On-Line Monitoring of Biofilm Forming Pseudomonas Sp on Stainless Steel Electrodes by Repetitive Cyclic Voltammetry

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Abstract

Biofouling monitors available presently do not detect biofilm formation directly. Some measure the reduction in heat transfer and pressure drop which can happen after considerable growth of biofilm formation typically greater than 30µm. By that time chlorine cannot penetrate these biofilms. There are some electrochemical techniques that detect corrosion initiation under the biofilms. This is a concern for the life and stability of biofilm probes. The main objective of the present study was to develop an electrochemical detector based on cyclic voltammetry on electrodes made of materials used in cooling water systems. Repetitive cyclic voltammetry is a technique that provides information on the redox reactions taking place at the metal electrolyte interface. Earlier work on biofilmed platinum electrodes has shown that area reduction on the surface due to biofilm formation suppresses the peak for oxidation of adsorbed hydrogen thereby indicating direct biofilm formation. In this study we used stainless steel electrodes with biofilms of a gram negative bacterium Pseudomonas sp. Studies with stainless steel electrodes exhibited very distinct responses between clean and biofilmed conditions providing several parameters for biofilm monitoring like lowering of peak height at the oxidation of adsorbed hydrogen peak, lower current density at the peakand lower charge density under 3h, 6h, 24h and 48h biofilms. Thus this preliminary study has confirmed the potential of cyclic voltammetry for online monitoring of early biofilm formation.

Key words - *Biofilms, MIC (Microbial Influenced Corrosion), cyclic voltammetry, Stainless steel electrodes.*

I. Introduction

Fouling refers to the adverse construction of the inorganic and/or organic matters which is deposited on the material surfaces. These deposits can impede the flow of heat across the surface, increase the fluid frictional resistance at the surface, and increase the rate of corrosion at the surface. It results in unsatisfactory performances or a detrimental effect leads to be reduced life time of equipment [2]. The Surface-associated microbial growth, i.e. a Biofilm is known to instigate biofouling. The presence of biofilm may promote Interfacial physico-chemical reactions that are not favored under abiotic conditions. In the case of metallic materials, undesirable changes in material properties due to a biofilm (or a biofouling layer) are referred to as biocorrosion or Microbial Influenced Corrosion (MIC).To monitor these microbial attachment and biofilm formation, electrochemical techniques have used which are very sensitive to detect, and the data can be rapidly processed. In addition, they are environmental friendly and simple to operate because only tiny electrical energy is required to run the system.They may also provide a convenient means of detecting the early stages of formation of biofilms in heat exchangers and water treatment system [1]. In these studies, one or more electrochemical parameters such as the current density, potential difference, impedance, potential noise, potential resistance [3]or the charge density of the surface before and after biofilm formation can also be measured [1] .In the present work we set out to develop an electrochemical detector to monitor in situ formation of biofilms in flow systems by repetitive cyclic voltammetry.

II. Materials and Methods

A. Specimen preparation:

AISI type 304 Stainless steel specimens were obtained by cutting the sheets into medium sized coupons (60mmx30mmx1mm). The specimens were polished with successive grades of emery paper to have a final surface finish of 1000 grit and were cleaned thoroughly with soap solution and degreased in acetone. After air-drying the coupons were kept in desiccators and the samples were sterilized under the UV light for 15 minutes before exposing them into the nutrient medium containing the microorganisms.

B. Microorganism culture and Biofilm Setup

A culture of *Pseudomonas sp* were grown in a conical flask containing 500ml of 10 % Nutrient broth (Himedia) and incubated at 35°C. After the culture reaches its exponential growth phase, the Stainless Steel electrodes (Working Electrode) were exposed to the medium containing

Pseudomonas sp for the time interval of 3h, 6h, 24h and 48h. These electrodes were used for further electrochemical measurements and microscopy studies

C. Electrochemical Monitoring

The electrochemical technique used was repetitive cyclic voltammetry applied to a working electrode with and without bio-film on the surface. A triangular potential sweep was continuously applied to the freshly polished specimen (working electrode, WE) and the current caused by the breakdown of water molecules was recorded as a function of the applied potential [5]**.** The potential profile is, therefore, a linear function of time and can be described as Eapp = Ei±vt, where Eapp is the applied potential at a time t, Ei is the initial potential and v is the scan rate in V $s^{-1.}$ At a pre-set value, the scan rate is reversed and the potential is scanned to the initial value. This cycle can be repeated as many times as required. The working electrodes (WE) were the substratum for bio-film formation. The WE were 1 mm diameter stainless steel discs (area of the sensor 7.85x10-^{7} m^2), prepared by sealing a platinum wire into a glass tube and polishing the surface of the cross-section using a polishing cloth and alumina powder. The internal end of the platinum wire was sealed to a copper wire that provided the external contact. The reference electrode (RE) was a Calomel electrode and the auxiliary electrode (AE) was a platinum spiral. After polishing, the electrode was cleaned with deionised water and polished again using a clean polishing cloth.

The batch electrochemical cell consisted of a three compartment 15 ml all-glass cell, which contained the platinum WE, the platinum AE and the Calomel RE. For the experiment to carried out, phosphate buffer (pH 7; 0.2 mol l^{-1} Na_2HPO_4 and 0.2 mol l^{-1} NaH_2PO_4) was chosen as the electrolyte since it provided an appropriate medium for the bacteria and a convenient solution pH. The electrochemical experiments were carried out using a potentiostat Auto lab type PGSTAT 20, Ecochemie. Each electrode was subjected to the electrochemical treatment by immersion in the solution of interest; the potential was recycled between the appropriate limits. All chemicals used were of analytical grade. The solutions were not degassed in order to get similar conditions to those observed in real systems. The voltammograms were recorded using the data acquisition program GPES 4.6. For each condition tested, the electrochemical measurements were carried out using different working electrodes and a comparison was made between cyclic voltammograms obtained with the uncolonized electrode surfaces and after bio-films of different ages was grown on the electrodes. Prior to their utilization, the electrodes were degreased with ethanol, rinsed twice with sterile deionized water and sterilized using ethanol. They were rinsed with sterile deionized water before being cleaned electrochemically, by recycling the potential between -0.5 and 1.0 V for 30 min at 250 mV s^{-1}.

D. Epi-Fluorescence Microscopy

The specimens were gently washed with sterile water and air-dried in a sterile chamber and a few drops of acridine orange (0.1 % solution in distilled water) were placed on the specimen. After 2 minutes, the excess stain was drained off and the specimens were washed in sterile water, dried and observed under a Nikon Eclipse E600 epi-fluorescence microscope (excitation filter BP 490; barrier filter O 515). Bacterial cells fluoresced green / orange and were easily differentiated from inorganic particles and detritus.

E. Analysis of Microbial Density

The coupon was placed in a small sterile beaker, placed in a water bath sonicator to displace the cells from the surface. Optimum time of sonication was decided by trial and error and was found to be 3 to 5 minutes [4].Serial dilution were made upto 10^{-5} using 0.1M of phosphate buffered saline with pH 7.0.

III. Results and Discussion

A. Microscopic observation

The Stainless Steel samples exposed to *Pseudomonas sp* culture shows an increase in the bio-film formation with an increase in the exposure time. The epi-fluorescence micrograph (Fig 1a, 2b, 3c and 4d) of stainless steel shows the increase in number of cells when exposed for the period of 3h, 6h, 24h and 48h respectively. Abundant amounts of polysaccharide can be observed on some areas of the surface, constituting EPS fronds (the flurochromes used stained not only the bacterial cells but also the EPS matrix), masking the bacterial cells. Other zones still have discrete bacterial cells.

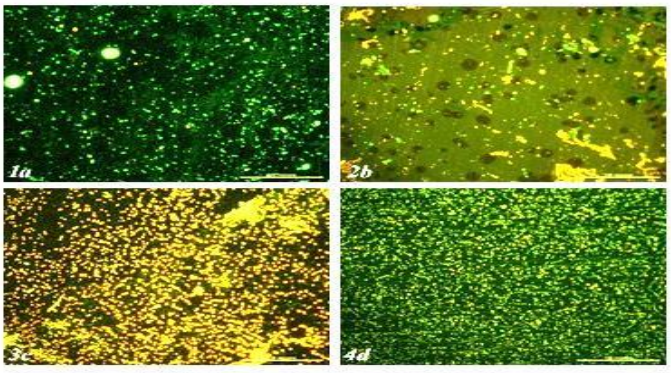


Fig. 1a, 2b, 3c and 4d: Epifluorescence microphotographs of the Stainless Steel surface before the electrochemical treatment. Electrode covered with a biofilm grown for 3h, 6h, 24h

and 48h (*Pseudomonas sp*) respectively. Scale bar - 100 µm.

B. Total viable count

The values of TVC for the adhesion studies with Gram negative bacteria *Pseudomonas sp* Stainless steel showed that there is an increase in the bacterial adhesion with respect to time.

Table 1. The total viable count (TVC) results of *Pseudomonas sp.* biofilm on Stainless Steel (SS) coupons.

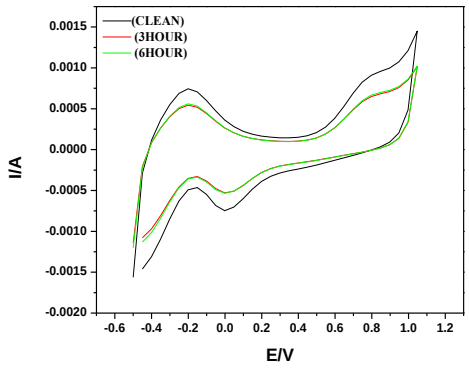
Sample	Cfu/cm^2
3 hour	391.16 x10^4
6 hour	526.93 x10^4
24 hour	550.60 x10^4
48 hour	600.13 x10^4

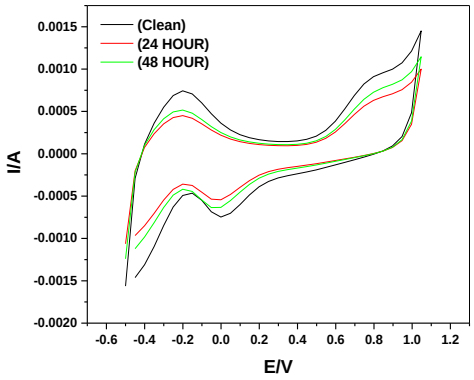
C. Cyclic voltammogram studies

The pattern of voltammogram of uncolonised stainless steel is clearly differentiated from that obtained for colonized electrode as shown infig 2. The voltammogram of 3h, 6h, 24h and 48h colonized electrodes were very much similar in pattern. From the table, it was observed that there was a gradual decrease in current. The charge density corresponding to different hours shown in fig 2, 3 and 4 infers that there is a decrease in peak area on colonized surfaces.

[1]Showed cyclic Voltammetry of platinum electrodes to study bacterial adhesion to surfaces over a 4hr period. In this study, where cyclic Voltammetry was conducted at 0.5VS^{-1}, the surface charge density resulting from the adsorbed bacteria was calculated from the integrated current–potential response corresponding to the anodic oxidation in the presence of bacteria and to oxide reduction. The difference between these two charges was considered to be related to the number of adsorbed bacterial cells.

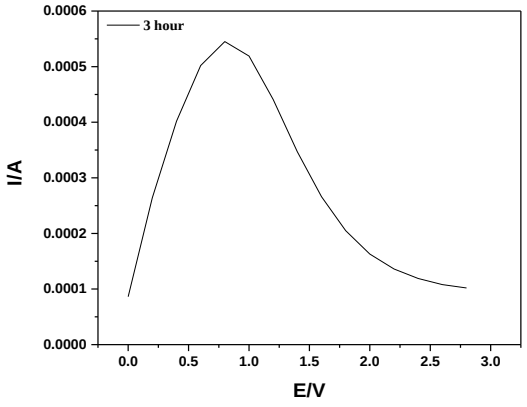
The oxidation of the adsorbed hydrogen peak is very sensitive to the age of bio-film only during early bio-film formation. When the bio-film was older than 24 hr the effect was similar to the early bio-films. With increase in bio-film age the response was not linear.

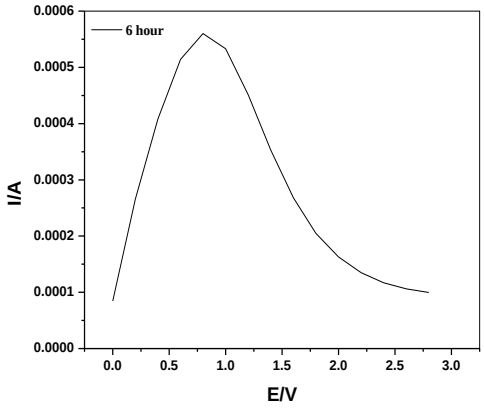




Samples	Potential	Current
Clean	-0.201	0.074e-2
3hr	-0.201	0.054e-2
6hr	-0.201	0.056e-2
24hr	-0.201	0.045e-2
48hr	-0.201	0.052e-2

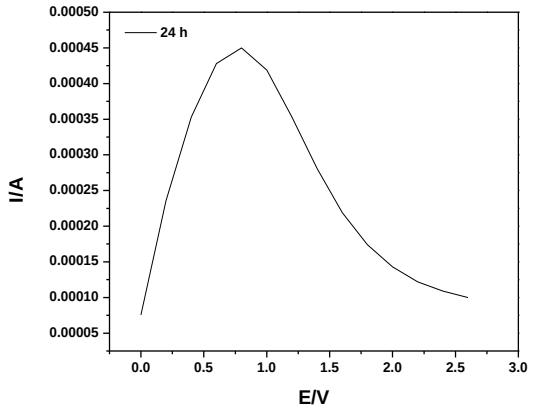
Fig 2. Cyclic Voltammograms obtained at Stainless Steel electrodes in phosphate buffer solution (scan rate 0.25Vs-1, potentials between 0.5 and 1.0vs a Calomel reference electrode). Line Black- uncolonized electrode, Line Red- electrode covered with a biofilm for 3hr, Line Green- electrode covered with a biofilm for 6hr.

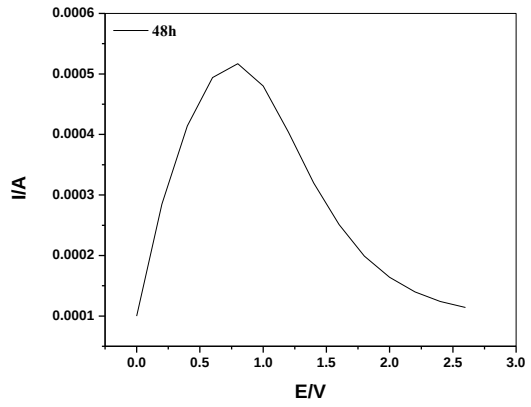




Samples	Area	Peak	Width	Height
3 hour	8.0122E-4	0.8	1.2	5.45E-4
6 hour	8.1366E-4	0.8	1.2	5.6E-4

Fig. 3 Surface Charge Density obtained at Stainless Steel electrodes in phosphate buffer solution (scan rate 0.25Vs-1, potentials between 0.5 and 1.0vs a Calomel reference electrode). Line Black –electrode covered with biofilm for 3 hour and 6 hour of scan 100.





Samples	Area	Peak	Width	Height
24 hour	6.7495 E-4	0.8	1.4	4.5 E-4
48 hour	7.798 E-4	0.8	1.4	5.17 E-4

Fig. 4 Surface Charge Density obtained at Stainless Steel electrodes in phosphate buffer solution (scan rate 0.25Vs-1, potentials between 0.5 and 1.0vs a Calomel reference electrode). Line Black –electrode covered with biofilm for 24 hour and 48 hour of scan 100.

Conclusion

The result showed that Cyclic Voltammetry applied to AISI type 304 SS electrode can be used as effective monitoring method to detect early bio-film formation. The change in the shape of the voltammograms as potential is cycled may constitute a means of providing information on the coverage of the surface, since the area under the peaks decreases as the coverage of the surface increases.

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