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Microbial induced corrosion by ferric–reducing bacteria isolated from an oil separation tank

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Keywords

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RESUMEN

Se requiere identificar a las poblaciones microbianas que participan en la Corrosión Inducida por Microorganismos, con la finalidad de implementar estrategias de monitoreo eficiente y de control. Las poblaciones de microorganismos anaerobios presentes en la industria petrolera, particularmente en la producción de gas y petróleo, así como en las líneas de transporte y en los tanques de almacenamiento, han sido estudiadas muy pobremente y los estudios presentes se han enfocado principalmente en bacterias sulfatorreductoras de los géneros *Desulfovibrio* y *Desulfobacter*. Sin embargo las bacterias fermentativas también tienen gran relevancia en la corrosión de metales, como se describió en 1997, por el grupo de Magot y colaboradores, quienes caracterizaron una bacteria no sulfidogénica pero con capacidad de producir corrosión. En este estudio se aisló de un tanque de separación, una bacteria anaerobia, fermentativa y reductora de fierro, perteneciente al género *Sedimentibacter*, con capacidad de producir corrosión en el acero al carbón SAE1018.

ABSTRACT

It has required the characterization and identification of the microbial populations responsible for Microbial Induced Corrosion (MIC), and their interactions with distinctive microorganisms allocated on metallic surfaces, in order to implement efficient monitoring and control strategies. Microbial anaerobic communities present at oil and gas producing, transporting and storage facilities have been poorly characterized and studies had mainly focused on *Desulfovibrio* and *Desulfobacter* genus. However, fermentative bacteria have important participation on corrosion metals as described by Magot et al. (1997), which characterization of non-SRB sulfidogenic bacteria was able to produce corrosion. In this study, it was isolated of an oil-water tank separation, an anaerobic bacterium, fermentative and ferric-reducing, belong to *Sedimentibacter* genus with corrosion capability on Carbon Steel SAE1018.

Introduction

The total estimated direct cost of metallic corrosion in the US represented approximately 3.1% of the gross domestic product in the year 2002 (276 billion dollars) according to the U.S. Federal Highway Administration, specifically in the oil and gas exploration and production sectors, the cost has estimated at 7 billion dollars to monitor, replace and maintain these assets (Koch et al., 2002). While these estimates did not distinguish between abiotic and biotic corrosion phenomena, it has suggested that Microbial Induced Corrosion (MIC) represents up to 20% of the corrosion occurring in industrial systems (Ferrari, 1995). Monitoring of the bacterial populations in oil and gas industry pipelines is of importance since it is well recognized that some anaerobic bacteria can cause biofouling and biocorrosion of steel pipelines (Mc Kinerney y Sublette, 1997). The microorganisms influence the corrosion by altering the chemistry at the interface between the metal and the bulk fluid (Jones and Amy, 2002). Traditionally the monitoring of bacteria in oil and gas industry facilities had focused on sulfate-reducing bacteria (SRB) (Cord-Ruwisch et al., 1985). These bacteria are the most extensively studied bacteria in relation to MIC and their participation in corrosion processes was evidenced decades ago (von Wolzogen Kuhr y van der Vlugt, 1934). Corrosion by SRB has mainly attributed to the production of corrosive hydrogen sulfide (H_2S), cathode depolarization by biological consumption of hydrogen at the cathode and production of sticky exopolymers. Some studies reported the characterization of oil field microbial communities, both by culture-dependent and culture-independent methods, revealing the presence of several physiological types of bacteria as sulfate-reducers, sulfidogens, fermentative bacteria, metal reducers, methanogens and acetogens, depending on the physicochemical conditions of the reservoirs (Magot, 2000; Van Hamme et al., 2003). Other species belonging to the order *Clostridiales* have so far been isolated from oil wells, they include *Dethiosulfovibrio peptidovorans*, family *Syntrophomonadaceae*, and these bacteria produce the highest rate corrosion in pipelines, about 4 mmy and produce sulfides from thiosulphate (Magot et al., 1997).

The goal of this study was to characterize a fermentative bacterium isolated from oil environment and todetermine its participation on the carbon steel corrosion.

Methodology

Samples were taken from a Station 1(oil separation tank) and the exit of gas pipelines of the line Atasta-Cd. PEMEX of *Petróleos Mexicanos* industry. The samples were collected in sterile plastic bottles and kept at room temperature until used. The *in situ* temperature on the gas pipelines and the separator tank was ranged from 30-35 °C.

Isolation. The initial enrichment culture was conducted by inoculating 75 mL of anaerobic API-RP38 medium prepared under N_2 atmosphere (API recommended practice for biological analysis of subsurface injection waters. American Petroleum Institute, Washington D.C., March 1992) with 25 g of encrustation's sour gas pipelines and were incubated 30 days at 30 °C. Stable enrichment culture was obtained after 3 consecutive transfers. The isolation of pure cultures using the roll tube method (Hungate, 1969), at 30 °C, was conducted in the basal medium (BM) contained (per L distilled water): 1 g, NH_4Cl; 0.3 g, K_2HPO_4; 0.3 g, KH_2PO_4; 0.2 g, $MgCl_2\ 6H_2O$; 0.1 g, $CaCl_2\ 2H_2O$; 0.1 g, KCl; 0.1 g, 0.1 g, yeast extract; 0.5 g, cystein-HCl; 1 g, NaCl; 1 mL, rezarsurine 0.1% p/v; 10 mL, trace mineral solution (Balch et al., 1979), 5 g, sodium lactate was used as source carbon; The pH was adjusted to 7 with KOH 10 M or HCl 1 N, and then was filtered through 0.22 µm membrane. The medium was boiled under a stream of O_2- free N_2 gas, and cooled to room temperature.
Five mL aliquots were dispensed into Hungate tubes and 75 mL in serum bottles, under a stream of N_2 gas and sealed vessels were autoclaved for 45 min at 116 °C. Prior to inoculation $Na_2S{\cdot}9H_2O$, was injected from anaerobic sterile stock solutions to final concentration of 0.04% (w/v) and pH was adjusted to 7.0 with sodium bicarbonate 10% w/v, anoxic solution (Ravot et al., 1995). This basal medium was supplemented with Noble agar (1.8% w/v) (Difco Laboratories, Detroit, MI, USA).
Growth experiments were performed in duplicate, using the basal medium (BM) with 2.0 g per liter of yeast extract. The carbon source was conducted using Glucose, Saccharose, Arabinose, Maltose, Fructose, Ribose, Gluconate, Raffinose, Manose, Lactate, Acetate, Formiate, Hidroxycinamic Acid at 20 mM.

Cell morphology and physiological characterization. Morphological characteristics of IMP6C3 isolate was observed with a phase and luminous microscope (Nikon). The presence of spores was determined by microscopic cells observation.

The isolate ability to use sodium nitrate, sodium thiosulfate and ferric chloride as electron acceptor other than sulphate was tested in basal media with lactate as carbon and energy source, where each electron acceptor was added as in a final concentration (20 mM). The growth ability with different concentration of NaCl and optimal temperature was determined. The cultures were incubated at 30 °C during 8 days, in anaerobic conditions. The isolates were subcultured at least twice under the same experimental conditions. Growth was determined by measuring the optical density at 580 nm using a UV-visible spectrophotometer (WPA light wave S2000).

***Biofilms and corrosion activity*.** Corrosion coupons of carbon steel SAE1018 (18 mm x 20 mm x 0.7 mm) surface finished with sandblast were used for this test (Mack et al., 1983). Autoclavable plastic racks containing 9 metallic coupons were placed into recirculating anaerobic reactors (Ramos Casillas et al., 2004) containing 180 mL of filtered basal medium (BM) with lactate as source carbon. *D. peptidovorans* DSM 11002 was utilized as positive control with corrosive activity and the test fluid was the basal medium with glucose as source carbon and sodium chloride, 25 g/L. The system was sterilized at 116 °C by 45 min. Medium recirculation was obtained using a peristaltic pump at a flow rate of 8 mL/min. The reactors were inoculated with 20 mL of exponential phase cultures and incubated at 30 °C to IMP6C3 isolate and 42 °C to *D. peptidovorans*, during 30 days.

For weight loss experiments, biofilms and corrosion products accumulated on the metallic coupons were completely removed by extensively cleaning under a stream of tap water and boiled during 5 min in NaOH 20%, extensively washed with tap water after with 95% ethanol and acetone and finally dried to constant weight (ASTM G1-81). Weight loss was then measured and reported in millimeters penetration per year (mmy). After the weight loss measurements, the clean coupons were also examined by optical microscopy (Nikon), at 40 X, with oblique illumination to delimit the affectations (Jones, 1996)

***Electron microscopy*.** Biofilms formed on the metallic coupons in the recirculating reactors were fixed for 2 h with 5% glutaraldehide in phosphates buffer pH 7.2 at 8 °C, and osmium tetroxide before being dehydrated by successive washes in increasing concentrations of ethanol as previously described (Bozzola and Russell, 1991) and were observed in a JSM-5900L low-vacuum electron microscope at an accelerating voltage of 10 kV. Biofilms Elemental analyses were performed by Rays X Energy Dispersive Spectroscopy (EDX).

***16S rDNA gene sequence analysis*.** Total genomic DNA was isolated from lysed bacterial cells by treatment with proteinase K and cetyl trimetyl ammonium bromide prior to extraction with phenol/chloroform/isoamyl alcohol and precipitated with isopropanol, as described by Ausubel et al., (1995). The 16S rDNA gene of the strains were amplified by adding 2 µL of DNA to a thermocycler microtube containing 5 µL PCR buffer 10X, 3 µL of 25 mM $MgCl_2{\cdot}6H_2O$, 1 µL of 10 mM deoxynucleoside triphosphate mix, 0.5 µL of 50 µM primers Fd1 and Rd6, 37.75 µL of sterile distilled water and 0.25 µL of 5U/µL *Taq* polymerase (Promega). The universal primers Fd1 (5'-AGAGTTTGATCCTGGCTCAG-3') and Rd1 (5'AAGGAGGTGATCCAGC C-3') were used to obtain the PCR product. PCR was performed by initial denaturation at 95 °C for 3 min, followed by 30 cycles of denaturation at 95 °C 1 min, annealing at 53 °C for 1min, extension at 72 °C for 2 min and finally an extension cycle of 72 °C for 10 min. Amplification fragments were cloned in the pCR® 2.1 –TOPO according to the manufacturers protocols (TOPO TA Cloning®, Invitrogen™, Life technologies, Carlsbad, California, USA). Plasmid of the clones was isolated by the Ultraclean™ 6 minute Mini Plasmid prep Kit™ according to the manufacturer's instruction (Mo Bio Labs, Solana Beach, CA 92075). The inserts were amplified using primers set M13 forward (5'-GTAAAACGACGGCCAG-3') and M13 reverse (5'-CAGGAAACAGCTATGAC-3'). The PCR products were sequenced in an ABI PRISM sequencer model 3100, 3.7 version, according to the manufacturer's protocols.

Results and discussion

Cell morphology and characterization of isolate. Anaerobes have always been considered as the dominant micro-organisms of appeared in oil reservoirs (Magot et al., 2000). Among them, sulfate- reducing and fermentative bacteria constitute an important microbial community of the oilfield environment. In this study an anaerobe isolate IMPC3 was obtained on lactate-containing medium at 30 °C from a station 1 (oilfield separator tank). IMP6C3 are bacilli, occurring singly or in chains of two cells up to 12 or more cells (Figure 1A) and positive Gram reaction. In addition, long filaments of up to 100 µm, sometimes having only a few or no, visible septations (Figure 1 B). Cells of strain IMPC3 were rod-shaped (0.5-0.7 µm wide; 5-6 µm long) (Figure 1C). Cells were motile under the light microscope when a freshly withdrawn sample was observed. Heat- resistant (80 °C, 30 min), oval endospores were observed by phase contrast Microscope (Nikon E800).

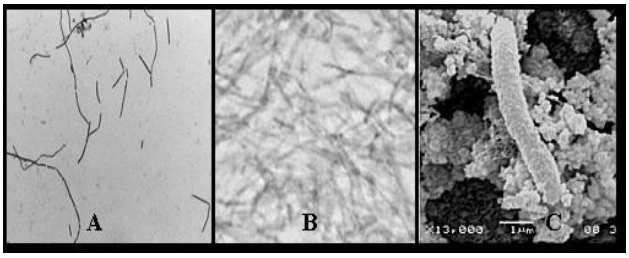

Figure 1. Isolate IMPC3: A) Bacilli, Gram positive at 600 X; B) larges ramifications at 1000X; C) on carbon steel corrosion coupon by SEM at 13 000 X, with mineral salts.

The isolate IMP6C3 required yeast extract for growth, the maximum growth yield was obtained when 2g% yeast extract and thiosulfate (20 mM) was added to basal medium,similar results were obtained by Magot et al. (1997) with *Thermoanaerobacter* and Surkov et al. (2001) with other *Dethiosulfovibrio* species, they have demonstrated that during thiosulfate utilization in presence of yeast extract there were considerable improvements in growth rates and biomass yields (Figure 2). The IMP6C3 isolate utilize, glucose, saccharose, lactate and acetate, but not utilize, arabinose, maltose, fructose, hidroxicinnamic acid, ribose, gluconate, raffinose, manitol, mannose, formiate. Its growth was increased in one logarithm in presence of ion ferric as electrons acceptor final (Figure 3). Isolate IMP6C3 growth with sodium thiosulfate in presence of yeast extract, the maximum H_2S production was of 5.5 mmol/L but not utilize sulfate, nitrate and sulfur.

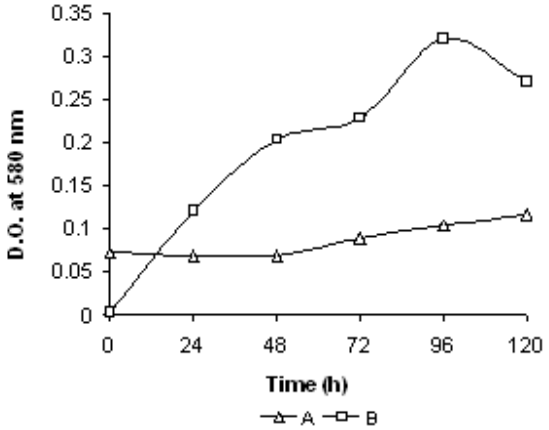

Figure 2. Kinetic growth in basal medium with lactate as carbon source and thiosulfate as final acceptor electron, optical density at 580 nm: A) 0.1g/L yeast extract; B) 2.0 g/L yeast extract.

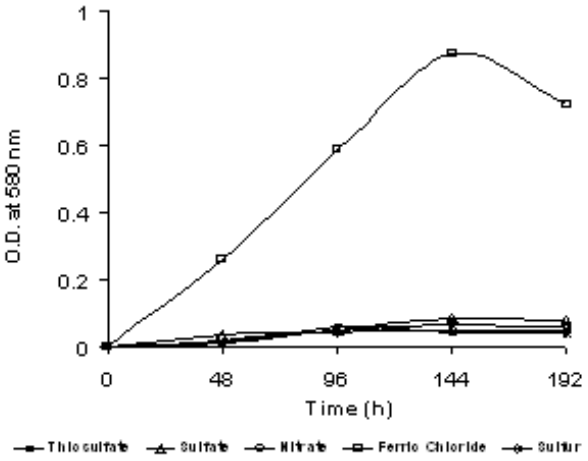

Figure 3. Kinetic growth in basal medium with glucose as source carbon and different acceptor final electrons.

Biofilms . In the mineral basal medium with lactate as carbon source and sulfate as final acceptor, the biofilms produced by IMP6C3 isolate were uniformLy distributed on the surface corrosion coupon. The biofilms were constituted by two films about 3 μm each one (Figure 4-1). On the biofilms surface were detected corrosion products (Figure 4-2C), and by the energy dispersive X-ray analysis (EDX) were detected iron, carbon, sodium, magnesium, aluminum, oxygen, phosphorus, sulfur and calcium (Figure 5C); sodium, magnesium, calcium and phosphorus, were found by De Romero et al. (2002) in biofilms produced by SRB, but these products were culture medium constituent. Under surface, there were abundant microorganisms compacted and attached on the metal (Figure 4-2B) and by EDX analysis, were detected phosphorus, carbon and oxygen, magnesium, sodium and siliceous, calcium and little quantity of aluminum, iron and sulfur probably as ferric sulfide (Figure 5B).

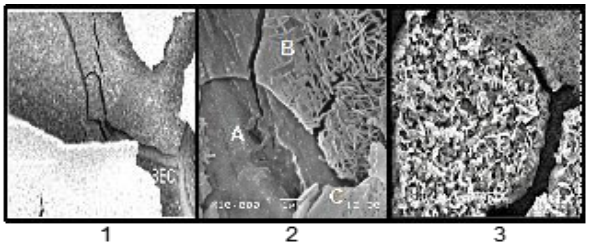

Figure 4. IMP6C3 biofilms by scan electronic microscopy: 1) Biofilm at 6 000 X, showing a crevice on the metal; 2) Two films at 10000 X: A: corrosion coupon with a pit and others affectations; B: in the internal film there are compacted bacteria with mineralization; C: Biofilm surface totally mineralized 3) cells on the surface biofilm at 4000 X.

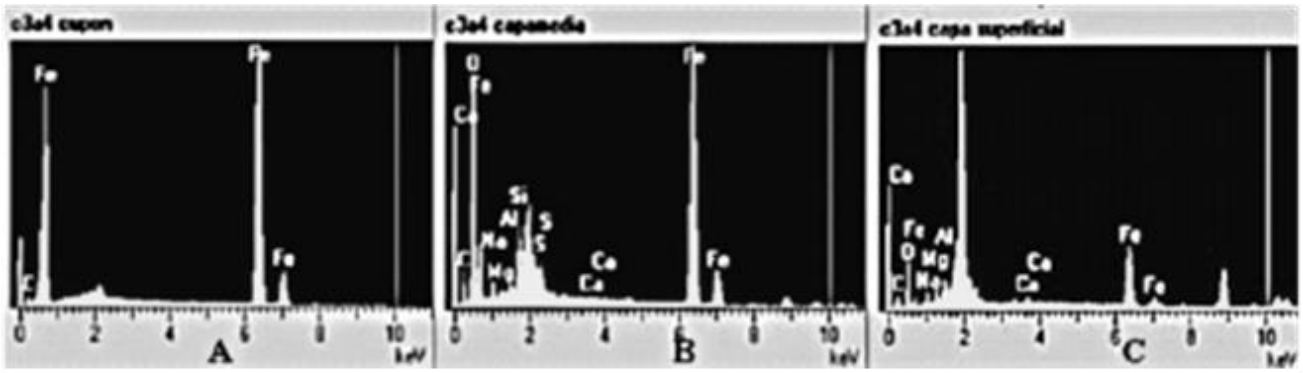

Figure 5.- *Sedimentibacter* sp. C3 EDX análisis: A) coupon corrosion; B) under biofilm surface; C) surface biofilm.

Yu Chan et al. (2002), attributed the carbon and oxygen presence to proteins, polysaccharides and hydrocarbon, suggested that they may play important roles in biofilmm formation and pit corrosion. On the metal surface was observed pits and crevice (Figure 5-2A), By EDX analysis only were found abundant iron and small quantity of carbon (Figure 5A).

Sedimentibacter sp. IMP6C3 removed fragments in thin films producing scarce but extended affectations, this corrosion type has not reported previously for this microorganism. *Sedimentibacter sp.* IMP6C3 produced a rate corrosion of 0.29 ± 0.007 mm/year, the affectations that were observed by optic microscopy at 40X, showed important deterioration. Under the test condition

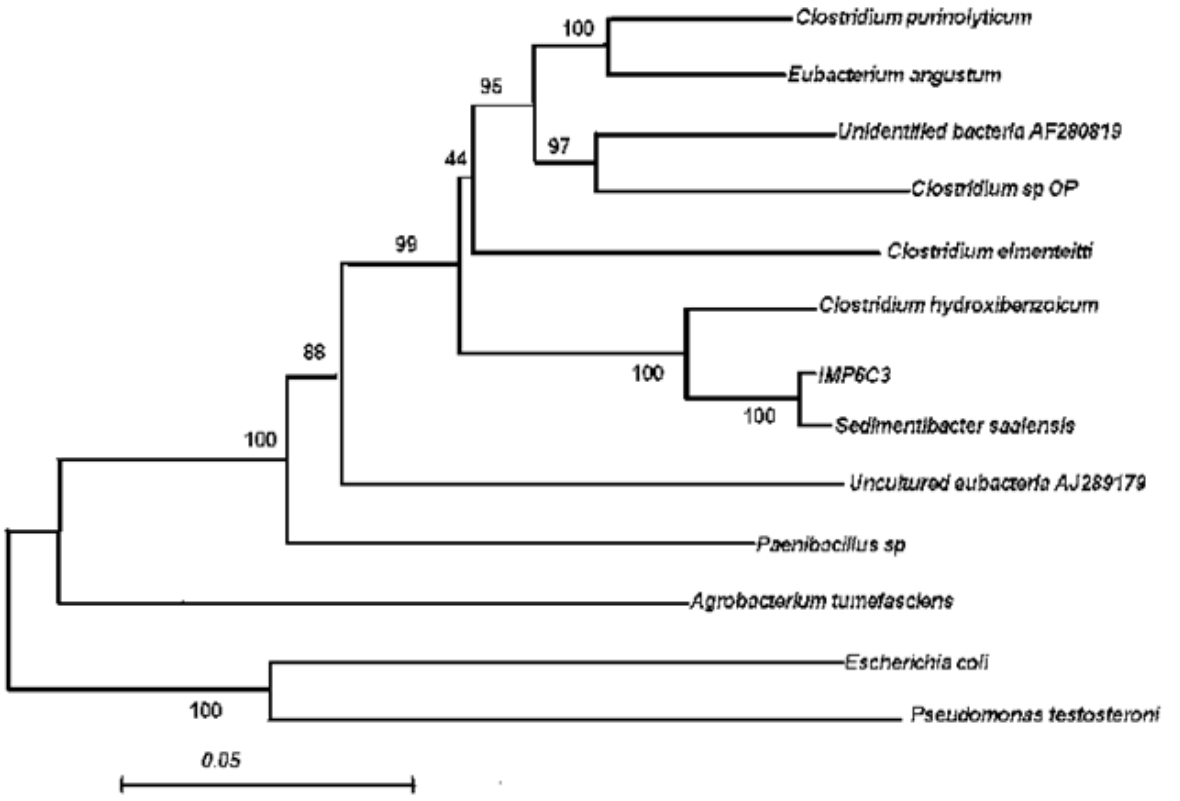

Figure 8 Isolate IMP6C3 Dendogram (own elaboration)

Corrosion activity

The rate corrosion was determined by lost weight and was calculated by D 2688-94 formula (ASTM Standard). described in this study, *D. peptidovorans* was the strain with lower rate corrosion, its produced 0.17 mm/y on the carbon steel SAE1018.

Identification of the isolate by 16S rDNA gene sequence

This sequence was used for identification of the most closely related bacterial strains in a database search to determine the taxonomic position of isolate IMPC3, as shown in the phylogenetic tree in figure 6. Only the 16S rRNA gene sequence of *S. saalensis* (Breitenstein et al., 2002), had a high level of identical nucleotides (98% over 1,475 bp). Therefore, isolate IMPC3 is referred to here as *Sedimentibacter sp.* 16S rDNA sequence analysis revealed that isolate IMP6C3 was a member of the order Clostridiales. The most closely related other species belonging to the genus *Sedimentibacter*, like *Sedimentibacter saalensis, S. hydroxybenzoicus* (formerly *C. hydroxybenzoicum* according to Breitenstein et al., 2002, and S*edimentibacter sp*. strain BRS22, exhibited levels of sequence identity to isolate KI between 94 and 95%. Much lower levels of homology to bacilli (89 to 90%) (Figure 6, right branch) were found. Other related bacteria were Clostridiaceae belonging to clostridial cluster XI (for example, *Clostridium felsineum* DSM 794) and clostridial cluster XII (e.g., *Clostridium purinolyticum* ATCC 33906 and *Clostridium acidurici* ATCC 7906). *Sedimentibacter* genus has three species, *S. hydroxybenzoicus* and *S. saalensis*, both were isolated from a freshwater pond and river sediment, respectively (Breitenstein et al., 2002), and *S. hongkongensis* isolated from a patient with bacteremia and colonic carcinoma (Woo, P.C.Y, 2001, not published). Figure 6, presents a dendogram generated by the neighbor- joining method (Saitou y Nei, 1987).

Discussion

The anaerobe bacteria isolation from gas pipelines encrustations indicates the corrosion and plugging risk (Iverson, 1987; Hamilton, 1998). One of the genera most isolated from metallic structure that had major capability to produce biodeterioration was *Desulfovibrio* (Beech et al., 1991). But the aggressive attack of the fermentative bacteria to the metallic structure, its principally because they can assimilate sulfur, organics acids (lactic, acetic, butyric, propionic) and produce sulphidric acid, by glicolytic way from glucose, lactose, sucrose or peptones; also, they utilize iron as final acceptor electrons (Erlich, 2009), and this element are in high concentration on pipelines.

On the oil separation tank and pipelines, the carbon and energy source were hydrocarbon and organics acids as product of oil degradation and this conditions were favorable to microbial growth. *Sedimentibacter* genus , had been isolated in others ecosystems as mats (Breitenstein et al., 2002), and colonic carcinoma (Woo, 2001, not published), but not was isolate from oil industry. Other fermentative bacteria were isolated on the metallic structures as *Petrotoga* (Davey et al., 1993), *Thermotoga* (Fardeau et al., 1997), *Garciella nitratireducens* (Miranda-Tello et al., 2003), *D. peptidovorans* (Magot et al., 1997), and *Thermoanaerobacter (*Fardeau et al., 2000) are fermentative bacteria isolated from oil environments, they produce sulfides from thiosulphate, but not utilize ferric ions as final acceptor electrons as *Sedimentibacter sp*. IMPC3; *Garciella, Dethiosulfovibrio* and *Sedimentibacter* are belonging to the order *Clostridiales* that can survive in oil environments.

Conclusions

The Isolate IMP6C3 was identified by 16SrDNA gene sequence as *Sedimentibacter* sp. with the closest homology with *Sedimentibacter saalensis*, for that, it suggests that this is a new species

In this study, *Sedimentibacter* sp., was determined his important corrosive capability, utilize Iron as final electron acceptor and produce biofilms on SAE1018 carbon steel. It growth is increased in 20% NaCl, and utilize sodium thiosulfate in presence of yeast extract. With these characterize, this microorganism is considered with higher risk for structure metallic, similar to *D. peptidovorans*. *Sedimentibacter* genus has not isolated from hydrocarbon pipelines and there is no evidence about its participation in metal corrosion. In this study demonstrated that *Sedimentibacter sp.* IMP6C3 has ability to biofilm production and corrosion on the carbon steel and to utilize iron as final acceptor electron.

Acknowledgments

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