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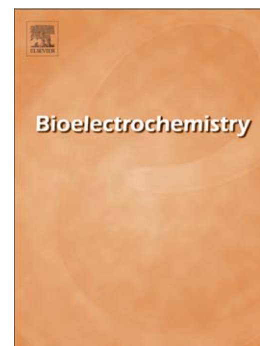

Electron transfer mechanism in *Shewanella loihica* PV- 4 biofilms formed at graphite electrode

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Electron transfer mechanism in *Shewanella loihica* PV- 4 biofilms formed at graphite electrode

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Abstract

Electron transfer mechanisms in *Shewanella loihica* PV- 4 viable biofilms formed at graphite electrodes were investigated in potentiostat-controlled electrochemical cells poised at oxidative potentials (0.2 V vs. Ag/AgCl). Chronoamperometry (CA) showed a repeatable biofilm growth of *S. loihica* PV- 4 on graphite electrode. CA, cyclic voltammetry (CV) and its first derivative shows that both direct electron transfer (DET) mediated electron transfer (MET) mechanism contributes to the overall anodic (oxidation) current. The maximum anodic current density recorded on graphite was 90 µA cm^{-2}. Fluorescence emission spectra shows increased concentration of quinone derivatives and riboflavin in the cell-free supernatant as the biofilm grows. Differential pulse voltammetry (DPV) show accumulation of riboflavin at the graphite interface, with the increase in incubation period. This is the first study to observe a gradual shift from DET to MET mechanism in viable *S. loihica* PV- 4 biofilms.

Keywords: Extracellular Electron Transfer, *Shewanella loihica* PV- 4, Electroactive biofilms, Graphite electrode, mediated electron transfer.

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1. Introduction

Shewanella sp. is a Gram-negative, biofilm-forming gamma-proteobacterium. Most members of *Shewanellaceae* family, with the relevant exception of *S. denitrificans*, are capable of extracellular electron transfer (EET) to reduce insoluble metal oxides and hydroxides at circumneutral pH as a part of their energy conservation strategy [1-4]. *Shewanellaceae* are relevant to metal bioremediation, microbially influenced corrosion, and bioelectricity production in Microbial Fuel Cells (MFCs) [5-7]. Because of their EET properties, *Shewanellaceae* has been included in the group of electroactive bacteria and their biofilms are often termed electroactive biofilms (EABs) [8-10]. With respect to other well-known EAB-forming bacteria, such as *Geobacter* sp., *Shewanella* sp. has a more adaptable metabolism, since it is a facultative strain and can grow on many substrates [11]. *Shewanella* sp. expresses numerous multi-heme cytochromes on the outer membrane that enables DET to the electrodes [12], but also produce redox mediators that facilitate MET [8, 9, 13]. The concurrence of DET and MET in *Shewanella oneidensis* MR-1 was extensively proven both in metal reduction and bioelectricity production [4, 7, 8, 10]. In this study, we focus on the lesser known *S. loihica* PV- 4, which was recently isolated from an iron-rich microbial mat near a deep-sea hydrothermal vent located on the Loihi Seamount in Hawaii [14]. This strain has received attention because it generates higher current density than other *Shewanella* strains [13]. Interestingly, it has been reported that both *S. oneidensis* MR-1 and *S. loihica* PV- 4 produce derivatives of quinones [13], and riboflavin (RF) [10] under anaerobic growth conditions. However, while *S. oneidensis* MR-1 strain is well known to utilize flavins as redox mediator to shuttle electrons to electrode/metal oxide [8-10], the involvement of redox mediators in electron transfer to electrode/metal oxide was not fully demonstrated for *S. loihica* PV- 4 strain [13]. The applicability of an electroactive strain in bioelectrochemical systems (BES) depends on full characterization of the EET mechanism and the ability to form stable EABs at different electrode materials [15]. Graphite has the high surface roughness, high conductivity, and is the most cost-effective material for BES. In this study, commercial graphite was tested for the growth and characterization of EET mechanism in *S. loihica* PV- 4 biofilms.

2. Materials and methods

2.1. Bacteria and growth medium

Shewanella loihica PV- 4 strain (DSM 17748) was purchased from German Collection of Microorganisms and Cell Cultures (DSMZ), Germany. The culture was grown aerobically for 24 hours (h) at 30 ºC in Luria-Bertani medium (LB). Subsequently, the culture was centrifuged at 13,400 rpm for 20 min, and the LB medium was replaced with 10 ml of defined medium (DM) containing per litre: $NaHCO_3$ 2.5 g, $CaCl_2{\cdot}2H_2O$ 0.08 g, NH_4Cl 1.0 g, $MgCl_2{\cdot}6H_2O$ 0.2 g, NaCl 10 g, HEPES 7.2 g. Vitamins mixture (1 ml) and trace metal solution (10 ml) were added to the DM as previously described, and 15 mM lactate was added to the medium as electron donor [4]. The cells were grown aerobically in DM at 30 ºC for 2 days, under shaking condition at 150 rpm. Following centrifugation for 20 min at 13,400 rpm, the pellet was washed three times with DM medium, to remove soluble redox mediators from the inoculum.

2.2. Electrode preparation

The graphite sheet (Tokai Co, Japan) was cut into 2 cm ×1 cm × 0.2 cm size electrodes. The graphite electrodes were polished by sandpaper (400 particles/ inch), cleaned in 1M HCl overnight, and stored in deionised (DI) water. Throughout this paper, all electrochemical potentials are described versus Ag/AgCl reference electrode (Fisher Scientific, Ireland), unless otherwise indicated.

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2.3. Electrochemical setup and analyses
Single chamber jacketed electrochemical cells (EC) of 10 ml working volume with three electrodes configuration were used as previously described [8]. The Ag/AgCl reference electrode was connected to the EC via a saturated KCl salt bridge ending in a 3 mm Vycor glass membrane (Bioanalytical Systems, UK). A 0.1 mm Pt wire (Sigma–Aldrich, Ireland) was used as the counter electrode. The working electrodes (graphite or ITO) were attached to the potentiostat via Pt wire, nylon screw and nut (Small Part, USA). The assembled EC were mounted on the magnetic stirrer. The headspace of the EC was continuously flushed with humidified, sterile N_2, which had been passed over a heated copper column to remove trace oxygen. The EC, maintained at 30 ºC throughout the experiment, was connected to a 5-channel potentiostat (VSP Bio-Logic, USA). Chronoamperometry (CA), cyclic voltammetry (CV) and differential pulse voltammetry (DPV) were used to analyze the *S. loihica* PV- 4 biofilm formed at graphite electrode, as previously described [8]. The magnetic stirrer was maintained at a constant speed of 150 rpm during CA, and was turned off during CV and DPV. The parameters for the techniques were chosen as it follows CA: $E_{applied}$ = 0.2 V vs Ag/AgCl; CV: equilibrium time, 5 s; scan rate, 1 mV/s; E_i = -0.8 V vs Ag/AgCl; E_f = 0.2 V vs Ag/AgCl; DPV: $E_{initial}$ (E_i) = -0.8 V vs Ag/AgCl and E_{final} (E_f) = 0.2 V vs Ag/AgCl; pulse height, 50 mV; pulse width, 300 ms; step height, 2 mV; step time, 500 ms; scan rate, 4 mV/s; ; accumulation time, 5 s. CV and DPV tests were performed approximately after every 24 h throughout each experiment.

2.4. Biofilm growth on graphite electrode
The washed *S. loihica* PV- 4 cell suspension was adjusted to a known O.D $_{520\ nm}$= 2.0 ± 0.2, then purged for 30 min with purified N_2, and finally 5 ml of this suspension was added to the electrochemical cell filled with 5 ml of DM medium. Lactate was added to a final concentration of 15 mM. After 24 h, the spent growth medium was replaced with the fresh DM medium, to promote EAB growth. Following the first medium change (MC), 15 mM lactate was injected twice at about 48 h and 72 h, to maintain non-limiting electron donor concentration in the EC.

2.5. Fluorescence spectroscopy
Fluorescence spectroscopy of the spent medium collected from the EC containing graphite anode was performed using a LS-50*B* luminescence spectrometer (Perkin Elmer, UK). Before analysis, the spent medium was centrifuged at 13,400 rpm for 20 minutes and filter-sterilized via 0.22 µm filter (Millipore, USA). The fluorescence excitation spectra (200 – 400 nm) at 430 nm emission wavelength and emission spectra (350 – 600 nm) at 360 nm excitation wavelength were recorded. The excitation and emission slit widths were 2.5 nm with photomultiplier tube (PMT) voltage of 600V.

2.6. Confocal microscopy
S. loihica PV- 4 biofilms grown at graphite electrode was collected after 96 h of the EC operation. The samples were removed from the EC in an anaerobic chamber (Coy Laboratory, USA), followed by staining for 30 minutes in 1 mg ml^{-1} acridine orange. After rinsing to eliminate excess dye, the samples were fixed to a glass slide. The confocal images were captured with a laser scanning microscope (Zeiss LSM 510, USA), using argon laser 488 nm as excitation source. The objective was a PLAN apochromatic 63 x oil immersion, with numerical aperture 1.40. Fluorescence was recorded with a low pass filter at 505 nm. A series of images were taken along the biofilm thickness (Z axis) at regular intervals (0.5 µm).

2.7. Scanning electron microscopy

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S. loihica PV- 4 biofilm coated graphite electrodes were removed from the EC after 96 h of operation in the laminar air flow. The biofilm sample was fixed with 2% glutaraldehyde in filtered (0.22 µm) phosphate buffer saline (PBS) for 2 hours and dehydrated using ethanol gradient (beginning with 20 %, 40 %, 60 %, 80 % and ending with 100 % ethanol). The samples were then air-dried, sputter coated with gold using a sputter coater, and then the samples were observed with Zeiss scanning electron microscope.

3. Results and discussion

3.1. Chronoamperometry of S. loihica **PV- 4** *biofilms on graphite electrode*

Chronoamperometry of *S. loihica* PV- 4 biofilms on graphite electrode is shown in Fig. 1. A current density of 5 ± 1.2 µA cm^{-2} was immediately observed after inoculation of *S. loihica* PV- 4 cell suspension. The anodic (oxidation) current grew steadily at a rate of 3 µA cm^{-2}/ h, and then reached a maximum of 45 ± 12 µA cm^{-2} within 24 h. The experiment was performed using 5 independent replicates. An average standard deviation of 13 µA cm^{-2} in the current density was calculated within the replicates, indicating a good reproducibility among replicates. The anodic (oxidation) current shows catalytic oxidation of the lactate and simultaneous reduction of the graphite electrode. After first MC, the chronoamperometry shows a 60 ± 10 % drop in the original current (Fig. 1). This current pattern shows a contribution to the current generation by suspended *S. loihica* PV- 4 cells and/or by soluble electron transfer agents. Previously, Marsili et al [8] reported 80 % drop in the current by *S. oneidensis* MR-1 on graphite electrode after replacing spent growth medium with fresh medium. This current drop was then attributed to the presence of flavins in the spent medium that acts as redox mediators, increasing current production. Recently, Newton et al [13] reported that the anode attached and suspended *S. loihica* PV- 4 cells contributes equally to the current generation at graphite felt electrode. Therefore, to promote *S. loihica* PV- 4 biofilm growth and to eliminate the contribution from suspended cells, the spent medium was replaced with fresh medium after 24h of the inoculation (Fig.1). After first MC current increase from 23 ± 10 µA cm^{-2} (at 26h) to around 56 ± 15 µA cm^{-2} (at 40h) and decreased thereafter but recovered quickly after lactate injection (15 mM) at 48 h. The current increase quickly to 76 ± 14 µA cm^{-2} and 90 ±18 µA cm^{-2} within 6 h of lactate addition (15 mM) at 48 h and 72 h, respectively (Fig. 1), indicating that lactate was limiting in the EC. Subsequent lactate addition (15 mM) did not result in any further increase (data not shown). The maximum current density of 90 ± 18 µA cm^{-2} was higher than the maximum current density (74 µA cm^{-2}) reported earlier for *S. loihica* PV- 4 on graphite felt electrode [13]. Similar current pattern for *S. loihica* PV- 4 was reported earlier on nano-networked polyanline (NN-PANI) modified ITO electrodes, with maximum current density reaching up to 115 µA cm^{-2}. The higher current density on NN-PANI modified ITO electrode compared to the plain ITO was attributed to the enhanced electron transfer via mediator molecules in the nanoporous surface structures of the electrode [16].

Higher surface roughness of graphite allows faster initial attachment and favour early biofilm formation. The average surface roughness (rms) and average peak to valley roughness of the graphite used in this study were measured from AFM images as 1.8 ± 0.4 µm and 3 ± 0.3 µm, respectively (details not shown). Scanning electron microscopy of the bare graphite electrode also shows clearly the presence of micron size cracks on the surface (Fig. 2A). *Shewanella loihica* PV-4 cells are rod shaped with length of ~1.6 µm and diameter of ~1 µm, as reported earlier [4]. The rms value of graphite and cell size of *S. loihica* PV-4 are comparable. In general, higher values of surface roughness favour bacterial settlement, when the roughness values are comparable with the size of the microbial cells [17]. Further, microbial cells accumulated in the cracks and crevices of graphite electrodes can access more of the

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electrode surface area and are in closer proximity with other cells, i.e., the biofilm is better "packed". This assumption was confirmed by scanning electron (Fig. 2B) and confocal microscopy (Fig. 2C), which clearly reveals a 2-3 µm thick and uniformly packed *S. loihica* PV- 4 biofilms on the graphite electrode.

3.2. Cyclic voltammetry and first order derivatives

The cyclic voltammograms of *S. loihica* PV- 4 biofilm on graphite collected after MC shows two overlapping catalytic waves, one onset at -0.6 V, centred at -0.44 V vs. Ag/AgCl, and the second onset at -0.2V, centred at -0.07 V, indicating two simultaneous catalytic electron transfer process at the graphite interface (Fig. 3A). Recently, Carmona-Martinez et al [18], observed the presence of two catalytic centres with E_m value -0.33 V and -0.07 V vs. Ag/AgCl in the turnover CV of *S. oneidensis* MR-1 strain on graphite electrode. They attributed the first centre to MET and second to the DET. First derivative of the corresponding CVs shows the presence of three redox centers RC (I) = -0.07 V; RC (II) = -0.35V; and RC (III) = -0.44V vs. Ag/AgCl. The redox potential of RC (I) (-0.07 V vs Ag/AgCl) obtained in our study is close to the midpoint potential of the outer membrane *c* type cytochrome reported earlier from the whole cell analysis of *S. loihica* PV- 4 (-0.054 V vs. Ag/AgCl) [15] as well as for *S. oneidensis* MR-1 (-0.07 V vs. Ag/AgCl) [29]. Newton et al [13] showed that Shew 2525 acts as terminal reductase in *S. loihica* PV-4 and the PV- 4 mutant lacking Shew2525 was severely impaired of the current generation. Therefore, it is likely that the RC (I) may refer to the Shew2525. Cyclic voltammetry of Shew2525 mutants has not been reported. The comparison of wild type *S. loihica* PV- 4 and Shew 2525 mutants CVs may confirm our results. The potential of RC (II) and RC (III) are close to the midpoint potential of the quinone derivatives (-0.27 V vs. Ag/AgCl) [13] and riboflavin (-0.42 V vs. Ag/AgCl) [8], respectively, which are common redox mediators secreted by *Shewanella sp*. The standard redox potentials of quinones are widely distributed from -0.02 V to -0.38V vs. Ag/AgCl [13] and governed by the functional groups. However, closer look at the first derivatives reveal an interesting pattern about the shift from DET to MET by *S. loihica* PV- 4 biofilms at graphite electrode. Immediately after first MC (at 24 h after inoculation) the derivative of the CV shows that the electrons are transferred mostly via RC (I) directly to the electrode and RC (III) plays a minor role in the mediated electron transfer (Fig. 3B). Interestingly, first derivative at 48 h shows comparable peaks from both DET and MET at RC (I) and RC (III), respectively. However, with the further increase in the incubation period (at 72 h and 96 h) the electrons are transferred preferentially by RC (III) via MET mechanism, which was evident from the Fig. (3B). This suggests that with the increase in the incubation period the redox mediators (flavins) produced by *S. loihica* PV- 4 biofilm cells accumulate at the interface and are subsequently used to mediated electrons at graphite electrode. Selection of ET mechanism by electroactive bacteria depends on the several factors of which surface properties of the electrode material may play a significant role. Graphite/carbon electrode has the high adsorption affinity for the biomolecules such as flavins [8]. Therefore, it could be possible that adsorbed flavins at the graphite interface decrease the interaction between outer membrane cytochrome of attached *S. loihica* PV- 4 cells and the electrode surface, thereby switching the DET to MET. Peng et al [19] and Liu et al [20] showed the possibility of decreased interaction between outer membrane cytochrome complex in *S. oneidensis* MR-1 and *S. loihica* PV- 4 with an increase in the catalytic current, respectively.

3.3. Differential pulse voltammetry

DPV of the *S. loihica* PV- 4 biofilm formed at graphite electrode confirms the above results and shows the accumulation of flavins represented by the increase in the peak height at RC

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(III) with the increase in the incubation period (Fig. 4). Most of *Shewanella sp* are found to secrete redox-mediators such as flavins and quinones that mediated electron transfer, and increase in the DPV peak height may represent the accumulation of redox-active mediator at the interface, while decrease in peak height represent the loss of these compounds [8]. A direct correlation between increases in flavins peak height in DPV with incubation period was observed (data not shown), as reported earlier for *S. oneidensis* MR-1 [8]. DPV shows the similar pattern as observed in the first derivatives of the CVs, i.e. the peak height at RC (I) decrease relative to the increase in the peak height at RC (III) with the biofilm growth. At 48h DPV shows comparable peak intensity at RC (I) and RC (II).

3.4. Fluorescence spectroscopy of the cell-free supernatants

To further validate the presence and accumulation of redox mediators in the medium associated with *S. loihica* PV- 4 biofilm formed on graphite electrode, the cell-free supernatants were subjected to the fluorescence spectroscopy. The excitation and emission spectra recorded are shown in Fig. 5. The fluorescence spectra of the supernatant collected at different stages of the experiment from graphite electrode shows two peak emission wavelengths at 440 nm and 520 nm. The first peak emission wavelength is similar to the wavelength of quinone derivatives (430 nm) and second is close to the flavins (520 nm), as reported earlier [16]. The presence of quinone derivatives in the graphite associated supernatants was confirmed by excitation spectra, where the major peak at 350 nm corresponds to the quinone derivatives [13]. Interestingly, the relative peak intensity (excitation and emission) of the supernatants collected at every 24 h from *S. loihica* PV- 4 biofilm associated with graphite shows a gradual increase, indicating accumulation of quinone derivatives and flavins in the medium with the biofilm growth. It is worthwhile to mention that as the *S. loihica* PV- 4 biofilm grew on graphite electrode more quinone derivatives accumulate in the culture medium than flavins (Fig. 5A, B). Conversely, DPV of *S. loihica* PV- 4 biofilm on graphite shows the accumulation of flavins at the electrode interface with the incubation period (Fig. 4). This may be because of the high adsorption affinity flavins such as riboflavin for carbon/graphite electrodes and to the cell materials [12], which allows higher accumulation of flavins at the interface. While most quinones diffuse into the bulk solution. These results support the first derivative of CVs (Fig.3B) and DPV data obtained for *S. loihica* PV- 4 biofilm on graphite electrode (Fig. 4).

4. Conclusions

We grew *S. loihica* PV- 4 biofilm at graphite electrodes in potentiostat-controlled electrochemical cells. The results indicated that DET and MET, through membrane cytochromes and microbially produced redox mediators, respectively, occur jointly with similar relevance at the biofilm/graphite interface. However, *S. loihica* PV- 4 biofilm switch from DET to MET as the microbially produced redox mediator (flavins) accumulates at the biofilm/graphite interface. Fluorescence spectroscopy and DPV supports accumulation of flavins and their role as EET mediators on graphite. Graphite has the high surface roughness which favors higher microbial adhesion and flavins adsorption/accumulation. Our results provide considerable evidence on the presence of DET and MET mechanism in *S. loihica* PV- 4 biofilms formed at graphite electrode.

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Figure captions

Figure 1. Chronoamperometry of *S. loihica* PV- 4 (AI) after inoculation at graphite electrode poised at oxidative potential (0.2 V vs. Ag/AgCl), (MC) medium change after 24 h with the fresh DM medium was followed by (LA) 15 mM lactate addition at 48h and 72h, respectively.

Figure 2. (A) Scanning electron microscopy picture of a bare graphite, (B) and *S .loihica* PV- 4 biofilm on graphite. (C) Confocal microscopy picture of the *S. loihica* PV- 4 biofilm on graphite electrode collected after 96 h of cultivation at 0.2 V vs. Ag/AgCl.

Figure 3. (A) Cyclic voltammograms at scan rate = 1 mV s^{-1} {obtained at (a) 24 h, (b) 48 h, (c) 72 h and (d) 96 h after MC}, and (B) first order derivatives of corresponding CVs {obtained at (a) 24 h, (b) 48 h, (c) 72 h and (d) 96 h after MC} of *S. loihica* PV- 4 biofilms formed at graphite electrode. (B) The major redox centers in first order derivatives of CVs were identified as RC- I = -0.07 V, RC- II = -0.35 V, and RC- III = -0.44 V vs. Ag/AgCl.

Figure 4. DPV of *S. loihica* PV- 4 biofilms associated with graphite electrode, collected at regular time intervals (a) 24 h, (b) 48 h, (c) 72 h and (d) 96 h after MC. The major redox centers were identified as RC- I = -0.07 V, RC- II = -0.35 V, and RC- III = -0.44 V vs. Ag/AgCl.

Figure 5. (A) Fluorescence excitation and (B) emission spectra of the cell free supernatant {collected at (a) 24 h, (b) 48 h, (c) 72 h and (d) 96 h after MC} from *S. loihica* PV- 4 biofilms associated with graphite.

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Figure 1.

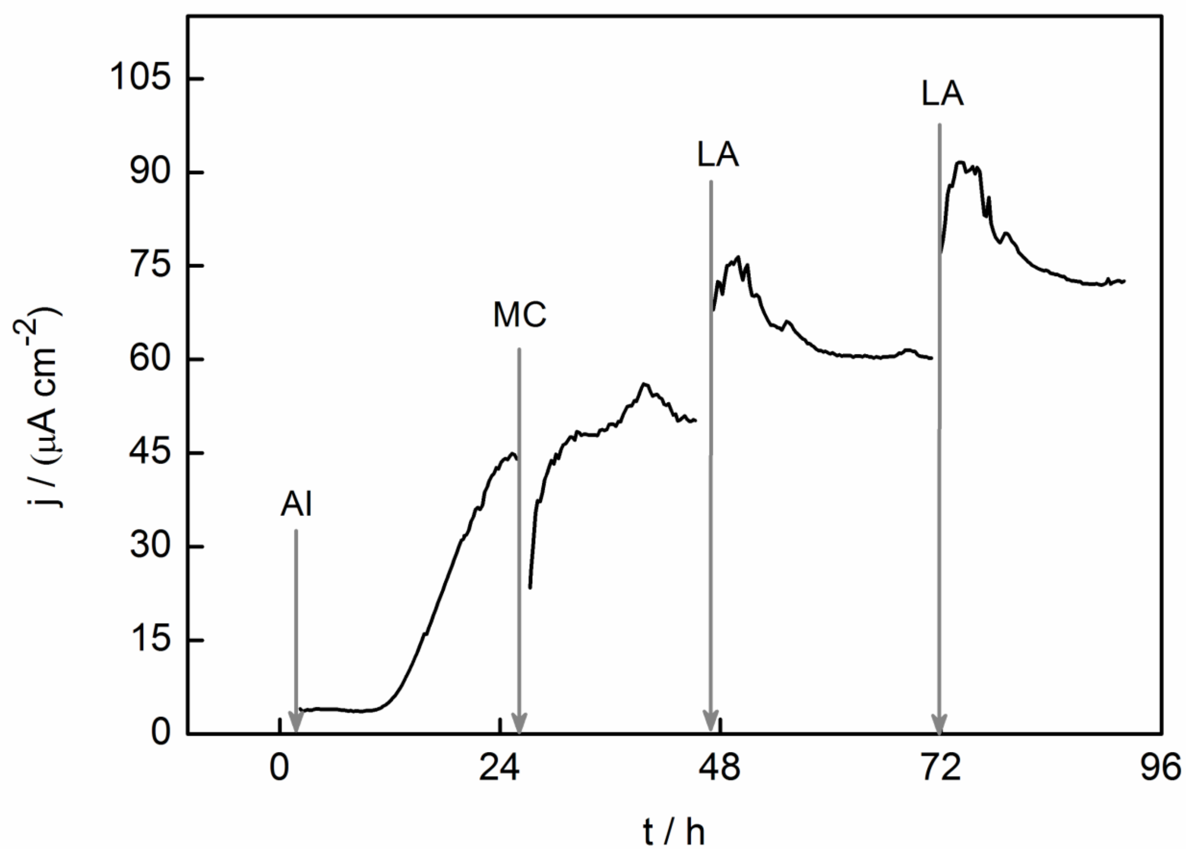

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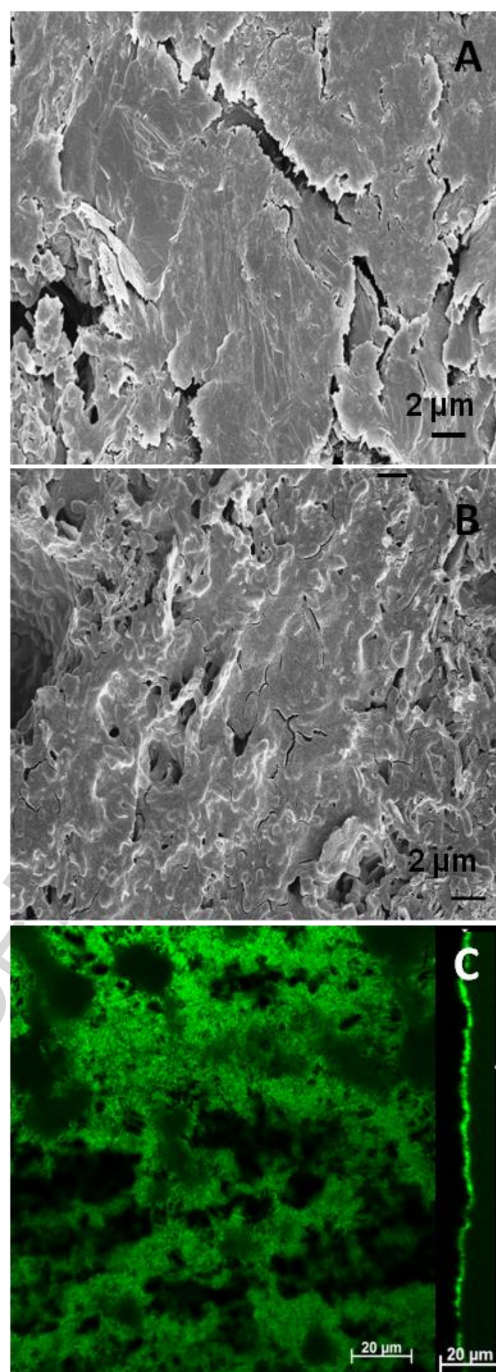
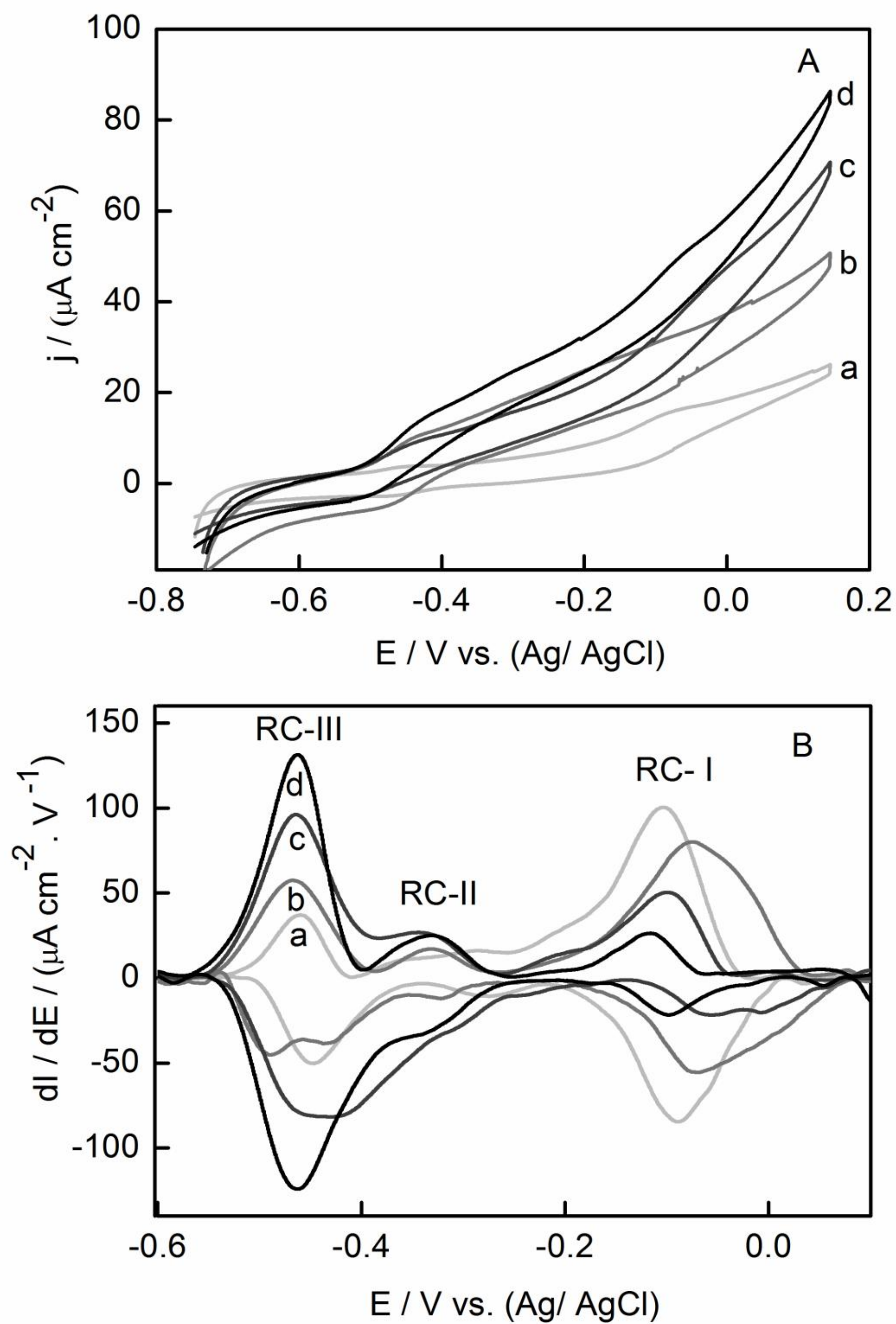
Figure 2.



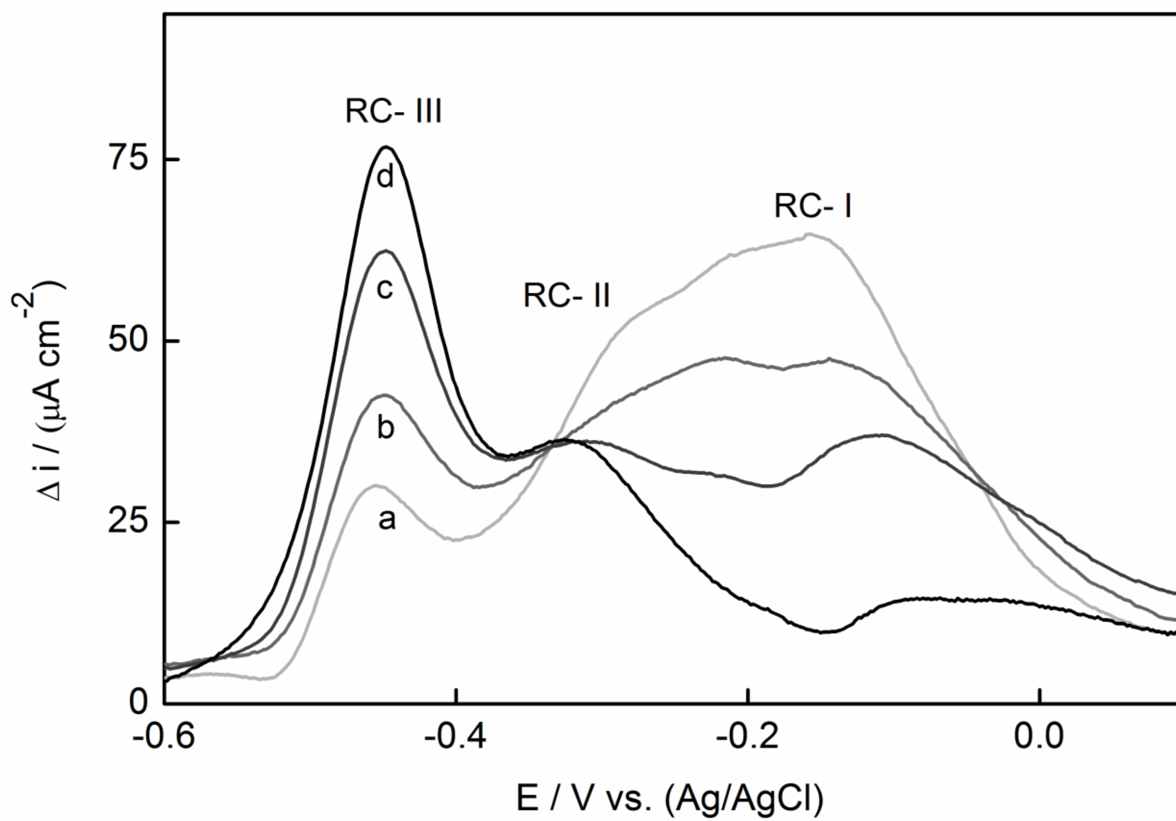
ACCEPTED MANUSCRIPT

Figure 3.



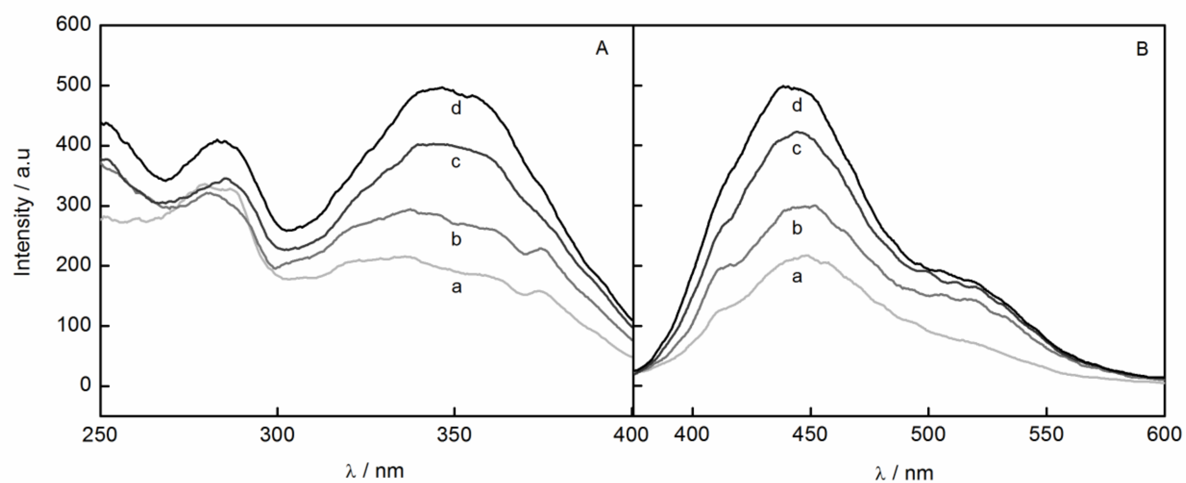
ACCEPTED MANUSCRIPT

Figure 4.



ACCEPTED MANUSCRIPT

Figure 5.



ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Highlights

- DET shift towards MET mechanism as *S. loihica* PV-4 biofilm grew on graphite electrode
- Accumulation of riboflavin at *S. loihica* PV- 4 biofilm/graphite electrode interface

ACCEPTED MANUSCRIPT