

The influence of hydrogen bubble formation on the removal of *Pseudomonas fluorescens* biofilms from platinum electrode surfaces

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Abstract

Hydrogen bubble formation on the surface of platinum electrodes as a means of removing biofilms was studied. Biofilms of *Pseudomonas fluorescens* of different ages were grown on platinum electrodes and challenged with hydrogen bubbles formed at the surface of the electrodes, by cycling the potential at -2.0 V. The removal of the biofilms from the surfaces was assessed by direct epifluorescence microscopy. The removal of the biofilm from the surface was dependent on the biofilm age. As the biofilm became older, the duration of bubble formation needed to achieve complete removal changed, but in some cases, it was not possible to obtain a completely clean surface. An enhancement of biofilm removal was obtained if the potential was cycled between -0.5 and 1.0 V for 30 min prior to bubble generation, probably due to the weakness of the forces established between the surface and the biofilm and within the biofilm.

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Keywords: *Pseudomonas fluorescens*; Biofilms; Cyclic voltammetry; Hydrogen bubbles; Detachment

1. Introduction

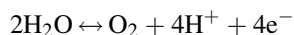
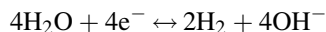
Bacterial biofilms in industrial equipment can cause serious problems, such as corrosion of pipe materials, resistance to heat transfer in heat exchangers and spoiled end products, which lead to large economic burdens in industry. These phenomena occur since in the interior of some industrial equipment flows biodegradable matter that enhances microorganism growth on surfaces, causing biofouling. This situation must be avoided due to the high costs associated with the need for biofilm control and the effects on the final product quality and deterioration of the equipment [1]. However, the search for both an inexpensive and effective way to reach that goal has not yet produced such a method. Adhering microorganisms and biofilms can be released from surfaces by a number of biological, chemical and physical processes.

In the course of a work carried out to optimise the use of platinum electrodes to detect biofilm accumulation, it was shown that cyclic voltammetry applied to the platinum electrodes with a biofilm grown on its surface alters the structure and activity of the biofilm [2]. Illsley et al. [3] and Gião et al. [2] verified that the amount of cells adhered on a platinum electrode surface can be reduced by cyclic voltammetry suggesting that the optimisation of cyclic voltammetry conditions could be used as a means of cleaning the surface of the platinum sensor. Nevertheless, previous results showed that it was not possible to clean the surface completely using only cyclic voltammetry [2] but when hydrogen bubbles were formed on the surface of the platinum electrode by cycling the potential between -2.0 and 1.0 V during 1.5 min, a 2-h biofilm was removed.

When the potential is cycled at very low or very high limits, the formation of bubbles takes place. At those limits, the electrolysis of water occurs at the positive or negative limits causing, respectively, the formation of oxygen or hydrogen bubbles, since the electrode acts either as a

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cathode or an anode. The principal cathodic and anodic reactions are, respectively [4]:



Several years ago, Dhar et al. [5] showed that bubbles formed on an electrode hampered the adhesion of bacterial cells to that surface. Other studies confirmed that the detachment of cells and other particles from surfaces can be promoted by the passage of air bubbles introduced in the system [6,7].

The purpose of this study was to investigate the potential of hydrogen bubble formation on the surface of platinum electrode sensors, as a means of removal from those surfaces biofilms of different ages formed by *Pseudomonas fluorescens*. *Pseudomonas* were chosen as model microorganisms since they are good biofilm producers and are involved in many biofouling episodes.

2. Material and methods

2.1. Microorganism, cell growth and biofilm development

Pseudomonas fluorescens ATCC 13525 were used in this work to form biofilm on platinum electrode surfaces. These bacteria were maintained in the exponential phase of growth, in a 0.5-L volume fermenter. This fermenter was continuously fed with a sterile medium containing 5 g L⁻¹ glucose, 2.5 g L⁻¹ peptone and 1.25 mg L⁻¹ yeast extract in phosphate buffer (4.3 g L⁻¹ Na₂HPO₄·2H₂O and 3.75 g L⁻¹ KH₂PO₄). This culture was used to continuously inoculate a 0.5 L reactor fed at a dilution rate of 0.84 h⁻¹ with a medium consisting of 50 mg L⁻¹ glucose, 25 mg L⁻¹ peptone and 12.5 mg L⁻¹ yeast extract. Platinum electrodes were immersed in this reactor for different intervals of time. These electrodes with biofilm on their surface were used to study the effect of hydrogen bubbles formation on biofilm detachment. Prior to their utilisation, the electrodes were degreased using a detergent, rinsed twice by immersion in distilled water with agitation for 10 min and sterilised by immersion in ethanol for 20 min, before being cleaned electrochemically by cycling the potential between -0.5 and 1.0 V limits at 250 mV s⁻¹ for 30 min as described by Vieira et al. [8].

2.2. Electrochemical experiments

The working electrodes were platinum discs with 1 mm diameter (area of the platinum: 7.85 × 10⁻⁷ m²). The electrodes were prepared by sealing a platinum wire into a glass tube, and the internal end of the platinum wire was sealed to a copper wire that provided the external contact. The surface of the cross-section of the platinum was polished with alumina powder (grain of 0.05 μm), on a

polishing cloth. The reference electrode was a Metrohm silver/silver chloride electrode and all the data are reported versus this reference. The auxiliary electrode was a platinum spiral. The electrochemical experiments were carried out in a two-compartment, three-electrode cell at room temperature, using a potentiostat Autolab type PGSTAT 10, Ecochemie that produced a repeating triangular function.

Each electrode, before immersion in the fermenter, was electrochemically treated by introduction in an electrochemical cell containing phosphate buffer pH 7 and the potential was cycled between -0.5 and 1.0 V limits, at 250 mV s⁻¹. The electrodes were then immersed in the fermenter to form biofilms of different ages on the surface of the electrodes. These electrodes (WE) were then inserted in the electrochemical cell, and the electrode potential was cycled at -2.0 V in order to form hydrogen bubbles at the surface of the platinum electrodes during different intervals of time. For older biofilms, this procedure was preceded by cycling the potential between -0.5 and 1.0 V for 30 min, at a sweep rate of 250 mV s⁻¹.

2.3. Epifluorescence observations

The biofilm on the surface prior and after the electrochemical procedures was assessed by visualizing the platinum electrodes under epifluorescence microscopy after staining the surface with the *BacLigh* viability kit, Molecular Probes. The two *BacLigh* stains, SYTO 9 and propidium iodide, dissolved in dimethylsulphoxide (DMSO), were mixed together (130 μL + 130 μL) and used to stain the surface of the electrode during 15 min in the dark. The surface was observed with a Zeiss (AXIOSKOP) microscope fitted with fluorescence illumination. The optical filter combination for optimal viewing of stained preparations consisted of a 480–500 nm excitation filter in combination with a 485 nm emission filter. The micrographs were obtained using a microscope camera (AxioCam HRC, Carl Zeiss).

According to the manufacturer's protocol, viable cells fluoresce green, while non-viable cells fluoresce red. In these microphotographs, the platinum surface appears black, while the glass surface appears brighter.

3. Results and discussion

When the potential is cycled at very low or very high limits, the formation of bubbles on the electrode surfaces takes place. At those limits, the electrolysis of water occurs causing, respectively, the formation of hydrogen or oxygen bubbles since the electrode acts either as a cathode at low potentials or an anode at high potentials. When the potential is cycled at -2.0 V, the working electrode (WE), which is the electrode with biofilm on its surface, acts as the cathode and hydrogen bubbles are formed according to equation [4]



At the auxiliary electrode (platinum spiral), oxygen bubbles are formed according to equation:



It was observed that initially, very small bubbles were formed on the platinum surface. Those bubbles became bigger after some minutes and some were released into the fluid. The formation of bubbles at the spiral platinum electrode was also observed.

For an uncolonised electrode, the formation of bubbles occurs at the whole platinum surface. When the surface is partly covered with biofilm, the formation of those bubbles will occur not only on the platinum surface free of cells and biofilm (that is smaller than the uncolonised surface) but also on the surface covered with biofilm (probably at a lower extent than at the uncolonised surface). Actually, as the biofilm is mainly constituted of water (more than 98%), the electrolysis of water contained in the biofilm may also occur when the electrode is subjected to very low or very high potentials.

The hydrogen bubbles formed on the platinum electrode surface, by cycling the potential at -2.0 V, are released into the surrounding medium. These bubbles may contribute to the detachment of adhering bacteria or the biofilm grown on the surface. Two mechanisms may account for the detachment of the cells or the biofilms from the surfaces:

- when a bubble is formed beneath the bacterial cells or the biofilm, it may slough off the deposits, which may be released into the medium;
- when the bubbles are generated at the uncovered platinum surface of the electrodes, they accumulate (giving rise to bigger bubbles) before being released. During the passage of the bubbles through the surface, bacteria and biofilm may be removed, by the sweeping of the surface by the bubbles.

Concerning the effects of bubble formation on the biofilm accumulated on the surfaces, several experiments were carried out, changing the age of the biofilm and the duration of bubble formation, and in some cases, cyclic voltammetry between -0.5 and 1.0 V was also carried out. The effect of the electrochemical treatment was assessed by means of the observation of the biofilms by epifluorescence microscopy before and after the treatment. In these microphotographs, the platinum surface appears black, while the glass surface appears brighter.

For a 2-h old biofilm, which still has discrete microorganisms and micro colonies on the surface, 1 min of hydrogen bubbles formation was enough to remove completely the cells from the electrode surface. Conversely, a 4-h biofilm was not completely cleaned after this treatment. Fig. 1a shows a microphotograph of an electrode colonized with a 4-h biofilm obtained after staining the biofilm with live/dead viability kit before the electrochemical treatment. It can be seen that the electrode still has discrete microorgan-

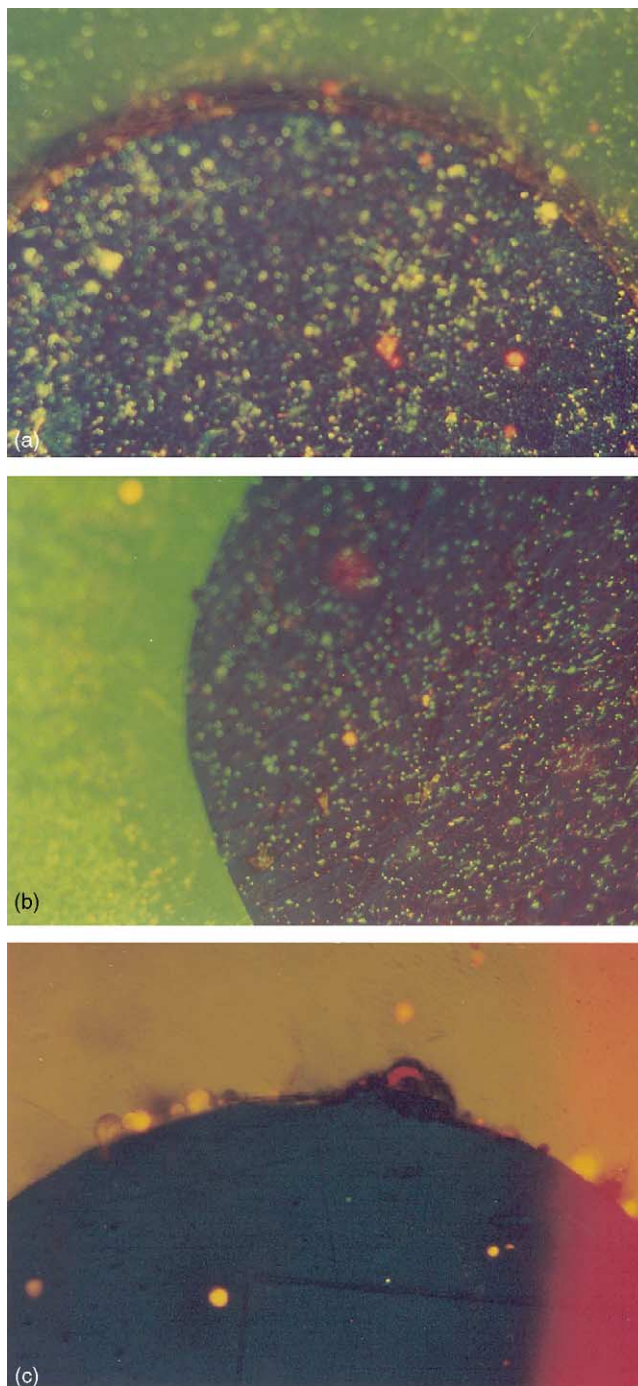


Fig. 1. Epifluorescence micrographs of a platinum electrode colonised with a 4-h old biofilm: (a) before cyclic voltammetry; (b) after hydrogen bubbles formation during 1 min; (c) after hydrogen bubbles formation during 2 min.

isms and micro colonies on the surface, mainly alive, since they appear green, and that an EPS matrix is not present for a biofilm with this age. After cycling the potential at -2.0 V during 1 minute, forming hydrogen bubbles at the platinum surface, the electrode still appears with cells, some of them dead that fluoresced red (Fig. 1b). However, an increase of the interval of time of bubble formation for 2 min completely cleaned the electrode (Fig. 1c).

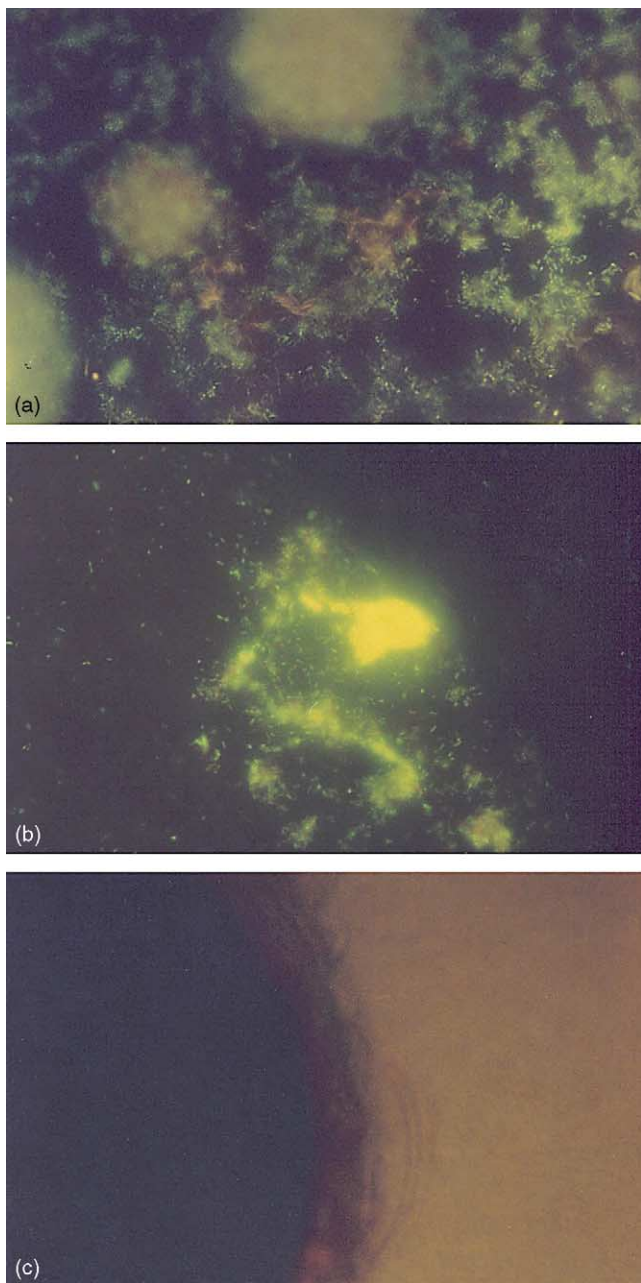


Fig. 2. Epifluorescence micrographs of a platinum electrode colonised with a 24-h old biofilm: (a) before cyclic voltammetry; (b) after hydrogen bubbles formation during 4 min; (c) after cycling the potential between -0.5 and 1.0 V for 15 min followed by hydrogen bubbles formation during 4 min.

For very young biofilms, such as biofilms constituted only by adhered cells, the detachment of cells can be attained just by the formation of hydrogen bubbles on the platinum surface. As previously mentioned, those bubbles may pull the cells away from the surface or may release them due to the passage of the air–liquid interface. Additionally, electrolysis will also cause the production of hydroxyl ions and protons. These will change the pH of the aqueous phase and may cause the reduction of electrostatic force between microbial cell and electrode surface.

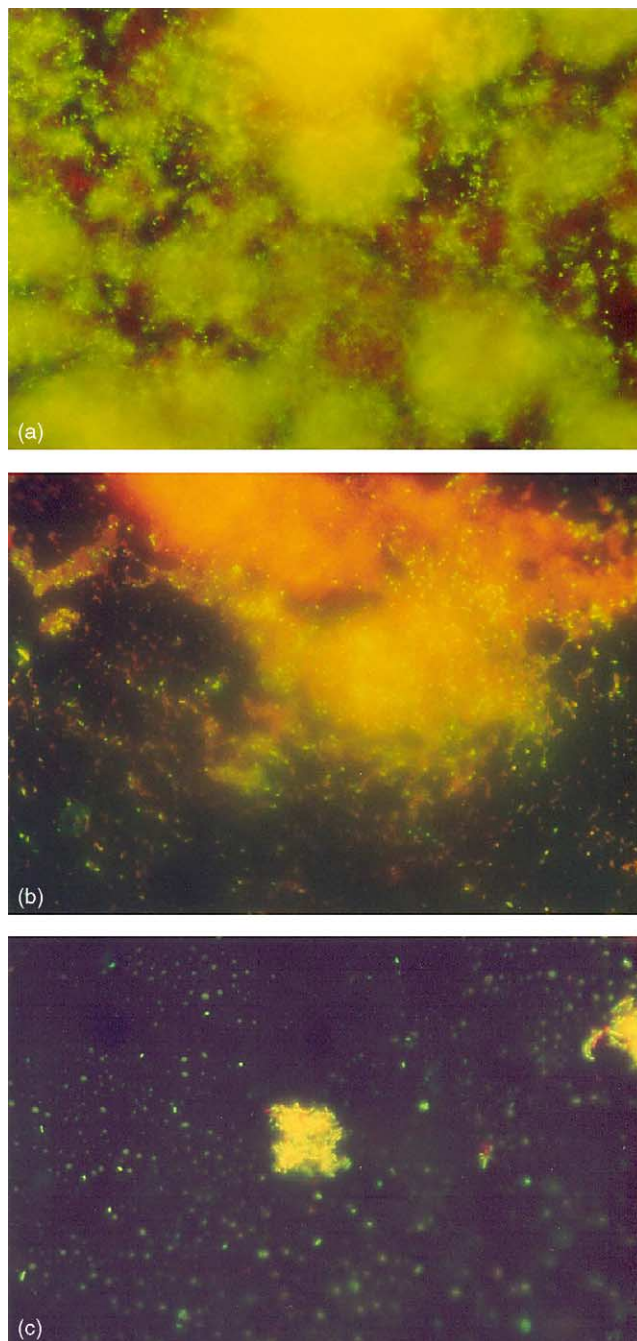


Fig. 3. Epifluorescence micrographs of a platinum electrode colonised with a 3-day old biofilm: (a) before cyclic voltammetry; (b) after hydrogen bubbles formation without cycling the potential between -0.5 and 1.0 V; (c) after cycling the potential between -0.5 and 1.0 V for 30 min followed by hydrogen bubbles formation.

It was observed, however, that the time of bubble formation needed to release the adhered cells was related with the age of the biofilm, probably due to the stronger interactions established between the cells and the surfaces and also by the higher number of bacteria adhered on the surface. Gómez-Suárez et al. [6] made a study on the detachment of bacteria adhering to substratum surfaces upon the passage of an air–liquid interface. The air bubbles were

Table 1
Removal of biofilm of different ages from the surface

Biofilm age	Duration of potential cycling ^a (min)	Duration of hydrogen bubbles formation on the platinum surface (–2.0 V) (min)	Removal of the biofilm from the surface
2 h	–	1	Total
4 h	–	1	Partial
	–	2	Total
24 h	30	2	Partial
	–	4	Partial
	30	4	Total
3 days	30	8	Partial
	–	10	Partial
	30	10	Total
5 days	–	10	Partial
	30	10	Total
7 days	30	10	Partial
	30	10	Partial
	30	15 + 15	Partial

^a The electrode potential was cycled between –0.5 and 1.0 V at the scan rate of 0.250 V s^{–1}.

introduced in the system by means of injection of air. They concluded that several parameters accounted for the detachment of cells from the surface including the velocity of the air bubbles (the higher the velocity, the smaller was the detachment from the surface), the shape of the bacteria (rod-shaped bacteria were more difficult to detach than coccoid bacteria), the presence or absence of conditioning film (in the presence of this film, it was difficult to remove all the adhering bacteria, and they were displaced). It must be stressed that the above-mentioned work was carried out with bacteria harvested from a growth medium and deposited on a surface through flow in a flow-cell system, while in the case under study the biofilms are formed on a surface by introducing that surface in a vase with conditions prone to biofilm formation.

For a 24-h biofilm, polymers imbuing the cells are already present, as can be seen by the green fluorescent spots presented in Fig. 2a. For this condition, it was not possible to eradicate completely the biofilm from the platinum surface by the formation of hydrogen bubbles for 4 min, since cells were remaining on the electrode surface after this treatment (Fig. 2b). However, it was observed that if the potential was cycled between –0.5 and 1.0 V, for at least 30 min, before the formation of the bubbles, a complete removal of the biofilm was achieved (Fig. 2c).

Fig. 3a shows a 3-day old biofilm, which has a very heterogeneous structure. Abundant amounts of polymers can be observed on some areas of the surface, constituting EPS strands (the fluorochromes used stained not only the bacterial cells but also the EPS matrix), masking the bacterial cells [9]. Other zones still have discrete bacterial cells. For a 7-day old biofilm, it was not possible to clean the electrode surface completely, even by increasing the time of bubbles formation up to 15 min. Observations (results not shown) demonstrate that when the potential was not cycled

between –0.5 and 1.0 V before bubble formation, there were a large amounts of bacterial cells and EPS matrix and some zones with discrete cells. Furthermore, after cycling the potential between –0.5 and 1.0 V for 30 min, followed by the production of bubbles during a period of 10 min, followed by a second period of 10 min of bubbles the detachment was not complete and bacterial cells and small amounts of polymers can still be observed on the surface, demonstrating that it was not possible to attain a complete cleaning of the surface.

Table 1 gathers the results obtained for the removal of biofilms of different ages from the surface, as a function of the duration of hydrogen bubbles formation on the platinum surface and the situations when a previous cycling of the potential between –0.5 and 1.0 V was carried out. Removal of the biofilm from the surface was dependent on biofilm age. In fact, as the biofilm becomes older, the conditions needed to achieve a complete removal from the surface changed and in some cases, it was not possible to obtain a completely clean surface. Several reasons may account for this behaviour. As the biofilm gets older the adhesion forces between the biofilm and the surface are higher, and thus, it is more difficult to remove it from the surface. Even though, the generation of bubbles may occur beneath the biofilm, this phenomenon may slough off less biofilm since the amount of the biofilm on the surface is very high. In addition, there is less uncolonised surface and thus fewer zones where bubble formation may occur and subsequently being released into the medium. As a result, it is expected to have less removal due to sweeping of biofilm from the surface.

On the other hand, it was observed that an enhancement of biofilm removal was obtained if the potential was cycled between –0.5 and 1.0 V during 30 min prior to bubble generation. The possible explanation for this behaviour is

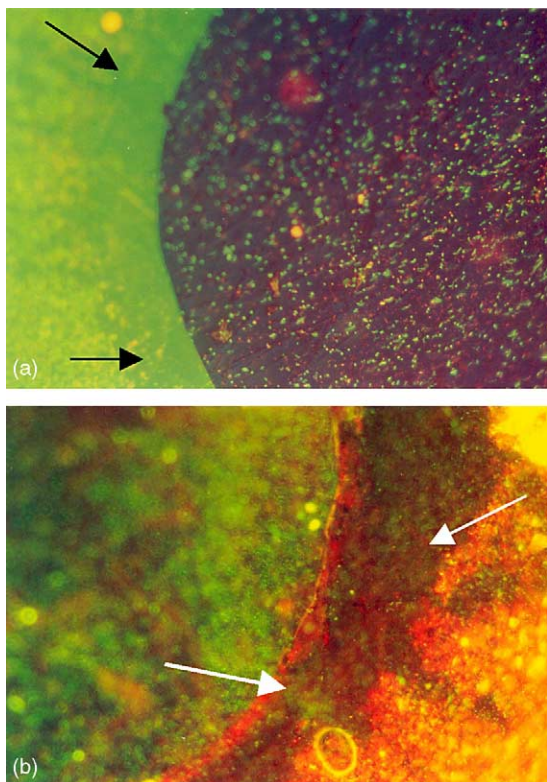


Fig. 4. Examples of the effect of the passage of hydrogen bubbles (generated on the platinum surface) through the glass surface. Pointed out by the arrows can be seen the biofilm displaced in the direction of the glass: (a) biofilm with 4 h; (b) biofilm with 3 days.

that when the potential cycles are applied to the platinum surface the forces between the biofilm and the surface are weakened. Most likely, the distance between the biofilm and the surface increases and thus the liquid film between the bacterial cells and the surface becomes thicker. Consequently, bubble generation beneath the biofilm may be easier and will slough off the biofilm to a greater extent.

Regarding the biofilm formed on the glass surface in most of the situations the sweeping of the biofilm by the bubbles was not enough to remove the biofilm from that surface but caused a re-disposition and displacement of the biofilm. Fig. 4a and b show the surface of the platinum electrodes with, respectively, a 4-h and a 3-day biofilm, after the bubble formation treatment. In these pictures, the darker platinum surface and the brighter glass surface were distinguishable. The arrows in the pictures point out the biofilm displaced in the direction of the glass, by the bubbles formed at the platinum surface. A cleaner surface is obtained at the interface between the platinum and the glass surface, but a denser surface is obtained immediately after this area. Fig. 5c also demonstrates that the glass surface obtained after the treatment with the bubbles has less biofilm accumulated than the surface prior to the treatment, suggesting that some removal of the biofilm occurred due to the passage of the bubbles on the surface. As previously

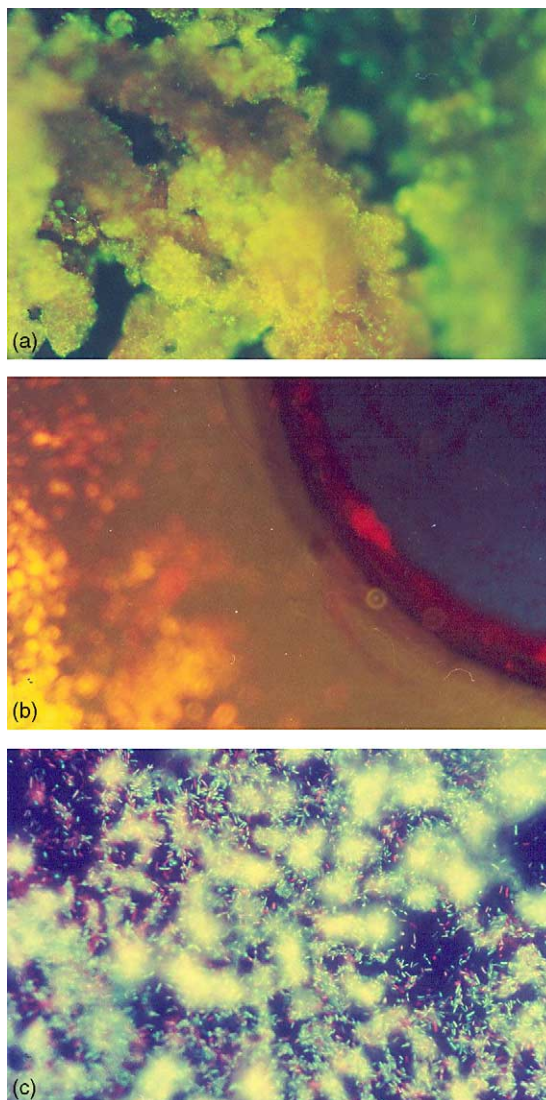


Fig. 5. Microphotographs of biofilm with 5 days: (a) without treatment; (b) after cyclic voltammetry and bubble generation at the platinum surface; (c) biofilm on the glass after cyclic voltammetry and bubble generation at the platinum surface.

mentioned, Gómez-Suárez et al. [6] also observed a displacement of the biofilm due to the passage of air bubbles.

4. Conclusions

The removal of the biofilm from the surface caused by the generation of hydrogen bubbles on the platinum surface was dependent on the biofilm age. In fact, as the biofilm becomes older, the duration of bubble formation needed to achieve complete removal from the surface changed, but in some cases, it was not possible to obtain a complete clean surface. On the other hand, it was observed that an enhancement of biofilm removal was obtained if the potential was cycled between -0.5 and 1.0 V during 30 min prior to bubble

generation, probably due to the weakness of the forces established between the surface of the biofilm and within the biofilm.

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