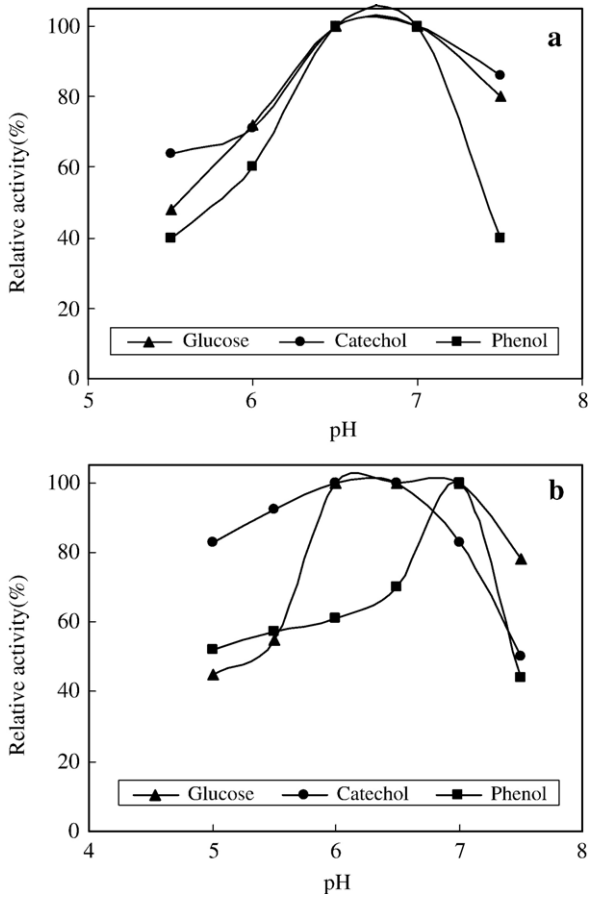

Fig. 4. Variation of response versus pH for (a) *P. putida* and (b) *P. fluorescens* based systems with osmium redox polymer II, [pH 5.5–7.5; potassium phosphate buffer (50 mM), (For *P. putida*; catechol concentration; 0.08 mM, glucose concentration; 0.2 mM, phenol concentration; 0.08 mM), (For *P. fluorescens*; catechol concentration; 0.02 mM, glucose concentration; 0.2 mM, phenol concentration; 0.02 mM), applied potential; −180 mV].

Table 1
Analytical characteristics of the osmium redox polymer I and II based microbial sensors fabricated with *P. putida* and *P. fluorescens* in batch amperometric measurements

Microbial sensors		Substrate	Linear range/mM	Calibration equation*	R^2
Osmium redox polymer I	*P. putida*	Glucose	0.2–1.4	$y=13.7x-0.091$	0.997
		Catechol	0.005–0.025	$y=1548.6x+0.143$	0.997
	P. fluorescens	Glucose	0.05–1.0	$y=100.75x-0.72$	0.999
		Catechol	0.005–0.02	$y=2524x-2.06$	0.989
Osmium redox polymer II	*P. putida*	Glucose	0.1–2.2	$y=26.2x+1.3$	0.987
		Catechol	0.02–0.12	$y=142.7x+0.18$	0.998
		Phenol	0.02–0.08	$y=394.5x+0.04$	0.999
	P. fluorescens	Glucose	0.2–2.0	$y=28.36x-2.6$	0.988
		Catechol	0.02–0.08	$y=278.53x-0.58$	0.993
		Phenol	0.02–0.2	$y=377.4x+0.84$	0.995

Conditions: electrolyte solution; 0.1 M phosphate buffer pH 7.0; applied potential for type I and type II polymer based sensors; +300 mV and −180 mV vs. SCE, respectively.
*x; concentration in mM, y; signal intensity in nA.

Table 2
Analytical characteristics of the osmium redox polymer I and II based microbial sensors fabricated with *P. putida* and *P. fluorescens* in flow injection amperometric measurements

Microbial sensors		Substrate	Linear range/mM	Calibration equation*	R^2
Osmium redox polymer I	*P. putida*	Glucose	1.0–7.5	$y=0.281x+0.082$	0.995
		Catechol	0.025–0.2	$y=25.26x-0.24$	0.994
	P. fluorescens	Glucose	2.5–15	$y=1.26x-2.3$	0.996
		Catechol	0.1–1.25	$y=5.01x-0.1227$	0.981
Osmium redox polymer II	*P. putida*	Glucose	1.0–7.5	$y=0.79x+0.22$	0.995
		Catechol	0.1–1.0	$y=3.22x+0.06$	0.998
	P. fluorescens	Glucose	1.0–8.0	$y=0.58x-0.08$	0.989
		Catechol	0.1–0.8	$y=1.47x+0.061$	0.986

Conditions: carrier solution; 0.1 M phosphate buffer pH 7.0; flow rate; 0.5 mL min^{-1}, applied potential for osmium redox polymer I and II polymer based sensors; +300 mV and −180 mV vs. SCE, respectively.
*x; concentration in mM, y; signal intensity in nA.

production of phenol hydroxylases (phenol monooxygenases; E. C 1.14.13.7) during the adaptation phase and further metabolic enzymes that are produced in the bacterial cells only in the presence of such substrates in the adaptation phase. Phenol monooxygenases also known as flavoprotein monooxygenase are single-component or multicomponent enzymes that catalyse the initial steps in a variety of aromatic biodegradation pathways and are of interest for bioremediation strategies as well as for biocatalytic applications, because the regioselective hydroxylation of phenols to catechols is notoriously difficult to achieve by means of chemical methods [31]. The catechol dioxygenases; catechol 1,2-dioxygenase (E.C 1.13.11.1) and catechol 2,3-dioxygenase (E. C 1.13.11.2) are non-haem iron-dependent metallo-enzymes, which catalyse the oxidative cleavage of the enzymatic product of phenol hydroxylases by using molecular oxygen in *Pseudomonas* sp. as a part of the bacterial catabolic pathways [32]. Due to these facts, till now various *Pseudomonas* sp. have been combined with a number of different biotechnological processes that have been employed in several industrial productions, in biomedical applications and in environmental remediation [33].

Electron transfer mediators are usually needed to be able to establish an efficient electron transport between redox enzymes with bound cofactors such as flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), pyrroloquinoline quinone (PQQ), and also for $NAD(P)^+$-dependent dehydrogenases (that require soluble $NAD(P)^+$ cofactor and an electrode catalytically active for the oxidation of NAD(P)H and regeneration of $NAD(P)^+$) and an electrode [34–36]. The conductive properties of $Os^{2+/3+}$ redox polymers promote a good electrical communication between the electron donating systems in the bacteria and the electrode surface. Because of the good contact between *G. oxydans* cells and osmium redox polymer I, an efficient electron transfer between the bacterial cells and a cysteamine modified Au-electrode could be established. Modification of the electrode with a protective self-assembled cysteamine layer prevents any possible strong adsorption of bacterial cells onto the surface of the gold electrode, which could cause cell destruction. The charged character of the cysteamine layer minimises any possible destructive hydrophobic interactions between the plain gold electrode and the bacterial cells [24]. Strong electrostatic interactions between the negatively charged *Gluconobacter* cells and the positively charged $Os^{2+/3+}$-polymer further helped in facilitating electron transfer from the cells to the electrode.

In this work, similar observations to those reported for *G. oxydans* [24] have also been found for both *P. putida* and *P. fluorescens* cells. In the presence of an $Os^{2+/3+}$-polymer a response current can be observed in the presence of a carbon source. When using osmium redox polymer I, no signal was observed for phenol in contrast to catechol. Hence, the amperometric response might arise from the activity of catechol dioxygenases instead of phenol hydroxylases in both cells. Moreover, glucose was also tested as a substrate and caused a response in all

Table 3
Reproducibility of osmium redox polymer I and II based microbial sensors fabricated with *P. putida* and *P. fluorescens*

Microbial sensors		[Substrate]/μM	RSD (%)
Osmium redox polymer I	*P. putida*	Catechol (5)	2.9
	P. fluorescens	Catechol (5)	1.9
Osmium redox polymer II	*P. putida*	Phenol (20)	2.6
	P. fluorescens	Phenol (20)	3.2

RSD, relative standard deviation for 5 times repetitive measurements.

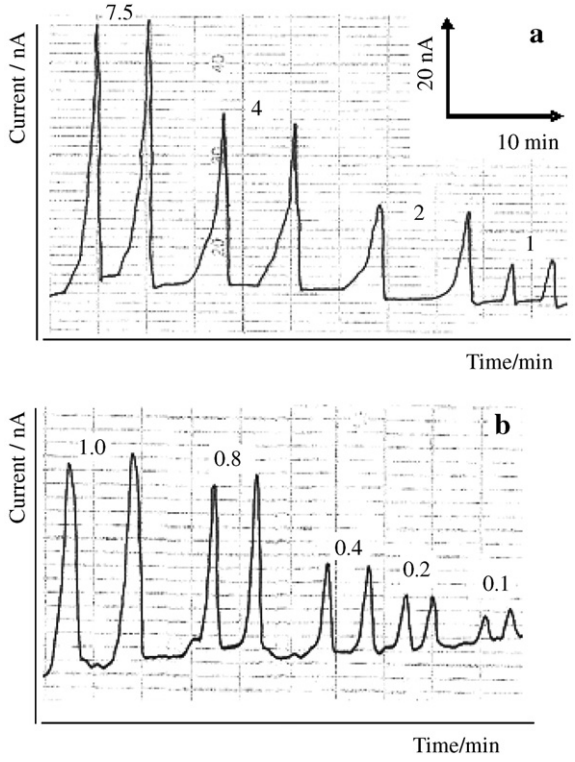

Fig. 5. Calibration trace obtained for (a) glucose and (b) catechol using a microbial sensor fabricated with *P. putida* and osmium redox polymer II. Conditions: carrier solution; 0.1 M phosphate buffer pH 7.0, flow rate; 0.5 mL/min, applied potential; −180 mV vs. Ag|AgCl. Number above peaks denotes concentration of substrate in mM.

systems probably because of quinoprotein glucose dehydrogenases [E.C 1.1.5.2], found in the outer surface of the cytoplasmic membrane [37] in *P. fluorescens* and NAD^+ dependent glucose 1-dehydrogenases [E.C 1.1.1.118] in *P. putida* [38]. However, lower responses for glucose were obtained compared to those for catechol most likely as the result of the adaptation process. Cyclic voltammograms of bacterial cells wired with osmium redox polymer I and II, respectively, on cysteamine modified gold electrode in the absence and presence of substrates are shown in Figs. 1 and 2, respectively.

The formal redox potentials of the two polymers were originally determined to be +140 mV and −195 mV, respectively [19,23], very similar to values reported in our previous work, i.e., +140 mV and −185 mV for osmium redox polymer I and II [22].

The mentioned data were used to choose the proper working potentials of our systems and the potential dependence of the sensitivity toward phenol as substrate was investigated (results not shown). High and stable responses were obtained at +0.300 V and −0.180 V vs. SCE for osmium redox polymer I and II, respectively, and used throughout all amperometric measurements.

3.1. Effect of pH

The effect of pH on the sensor response was tested using phosphate buffers (0.1 M) between pH 5.5 and 7.5. The results of the relative activity versus pH are shown in Figs. 3 and 4.

The optimum pH of the osmium redox polymer I based systems was obtained as 7.0 for both catechol and glucose. A decrease in the response was observed at lower and higher pH values for both types of bacterial cells. However, in the case of using the other redox polymer, osmium redox polymer II, the pH optimum was found to be between 6.5 and 7.0 for *P. putida* and between 6.0 and 7.0 for *P. fluorescens* and a drop was also observed for lower and higher pH values. As can be seen in Figs. 3 and 4, the pH optima were dependent on both the metabolic process i.e., glucose or catechol consumption, as well as type of redox polymer. It seems as though differences in ionic properties and degree of hydrophobicity of the two redox polymers due to the change of pH affect the interactions of the polymers with the redox enzymes in the catabolic pathway of the bacteria. A variation in pH optima depending on redox polymer was also observed in our previous study on "wiring" pyranose oxidase [22]. As a result, pH 7.0, which is also very close to the pH of the growth medium, was chosen as optimum and used for further studies.

3.2. Analytical characteristics

Calibration equations and linear dynamic ranges were obtained using catechol and glucose as substrate for both osmium redox polymer I and II based sensors fabricated with *P. putida* and *P. fluorescens* at the selected conditions. As has been indicated in Table 1, the use of the alkylated biimidazole complex (osmium redox polymer II) with the longer tethers with respect to osmium redox polymer I, caused approximately a ten

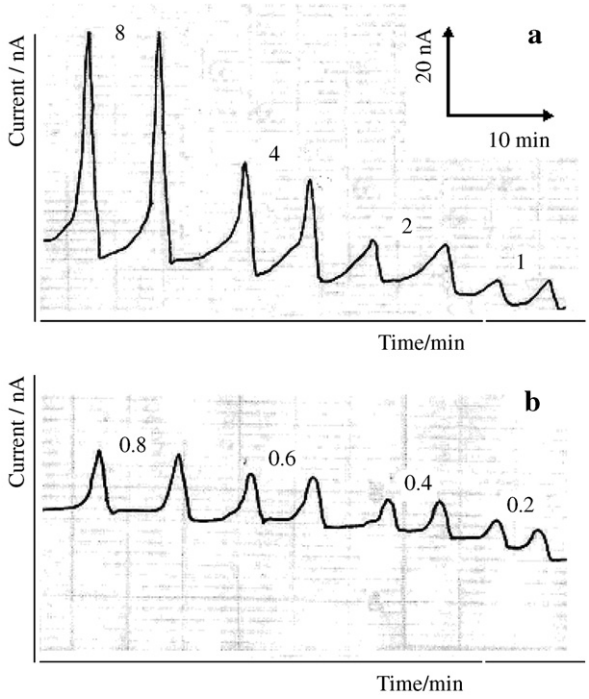

Fig. 6. Calibration trace obtained for (a) glucose and (b) catechol using a microbial sensor fabricated with *P. fluorescens* and osmium redox polymer II. Conditions: carrier solution; 0.1 M phosphate buffer pH 7.0, flow rate; 0.5 mL/min, applied potential; −180 mV vs. Ag|AgCl. Number above peaks denotes concentration of substrate in mM.

times higher sensitivity similar to those results obtained in a previous study [22]. Also, phenol could additionally be detected besides catechol and glucose using the osmium redox polymer II based sensors. This might be due to the good electrical communication between the longer tether of the osmium redox polymer II and phenol hydroxylase enzymes in the cells.

Amperometric responses of the microbial sensors for glucose and catechol were also investigated in the flow injection mode. The effect of the flow rate on the microbial amperometric response was explored for the osmium redox polymer II based sensor fabricated with *P. putida* using 2 mM glucose as substrate. As a result of this experiment, current values were found to be 3 nA, 2.2 nA and 1.1 nA for 0.25 ml min^{-1}, 0.5 ml min^{-1} and 1.0 ml min^{-1}, respectively, reflecting the strongly kinetically controlled response. For a flow rate of 0.25 ml min^{-1}, a higher response peak was obtained but the peak width was about 4 min. For further experiments a flow rate of 0.5 ml min^{-1} was chosen as a compromise between response intensity and sample throughput. The results are given in Table 2.

As was obvious, when the biosensors were used in the flow injection system because of the restricted contact time of the substrate with the bioactive microorganism layer in combination with dilution of the sample concentration in the flow system before reaching the electrode surface, lower responses were observed in comparison with responses obtained for batch measurements. Additionally, even though all solutions were carefully degassed and kept under N_2 some O_2 may leak into the carrier solution and may compete with the osmium redox polymer as the electron acceptor, which would cause a decrease in the response.

The reproducibility of the fabricated sensors was determined by repetitive injection of catechol and phenol solutions. The results are given in Table 3.

Studies of the operational stability of the fabricated sensors in batch analysis showed that the osmium redox polymer I based sensors fabricated with *P. putida* and *P. fluorescens*, lost 58% and 41% their activity, respectively, in repeated use over 480 min (40 assays) and the corresponding values for the osmium redox polymer II based sensors fabricated with *P. putida* and *P. fluorescens* were about 25% and 35%, respectively. The improvement of the operational stability, which was observed for microbial sensors based on osmium redox polymer II can be an important advantage for practical use of the wired bacterial systems as a part of microbial fuel cell as well as part of sensor for BOD measurements. In Figs. 5 and 6 some flow injection peaks for both *P. putida* and *P. fluorescens* using various concentrations of both glucose and phenol are shown using osmium redox polymer II. The figures also show the reproducibility between the response for the injected samples and additionally that the shape of the peaks are different for the two substrates possibly reflecting different kinetic restrictions for the metabolism of glucose compared with that for phenol.

4. Conclusion

The concept of directly electrically ‘‘wiring’’ the enzymes in the respiratory chain of intact bacteria has already been studied [24]. In the case reported here, the basis of “wiring” whole bacterial cells is mainly based on electron transfer between redox enzymes, which are not membrane bound, and artificial polymeric electron acceptors. The bacterial cells used in this work were grown in the presence of phenol for the adaptation process. Since, phenol was reported to act as a membrane active agent that increases the permeability of the cytoplasmic materials such as amino acids, purines and pyrimidines [39], hence, the adaptation process enables cytosolic enzymes such as phenol hydroxylase and catechol dioxygenases to be accessible somehow for the redox polymers. The mechanism behind this is not known. Besides porins, the presence of accessory proteins in the cytoplasmic membrane, which might act as channels for redox polymers to reach the cytosolic enzymes, has also been reported in early studies to explain the phenomena by which microbes respond to environmental changes [39]. Natural charge transfer pathways between the cytosolic redox enzymes and the respiratory redox enzymes located in the periplasmic membrane may also be involved and explain the possibility to “wire” the cells studied here and obtain a response originating from redox reactions occurring in the cytosol. All these facts might be effective to be able to receive a response from the fabricated microbial sensors. However, as far as *P. fluorescens* based sensors were concerned, the response for glucose in contrast to the responses to phenol and catechol was probably due to the interaction of the membrane bound quinoprotein glucose dehydrogenases with Os polymers as already observed for *G. oxydans* cells [24].

Moreover, several microbial species have been reported to release electrons to an electrode directly or with the use of their electroactive metabolites [5,40]. However, bacterial species do not readily release electrons directly with electrodes and hence the intervention of synthetic and/or natural compounds, i.e., redox mediators, is required [41,42]. A different type of microbial fuel cell has been described, designed for the treatment of sewage and landfill effluent wastewater [41]. It could be also possible to use *Pseudomonas* sp. and osmium redox polymers, which provide oxygen independent measurement with high sensitivity, owing to the fast electron collection efficiencies of the osmium redox polymers that compete very well with molecular oxygen, as a part of microbial fuel cell studies as well as BOD measurements.

Acknowledgements

The authors thank the following agencies for the financial support; The Swedish Research Council (VR), The Swedish International Development Cooperation Agency (SIDA) and The Turkish Scientific Technical and Research Consultation (TUBITAK).

References

[1] T.T. Bachman, U. Bilitewski, R.D. Schmid, A microbial sensor based on *Pseudomonas putida* for phenol, benzoic acid and their monochlorinated derivatives which can be used in water and *n*-hexane, Anal. Lett. 31 (1998) 2361–2373.

[2] B. Beyersdorf-Radeck, U. Karlson, R.D. Schmid, A microbial sensor for 2-ethoxyphenol, Anal. Lett. 27 (1994) 285–298.
[3] B. Beyersdorf-Radeck, K. Riedel, U. Karlson, T.T. Bachman, R.D. Schmid, Screening of xenobiotic compounds degrading microorganisms using biosensor techniques, Microbiol. Res. 153 (1998) 239–245.
[4] J. Racek, Cell-based Biosensors, Technomic Publ. Co., Lancaster, PA, USA, 1995.
[5] K. Rabaey, W. Verstraete, Microbial fuel cells: novel biotechnology for energy generation, Trends Biotechnol. 23 (2005) 291–298.
[6] T. Ikeda, K. Kano, An electrochemical approach to the studies of biological redox reactions and their applications to biosensors, bioreactors, and biofuel cells, Rev. J. Biosci. Bioeng. 92 (2001) 9–18.
[7] H.P. Bennetto, J. Box, G.M. Delaney, J.R. Mason, S.D. Roller, J.L. Stirling, C.F. Thurston, Redox-mediated electrochemistry of whole-micro-organisms: from fuel cells to biosensors, in: A.P.F. Turner, I. Karube, G.S. Wilson (Eds.), Biosensors — Fundamentals and Applications, Oxford University Press, Oxford, 1987, pp. 291–314.
[8] N.J. Richardson, S. Gardner, D.M. Rawson, A chemically mediated amperometric biosensor for monitoring eubacterial respiration, J. Appl. Bacteriol. 70 (1991) 422–426.
[9] G. Ramsay, A.P.F. Turner, Development of an electrochemical method for the rapid determination of microbial concentration and evidence for the reaction mechanism, Anal. Chim. Acta 215 (1988) 61–69.
[10] T. Ikeda, T. Kurosaki, K. Takayama, K. Kano, K. Miki, Measurements of oxidoreductase like activity of intact bacterial cells by an amperometric method using a membrane-coated electrode, Anal. Chem. 68 (1996) 192–198.
[11] D.M. Rawson, A.J. Willmer, A.P.F. Turner, Whole-cell biosensors for environmental monitoring, Biosensors 4 (1989) 299–311.
[12] S.D. Roller, H.P. Bennetto, G.M. Delaney, J.R. Mason, J.L. Stirling, C.F. Thurston, Electron-transfer coupling in microbial fuel cells 1. Comparison of redox-mediator reduction rates and respiratory rates of bacteria, J. Chem. Technol. Biotechnol. 34B (1984) 3–12.
[13] L.P. Hadjipetrou, T. Gray-Young, M.D. Lilly, Effect of ferricyanide on energy production by *Escherichia coli*, J. Gen. Microbiol. 45 (1966) 479–488.
[14] N.F. Pasco, K.H. Baronian, C. Jeffries, J. Hay, Biochemical mediator demand — a novel rapid alternative for measuring biochemical oxygen demand, Appl. Microbiol. Biotechnol. 53 (2000) 613–618.
[15] N. Pasco, K. Baronian, C. Jeffries, J. Webber, J. Haya, MICREDOX® — development of a ferricyanide-mediated rapid biochemical oxygen demand method using an immobilised *Proteus vulgaris* biocomponent, Biosens. Bioelectron. 20 (2004) 524–532.
[16] K. Kano, T. Ikeda, Fundamentals and practices of mediated bioelectrocatalysis, Anal. Sci. 16 (2000) 1013–1021.
[17] K. Takayama, T. Kurosaki, T. Ikeda, T. Nagasawa, Bioelectrocatalytic hydroxylation of nicotinic acid at an electrode modified with immobilized cells of *Pseudomonas fluorescens* in the presence of electron transfer mediators, J. Electroanal. Chem. 381 (1995) 47–53.
[18] Y. Degani, A. Heller, Electrical communication between redox centers of glucose-oxidase and electrodes via electrostatically and covalently bound redox polymers, J. Am. Chem. Soc. 111 (1989) 2357–2358.
[19] A. Heller, Electrical connection of enzyme redox centers to electrodes, J. Phys. Chem. 96 (1992) 3579–3587.
[20] T.J. Ohara, R. Rajagopalan, A. Heller, Glucose electrodes based on cross-linked $[Os(bpy)_2Cl]^{+/2+}$ complexed poly(1-vinylimidazole) films, Anal. Chem. 65 (1993) 3512–3517.
[21] H. Karan, Enzyme biosensors containing polymeric electron transfer systems, in: L. Gorton (Ed.), Biosensors and Modern Biospecific Analytical Techniques, Book Series: Comprehensive Analytical Chemistry, vol. XLIV, Elsevier, Amsterdam, 2005, pp. 131–178.
[22] S. Timur, Y. Yigzaw, L. Gorton, Electrical wiring of pyranose oxidase with osmium redox polymers, Sens. Actuators, B, Chem. 113 (2006) 684–691.
[23] F. Mao, N. Mano, A. Heller, Long tethers binding redox centers to polymer backbones enhance electron transport in enzyme "wiring" hydrogels, J. Am. Chem. Soc. 125 (2003) 4951–4957.
[24] I. Vostiar, E.E. Ferapontova, L. Gorton, Electrical wiring of viable *Gluconobacter oxydans* cells with flexible osmium-redox polyelectrolyte, Electrochem. Commun. 6 (2004) 621–626.
[25] S. Timur, N. Pazarlioglu, R. Pilloton, A. Telefoncu, Detection of phenolic compounds by thick film sensors based on *Pseudomonas putida*, Talanta 61 (2003) 87–93.
[26] S. Timur, L.D. Seta, N. Pazarlioglu, R. Pilloton, A. Telefoncu, Screen printed graphite biosensors based on bacterial cells, Process Biochem. 39 (2004) 1325–1329.
[27] E.A. Barnsley, The bacterial degradation of fluoranthene and benzo[a] pyrene, Can. J. Microbiol. 21 (1975) 1004–1008.
[28] R. Appelqvist, G. Marko-Varga, L. Gorton, A. Torstensson, G. Johansson, Enzymatic determination of glucose in a flow system by catalytic oxidation of the nicotinamide coenzyme at a modified electrode, Anal. Chim. Acta 169 (1985) 237–247.
[29] N. Yoshida, K. Yano, T. Morita, S.J. McNiven, H. Nakamura, I. Karube, A mediator-type biosensor as a new approach to biochemical oxygen demand estimation, Analyst 125 (2000) 2280–2284.
[30] J. Tkac, V. Stefuca, P. Gemeiner, Biosensors with immobilised microbial cells using amperometric and thermal detection principles, in: V. Nedovic, R. Willaert (Eds.), Applications of Cell Immobilisation Biotechnology, Book Series: Focus in Biotechnology, vol. 8B, Springer, 2005, pp. 549–566.
[31] M.H.M. Eppink, E. Cammaart, D. van Wassenaar, W.J. Middelhoven, W.J.H. van Berkel, Purification and properties of hydroquinone hydroxylase, a FAD-dependent monooxygenase involved in the catabolism of 4-hydroxybenzoate in *Candida parapsilosis* CBS604, Eur. J. Biochem. 267 (2000) 6832–6840.
[32] C. Nakai, G. Yamazaki, H. Kagamiyma, M. Nozakai, Amino acid sequence of catechol 1,2-dioxygenase (pyrocatechase) isozymes alpha alpha from *Psedomonas arvilla* C-1, Biochem. Mol. Biol. Int. 39 (1996) 781–788.
[33] H.J. Heipieper, R. Diefenbach, H. Keweloh, Conversion of *cis* unsaturated fatty acids to *trans*, a possible mechanism for the protection of phenol-degrading *Pseudomonas putida* P8 from substrate toxicity, Appl. Environ. Microbiol. 58 (1992) 1847–1852.
[34] L. Habermüller, M. Mosbach, W. Schuhmann, Electron-transfer mechanisms in amperometric biosensors, Fresenius', J. Anal. Chem. 366 (2000) 560–568.
[35] A. Chaubey, B.D. Malhotra, Mediated biosensors, Biosens. Bioelectron. 17 (2002) 441–456.
[36] W. Schuhmann, Amperometric enzyme biosensors based on optimised electron-transfer pathways and non-manual immobilisation procedures, Rev. Mol. Biotechnol. 82 (2002) 425–441.
[37] K. Matsushita, M. Ameyama, D-Glucose dehydrogenase from *Pseudomonas fluorescens*, membrane-bound, Methods Enzymol. 89 (1982) 149–154.
[38] A.S.L. Hu, A.L. Cline, The regulation of some sugar dehydrogenases in a *Pseudomonad*, Biochim. Biophys. Acta 93 (1964) 237–245.
[39] A. Sharma, D. Kachroo, R. Kumar, The dependent influx and exflux of phenol by immobilized microbial consortium, Environ. Monit. Assess. 76 (2002) 195–211.
[40] D.E. Holmes, D.R. Bond, D.R. Lovley, Electron transfer by *Desulfobulbus propionicus* to Fe(III) and graphite electrodes, Appl. Environ. Microbiol. (2004) 1234–1237.
[41] W. Habermann, E.-H. Pommer, Biological fuel cells with sulphide storage capacity, J. Appl. Microbiol. Biotechnol. 35 (1991) 128–133.
[42] G.T.R. Palmore, G.M. Whitesides, Microbial and enzymatic biofuel cells, Enzymatic Conversion of Biomass for Fuels Production, Oxford University Press, 1994, pp. 271–290.