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Isolation and Identification of anaerobic cultivable iron reducing bacteria from crude oil and detection of biofilms formation on carbon steel surfaces

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ABSTRACT

Microbiologically influenced corrosion (MIC) that responsible for corrosion problems, especially in oil and gas industry. The microorganism capable of iron reduction (IRB) that involving in MIC mechanisms, was contributing as a main problem for localized corrosion. *Shewanella* species are considered to be major iron-reducing bacteria (IRB) of crude oil; the aim of this study is to determine whether any of the *Shewanella sp.*are important in formation of biofilm on carbon steel surfaces. Over 158 colony-forming units (CFU) associated with anaerobic cultivable and high producing H_2S strains were isolated in crude oil from Melaka, Malaysia. A total of 59 bacterial strains were identified as *Shewanella* species by morphologically and biochemically analyzed. The genus *Shewanella* was the most commonly encountered, among the genera of bacteria observed. *Shewanella chilikensis* specie was the most common specie, followed by *Shewanella algae*.34 isolates were selected as *Shewanella* isolates due to their ability of the isolates to grow well at 30°C and change VMNI color to black during 1-3 days Analysis of biofilm formation profile of the 34 IRB strains was determined by the crystal violate method, we observed that the 34 strains were able to produce biofilm. The 34 strains were divided into 18 (12 and 6 strains from *S. chilikensis* and *S. algae*, respectively) strong biofilm producers, 15 (8 and 7 strains from *S. chilikensis* and *S. algae*, respectively) moderate biofilm producers, and only 1 from *S. chilikensis* was observed to be weak biofilm producers. The 16S rRNA gene sequence analysis confirmed the identification of these two major groups. This finding is important to increase the information to isolate, identify and perform initial characterization on these associated bacteria found in the crude oil of Malaysia.

KEY WORDS
Crude oil, *Shewanella* species, biofilm formation, 16S rRNA, Malaysia

INTRODUCTION

Carbon steel metals are considered by the oil and gas industry to be the most efficient and economical metals for the storage and transportation of hydrocarbon all over the world, this is because during oil and gas operations, carbon steel metals are subjected to different corrosion deteriorating mechanisms which is as a result of the interaction that exist between the carbon steel container and fluid. Among these corrosion mechanisms involved in the oil industries is microbiologically influenced corrosion (MIC). MIC is considered to be responsible of the 20% or even higher of the total corrosion in the oil and gas industry worldwide [12].

Petroleum hydrocarbons are considered to be an excellent carbon source (source of food) of several microbes in the three domains of life which include bacteria, Archaea and Eucarya. The microbial representatives of the three domains (bacteria, Archaea and Eucarya) may play important roles in MIC [18,41]. Acid producing bacteria (APB), iron and manganese oxidizing bacteria (I/MOB), Sulfate reducing bacteria (SRB), iron reducing bacteria (IRB), and methanogensis, are the major group of organism and process considered to be associated with pipeline and storage tank system corrosion of metal [18,41]. Different researchers have reported that SRB is a major MIC causative bacteria in oil and gas industry [12]. Practically MIC is as a result of interactions of different form of microorganism coexisting in the same environment and has the ability to positively affect the chemical and electrochemical metabolic processes. Much attention has been focus on SRB in MIC studies which underestimates the role of other important microorganisms in the corrosion process for example IRB [12]. IRB are bacteria capable of reducing iron [25]. These bacteria are facultative anaerobic, they uses oxygen under aerobic conditions and also reduces ferric iron (Fe^{3+}) [12,18,41]. Reports from different researcher have shown that IRB have the ability to metabolize over 166 different electron acceptors which includes among others, NO^{3-}, NO^{2-} , $S_2O_3^{2-}$, SO^3 and Mn(IV) [12,18,39]. Generally IRB are isolated from different habitats, *Shewanella* are common to marine environment, estuary, intertidal areas, and freshwater [42,44].

Shewanella was defined to be new genus in 1985 [20] which later was named after James Shewan due to his work on microbiology of fish [32]. This organism belongs to the Order alteromonadales, Family Alteromonadceae in the gamma subdivision of the Proteobacteria. *Shewanella* species are characteristic motile rods, gram-negative bacteria which are nonfermentative, even though some researchers have reported glucose fermentation ability of the few species of these bacteria [4,10] . These species produces H_2S from inorganic and organic sources. *Shewanella* are anaerobic bacteria which uses different electron acceptors, most of *Shewanella* strains have the capacity to reduce trimethylamine- N-oxide (TMAO) [6,38]. These bacteria can be isolated in different habitats. *Shewanella* species could be psychrotrophic (S. putrefaciens, S. baltica, S. frigidimarina, S. woodyi, and S. dinitrificans), mesophilic (S. algae, S. amazonensis, S. colwelliana, S. oneidensis, and S. putrefaciens), psychrophilic, barophilic (S. benthica) or psychrophilic (S. gelidimarina and S. hanedai), [5,11,27,38].

Aside corrosion of metal, *Shewanella* bacteria are also found to be responsible for the organic material decomposition, this include forming thick biofilm layer on stainless steel, cracking of crude petroleum, bioremediation of contaminated material and reduction of magnesium and iron oxide, these activities of the bacteria shows its importance in microbiological influenced corrosion (MIC) [24,28,29,31].

In Malaysia, little or no work has been done on IRB, particularly the genus *Shewanella*. Up till now, there is no work done that focuses on IRB isolation from the crude oil environment of Malaysia. Looking at the importance of IRB in oil industry, it is very important to provide baseline data for further research of IRB in both oil and non oil producing industries in the future. Therefore, this study is aim at isolation, identification, characterization and detection of biofilms formation on carbon steel surfaces of IRB in crude oil environment in Malaysia.

MATERIALS AND METHODS

2.1 Sampling:

Crude oil samples were collected in September 2012, at a Petronas refinery Sdn. Bhd Sungai Udang, Melaka, Malaysia. About 4.5 L of Samples were taken from bottom part of local storage depot situated in the petroleum refinery in Melaka, Malaysia. The oil samples from the reservoirs were collected in triplicate, using sterile Schott bottles (500 ml). The bottles were completely filled with the oil samples, hermetically closed by screw caps to avoid contamination and oxygen intrusion, and kept on ice during transportation to the laboratory.

2.2 Culture media:

The bacteria were cultured using VMNI medium as proposed by [47]. The medium was prepared by using filtered seawater. The composition (g/L) of VMNI medium consists of 0.5 KH_2PO_4, 1.0 NH_4Cl, 4.5 $NaSO_4$**,** 0.3 sodium citrate, 0.04 $CaCl_2.6H_2O$, 0.06 $MgSO_4.7H_2O$, 2.0 casamino acids, 2.0 tryptone, 6.0 lactate, 0.1 ascorbic acids, 0.1 thioglycollic acids and 0.5 $FeSO_4.7H_2O$. The pH of the medium was adjusted in range of 7.0 to 7.2 using 1.0 M NaOH prior to autoclaving at 121 ^oC. 1.0 mL of trace element and 2.0 mL of vitamins were added after autoclaving, the medium was left to cool at room temperature before inoculation.

2.3 Isolation of bacteria from crude-oil samples:

Crude oil (50 ml) was centrifuged at 3500_{rpm} and 4^0C for 5 min. The Precipitated layer obtained was added to (50 ml) of VMNI broth in a centrifuge tube and incubated at 30^0C in a shaker at 200_{rpm} for several days anaerobically. Centrifuge tube was filled with VMNI broth until there is no space for air to achieve anaerobic conditions. The enrichment cultures were transferred to agar plates, through the streak plate method after six

serial dilutions the fingerprints were less complex and clustered at higher similarities. After individual colonies were produce on VMNI agar, it was then incubated at 30°C. The anaerobic conditions were achieved using AnaeroGen™ KENKI (Thermo Scintific,) and each pure colony was referred as isolate. Selection of pure culture that will be used for further analysis was done. Stock cultures were stored at 4 °C and regularly transferred to fresh medium to maintain viability. The indication of successful incubation was the blackening of the medium within 3-7 days, and generation of H_2S which was also recorded as positive for IRB presence. Serial dilution of the culture was carried out and pure cultures were obtained using pour plates and streak techniques on agar plates. Plates were incubated at 30°C for 2-3 days, colonies formed were counted. Total anaerobic counts were determined, combining the counts of black and red or pink colonies. All samplings were carried out in triplicate. The isolates were further purified in veal VMNI broth and on plate agar at 30°C. The strains were stored at -20°C in broth medium with 4% (vol/vol) glycerol for further characterization.

2.4 Identification of bacteria:

All strains were tested at 30°C for the following key characteristics: gram reaction [9], motility and cell shape (phase-contrast microscopy after growth in VMNI for 24 h), cytochrome oxidase (BBL DrySlide oxidase, catalog no. 231746; Becton Dickinson, Detroit, MI) [15], catalase reaction (3% H2O2), [8], and production of H_2S from thiosulfate [8]. Fermentation of glucose was tested in the VMNI medium at 30°C, assimilation of several carbohydrates (citrate, glucose, lactate, and sucrose) [45] were used to group the isolates as *Shewanella* species. Briefly, 0.1% (wt/vol) of the tested carbohydrate was added to a VMNI broth as a carbon soruse. A total of 138 of an overnight grown culture were spotted in the broth. Cultures spotted in broth containing no carbohydrate were used as controls for the growth due to carryover from the media of the overnight culture. Assimilation of carbohydrate was determined by visual inspection of the medium for growth after 1 to 7 days at 30°C.

2.5 Molecular Identification and Phylogenetic Analysis:

Extraction of genomic DNA:The strain of IRB was grown on VMNI agar. Bacteria was prepared by taken single colony from 1.5 mL of 72-hour culture (approximately $6x10^7$ cells) in VMNI which was previously incubated at 30^O C with agitation at 200_{rpm}. The genomic DNA was extracted using wizard® Genomic DNA purification kit (Promega Corporation, USA) according to the manufacturers guide

2.5.1 PCR Amplification:

Amplification of the gDNA was done according to EconoTaq® PLUS GREEN 2X Master Mix protocols. A total of 50 µl PCR reaction mixtures containing 1µl of DNA template (10 ng/µl) 25µl of EconoTaq® PLUS GREEN 2X Master Mix (the Master Mix, which contains: 0.1 units/µl of EconoTaq DNA Polymerase, Reaction Buffer (pH 9.0), 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dATP, 3 mM MgCl2, and a proprietary mix of PCR Enhancer/Stabilizer and blue and yellow tracking dyes), 0.5 µl of 100 pmol/ µl Forward primers F (5'AGAGTTTGATCCTGGCTCAG-3), 0.5 µl of 100 pmol/ µl Reverse primers (5' ACGGTTACCTTGTTACGACTT-3), and 23 µl of Nuclease-free water. Thermal cycling conditions were (i) 94 ^{O}C for 2 min; (ii) 35 cycles of 94 ^{O}C for 30 sec, 65 ^{O}C for 30 sec, 72 ^{O}C for 1 min; followed by (iii) a final extension of 10 min at 72 ^{O}C.

2.5.2 Agarose Gel Electrophoresis:

PCR products were electrophoresed in a 1.0% agarose gel in 1X TBE buffer as the running buffer. The gel was electrophoresed at 80 V/cm for 120 minute. A 1 kb DNA ladder (Lucigen Corp,USA.) was used as a marker to estimate the size of DNA bands. The gel was stained with ethidium bromide-TBE solution for 20 - 30 minutes and later de-stain in distil water. The gel was then visualized under UV using SeaKem® LE Agarose (CAMBREX, USA.)

2.5.3 DNA sequencing:

The amplified PCR product was commercially sequenced by 1st BASE Pte Ltd. The generated DNA sequence was edited and aligns using bio-edit software.

2.5.4 Blast:

Sequence identities were verified by a BLASTX (Altschul *et al* 1997)(NCBI-BLAST) search against available molecular databases.

2.5.5 Sequence analysis and phylogenetic:

A phylogenetic tree that consisted of organisms covered in the present study and other cutinases individual rRNA gene sequences to those in the NCBI database (http://www.ncbi. nlm.nih.gov/BLAST) was constructed using ClustalW2 http://www. ebi.ac.uk/Tools/msa/clustalw2/ [36]. A maximum-parsimony evolutionary history

was inferred using the neighbour-joining method in MEGA6. The 1000 bootstrap replicates of the original sequence data were run to assess the confidence value of individual branches [7,30]. The sequences of the 16S rRNA gene of the strains have been registered in GenBank with accession numbers KU892722, and KU892723.

2.6 Biofilm formation and stability on carbon steel coupon surfaces:

Biofilm formation was analyzed according to [34] with same modifications. Biofilms of IRB were prepared by inoculating carbon steel coupon (Chemical composition (wt %) of this steel was C 0.12, Mn 0.5, S 0.045, P 0.04 and Fe is balance). At each grinding steps with culture of mid-log phase (10^5 cells /ml) grown in VMNI medium. At the start of each experiment, the treatment of the surface of the carbon steel coupon (15mm×10 mm×1.5 mm) was done using sandblasting technique. The coupon for biofilm formation assay was ground with SiC grit paper grade 240, 320, 400, 600, 800 and 1200 and the surface were also cleaned before the biofilm assay [26]. Briefly coupons were cleaned in 18% HCl, and then neutralized by immersion using saturated sodium bicarbonate solution after that, coupons were then washed with sterile distilled water, rinsed in acetone, and re rinsed again with sterile distilled water and air-dried before being placed into hot air oven at 75°C for 30 mins an air stream. The biofilm formation device was a 24-well-plate with a carbon steel coupon each. IRB cells were introduced in the biofilm device. A control without IRB was performed in this assay. Two milliliters of the cell suspension was added to each well, covering the carbon steel coupon surfaces, and incubated at 30°C for 7 days [14]. At the end of the biofilm formation incubation period on the carbon steel coupon surfaces, the carbon steel coupon were removed aseptically from the broth culture for biofilm quantification using the crystal violet binding assay described by [34]. Loosely or Unattached attached cells were removed by washing with distilled sea water; adhered cells on the carbon steel coupon were stained with 1% crystal violet and were let to stain for 20 minutes. The cells were rinsed again with distilled water. The dye bound to adherent cells was re-solubilized with 5 mL of 97% (v/v) ethanol per well. Ethanol with re-solubilized dye in each well was transferred to new sterile 96-well flat-bottomed polystyrene plate. The OD of each well was measured at 630 nm. The test was done in triplicate. The procedure was repeated thrice for each coupon and the obtained results were expressed as the mean value, and standard deviation as required

All tests were carried out in triplicate, and the strains were divided into the following categories: no biofilm producer, weak biofilm producer, moderate biofilm producer, or strong biofilm producer [34]. For this, it was necessary to establish the cutoff value (ODc). The ODc was defined as three standard deviations (SD) above the mean OD of the negative control (uninoculated medium): ODc = average OD of negative control + (3×SD of negative control). Based upon the OD values: $OD \leq ODc$ = no biofilm producers $ODc < OD \leq 2 \times ODc$ = weak biofilm producer, $2 \times ODc < OD \leq 4 \times ODc$=moderate biofilm producers and $4 \times ODc < OD$=strong biofilm producers.

2.7 Statistical analysis:

The Excel data analysis package was used to calculate mean, standard deviation of the mean. Statistical comparisons of the results were performed by one-way ANOVA using SPSS ver.20. Significant differences ($P<0.05$). All the results were calculated from the mean of three replicate samples for each data point.

Results

3.1 Characterization of IRB isolation:

A total of 158 CFUs were obtained from crude oil under anaerobic condition. Among these more than 12 morphologically different (based on size and colony appearance smooth or rough and presence of pigment) bacterial strains were analyzed. Fifty-eight bacterial strains grew on VMNI agar with pink-orange, Pale brown or pink colonies appeared after 1-3 days (Fig 1a), H_2S producing, gram negative, motile rods with positive oxidase and catalase reactions. Among the 59 bacteria isolated, 34 were selected as *Shewanella* isolates due to their ability of the isolates to grow well at 30°C and change VMNI color to black during 1-3 days (Fig 1b), and were further characterized for identification and biofilm formation. Based on these traits, the strains were tentatively classified as *S. chilikensis* according to the method of Sucharita *et al* [35], and *S. algae* according to the characteristics mentioned by Venkateswaran *et al* [40]. However, these characteristics were not sufficient to allow for a complete differentiation between *S. chilikensis* and *S. algae*. A simple phenotypic scheme derived from various biochemical tests was used to further distinguish the 34 strains (Table 1). Based on these phenotypic traits, the changes in the abundance of the *Shewanella* species from crude oil at 30°C were noted and 21 isolates were delimited as a *S. chilikensis* and 13 isolate as *S. algae*.

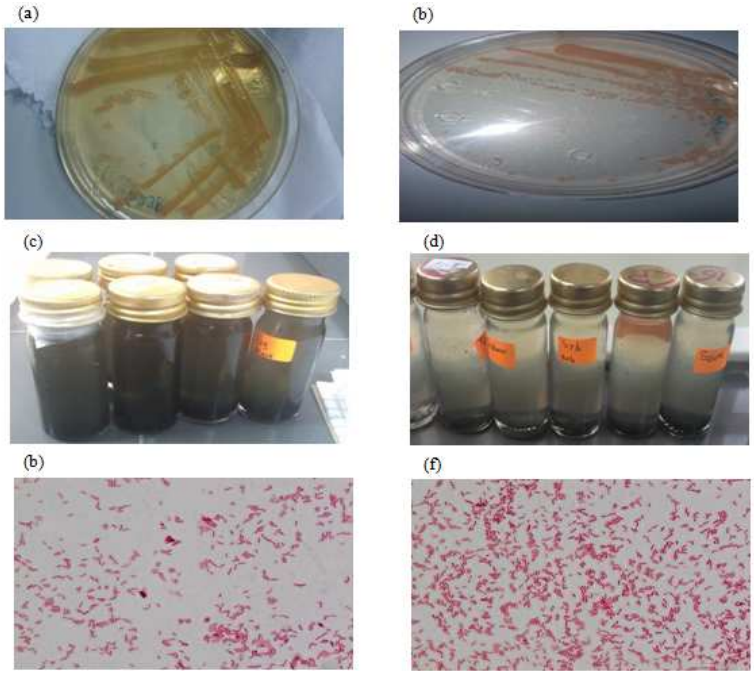

Fig. 1: Photographs obtained from the IRB isolates: (a) Colony of IRBc1 (b) Colony of IRBa24 (c) H2S production positive (d) H_2S production negative (e)Gram stain of IRBc1 (f) Gram stain of IRBA24 (Gram reaction was observed under oil immersion using 1000x magnification of compound microscope

Table 1: Phenotypic characteristics that differentiate *Shewanella* strain

Characteristic	*S. chilikensis* (21)	*S. algae* (13)
Colony colour	Pale brown	Pink
Oxidase	+	+
catalase	+	+
Growth at: 30 ^{O}C 37 42 ^{O}C	 + + +	 + + +
Carbon source utilization Malate Mannose Glucose Lactate Citrate Sucrose Pyruvate acetic acid	 + - - + - - + +	 - + + + - - - -
Urease	+	-
Haemolysis	+ (α)	+
6%NaCl	+	+

3.2 Biofilm Production Analysis:

In our study, we observed that all 34 strains that were selected of 59 strains belonging to Shewanella sp. were able to produce biofilm. These 34 strains were divided into 17 strong biofilm producers, 15 moderate biofilm producers, and 1 weak biofilm producers (Fig. 2a and b). The classification was based on established cutoff value (ODc) as described in item 2.6. The ODc was 0.4829. Among the *S. chilikensis* and *S. algae*, significant result ($p < 0.05$) in biofilm formation was observed. The greatest number of strong producing strains was seen in *S. chilikensis* (12) and (5) in *S. algae.*

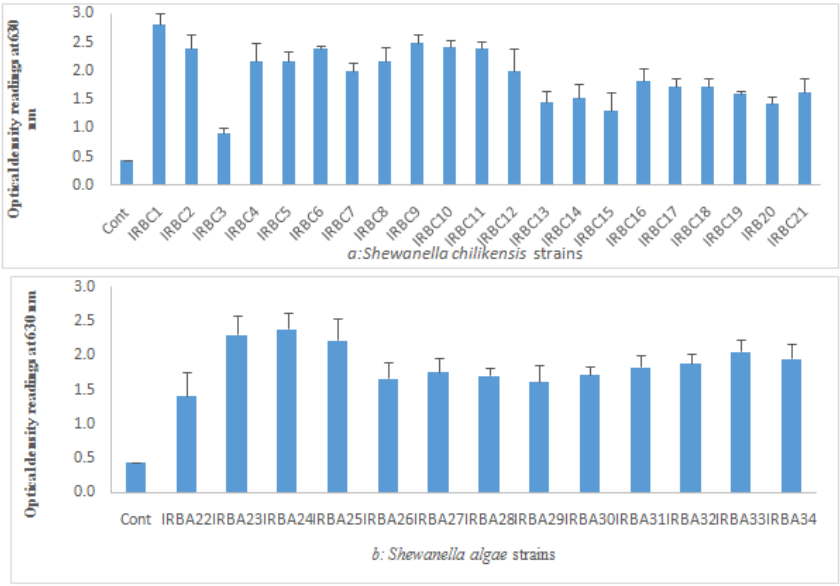

Fig. 2: Biofilm formation on carbon steel coupon surface. IRB strains grew on a VMNI medium. The bars represent means and the whiskers standard deviations.

3.3 Moleculer characterization and phylogenetic analysis based on 16s rRNA gene sequence:

The 16S rRNA gene sequences of 2 selected strains representing main two groups of different phenotypic traits were compared to the sequences of other *Shewanella* species. These strains and most active isolates were identified as *Shewanella* sp. which belongs to iron-reducing bacteria group. The purified 16S rRNA gene sequence were successfully recovered for IRBC1 (coded as IRB1) and IRBA24 (coded as IRB2) (Fig 3a). The PCR products were approximately 1500 kb in size corresponding to the group of bacteria.The comparison of DNA sequences with sequences in GenBank was also compiled with number series and maximum identification percent. All sequences give similarity between 97% - 99%. According to 16S gene sequences in GenBank , the strain (IRBC1) cluster with *Shewanella chilikensis* strain JC5, while the other strain (IRBA24) cluster with *Shewanella algae* strain MAS2741.

The phylogenetic tree (Fig 3b) was constructed to show the relationship and comparison of our crude oil bacteria sequences data and previously published data from NCBI (GenBank). IRBC1 and IRBA24 were clustered together (bootstrap 87%) and formed these isolates clustered with *Shewanella chilikensis* and *Shewanella algae* (bootstrap 100%).These strains are from the same family (Shewanellaceae and under phylum Proteobacteria).

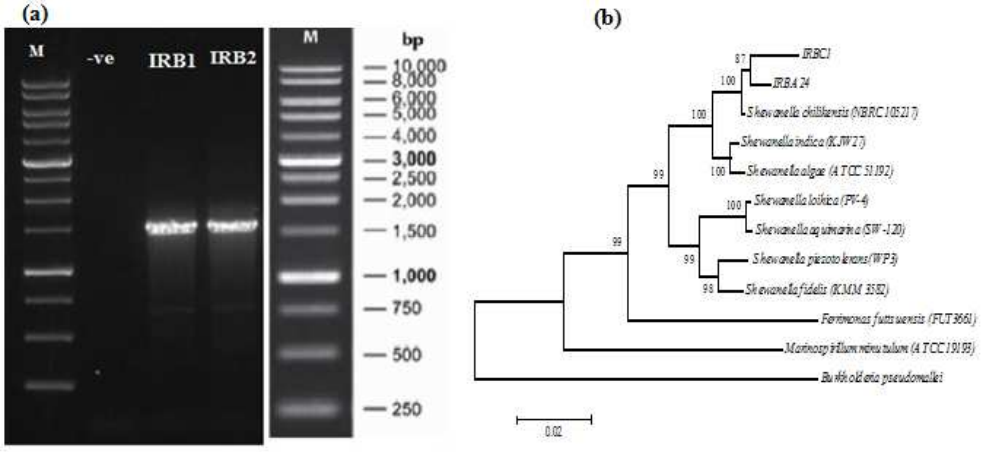

Fig. 3: (a): A garose gel electrophoresis of purified 16S Rdna for IRB1 and IRB2, **(b)** Phylogenetic tree displaying evolutionary relationships between genus *Shewanella* in this study and other 7 selected strains representing each subgroup of the different phenotypic traits within the genus *Shewanella*. The evolutionary history was inferred using the Neighbour-Joining method. Confidence values were assessed from 1,000 bootstrap replicates of the original sequence data and are shown next to the branches. The scale indicates the evolutionary distances computed using the p-distance method and are in the units of the number of amino acid differences per site. The analysis involved 7 amino acid sequences. Evolutionary analyses were conducted in MEGA6.

Discussion:

Bacterial isolates that have until recently been identified as *S. chilikensis* and *S. algae* by standard microbiological key characteristics as being gram-negative motile rods with positive oxidase and catalase reactions that produce H_2S have strictly respiratory metabolism and are able to reduce several electron-acceptors. These strain showed good growth anaerobically in VMNI medium and on agar plates. *Shewanella* belong to the group of iron reduction bacteria because of its ability to reduce Fe^{+3} to Fe^{+2}. Furthermore, these bacteria are facultative anaerobic bacteria that can use various electron acceptor in anaerobic conditions such as Fe^{+3}, Mn (IV), [13]. Rapid growth of bacteria was observed on this medium as it only took 1-3 days on the liquid medium and more than three days on agar plates to be blackened. Black precipitate was notice at the bottom of the bottle after three days of culturing. The observation of black color and precipitate could imply the presence of H_2S production by the bacteria. It has been established that the culturing of *Shewanella* bacteria on agar VMNI produces orange pigment colored colony after 24 hours while culturing of this bacteria for 3 days or more than changes the orange color to black which is as a result of hydrogen sulfide H_2S produced by the bacteria (*Shewanella* species). The established fact is not different from the result obtained in this study in which black precipitate and color was observed in the culture after 3 days, this confirmed our observation to be similar to the previous work done in other parts of the globe. We carefully used selected criteria to differentiate species of *Shewanella* bacteria as previously done by other studies [33,38,46]. Also it may be possible to systematize any group of isolates base on the phenotypic characters; Nevertheless, the comparative value of a given trait or group of traits over another remains difficult to assess [38]. Also, variations in the approaches used for the culture and testing of isolates can produce different results. Hence, we also consider 16S rRNA sequence analysis and it was evident that some of the strains examined in the present study may encompass two *Shewanella* species (*S. chilikensis* code IRBC1and *S. algae*, code IRBA2). A detailed taxonomic approach is necessary to further describe these species.According to Sacchi (2002) CEF, 16S rRNA gene sequences was used to estimate the relationship between identified unknown bacteria and other bacteria in NCBI database. All prokaryotes have this type of gene in themselves and it would be easier identification of bacteria by using gene sequences. The application of 16S rRNA was widely in order to identify bacteria in an open environment including liquid environment [17]. Our results of 16S rRNA gene sequence and phylogenetic tree analysis confirmed the phenotypic grouping established in the present study and identified the strains as *Shewanella* species. These strains were belong to the Shewanellaceae family and under the class of Gammaproteobacteria [2]. These bacteria are able to grow in anaerobic and aerobic conditions and recognized as facultative anaerobic bacteria, having short rod shaped forms and belong to gram negative bacteria and according to Bowman (2005) CEF, genus Shewanella comprises of Gram negative, rod shaped, motile and facultative anaerob. These bacteria also have been isolated from different sources environment [43] and this has been demonstrated in this study where the shawell were One of groups of bacteria prevailing in the crude oil samples.

Several liquid ecosystems bacterial life is the formation of biofilm which is common, i.e., the gluing of sessile high-density cell consortia together by an exopolymeric attributed to various factors, which include greater resistance or tolerance to exogenous stresses or nutrient accumulation at surfaces [21]. In our study we find out that *Shewanella* genus which is one of the IRB's groups are able to produce biofilm on the surface of carbon steel which can lead to biocorrosion process.

The formation of biofilm and EPS is assisted by several factors and one of them is the presence of oxygen [23]. In this study, IRB were cultured in anaerobic condition before being added with carbon steel coupon and based on the optical density (OD) reading on 630 nm wavelength of *Shewanella* obtained from crystal violet assay shows production of high value of biofilm and which is similar to the study by [22] which stated that biofilm formation can be structurally packed if *Shewanella* strain (one of the bacteria belonging to IRB group) is being used. A study by [37] shows that removal of oxygen enhances the attachment of bacteria towards any surfaces, besides that, there are some strains that produce a thin layer of biofilm and EPS. There is also a possibility that some mistakes may occur during the growth of IRB where little amount of oxygen diffuse into the solution. The presences of oxygen causes change to pH, redox and reducing ability. [3] stated that oxygen affects the formation and structure of three dimensional biofilm by *Shewanella* strains, it is also supported by [17], who stated that the presences of other elements including the oxygen could modify the chemical environment and thus enhance and mitigate the corrosion process by bacteria.

Conclusion:

It can be concluded that most of bacteria isolated from crude oil under anaerobic conditions in this experiment belongs to *Shewanella* genus. The two species of *Shewanella* encounter in this study are*S. chilikensis* and *S. algae*. *Shewanella chilikensis* and *Shewanella algae* are IRB's group of bacteria and are able to produce biofilm on the surface of carbon steel which can lead to biocorrosion process.

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