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Leptospirillum ferrooxidans based Fe^{2+} sensor

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ABSTRACT

A novel electrochemical biosensor integrating the strictly autotrophic bacterial strain *Leptospirillum ferrooxidans* as a recognition element and a Clark type oxygen probe as a transducer was designed, metrologically and analytically characterized and applied for the specific Fe^{2+} determination. The bacterial Fe^{2+} oxidation involves O_2 consumption, thus the quantification was performed registering the decrease of the oxygen reduction current. The limit of detection was found to be 2.4 μmol L^{-1} and the sensitivity of the determinations—3.94 nA L μmol^{-1}. The response time of the biosensor is 18 s for Fe^{2+} concentrations of 10^{-5} to 10^{-4} mol L^{-1}. The biosensor was applied as well for the indirect determination of Fe^{2+} oxidizing species such as $Cr_2O_7^{2-}$, reaching a sensitivity of 2.47 nA L μmol^{-1}.

The transducer characteristics were evaluated and optimized to obtain short response time and high sensitivity.

The analytical performances of the biosensor subject of the present work were found to be similar to that of the *At. ferrooxidans* based one developed by the authors earlier, avoiding however the sulfur compounds interference, because of the substrate specificity of the applied bacterial strain.

1. Introduction

Leptospirillum ferrooxidans is known as an acidophilic chemolithotrophic iron-oxidizing bacterium (Balashova et al., 1974; Harrison, 1984; Harrison and Norris, 1985; Hippe, 2000; Johnson, 2001; Markosyan, 1972; Norris, 1983). Its metabolic activity, similar to that of the most extensively characterized iron-oxidizing acidophil *Acidithiobacillus ferrooxidans* (Colmer and Hinkle, 1947; Harrison, 1984; Kelly and Wood, 2000; Leduc and Ferroni, 1994; Temple and Colmer, 1951; Trudinger, 1971), involves ferrous iron oxidation using molecular oxygen as electron acceptor:

$$4Fe^{2+} + O_2 + 4H^+ = 4Fe^{3+} + 2H_2O \quad (1)$$

Hence, this process could be exploited for Fe^{2+} determination measuring the bacterial oxygen consumption. However, while Fe^{2+} sensors based on *At. ferrooxidans* have been already designed, characterized and described (Mandl and Macholan, 1990; Zlatev et al., 2006a,b), a *L. ferrooxidans* based Fe^{2+} sensor has not been reported until now.

Unlike *At. Ferrooxidans*, growing in both ferrous iron and sulfur media, *L. ferrooxidans* is a strictly autotroph and uses Fe^{2+} as a sole source of energy. The substrate specificity of *L. ferrooxidans* promotes the expectation that the Fe^{2+} sensor based on this bacterium will provide no sensitivity to sulfur compounds, thus overcoming the main drawback of the *At. ferrooxidans* based Fe^{2+} sensors (Mandl and Macholan, 1990; Zlatev et al., 2006a). In addition, *L. ferrooxidans* displays greater affinity to ferrous iron, combined with about 10 times higher tolerance to ferric iron inhibition, compared to *At. ferrooxidans* (Norris et al., 1988).

The goal of the present work is the development and the analytical and metrological characterization of a *L. ferrooxidans* based electrochemical sensor for direct Fe^{2+} determination. Additionally, its application for indirect Fe^{2+} mediated $Cr_2O_7^{2-}$ determination is presented, as well as the characteristics of the Clark type oxygen probe used as a sensor transducer (Zlatev et al., 2006a), which were evaluated and optimized to obtain a maximal sensor response.

2. Experimental

2.1. Instrumentation

A special construction Clark type oxygen probe with a flat front end and uniformly distributed gold microcathodes equipped with a bacterial membrane holder (Zlatev et al., 2006a) connected to an appropriately modified Model LC-4B Amperometric Detector (BAS, USA) was applied for the sensor response registration. An especially developed noise suppression unit (subject of another publication) was connected between the current to voltage converter and the ADC, allowing about 50-fold suppres-

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sion of the noise without any signal distortion and response time degradation.

Model Unimax 1010 DT turntable (Heidolf, Germany) and Model 64R temperature controller (Brookfield, USA) were used for the bacterial culture development. Model 350 pH/ion analyzer (Corning, USA) connected to a PC was applied for pH measurements and redox potential monitoring. The Fe^{2+} photometric determinations were carried out applying Stasar III photometer (Gilford, USA).

2.2. Reagents

All the reagents were of analytical grade and were purchased from Merck and Sigma. Deionized water produced by MilliQ system of Milipore was used for the experiments and bacterial culture media preparation.

2.3. Bacterial strain and culture media

The bacterial strain *L. ferrooxidans*, DSM 2705^T, isolated and described by Markosyan (1972) was cultured in DSM medium 882 with the following composition: (i) solution A: $(NH_4)_2SO_4$—132 mg, $MgCl_2{\cdot}6H_2O$—53 mg, KH_2PO_4—27 mg, $CaCl_2{\cdot}2H_2O$—147 mg, H_2O—950 mL; (ii) solution B: $FeSO_4{\cdot}7H_2O$—20 g, H_2SO_4 0.25N—50 mL; (iii) trace element solution: $MnCl_2{\cdot}2H_2O$—62 mg, $ZnCl_2$—68 mg, $CoCl_2{\cdot}6H_2O$—64 mg, H_3BO_3—31 mg, Na_2MoO_4—10 mg, $CuCl_2{\cdot}2H_2O$—67 mg, H_2O—1000 mL. The medium, with final pH adjusted to 1.8, was prepared by mixing solution A and solution B and adding 1 mL of trace element solution. Prior to mix, the solutions were autoclaved separately at 112 °C for 30 min.

2.4. Bacterial cultures development

L. ferrooxidans bacterial cultures were developed in 0.5-L flasks mounted on a turntable at 100 rpm, ensuring thus a moderate bacterial culture oxygenation. The turntable was placed in air thermostated reactor maintaining 30 °C. Photometric determinations of the Fe^{2+} concentration applying the ortho-phenantroline method (Charlot, 1961) were carried out periodically and the redox potential was continuously monitored in parallel. The bacteria development was stopped at the end of the exponential—the beginning of the stationary growth phase (90% of the ferrous ions oxidized), and followed by culture centrifugation. The concentrated bacterial mass was washed, resuspended in deionized water, obtaining protein concentrations between 5 and 10 $\mu g\,L^{-1}$, and kept at 4 °C. It was used for bacterial membrane preparation.

2.5. Fe^{2+} determination and monitoring

2.5.1. Photometric method

Samples of 10 μL were taken from the bacterial culture periodically for Fe^{2+} determination by the ortho-phenanthroline spectrophotometric method. The formed extremely stable over the time red–orange complex absorbs light in the range of 380–580 nm (Charlot, 1961) with a maximal absorption at 505 nm in the pH interval from 2 to 9. The Fe^{2+} concentration was evaluated using a preliminary built calibration curve: light absorption vs. Fe^{2+} concentration.

2.5.2. Potentiometric method (Pesic et al., 1989)

Unlike the photometric method, the potentiometric one allowed continuous monitoring of the Fe^{2+} concentration during bacterial culture development. Very precise control of the bacterial culture development can be achieved, since the redox potential E depends on the Fe^{3+}/Fe^{2+} concentration ratio, according to the Peters–Nernst equation:

$$E = E^{\circ} + \left(\frac{RT}{F}\right) \ln\left(\frac{[Fe^{3+}]}{[Fe^{2+}]}\right) \quad (2)$$

where E° is the standard redox potential, R is the gas constant, T is the temperature, F is the Faraday constant, and $[Fe^{3+}]$ and $[Fe^{2+}]$ are the ionic concentrations at equilibrium.

A Pt wire sensing electrode and Ag, AgCl/3 M KCl reference electrode was applied connected to the PC controlled Corning pH/ion analyzer for Fe^{2+} concentration monitoring.

2.6. Determination of the protein concentration

The Bradford method (Bradford, 1976) was applied initially for the resuspended bacterial mass protein concentration determination. Since this method is complicated and time-consuming, it was replaced by the simpler and more rapid turbidimetric method (Layne, 1957) based on the preliminarily built calibration plot: light absorption–protein concentration determined by the Bradford method.

It was found experimentally that the maximal light absorption by the *L. ferrooxidans* concentrated bacterial mass occurs at 275 nm, in the ultraviolet light range. The absorption value however remained sufficiently high and usable even for wavelengths up to 400 nm. Thus, a standard curve was built at 340 nm using a simple visible photometer, by dilution of an initial concentrated bacterial mass whose protein concentration was previously determined by the Bradford method. The curve appeared to be linear in the concentration range under study up to 25 $mg\,L^{-1}$ of protein.

2.7. Sensor construction and bacterial membrane preparation

The biosensor, including a Clark type oxygen probe as a transducer and a bacterial membrane as a sensing element was fabricated like described in a previous work (Zlatev et al., 2006a).

The bacterial membrane was prepared by filtration of a defined volume of the resuspended bacterial mass through a 0.15 μm pore size Sartorius cellulose filter. After the filtration, the cellulose filter charged with bacteria was attached onto the plastic membrane of the Clark oxygen probe forming a sandwich type bacterial membrane. The bacterial mass was confined between the Clark probe plastic membrane and the cellulose filter, permeable for the oxygen and the analyte.

The biosensor construction was schematically presented and discussed by the authors earlier (Zlatev et al., 2006a; Stoytcheva et al., 2009).

Even kept at 4 °C, the bacteria mortality caused a diminution of the resuspended bacterial mass activity with time, resulting in decrease of the sensor sensitivity and LOD. Although the deviations could be corrected by sensor calibration, the proposed method for bacteria membrane preparation allowed avoiding the great dispersion of the initial sensor characteristics. For this purpose the filtered volume of the resuspended bacterial mass was determined as a function of the bacterial activity. A two steps procedure was applied for the bacterial membranes preparation: (i) determination of the response R_0 to 50 $\mu mol\,L^{-1}$ Fe^{2+} of the sensor with bacterial membrane prepared by filtering of 1 mL or known volume (V_0) of the resuspended bacterial mass; (ii) calculation of the volume V_1 to be filtered to achieve the desired sensor response R_1 applying the equation:

$$V_1 = \frac{(R_1/R_0)}{V_0}. \quad (3)$$

2.8. Measuring procedure

Bacterial oxygen consumption, proportional to Fe^{2+} concentration was measured registering, as a sensor response, the current of O_2 reduction. The Fe^{2+} concentration was evaluated by the current diminution resulting from the sample insertion, using a preliminary built calibration plot, taking into account the sample dilution. The measurements were performed in a cell, containing 28 mL of diluted H_2SO_4 aqueous solution (pH 1.8), under stirring.

Since very small current changes caused by the sample injection were measured in the presence of high background current due to the bulk oxygen concentration, a current compensation was applied. It allowed the potentiostat *I–E* converter sensitivity augmentation, in order to achieve sufficiently high amplitude of the measured response. Unfortunately, the current compensation does not eliminate the noise which is amplified too with sensitivity increase, thus causing signal-to-noise ratio (*S*/*N*) and hence—LOD and precision degradation. The application however of an especially developed noise suppression unit (subject of another publication) allowing increasing about 50 times the *S*/*N* ratio helped avoiding this problem.

3. Results and discussion

3.1. Bacterial culture growth monitoring

The development of active bacteria requires their growth to be terminated near the end of the exponential phase. The end point determination can be achieved by precise monitoring of the Fe^{2+} concentration applying the on-line potentiometric method after preliminary calibration done by results obtained by the photometric method application.

The redox potential vs. time curve (Fig. 1A) strictly corresponds to the Fe^{2+} concentration ratio vs. time (Fig. 1B) curve allowing the precise on-line monitoring of the bacterial growth instead of the time and reagent consuming off-line photometric method usually applied.

It was found (Fig. 1A and B) that 90% of the ferrous ions oxidation (determined by the photometric method) corresponds to redox potential value of 625 mV which is the end point of the bacterial culture development, followed by centrifugation and resuspension, as described above.

3.2. Oxygen probe characterization

3.2.1. Transducer response time determination

The oxygen probe (transducer) response time is an important component of the total biosensor response time. It is affected by the rate of the oxygen reduction on the Clark probe cathode, i.e. by the cathode area, by the permeability of the plastic oxygen permeable membrane, and by the oxygen gradient change mode, keeping all the other conditions constants (thickness of the electrolyte layer between the plastic membrane and the probe cathode, electrolyte composition, applied potential, electrode material, and temperature).

Response time refinement was performed by increasing the internal oxygen consumption and by membrane permeability improvement, choosing adequate plastic material and thickness. The increase of the internal oxygen consumption was achieved by increasing the effective diameter of the Clark probe cathode using for this purpose a multicathode, i.e. uniformly distributed on the entire acrylic support surface gold discs in contact with the Clark probe electrolyte (Zlatev et al., 2006a). The characteristics of this original Clark type oxygen probe were not published until now.

Two response times of the oxygen probe were determined experimentally creating positive and negative steps of the oxygen concentration by stirring rate change (from 0 to 800 rpm) and by Na_2SO_3 addition respectively. The stirring provokes an increase of the oxygen concentration at the solution/oxygen probe membrane interface intensifying also the mass exchange at the solution/air interface resulting in bulk equilibrium oxygen concentration increase. The addition of Na_2SO_3 which is easily oxidized by the dissolved oxygen yielding Na_2SO_4 immediately decreases its concentration. The two types of the oxygen probe current responses: positive and negative, are shown in Fig. 2.

The experimental results showed 8.7 s response time to 90% of the maximal response to positive concentration changes, while to

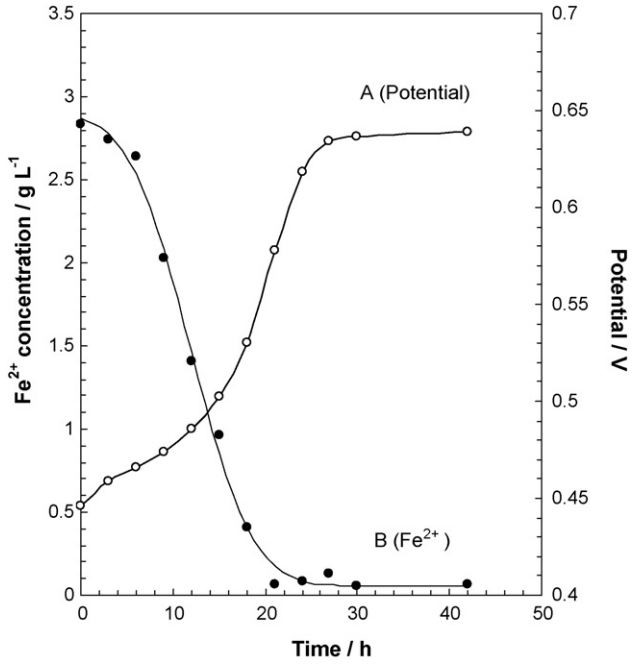

Fig. 1. Bacterial culture growth monitoring: on-line potentiometric (A); off-line photometric (B).

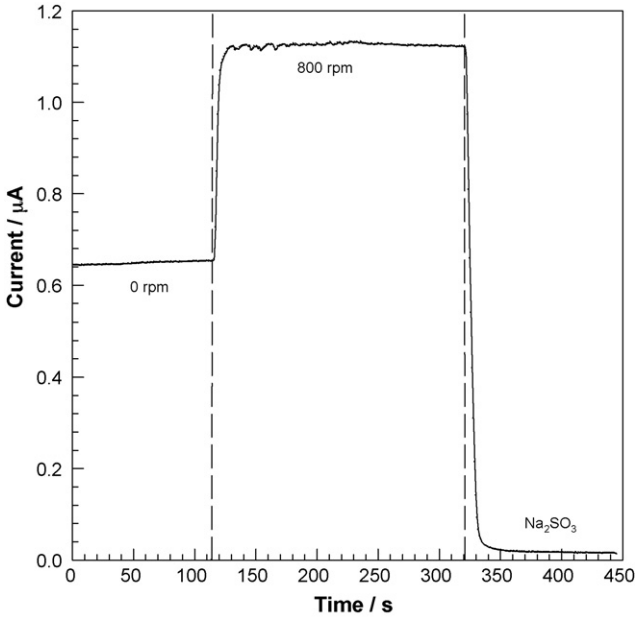

Fig. 2. Oxygen probe transducer current transient response to increasing and to decreasing oxygen concentrations (25 °C, −0.90 V).

the negative changes the response was found to be 12.5 s, i.e. about 1.5 times longer. Unfortunately, the bacterial sensor response is based on the transducer response to decreasing oxygen concentrations.

3.2.2. Membrane transport parameters

Since the biosensor response represents the reduction current of the oxygen, crossing the Clark type probe membrane, the transport parameters and the thickness of the latter strongly influenced the response time of the sensor and the sensitivity of the determinations. Thus, they were evaluated and optimized.

According to Eqs. (4) and (5) (Tarnowski et al., 1995), the sensitivity of the oxygen determination is a function of the membrane permeability and oxygen diffusion coefficients:

$$I_d = 4FA(P_m/Z_m)P_{O_2} \quad (4)$$

$$P_m = D_m S_m \quad (5)$$

where: I_d is the oxygen mass transport limited current, F is the Faraday constant, A is the area of the cathodes, P_m is the membrane oxygen permeability coefficient, Z_m is the membrane thickness, P_{O_2} is the partial pressure of the oxygen in the solution, D_m is the membrane oxygen diffusion coefficient, and S_m is the oxygen solubility in the membrane phase.

Thus, the membrane oxygen permeability coefficient was evaluated registering the steady-state current response of the probe in air-equilibrated deionized water under stirring at constant temperature. The chosen stirring rate of 1000 rpm maintained an oxygen flux, remaining almost constant with current rate increase. In these conditions, the diffusion layer is confined to the membrane and consequently, the steady-state current depends only on the oxygen transport properties of the membrane (Hitchman, 1978).

The oxygen permeability coefficient (P_m) was found to be $(1.19 \pm 0.05) \times 10^{-12}$ mol m^{-1} s^{-1} kPa^{-1} ($N = 12$) calculated applying Eq. (4) ($I_d = 1.123 \times 10^{-6}$ A; $A = 2.2 \times 10^{-6}$ m^2; $Z_m = 15 \times 10^{-6}$ m; $P_{O_2} = 1.8 \times 10^4$ Pa).

The membrane oxygen diffusion coefficient (D_m) was derived from the slope of the line, described by the following equation (Hitchman, 1978):

$$\ln \frac{1 - (I_t/I_s)}{2} = -\frac{\pi^2 D_m t}{Z_m} \quad (6)$$

where: I_t is the transient current response and I_s is the steady-state current response of the oxygen probe.

The transient current response was obtained by step changing of the solution stirring rate from 0 to 800 rpm (Fig. 2), sufficient to ensure maximal O_2 flux and able to maintain the oxygen concentration at the outer surface of the membrane at bulk levels. The logarithmic plot built to evaluate the oxygen diffusion coefficient in the membrane D_m and described by the equation $y = -0.3074x - 1.3056$ ($r^2 = 0.9875$) is presented in Fig. 3.

It was found that $D_m = (4.6 \pm 0.2) \times 10^{-5}$ cm^2 s^{-1} ($N = 7$).

All the values obtained are related to 15 μm thick polyethylene membrane. This membrane thickness, combined with a large cathode surface, was found to be the optimal to ensure a compromise between the transducer response time and sensitivity.

3.3. Analytical and metrological characterization of the *L. ferrooxidans* based Fe^{2+} sensor

Every series of measurements were carried out during the same day and no corrections were made for bacterial activity diminution, preliminary determined to be about 1% per day, applying the following procedure: the sensor response for a same value of Fe^{2+} concentration (50 μmol L^{-1}) was measured at identical experimental conditions (30 °C, pH 1.8, 100 rpm) within a time interval of 12 h

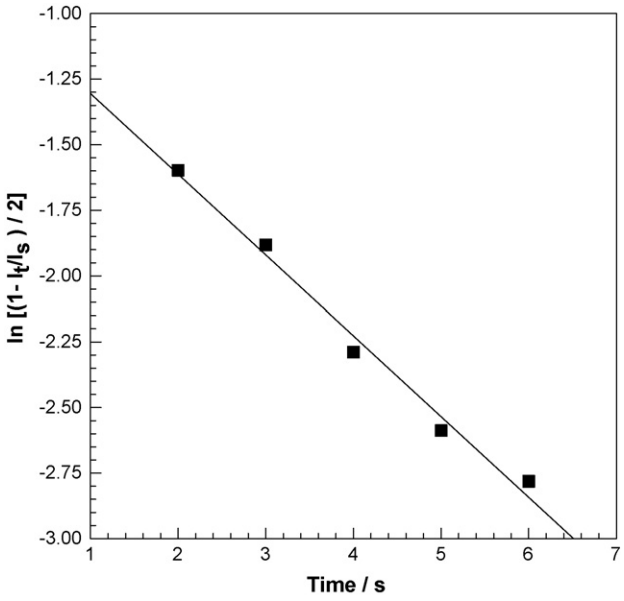

Fig. 3. Logarithmic plot built to evaluate the oxygen diffusion coefficient (D_m) in the polymer membrane of the oxygen probe.

and the response change was evaluated. A constant temperature was maintained, since parameters affecting the sensor response amplitude, such as transducer response to oxygen (about 2%/°C), equilibrium dissolved oxygen concentration (about 1.4%/°C), as well as the bacterial activity, are temperature dependent, thus provoking a 5%/°C sensor response change. Working at thermostatic conditions allowed avoiding fluctuations of the equilibrium dissolved oxygen concentration, while the constant stirring rate kept unchanged the oxygen concentration gradient at the bacterial membrane/solution interface.

The typical sensor response to Fe^{2+} is presented in Fig. 4. The corresponding calibration plot (steady-state current I_s/nA vs. Fe^{2+} concentration C_{Fe}/μmol L^{-1}) is linear in the investigated concen-

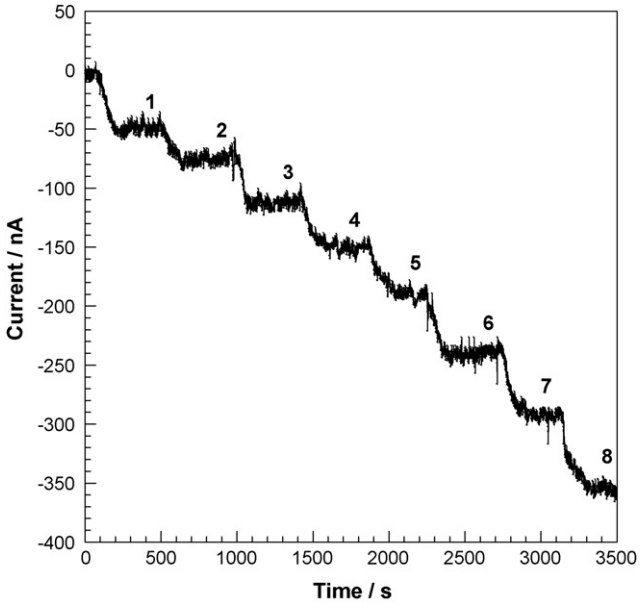

Fig. 4. Amperometric response of the *L. ferrooxidans* sensor for increasing Fe^{2+} concentrations: (1) 10 μmol L^{-1}; (2) 20 μmol L^{-1}; (3) 30 μmol L^{-1}; (4) 40 μmol L^{-1}; (5) 50 μmol L^{-1}; (6) 60 μmol L^{-1}; (7) 70 μmol L^{-1}; (8) 80 μmol L^{-1} (air-equilibrated deionized water at 30 °C, pH 1.8 and 100 rpm).

tration range up to 80 μmol L^{-1}, representing a part of the total dynamic linear range which was found to be 2.1 mmol L^{-1}, at the mentioned above experimental conditions. The calibration plot can be described by the equation: $I_s = 3.94C_{Fe}$, with $r^2 = 0.9935$, i.e. the sensor sensitivity is 3.94 nA L μmol^{-1}.

The limit of detection, experimentally determined in the same conditions was found to be 2.4 μmol L^{-1} when the signal-to-noise ratio equals to 3. According to the UPAC definition determining the limit of quantitation as LOQ = 3.3 LOD (Thévenot et al., 1999), the *L. ferrooxidans* based Fe^{2+} bacterial sensor LOQ was found to be 7.92 μmol L^{-1}.

The steady-state sensor response time was determined to be about 18 s for Fe^{2+} concentrations of 10^{-5} to 10^{-4} mol L^{-1}.

The *L. ferrooxidans* based Fe^{2+} sensor when not in use was stored in diluted aqueous solution of H_2SO_4 with pH 1.8, at ambient temperature. The sensor response to 50 μmol L^{-1} Fe^{2+} was measured daily at 30 °C, pH 1.8 and 100 rpm. After 5 days about 10% lost of activities was observed, while for 45 days the response was only 50% of the initial. Thus, the storage lifetime t_{L10} and t_{L50} were determined to be 5 and 45 days respectively, at ambient temperature. The long storage lifetime of the bacterial sensors at ambient temperature is one of their advantages compared to the enzymatic ones which loose activity due to the enzyme denaturation.

It was found that the sensor lifetime equals the free (non-immobilized) bacteria lifetime kept at the same conditions (H_2SO_4 aqueous solution, pH 1.8, ambient temperature), demonstrating that the nature of the membrane material does not affect this parameter.

The addition to the storage media of Fe^{2+}, involved in the bacterial metabolic activity, increased the sensor lifetime. However, this procedure was not applied to avoid the "cleaning" of the bacterial membrane before every application, by sensor immersion for a long time in running deionized water while the internal Fe^{2+} concentration decreases to zero.

The reproducibility of the bacterial sensors was determined by the ability of preparation of bacterial membranes with same bacterial activity, as presented in a previous authors work (Zlatev, 2002). Applying the procedure described above, combined with preliminary bacterial activity determination, a reproducibility of the response within 3% R.S.D. for 50 μmol L^{-1} Fe^{2+} was achieved at 30 °C and 100 rpm agitation speed ($I_s = 196 \pm 2$ nA, $N = 10$, $P = 95\%$).

Unlike *At. ferrooxidans*, it is known that *L. ferrooxidans* grows in Fe^{2+} containing media only and that is why it was not expected sulfur compounds influence on the sensor response. Nevertheless, because the *At. ferrooxidans* Fe^{2+} sensor reported by the authors (Zlatev et al., 2006a) is sensitive to thiosulfate, its influence on the *L. ferrooxidans* based Fe^{2+} sensor response was tested. The sensor response to 50 μmol L^{-1} Fe^{2+} and increasing up to 0.6 mmol L^{-1} thiosulfate concentrations made by successive microliter volumes additions of concentrated thiosulfate solution was registered and no effect was observed out of the negligible response fluctuations within the experimental error.

Because of the metabolic restriction of *L. ferrooxidans*, the sensor response to Fe^{2+} could be affected by bacteria metabolism inhibitors only. The Fe^{2+} oxidizing agents such as $Cr_2O_7^{2-}$ are not assumed to be interfering agents because they change the free Fe^{2+} concentration itself but not interfere with the ferrous iron determination.

As demonstrated by Sugio et al. (1987, 1992, 1994), the iron-oxidizing activity of *L. ferrooxidans* could be blocked in the presence of bisulfite ions, because of the iron oxidase inhibition. The bisulfite ions interference on the ferrous iron quantification was investigated by monitoring the sensor response amplitude to 10 μm L^{-1} Fe^{2+} and increasing HSO_3^- concentrations. Small volumes of Na_2SO_3 were added to the Fe^{2+} containing H_2SO_4 solution (pH 1.8) obtaining thus concentrations of: 0.1, 0.2, 0.5 and 1 μmol L^{-1}. In acidic media the sulfite ion is converted to bisulfite one according to the equation: $SO_3^{2-} + H^+ \rightleftharpoons HSO_3^-$. The Na_2SO_3 additions provoked respectively 27%, 52%, 81% and 92% diminution of the steady-state sensor response, measured 30 min after every addition. As known, SO_3^{2-} undergoes a rapid oxidation by the dissolved oxygen forming SO_4^{2-} and the rate constant increases with pH (Shi et al., 2006). This effect however was not taken into account since the experiments were performed at very low pH value (1.8).

As demonstrated (Table 1), the sensitivity of the Fe^{2+} quantification is almost the same, when using *L. ferrooxidans* and *At. ferrooxidans* based sensors. However, the *L. ferrooxidans* based sensor displays a shorter response time and ensures free of sulfur compounds interference determinations, because of the specific mechanism and kinetics of the Fe^{2+} oxidation (Sand et al., 1992).

Thus, the proposed Fe^{2+} sensor can be applied for continuous on-line monitoring of ferrous iron in acidic sulfate media without sample pretreatment. In contrast, the conventional photometric methods require preliminary sample treatment: Fe^{2+} extraction, pH adjustment, reagents addition, etc., which makes the on-line determination complicated or even impossible.

3.4. Application of the *L. ferrooxidans* based sensor for indirect $Cr_2O_7^{2-}$ determination

Since the bacterial sensor based on *L. ferrooxidans* is sensitive to Fe^{2+}, it can be used for indirect determinations of any agent reacting with Fe^{2+}, thus changing stoechiometrically its concentration without affecting the bacteria metabolism. $Cr_2O_7^{2-}$ completely fulfill these two conditions. The process which mechanism is investigated in details (Espenson and King, 1963; Laitinen and Harris, 1979) is described by the following equation:

$$Cr_2O_7^{2-} + 14H^+ + 6Fe^{2+} \rightarrow 2Cr^{3+} + 6Fe^{3+} + 7H_2O \quad (7)$$

One mol of $Cr_2O_7^{2-}$ oxidizes 6 mol of Fe^{2+}, thus lowering its concentration causing decrease of the bacterial oxygen consumption, resulting in biosensor response change proportional to the $Cr_2O_7^{2-}$ concentration, as shown in Fig. 5.

The sensor response was registered during successive additions of $Cr_2O_7^{2-}$ aliquots to a solution containing a constant Fe^{2+} concentration (60 μmol L^{-1}), at 30 °C, pH 1.8, and 100 rpm stirring rate. The corresponding calibration curve (steady-state current I_s/nA vs. $Cr_2O_7^{2-}$ concentration C_{Cr}/μmol L^{-1}) was described by the linear equation: $I_s = 2.47C_{Cr}$, with $r^2 = 0.9817$.

The linear range of $Cr_2O_7^{2-}$ determination depends on the initial Fe^{2+} concentration. Since 1 mol of $Cr_2O_7^{2-}$ oxidizes 6 mol of Fe^{2+}, the linear range of the sensor toward $Cr_2O_7^{2-}$ is 1/6 of the linear range of the Fe^{2+} determination.

The proposed *L. ferrooxidans* sensor is not sensitive to $Cr_2O_7^{2-}$. It only senses the changes of the mediator Fe^{2+} concentration resulting from the reaction (7). Thus, the $Cr_2O_7^{2-}$ determination was

Table 1
Main characteristics of the *L. ferrooxidans* and *At. ferrooxidans* (Zlatev, 2002) based sensors for Fe^{2+} quantification.

Bacteria	Sensitivity (nA L μmol^{-1})	LOD (μmol L^{-1})	Response time (s)	t_{L50} days	R.S.D. (%)	Interferences
L. ferrooxidans	3.94	2.4	18	45	<3	No
At. ferrooxidans	4.09	0.9	45	–	<3	Thiosulphate

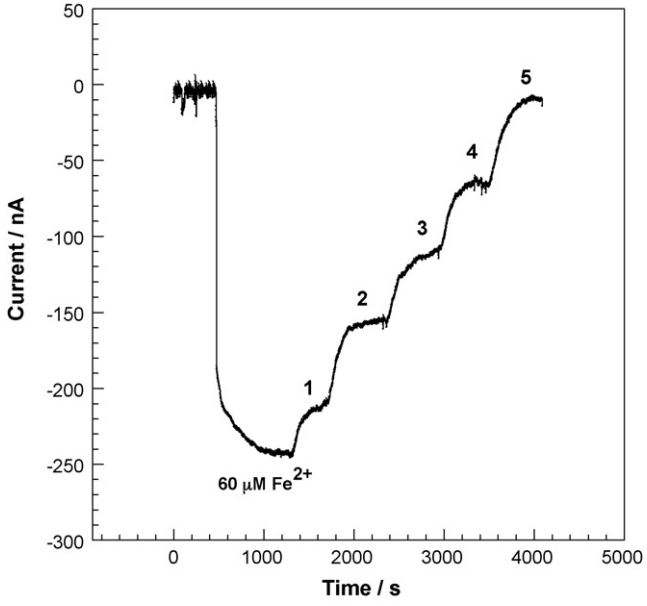

Fig. 5. Amperometric response of the *L. ferrooxidans* sensor for increasing $Cr_2O_7^{2-}$: (1) 2 μmol L^{-1}; (2) 4 μmol L^{-1}; (3) 6 μmol L^{-1}; (4) 8 μmol L^{-1}; (5) 10 μmol L^{-1}, in air-equilibrated deionized water at 30 °C, pH 1.8, 100 rpm, and Fe^{2+} concentration of 60 μmol L^{-1}.

influenced by the factors affecting the Fe^{2+} determination described above.

4. Conclusion

Taking advantage of the metabolic restriction of the bacterium *L. ferrooxidans*, a novel electrochemical biosensor was developed, providing specific Fe^{2+} quantification not interfered by sulfur compounds presence, thus overcoming the main drawback of the *At. ferrooxidans* based Fe^{2+} sensors.

The biosensor metrological and analytical characteristics, such as LOD, sensitivity, reproducibility, lifetime t_{L50}, and steady-state response time were evaluated and reported.

The Clark type oxygen probe parameters were optimized to obtain transducer short response time and high sensitivity.

The sensor was applied as well as for the indirect $Cr_2O_7^{2-}$ determination, using the Fe^{2+} as a mediator.

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