BACTERIAL CORROSION INDUCED BY AEROBIC AND ANAEROBIC MICROORGANISMS

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Abstract: The biocide is a chemical substance capable to killing different forms of microorganisms which is widely used in oilfields to control microbial problems. In this study, the efficacy of biocide (Quaternary-ammonium compounds 'QACs' blended with Glutaraldehyde) that is commonly used in Libyan oilfields was investigated against natural micro-flora isolated from open re-circulating cooling water system. The effects of those chemicals were studied on bacterial isolates used to build up biofilms on stainless steel coupons (AISI 304 and AISI 316). Two bacterial strains have been selected to be carried out in this study and identified as *Pseudomonas aeruginosa* and Sulfate-reducing bacteria (*Desulfobulbus sp*).

The strains were grown with increasing concentrations of the Quaternary-ammonium compounds blended with glutaraldehyde. Atomic Force Microscope (AFM) was used to visualize the topographic of biofilms developed on metal surfaces. The efficiency of the biocide was determined by acridine orange (AO) staining. In addition, electrochemical technique (linear Polarization Resistance) gave information on corrosion rate associated with the microbial activity and the biocide effects. The results show that microbial strains have different responses against biocide. While *Pseudomonas aeruginosa* shows ability to grow at high concentrations of biocide, *Desulfobulbussp* has no resistance-where 25ppm was enough to cause a shock for the growth of microbes-not only the microbial growth but their adhesion and the biofilm formation decrease dramatically.

Keywords: Disinfectants, biocide, Quaternary-ammonium compounds, Glutaraldehyde, biofilm

INTRODUCTION

Biofilms (Microorganisms attaching to the surfaces) are involved in many industrial fouling, corrosion, and hygiene problems. The main strategy of biofilm control to use chemicals (called biocide) to that are kill the attached microorganisms and/ or remove them from the surface. Unfortunately, bacteria in biofilms are usually found to be many times more difficult to eradicate than their planktonic counterparts. Actually, over 80% of microorganisms have shown ability of associating to surfaces. (Dinty *et al*, 2006; Wingender and Flemming, 1999). The relative resistance of biofilm microorganisms has tremendous economic and environmental ramifications in applications as diverse as cooling water, medical implants, drinking-water distribution, metalworking, and products quality (Keresztes *et al*, 2001; Zarnea, 1994; Flemming, 1996 and Beech, 2004).

Inside the biofilm, living cells belonging to various groups of microorganisms live together in a slimy matrix made of extracellular polymeric substances (EPS). That is produced by one or several species that includes organic and inorganic substances captured from the environment. This structure constitutes a protected mode of growth that allows survival in aggressive environment (Bandoni and Koske, 1974; Busscher and Weerkamp, 1987). There are four stages through the development of a mature biofilm: initial attachment, irreversible attachment with the production of extracellular polymeric substances (EPS), early development, and maturation of biofilm architecture (Stoodley *et al*, 2002; Kimberly, 2004).They differ from their planktonic form in their growth rate, composition and increased resistance to biocide. This makes

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them highly difficult to eliminate with treatment regime of antimicrobial agents. Many studies have stated that the bacteria enclosed within the biofilm are extremely resistance for antimicrobial treatments (Russell, 1995; Heinzel, 1998; McBain, and Gilbert, 2001; Chapman, 2003). One of the most important bacteria in oilfields is Sulphate Reducing Bacteria (SRB). Those bacteria involved in many problems in petroleum industries such as Microbial Influenced Corrosion (MIC) by cathodic depolarization of metal surfaces resulting in formation of localized pitting. Hydrogen sulphide as a metabolic by-product can result in the formation of iron sulphide deposits which depolarizes the anode and increases the corrosion rate. And slime forming bacteria that has potential to produce large volumes of exopolymers which is ideal for biofilm formation. Slime production can result in fouling and blocking of filter. Establishment of oxygen concentration cells, which can lead to under deposit corrosion and promote an ideal environment for SRB.

For that, a wide range of biocides (active chemical agents) has been used to control biofilms (microbial). These are including oxidizing and non-oxidizing biocides such as chlorine, hypochlorite, glutaraldehyde, and quaternary ammonium salts (Hector, 2002 and Russell, 2003). Glutaraldehyde and QACs have intensively studied. Currently, the largest application of glutaraldehyde is in the industries, whereas, it is employed, to a lesser degree, for oil drilling applications and gas pipelines to reduce populations of sulfate bacteria. Where, Glutaraldehyde characterized as strong antimicrobial (biocidal) properties. Like other compounds in the aldehyde family, glutaraldehyde possesses carbonyl groups that react readily with nucleic acids and proteins by alkylating sulfhydryl, hydroxyl, carboxyl, and amino groups (Chambon *et al*, 1992; Rutala, 1990). But glutaraldehyde cannot cross the cell's lipid membrane. Thus, it can only act outside these micro-organisms and has consequently a rather slow action. On the other hand, QAC's are cationic tensio-active agents. At low concentrations, they have cidal properties on a wide range of microorganisms. Their major site of action is the cell membrane, where they create dissolution of phosphorlipids and cause changes in permeability that allow the escape of cell constituents and cause cell disorientation (Russel, 2003). These changes in permeability also allow glutaraldehyde to penetrate inside the biofilms.

However, the additive biocide with QAC's show effective results but it needs high concentrations to control microbial activities. Several references showed that many types of gram-negative organisms such as P. *aeruginosa* have high levels tolerance to QACs. (Guerin-Mechin *et al*, 2000; Sundheim *et al*, 1998).

The present study was investigated the antimicrobial efficacy of Quaternary-Ammonium Compounds (QACs) blended with Glutaraldehyde against natural micro-flora from open re-circulating cooling water system in Libyan oil field.

MATERIAL AND METHODS

Bacterial strains and growth conditions: The consortium used in this work was isolated from deposit fouling samples from open re-circulating cooling water system in Libyan oil field. *Pseudomonas* species was chosen for this study, because it is known to be responsible for the microbial fouling on various surfaces. These bacteria are the most widely recognized in resistant of antimicrobial agent (Sundheim *et al*, 1998). Sulphate reducing bacteria (SRB) was also chosen because of their role to accelerate the corrosion process for metal surfaces. *Pseudomonas* sp cultured on R2A medium consisting of: yeast extract 0.5g,proteose peptone 0.5g,casamino acids 0.5g,glucose 0.5g, soluble starch 0.5g,sodium-pyruvate 0.3g,dipotassium hydrogen orthophosphate (K_2HPO_4) 0.3g, magnesium sulfate ($MgSO_4.7H2O$) 0.05g, distilled water 1,000mL (final pH 7.2).SRB cultured on Postagte Medium B-Lactate Baseconsisting: Potassium Phosphate, monobasic (KH_2PO_4) 0.5g,Ammonium chloride (NH_4Cl) 1.0g,Calcium sulfate ($CaSO_4$) 1.0g, Magnesium sulfate ($MgSO_4.7H_2O$) 2.0g,Sodium lactate ($C_3H_5NaO_3$) 3.5ml,Yeast extract 1.0g, Sodium chloride (NaCl) 1g, Ascorbic acid ($C_6H_8O_6$) 0.1g,Thioglycolic acid ($C_2H_4O_2S$) 0.3g, Ferrous sulfate ($FeSO_4.7H_2O$) 0.5g,distilled water 1,000ml (final pH 7.6) All experiments were incubated at 25°C.

Biocide: The chemical treatment that used in this study was Quaternary-Ammonium Compounds (QACs) blended with Glutaraldehyde (GA).

Metal sample preparation: AISI 304 and AISI 316 stainless steel samples (10mm x 10mm x2mm) were used. Table 1 presents the chemical composition of the steel. All samples have been polished firstlywith emery paper grades 600, 800, 1000 and 2000 grit,

Table 1. Chemical composition of the AISI 304 and AISI 316 austenitic stainless steel

Composition (%) type	C	Si	Mn	Ni	Cr	Mo	N	S	P	Cu
AISI 304	0.052	0.510	0.900	8.05	18.050	0.1900	0.0057	0.0025	0.023	0.180
AISI 316	0.08	0.75	2.0	14.0	18.0	3.0	0.1	0.03	0.045	-

then with diamond paste of 6, 3, 1 and 0.25μm, degreased with acetone, and dried on air.

Biofilm formation: These experiments were performed in R2A and postgate medium B at 25°C in the presence of *pseudomonas* species and sulphate reducing bacteria (SRB). R2A agar was used to monitor the growth of bacteria and to check for contamination for *Pseudomonas* species and postgate medium E for SRB. The short term submersion of AISI 304 and AISI 316 coupons with pure cultures of isolated bacteria was carried out in 250 ml Erlenmeyer flasks containing 100 ml of medium. The coupons were exposed for 5 days up to three weeks for sulphate reducing bacteria (SRB) with/without adding biocide at different concentrations (25,100 and 500 ppm).

Characterization of Bacterial Stains: The Preliminary characterization of bacterial strains was achieved according to standards for microbial identification in Bergy's manual of systematic bacteriology, on the basis of colony morphology, biochemical tests and microscopic examination.

Fluorescent microscope: Coupons from inoculated media with/without biocide were removed after 5 days of incubation, and then rinsed with distilled water to remove any unattached bacteria. Then the following staining procedures were used. Coupons were bathed in a 0.01% aqueous (w-v) solution of Acridine Orange (AO) (SCP LTD., UK) for 3 min before being rinsed twice in non-flowing distilled water (Ladd and Costerton,1990), and air dried.

The coupons were examined by a Fluorescent Microscope (ZEISS, AXIO, and Imager A1 with digital camera under 100x magnification).The images were taken at different places on the surface.

AFM operation for measurement of bacterial biofilm: Atomic force microscope (AFM) (Nanoscope IIIA contact mode, Digital Instruments) was used to obtain topographic images of sample surfaces on air. Treated and untreated coupons were examined after removing from medium and left to air-dry. Images were taken close to the middle of the samples, at least on three different places.

Electrochemical technique (Polarization Resistance technique): A potentiodynamic method was used to obtain the potential-current (E-I) ratio, with respect to the free corrosion potential, Ecorr Steel rod electrodes were placed in 300-ml Erlenmeyer flasks filled with 250 ml of descried medium with and without inoculums and biocides. The flasks were inoculated with bacterial isolates as following: control with inoculated medium without adding biocide, and adding biocide at 25ppm, 100ppm, and 500ppm. At the same time, the blank was non-inoculated medium. The steel rods screwed to the samples holder, connected to the potential measuring instrument, were polarized by 50mV and the corrosion rate calculated automatically from the E values and was presented digitally on the screen. Measurements were made daily and at extended time periods depending on potential changes.

RESULTS AND DISCUSSION

Characterization and identification of the isolate: The characteristic of *pseudomonas* species was aerobic, gram-negative, and motile, rod shaped, producing green pigments and biochemical tests were Catalase and Oxidasepositive. According to Bergey's manual, it clearly seems to be *pseudomonas aeruginosa*. The characteristic of sulphate reducing bacteria (SRB) was anaerobic, gram negative, lactate oxidation, not utilize the acetate and motile, the deslfvoirdin was negative, it might be *Desulfobulbussp.*

Fluorescent micrographs: The most widely used stain is the Acridine Orange (AO). It is a nucleic acid selective stain. AO interacts with DNA and RNA by intercalation or electrostatic attraction respectively. Cells may appear red-orange or green. It may be distinguished based on

color as live vs. dead cells; live cells contain more RNA and appear orange/red, dead cells lack RNA but may contain DNA and appear green.

In experiments of this study, the stained coupons showed that fluorescent colors were changed, depending on adding biocide. (Fig. 1) illustrates a typical image of *Pseudomonas aeruginosa* settlement on stainless steel coupons AISI 304 after immersion in R2A medium with different concentration of biocide for 5 days of incubation. Attached bacteria have shown ability to grow under stress condition (increasing concentrations of biocide), which indicate the acclimation, while 25 and 100ppm have shown slighter effect on embedded bacteria, 500ppm showed effective result, but takes longer time for proper effectiveness.

Figure (2) illustrates image of *Desulfobulbussp* settlement on stainless steel coupons AISI 316 after immersion in postgated medium B with different concentrations of biocide for 5 days to three weeks. The results show that 25ppm of biocide concentration is enough to prevent the bacterial attachments. In order to confirm previous results, the electrochemical technique (LPR) was used.

Electrochemical results and biocide assessment: The use of electrochemical techniques for the evaluation of microbial corrosion, as well as the biocide selection and evaluation, has been recently studied by Mansfeld, 2003.

The polarization resistance (PR) is the most common electrochemical technique used to evaluate corrosion rates on metals surface. This evaluation technique allows measuring instantaneous corrosion rates, and its monitoring can be continuous. Therefore, the technique is useful when the corrosion rate changes. A simplification of the Polarization resistance technique is the linear polarization techniques which assumed that the relationship between the potential (E) and current density (i) is linear.The results of *Pseudomonas aeruginosa* have shown a different trend as can be seen in (Fig. 3) the result confirm that, the corrosion rate of the inoculated medium without any biocide was dramatically increased and reached after 3 days for maximum value. Regarding the biocide action, 25 and 100ppm concentration of biocide has showed lower effect on the corrosion rate, while 500ppm concentration of biocides decreased significantly the corrosion rate. The corrosion rate was steady after 9 days, this due to the adhering bacteria and their polymeric material. At the same time, when the microorganisms were inhibited due to the biocide action, there is a reduction on the corrosion rate values. In Fig.4 the corrosion rate was increasing with inoculated medium (corrosion rate is reflection to bacterial activities) but at 100 and 500ppm concentrations of biocide no bacterial activities has been shown. This is an indicator for biocide efficiency against bacterial attachment.

These results confirm that presence of 500 ppm significantly reduce the corrosion rate and has most effect against *Pseudomonas aeruginosa*. But in case of SRB the 25ppm is enough to prevent attached bacteria.

The effect and action of antimicrobial compounds: Atomic force microscopy (AFM) has been used in several studies for the investigation of the effect and mode of action of antimicrobial agents upon bacterial cells. These studies including the action of Penicillin on *Bacillus subtilis* (Kasas *et al*, 1994), the effect of pretreatment of steel with glutaraldehyde on aerobic marine biofilms formed upon stainless steels (Tapper *et al*, 1998; Li-Chong *et al*, 2002, Beech *et al*, 2002; Beech and 2004), as well as the microorganisms embedded into biofilm were visualized by AFM: this technique is good for the visualization of shape and size of microbes embedded into biofilms and for determination of thin layer thickness.

This research have used the AFM to study the adhesion of cells and the formation of biofilms on AISI 304 and 316 AISI stainless steel surfaces in the absence and present of the biocide and on other way, to investigate the action of biocide on pure bacterial cells. Fig.5 illustrates a typical image of *Pseudomonas aeruginosa* cells attached to the stainless steel surface after immersion in R2A medium for 2 days. The dimensions of the cell were 3.406 x 0.281μm (length x width). The shape of the bacteria is clearly visible. Fig.6 illustrates aggregates growth of sulphate reducing bacteria on surface after immersion in postagte medium B for 5 days and the mature biofilms on surface where the attached bacteria surrounded by by-products (polymeric materials "EPS" and sulphied, Fig.7). However, the obtained results in present work confirmed that, *Pseudomonas aeruginosa* has tolerance to increasing biocide, and optimum efficacy of biocide at 500ppm. But in case of SRB, 25ppm is enough to control bacterial activities

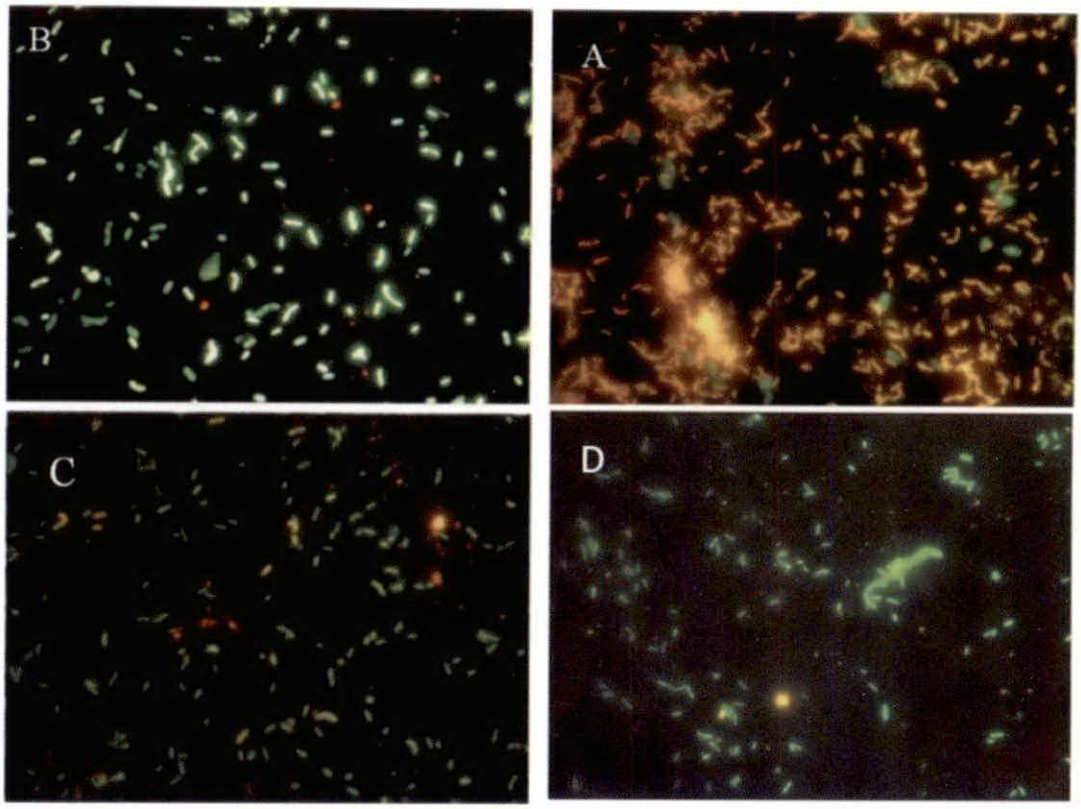

Fig. 1 The floursent images of *Pseudomonas aeruginosa* onAISI 304 after 5 days with different concentrations of biocide stained with AO (A) without biocide (B) with 25ppm (C) with 100ppm (D) with 500ppm – green could be indication for dead cells and red-orange for live cells .

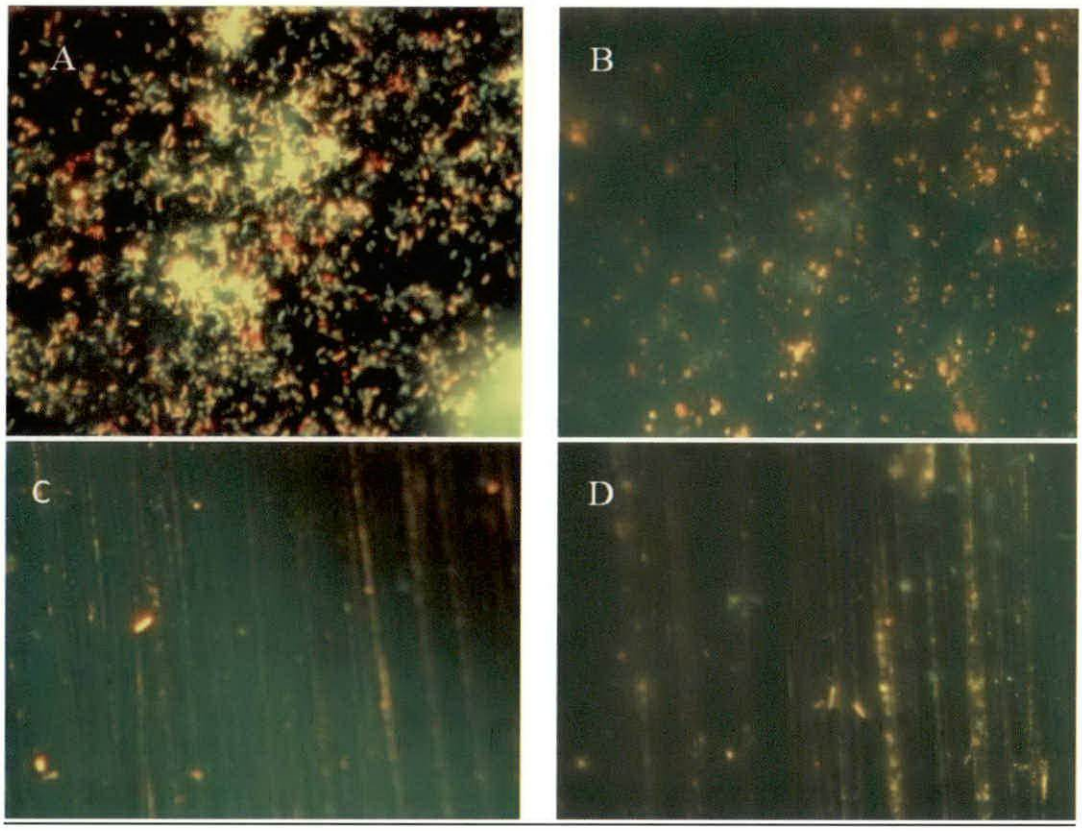

Fig. 2 The floursent images of *Desulfobulbus* sp onAISI 316 after 21 days with different concentrations of biocide stained with AO (A) without biocide (B) with 25ppm (C) with 100ppm (D) with 500ppm.

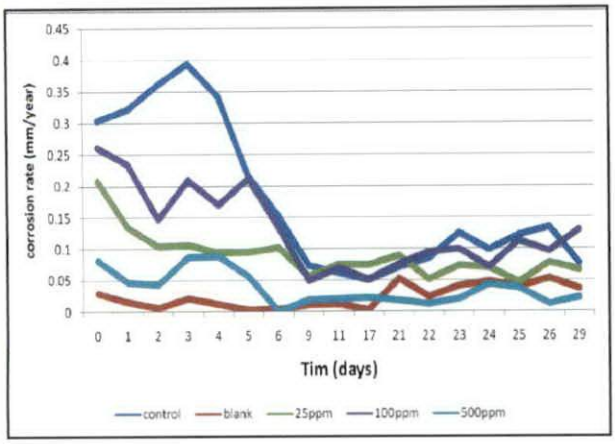

Fig. 3 Effect of *Pseudomonas aeruginosa* with and without biocide (at different concentrations; 25ppm, 100ppm and 500ppm) on the corrosion rate.

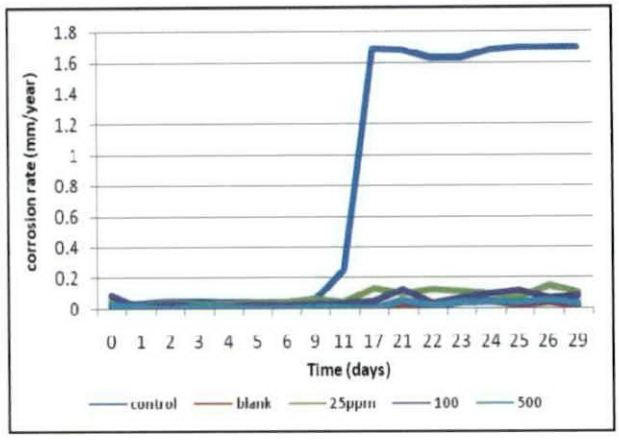

Fig. 4 Effect of *Desulfobulbus* sp with and without biocide (at different concentrations; 25ppm, 100ppm and 500ppm) on the corrosion rate.

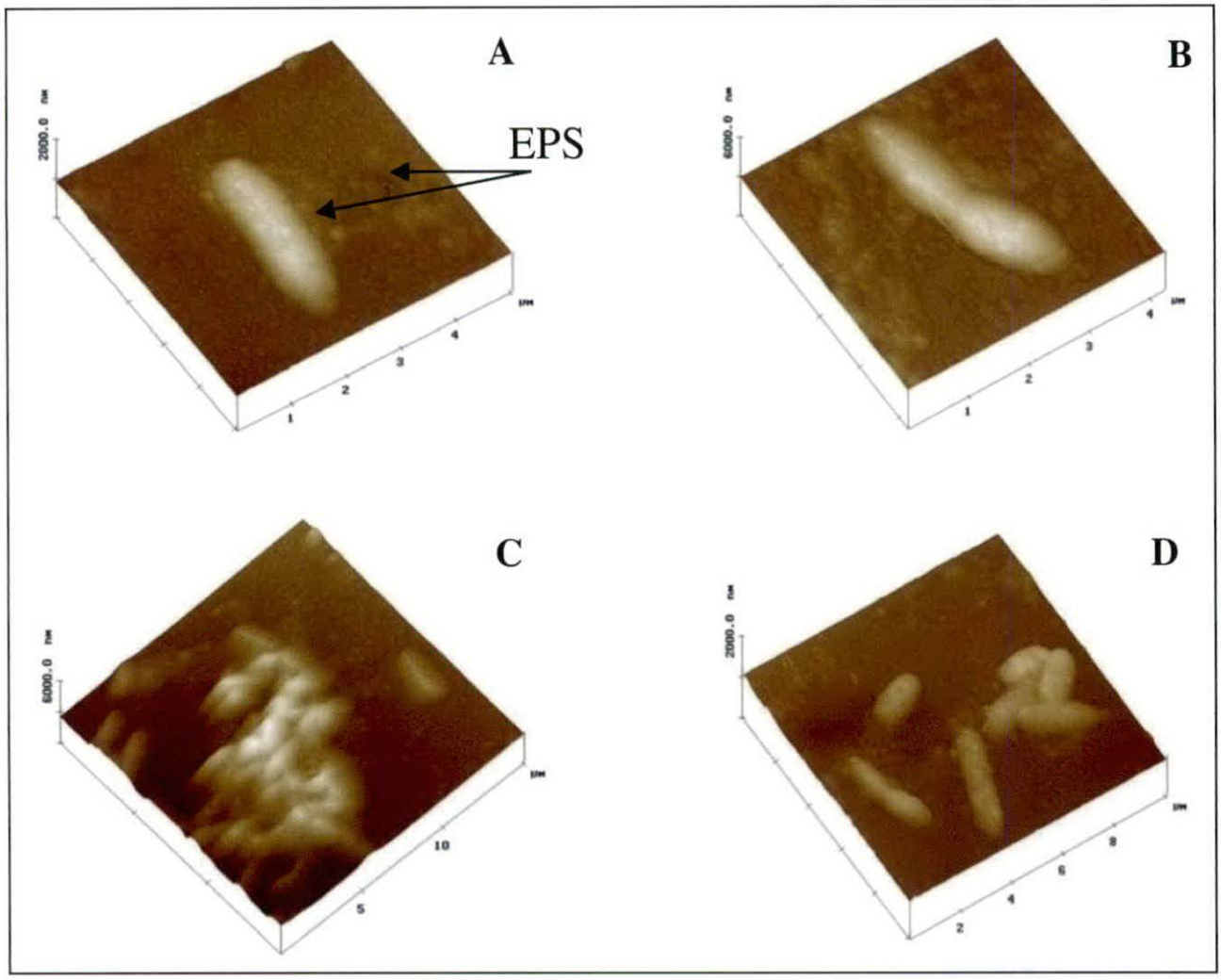

Fig. 5 The typical shaped of *pseudomonas aeurgnoa* cells attached toAISI 304 stainless steel surfaces (A) single cell on the metal surface (B) multiplying cells on the metal surface and EPS produces are all over the surface (C)several microbes pre-treated(D) biofilm covering the stainless steel 304 surface.

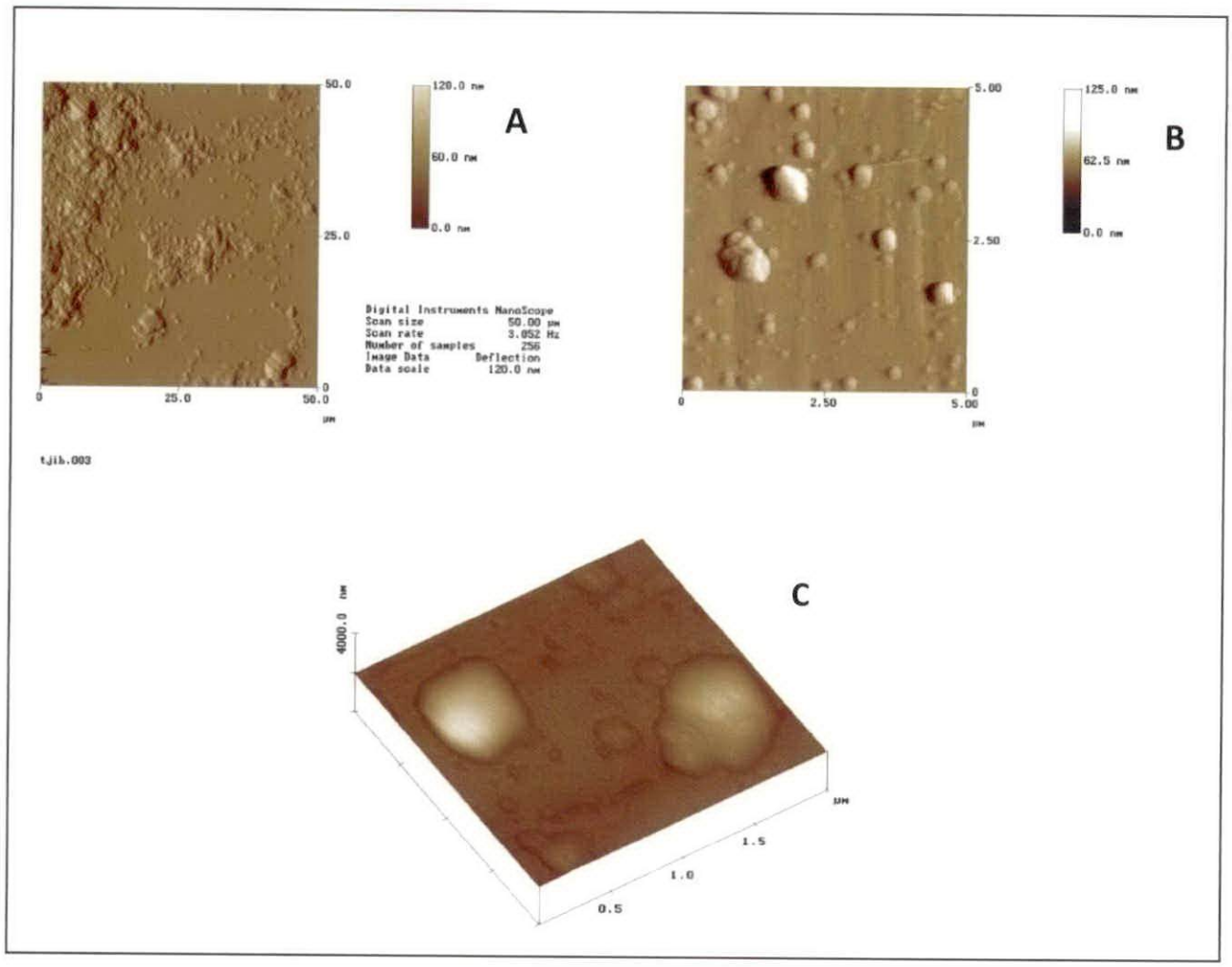

Fig.6 The *Desulfobulbus* sp cells attachedtoAISI 316 stainless steel surfaces (A) aggregates growth on the metal surface (B) several microbes pre-treated(C)biofilm covering the stainless steel 316 surface

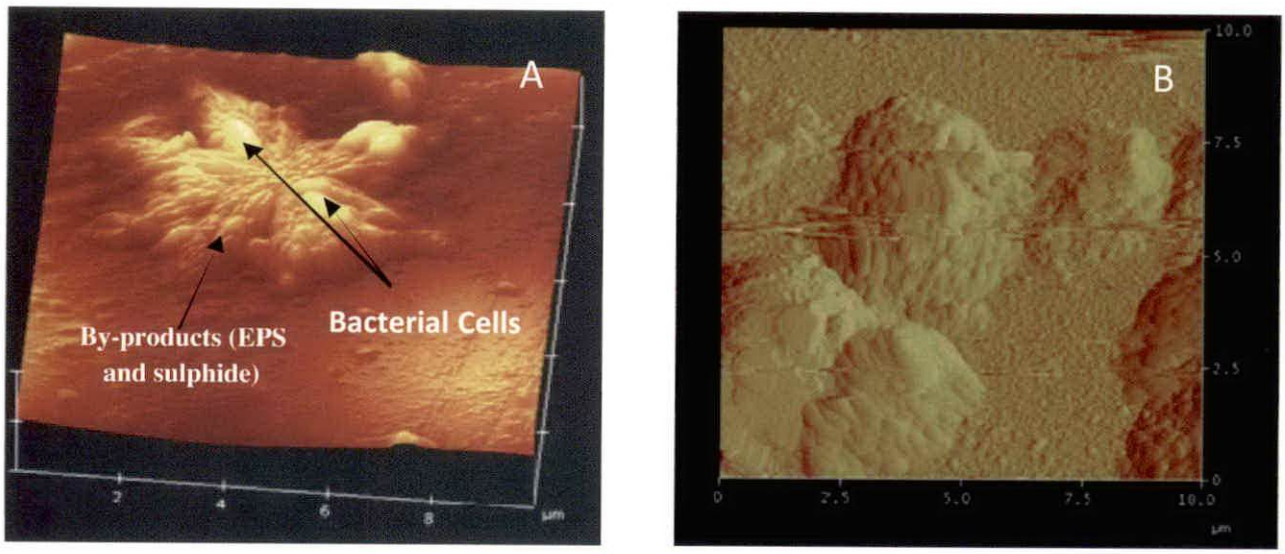

Fig. 7 The mature biofilms of *Desulfobulbus* sp cells attachedtoAISI 316 stainless steel surfaces after three weeks (A) multiplying cells on the metal surface surrounded by EPS and some sulfides (B)biofilm covering the stainless steel 316 surface.

CONCLUSION

Results showed that pesudomonas aergiunos has the ability to grow at high concentration of QACs and GA biocide and has tolerance up to 500ppm. The fluorescent images showed that bacteria were attached on surface even at high concentration, and the responseto biocide, however showed low efficiency versus time. But, in case of SRB, 25ppm is sufficient to prevent attached bacteria.

The electrochemical results showed that pesudomonas aergiunos has effect on the corrosion rate where the corrosion rate of control without any biocide was dramatically increased. While the concentrations of 500ppm has showed decreases of the corrosion rate significantly for pesudomonas aergiunos. But for Desulfobulbus sp, all concentrations showed the same efficiency on the corrosion rate.

AFM showed the visualizing bacteria on stainless steel surface. The maturation of biofilms is difficult to visualize because of microbial by-products.

More investigations need to be carried out to study the effect of the blending biocides on mixed culture.Also simulation of continuous culture by using biofilms reactor should be considered.

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