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Development of Biofilm (bf) on the Mild Steel Surfaces Immersed in Suez Gulf Sea Water

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Abstract: Biofilms (BF) were left to develop on the mild steel surface coupons under natural Gulf Suez seawater for 15 days. The site is near the General Petroleum Company (GPC). The development of BF contained a bacterial population with black and rusty appearance. The BF bacteria were collected from the mild steel surfaces and a stabilized mixed culture containing sulphate reducing bacteria (SMC-SRB) was obtained through enrichment on Postage medium B. Eighteen isolates were obtained using Postage medium C. The isolates were characterized and identified using API system.The amount of polysaccharide determined as total carbohydrate was taken as indicator for the development of microbial biofilm. The amount of the total carbohydrates ranged from 5.1 to 10.4 µg /cm^2 of the metal surface.

Key words: Biofilm, sulphate reducing bacteria, microbial influenced corrosion, mild steel, expolysaccharide

INTRODUCTION

The biofilm can be defined as microbial communities, containing large different numbers of microorganisms which produce a wide range of biopolymers Pope and Zintal[27]. This leads to adhesion to surfaces, within hours of immersion. A preliminary phase can take place where organic matter, glycoproteins and polysaccharides are adsorbed to the surface to a depth of 10-80 µm. this is called surface conditioning Newman *et al*.[24]. The second phase of fouling development is the attachment of bacteria to the conditioned surface. This occurs within few days of immersion Costerton *et al*.[12] and Corrosion management[10].

Organisms multiply during the next few days of exposure and forms of microcolonies enclosed in a slime of their extracellular polymer Pope and Zintel[27]. These bacteria including SRB are embedded into slimy polysaccharide capsule Mittelman and Geesey[22] can cause pitting of stainless steel in an adjacent location when air is present Greesey *et al*.[16]. Expolysaccharides released by biofilm population had a higher content of uronic acids compared to expolysaccharides (EPS) released into the bulk phase Beech *et al*.[4], Fleming[15]. In addition, EPS are involved by their ability to bind metal ions. They are responsible for the increase of friction resistance changes of surface properties such as hydrophoobicity, roughness, and color Percival[26]. Rapid succession of species within the community of the BF seems to be mainly Gram Negative rods that produce copious quantities of extracellular binding materials Corpe[9]; Gerchakov *et al*.[17]; Baier *et al*.[3] and NACE[23].

SRB belong to anaerobic group bacteria that uses sulphates as an electron acceptor and produced hydrogen sulphide, Soimajakvi *et al*.[31]; Postgate[28] and Hansen[20]. Hydrogen sulphide produced by the SRB can be oxidized by other organisms (sulfur oxidizers) to produce sulfuric acid, which destroy the municipal sewage pipe systems Bos and Kuenen[6]. Slime forming bacteria were mostly aerobic and frequently represent the following families: Pseudomonaceae, Enterobacteriaceae, Micrococcaceae, and Bacillaceae, NACE[23].

However, in Egypt the petroleum industries suffer from microbial influenced corrosion (MIC). It was estimated that one company (Gulf Suez petroleum company (GupCo)) spent more than million dollars/year to combat bacteria causing MIC, El-Raghy *et al*.[14]. The quaternary ammonium compounds have been in use for many tears Brunt[7] to combat MIC.

MATERIALS AND METHODS

Mild Steel: Mild steel used for biolfilm growth and/or electrochemical tests was kindly analyzed by the Iron & Steel Egyptian Company. It had the following composition (% w/w): C, 0.08; Si, 0.08; Mn, 0.36; P, 0.02; S, 0.018; Al, 0.004 and Fe, 99.438.

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Microorganisms: Biofilm bacteria included in the SMC-SRB (stabilized mixed culture of sulphate reducing bacteria and coexisting aerobic bacteria) were isolated from Biofilms developed on mild steel surfaces, which were exposed to Gulf Sue seawater, for 15 days near GPC (General Petroleum Company).

Culture Media: The following media were used for enrichment and isolation of SMC-SRB and biofilm bacteria according to Postgate[28]. Media were sterilized for 20 min. at 121°C.

Postgate Medium B (g/L) Was Used for Enrichment of SRB: 0.5, KH2PO4; 1.0, NH4Cl; 1.0, CaSO4; 2.0, MgSO4.7H2O; 3.5, Sodium lactate; 1.0, Yeast extract; 0.1, Ascrorbic acid; 0.1, Thioglycollic acid; 0.5, FeSO4.7H2O; 25, NaCl and tap water 1 liter. The pH was 7.5. This medium always be turbid. Sodium chloride was added to simulate salinity.

Postgate Medium C (g/L) Used for Isolation of Biofilm Bacteria: 0.5, KH2PO4; 1.0, NH4Cl; 4.5, Na2SO4; 0.06, CaCl.6H2O; 0.06, MgSO4.7H2O; 6.0, Sodium lactate; 0.1, yeast extract; 0.1, Ascorbic acid; 0.1, Thioglycolate; 0.004, FeSO4.7H2O; 0.3, sodium citrate; 25, sodium chloride and distilled water (1 liter).

Postgate Medium E (g/L) Used for Isolation of SMC-SRB: 0.5, KH2PO4; 1.0, NH4Cl; 1.0, NaSO4; 2.0, MgCl2.6H2O; 1.0, CaCl2.6H2O; 3.5, sodium lactate; 1.0, yeast extract; 0.1, Ascorbic acid; 0.1, Thioglycolate; 0.5, FeSO4.7H2O and 15, agar. The pH value was 7.6 sodium chloride was added to simulate salinity.

Nutreint Agar Medium, APHA[1]: Beef extract 3.0, NaCl 5.0, Peptone 5.0, Agar 20.0, pH 7.0 was used for isolation and characterization of aerobic bacteria BF. Aux medium Elmer *et al.*,[13], this medium was provided by API 20 NE system. Which applied for the identification of isolated bacteria by using the API20E strips. The inoculums density of each isolate was adjusted by visual comparison to the Mcfarland stander suspension, which supplied component of API20E identification system.

Analysis of Seawater: Seawater analysis was kindly supplied with its analysis by GPC according to APHA[1].

Preparation and Cleaning of the Mild Steel: The mild steel was divided into coupons each had dimensions of 5x20x0.2 cm. The coupon surfaces were abraded by emery paper beginning with 120 then, 240; 300; 400 and finished with a fine one (number 600). The edges were abraded lightly to reduce the preferential edge corrosion tendency Ringas and Robinson[29]. The coupon is then degreased with acetone followed by ethanol, dried in air and preserved in desiccators.

BF Formation: The prepared coupons were washed and immersed in fresh Suez Gulf seawater in a glass basin (250 ml capacity). The water was changed every three days and kept at room temperature (20°C) Shams El-Din *et al.*[30], during 15 days. During this period the Enrichment and isolation of Bacteria. The total microbial population of the biofilm was obtained from the coupon surface by scraping it with a sterile shaving razor blade. The BF was transferred to sterile screw capped bottles having sterile Postgate medium B and was incubated for 3 days at 30°C. By repeated sub culturing 25 times on the same medium we get what we call Stabilized Mixed Culture of sulfate reducing bacteria (SMC-SRB) Abd El-Samie *et al.*

Preparation of Stabilized Mixed Culture of SRB (SMC-SRB) Isolated from Biofilms Formed on Mildstell Coupons: The bacterial biofilms sample which was collected from any metallic surface. A sterile new safety razor can be used for collecting the biofilm. In the present work the successive enrichment and isolation of separate black colonies was done to obtain SMC-SRB. Enrichment was done using medium B. Isolation using deep agar E, proved to obtain the required mixed cultures from each separate black colonies (Fig. 1). In other words, the enrichment medium B culture was used to inoculate molten and cooled (40°C) medium E followed by more gentle shaking, cooling and incubation at the optimum temperature of anaerobic bacteria. This is what is meant by the SMC-SRB. The following figures illustrate a step-by-step procedure for the isolation processes (Figs. 2 and 3).

Characterization of Aerobic Bacterial BF: Eighteen aerobic isolates from the SMC-SRB were isolated using Postgate medium C, and subjected to the identification using the API 20 E system which was translated into a numerical code consisting of seven digits leading to complete identification of each isolate Elmer *et al.*[13]. The eighteen isolates were alos studied to elucidate their colony and morphological characteristics Gerherdt *et al.*[18].

Isolation of Aerobic Bacteria from SMC-SRB: BF aerobic bacteria which was co-existing with SRB was isolated from enriched SMC-SRB by normal streaking on sterile Postgate medium C-agar plates and incubated at 30°C for 24 hour. Enrichment of biofilm aerobic bacteria was done using sterile liquid Postgate medium C.

Fig. 1: Separate Colonies of SEB on Postgate Medium E.

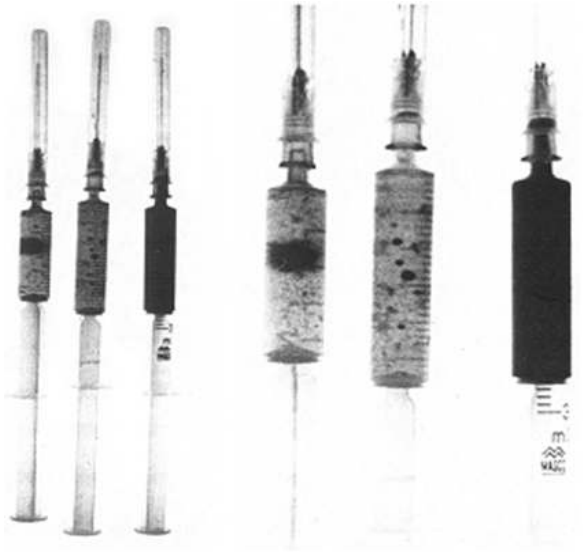

Fig. 2: The new culturing technique for the SRB using the syringe agar tube.

Reformation of Biofilm: The efficiency of the isolated biofilm bacteria individually and in combination to form biofilm on mild steel surfaces was determined, by incubating the prepared cleaned polished, sterile mild steel coupon (1x5cc) in screw capped bottles containing sterile Postgate medium C, then inoculated with one ml of SMC-SRB or the tested pure BF isolates. The inoculated bottles were incubated at 30°C for 7 days. The reformed biofilms were collected as mentioned before and subcultured again in the Postgate medium C, at 30°C for 24 h for aerobic BF isolates and 3-4 days for SMC-SRB growth. The planketonic bacteria were removed before scrapping biofilms from the metal surfaces by gently inverting the bottles by hand for two minutes to loosen and suspended the planktonic bacteria. The biofilms reformed on the mild steel surfaces were collected and enriched as mentioned. After the incubation period, the absorbance of biofilm specimens were measured at 660nm Gerhardt *et al*.[18].

Determination of Total Carbohydrate of the Biofilms: Total carbohydrates of the BF bacteria were determined by the phenol sulphuric method[18].

RESULTS AND DISCUSSION

Analysis of Suez Gulf Seawater: The cementing clumping of the polysaccharides and agglomeration of inorganic crystals and bacterial cells encouraged further bacteria growth over the metal surface SRC could grow within these layers and produced high localized concentration of H2S which cause sever pitting corrosion (31). Therefore was agood source for SRB isolation especially it contained high sulphate ion concentration (3200 ppm) which was considered to be a sole energy source for SRB growth Billman[5]. Table (1) demonstrated the results of physicochemical analysis of Suez Gulf seawater.

The water analysis was approximately similar to that obtained by Ateya *et al*.,[2] who found that the SRB increased the corrosion rate of mild steel under exposed seawater, which differed markedly in the salts concentrations according to its source Shams El-Din *et al*.[30].

Generation of the Biofilm: After 3 days from the exposure the entire mild steel coupon surface was covered by black slimy and rusty layer. This layer became thicker at end of incubation period (15 days). The black film might be due to sulfide resulting from sulfate reduction as a metabolic activity of SMC-SRB. On the other hand, the rusty layer might be due to oxides formation as a chemical corrosion product Kuhr and Vulgt.

Isolation of Bacteria Usually Done by Either of Two Techniques, Namely:

- Streaking the microbial sample on the surface of a solidified nutrient agar medium using a sterile, nickel-chrome wire or a sterile bent glass rod.
- Gentle mixed the microbial sample with molten nutrient agar medium (40-50°C) following by rapid coding to the room temperature.

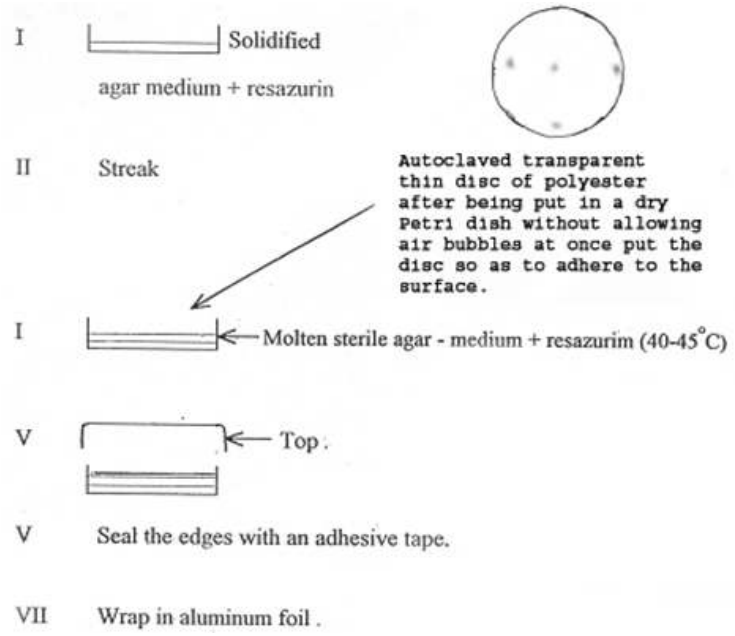

Fig. 3: The double agar plate technique.

Table 1: Physicochemical analysis of Suez Gulf seawater.

PH AT 20°C	8.0
SP.gr at 20°C	1.034
Ca2+	481 ppm
Mg2+	1642 ppm
Fe3+	0.04 ppm
Na+ and K+	15018 ppm
Cl-	22000 ppm
CO3--	0.0 ppm
HCO3-	122 ppm
SO4--	3200 ppm
TDS	48720 ppm

Streaking ensures the mechanical shearing of the sample so that nearly every individual bacterium is separated from one another and is located at a fixed place of the surface of agar. Upon incubation, at a suitable temperature, each bacterium will divide many times to give a visible colony.

On the other hand, gentle mixing, of a tube or a Petri-dish does not create enough shearing to separate each bacterium from each other.

Naturally, bacteria live in mixed communities and might form different types of physical association. Biofilm formation, on solid surfaces, is a good example of this association. This takes place through the formation of a matrix of biopolymers. Gentle mixing in a viscous medium like melted nutrient agar medium proved to keep the physically associated bacteria within a separate colony. When biofilms formed on mild steel coupons were subjected to the isolation procedure using mixing in melted agar medium, the obtained separated black colonies proved to contain SRB associated with facultative anaerobic acid producing bacteria.

Table 2: Total carbohydrate and optical densities for the bacterial biofilm culture.

Bacterial biofilm culture	Total carbohydrate concentration ($glcm^2$)	Optical density
SMC-SRB	8.9	1.35
1	9.8	0.47
2	9.34	0.58
3	9.3	0.18
4	7.6	0.13
5	6.8	0.56
6	8.9	0.14
7	8.6	0.91
8	9.4	0.32
9	8.9	0.23
10	10.4	0.56
11	8.5	0.46
12	8.9	0.42
13	9.3	0.46
14	8.9	0.49
15	9.8	0.32
16	8.2	0.32
17	5.1	0.39
18	9.8	0.43

Establishment of Coexisting Stabilized Mixed Culture (SMC-SRB): SMC-SRB was isolated and enriched in sterile Postgate medium C and B respectively, using deep agar method. The process was repeated for about 25 times to get SMC-SRB.

Isolation of Aerobic Biofilm Forming Bacteria: The isolation process was carried out from the SMC-SRB, on nutrient agar medium using streaking plate technique. Individual separate 18 colonies were picked up and subcultured several times to obtain a pure colony forming unit.

Table 3: Morphological characterization of microbial isolates and their colonies after 24 h growth at 30°C and 37°C on nutrient agar medium.

Isolates number	Colony shape	Colony color	Cell size
1,2,3 & 4	Circular flat	Beige	6-8x1
5,6,7,10 & 11	Circular flat	Creamy	4-6x1
8 & 9	Circular flat	Creamy	3x1
12, 13 & 18	Convex circular	Beige	4-6x1
15 & 16	Convex circular	Beige	3x1

Table (2) showed the optical densities at 660 nm for the growth of the aerobic and SMC-SRB responsible for biofilm formation. SMC-SRB showed the highest optical density (O.D. 1.35), compared to the other values which ranged from 0.13 to 0.91. These results revealed that all isolates had the ability to reform the biofilm on the mild steel surface singly or in combination.

On the other hand Table (2) showed the values of total carbohydrate, which were determined in the collected Biofilm of SMC-SRB and aerobic bacterial isolates. The results revealed that the total carbohydrate concentrations ranged from 9.8 to 5.1 (µg/cm). Besides, the results showed that some aerobic bacterial isolates revealed high concentration of total carbohydrate than SMC-SRB themselves.

Morphological Characterization of Biofilm Aerobic Bacteria: The eighteen isolates were gram negative with different shapes and sizes as shown in Table (3). The oxygen requirement was tested by stab technique Gerhardt *et al.*[18]. The other physiological characteristics were defined using the API 20 NE system, Elmer *et al.*[13] and the characterization of the individual isolates were accomplished through the API NE20 profile index. These 18 isolates are numbered from 1 to 18.

Biochemical tests were recorded, although the results revealed that the 18 isolates were identified according to the API 20NE system, the 18 isolates were positive for catalse, motility, esculin fermentation, gelatin liquefaction except isolate number 8. Growth occurred in all the isolates on mannose, mannitol containing media. All were oxidase positive except isolates number 4, 6, 10, 11, 13, 14 and 18.

These isolates might belong to the family Pseudomonaces on the basis that they were straight rods, motile, catalase positive and oxidase positive, Pelezar and Micheal[25].

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