

# APPLICATION OF MERCURY RESISTANT BACTERIA ISOLATED FROM ARTISANAL SMALL-SCALE GOLD TAILINGS IN BIOTRANSFORMATION OF MERCURY (II) - CONTAMINATED SOIL

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**ABSTRACT:** This research aims to study the detoxification of Hg by a consortium of bacteria throughout bioremediation technology. The consortium of bacteria was identified to be the strain of *Bacillus cereus* TL-01, *Bacillus* Sp TL-02 and *Brevundimonas diminuta* TL-03 by 16s rRNA sequencing. The result presented the potential of growth in the used medium and bacteria grew faster orderly in no/less to high Hg concentration. Bacteria started its log phase after 4 and 20 hours inoculation in pure culture and 50 ppm Hg while the growth prolonged its lag phase to 52 hours in 100 ppm Hg. Experimental treatments were done using synthetic soil under an anaerobic condition with 60 days of observation. For a microcosm of soil treatment, the highest removal of mercury under influence of temperature was 73 % at 45°C while the highest removal under influence of pH was 69% at pH of 4-5 and the highest removal under nutrient amendment was at CNP of 100:10:1 with an efficiency of 75%. There was 0.02% of Hg<sup>0</sup> found to be adsorbed onto the activated carbon analyzed by SEM-EDS. The oxidation-reduction potential (ORP) was measured in the range of -60 mv to 80 mv ranging in reduced soil considered into anaerobic condition. To sum up, the isolated bacteria represents to potential in detoxifying Hg while Hg removal was better with the anaerobic condition. A high concentration of Hg did not inhibit the growth of isolated bacteria but it just prolonged the adaptation stage of bacteria.

*Keywords: Mercury biotransformation, Soil remediation Biological treatment, Mercury resistance bacteria*

## 1. INTRODUCTION

Mercury (Hg) is considered as one of the main global pollutants due to its persistence, bioaccumulation, and toxicity in the environment [1]. Mercury pollution can be caused by natural sources such as volcanic eruptions, forest fires, re-emissions and anthropogenic sources including gold and silver extraction, mining activities, coal-fired power plants, and either medical and electronic waste products. Even a small amount of Hg can cause serious damage to all organisms. Excessive mercury in the human body is neurotoxins, which mostly interfere with the brain and nervous system. Even in low doses, mercury can affect a child's development. For adults, mercury poisoning can affect blood pressure regulation, fertility, memory loss, tremors, and vision loss. People affected by mercury pollution are also found to be resistant to some antibiotics [2].

Bioremediation is the application of microorganisms or microbial processes or products to remove or degrade pollutants from a contaminated area. A more rigorous definition is the intentional use of biological degradation procedures to remove or reduce the concentration of environmental pollutants from a site where they can

be released [3]. Therefore, microorganism plays a crucial role in detoxifying the hazard of mercury. Hg resistant bacteria have mer operon that carries a number of genes and products of which can transport (MerP and MerT) Hg compounds into the cell and reduce both organic and inorganic Hg<sup>2+</sup> to insoluble elemental Hg<sup>0</sup> that subsequently diffuses from the cell [1]. The catalytic process involved two intracellular enzymatic reductions such as organomercurial lyase enzyme (MerB) catalyze organic mercury compound to Hg<sup>2+</sup> followed by the mercury reductase enzyme (MerA) catalyze Hg<sup>2+</sup> to Hg<sup>0</sup> [4]. Inorganic Hg can be methylated to organic Hg through the activity of microorganisms.

This research aimed to isolate and identify mercury resistance bacteria (MRB), observe the growth of bacteria in various Hg concentrations, study the bioremediation of mercury in soil under anaerobic conditions, and analyzed the volatilization of elemental mercury.

## 2. METHODOLOGY

### 2.1 Research Materials

The research used the synthetic agricultural soil to study the potential of selected bacteria for

bioremediation. Nutrient Agar (NA), prepared by dissolving NA powder of 28 g into 1000 ml of distilled water composited by 5 g of Peptone, 5 g of NaCl, 1.5 g of Beef extract, 1.5 g of Yeast extract and 15 g of Agar. Nutrient Broth (NB), composited by 5 g of Peptone, 5 g of NaCl, 1.5 g of Beef extract and 1.5 g of yeast extract dissolving into 1000 ml of distilled water.  $\text{HgCl}_2$  was used as the pollutant for synthetic sample.

## 2.2 Isolation of Mercury Resistance Bacteria

The isolation of mercury resistance bacteria was started by homogenizing 1 g of soil sample into 10 ml of sterilized water. Then, Samples were diluted into seven concentration ( $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$ ,  $10^{-5}$ ,  $10^{-6}$  and  $10^{-7}$ ). Homogenization of sample was done by Vortex. After that, 100  $\mu\text{l}$  of samples were inoculated onto sterilized petri dish contained medium of NA. Bacteria are inoculated by spreading using rod spreading L onto the agar plate. The incubation was done in 48 hours at room temperature. This was done in purpose of getting the dominant bacteria that could live, grow, and detoxify the contaminated environment with sources of mercury pollutant.

## 2.3 Adaptation of Mercury Resistance Bacteria

Bacteria from the enrichment stages derived from the solid medium was transferred aseptically using an ose needle into a tube medium containing NA to receive anaerobic/facultative anaerobic bacteria during 24-72 hours. The bacteria grown in the agar medium was later inoculated again onto a new petri dish containing NA amended with concentration of 10 ppm  $\text{HgCl}_2$ . Transferring grown colonies by an ose of 48-hours isolate was inoculated into 10 ml of nutrient broth (NB) liquid medium in a test tube and incubated in room temperature for 24 hours followed by extraction 5 ml from previous stage to inoculate into 45 ml NB with 10 ppm  $\text{HgCl}_2$  and continuously inoculated into NB with 50 ppm, 75 ppm and 100 ppm of  $\text{HgCl}_2$ . The incubation of each inoculation was in every 48 hours. The bacteria that grew in this liquid medium was able to be used for bioremediation of soil. Sample for isolation and growth anaerobic bacteria was placed into anaerobic jar.

## 2.4 Bacterial Growth Curve

Determination of growth curve was done by observing the growth of bacteria in NB dissolved with various concentration of Mercury such as Pure Culture, 50 ppm Hg, 100 ppm Hg, 200 ppm Hg, 300 ppm Hg and 500 ppm Hg. Determining the growth was done with the indirect method using a Spectrophotometer measured as Optical Density

(OD) at a maximum wavelength ( $\lambda_{\text{max}}$ ) of 600nm [5]. Optical Density was performed until the stationary stage of bacteria growth with 6 hours time interval.

## 2.5 Remediation of mercury in soil

Experimental setup was done using synthetic sample by mixing soil with 200 ppm Hg (approximately 270 ppm  $\text{HgCl}_2$ ). Observation of the performance was determined within 60 days under anaerobic condition. The treatment sample contained soil, Hg, bacteria (inoculated from mid of log phase). The moisture was maintained for all of the reactors as the ideal water content is range from 30%-60% which is reported by [6]. Anaerobic condition of reactors performance was maintained by flashing nitrogen gas. Activated carbon column was used to absorb the elemental mercury ( $\text{Hg}^0$ ) undergoing of biotransformation mechanism. The research was conducted under the variation of temperature (25°C as the control, 30°C, 37°C and 45°C), pH (4-5, 5-6 as the control, 6-8 and 8-9) and influence of nutrient amendment of CNP (300:10:1, 200:10:1, 100:10:1, 100:40:1 and 100:20:1). Adjustment to acidic soil was used Oxalic acid ( $\text{H}_2\text{C}_2\text{O}_4$ ) to decrease the pH while alkaline soil was used  $\text{CaCO}_3$  to increase the pH. Glucose ( $\text{C}_6\text{H}_{12}\text{O}_6$ ), Potassium Nitrate ( $\text{KNO}_3$ ) and Potassium di-hydro phosphate ( $\text{KH}_2\text{PO}_4$ ) were amended as the carbon and nutrient sources of the bacteria. The reactor configuration for microcosm scale of the soil treatment is shown in Figure 1.

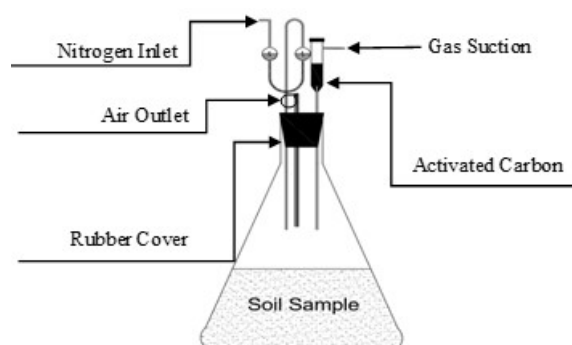


Fig. 1 Reactor for soil remediation of mercury.

## 3. RESULTS AND DISCUSSION

### 3.1 Bacterial Growth Observation

The growth pattern in six variation of mercury concentration represented by the absorbance valued in the range of 0.05 until 0.7 where the bacteria growth faster orderly from no or less toxic to high toxic solution (Figure 2). In pure culture, bacteria started growing since 4 hours of inoculation. This result present the potential of growth in the used

medium as the fastest growth since there is no inhibition to disturb bacteria growing. In Hg 50 ppm, the bacteria started its log phase in 20 hours. With addition of mercury, the higher concentration of toxic element, bacteria also need longer time to perform the lag phase. As seeing from the Figure 2, the bacteria grow in 100 ppm of mercury spend around 52 hours to start its exponential phase while the bacteria growing in 500 ppm of mercury spend almost 120 hours for this adaptation phase.

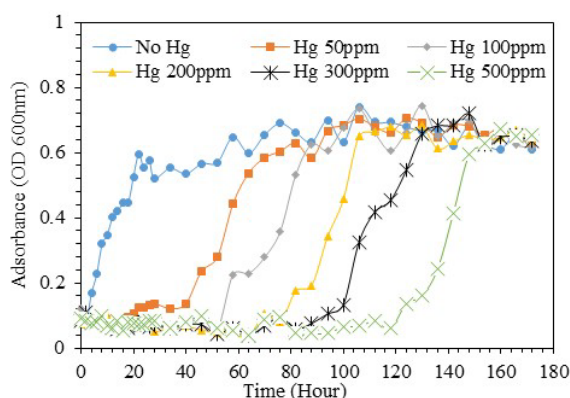


Fig. 2 Bacterial growth in various mercury concentration.

Bacteria prolonged lag phase because cells require adaptation against the toxicity [7]. Additional of  $\text{HgCl}_2$  or higher concentration of

mercury in culture media increased the lag phase but did not affect the other growth phase of the bacteria [8]. Result was found that specific growth of bacteria in pure culture was 0.0225/h showing the highest rate among others. This is because bacteria used nutrient in medium as the energy source and there was no disturb from waste/toxicity. With additional of Hg, specific growth rates of bacteria were shown to be less comparing to pure culture which were in the range of 0.0125/h to 0.163/h. Result presented of slightly different of specific growth rate (Table 1). This is because growth rate of mixed cultures depended on the concentration of the carbon substrate (nutrient) present in culture media and was unaffected by the type of mercury added [9].

Table 1. Specific growth rate of mercury resistance bacteria.

Hg (ppm)	$\mu$ (1/h)	Lag Phase Duration (h)
0 (w/o Hg)	0.0225	4
50	0.014	26
100	0.0125	52
200	0.0141	58
300	0.0145	82
500	0.0163	120

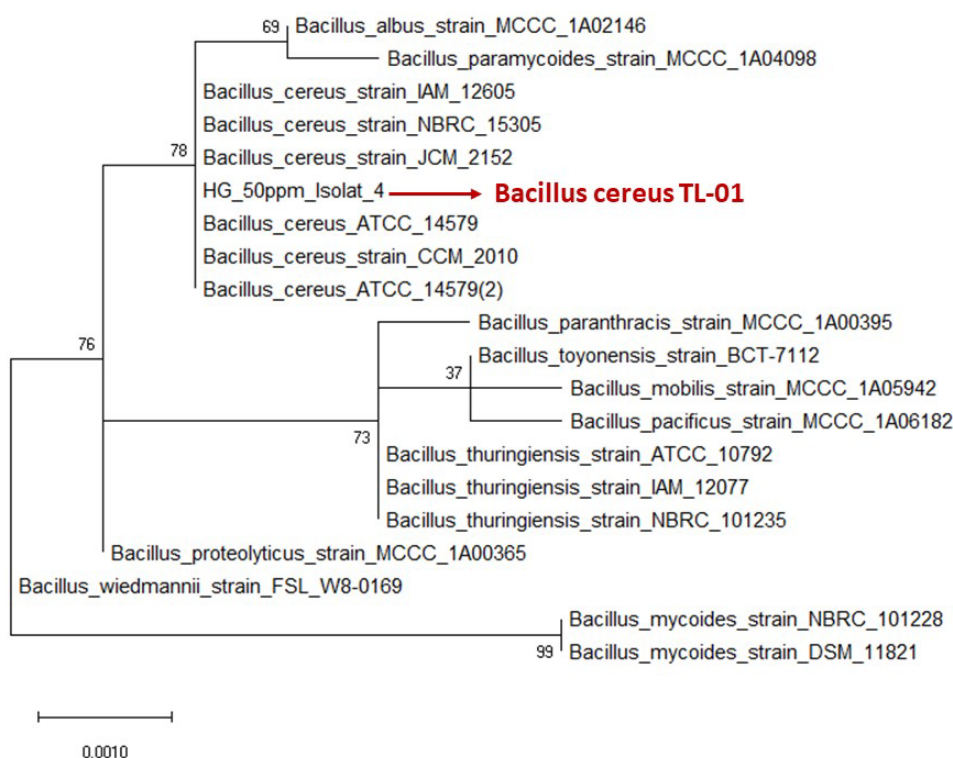


Fig. 3a Phylogenetic tree of the mercury resistance bacteria (Bacillus Cereus TL-01)

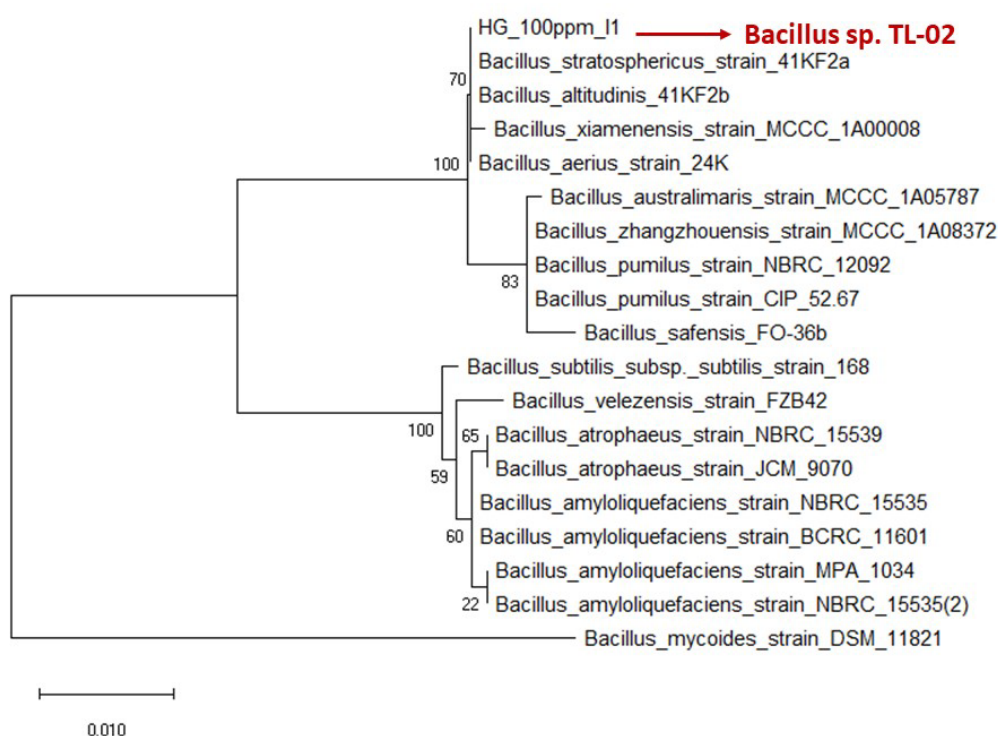


Fig. 3b Phylogenetic tree of the mercury resistance bacteria (*Bacillus Cereus* TL-02)

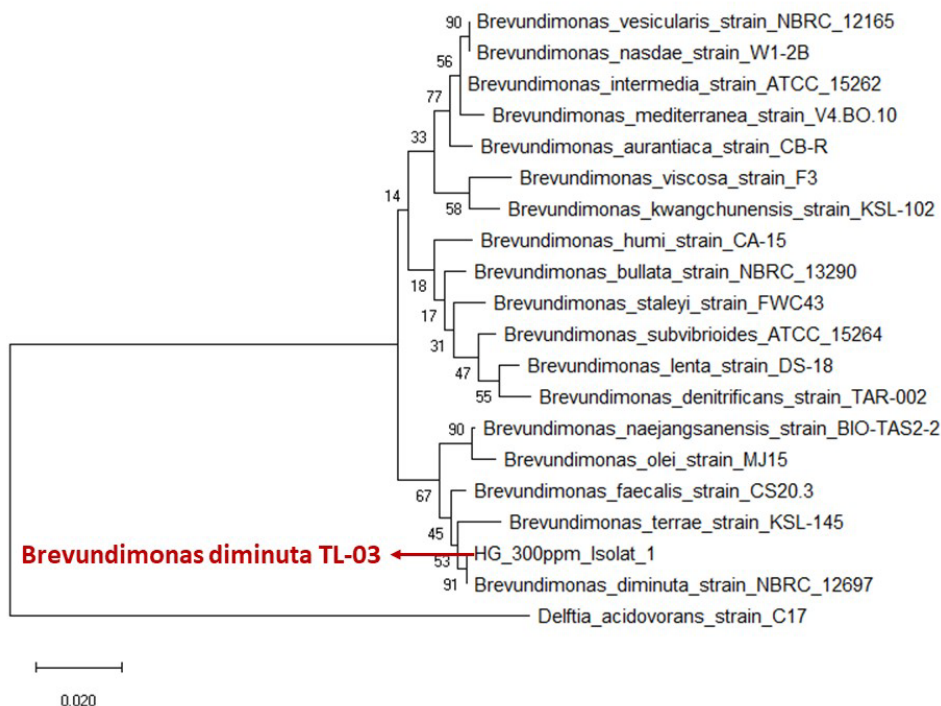


Fig. 3c Phylogenetic tree of the mercury resistance bacteria (*Brevundimonas diminuta* TL-03)

### 3.2 Identification of Mercury Resistance Bacteria

Bacteria was first characterized and classified by microscope based on their morphology and straining properties [10] and finally identified by

16s rRNA sequencing. Figure 3 shows the phylogenetic tree of the mercury resistance bacteria. The blast result from DNA of the isolates showed the bacteria to be the strain of *Bacillus cereus* TL-01, *Bacillus sp* TL-02 and *Brevundimonas diminuta* TL-03. The three types of bacteria were found to be

resistance in Hg 50 ppm and 100 ppm Hg. However, the resistance strain was dominant of the *Bacillus* sp and *Brevundimonas diminuta* in 300 ppm Hg. *Bacillus* sp was the bacterial strain found to be dominant at 500 ppm Hg.

### 3.3 Mercury Reduction from Soil

Figure 4 presents the result of mercury reduction under process of biotransformation. Temperature has a great influence to biotransformation of mercury from soil resulting of decreasing concentration from approximately 251 mg/kg to 67 mg/kg as the highest removal with efficiency of 73.3% at temperature of 45°C. The result was followed by the incubation of 37°C with efficiency of approximately 60% while at 25°C and 30°C had similar removal of 51%. In compost reactor, increasing of temperature could be resulted in increasing the microbial population until the peak which was depending on the type of organic material involved [11]. Temperatures between 90° and 140°F equivalent to approximately of 32°C and 60°C indicate rapid decomposition by the strong activity of microorganism. *Bacillus* sp was found to be the dominant bacteria that could survive with temperature above 45°C [11]. However, the strain of *Bacillus cereus* was found to be the moderately thermophilic bacteria and has the potential in detoxification of several types of metal at 45°C [12]. Increasing temperature to 45°C had the positive affect on the enzyme activity [13].

Acidic soil at pH 4-5, concentration of mercury reduced from 215 mg/kg to 67 mg/kg taking into approximately 68.8 % removal. Lowest mercury reduction in soil among pH variation was in treatment of pH 6-8 showing efficiency of about 42 %. With the treatment at pH 5-6, efficiency of removal was around 51 %. Treatment of alkali soil with pH of 8-9 gave the result of 51 %. It is well established that the biotransformation was better in acidic soil where pH value is important in mobility and availability of the heavy metal (including Hg) in the soil, not only by the effect on the metal speciation in the soil solution but also the changing of soil characteristic [14]. At base pH, the activity of enzyme decrease because this condition may cause denaturation of activity of enzyme [15].

The result indicated that addition of much carbon and nitrogen did not give higher efficiency of Hg removal. Therefore, only a proper ratio of CNP that give effectively yield. Highest carbon addition seen with the ratio of 300:10:1 resulted with the efficiency of approximately 42 %. Highest nitrogen addition, CNP ratio of 100:40:1, gave removal efficiency of about 53%. Ratio of 200:10:1

and 100:20:1 give the efficiency of probably 60% and 51%.

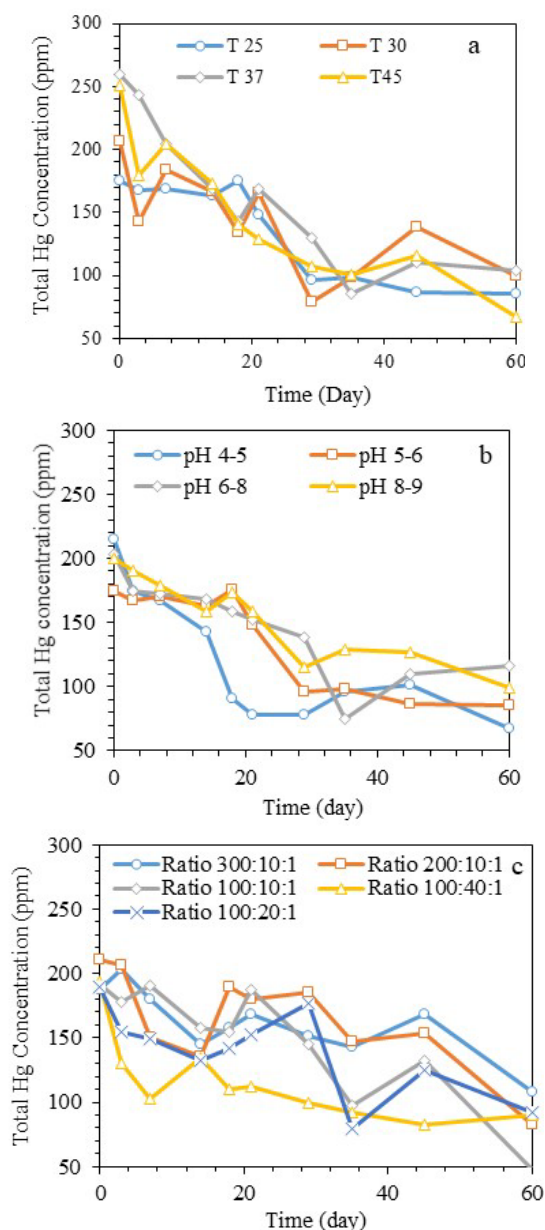


Fig. 4 Results of mercury reduction in soil remediation with variation of (a) temperature, (b) pH, and (c) C:N:P ratio.

However, mercury was much reduced with the ratio of 100:10:1 with efficiency up to 75 %. The excessive of C or N does not prove to be always better for the bacteria. Too much of N addition may result in inhibition of bacteria growth where the highest N addition decreased the bacterial biomass production [16]. Higher amounts of bioavailable C did not lead to higher biomass, instead the microbial community and associated metabolic response has shifted toward more copiotrophic organisms [17]. Hg binding to OM with high affinity, thus



decreasing its availability and mobility which were quite important for the formation of elemental Hg from the reduction of oxidized mercury [14].

### 3.4 Volatilization of Elemental Mercury

Elemental mercury ( $Hg^0$ ), the non-toxic form of mercury, was produced after the catalyzation process of mercury ion in soil by mercury reductase enzyme. In this research, Activated carbon was used to capture  $Hg^0$  volatilized from soil. Figure 5 shows the morphology and composition of the activated carbon used before soil treatment by SEM-EDS.

The ZAF method standardless quantitative was analyzed the composition of the element with 800 zooming of the SEM image. The result showed the major element was carbon and Oxygen with 89.5 %

and 8.2 % in atom accordingly. Hg was found to 0.02 % in atom represent in the sample. This mean that Hg were volatile and absorb onto the carbon. The Hg can be quantity through many factors. Carbon from various sources shows entirely different performance [18]. Results uniquely demonstrated that  $Hg^0$  was completely oxidized to divalent mercury ( $Hg^{2+}$ ) during the adsorption process of  $Hg^0$  by activated carbon [19]. Adsorption of  $Hg^0$  can be influence changing of temperature while previous research found the adsorption of  $Hg^0$  from 20°C until 270°C. High temperature caused a reduction in the adsorption capacity of the carbon samples, which is consistent with physical sorption theory [18]. The adsorption of  $Hg^0$  was better with the impregnated of activated carbon [20].

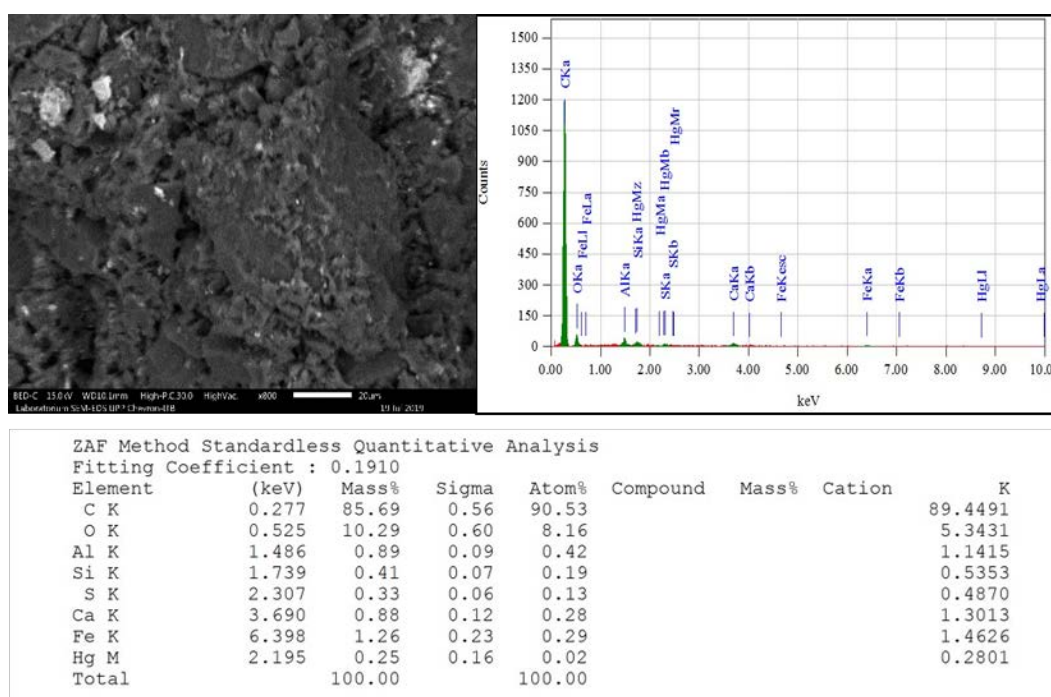


Fig. 5 Morphology and chemical composition of the used activated carbon after soil treatment.

### 3.5 Oxidation Reduction Potential (ORP)

The oxidation-reduction potential (redox potential or Eh) is a measure of the degree of reduction of soil and the number of free electrons exchanged in redox reactions. Figure 6 shows the result of redox potential of the reactors.

Redox potential profile of the remediation were in the range of 1 mv to 60 mv except in variation of pH 6-8 and pH 8-9 where the ORP were in the range of -50 mv to 5 mv. According [21], the redox potential value determined the performance of microcosm into reduced soil condition as it was in the range of -100 mv to 100 mv. This meant that the

performance was in anaerobic condition. Redox potential can be measured as the result of chemical reaction in reducing and accepting electrons. In bioremediation, Organic matters has the capability to form complexes with  $Hg^{2+}$ . Hg-organic complex, electron can be transferred from the organic matter to  $Hg^{2+}$  in order to reduce it to  $Hg^0$  [22]. In another pathway, the mercury resistant bacteria can transform inorganic  $Hg^{2+}$  species into  $Hg^0$  by enzyme mercury reductase. The reaction is carried out by the enzyme NADPH-dependent flavoenzyme mercuric reductase. This enzyme is capable of reducing  $Hg^{2+}$  to  $Hg^0$  by catalyzing the electron transfer from a thiol complex form with  $Hg^{2+}$  [22]. Additionally, electron transfer chain can

also cause from anaerobic respiration. Microorganism use organic carbon to produce energy. Here, it plays a role as electron donor while nitrite, nitrate and carbon dioxide are used as the electron acceptor. Nutrient amendment enhanced availability of electron acceptors in anaerobic respiration thus enhance mineralization [23]. Additional nutrient in soil increased organic matter in soil resulted in enhancement of metabolism process in utilizing substrate to produce energy, CO<sub>2</sub> and CH<sub>4</sub>. Results showed that nutrient amendment decreased soil redox potential [24]. This is maybe the reason to make ORP value decreased into reduced soil upon the time as prove from the result.

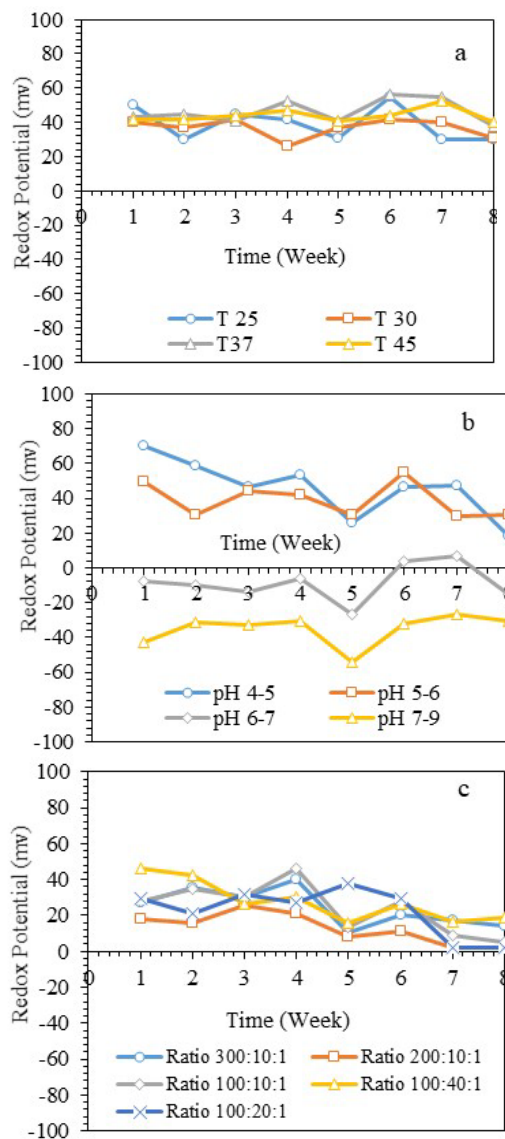


Fig. 6 Redox potential of the soil treatment with variation of (a) temperature, (b) pH, and (c) C:N:P ratio.

### 3.6 Enumeration of Bacteria in Soil

Mercury resistant bacteria can remove mercury and grow in the presence of this toxicant by enzymatic reduction activity of the enzyme mercuric reductase (merA gen product), while mercury sensitive bacteria do not have any mechanism for detoxification of bacteria. Figure 7 presents the bacteria population profile growing in soil.

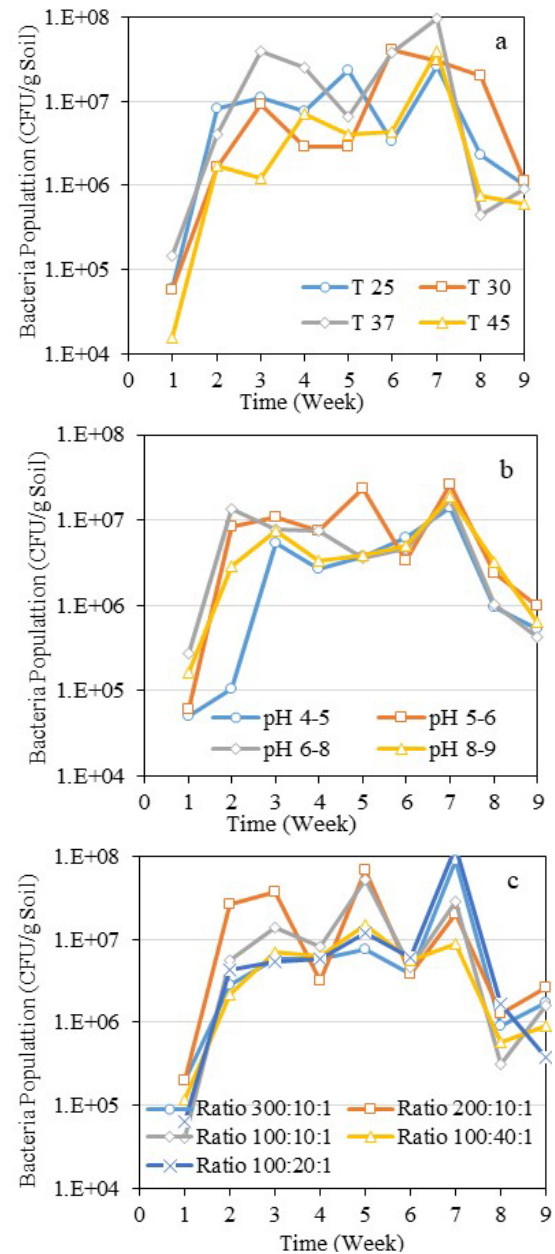


Fig. 7 Population of the bacteria growth in soil with variation of (a) temperature, (b) pH, and (c) C:N:P ratio.

The growth curve of the bacteria showed similar pattern in all variation where the log phase started from week 1 until week 3. Additionally, the stationary phase followed to week 7 of the experiment and the population of the bacteria started decreasing in week 8 shown to be the decay phase. In the variation with temperature, the population of bacteria the highest with temperature of 37 °C as it was in the range of 1.4E+05 cfu/g soil to 9.7E+08 cfu/g soil. The temperature affect the chemical reactions in the process of bacterial growth, growth rate, and the total amount of the growth of microorganisms [25]. In the variation with pH, the population of bacteria grew from 6E+04 cfu/g soil to 2.4E+07 cfu/g soil. In all the result from pH variation, bacteria grew similarly just pH 5-6 showed a bit better comparing to others. The enzyme works at neutral pH and will become inactive when the environment becomes very acidic or very alkaline [25]. The degree of acidity affects the growth of bacteria because the pH affects the enzymes in the metabolism of bacteria. Variation with nutrient amendment, the range of bacteria from the treatments process were from 4.1E+04 cfu/g soil to 1.3E+08 cfu/g soil. The result specifically present that addition of nutrient to the soil cause higher bacteria population. Hence, bacteria can produce more enzyme for catalyzing the pollutant so that it increase removal of  $Hg^{2+}$ . The increasing of population of bacteria prove to the possibility of growing condition and this is a good sign that the bacteria has potential for the transforming the harmful substance as mercuric to less toxic of elemental mercury. The lack of proportionality between respiration rates and DOM released in this study suggests that higher amounts of bioavailable C did not lead to higher biomass, instead the microbial community and associated metabolic response has shifted toward more copiotrophic organisms [17]. Addition of low molecular weight C compounds (glucose, citric acid, glycine) to soil has been previously observed to shift the structure of bacterial communities to more copiotrophic organisms with no strong correlations between respiration rates and community structure [26].

#### 4. CONCLUSION

This bioremediation technique is a green and economical technology which is seen as an efficient method in detoxifying mercury which could be applied in water, sediment and soil. The indigenous consortium of bacteria has enough capability to transform the toxicity form of mercury to the less toxic form. Additional of  $HgCl_2$  or higher concentration of mercury did not affect the growth of the bacteria but it prolonged the lag phase

because cells require adaptation against the toxicity. This is because growth rate of mixed cultures depended on the concentration of the carbon substrate (nutrient) present in culture media and was unaffected by the type of mercury added. Environmental factors such as temperature, pH and nutrient amendment take a crucial role in enhancement of bioremediation of mercury from soil.

#### 5. ACKNOWLEDGMENTS

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