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Electrochemical polymerization of 1-(4-nitrophenyl)-2,5-di(2-thienyl)-1 *H*-pyrrole as a novel immobilization platform for microbial sensing

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ABSTRACT

Two types of bacterial biosensor were constructed by immobilization of *Gluconobacter oxydans* and *Pseudomonas fluorescens* cells on graphite electrodes modified with the conducting polymer; poly(1-(4-nitrophenyl)-2,5-di(2-thienyl)-1 *H*-pyrrole) [SNS(NO_2)]. The measurement was based on the respiratory activity of cells estimated by the oxygen consumption at −0.7 V due to the metabolic activity in the presence of substrate. As well as analytical characterization, the linear detection ranges, effects of electropolymerization time, pH and cell amount were examined by using glucose as the substrate. The linear relationships were observed in the range of 0.25–4.0 mM and 0.2–1.0 mM for *G. oxydans* and *P. fluorescens* based sensors, respectively.

1. Introduction

Since 1977, increasing interest has been devoted to the electrical properties of a new-class material, organic conducting polymers due to their facile synthesis, good environmental stability and electrochemical properties [1–3]. They can be used as light emitting diodes [4], batteries [5], solar cells [6], enzyme immobilization matrices [7,8] sensors [9,10], photovoltaics [11], ion exchange membrane in fuel cells [12], drug release systems [13] and electrochromic devices [14] and [15]. Polypyrrole (PPy) is one of the most extensively used conducting polymers in design of bioanalytical sensors as well as for other purposes due to their good electrical properties, long-term stability of its conductivity and to the possibility of forming homopolymers or composites with optimal mechanical properties [16]. In previous works, glucose oxidase, lactate oxidase, and galactose oxidase [17] and alcohol oxidase [18] were immobilized using pyrrole or derivatives and their biosensing capacity has been also widely investigated. As well as catalytic biosensors [19], immunosensors [20] and DNA biosensors [21] based on PPy have been reported. Moreover, PPy which protects electrodes from interfering with electrochemically active materials [22] is mostly used in affinity sensors due to its biocompatibility. It also offers an easy way for immobilizing various biologically active compounds. Preparation and basic characterization of polypyrrole-based molecularly imprinted polymer (MIP) for label-free detection of bovine leukemia virus glycoprotein *gp*51 were also described in the previous work where PPy was successfully used as a matrix for the MIP preparation [23]. On the other hand, use of this conducting polymer was reported in the design of a fluorescence-based immunosensor where PPy induced fluorescence quenching was utilized [24]. Apart from its biocompatibility, PPy is capable to transduce energy due to the interaction of analyte and analyte-recognizing-site into electrical signals that are easily monitored. Currently, this polymer becomes one of the major tools for nanobiotechnological applications [19].

Immobilized microbial cells have been used in a variety of applications such as bioreactors [25], biocontrol [26], biodegradation [27] and biosensors [28]. Using microbial cells as the biocomponent in biosensors has several advantages; for instance they are present ubiquitously and are able to metabolise a wide range of chemical compounds. Furthermore, microorganisms have a great capacity to adapt to adverse conditions and to develop the ability to degrade new molecules with time. Microbes are also amenable for genetic modifications through mutation or through recombinant DNA technology [29]. Although purified enzymes have very high specificity for their substrates or inhibitors, their application in biosensors construction may be limited by the tedious, time-consuming and costly enzyme purification, requirement of multiple enzymes to generate the measurable product or need of cofactor/coenzyme. Microorganisms provide an ideal alternative to these bottle-necks [30].

Various immobilization matrices were previously obtained by the copolymerization of [SNS(NO_2)] with pyrrole and used for enzyme immobilization [8]. Large number of papers on the advantages of using conducting polymers (CP) for the fabrication of novel catalytic surfaces has been published. However, use of CP based microbial sensors has not been widespread to date [31]. On the other hand,

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future biosensors based on CPs will be increasingly miniaturized due to the adaptability of electrodeposition with respect to size (even in micro or nano-order), it can be possible to obtain microbial sensors in desired dimensions using the proper cell immobilization during this process.

In this study, the conducting polymer of [SNS(NO_2)] was used as an immobilization platform for two different bacteria. *Gluconobacter oxydans* and *Pseudomonas fluorescens* cells were adsorbed on the conducting polymers onto the graphite electrodes and then covered with a dialysis membrane to avoid the cell leakage during the operational conditions. Finally, proposed systems were analytically characterized.

2. Materials and methods

2.1. Reagents

Calcium carbonate, D-glucose, D(+)-galactose, D(+)-xylose, agar and dialysis tubing cellulose membrane (25 mm × 16 mm) were purchased from Sigma (St. Louis, USA; www.sigmaaldrich.com). Methanol, dichloromethane, acetonitrile, and sodium hydroxide were purchased from Merck (Darmstadt, Germany; www.merck.com). $AlCl_3$, succinyl chloride, propionic acid, nitromethane, ferric (III) chloride, $NaClO_4$, and $LiClO_4$ were purchased from Aldrich (www.sigmaaldrich.com) and were used without further purification. Yeast extract was purchased from Oxoid (Hampshire, England; www.oxoid.com). All other chemicals were of analytical grade.

2.2. Apparatus

Chronoamperometric measurements were carried out with a Radiometer electrochemical measurement unit (Lyon, France, www.radiometer.com). Ag/AgCl (3 M KCl saturated with AgCl as an internal solution, Radiometer Analytical, REF321) and a Pt electrode (Metrohm, Switzerland, www.metrohm.com) were used as the reference and counter electrodes, respectively. Palm Instruments (PalmSens, Houten, The Netherlands, www.palmsens.com) with three electrode configurations was used for cyclic voltammograms. Graphite electrodes modified with conducting polymer and bacterial cells were used as the working electrodes.

JEOL JSM-6400 model scanning electron microscope (SEM) was used for surface imaging of the microbial electrodes.

2.3. Synthesis of [SNS(NO_2)] monomer

The monomer [SNS(NO_2)] was synthesized from 1,4-di(2-thienyl)-1,4-butanedione and 4-nitroaniline in the presence of *p*-toluenesulphonic acid (PTSA) as reported previously. A round-bottomed flask equipped with an argon inlet and magnetic stirrer was charged with the 1,4-di(2-thienyl)-1,4-butanedione, 4-nitroaniline, PTSA and toluene. The mixture was stirred and refluxed (110 °C) for 24 h under argon. [SNS(NO_2)] monomer was obtained as pale brown powder after evaporation of the toluene, followed by flash column chromatography (SiO_2 column, elution with dichloromethane) [8,32].

2.4. Biological material

The strain of *G. oxydans* DSM 2343 and *P. fluorescens* (*Pseudomonas putida* DSM 6521) was obtained from DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany, www.dsmz.de). *G. oxydans* was maintained on the agar containing (g L^{-1}): D-glucose, 100; yeast extract, 10; calcium carbonate, 20; agar, 20 [33]. The cell biomass was prepared by aerobic cultivation at 28 °C on a rotary shaker in 250 mL flasks filled with 50 mL of media. The growth medium contained glucose, 5 g L^{-1} and yeast extract, 5 g L^{-1}. The culture, inoculated from the slant agar, was incubated for 16 h to reach the late exponential phase. The cell growth was followed spectrophotometrically via measuring optical density at 600 nm [34]. Then, the cells from one of the cultivation flasks were collected by centrifugation (10 min, 3500 ×*g*), resuspended in sterile 0.9% NaCl solution and centrifuged again [35].

P. fluorescens was sub-cultured in nutrient agar, and transferred to mineral salt medium (MSM) containing 1 g/L glucose. MSM with the following compositions were used as growth media for *P. fluorescens*: 0.244% Na_2HPO_4, 0.152% KH_2PO_4, 0.050% $(NH_4)_2SO_4$, 0.02% $MgSO_4 \cdot 7H_2O$, 0.005% $CaCl_2 \cdot 2H_2O$ and trace element solution (10 mL/L). B. 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). The pH of the growth media was adjusted to 6.9. After 16 h, the biomass was harvested by centrifugation and suspended in MSM and then re-centrifuged [36]. Bacterial cells in logarithmic phase were used during the experiments and the cell growth was followed spectrophotometrically via measuring optical density at 560 nm [37].

After centrifugation, biomasses were used for the biosensor preparation. Daily prepared electrodes with fresh cells were used in all experimental steps.

2.5. Preparation of microbial sensors

Prior to the electropolymerization, spectroscopic grade graphite rods, (Ringsdorff Werke GmbH, Bonn, Germany, type RW001, 3.05 mm diameter and 13% porosity) were polished on wet emery paper and washed thoroughly with distilled water.

Electrochemical polymerization of [SNS(NO_2)] was potentiodynamically carried out between the potential range 0.0 V and 1.1 V in $NaClO_4$ (0.1 M) and $LiClO_4$ (0.1 M)/acetonitrile medium. For immobilization of bacterial cells, 25 μL of intact cells (*G. oxydans* with 35×10^9 cell titer and *P. fluorescens* with 15×10^{10} cell titer) was spread over the polymer coated electrode and allowed to stand at ambient conditions for drying for 1 h. Then, the layer was covered with a dialysis membrane, pre-soaked in water. The membrane was fixed tightly with a silicone rubber O-ring [38].

2.6. Measurements

All experiments were carried out at ambient conditions in a vessel containing 10 mL of working buffer solution. The electrode was washed with distilled water and kept in potassium phosphate buffer for 5 min after each measurement. The microbial sensors were initially equilibrated in buffer and then, substrate was added to the reaction cell. The biosensor responses were registered as the current densities (μA/cm^2) by following the oxygen consumption at −0.7 V as a result of metabolic activity. Buffer was refreshed after each measurement [39].

3. Results and discussions

G. oxydans is a gram-negative and rod-shaped acidophilic bacterium belonging to the Acetobacteraceae family; the α-subclass of the Proteobacteria [40,41]. *Gluconobacter* strains are strict aerobes and use a variety of sugars, alcohols and polyols as substrates which are oxidized incompletely [42]. *Gluconobacter* spp. grow very fast in simple cultivation media, contain high enzymatic activities, are relatively stable during immobilization, and their pyrrolo-quinoline quinine-dehydrogenases (PQQ-DH) do not require an external cofactor, hence these features make them particularly suitable for biosensor construction [43].

PQQ enzymes are attractive not only because of their oxygen independence, but also, they exhibit a direct electron transfer between their active site and certain electrodes thus, making the construction of biosensors based on such enzymes simpler [44,45]. Polypyrrole-

integrated quinohemoprotein alcohol dehydrogenase (QH-ADH) from *Gluconobacter* was used in a previous work and QH-ADH was found to exhibit a variety of specific features depending on the biosensor architecture [45]. The same enzymes were also successfully used in potentiometric biodevice design based on PPy [46]. Moreover, a mediated biosensing application of the PQQ-dependent glycerol dehydrogenase purified from the mutant strain of *Gluconobacter* sp. 33 was also described [47].

On the other hand, the genus *Pseudomonas sensu stricto* belongs to the γ-subclass of the Proteobacteria and contains mostly fluorescent *Pseudomonas* spp. as well as a few non-fluorescent species [48]. *P. fluorescens* is a gram-negative, rod-shaped bacterium present in water, soils and in the plant rhizosphere [48,49]. Various microbial sensors have been characterized by the integration of mentioned bacterial cells into the different immobilization matrices [33,35–39,43].

A variety of different biological materials ranging in size from whole cells to large fragments of proteins have been immobilized in conducting polymers (CP). Most work has been done with enzymes, antibodies, haptens, oligonucleotides and chemical receptors [31]. Recently, new CPs based on poly(3,4-ethylenedioxythiophene) (PEDOT) have been used to construct very conductive and stable materials in aqueous environment, which is ideal for biosensing purposes. It has been also used for enzyme immobilization for glucose biosensing [50]. Immobilization of intact *P. fluorescens* cells was also successfully achieved on a thiophene-based conducting polymer and a good linearity as well as repeatability and operational stability was observed in our previous study [38]. Moreover, the polymerization of a functionalized pyrrole produces polymers with interesting properties and is a way of immobilizing (bio)-molecules on the electrode surface [51].

In this work, electrochemical polymerization of 1-(4-nitrophenyl)-2,5-di(2-thienyl)-1 *H*-pyrrole [SNS(NO_2)] was carried out. It is known that electropolymerization enhances homogeneous film formation on the electrode surface, regardless of its shape or size, and can be carried out with large electrode surfaces with a control of thickness. Moreover, the film thickness can easily be followed with the measurement of the total charge during the deposition of CP [31]. Electrochemical polymerization was carried out by cycling the potential. Cyclic voltammograms before and after the electropolymerization of [SNS(NO_2)] on the graphite electrode were shown in Fig. 1.

Electropolymerization time affects both the rate of growth and the quality of the conducting polymer films produced [52]. After 5, 10, 15, and 20 min of electropolymerization (referring to 50, 100, 150 and 200 scan numbers), the total charges involved in the film formation were measured as 2.75×10^{-4}, 4.92×10^{-4} and 5.21×10^{-4}, 2.16×10^{-3} C,

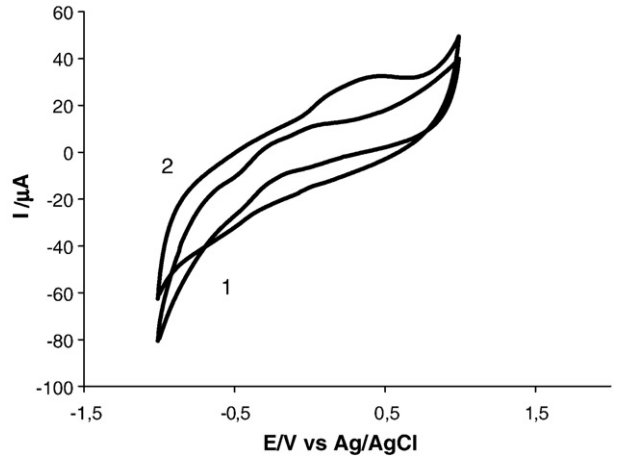

Fig. 1. Cyclic voltammograms on graphite electrodes (in potassium phosphate buffer; 50 mM, pH 6.5) before (1) and after (2) electrochemical polymerization of [SNS(NO_2)], number of scans: 100).

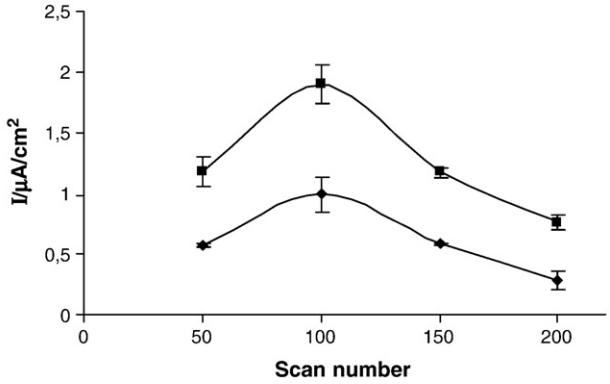

Fig. 2. Effect of number of scan on the biosensor responses (in potassium phosphate buffer: 50 mM, pH 7.0 for *G. oxydans*; pH 7.5 for *P. fluorescens*, room temperature, −0.7 V, 0.5 mM glucose; ◆ *P. fluorescens*, ■ *G. oxydans*).

respectively. These electrodes were used to prepare microbial sensors as mentioned in the experimental part. As shown in Fig. 2, maximum activity was registered when the working electrode was coated using 4.92×10^{-4} C. According to our findings the most convenient thickness was obtained with an electropolymerization time of 10 min for the immobilization of both intact bacterial cells. After 10 min, incompact matrix structure develops and causes lower current signals as reported in previous works [38].

Morphologies of matrices with bacterial cells were also observed and shown in Fig. 3(A), (B). Unbound cells were removed by washing the electrode surfaces several times before analysis. As seen from the micrographs, [SNS(NO_2)] matrix enabled cells to attach to the surface and provided an efficient platform. Hence, intact cells could keep their metabolic activities during the operational conditions.

3.1. Effect of pH

According to the optimization studies, the effect of pH on the electrode response was investigated for glucose in 50 mM potassium phosphate buffer (pH 6.5–8.0) as shown in Fig. 4. Maximum biosensor responses were observed at pH 7.0 and 7.5 for *G. oxydans* and *P. fluorescens* based systems, respectively. Sharp decreases were observed at pH values higher than 7.5. As a result, pH 7.0 and 7.5 were chosen as the optimum pH for *G. oxydans* and *P. fluorescens* respectively and further studies were conducted with these values.

3.2. Analytical characteristics

The analytical characteristics of the microbial sensors were examined under optimized conditions using glucose as the substrate (in the potassium phosphate buffer (50 mM; pH 7.0 and pH 7.5 for *G. oxydans* and *P. fluorescens*)). Calibration curves were plotted for current density versus substrate concentration (where y is the sensor response in terms of current density (μA/cm^2) and x is the substrate concentration in mM). For the *G. oxydans* biosensor, a linear relationship was observed between 0.25 and 4.0 mM glucose concentration satisfying the equation $y=0.359x+0.420$ ($R^2=0.985$). On the other hand, *P. fluorescens* sensor exhibited a narrow linearity between 0.2 mM and 1.0 mM glucose represented with an equation $y=3.241x-0.251$ ($R^2=0.992$). At higher concentrations for both systems, current signals remained constant revealing that the systems reached to saturation.

The repeatability of the microbial sensors was estimated by repetitive measurements in the presence of glucose (0.8 mM for *G. oxydans* and 0.8 mM for *P. fluorescens* biosensors), ($n=5$). The standard deviations (S.D) and variation coefficients (c.v) were found to be 0.815 ± 0.034 and 4.2% for *G. oxydans* based and 0.539 ± 0.006 and 1.0% for *P. fluorescens* based biosensors.

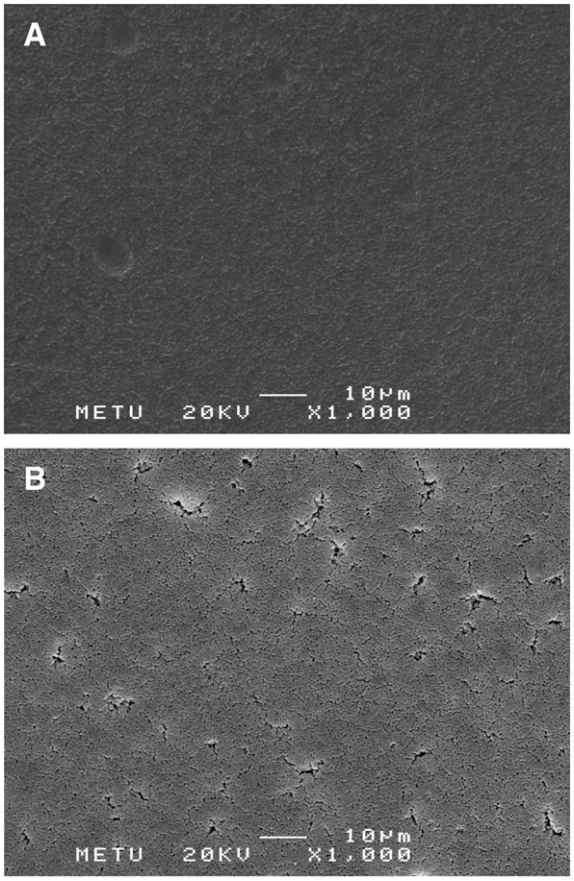

Fig. 3. SEM images of *Gluconobacter oxydans* (A) and *Pseudomonas fluorescens* (B) cells on [SNS(NO_2)] modified graphite electrodes.

Calibration curves were also obtained for the microbial electrodes containing no [SNS(NO_2)] behind the dialysis membrane. The current signals were smaller and irreproducible in this case as already reported in a previous work [38].

To examine the operational stability of both systems, microbial biosensors were immersed in the reaction cell containing the buffer solution at the optimized conditions. *G. oxydans* based system lost only 6.0% of its activity after 5 h. In case of *P. fluorescens* sensor, decrease in the activity was limited to 3.0% after 3.5 h.

Furthermore, substrate specificity of the proposed microbial biosensors to various substrates such as mannose, galactose, xylose, methanol and ethanol was tested (Fig. 5). *G. oxydans* biosensor showed appreciable response only towards ethanol. However, *P. fluorescens* biosensor revealed response towards galactose and xylose. No response was observed for mannose, methanol and ethanol in given conditions.

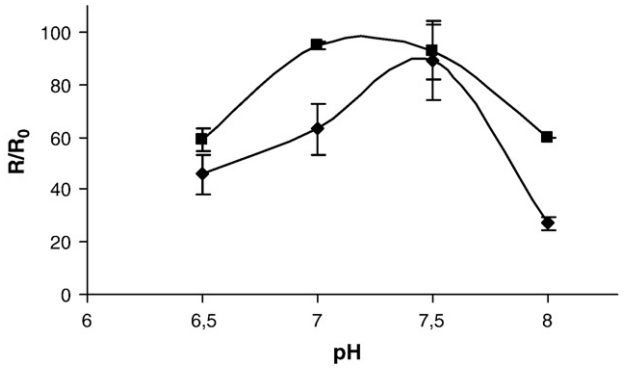

Fig. 4. Effect of pH on the biosensor response (6.5–8.0: potassium phosphate buffer (50 mM), −0.7 V, 0.5 mM glucose; for the biosensor *x* and *y* show pH and biosensor response %, *R*: current and R_0: maximum current, respectively. ♦ *P. fluorescens*, ■ *G. oxydans*).

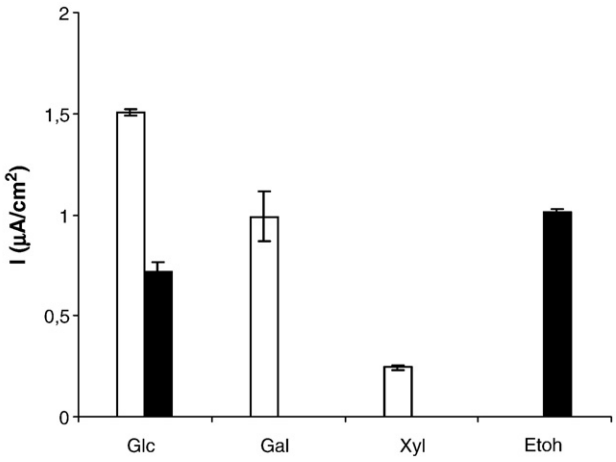

Fig. 5. Substrate specificity of the biosensor (in potassium phosphate buffer, 50 mM, pH 7.0 for *G. oxydans*; pH 7.5 for *P. fluorescens*, −0.7 V, substrate concentrations: 0.5 mM), (Data are presented as value ± S.D, $n = 3$; □ *P. fluorescens*, ■ *G. oxydans*).

Additionally, *G. oxydans* sensor was calibrated against ethanol and a linear response was observed in the range of 0.1–5.0 mM given by the equation $y = 0.163x + 0.425$ ($R^2 = 0.978$). Since the system has no response to methanol, it can be concluded that it could be possible to make selective ethanol analysis in the presence of methanol. On the other hand, the proposed sensor may be used for glucose and ethanol analyses in the same sample after chromatographic separation.

4. Conclusion

In this work, electrochemical polymerization of 1-(4-nitrophenyl)-2,5-di(2-thienyl)-1 *H*-pyrrole was achieved via cyclic voltammetry and bacterial cells were immobilized successfully on the conductive matrix. The biosensor preparation method was a two-step procedure in which the cells were immobilized by adsorption on the surface after the electropolymerization step. Use of dialysis membrane to cover the surface after immobilization turned out to be a way of conserving the bioactive surface during the operation. The preparation is simple and not time consuming. Besides, systems proposed showed good linearity and repeatability as well as high operational stability.

Gluconobacter has been extensively used for biosensor development and, recently, biofuel cells. All of these applications rely on *Gluconobacter*'s distinctive metabolism. As new applications are being developed, there is also a shift from the practical use of *Gluconobacter* as a biocatalyst to the targeted use of dehydrogenases, respiratory chain components, electron transfer and other processes at the subcellular level [53]. Microbial fuel cells (MFCs) have drawn great research attention in recent years [54]. Important advances in the performance of MFCs have been realized during the last years [55,56] resulting in a comparable power density to that of enzyme based fuel cell [57]. Microorganisms in MFCs offer some major advantage over enzymes in that they can be less susceptible to poisoning and loss of activity under operating conditions and can catalyze thorough oxidation of various organic substrates, thus provide MFCs greater application potential in energy production from organic waste and renewable biomass [54,58]. Additionally, MFCs have the ability of adapt the respiration machinery of the microbial cells to new conditions including new substrates as biofuels [53]. For instance, a different type of MFC inoculated with sludge containing a mixed population of bacteria has been designed previously for the biodegradation of phenol and electricity was successfully generated [59].

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 due to their capability to degrade phenolic compounds after the adaptation step [37]. Thus, it could be possible to use the proposed system (*P. fluorescens* cells immobilized on SNS(NO_2) matrix) as a part of microbial fuel cell studies which might be utilized for the degradation of phenolic substrates as already described before.

The functionalization technique of pyrrole monomers and their subsequent electropolymerization is a straightforward procedure for the immobilization of bioreagents on electrode surfaces, a procedure being widely used for the enzyme immobilization [31]. It is clear that to choose the material and design of a surface are very important criteria to improve the performance of microbial biosensing system as well as enzyme based biosensors. The biocompatibility between bacteria and the matrix should be carefully considered since it markedly affects metabolic activity and viability of the cell, and hence, the performance of the systems. Especially, electroconductive polymers with microporous structures could be a good alternative to comply with the size of bacteria [60].

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