Cultivation and Detection of Sulfate Reducing Bacteria (SRB) in Sea Water

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Abstract: Sulfate-reducing bacteria (SRB) represent a class of anaerobic microorganisms that conduct dissimulatory sulfate reduction to obtain energy. The present study aimed to detect and control SRB activities using a very rapid detectable culture medium and reduction of potential economic loss in the petroleum sector. This study is an attempt to isolate SRB from sea water by rapid and sensitive culture media and to control their effect using eight commercial biocides (Aldehydes and quaternaries). The present work studies the effect of composition of four recommended culture media (Postgate medium B, Starkey, Baar's and API media), besides, the presence of metal coupons in these media to enhance the growth of sessile SRB. Furthermore, the present study evaluates the efficiency of filtration of these culture media on the growth of SRB. The results revealed that modified Postgate medium B was the recommended medium for SRB growth. In addition, the results showed that rapid and abundant growth of SRB when the metal coupons were immersed in the culture media which were deficient in iron. The unfiltered culture media improved the SRB growth. The growth of SRB was depressed by 15 ppm of the commercial quaternaries rather than 20 ppm of the aldehydes.

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Key words: SRB, Culture media, Biocides, Minimal inhibitory concentration

1. Introduction:

One of the important practical problems is the control of SRB growth in economically important situations in the petroleum sector. Consequently, considerable research has been devoted to testing various potential micro biocides and the results have been displayed throughout the scientific literature (Kumaraswamy *et al.* 2010). SRB, which generate large amounts of toxic hydrogen sulfide in aquatic ecosystems, are important not only for ecological reasons but also economically. The activities of SRB in natural and man made systems are of great concern to engineers in many different industrial operations (Gibson, 1990; Odom, 1990; Odom and Singleton, 1992). Oil, gas and shipping industries are seriously affected by the sulfide generated by SRB (Battersby, 1988; Hamilton, 1994; Peng *et al*., 1994; Okabe *et al*., 1995 and Cullimore, 2000). In the oil industry most monitoring of microbiologically influenced corrosion (MIC) has in the past only been conducted on sulfate-reducing Bacteria (SRB) carried out by cultivation based techniques. (Jan Larsen, 2010).

Sulfate reducing bacteria (SRB) are a group of genetically similar anaerobic organisms that were first discovered by Hamilton (1994). The SRB form an integral part of a group referred to as "sulfur bacteria" and are sometimes considered to be nuisance bacteria in a number of ways, (Tiller, 1990 and American Water Works Association, 1995). These bacteria are seldom isolated because of their slow growth. Colonies appear after more than three days of incubation and are generally not noticed, being overgrown by the accompanying flora. Accordingly, their isolation requires specific or selective growth medium (Julien Loubinoux *et al*., 2003).No growth takes place in media rendered "biologically free" of iron, (Postgate, 1984; Widdel, 1988; Parkes, *et al.*, 1989).

The present study was conducted to show the efficacy of the impure (turbid) media on the detection of SRB growth.

2. Material and Methods:

2.1. Organisms

A stabilized mixed culture of sulfate reducing bacteria (SMC-SRB) was isolated from the failure shipping pipe line for treated oil (Esh El-Mallaha. Petroleum Company).

2.2. Culture media

Four recommended media of the most commonly used ones were evaluated for SRB growth. The compositions of these media were nominated in table (1). Thioglycollic and Ascorbic acid were added to all media to increase their reducing power. Saline water was (50%of the total volume) used in replacement of tap and distilled water.

2.3. The electrode

The electrode used was derived from mild steel sheets with the following composition:
0.09 % (C), 0.07 % (Si), 0.37 % (Mn), 0.017 % (S), 0.028 % (P), 0.005 %(Al), 0.015 %(Ni), 0.11 %(Cr), 0.004 % (Mo), 0.006 % (Cu), and 0.007 % (V).The electrodes were polished with emery papers 200.400, 600, grade for fine polishing .They were washed with distilled water then degreased with acetone and finally dried till use.

2.4. Pipe line description by ESHPETCO

Length 7 Km (above the ground surface); diameter 18", construction date since 1982; fluid was crude petroleum oil with water content 0.05 % Vol. sulfur content 4.5 % wt, pH value was 6-6, 5 and temperature 20-30°C and the pipe line grade was API–42.Pipe line operation data was as following pressure was 14 bar, stagnant fluid periods since operation were at 1st time (9 months in 2006) the 2nd time and 6 months in 2007.

2.5. Pipe line failure

The pressure dropped and cured oil shortage delivered at point was noted at rapid date. Pipeline track was surveyed and spelled oil was found in one of the train depression along 7 Km train, the pipes lie directly on the

ground according to its natural topography of elevations and depressions.

One pipe was found ruptured open to a perfect longitudinal line extending to about 2 meters, slight bulging is clear in the middle of the opening. Huge amounts of crude oil were spilled due to the failure estimated by 10.000 barrels. The opening was in the position 6 o'clock in contact with the ground.

2.6. Field Inspection

Field inspection was done by Central Metallurgical Research and Development Institute (CMRDI).The inspection result reported that the pipe line failure is due to badly fabricated welded pipe. Internal pitting corrosion due to sour oil (along 25 years of service) was a trigger of cracking in the infused weld grooves which ended to complete rupture of the pipe. Two other factors contribute to the failure; a) positioning of the weld line (seam) at the bottom (6'oclock) where water can best accumulate, and b) stagnant long (shut down) periods. They did not ignore the possible malfunction of the treatment plant with respect to water and salt content in the treated oil.

2.7. Isolation and enrichment of SMC-SRB

SMC-SRB was obtained by transferring 1ml of the received internal sludge sample of the failure shipping pipe line into sterile screw capped vials (1.5 × 5 cm) containing modified Postgate medium B as mentioned in table (1).The bottle was incubated for 7 days at 30°C. Blackening of the bottle meant a positive growth for SRB. This step was repeated at least 10 times to obtain a SMC-SRB (figure1).

2.8. Physicochemical analysis of saline sea water

The water sample was completely analyzed according to APHA (1989) as recorded in table (2).

2.9. Evaluation of the culture media on the SRB growth

Four different recommended culture media (Postgate B, API, Starky and Baar's) were prepared according to their compositions as shown in table (1). Thioglycollic and ascorbic acid were added to increase the reducing power of the medium. All media were autoclaved at 121°C for 20 min. Observations of the culture media were recorded in table (3). API medium was the only clear one. These media were distributed in 10 ml sterile screw capped vials (9ml) in each one of them. Enriched SMC-SRB 4 day's old culture was inoculated into the previous culture vials. Then all vials were incubated at 30°C for 7 days and observed by naked eye (table 4). The sulfide production was determined via the SRB activity during 7 days to record the time course of the sulfide production by SMC-SRB (figure 2). Sulfide was determined iodometrically according to APHA (1989).

Table (1) Chemical composition of the modified culture for SMC-SRB growth in g/L

Chemical ingredient	Postgate B	API	Starkey	Baar's
KH_2PO_4	0.5	0.01	0.5	0.5
NH_4Cl	1.0	-	1.0	1.0
Na_2SO_4	1.0	-	1.0	-
$CaCl_2.6H_2O$	0.1	-	0.1	-
$MgSO_4.7H_2O$	2.0	0.2	2.0	2.0
Sodium Lactate (60 –70%)	5 ml	4ml	5 ml	5ml
Yeast extract	1.0	1.0	-	-
Ascorbic acid	0.1	0.1	0.1	0.1
Thioglycollic acid	0.1	0.1	0.1	0.1
$FeSO_4.7H_2O$	0.5	-	-	-
NaCl	26	26	26	26
$Fe(SO_4)_2(NH_4)_2.6H2O$	-	0.2	0.5	0.5
$CaSO_4$	-	-	-	1.0
Sea water Distilled water	500 ml 500 ml	500 ml 500 ml	500 ml 500 ml	500 ml 500 ml
pH	7-7.5	7-7.2	7-7.3	7-7.5

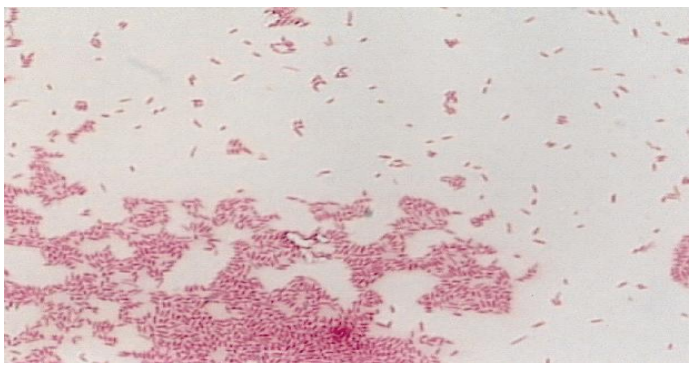

Figure (1): Gram negative of stabilized mixed culture SRB

Table (2): Physical chemical analysis of sea water received from ESHPETCO.

pH at 20ºC	6.73
Sp. Gr. at 20ºC	1.017
Resistivity at 20 ºC	0.227 Ohm. m
Sodium (Na^+)	4904 ppm
Potassium (K^+)	130 ppm
Calcium (Ca^{2+})	1231 ppm
Magnesium (Mg^{2+})	1763 ppm
Iron (Fe^{++})	0.02 ppm
Manganese (Mn^{2+})	0.09 ppm
Barium (Ba^{2+})	0 ppm
Strontium (Sr^{++})	26 ppm
Zinc (Zn^{++})	0 ppm
Lead (Pb^{++})	0 ppm
Chloride (Cl^{-})	13000 ppm
Sulphate(SO_4^{--})	2400 ppm
Bicarbonate(HCO_3^-)	117 ppm
Carbonate (CO_3^{--})	0 ppm
Total dissolved Solids	23601 ppm

Table (3): Observations of the modified culture media for SMC-SRB in sea Water.

Media / Properties	Postgate (B)	API	Starky	Baar's
Color	Yellow with faint gray	Yellow	White	White
Turbidity	Turbid	Clear	Turbid	Turbid
pH before autoclaving	7.3	7.2	7.2	7.2
pH after autoclaving	6.7	6.4	6.4	6.4
E_h(mV)	-345	-260.3	-200.7	N.D

N.D Not determined.

Table (4) Naked eye observation of SMC-SRB growth using the modified culture media in sea water during 7 days incubation at 30°C

Time (days)	Media			
Zero Time	**Postgate (B)**	**API**	**Starky**	**Baar's**
1	-	-	-	-
2	+	-	-	-
3	++	-	-	-
4	+++	-	+	±
5	+++	-	++	+
6	+++	-	+++	+
7	+++	+	+++	+

- No growth + Moderate growth ++ Good growth +++ Severe growth

2.10. Effect of metal coupons on the enhancement of SRB growth

The mild steel coupons of 0.2 × 1 × 5 cm were prepared using emery papers with very fine grade as mentioned before. The prepared clean mild steel coupons were immersed in the screw sterile vials which contained the previous culture media. One ml of the enriched SMC–SRB was inoculated into the previous vials. The vials were incubated and observed visually (table 5) for 3 days at 30°C. Then the sulfide produced was determined as mentioned before (figure 3).

Figure (2): Time course of sulfide production by SMC SRB during 7 days incubation at 30ºC in different culture media

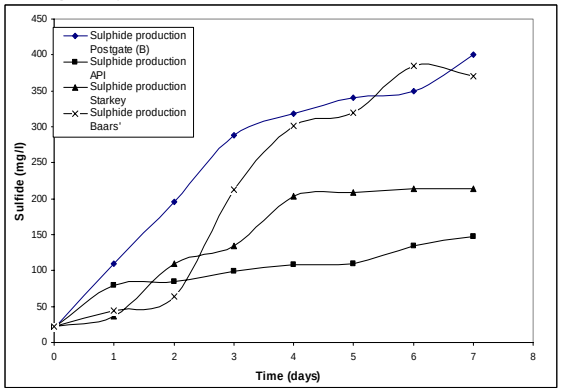

Table (5): Naked eye Observation of SMC-SRB growth in the presence of metal coupon in the culture media after 3 days incubation at 30°C

Media / Time (days)	Postgate (B)	API	Starky	Baar's
7	++++	++	++++	+++

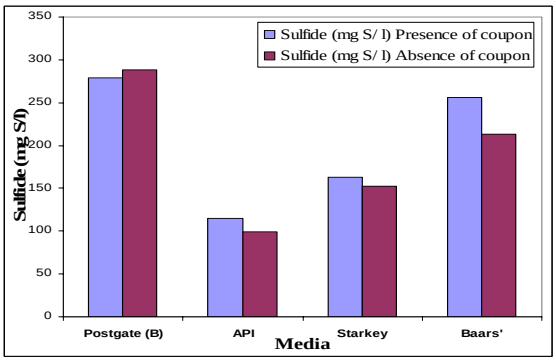

Figure (3): Effect of presence of metal coupon on the SMC-SRB growth using culture media after 3 days incubation at 30ºC.

2.11. Effect of the weight loss measurements

Weight loss measurements were carried out in screw capped vials containing the previous sterile culture media. These vials were inoculated with 1 ml of enriched SMC-SRB. The clean weight mild steel coupons (W1) were immersed completely in the medium and incubated for 7 days at 30°C. After the incubation period ended the coupons were picked up and immersed in a washing solution (1 % HCl + 0.5 %Thiourea) for 5 min to remove the corrosion product layer. Then the coupons were washed by distilled water and dried, then reweighed and the weight loss was recorded to calculate the corrosion rate as showed in (table 6) according the following equation.

MPY = (Area factor)* X (Wt.loss in mg)/ (Days exposed)

*(The area factor is computed from the exposed surface area and density)

Table (6): Effect of different culture media on the corrosion rate of the mild steel. Coupon after 7 days of incubation at 30°C by weight loss technique (MPY)

Post gate (B)	API	Starkey	Baar's
0.87	5.2	14.8	0.534

2.12. Effect of filtration of the culture media on the SRB growth

The turbid culture media Postgate B, Starky and Baar's were filtered through filter paper before autoclaving. After that the culture media were distributed in sterile screw capped vials and then inoculated with 1 ml of enriched SMC-SRB and incubated at 30°C for 7 days. Sulfide production was determined as mentioned before (table 7).

Table (7): Effect of clear medium on the SMC-SRB growth after 3 days incubation at 30°C using different culture media

Media	Sulfide concentration mg S/L	Without filtrate after 3-days
Postgate B	115	288
API	167	99
Starkey	11	135
Baar's	13	213

2.13. Biocide test

The Baar's medium as mentioned in table (1) dispensed in 9.0 ml amounts into a series of 10 ml capacity screw capped glass vials. These vials contained various concentrations ranged from 5 to 20 ppm of commercial biocides (four quaternaries and four aldehydes) coded as Q1, Q2, Q2, Q3, Q4, A1, A2, A3, and A4, respectively. The vials were autoclaved at 121°C for 15 min .After cooling the enriched SMC-SRB was inoculated and the SRB growth was detected after 7 days incubation calorimetrically by measuring the absorbance at 580 nm (table 8). Control vial was inoculated with sterile H_2O. The efficiencies (E %) of biocides (table 9) were calculated according to the following equation:

E % = E uninhibited – E inhibited/Euninhibited × 100

Table (8): Determination of the mic of the tested biocides against SMC-SRB by using colorimetric measurement at 580 nm.

Conc. ppm	Tested samples							
	Q1	**Q2**	**Q3**	**Q4**	**A1**	**A2**	**A3**	**A4**
Control	0.230	0.230	0.230	0.230	0.230	0.230	0.230	0.230
5	0.209	0.200	0.229	0.205	0.211	0.203	0.213	0.219
10	0.107	0.103	0.119	0.120	0.073	0.088	0.079	0.086
15	0.078	0.055	0.066	0.060	0.011	0.012	0.011	0.013
20	0.011	0.009	0.012	0.013	0.006	0.006	0.004	0.007

Table (9) Efficiencies (%) of the tested biocides against SMC SRB

Conc. ppm	Tested samples							
	Q1	**Q2**	**Q3**	**Q4**	**A1**	**A2**	**A3**	**A4**
Control	0	0	0	0	0	0	0	0
5	9	13	4	11	30	38	45	45
10	53	55	48	48	68	62	65	63
15	66	76	71	74	95	95	95	94
20	75	80	79	80	97	97	98	97

3. Results and Discussion:

Physicochemical analysis of saline sea water as recorded in table (2) revealed that it was a very good source for SRB due to the sulphate contents (2400 ppm).This was confirmed by the field inspection done by (CMRDI); they did not ignore the possible malfunction of the treatment plant with respect to water and salt content in the treated oil. Thus the pipe line failure might be due to biological effect in addition to physical one. Besides the Blacking of modified Postgate medium B revealed the positive growth of SMC-of SRB.The results detected an improvement for SRB growth due to addition of supplied sea water in comparison with the previous studies .Figure (1) showed that SRB was a pure Gram negative short rods. SRB are present in most soils and water, but are outnumbered by other types of microbes except in special environments. Accordingly enrichment of the needed environments with these bacteria is usually necessary before isolation is attempted (Widdel, 1988). On the other hand the presence of reductants in culture medium makes isolation a much less formidable task (Postgate, 1984).

Postgate medium B is a multipurpose medium for detecting and culturing *Desulfovibrio* and *Desulfotomaculun*. Most of the ingredients can be prepared and held as a stock, but the thioglycollate and ascorbate, which may be omitted if the inoculua are fresh, flourishing culture, should be added and the pH adjusted just before autoclaving. The medium should then be used as soon as it cools down because the reductants deteriorate in air at neutral pH values. This process accompanied by a transient purple color. The precipitate in medium B aids growth of tactophilic strains. This medium is recommended for long term storage of strain. In cultivation of the SRB in pure culture, the major prerequisite is simple. The redox potential (E_h) of the environment must start around -100 mV. This means that mere exclusion of air is not sufficient to ensure growth (a boiled-out Lactate + sulfate medium would have an E_h of about +200 mV under N_2 be about with 5–m M Na_2S the value would be about (-220 mV) (Postgate, 1984).This was mentioned in tables (4) which showed that modified Postgate medium B was the recommended medium for SRB growth followed with Starky then Baar's one because the precipitate in these media aids growth of tactophilic strains and this was confirmed by the effect of filtration of the culture media on the SRB growth as showed in table (7) ,the filtration of the turbid medium reduced the SRB growth . In addition the results in table (5) and figure (3) revealed that rapid and abundant growth of SRB after 3-days incubation when the metal coupons were immersed in the culture media which were deficient in iron. This due to the utilization of the hydrogen evolved when metallic iron immersed in mineral medium provides additional evidence for the presumed role of SRB in anaerobic corrosion of ferrous pipes namely that the depolarizing of cathodic elements of electrochemical systems on the surface of the metal. Weight loss measurement recorded that Starky medium was the most aggressive medium to mild steel. This might be due to the anodic dissolution of mild steel and cathodic depolarizer's effect of FeS film which formed due to SRB activity. So for diagnostic purposes media often prescribed are those which contain about 0.5 % of a ferrous salt. This forms a black precipitate of FeS when sulfide is formed, so blacking of the medium as a whole, or the zone round a colony, is an evidence for bacterial sulfate reduction as shown in equation (1) (RzecZycka and Blaszczyk, 2005).On the other hand API medium showed a high (mpy) corrosion rate in comparison to modified Postgate B and Baar's media. This might be due to metal exposure to chemical dissolution in that medium (general corrosion) not for SRB activity. This illustrated that the growth of SRB by (using API medium) conventional methods is very time consuming, (Iverson, 1987; Taylor and Parkes, 1983).

$$SO_4^{2-} + \text{Organic matter} = HS^- + H_2S + HCO_3^- \quad (1)$$

Due to the economic losses as well as environmental health and safety hazards caused by the activity of stabilized mixed culture containing sulfate reducing bacteria,(SMC-SRB)in many industrial sector such as the oil and gas industry,

it was important to minimize the risks resulting from SRB activity. These bacteria are mainly sulfate reducers, and their growth frequently causes severe corrosion problems in oil well pipes. One of the simplest ways to measure the effect of biocides on an organism is by determining the Minimal Inhibitory Concentration (mic) which just prevents growth in a suitable medium. The antibacterial agent is serially diluted in the medium and standardized inocula of the test strain are added. After incubation for a predetermined growth, the cultures are examined and the mic for the biocide is detected (Sharma, *et al.*, 1986). The mic of microbicides and bacteriostatic substances are usually governed by the nature of the medium in which the substance is tested and also by the size of the inocula. Iron salts can increase the apparent resistance of cultures to inhibitors, and in case of *Desulfovibrio* species the presence or absence of NaCl may influence inhibition (example a quaternary biocide) (Bessems, 1983). In the present work the effect of tested biocides on a cell is normally dependent on its concentration and it's can be seen from the slight increase and leveling of A_{580} at growth of SRB was depressed by 20 ppm of the commercial quaternaries rather than 15 ppm of the aldehydes. The results in tables (8&9) showed the biocidal activity and biocidal efficiencies and recorded that up to 97% for commercial aldehydes and up to 75% for commercial quaternaries at 20 ppm.The cell membrane of microorganisms is composed of several lipids and protein layers arranged together in a specific arrangement called the bilayer (or multi layer lipoprotein structure). The presence of the lipids as a building unit in the cell membrane acquires them their hydrophobic Character (Bessems, 1983). The selective permeability of the lipoprotein membrane represents the main function, which control the biological reaction in the cell. Hence any factor influences that permeability causes a great damage to the microorganisms, which leads it to die.

4. Conclusion:

The isolation of SRB by conventional methods is very time consuming. The present work study recommended modified Postgate B and Starkey media because the precipitate in these two media aids growth of tactophilic strains. Also the use of the supplied water sample by 50% of the total volume of the culture medium improved the SRB growth. Besides, the presence of metal, improved the SRB growth.Ontheotherhand the growth of SRB was depressed by 15 ppm of the commercial quaternaries rather than 20 ppm of the aldehydes. The present study aimed to detect and control SRB activities using a very rapid detectable culture medium .In addition to reduction of their economic loss in the petroleum sector.

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