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## Effects of nutrient composition on the formation of biofilm and biocorrosion in MEOR biostimulation medium based on response surface methodology

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# Effects of nutrient composition on the formation of biofilm and biocorrosion in MEOR biostimulation medium based on response surface methodology

I A Purwasena<sup>1\*</sup>, D I Astuti<sup>1</sup>, T Intan<sup>1</sup>, D Lani<sup>1</sup> and W N Rodiana<sup>1</sup>

<sup>1</sup>School of Life Sciences and Technology, Institut Teknologi Bandung (ITB), Jalan Ganesha 10 Bandung, West Java, 43253, Indonesia

\*E-mail: isty@sith.itb.ac.id

**Abstract.** Nutrition addition in Microbial Enhanced Oil Recovery (MEOR) application is one of the methods used to increase oil production. Nutrition injection in MEOR application must be done carefully because the lack of proper nutrition addition can induce biofilm and biocorrosion formation. The composition of biostimulation medium for optimizing beneficial indigenous bacterial growth in Microbial Enhanced Oil Recovery (MEOR) was evaluated using Response Surface Methodology (RSM) with Central Composite Design (CCD). Three main factors for the medium composition were molasses, Diammonium Phosphate (DAP), and NPK. The RSM was used to know the most effective medium composition towards biofilm production and strength that high potential leads to biocorrosion. Molasses, NPK, and diammonium phosphate (DAP) are utilized as carbon, nitrogen, and phosphate supplementary substrate in Brine water as basal medium. Molasses concentration was 0%-10% while DAP and NPK was 0%-0.5%. Both aerobic and anaerobe sessile bacteria, as well as acid producing bacteria, were enumerated by total plate and turbidity methods. Statistical analysis with  $\alpha=0.05$  proves molasses give significance effect to biofilm strength and sessile anaerobic bacteria. DAP gives significance effect to biofilm strength and sessile aerobic and anaerobic bacteria. NPK give significance effect to sessile anaerobic bacteria. Molasses addition decrease brine pH by microbial activity, make the carbon steel coupon ST-37 corrosion rate increased. Meanwhile, the addition of DAP increased the pH of brine medium and decrease the corrosion rate.

## 1. Introduction

Indonesia's energy demand is increasing along with economic and huge population growth. On the other hand, oil production is decreasing from 287.1 million barrels in 2006 to 251.87 million barrels in 2015 [1]. Microbial Enhanced Oil Recovery (MEOR) is one of the tertiary methods that increase oil production using microorganism activity that benefits the oil uptake process from the reservoir. Biostimulation is one of the MEOR approaches by adding suitable nutrient into the reservoir to increase indigenous microbial growth as well as increase the production of its metabolites [2, 3].

Unfortunately, the injection of those nutrients should be considered carefully because it could lead to microbial accumulation along the metal-carbon steel pipeline and also equipment that induces Biofouling and biocorrosion deterioration process. The microbial cells tend to form a biofilm which has caused many problems such as pipe clogging, biofouling, oil souring and Microbiologically Influenced Corrosion (MIC) [4]. The MIC causing crucial problems in industries that estimated 40% of internal pipeline corrosion in petroleum industry [5].



The improper concentration of nutrients addition in MEOR Biostimulation could affect excessive microbial growth and microbial accumulation in the system that leads to biocorrosion. Microbial growth depends on the supply of carbon (C), nitrogen (N), phosphor (P), and minor trace elements. The previous study showed that molasses, NPK, and diammonium phosphate were suitable to be applied as a medium for MEOR biostimulation. This study aimed to analyze the effect of several main nutrients concentration formula developed by response surface methodology experimental design as a tool of biostimulation approach towards biofilm formation and its biocorrosion potential on a metal surface.

## 2. Materials and Methods

### 2.1. Microorganisms

An indigenous microbial consortium in this study was obtained from Brine water of oil reservoir, located in South Sumatera, Indonesia. The physical and chemical characteristics of the brine sample are shown in Table 1.

**Table 1.** Chemical characteristics of brine sample

Parameter Analysis	Quantity
Salinity (‰)	18.34
Conductivity (μS/cm)	29,900
Hardness CaCO <sub>3</sub> (mg/L)	140
Calcium (mg/L)	39.7
Bicarbonate (mg/L)	31.14
Carbonate (mg/L)	274
Nitrate (mg/L)	0.010
Nitrite (mg/L)	0.022
Ammonium (mg/L)	24
Sulphate (mg/L)	20.8
Orthophosphate (mg/L)	0.048
Total Phosphate (mg/L)	0.088
Sulfide (mg/L)	0.019
Total Fe (mg/L)	1.33
Fe <sup>2+</sup> (mg/L)	1.29
Fe <sup>3+</sup> (mg/L)	0.041
Organic Compound (mg/L)	572
Cl <sup>-</sup> (mg/L)	8,960

### 2.2. Carbon steel coupon preparation

The Carbon-steel coupon used in this study was ST-37 type with dimension (10x10x±1.8) mm<sup>3</sup> treated with gradient scoured of 200, 400, 600, 800 grade sandpapers [6]. The coupons decreased with 98% ethanol and soaked in acetone. Following with dried for 2 hours in 70°C oven and autoclaved at 121°C for 15 minutes before used. The coupon composition confirmed by Scanning Electron Microscopy-Energy Dispersive Spectroscopy (SEM-EDS).

### 2.3. Experimental design to evaluate the effect of nutriron composition

The concentration of Carbon, Nitrogen, and Phosphate combination was assessed for brine medium formulation with surface response methodology. The 20 variations (Table 2) of design experiment was obtained from surface response methodology (two order) using a central composite design of Minitab 17 Statistical Software™. The nutrients concentration used are 2-8% (w/v) of molasses as carbon source and 0.1%-0.4% (w/v) diammonium phosphate (DAP) and NPK use as phosphate (P) and nitrogen (N) source [7-9]. All of these three sources of nutrients were diluted in brine water.

#### 2.4. Analysis of biofilm strength

The 20 variations of Brine water and nutrients formula incubated in sterile 24 well microtiter plate with 3 mL final volume of each well. Brine water without additional nutrients used as a negative control. After incubated at 70°C for 14 days sessile microbial cell was counted on a different specific medium. After 14 days of incubation time, a planktonic cell was removed and the remaining cell on the surface of each microtiter plate was washed with Phosphate Buffer Saline (PBS) pH 7.2 for 3 times. The remaining sessile microbial layer on the bottom of each well was applied for heat fixation at 70°C for 45 minutes. Then, remained microbial layer stained with 1mL 0.5% crystal violet for 5 minutes. When the crystal violet was removed thoroughly, the surface was washed with PBS for three times. The remaining stained-sessile cell was diluted with 1mL ethanol 70%. The amount of 200µL of those homogenized stain solutions was measured using 96 well microplate reader by BIO-RAD ELISA Reader (595nm) in triplicates for each sample [10, 11]. The absorbance was categorized using interpretation from Stepanović *et al.* [12].

#### 2.5. Microbial enumeration

Planktonic and sessile microbial enumeration was tested. Planktonic microbial enumeration was counted using absorbance on a spectrophotometer. While sessile microbial enumeration was quantified using the total plate count method on several growth media. Nutrient Agar (NA) for aerobic microbes, Postgate B for Sulfate-reducing and anaerobic bacteria. All of the bacterial growth medium was incubated at 70°C with initiation time of 1-2 days for aerobic bacteria and 7-14 days for anaerobic bacteria. While all anaerobic bacteria incubated in the same temperature for 7-14 days with special Nitrogen gas pump to create anaerobic condition [13].

#### 2.6. Biocorrosion analysis

Brine water supplemented with nutrients formula (shown in Table 3) was inoculated in 250 mL of the final volume in a vacuum glass bottle. In order to create an anaerobic condition, those brine water mediums were gassed with Nitrogen for three minutes to eliminate the diluted. The prepared Carbon Steel ST-37 coupon was then placed to each bottle and incubated for 14 days at 70°C. The bacteria were inoculated aseptically in anaerobic-conditioned laminar. Autoclaved brine (Treatment A) used as a control to eliminate the role of biocorrosion process. After days of incubation, three carbon-steel coupons from each formula were harvested and rinsed with pickling methods using HCl 26% (v/v), and neutralized with NaOH 10% (w/v) and washed with sterile distilled water. Isopropyl alcohol and acetone were used to eliminate biofilm extracellular polymeric substances (EPS) which were remaining attached on the coupon. The coupon was dried at the 70°C oven for 30 minutes and moved to desiccator to eliminate Oxygen contact and weighed with an analytical scale. Corrosion rate count with equation (1) [14].

$$\text{Corrosion rate} = \frac{k \times W}{A \times T \times D} \quad (1)$$

$k$  = constant

$T$  = Exposure period (h)

$A$  = Area (cm<sup>2</sup>)

$W$  = weight loss (g)

$D$  = density (g/cm<sup>2</sup>)

#### 2.7. EPS analysis

Biofilm was scrapped from the Carbon Steel Coupon using laboratory spatula and washed with 1.5mL TE Buffer pH 7.5, 10mM Tris base, 10mM EDTA, 2.5% NaCl, and then resuspended. Biofilm suspension centrifuge in 4°C, 4x10<sup>3</sup> g for 20 minutes. Concentrated biomass was resuspended in 1.5mL 0.85% NaCl and 0.22% formaldehyde in 80°C for 30 minutes for EPS (Extracellular Polymeric Substance) extraction. EPS was then harvested after centrifugation (4°C, 1x10<sup>4</sup>g) for 60 minutes [15].

The carbohydrate concentration contained in EPS was quantified by phenol-sulfuric methods [16]. The amount of 500 $\mu$ L sample was inserted into a sterile reaction tube, supplemented with 500 $\mu$ L 5% phenol and 2.5mL 96% sulfuric acid and mixed with a vortex. The reaction was incubated in 30°C for 30 minutes. The absorbance was measured at 490nm in triplicates with standard curve which was generated by using 0.01mg/mL standard glucose suspension.

### 2.8. Scanning electron microscopy

Each coupon samples were soaked in 4% glutaraldehyde in PBS for 12 hours for fixation. The coupon was dehydrated using ethanol gradient (70%, 75%, 80%, 85%, 90%, 98%) and dried for 12 hours in room temperature desiccator before coated with gold for scanning electron analysis using JSM-6360LA SEM [14].

## 3. Result and Discussion

### 3.1. Effect of nutrients composition on biofilm formation

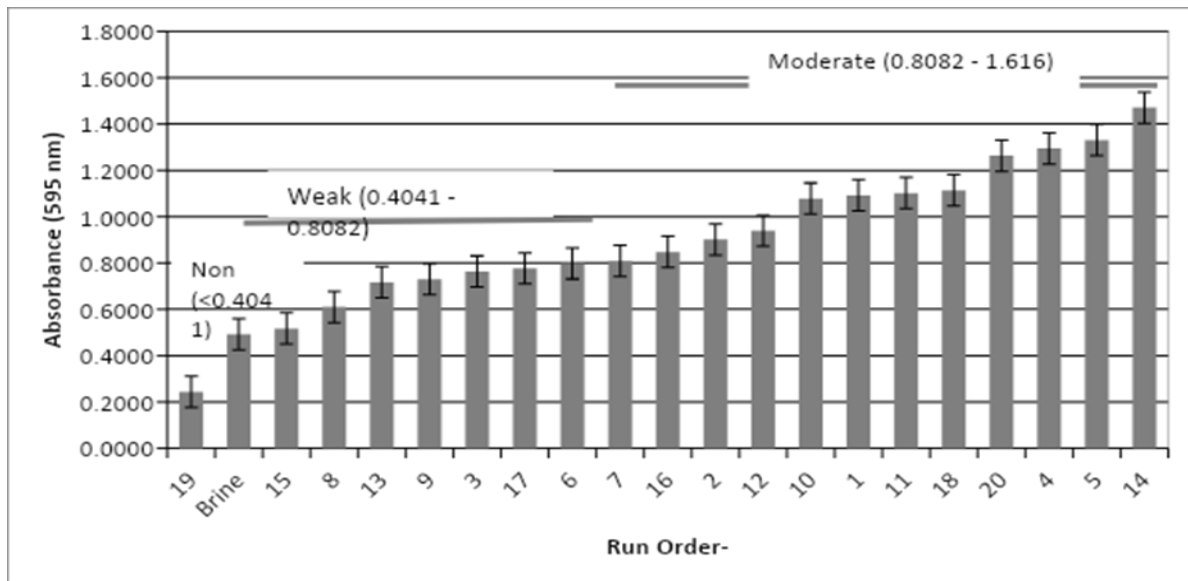
The varied concentration of Molasses DAP, and NPK as main factors were used in Response Surface Analysis and resulting in 20 variations of experiments such as mentioned above (Table 2). The results of those variations on biofilm strength and bacterial cell numbers are shown in Table 4. Based on multiple regression analysis on Central Composite Design matrix, and taking into account the response variable of OD, aerobe, and anaerobe bacterial cell numbers given in Table 2, a second-order polynomial equation was obtained as the equation (2), (3), and (4).

$$\text{OD} = 0,877 + 0,1507 X_1 - 0,989 X_2 - 0,01543 X_1^2 \quad (2)$$

$$\text{Aerobic bacteria} = 209,0 + 451 X_2 \quad (3)$$

$$\begin{aligned} \text{Anaerobic bacteria} = & 18358 + 7397 X_1 - 959 X_2 - 7928 X_3 - 9926 X_1 * X_2 - 16563 X_1 * X_3 \\ & + 279901 X_2 * X_3 \end{aligned} \quad (4)$$

p-value for OD, aerobe bacteria and anaerobe bacteria are 0.042, 0.017 and < 0.001 respectively with significant value  $\alpha = 0.05$ .  $X_1$ ,  $X_2$  and  $X_3$  stand for variable molasses, DAP and NPK. Biofilm analysis categorized into three biofilm strength interpretation based on the absorbance of each variation. Non-biofilm < 0.4041; weak biofilm  $0.4041 < \leq 0.8082$ ; and moderate biofilm  $0.8082 < \leq 1.616$  (Figure 1). All variations that use molasses as the carbon source resulting in biofilm formation. While the weak biofilm formed when the addition of DAP concentration was more than or as much as the NPK concentration. While the moderate biofilm formed with DAP concentration lower than that of NPK concentration.

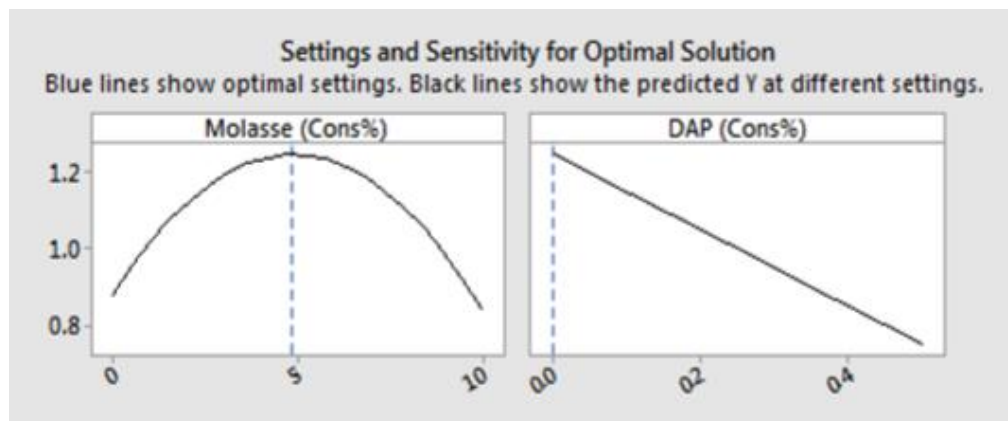


**Figure 1.** Biofilm strength interpretation from 20 nutrients variations formula.

**Table 2.** 20 Nutrients variations formula from surface response experimental design with OD (optical density) and microbial enumeration result.

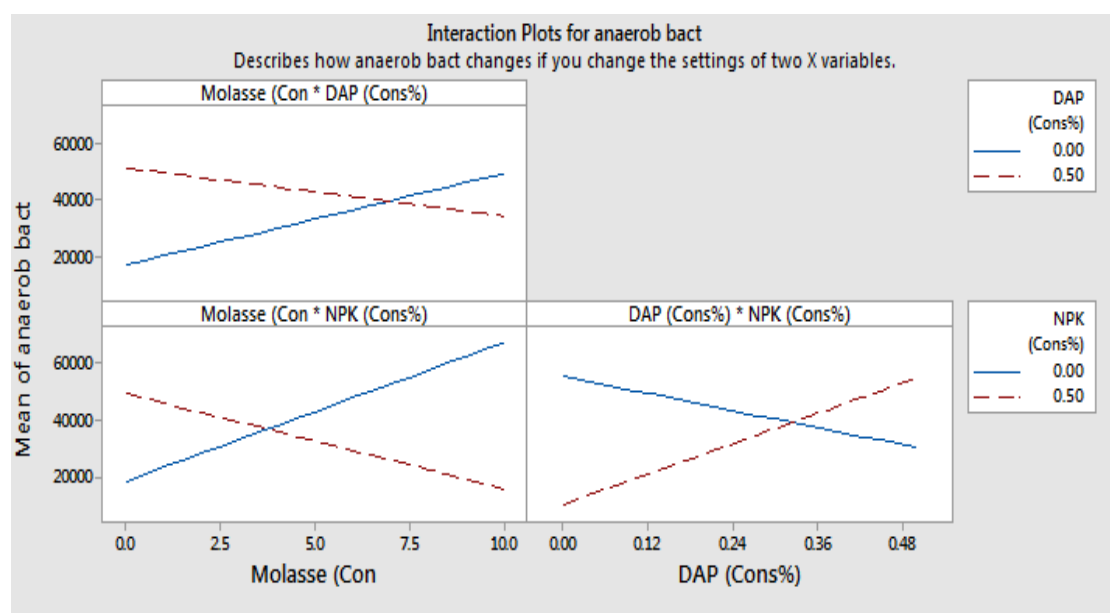
Run Order	Nutrient Concentration (%)			OD	Aerobic Bacteria	Anaerobic Bacteria	pH
	Molasses	DAP	NPK				
1	2	0.40	0.40	1.0923	$2.87 \times 10^2$	$5.40 \times 10^4$	5.371
2	8	0.10	0.10	0.9016	$2.25 \times 10^2$	$5.48 \times 10^4$	5.070
3	5	0.25	0.25	0.7634	$2.57 \times 10^2$	$3.37 \times 10^4$	4.979
4	5	0.25	0.50	1.2946	$2.65 \times 10^2$	$3.56 \times 10^4$	4.934
5	5	0.25	0.25	1.3310	$3.98 \times 10^2$	$3.68 \times 10^4$	5.076
6	2	0.40	0.10	0.7982	$3.27 \times 10^2$	$3.00 \times 10^4$	5.638
7	5	0.25	0.00	0.8091	$2.73 \times 10^2$	$4.62 \times 10^4$	5.244
8	5	0.50	0.25	0.6099	$5.10 \times 10^2$	$4.37 \times 10^4$	4.974
9	5	0.25	0.25	0.7299	$4.58 \times 10^2$	$3.08 \times 10^4$	5.051
10	5	0.00	0.25	1.0783	$2.1 \times 10^2$	$3.54 \times 10^4$	5.080
11	5	0.25	0.25	1.1021	$4.12 \times 10^2$	$4.37 \times 10^4$	5.061
12	5	0.25	0.25	0.9391	$1.98 \times 10^2$	$4.07 \times 10^4$	5.030
13	10	0.25	0.25	0.7166	$3.93 \times 10^2$	$4.23 \times 10^4$	5.090
14	8	0.10	0.40	1.1372	$2.07 \times 10^2$	$2.37 \times 10^4$	4.910
15	8	0.40	0.40	0.5173	$3.50 \times 10^2$	$3.11 \times 10^4$	4.970
16	5	0.25	0.25	0.8482	$4.87 \times 10^2$	$4.00 \times 10^4$	5.127
17	8	0.40	0.10	0.7770	$4.63 \times 10^2$	$4.22 \times 10^4$	5.043
18	2	0.10	0.10	1.1139	$3.33 \times 10^2$	$2.99 \times 10^4$	6.537
19	0	0.25	0.25	0.2434	$1.40 \times 10^2$	$3.20 \times 10^4$	6.546
20	2	0.10	0.40	1.2631	$2.37 \times 10^2$	$2.35 \times 10^4$	6.007

From the data, the biofilm strength raises when the Molasses concentration added was up to 5% (v/v) (Figure 2). Addition of molasses concentration lower than 5% lower the biofilm strength. Molasses needed as the main carbon source that was very important in the biofilm formation. While the addition of DAP concentration will weaken the biofilm formation. Ammonium ion in DAP reacts with Chloride ion resulting Chloramine compound that has a disinfectant effect towards the microbial cell in the Brine Water that formed the biofilm [17]. While NPK concentration did not affect biofilm strength.



**Figure 2.** Molasses and DAP concentration effect towards biofilm strength analysis

The anaerobic condition of growing microbes in several media performed and resulting in the anaerobic cell amount higher than aerobic cell amount. Statistical analysis resulting linear equation and quadratic that was proofing the concentration of DAP has a significant effect towards both anaerobic and aerobic bacteria on biofilm. NPK and Molasses concentration give a significant effect on the growth of anaerobic cell amount. While statistical analysis on acid producing bacteria cells, the amount did not result in the significant effect.



**Figure 3.** Interaction plot of anaerobic bacteria

As shown in Figure 3, the effects of nutrient addition towards anaerobic bacteria amount conclude. Increasing concentration of Molasses when DAP and NPK concentration were low could raise the anaerobic bacteria number of cells, while the increasing Molasses concentration while DAP and NPK were high could decrease the number of anaerobic bacteria cells. DAP concentration raises the total anaerobic bacteria when NPK concentration serves at a high level. Each substrate effect towards anaerobic cell growth affected with the other substrate concentration that was added. Basically, Biofilm has consisted of diverse microorganism that has varied physiological activity and also nutrient needs that caused complex microbial and chemical interaction. The high concentrations of Carbon, Phosphate, and Nitrogen cause an increasing amount of varies bacterial communities that affect nutrient

competition. In order to get a high number of anaerobic bacteria, high C/N ratio of nutrients needed. It also can allow the growth of nitrifying bacteria, ammonification bacteria, phosphate utilizing bacteria [18]. That is why proportional substrate addition should be done carefully in the right concentration.

We can see the effect of substrate addition towards biofilm and anaerobic bacterial growth showed a similar pattern, that increasing DAP amount decrease the growth and increasing molasses amount increase the growth, in this study. This might happen because all the inoculation technique performed in this study using anaerobic approach by Nitrogen gassing and use of Anaerobic chamber. The biofilm cell quantification basically represents total microbes that involved in the biofilm formation process, which in this experiment dominated by anaerobic bacteria.

### 3.2. Effect of optimized nutrients formula towards biocorrosion potential in carbon-steel coupon

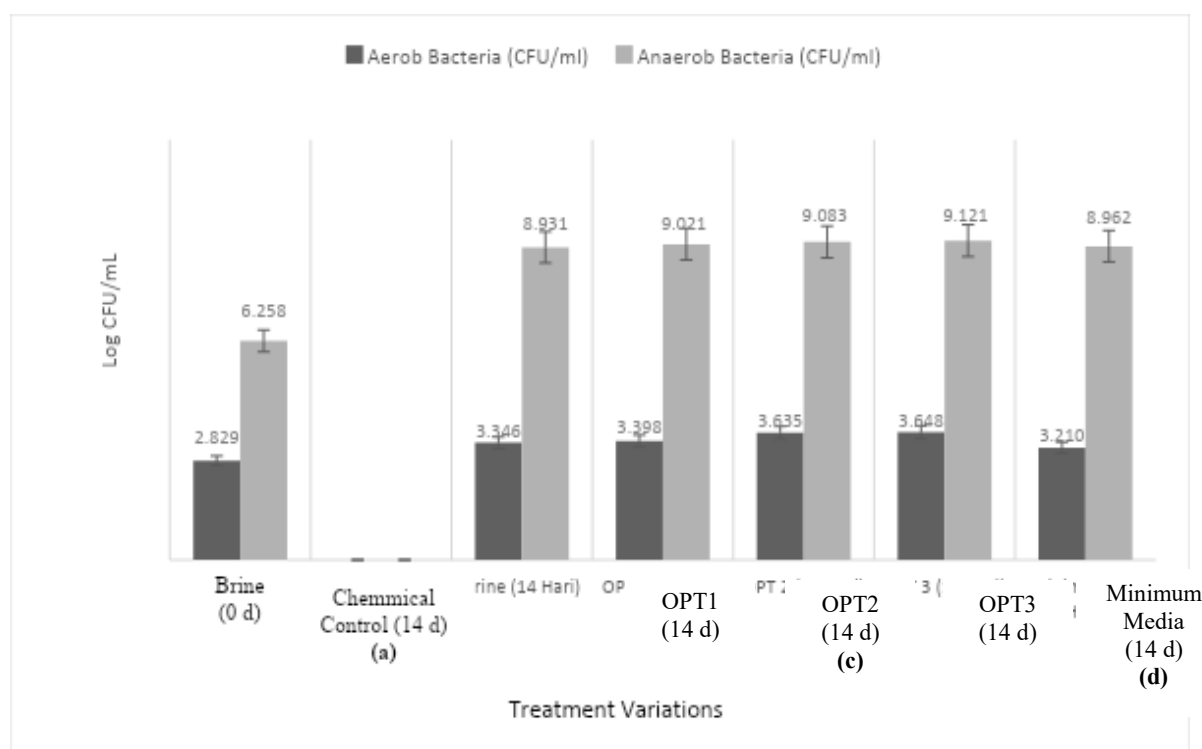
The statistical analysis was then given the result for the optimum medium that stimulated the biofilm formation consisted of molasses, DAP and NPK 4.6%; 0.4754% and 0.5% respectively. On the other hand, the medium formulation to minimize the biofilm formation has consisted of 0.1% DAP without molasses and NPK supplement. The results for those treatments variation as illustrated in Table 3.

**Table 3.** Result of biofilm and corrosion analysis on treatment variations.

Variations	Molasses (%)	Diammonium Phosphate / DAP (%)	NPK (%)	EPS Carbohydrate ( $\mu\text{g}/\text{cm}^2$ )	Corrosion Rate (mm/year)	pH
<b>Chemical Control (a)</b>	<i>Autoclaved Brine water without substrate addition</i>			39.38	0.685	8.81
<b>Formation Water (b)</b>	<i>Unsterile Brine water without substrate addition</i>			47.08	0.792	8.5
<b>OPT (c)</b>	4.6%	0.4754%	0.5%	59.58	3.615	5.98
<b>Minimum (d)</b>	0%	0.1%	0%	24.58	0.365	8.48

The carbon-steel ST-37 coupon composition was analyzed using SEM-EDX. The composition is shown in peak concentration of each metal alloys. The carbon-steel coupon prepared with manual scouring using gradient sandpaper. It is shown from the SEM-EDX result that 74.08% composition of Carbon-steel ST-37 coupon is Ferrous (Fe); 0.07% is Carbon (C), and 0.96% is Nitrogen (N). Table 3 shows that the corrosion rate increase by the high DAP amount, as well as the increasing of anaerobic and aerobic bacterial cell numbers (Figure 4). Based on the statistical analysis, DAP performed as an independent variable that affects the corrosion rate as it increases (shown in Table 3).





**Figure 4.** Microorganism total plate count of biocorrosion potential analysis

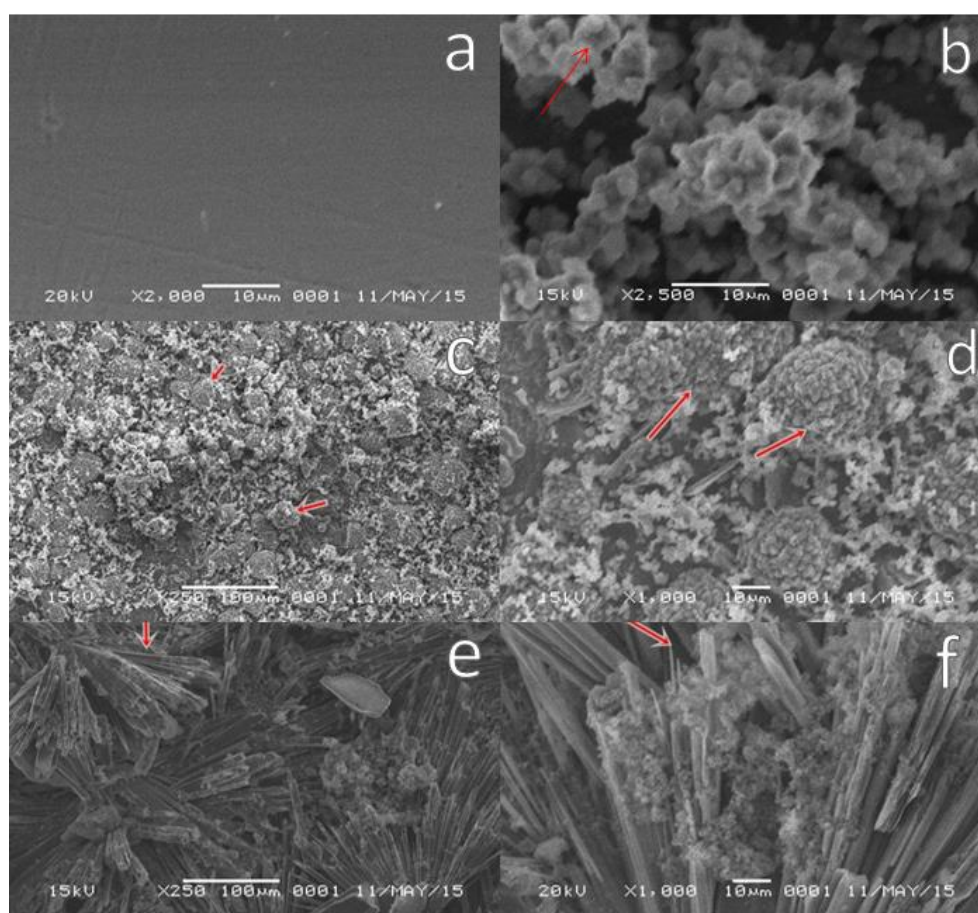
The increasing concentration of molasses affected the increasing microbial growth because it decreases pH in the environment due to microorganism activity that metabolizes molasses into acetic acid [16]. When environment pH is low, abundant hydrogen ions ( $H^+$ ) found in the environment and it induces oxidation-reduction reaction or else, the hydrogen ions interact in cathode or metal in this experiment and erodes protective layer in the metal surface. This reaction causing metal oxidation into  $Fe^{2+}$  that interact with hydroxide ions forming tubercle in metal surface and continuing its oxidation into  $Fe^{3+}$  with help of Iron-oxidizing bacteria that present in the system.  $Fe^{3+}$  ions could form other solid crystal forms of  $FeOOH$  or goethite in the surface of the metal when interacting with oxygen and hydroxide [19]. The SEM-XRD analysis performed after 14 days incubation vivianite was found in C formula. While SEM imaging of C did not show any pitting corrosion, assumed that the surface of the metal in both formulas covered with crystal existence.

Goethite and vivianite are the protective crystal that protects the metal surface from corrosion process, while hematite can lead to further corrosion. The Phosphate substrate addition into the system can reduce the existence of goethite crystal in the metal surface and form vivianite and struvite crystal that soluble in the acidic pH. The acidic environment because phosphate addition into the system can cause formation reactive Phosphate compound, including iron phosphide. Phosphate is an anamorphic compound that it hardly detects by XRD analysis. This kind of compound is reactive that could increase metal corrosion rate [20].

The minimum formula has an acidic pH environment because of passivity in the metal surface. DAP contains Phosphate (P) that act as a corrosion inhibitor [21]. Phosphates act as inorganic anodic inhibitor so that could form insoluble protection layer and cohesively protect the metal surface, called passivity [22]. The formation of passivity in the metal surface could decrease organic compound accumulation and conditioning alkaline pH. The Brine water contains  $Fe^{2+}$  and  $Fe^{3+}$  that could react with Oxygen and Hydroxide ions to form goethite crystal. The corrosion rate in the chemical environment and Brine water alone systems show smaller result compare to several optimized formula (c) treatments (Table 3). This could happen because the existence of microbial activity that also releasing organic acids metabolites in OPT treatments induced chemical corrosion process in the system. Consequently, on chemical condition

and water formation treatments the corrosion rate measured is the result of metal weight loss because of the formation of a protective crystal, such as goethite and vivianite.

Microorganism might not affect the corrosion in an instance, but indirectly affect the corrosion process as a system, microorganism affect the corrosion by producing metabolites such as organic acid from carbon source utilization, such as molasses in this experiment, that form acidic environment in the system. NPK affected corrosion has not clearly known, but the existence as Nitrogen, Phosphor and Potassium source made changes in aerobic and anaerobic growth that also affect biofilm formation in the system. Even though the different result of NPK existence on the system depends also on different molasses and DAP used due to changes on the C:N:P ratio of media.



**Figure 5.** SEM Visual Analysis of Biocorrosion Potential of Treatments Formula. Minimum (a) 2000x, Chemical Control (b) 1000x, Formation Water (c) 250x, Formation Water (d) 1000x, OPT2 (j) 250x, and OPT2 (k) 1000x. Red arrows show either biofilm, crystal formed, or pitting corrosion surface.

#### 4. Conclusion

From this study, we can conclude that molasses addition as a main carbon source in the nutrients increase anaerobic bacteria and add up to 5% molasses concentration increase the biofilm strength. The molasses addition of more than 5% could decrease biofilm strength. While, the addition of DAP (Diammonium Phosphate) concentration could increase aerobic microbial total cell, lower the biofilm strength, lower anaerobic bacteria amount when molasses concentration increases and raise anaerobic bacteria amount when molasses concentration decrease. Increasing NPK addition raises anaerobic bacteria amount when molasses is low and DAP is high. Molasses, DAP, and NPK optimum for the biofilm formation are 4.6%; 0.4754%; and 0.5% and for the minimum biofilm formation are 0%; 0.1% and 0%, in a respective order. The increasing microorganism amount correlated with increasing corrosion rate observed on

optimized media compares to one that observed in minimum media in this study. This biofilm formation might cause the increasing potential of biocorrosion process in the static environment in this study.

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