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Statistical Investigation on Anaerobic Sulphate-Reducing Bacteria Growth by Turbidity Method

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ABSTRACT

In oil and gas industry, corrosion due to activity of microorganism is one of the main factors, which contribute to catastrophic structural failure. Previous study always linked Sulfate-Reducing Bacteria (SRB) upon the mechanism of Microbiologically Influenced Corrosion (MIC), as the major contributors. In this study, mechanisms of SRB genus *D. vulgaris* in terms of bacterial growth under influence of environmental factors were investigated. The growth of pure strain ATCC 7757 and SRB isolated from the soil in suspected areas in Peninsular Malaysia were investigated by using turbidity measurement. Results from the study were analyzed statistically to show the significant influence due to various environmental factors. The results agreed that variation of each environmental parameter tested gives strong influence upon bacterial growth for SRB strain individually.

Key words: Sulphate-reducing bacteria, growth rate

INTRODUCTION

Underground pipeline network system is recognized as the most efficient, reliable and cost-effective way of transporting large volumes of oil and gas worldwide. However in long-term, underground buried pipelines are continuously exposed to many serious attacks, such as; external impact, abrasion and corrosion, which later lead to pipeline leakage and rupture (Noor *et al.*, 2008). Corrosion is identified as one of the main issues in pipeline systems, which gradually decreases the strength of its material due to the continuous exposure to an environment, which is reactive in many ways for many years (Javaherdashti, 2011; Noor *et al.*, 2011). All surroundings including; atmospheric, aqueous, underground soil and microbial attack are having the potential to gradually destroy the pipeline material, which is commonly made of carbon steel.

Microbial corrosion, also known as Microbiologically Influenced Corrosion (MIC) is a corrosion caused or promoted by both macro and microorganisms including; bacteria, which feed on nutrients and other elements found in the soil or water (Ringas, 2007; Abdullah *et al.*, 2014). Most of the bacteria live in the surrounding environment, usually soil or water but prefer to attach and grow on the steel surface and contributing to severe metal corrosion (Muthukumar *et al.*, 2003). The

biological activities of these microorganisms modify the local chemistry or environment and render it more corrosive to the metals. Pipeline failure by MIC attack represents a significant loss in various aspects especially profit, assets and reputation.

Previous studies recognized SRB as the principal causative organism responsible for corrosion on steel structures buried underground which cause unexpected failures (Cord-Ruwisch *et al.*, 1987; Tardy-Jacquenod *et al.*, 1996; Al-Jaroudi *et al.*, 2011). This microorganism may expedite MIC in pipeline systems primarily due to their predominantly anaerobic lifestyle and continuous production of corrosive hydrogen sulphide (Javaherdashti, 2009; Al-Abbas *et al.*, 2013). Generally, SRB has been distinguished by its ability to conduct dissimilatory sulphate reduction, using sulphate, as the terminal electron acceptor and reducing it to sulphide, for the energy source (Iverson, 1987; Mudryk *et al.*, 2000; Pires *et al.*, 2003). Each bacterial species has a specific tolerance range for specific environmental factors. Factors that might influence the bacterial growth rate are classified into two classes, which are physical and nutritional factors (chemical factors). Metabolism of SRB can be influenced by these factors because microorganisms are sensitive to changes in the environment.

Therefore, an accurate estimation of growth rate is essential to further estimation of the corrosion allowances for structural designs, planning for inspections and scheduling maintenance (Wang *et al.*, 2003). This work explored the performance of two SRB strains with the same genus *Desulfovibrio vulgaris*, namely pure strain and local isolated SRB. Their performances were evaluated by including four most vital environmental parameters.

MATERIALS AND METHODS

Sulfate-reducing bacteria: This study emphasizes two different sources of SRB, yet same genus of *Desulfovibrio vulgaris*, formerly known as *Desulfovibrio desulfuricans*. The first bacterial strain was sourced from the American Type Culture Collection (ATCC), known as ATCC 7757, while the second strain was isolated SRB from soil sample near the natural gas transmission line in Peninsular Malaysia, named as Sungai Ular. According to the pigging record gathered by pipeline operator, severe external corrosion was recorded around this area. The process of isolation was done by the certified laboratory, to get the single colony of *Desulfovibrio vulgaris*. Both strains were cultured in different media, namely modified Baar's and Postgate C medium. Both strains cultured were then tested, using Sani SRB test kit from Sani-Check product number 100 (Sani Check, Biosan Lab Inc., USA), as shown in Fig. 1.

Experiment method: The experiments were performed in a 125 mL sealed anaerobic vial, which need to be purged with filtered oxygen-free nitrogen for about 2 min for each vial and sealed by rubber and aluminium cap. All the vials were sterilized by autoclaving at 121°C for about 30 min to avoid any contamination. Modified Baar's and Postgate C medium were prepared according to standard. Table 1 shows the chemical composition for modified Baar's medium, which was recommended by ATCC, as the culturing media for ATCC 7757. All the ingredients except for No. 8 were mixed together with 1 L of distilled water and stirred for 30 min. The pH was adjusted to 7.5 before autoclaving at 121°C for 15 min. The medium was then purged with nitrogen gas for approximately, 1 h to remove oxygen prior to addition of $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$, which was not to conduct autoclave due to heat sensitivity.

Postgate C medium was prepared according to the chemical composition as shown in Table 2. The prepared medium was adjusted to pH 7.5 and sterilized in autoclave for about 30 min.



Fig. 1: Test on detection of SRB by sani test kit

Table 1: Chemical composition for modified Baar's medium

Ingredients	Concentration
Magnesium sulfate (g L^{-1})	4.096
Sodium citrate (g L^{-1})	5.700
Calcium sulfate (g L^{-1})	1.000
Ammonium chloride (g L^{-1})	1.000
Dipotassium phosphate (g L^{-1})	0.500
Sodium lactate (mL)	4.500
Yeast extract	1.000
Iron (II) sulfate (g-50 mL)	6.720

Table 2: Composition of postgate C medium (Postgate, 1984)

Ingredients	Concentration (g L^{-1})
Sodium lactate	6.000
Sodium sulfate	4.500
Ammonium chloride	1.000
Yeast extract	1.000
Monopotassium phosphate	0.500
Sodium citrate	0.300
Calcium chloride	0.060
Magnesium sulfate	0.060
Iron (II) sulfate	0.004

The medium was purged with nitrogen free oxygen gas for a period of 30-60 min in order to create anaerobic medium before being transferred to the vials. After which, 2% of pure strain was injected into the medium as contained in the vials. The remaining vials were injected with the isolated SRB. Then, all the vials were kept inside the incubator at 37°C and the turbidity measurement was taken each day for a week.

Turbidity method: Turbidity is one of the standard methods to detect the presence of microorganisms in liquid. Spectrophotometer (DR4000) was used to measure the Optical Density (OD) of the assigned broth culture at 600 nm wavelength in this study. A sample's ability to absorb light is referred, as Optical Density (OD). It can quantify the amount of light from a known internal source that is being transmitted through a sample of detector. Reading of 100% transmittance or zero absorbance of light can be noticed if no sample is present. Whereas, if sample is inserted, some

source of light is absorbed, as it passes through the cell providing reduction in transmittance. For this experiment, dilution was prepared in the ratio of 1 mL sample: 9 mL distilled water to observe the bacterial growth subject to change of environmental parameters.

Environmental factors: Four parameters were selected in this study in order to compare the performance of both SRB strains in terms of growth pattern, which are pH, temperature, salinity and iron content. According to standard, pH for both mediums was adjusted to 7.5, which is considered as neutral. However, this study set a range of pH between acidic and alkaline solution which is pH 5.5, 6.5, 7.5, 8.5 and 9.5. The purpose of this range is to investigate the performance of each strain upon variation of pH set for medium. Average incubation temperature for culturing bacteria is 37°C. Theoretically, temperature inside buried pipeline is 70°C and lower. Therefore, the temperature set for the experiment varied between 5, 20, 37 and 60°C according to limited equipment. Third parameter is salinity where chemical composition of magnesium sulphate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) for every medium is diversified from 0 to 100% for the mixture of the medium. Similar to salinity, iron content for every medium is varied through addition of $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ composition. Range between 0 and 100% of the chemical composition is prescribed in order to investigate the influence of variation of parameter iron upon bacterial growth.

RESULTS AND DISCUSSION

In order to investigate whether there are significantly different intensities between SRB strains, environmental parameters and time that influence turbidity measurement, both graphical and statistical analysis were conducted. Factorial analysis was carried out at a confidence interval of 95%, using SPSS20. Seven null hypotheses are assumed at first. Table 3-6 show the significant value of ANOVA-test result for each environmental parameter tested, namely pH, temperature, salinity and iron concentration. If the significance value is greater than 0.05 and the calculated F is low, null hypothesis will be accepted.

pH level: Figure 2a-b illustrate the turbidity measurement under influence of parameter pH over time according to incubation for a one month period. Through direct observation, pH 9.5 contributed to the highest turbidity for ATCC 7757 followed by pH 8.5, 6.5 and 7.5. Growth of SRB

Table 3: ANOVA-test for different pH level

Sources	Type III sum of squares	df	Mean square	F	Sig.
Bacteria	1.056	1	1.056	454.733	0.000
pH	2.573	4	0.643	277.126	0.000
Time	0.205	3	0.068	29.456	0.000
Bacteria×pH	1.084	4	0.271	116.775	0.000
Bacteria×time	0.016	3	0.005	2.246	0.084
pH×time	0.070	12	0.006	2.500	0.004
Bacteria×pH×time	0.140	12	0.012	5.017	0.000

a. R squared = 0.917 (Adjusted R squared = 0.901)

Table 4: ANOVA-test for different temperature level

Source	Type III sum of squares	df	Mean square	F	Sig.
Bacteria	0.627	1	0.627	199.660	0.000
Temperature	0.585	3	0.195	62.109	0.000
Time	0.042	3	0.014	4.480	0.005
Bacteria×temperature	0.655	3	0.218	69.443	0.000
Bacteria×time	0.104	3	0.035	11.004	0.000
Temperature×time	0.233	9	0.026	8.239	0.000
Bacteria×temperature×time	0.154	9	0.017	5.461	0.000

a. R Squared = 0.827 (Adjusted R Squared = 0.793)

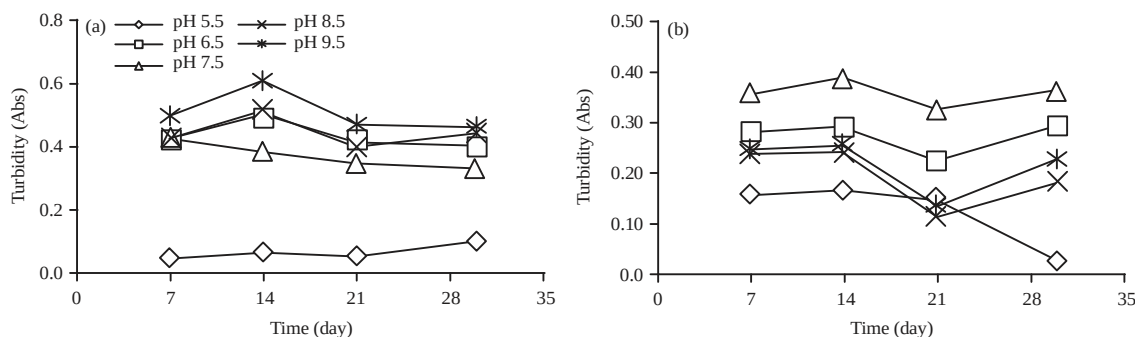


Fig. 2(a-b): Turbidity over time under influence of pH for (a) ATCC 7757 and (b) SRB Sungai Ular

Table 5: ANOVA-test for different salinity concentration

Source	Type III sum of squares	df	Mean square	F	Sig.
Bacteria	0.147	1	0.147	47.038	0.000
Salinity	0.254	4	0.064	20.294	0.000
Time	0.111	3	0.037	11.754	0.000
Bacteria×salinity	0.045	2	0.023	7.185	0.001
Bacteria×time	0.048	3	0.016	5.068	0.002
Salinity×time	0.313	12	0.026	8.330	0.000
Bacteria×salinity×time	0.100	6	0.017	5.299	0.000

a. R Squared = 0.680 (Adjusted R Squared = 0.618)

Table 6: ANOVA-test for different iron concentration

Source	Type III sum of squares	df	Mean square	F	Sig.
Bacteria	0.106	1	0.106	44.858	0.000
Iron	1.360	4	0.340	143.222	0.000
Time	0.097	3	0.032	13.669	0.000
Bacteria×iron	0.117	2	0.059	24.715	0.000
Bacteria×time	0.044	3	0.015	6.176	0.001
Iron×time	0.040	12	0.003	1.411	0.166
Bacteria×iron×time	0.017	6	0.003	1.209	0.304

a. R Squared = 0.824 (Adjusted R Squared = 0.790)

shows slow progress at pH 5.5 with explicit similarity in terms of pattern. However, the highest progress for SRB Sungai Ular was recorded at pH 7.5, followed by pH 6.5, 9.5 and 8.5. Similar to SRB ATCC, pH 5.5 also shows slow progress towards the growth pattern. In terms of preferable pH, both ATCC and Sungai Ular grow higher in alkaline solution compared to acidic. However, the growth of SRB Sungai Ular is the highest at neutral-alkaline solution, while ATCC reach the highest growth in alkaline solution.

Table 3 presented the ANOVA-test for the first parameter pH, where all the significant values except for H5 are less than 0.05. This generally indicates that null hypothesis for H1, H2, H3, H4, H6 and H7 are rejected and there is significant different between them, as stated previously. The analysis shows that different SRB strain exposed to certain range of time are not significantly, different in terms of growth of SRB. However, it can be concluded that there is significant different for each SRB strain upon parameter pH towards SRB growth pattern as null hypothesis for H4 is rejected.

Previous study highlighted the influence of physical parameters of pH towards cell adhesion of the bacteria (Sheng *et al.*, 2007). The SRB is reported active over wide range of pH from acidic to alkaline. Suitable pH is crucial for bacteria growth as it affects ionization and therefore the

interaction of a myriad of molecular processes. In addition, pH range plays a main role in the solubility of various substances that bacteria need. Thus, both SRB strains show higher growth in medium with pH 6.5 and above which is considered a neutral-alkaline environment. These bacteria prefer alkaline environment and increase the alkalinity by reducing sulfate ions to consume the organic acids, which is hydrogen sulphide, H₂S (Braissant *et al.*, 2007; Ismail *et al.*, 2014).

Temperature: Figure 3a-b illustrate the turbidity measurement under influence of parameter of temperature over time of exposure. Graph of ATCC 7757 clearly shows that growth pattern of the bacteria for all temperature variations are similar from the first week of incubation till the end. Highest increase in growth rate is recorded at the first week, constantly increasing at a smaller rate until day 21 before it drops a bit and then continues to increase until the final day of incubation. However, the growth rate is in constant pattern where the rise and drop is around 0.1 Abs. Through, direct observation of SRB Sungai Ular, temperature seems influential towards its growth. Temperature 5 and 60°C recorded a similar pattern with constant growth less than 0.2 Abs, temperature 37°C gives a similar pattern but a sudden drop is recorded on day-21 before increasing again till the final day of incubation. On the other hand, temperature 20°C increased substantially, during the first 2-weeks and reached a peak on day-21, dropping 0.07 Abs. It can be concluded that both SRB strains react differently towards temperature, whereby ATCC 7757 grew at an almost similar rate for all temperature compared to local isolated SRB, which grew highest at 20°C.

Table 4 presented the ANOVA-test for the second environmental parameter, which is temperature. The analysis shows that all the significant values for H1, H2, H3, H4, H5, H6 and H7 are less than 0.05. This generally indicates that the entire null hypothesis are rejected and there are significantly different between each of them. The result also indicates that SRB growth seems to be very sensitive to different SRB strain, change of temperature and time of exposure since the significant value for all tested pairs were found far less than 0.05. Thus, the difference for each SRB strain upon parameter pH towards SRB growth pattern is very significant.

Analogous to the wide pH range at which SRB activity can be detected, SRB are also active over a wide range of temperature. With regard to their optimal growth temperatures, SRB can be classified into five categories, which are psychrophiles, psychrotrophs, mesophile, thermophile and hyperthermophile. Therefore, results on pure strain ATCC 7757 proved that it belongs to the category of mesophiles and moderate thermophiles. However, local isolated SRB prefer to grow at

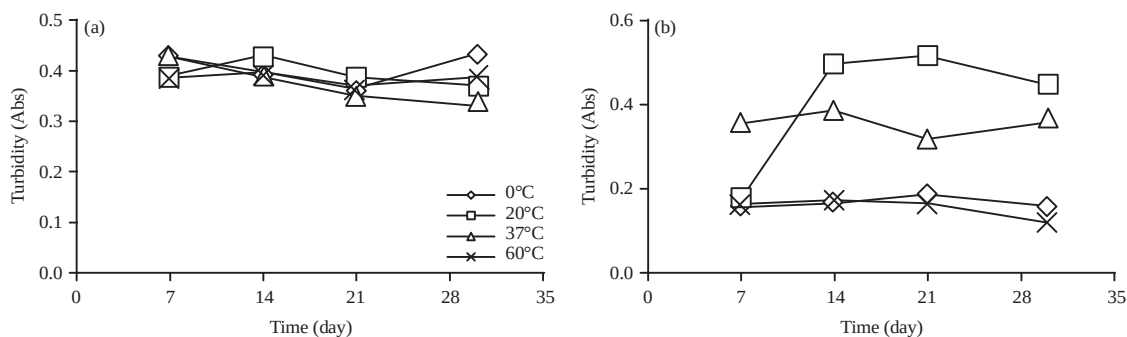


Fig. 3(a-b): Turbidity over time under influence of temperature for (a) ATCC 7757 and (b) SRB Sungai Ular

the maximum rate at temperature of between 20 and 37°C, which is considered to belong in the mesophiles category. Therefore, there will be an over-estimate if the developed model for pure SRB is used to determine the growth for local SRB.

Salinity concentration: Salinity for both SRB strains varies depending on the composition of Magnesium Sulphate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) in the medium used. Modified Baar's contains 4.096 g and Postgate C contains 0.06 g for every 1 L solution. Therefore, the variation of salinity differed between both mediums used; every 25% for modified Baar's and 50% for Postgate C. Figure 4a-b indicate turbidity measurement under environmental parameter salinity for each SRB strain. Growth of pure strain ATCC 7757 clearly show a similar pattern recorded for every salinity fixed for this test. The difference is huge in the first week with 100% salinity leading at 0.429 Abs compared to the others and there is a small difference between turbidity for the following weeks. In contrast, growth of SRB Sungai Ular shows significant difference for salinity 0, 50 and 100%. The 100% salinity recorded the highest turbidity throughout the incubation for a one month period. Therefore, according to bacterial growth, both strains react differently towards variation of salinity but local isolated SRB shows obvious pattern.

The ANOVA-test on the sample for the third environmental parameter, which salinity is presented in Table 5. A similar finding was observed for parameters salinity and temperature with most consistent pattern, whereby none of the variables yielded significant values of more than 0.05 and all the null hypothesis of H1, H2, H3, H4, H5, H6 and H7 are all rejected. The rejection of null hypothesis means that variation of SRB strain, temperature and time of exposure contribute to the SRB growth.

In the oil and gas industry, high salinity water is routinely injected into formation for pressure maintenance by water flooding (Zapata-Penasco *et al.*, 2013). Therefore, the salinity in the pipeline may vary at least during maintenance procedures. Contradictory results have been obtained from pure strain ATCC 7757 and local SRB Sungai Ular, where variation of salinity level does not influence ATCC 7757, as much as local SRB. However, the difference is significant enough for both SRB as the calculated p-values are less than 0.05. In addition, strong unpleasant smell of rotten-egg found during sampling process, which may be due to H_2S release. In addition, black precipitations of sulphides are the simplest way in determining the SRB growth (Yuzwa *et al.*, 1991; Beech *et al.*, 2000).

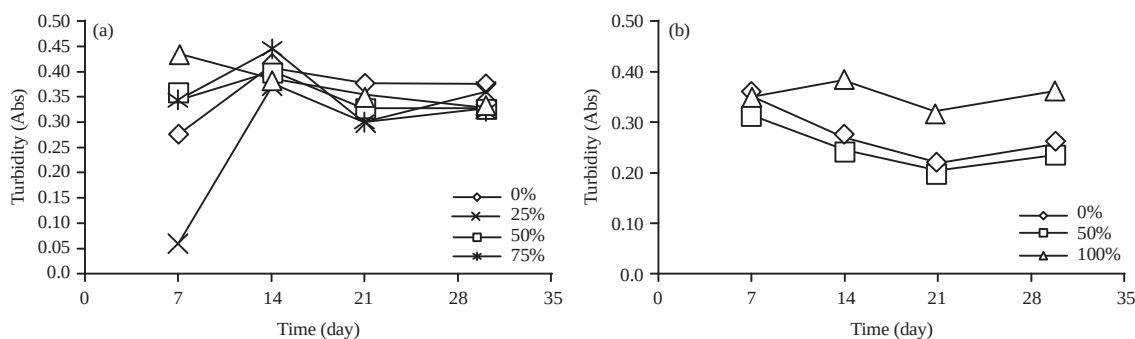


Fig. 4(a-b): Turbidity over time under influence of salinity for (a) SRB ATCC 7757 and (b) SRB Sungai Ular

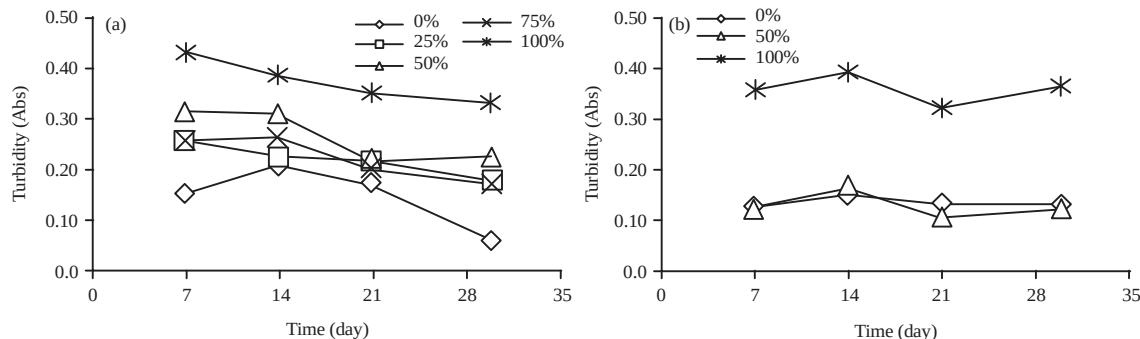


Fig. 5(a-b): Turbidity over time under influence of iron content for (a) ATCC 7757 and (b) SRB Sungai Ular

Iron (Fe) content: In accordance to salinity, component of iron for both mediums are also different, whereby composition of $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ in the modified Baar's is 6.72 g for every 50 mL and Postgate C contains 0.004 g for every 1 L solution. Therefore, the variation of salinity must differ between both mediums used, every 25% for modified Baar's and 50% for Postgate C because of different amount of iron composition in both medium. Figure 5a-b indicate turbidity measurement over time of exposure under environmental parameter iron concentration for each SRB strain. In day-14, increment for both strains were recorded, as the iron content getting higher. Obvious growth pattern is recorded for variation of iron content for both SRB strains. This concludes that the most favourable environment for both strains were at 100% iron content but with different turbidity reading.

While, the others parameter are proven to be influential in terms of SRB growth, iron content, on the other hand, exhibits dissimilar behavior for certain hypothesis. Null hypothesis H6 and H7 are accepted, where SRB strain for different time of exposure does not affect the SRB growth and also the difference of SRB strain on each parameter tested at different time exposure does not affect the SRB growth (Table 6). However, the objective of the study is achieved, where there is significant difference of the SRB strain upon each parameter towards SRB growth pattern.

Iron is one of the crucial elements that undergo extensive redox cycling in environment, as well as play major role in essential biochemical processes (Li *et al.*, 2006). The factorial analysis of experimental data agreed with the statement that there is significant difference in the variation of the parameter of iron upon bacterial growth for both SRB strains. However, growth data of SRB Sungai Ular obtained for iron concentration of 0 and 50% is much lower, as the composition of $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ in Postgate C medium is relatively small compared to modified Baar's medium. This supports the previous studies that iron is one of the major elements that undertakes extensive redox cycling in environment and plays a critical role in biochemical process (Li *et al.*, 2006).

CONCLUSION

Based on the laboratory works results and statistical analysis, it is reasonably well-defined that growth of SRB greatly influenced by environmental changes. The studies of OFAT approach upon variation of environmental parameters reveal that all parameters have strong influence on bacterial growth for SRB strain individually. The finding is significantly proven by factorial analysis taking the study of mechanism of local SRB strain into account. Based on the growth rate showed by both strain, it is possible for SRB to record high rate of corrosion towards the structure

material. Hence, cause destructive impact to the system. Therefore, further study upon the these bacterial strain is crucially needed to investigate their effect upon pipeline material.

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