Int. J. Electrochem. Sci., 14 **(2019)** 10162 – 10175, doi: 10.20964/2019.11.05

International Journal of ELECTROCHEMICAL SCIENCE
www.electrochemsci.org

Biofilm formation on Titanium and Titanium Oxide and its Characterization and Electrochemical Properties

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Received: 14 February 2019 / *Accepted:* 30 July 2019 / *Published:* 7 October 2019

This work studied the surface characterisation and the electrochemical evaluation of titanium (Ti) and anodised titanium (A-Ti) electrodes modified with a bacterial biofilm of a novel consortium composed of *Enterobacter cloacae complex*, *Enterococcus gallinarum*, *Escherichia coli*, *Klebsiella pneumoniae,* and *Enterococcus faecium* – the biological support media consisted of peptone casein nutrient broth (PCNB), and acid whey (AW) – with the purpose of evaluating the electrochemical response of non-carbonaceous materials with low interferences. This is useful for bio-electrodes, mainly biocathodes intended to operate in bioelectrochemical systems of substrates at neutral pH and acid pH. For this, scanning electron microscopy (SEM) and electrochemical techniques of chronopotentiometry and voltammetry were used, following the biofilm growth after the inoculation of the bacterial consortium on the Ti and A-Ti materials, at times of 24 h and 168 h. The results showed that it was possible to obtain biofilms of bacteria with a high efficiency in a period of 24 hours, noting that the A-Ti material favours the biofilm growth in terms of quantity, chemical stability and a biocathodic response. The biofilms demonstrated specific behaviours depending on the inoculation time and the biological support medium, with evidence of bacterial bodies coated by extracellular polymeric substances (EPS) in the AW media. These results allow the possibility of using Ti and A-Ti materials in bioelectrochemical systems whose purpose is to treat whey solutions with an acidic pH.

Keywords: Bio-electrode, Titanium, Anodised, Media, pH, Biofilm.

1. INTRODUCTION

Bioelectrochemical systems (BES) have been proven to be useful in the study of bioremediation and energy production from wastewater treatment [1]. In these systems, the bio-electrodes constituted

by microorganisms embedded in extracellular polymeric substances (EPS) and that are supported on metallic materials or compounds have crucial functions [2,3]. They behave like bioanodes when microorganisms transfer electrons to the material or as biocathodes when they receive electrons therefrom [2]. The mechanisms of these electronic transfers have been described according to the processes that occur environmentally, with more existing studies related to bioanodes than biocathodes. It is possible to have direct electron transfers in which cytochrome type c and hydrogenases are crucial. The mediated electronic transfers are those involving natural redox mediators produced by the same microorganisms of the bio-electrode, or artificial mediators when they are added by the researcher. However, the understanding of the BES is still limited and consequently it is essential to understand aspects that impact upon its functioning and performance [4]. Some authors have studied the influence of using a pure strain or a bacterial consortium [5,6] and the electrochemical response obtained during different biofilm growth stages [7]. The necessity of using endogenous or exogenous redox mediators to carry out the mediation electron transfer bacterium-electrode [8,9] and BES with both electrodes (anode and cathode) of a microbial nature has also been explored [10]. Other authors have studied the effect of the nature of the BES atmosphere [11], as well as the evaluation of the different materials that can be used to support the microorganisms in the BES [12-14]. It is important to mention that the modification of the electrode material to improve the bacterial adhesion and its respective electronic transfer has obtained greater importance. It should also be emphasised that the materials used to generate a bio-electrode need to be biocompatible, conductive, chemically stable, and resistant to corrosion. Furthermore, they must have surface characteristics, such as a greater surface area and high roughness, that allow bacterial adhesion and, consequently, the electronic transfer between the electrode and the microorganisms. These characteristics make it possible to obtain greater current efficiency [15]. Among the most commonly used materials are those of a carbonaceous nature, such as graphite felt [10,13,16,17], glassy carbon [17,18], solid graphite [17,19-21], carbon paper [13,22], carbon fibre [9], graphite cloth [14], and activated carbon cloth [11]. Other studies have used non-carbonaceous materials such as stainless steels [5,13,23], modified materials such as zinc oxide and indium tin oxide by Connolly [12], as well as polyurethane compounds [24,25] and titanium [26]. Due to its high biocompatibility titanium is a material that has been used in various biomedical materials, giving excellent results [27-31]. Some biomedical authors even use the oxidised form, which in some cases has been modified with other compounds in order to have a better affinity with the tissues [32,33], whose preparation is possible through various techniques such as anodising.

In the present investigation, the preparation of electrodes of titanium and titanium oxide obtained by anodising was carried out in order to use them as support materials for a novel bacterial consortium. The decision was made to work with a consortium considering that, as mentioned by Rebaey [21], Erable [5], Nevin [6] and Zuo [34], the microorganisms achieve higher current efficiencies in a consortium than as an isolated culture. This was composed of electroactive bacteria that, according to previous studies, do not require the addition of artificial redox mediators: *Enterobacter cloacae complex* [35] *Enterococcus gallinarum* [36], *Escherichia coli* [37], *Klebsiella pneumoniae* [38] and *Enterococcus faecium*. Although it is a Gram positive bacterium whose current production is low in pure culture biofilms, when added to Gram negatives crops have been shown to favour the current increase [39]. All this is done with the purpose of evaluating the bio-anodic or bio-cathodic electrochemical response of

the modified electrode and analysing the different growths of the biofilm as a function of the time of inoculation, in the media PCNB and AW. This represents a research focused on evaluating the electrochemical response of materials of a non-carbonaceous nature, which adds to those already existing and which in the future will be useful for bio-electrodes destined to operate in bioelectrochemical systems of substrates at neutral pH and acid pH.

2. EXPERIMENTAL PROCEDURE

2.1 Preparation of Ti and A-Ti electrode surface

The surfaces of titanium electrodes, with a geometric size of 12 cm^2 each, which were used as bio-electrodes and control electrodes, were polished (prior to the anodisation process) using 320 grit sandpaper. Subsequently, the electrodes were introduced into an ultrasonic bath containing deionised water, using a Cole Parmer 8890 device, for a period of 10 minutes. Next, the electrodes destined for anodising were pickled in an aqueous solution of sodium hydroxide (NaOH) at 30%, and then in a 5% NaOH aqueous solution (both at a temperature of 45–50°C). The anodising process was carried out using a potentiostatic technique with a two-electrode cell, connected to a BK Precision model 1670A power source. The electrolytic solution was 20% sulphuric acid, imposing 30 V of potential for 180 seconds. Finally, the materials were rinsed in a 5% NaOH aqueous solution at a temperature of 45–50°C. The Ti and A-Ti materials were characterised before and after their modification with the bacterial consortium by SEM/EDS analysis (images and acquisition of semiquantitative elemental microanalysis) using SEM (JEOL model 6300) equipped with a dispersive energy spectrometer detector (EDS). To carry out the characterisation of the materials after they were modified with the bacteria, it was necessary to coat each surface with gold in two blocks of six minutes using Denton Vacuum model Desk II equipment. It is important to note that the analysis of images after surface modification with the bacteria was carried out in order to correlate the electrochemical response.

2.2 Preparation of bio-electrodes and control electrodes

The bacterial consortium (*Enterobacter cloacae complex*, *Enterococcus gallinarum*, *Escherichia coli*, *Klebsiella pneumoniae* and *Enterococcus faecium)* intended to modify the surfaces was taken from a sample of cattle ruminal washing, which was isolated and adapted in acidic conditions as detailed in previous work [40]. The identification of the genus and the species of the bacteria present in the consortium was carried out via the Vitek® 2 system: automated cards with colourimetric reagents, inoculated with the suspension of each previously isolated pure microbial culture. The system identified the bacterial species and genus through metabolic activities and a bacterial development profile in the presence of inhibitory substances. The following media were used as biological support and carbon sources: PCNB BDBioxon pH 7 in a concentration of 1 gL^{-1} and AW taken directly from the residual of an industrial process with a pH 4.6. The PCNB was considered the standard growing medium in which the consortium could grow under ideal conditions. Meanwhile, the AW was considered as a system of

possible future application in the wastewater treatment of the cheese industry. Besides, two media with a different pH and organic content were used in order to evaluate the growth of the biofilm under the two different conditions. For the preparation of the bio-electrodes (BE), an anaerobic batch system was designed wherein Ti and A-Ti materials were separately imbibed for seven days in 100 mL of the two biological support media (PCNB and AW), which were inoculated with 1 mL ($2.2x10^8$ CFU) of the bacterial consortium in a stationary phase, previously cultivated in PCNB in a concentration of 1 gL^{-1} at a temperature of 37.5ºC, from *Enterobacter cloacae complex* ($2.4 x10^8$ CFU), *Enterococcus gallinarum* ($1.9x10^8$ CFU), *Escherichia coli* ($2.72.4 x10^8$ CFU), *Klebsiella pneumoniae* ($2.42.4 x10^8$ CFU) and *Enterococcus faecium* ($1.7x10^8$ CFU), all in a stationary phase. The preparation of the electrodes in each system was in triplicate. Simultaneously, preparation of the control electrodes (CE) was carried out by imbibing Ti and A-Ti materials for seven days in 100 mL of the biological support media PCNB and AW, that is, in the same conditions used for BE except for the inoculation of the bacterial consortium.

2.3 Electrochemical experiments

Electrochemical studies of the BE and CE were performed using a potentiostat/galvanostat Autolab PG-STAT-30, which uses NOVA 2.0 computer software. The PCNB and AW biological support media were used as an electrolyte solution with different bacterial growth times: initially, 24 h and 168 h after inoculation of the bacterial consortium, noting that the electrode analysed at the initial time was a CE, while the electrodes analysed at 24 h and 168 h were BE. This was done in order to compare the electrochemical responses during different periods of bacterial growth and, consequently, establish the modification generated by the growth of the formed biofilm. A three-electrode array was used employing the ME and CE as the working electrodes in each evaluation, with a platinum wire as a counter-electrode and a saturated calomel electrode (SCE) as the reference electrode. The electrochemical techniques of chronopotentiometry and voltammetry were used, maintaining the same electroactive area of the electrodes in all experiments. The chronopotentiometries were performed at zero current ($E_{i=0}$) for five seconds with the purpose of determining an equilibrium potential. The cyclic voltammetries were performed at a 25mV/s sweep speed, which is not harmful for the biofilm to characterise, as reported in previous studies by Marques [41], Rabaey [21], Zhang [13] and their respective collaborators, starting at $E_{i=0}$ in an anodic direction until an overpotential (η) of 500 mV is reached, subsequently inverting the direction of the sweep until an η of -500 mV is achieved, and ending in $E_{i=0}$. It is important to note that the voltammetric results were processed by establishing them in overpotential in order to obtain more comparable results between the two materials to be evaluated.

3. RESULTS & DISCUSSION

3.1 Characterisation of Ti and A-Ti electrode surface

Characterisation of the surface of the Ti and A-Ti electrodes was carried out by SEM/EDS before modification with the bacterial consortium. The Ti electrode presented typical metallic characteristics,

while A-Ti presented a homogeneous blue colouration film due to anodising when 30 V were applied. This behaviour is congruent with that reported by some authors [42] who indicate that the titanium oxide film presents such organoleptic characteristics before the imposition of said voltage.

Figure 1 shows the Ti (Fig. 1a) and A-Ti (Fig. 1b) electrode surface SEM analysis. In Figure 1a, a polished titanium surface with the absence of an oxide layer can be observed, while Figure 1b (corresponding to A-Ti) presents an oxidised surface with the formation of agglomerates, due to the anodising process.

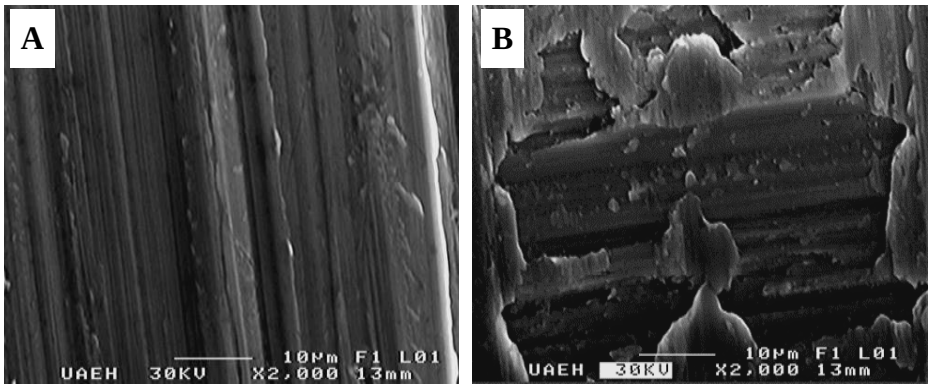

Figure 1. Surface scanning electron micrographs of the titanium (A) and anodised titanium (B) materials.

This was corroborated by the results of the semiquantitative analysis of chemical composition carried out by EDS, which demonstrated a composition of 100% of Ti for the material observed in Figure 1a, while in the material shown in Figure 1b, 81.4% titanium and 18.6% oxygen were obtained. This is indicative of the oxide layer formation on the surface.

3.2 Electrochemical response of Ti and A-Ti control electrodes (CE) in PCNB and AW biological support media

Table 1 shows the Ti and A-Ti CE null current chronopotentiometry results, which indicate that the Ti material has less positive (more cathodic) potential values, while the material of A-Ti has more positive potential (anodic). In addition, in the PCNB media for both electrodes, the potential was more positive (anodic) with respect to the AW media.

Table 1. Potential at zero current (Ei=0) of CE and BE at 24 h and 168 h after bacterial inoculation supported on Ti and A-Ti materials in the PCNB and AW media.

	PCNB			AW		
	CE	BE (24 h)	BE (168 h)	CE	BE (24 h)	BE (168 h)
Ti	39.3 ±1.8	203.3 ±40	-216.7 ±7.4	-121.1 ±1.3,	301.1 ±24	12.8 ±18
A-Ti	159.11 ±0.44	294 ±19	-83.8 ±7.4	98.3 ±2.3	230 ±81	13 ±18

Figure 2 presents the Ti and A-Ti CE voltammograms obtained in PCNB media (Figs. 2a and 2c, respectively) and in AW media (Figs. 2b and 2d, respectively). The voltammograms showed that the Ti material in both media presented an oxidation process at an overpotential of approximately 300–350 mV (Figs. 2a and 2b), which are predominantly attributed to the oxidation of the species of the medium and to a slight oxidation process on the Ti surfaces; these were not passivated since no limit currents were observed as in a passivation process. In addition, to obtain a passive layer, higher energy conditions are required than those given by the voltammetry studies in this work. In fact, further evidence that this relates to the oxidation of the species is that in the PCNB media (Fig. 2a) at an overpotential of 500 mV the Ti material presented a lower charge and anodic current compared to in AW media (Fig. 2b). This is because in the AW media there were a greater number of electroactive species, with high organic content composed by lipids, proteins and mineral salts. Meanwhile, in the A-Ti material in both media (Figs. 2c and 2d), no oxidation processes occurred during the entire range of anodic overpotentials studied. This behaviour is attributed to the fact that the material of the A-Ti electrode was previously anodised and this oxidation gave it the characteristic of being stable in both media (PCNB and AW).

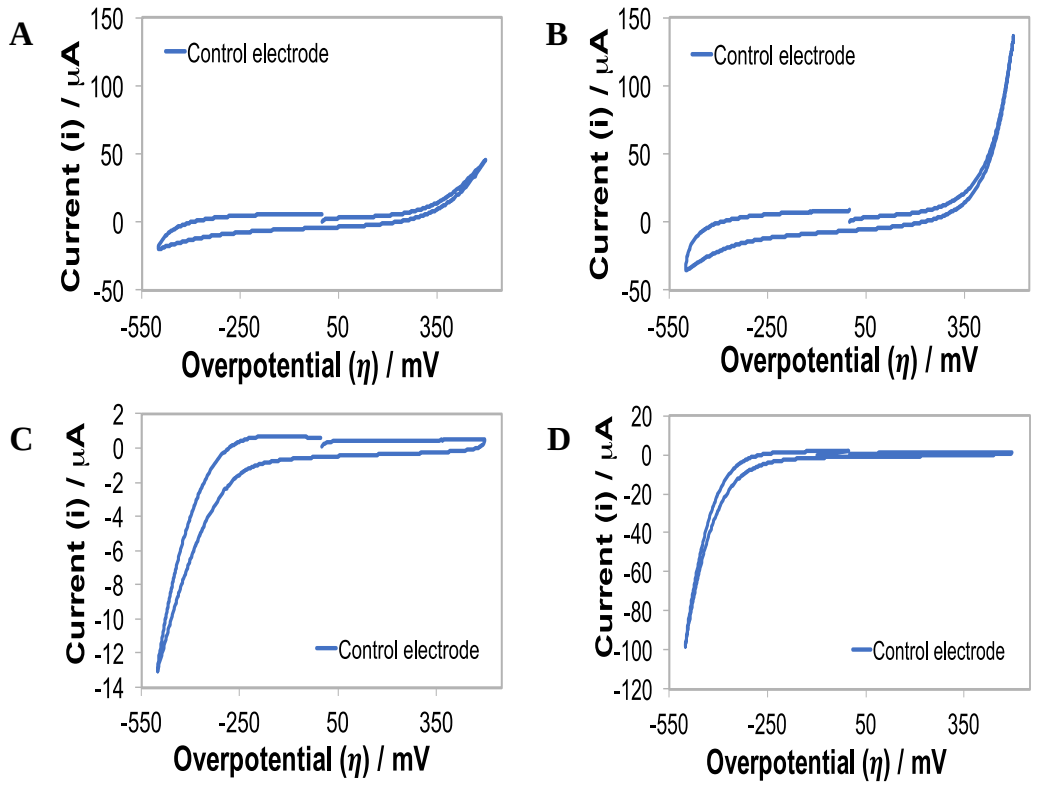

Figure 2. Voltammetric analysis of Ti materials in the biological support media PCNB (a) and AW (b) and of A-Ti in the biological support media PCNB (c) and AW (d).

However, when analysing the cathodic zone it was determined that the A-Ti material reached the highest current values in the AW media (-98.11 µA, Fig. 2d) compared to the PCNB media (-13.06 µA, Fig. 2c) at an overpotential of -500 mV, overcoming those obtained with the Ti material in the AW media (-35.73 µA, Fig. 2b) and PCNB media (-19.76 µA, Fig. 2a). This behaviour was attributed to the fact that, as mentioned previously, the AW media has a greater number of electroactive species,

including protons, than the PCNB, which could have been interacting with the A-Ti oxides for the process of species reduction and cannot be attributed to the reduction of the TiO_2 layer because the energy conditions used for anodisation were more energetic than the conditions given by the AW media. In addition, in the A-Ti material the oxides provide more electroactive area than in a Ti material without anodising. Lee and collaborators [43] mention that TiO_2 is useful for the evolution of hydrogen. It is important to mention that at the end of the experiments no macroscopic alterations were observed in the materials.

These results allow a possible advantage of the A-Ti material to be used in the reduction of the AW organic compounds due to its greater cathodic activity in the AW media. Therefore, it could be determined that the Ti materials had a more anodic behaviour while the A-Ti had a more cathodic behaviour in both media, although more extensive electrochemical activity was highlighted in the AW media for both materials.

3.3 Electrochemical response of the bio-electrodes (BE) on Ti material

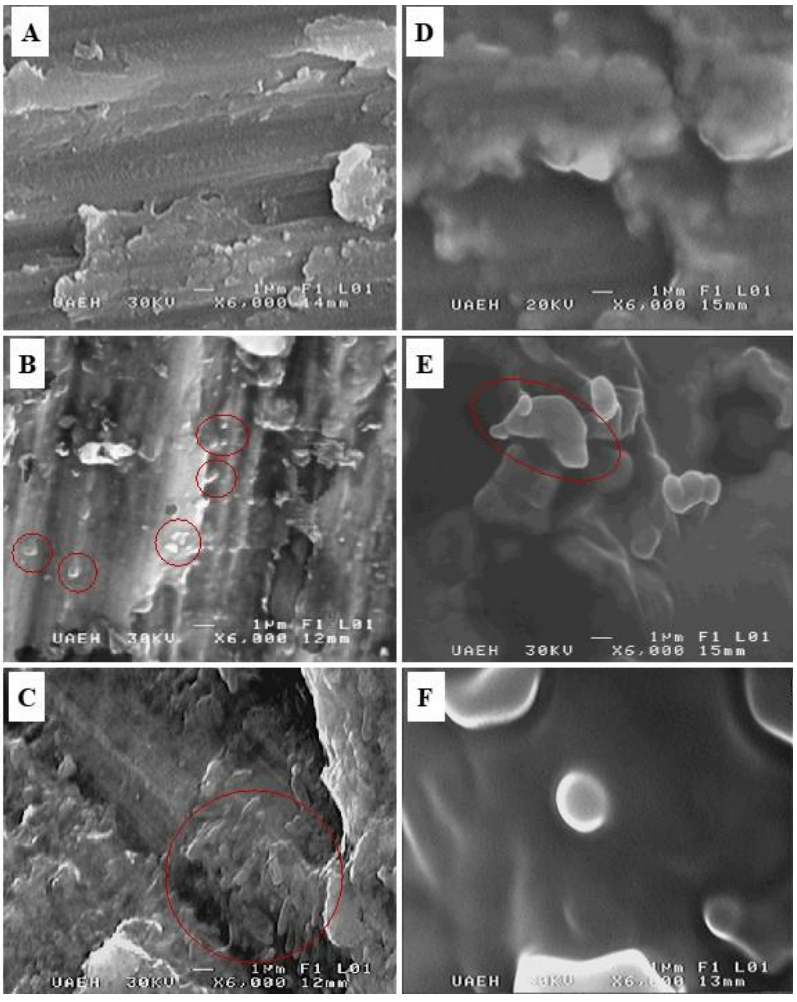

Figure 3. SEM micrographs of Ti electrodes, control electrodes (a and d) and bio-electrodes at 24 hours (b and e) and 168 hours (c and f) after inoculation of the bacterial consortium in biological support media PCNB (a–c) and AW (d–f).

Figure 3 shows the SEM analysis of the surfaces of the CE and BE at 24 h and 168 h after inoculation with the bacterial consortium with respect to Ti materials in PCNB media (3a–3c) and AW media (3d–3f). In Figure 3a, an unaltered surface of the Ti material can be observed since only a few agglomerates of oxides and scratches caused by mechanical polishing are evident. In Figure 3b the surface of an BE embedded in the PCNB media is observed after 24 hours of inoculation. In this image, a biofilm with distinguishable bacterial bodies can be observed, which are not observed on the surface of the CE. Furthermore, in micrograph 3b, as in the rest of the micrographs (Figs. 3 and 5), for illustrative purposes only some of the cells attached to the material are indicated with circles. Similarly, Figure 3c shows the surface of an BE embedded in PCNB after 168 hours of inoculation, which presents an increase in the number of bacterial bodies that are partially bound together and adhered to the surface of the Ti material.

Furthermore, in the AW media after 24 hours of inoculation (Fig. 3e), bacterial bodies that were not observed in the CE were adhered (Fig. 3d), whereas in the BE after 168 hours of inoculation (Fig. 3f), bacterial bodies coated by extracellular polymeric substances (EPS) were appreciated. That is to say, it is important to note that the groups of bacterial bodies observed after 24 hours and 168 hours of inoculation on the Ti material surface in AW media (Figs. 3e and 3f, respectively) can be seen to be embedded in EPS. In particular, as indicated in Figure 3f, the EPS generated in the BE after 168 hours of inoculation increased until the point at which the bacterial bodies became illegible. However, the characteristics of its roughness, as identified in Figure 3e, indicate that it was the EPS generated by bacteria and not the unmodified Ti surface. It is possible that this increase in the production of EPS observed in the Ti BE embedded in the AW media can be attributed to the environmental conditions of acidity within the AW media. As mentioned by de La Fuente and collaborators [44], under stress conditions the bacteria are protected in this structure. Another possibility is that an enriched medium promotes the production of EPS [45], most likely because this increases the possibilities of nutrient retention or simply because, by having an enriched medium, the bacteria have more favourable nutritional conditions.

In the chronopotentiometric null current ($E_{i=0}$) analysis of the BEs using Ti as a material in the biological support electrolytic media PCNB and AW (Table 1), the $E_{i=0}$ of the Ti BE in PCNB after 24 hours of inoculation of the bacterial consortium showed an increase in anodic potential with respect to the CE. Meanwhile, after 168 hours of inoculation, the $E_{i=0}$ of the BE shifted towards cathodic values, suggesting a surface modified by the bacteria. However, in the AW media, the CE of Ti started with a cathodic $E_{i=0}$ and moved towards more anodic values 24 h after inoculation; 168 h after inoculation, there was a decrease to a less anodic value. These results suggested that the $E_{i=0}$ of the Ti BE in both biological support media tends to move towards more cathodic values when the inoculation period is longer. This behaviour is attributed to the increase in the thickness of the biofilm formed on the material (Figs. 3c and 3f), making the surface of the evaluated material more resistive.

Figure 4 presents the voltammograms obtained from the CE and BE with Ti material after 24 hours and 168 hours of inoculation with respect to the biological support media PCNB and AW (Figs. 4a and 4b, respectively). In these voltammograms, as previously mentioned, it was observed that at overpotentials greater than 350 mV, the CE of Ti mainly presented the oxidation of species of the medium since no current values indicative of surface passivation were evident. On the other hand, the

BE in the PCNB media (Fig. 4a) after 24 hours of inoculation showed a higher anode current value (96.7 μA) than the CE (45.5 μA) because, in addition to the species of the media, bacteria that colonised molecules of their metabolism, mediators or waste products were present on the surface of the electrode. After 168 hours it was observed that the anodic current decreased (33.2 μA), reaching values lower than the CE. This can be attributed to the formation of the EPS layer; despite having conductive and semiconductor components, its polymeric nature gives it a lower conductivity than the Ti conductivity which lacks EPS on its surface, as does the CE, which generates greater opposition to electronic transfer. However, this does not mean that the electronic transfer between the bacteria and the electrode is compromised since EPS has active redox compounds present that allow these transfers to be made [46]. Although in the AW it was observed that the CE response presented an anodic current that decreased after 24 and 168 hours of inoculation, this can be attributed to the modification of the electrode. This is evident in the micrographs taken in the AW media (Figs. 3d, 3e and 3f) due to the obtention of the biofilm given by the large number of nutritious species of the AW media and as part of a bacterial protection strategy, as already mentioned. By reversing the direction of the sweep, one can observe the presence of a reduction process at overpotential values higher than -350 mV, repeating the same trend observed in the anodic processes for both the PCNB and for the AW.

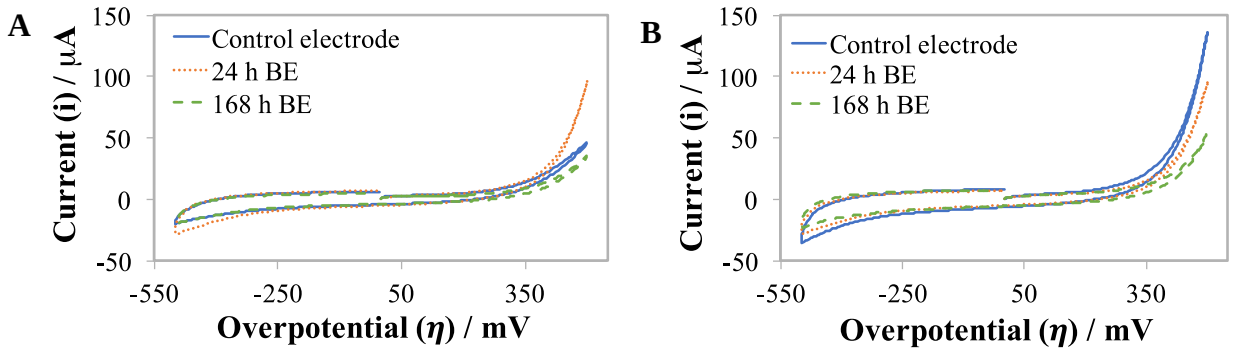

Figure 4. Voltammetric analysis of Ti materials in biological support media PCNB (a) and AW (b) at 24 h (orange line) and 168 h (green line) after inoculation and CE (blue line).

3.4 Electrochemical response of the bio-electrodes (BE) on A-Ti material

Figure 5 shows the images obtained by SEM for the CE and BE (after 24 hours and 168 hours of inoculation) with A-Ti material with respect to the biological support media PCNB (Fig. 5 a–c) and AW (Fig. 5 d–f). It was observed that the surface of the CE (A-Ti) in the PCNB media (Fig. 5a) was unaltered because the agglomerates of titanium oxide produced by the anodising process were still appreciated. Figure 5b shows the beginning of the bacterial colonisation after 24 hours of inoculation, albeit in an isolated manner; however, after 168 hours of inoculation, the colonisation of bacterial bodies multiplied on the A-Ti material in such a way that numerous bacterial cells were distinguished on the surface joined by the EPS generated by the bacteria (Fig. 5c). In this way, it can be confirmed that the longer the time elapses after inoculation, the greater the number of bacterial cells in the biofilm.

Figure 5d shows the formation of a film on the A-Ti CE surface when it was introduced into the AW media, without causing great superficial alterations due to the acidity. After 24 hours of inoculation of the A-Ti BE in AW media (Fig. 5e), the presence of bacterial bodies grouped in a similar manner to those observed in the BE of Ti without any anodisation (Fig. 3e). These bacterial groups were embedded in the EPS generated by themselves, which apparently appeared in greater quantity than in the A-Ti BE in the PCNB media (Fig. 5b). This, as discussed above for Ti BE without anodising in AW, was due to the acidity and organic content of the AW media. After 168 hours after inoculation in the AW media, the A-Ti BE surface showed a more abundant polymer layer (EPS) that imbibed bacterial bodies (Fig. 5f) than that presented in the A-Ti BE after 24 hours (Fig. 5e). There was even an intercommunication water channel within the generated EPS, confirming the presence of a three-dimensional EPS network within a mature biofilm.

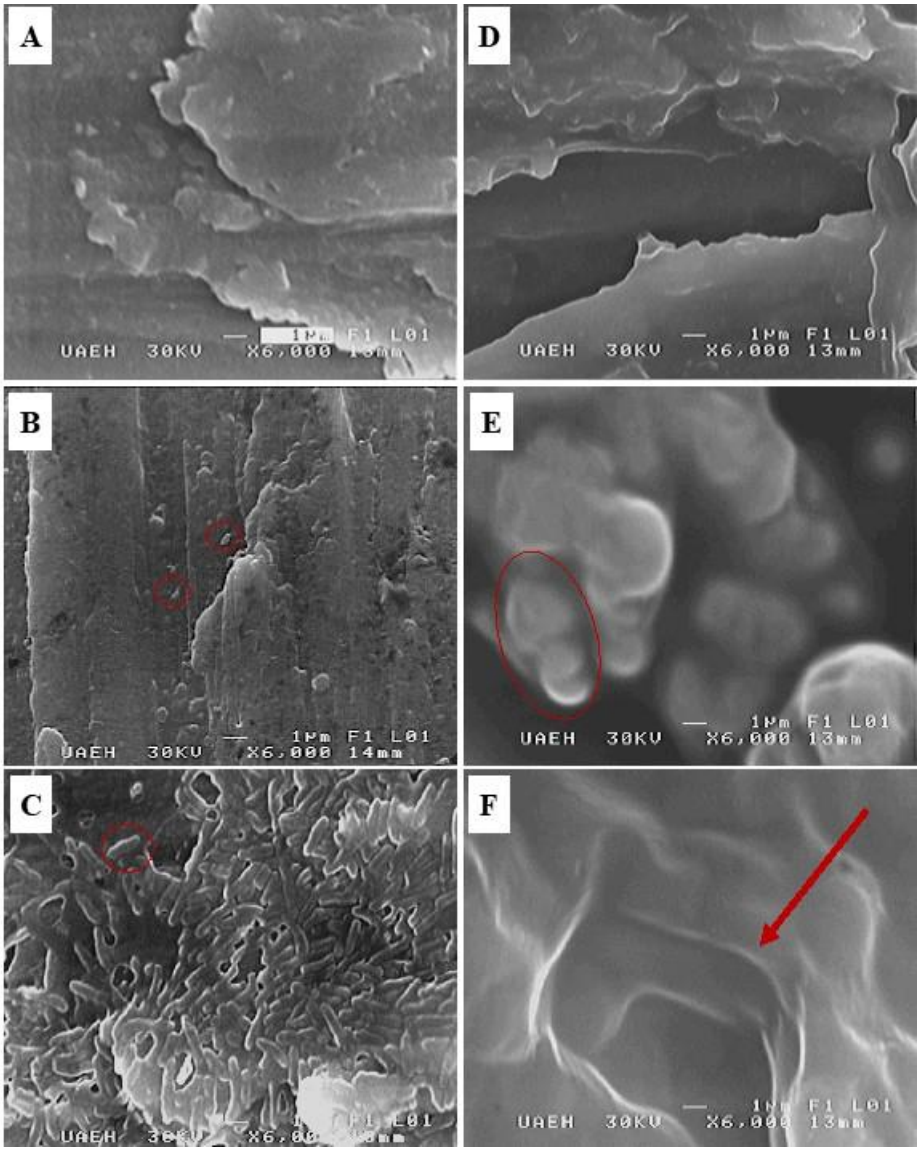

Figure 5. SEM micrographs of the A-Ti control electrodes (a and d) and bio-electrodes at 24 hours (b and e) and 168 hours (c and f) after the inoculation of the bacterial consortium in the biological support media PCNB (a–c) and AW (d–f).

It should be noted that, according to the SEM images presented in Figures 3 and 5, there were a greater number of bacterial cells on the A-Ti surface in comparison to those present on the Ti material surface in both biological support media. This is attributed to the fact that the oxide layer generated during the anodisation increases the roughness of the surface and, therefore, favours greater bacterial colonisation on the A-Ti material. This confirms what was reported by Arnold and Bailey [47], who determined that the number of bacterial cells and the growth of a bacterial biofilm on a stainless steel surface with a more polished finish are reduced in comparison to rough surfaces.

In the null current chronopotentiometric analysis of the A-Ti BE in the biological support electrolytic media PCNB and AW, after 24 hours of inoculation the A-Ti (as observed in the Ti BE) experienced $E_{i=0}$ increments until anodic values were achieved (see Table 1). However, after 168 h the $E_{i=0}$ values of the BE in AW were less anodic, while in the PCNB they reached cathodic values. This occurred in such a way that these variations in $E_{i=0}$ experienced by the A-Ti BE indicated that (as observed for the Ti BE) their values tended to move towards more energetic values as the inoculation period extended. This can be attributed to the fact that the increase in the thickness of the biofilms supported on the A-Ti material (as observed in the SEM images, Figs. 5c and 5f) made the evaluated material more resistive.

Figure 6 shows the voltammograms obtained with the electrodes A-Ti, CE and BE (at two inoculation intervals: 24 h and 168 h) in the biological support electrolytic media PCNB (Fig. 6a) and AW (Fig. 6b). It was observed that in both media and for the entire potential range, the CE and BE (after 24 hours and 168 hours after inoculation) did not show an oxidation process. This behaviour was attributed to the high stability in the anodic zone due to the oxidised surface by anodising. However, in the cathodic zone it was observed that BE after 24 hours and 168 hours of inoculation showed greater cathodic current than CE; this is because, as mentioned in section 3.2, the CE showed reduction processes of the species present in the PCNB media whose composition was not as complex as that of the AW and therefore the cathodic current reached was lower than the reached by the CE in AW media. When comparing the BE at 24 h and 168 h in the PCNB media (Fig. 6a), it can be observed that at 24 h, there was a greater cathodic current with respect to that of the BE at 168 h. This could indicate that, as mentioned previously, at 168 h the biofilm is thicker (Fig. 5c), making the electrode more resistive due to the amount of EPS generated; however, this did not inhibit the electronic transfer since it is possible that the bacteria located on the surface of the A-Ti were receiving the electrons to incorporate them into their metabolism.

On the other hand, in the AW media (Fig. 6b) after 24 hours of inoculation, the voltammetric response showed a different behaviour compared to that observed in the PCNB media (Fig. 6a), indicating that the cathodic current of the BE was lower than that of the CE, which is attributed to the factors mentioned in Section 3.2. In an enriched and protonated medium such as the AW, the oxide layer favours the reduction of species and evolution of hydrogen, thus reaching high cathodic currents. Likewise, at 168 h after the inoculation, the BE presented a cathodic current inferior to that obtained with the BE at 24 h after the inoculation, which again is attributed to a greater amount of EPS generated on the surface of the BE of A-Ti (as demonstrated in Fig. 5f), meaning that the electrode becomes more resistive.

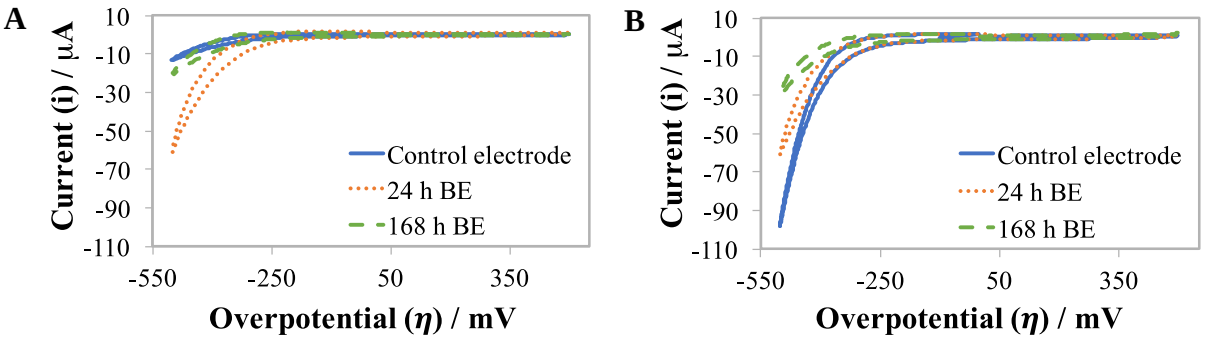

Figure 6. Voltammetric analysis of A-Ti materials in biological support media PCNB (a) and AW (b) at 24 h (orange line) and 168 h (green line) after inoculation and CE (blue line).

4. CONCLUSIONS

The results of this study clarified that the consortium of bacteria (*Enterobacter cloacae complex*, *Enterococcus gallinarum*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Enterococcus faecium)* was able to act as an electrotroph when supported on Ti and A-Ti surfaces at different times after the inoculation and imposition of a potential. The method of preparation of the modified electrodes (BE) with the bacterial consortium allowed bacterial biofilms with a high efficiency to be obtained in a period of 24 hours, noting that the A-Ti material favoured the growth of the biofilm with more bacterial cells than those shown in the Ti, reaching high cathodic current values with high stability in acid media. The Ti BEs showed a more defined bioanodic activity, while the A-Ti BEs presented biocathodic activity, with electrochemical responses attributed to the electronic transfer mediated by the bacteria that incorporated the electrons into their metabolism. Bacterial bodies coated by EPS on the Ti and A-Ti materials were observed after 24 hours and 168 hours of inoculation in the AW media, which was attributed to their high organic content and acidity. Consequently, the evaluation of the biofilms' growth in two media with a different pH allowed its influence on the type of biofilm growth and the electrochemical response obtained to be identified. Finally, the results highlight the possibility of using Ti and A-Ti as materials for BE applicable in various bioelectrochemical systems whose purpose is to treat whey solutions with neutral pH and acidic pH.

DISCLOUSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

ACKNOWLEDGEMENTS

The authors express their gratitude to CONACyT for the financial support granted in the doctoral grant. To the Institute of Health Sciences of the Autonomous University of the State of Hidalgo for the support provided for carrying out the biological tests and to the Polytechnic University of Francisco I Madero for their support in samples taking.

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