TECHNICAL NOTE

CULTIVATION OF SULPHATE REDUCING BACTERIA IN DIFFERENT MEDIA

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Abstract: Sulphate-reducing bacteria (SRB) represent themselves as a class of anaerobic bacteria that can reduce sulphate to sulphide for obtaining energy. This paper is aimed to detect sulphate-reducing bacteria activities using rapid detectable culture media. Two different strains of sulphate-reducing bacteria were used in this study, namely ATCC 7757 and local bacterial strain of SRB isolated from underground sample. Both strains were tested on three recommended culture media of modified Baar's, Postgate B and Postgate C. All three medium contained lactic acid which served to be as carbon source. The results showed that modified Baar's medium is the best medium for the growth of ATCC 7757 while Postgate C medium is recommended for the local SRB bacterial strain.

Keywords: *Sulphate-reducing bacteria, culture media, pure culture, corrosion, lactic acid*

1.0 Introduction

Microbiologically Influenced Corrosion (MIC) in one of the main concern in maintaining and managing the pipeline present in petroleum sector. The term MIC refers to corrosion due to the activities of live microorganisms (Zhang *et al.*, 2011; Yahaya *et al*., 2011). Previous study agrees that sulphate-reducing bacteria are the most dominant bacteria that lead to MIC (Okabe *et al.*, 1994; Fathul *et al.*, 2008; Zhang *et al.*, 2011). Sulphate-reducing bacteria are anaerobic and non-pathogenic bacteria, however powerful enough to cause problem both economically and ecologically according to Ghazy *et al.* (2011).

Sulphate-reducing bacteria reduce sulphur into sulphate, thiosulphate and sulphide before using it as final electron acceptor. Oxidation of organic compounds will provide energy for the growth of bacteria (Rzeczycka *et al.*, 2005):

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

To determine the maximum growth of sulphate-reducing bacteria, there are few requirements to be fulfilled. At first, the carbon source must be suitable for growth. Lactic acid is one of the examples of low molecular weight carbon source that is preferred by sulphate-reducing bacteria (Rzeczycka *et al.*, 2005). Secondly, as per the previous study, isolation of sulphate-reducing bacteria needs selective growth media (Ghazy *et al.*, 2011; Loubinoux *et al.*, 2003). Postgate (1984) stated that media with "biologically free" of iron will not be preferred for the growth of sulphate-reducing bacteria.

Thus, the present study is aimed to detect the growth of sulphate-reducing bacteria by using the method of impure (turbid) media.

2.0 Materials and Methods

2.1 Microorganisms

Same SRB genus of *Desulfovibrio* was used in this study. A stabilized mixed culture obtained from the culture collection (ATCC 7757) was used as the first SRB strain. The second SRB strain were collected and isolated from the underground site located in Pahang, Malaysia. Initial observation on the site discovered that this area is a potential area for pipeline installation because it was not far from the existing line and close to Kerteh, Terengganu. Kerteh known as the main oil gas hubs for Peninsular Malaysia. Soil condition for this site was reported as swampy, clay and fine sand, where the site is near to the river and mangrove forest. As field work was carried out to collect the sample, there was smell of rotten-egg represent the presence of SRB in the soil. The samples were then isolated in order to get a single colony of genus *Desulfovibrio* before culturing process.

2.2 Culture Medium

Three of the commonly used mediums were evaluated for the SRB growth, which are modified Baar's, Postgate medium B and Postgate medium C. These medium consisted of different chemical composition but with similar carbon source, which is lactic acid and highly recommended for the cultivation of SRB.

2.2.1 Modified Baar's medium

This type of medium is commonly used for the growth of SRB and the preparation of media was carried out according to the reports from previous studies (Akrima *et al.*, 2014; Mardhiah *et al.*, 2014). Table 1 shows the composition of the medium.

Table 1: Composition of modified Baar's medium.

No.	*Ingredients*	*Concentration(g/L)*
1	$MgSO_4$. $7H_2O$	4.096
2	$C_6H_5Na_3O_7.2H_2O$	5.7
3	$CaSO_4$	1.0
4	NH_4Cl	1.0
5	K_2HPO_4	0.5
6	Sodium lactate	4.5 ml
7	Yeast extract	1.0
8	$Fe(NH_4)_2(SO_4)_2$	6.72 g – 50 ml 5ml for 1000 ml medium

All the ingredients except for item No. 8 were mixed together with 1000 ml of distilled water and stirred for 30 minutes. The pH were adjusted to 7.5 before autoclaved at 121^0C for 15 minutes. The medium was then sparged with nitrogen gas for approximately one hour to remove oxygen from it prior to addition of $Fe(NH_4)_2(SO_4)_2$ which was not autoclaved due to heat sensitivity.

2.2.2 Postgate B Medium

As compared to other media, Postgate B medium is defined as "biologically free of iron". A steel coupon was put into each vial at the beginning of experiment to assist bacteria growth. Table 2 lists the composition of Postgate B medium.

Table 2: Composition of Postgate B medium. (Reis *et al.*, 1992; Sheng *et al.*,2007; Ghazy *et al.*, 2011)

No.	*Ingredients*	*Concentration(g/L)*
1	K_2HPO_4	1.0
2	NH_4Cl	2.0
3	$CaSO_4.2H_2O$	1.3
4	$MgSO_4 \cdot 7H_2O$	4.0
5	Lactic acid (88%)	2.7

The chemicals were mixed in 1 L of distilled water and stirred well before autoclaved as to ensure that all of the chemicals substances are mixed well. Then, pH 7.5 was recorded before autoclaved with the pressure of 1.2×10^4Mpa.

2.2.3 Postgate C Medium

Postgate C medium was prepared according to the chemical composition as shown in Table 3. The prepared medium was adjusted to pH 7.5 and sterilized in autoclave for about 30 minutes.

Table 3: Composition of Postgate C medium. (Postgate, 1984)

No.	*Ingredients*	*Concentration(g/L)*
1	Sodium Lactate	6.0
2	Na_2SO_4	4.5
3	NH_4Cl	1.0
4	Yeast Extract	1.0
5	KH_2PO_4	0.5
6	$C_6H_5Na_3O_7.2H_2O$	0.3
7	$CaCl_2.6H_2O$	0.06
8	$MgSO_4 \cdot 7H_2O$	0.06
9	$FeSO_4.7H_2O$	0.004

2.3 Experimental Procedure

The experiment was carried out under anaerobic condition. A number of 54 anaerobic vials were prepared containing steel coupon in each of the vial and spurged with nitrogen free oxygen gas for 2-minutes, before clamped with rubber and aluminium cap. The vials were then autoclaved in order to ensure sterile condition and to eliminate the possibility of contamination.

The medium was spurged with nitrogen free oxygen gas for a period of 30 to 60 minutes in order to create anaerobic medium before it is being transferred to the vials. After which, 2% of SRB strain (ATCC 7757) was injected into the medium as contained in 27 vials. The remaining vials were injected with the isolated SRB. Then, the vials were kept inside the incubator at 37°C and the turbidity measurement was taken on a daily basis for the next seven days.

(a) (b)

Figure 1: Sulphate-Reducing bacteria in Modified Baar's, Postgate B and Postgate C. a) ATCC 7757. b) SRB isolated from site.

2.4 Turbidity Method

Turbidity is one of the methods to detect the presence of microorganisms in liquid which includes sulphate-reducing bacteria. Spectrophotometer was used to determine the optical density (O.D.) of the assigned broth culture at 600 nm wavelength in this study. A sample's ability to absorb light was referred to be as optical density (O.D.). 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 was inserted, some source of light was 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.

Figure 2: Spectrophotometer DR4000 used for the study.

3.0 Results and Discussion

Theoretically, broth will become turbid due to the presence and growth of SRB in liquid culture (Maria and Csaba, 1999). The more turbid is the medium, the higher number of SRB present in the medium. Postgate (1984) stated that the characteristic of unpleasant smells from hydrogen sulphide and black coloured solution are mainly due to the evidence of SRB growth and its metabolism in the medium. Therefore, the turbidity measurement gives enough quantitative information which is needed in predicting the growth of SRB.

Table 4 shows the average turbidity reading for both sulphate-reducing bacterial strains present in three different media (modified Baar's, Postgate B and Postgate C). The data shows that the growth rate in modified Baar's medium for ATCC 7757 is found to be higher then the other medium. However, the isolated SRB exhibits better growth in Postgate C according to the turbidity of the medium which is slightly higher than the modified Baar's medium.

Table 4: Turbidity results of medium containing SRB.

Type of Medium	*Baar's Medium*		*Postgate B*		*Postgate C*	
Bacterial Strain	*ATCC culture*	*Isolated culture*	*ATCC culture*	*Isolated culture*	*ATCC culture*	*Isolated culture*
Day 1	0.065	0.400	0.061	0.091	0.044	0.139
Day 2	0.205	0.455	0.037	0.095	0.131	0.271
Day 3	0.226	0.495	0.069	0.138	0.161	0.482
Day 4	0.330	0.487	0.083	0.130	0.155	0.476
Day 5	0.336	0.437	0.088	0.099	0.167	0.447
Day 6	0.317	0.423	0.083	0.084	0.152	0.460
Day 7	0.252	0.298	0.072	0.108	0.119	0.453

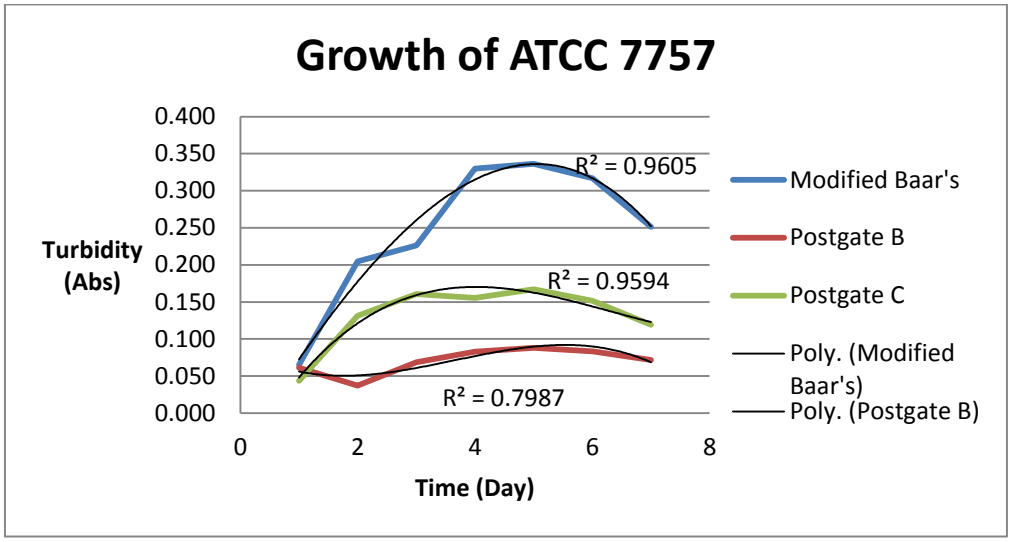

Figure 3: Growth of sulphate-reducing bacteria (ATCC 7757) at different time intervals.

Figure 3 presents the growth of ATCC 7757 over different time interval and the temperature set for growth was 37°C throughout the experiment. In order to get reliable growth pattern of SRB, turbidity was on a daily basis for a period of 7 days. The black colour of the medium and rotten egg smell is the indicator of the SRB growth within 2 to 3 days after the injection of SRB seed into the medium. The growth pattern of ATCC 7757 was confirmed by the increment number of turbidity over time for all three mediums. The theme identified in the graph showed that maximum turbidity number was observed at day-5, before it gradually decreased until the final day for all respected mediums. On the final day of the experiment, the turbidity in modified Baar's medium was recorded the highest at 0.252 Abs as compared to 0.072 Abs in Postgate B and 0.119 Abs in Postgate C medium.

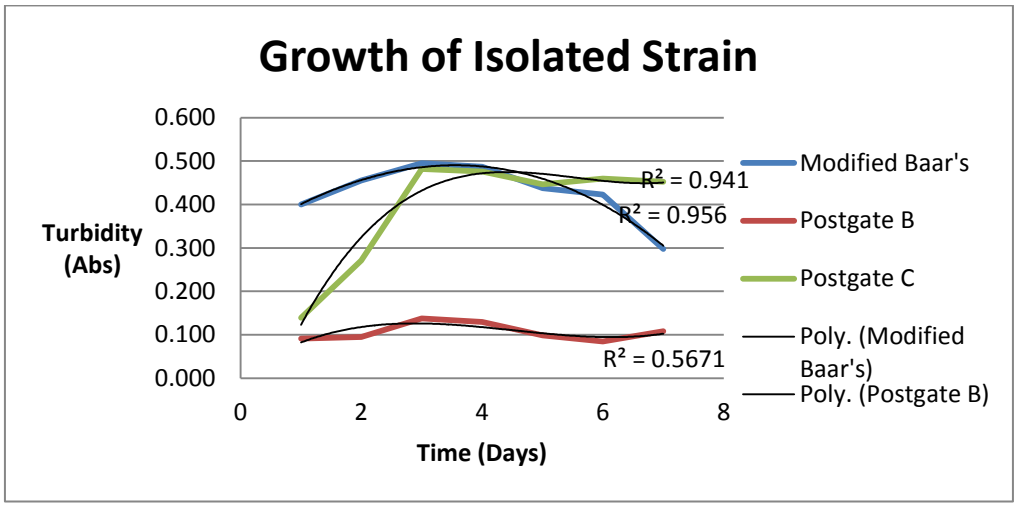

Figure 4: Growth pattern of isolated SRB Strain (isolated from local site) versus time.

Figure 4 provides the experimental data on the growth of SRB isolated strain from local site according to the different time intervals. From the graph pattern, it shows that during the first 3-days of cultivation period, the turbidity reading clearly illustrates higher growth rate for the isolated SRB present in modified Baar's medium, as compared to the Postgate medium. However on day-5, the growth of SRB steadily increased in Postgate C medium, whilst decreases in modified Baar's. The result also displays that from day-1, the growth of SRB in Postgate C slightly increases. Turbidity data at day-7 for Postgate C medium was the highest recorded at 0.453 Abs as compared to 0.298 Abs in modified Baar's and 0.108 Abs in Postgate B medium.

Although the same carbon source was used in all respective three mediums, yet different strain of SRB has their own preferable medium to their growth. These results interpreted that each chemical composition in the medium showed different effect on the growth of bacteria. Moreover, the presence of iron (Ferrum) in the medium is an essential component which is responsible for assisting the microbial activity of SRB. Dennis and Julia (2013) stated that iron sulphides (FeS) are one of the main characteristic products related to MIC.

4.0 Conclusions

This research extends our knowledge on the effect of different medium composition on the SRB growth pattern. The finding proposes that all three mediums could be used for the growth of SRB because of the similar type of carbon source in the composition. However, it is recommended to culture the SRB ATCC 7757 and the isolated SRB from site in modified Baar's and Postgate C medium respectively. The term of 'biologically

free Ferrum' cannot be applied for SRB because Ferrum (iron) is the most important component required for the growth of SRB. As a recommendation, the research may be extended for a longer period to investigate the growth pattern in long term condition. Moreover, local SRB strain can be isolated from crude oil sample so as to compare the dynamic growth of different SRB strain from onshore and offshore sites. Deep knowledge on MIC topic can guide industry for a better Structural Integrity Management (SIM) applied throughout the life of their assets to assure that the assets are fit for purpose and to maintain structural integrity throughout the designed life cycle and maybe longer (Narayanan *et al*., 2012; Narayanan and Sohaimi, 2013)

The research has successfully detected the presence of SRB from soil sample of local site indicating potential underground corrosion that may be experienced by steel pipelines. Any future study on this local strain of SRB and its effect on underground corrosion can utilise Postgate C for optimum growth.

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