Potential of Soil Bacteria as Mercury Bioremediation Agent in Traditional Gold Mining

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Abstract

Mandor Village has developed as a tradisional gold mining area since years ago. It involved activities that have led to extreme land condition and the release of mining residues, i.e., mercury, to the soils. The study examined the potential of soil bacteria as mercury bioremediation agent based on their population and activity in former mines with different ages. The bacterial population was measured by isolating soil bacteria on solid media using the pour plate method, and the colonies were enumerated during the incubation. The Nutrient Agar (NA) medium was used to obtain the total population, whereas the Salt Base Solution (SBS) was to determine the presence of mercury-tolerant bacteria. The addition of $HgCl_2$ affected the number of the colonies. The colony only grew until the concentration of $HgCl_2$ reached 5 mg/l, and the total colony was larger in older mines. The observation of bacterial activity showed that biotransformation performance was lower when the concentration of mercury was the same as its natural presence in soils (0.1-0.5 mg/l) compared with higher mercury level (1 mg/l). The research showed that lower mercury concentrations in nature reduced the natural ability of bacteria to transform pollutants. This study provides information that can assist the development of a technological approach to control mercury pollution in former traditional gold mines in an environmentally friendly manner using indigenous soil bacteria.

How to Cite

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INTRODUCTION

The traditional gold mining activities conducted by the community in Mandor Village, Landak Regency, West Kalimantan Province, known as PETI (*Penambangan Emas Tanpa Ijin*—Unlicensed Gold Mining), changed the physical, chemical, and biological characteristics of the soil in the environment. PETI is known to release harmful pollutants into the environment, namely mercury. Mercury is the most dangerous contaminant disposed to the environment, yet miners still use it as a gold binder to separate gold from sand and soil. Although mercury-based binders are not an efficient technology, they make the gold refining process cheaper, reliable and simple (Fahruddin, 2014). Aside from the high toxicity, in nature, mercury may be present at an alarming concentration because it can accumulate at various levels of the food chain (Jan et al., 2009).

As a result of mining activities, changes in soil characteristics like temperature, pH, nutrients, minerals, and heavy metal concentration, potentially affect the number and activity of soil microorganisms. This microbial parameter is a potential indicator in monitoring heavy metal-contaminated soil through two ways: first, by measuring the population size of the microbes and, second, by assessing their activity in removing the pollutants (Ghorbani et al., 2002). Good soil quality is characterized by a high level of microbial diversity (Sarma et al., 2010).

The interaction between microorganisms and the environment creates an interplay. Environmental conditions determine the selection of microorganisms by providing an enabling habitat and regulating food availability and product elimination. Meanwhile, the community of microorganisms controls the environmental conditions through the reactions of their metabolic products against the environmental components (Guimarães et al., 2010). Although there are limitations in extreme environments, soil microorganisms can remain highly active to quickly change the environmental conditions through the excretion of their metabolic products (Santini et al., 2015). The reduced condition extremity indicates favorable feedback from microbial communities to the environment (Schmalenberger et al., 2013). In reality, there is a negative and complex relationship between the population of microorganisms and metal concentrations (Ghorbani et al., 2002). At a certain concentration, metals disrupt the cellular functions and immediately cause apoptosis. These substances, then, gradually change the population based on the competitive ability and survival of the microorganisms.

Microorganisms activity can change the active metals in contaminated soil to inactive ones (Huang et al., 2006). However, not every species of microorganism can respond to the toxicity of heavy metals. For example, based on their nature, bacteria can be classified into Gram-positive and Gram-negative that differ in their interaction with metals, meaning that every microorganism has a different sensitivity to the toxicity of a pollutant. However, external factors, e.g., the characteristics of contaminants, also determine the microorganism activity against metals.

Bacterial growth depends on the type and size of metallic pollutants (Anyanwu et al., 2011). Therefore, the presence of mercury in the soil certainly affects soil bacterial population. Aside from type and concentration, the duration of pollutants remaining in the soil also controls bacterial growth. The longer the pollutants in the environment, the longer their interaction with bacteria leads to the higher tolerance level of the bacteria. Heavy metal-contaminated environment likely contains microorganisms that can adapt to and become resistant to the toxicity of heavy metals (Ahmad et al., 2005). Bacteria that are resistant to certain metals are also resistant to other metals (Jaysankar & Ramaiah, 2008). Mercury-tolerant bacteria can endure other heavy metals (Keramati et al., 2011). Bacteria that are resistant to non-metallic pollutants can also withstand many types of metallic contaminants. The presence of resistant bacteria in nature is an example of microorganisms' genetic and physiological adaptation to contaminants in their environment (Lima et al., 2009).

Indigenous bacteria can transform metal pollutants in the environment (Sasi et al., 2018). The enumeration of the population of mercury-tolerant bacteria is the first step in identifying the potential of soil bacteria as bioremediation agents in metal transformation. The age of mine shapes the soil properties that are assumed to affect the population and activity of mercury-tolerant soil bacteria. Therefore, the tolerance level of bacteria to mercury in the soil is necessary to be determined. This research aimed to examine the potential of soil bacteria as bioremediation agents from their population and activity in former mines with different ages. There is little information about this potential of soil bacteria. Researchers can use it as a basis for designing the recovery of former traditional gold mines with environmentally friendly in situ bioremediation, which has never been performed in Indonesia.

METHODS

The research method included the determination of soil bacterial population and activity from the collected samples in the laboratory. Each of these steps is explained as follows.

Sample Collection

The research took place in Mandor Village, Mandor District, Landak Regency, West Kalimantan Province. This area is the house of traditional gold mining activities that have long been developed to date. This quantitative research was conducted in a laboratory using the following parameters: soil characteristics at each age group of mines, the total population of soil bacteria, the population of mercury-tolerant bacteria, morphology test results, and mercury concentration in the media. The soil samples weighed ± 1.5 kg, collected at the depths of 1-10 cm (Schmalenberger et al., 2013; Simarro et al., 2013). Three sampling points were determined randomly at each age group of mines. The mercury concentration in the media was measured using the composite samples of each age group to identify the microbial mercury transformation activity.

Determination of Soil Bacterial Population

The soil bacterial population was isolated in solid media using a pour plate method, then the bacterial colonies were observed visually (shape and color) and enumerated during the incubation time (Anyanwu et al., 2011). The solid media used in this research were Nutrient Agar (NA) to obtain the total bacterial population during the following incubation time: 1, 2, 3, 4, 5, 6, 7, 14, 21, and 28 days and Salt Base Solution (SBS) to determine the presence of mercury-tolerant bacteria during the following incubation time: 1, 7 14, 21, and 28 days.

Determination of Bacterial Activity

The microbial mercury transformation activity was examined by adding 1% inoculum to the control media (without $HgCl_2$) and the nutrient-rich *Luria Bertani* media—which had been enriched with 0.1 mg/L, 0.5 mg/L, and 1 mg/L $HgCl_2$—and then measuring the resulted mercury concentration. The total mercury concentration in the media was measured every 0, 1, 2, 3, 4, 5, 6, and 7 days using a mercury analyzer. The color, shape, and type of the cells were observed microscopically and analyzed with the Gram staining in the laboratory.

RESULTS AND DISCUSSION

The abundance of an organism in the environment was determined by nutrient availability and various physical-chemical factors, e.g., temperature, redox potential, and pH (Wingfield et al., 2011). The extreme environmental conditions in the study area included soils with low pH (<4.5), relatively low water content (<30%), high temperature (>30ºC), low nutrition (C<3% and N<2%), high redox potential (>200 mV), and mercury content in the soil (0.1-0.5 mg/kg) (Table 1). These soil conditions potentially affect the soil bacterial population in the study area.

The enumeration of the bacterial colonies in the NA media indicated a rapidly growing population (Table 2). The size of the bacterial population in the soils of young mines (t<5 years) was much larger than in older sites. The measured number of colonies was expected to illustrate the presence of bacterial populations in a natural environment.

In the NA media, the population size in every age group of mines showed an increase since Day 2 and a significant rise on Day 4, 6, and 7. The bacteria reached their stationary growth phase in the third week of incubation (Day 14-21) before decreasing on Day 28. Nutrients were the main cause of this growth trend. On the early days of the incubation period, nutrients were not a limiting factor in bacterial growth, but later they existed in a limited amount and continuously decreased to a point where their low concentrations disrupted bacterial growth and even led to deaths in most of the population. Microorganisms require nutrients to grow and divide or replicate themselves (Adams et al., 2015). Nutrients are a basic element that becomes a growth-limiting factor that determines the ability of a microorganism to prepare the necessary enzymes in contaminant removal (Vidali, 2001).

The comparison between the soil bacterial population at the three age groups of mines (Figure 1 and Figure 2) shows that Point C (t<5 years) has a larger size of bacterial population than Point A (t>10 years) and Point B (5<t<10 years). The carbon concentration (C) and the C/N ratio in Point C are relatively higher than in the other two points. Carbon is a basic element in life, and it is needed in large numbers than any other elements. Carbon, together with hydrogen, oxygen, and nitrogen, makes up 95% of the cell weight (Vidali, 2001). In other words, the two soil properties in Point C induce a high abundance of the bacterial population. Therefore, when compared

Table 1. The Soil Characteristics of the Research Location

Age Groups of Mines	Parameters						
	pH	Water Content (%)	Temperature (°C)	C (%)	N (%)	Redox Potential (mV)	Hg (mg/kg)
>10 years	4.23	14.19	30.83	1.44	0.19	356.27	0.51
5-10 years	3.7	20.07	31.27	0.89	0.12	392.7	0.11
<5 years	4.05	13.72	31.5	3.00	0.35	415.63	0.27

Table 2. The Size of the Soil Bacterial Population in the NA Media

Incubation Periods (days)	Number of Colonies (CFU/ml) (x10³)		
	A (t>10 years)	B (5<t<10 years)	C (t<5 years)
1	0.0	0.0	0.0
2	1.0	0.3	306.0
3	1.0	0.3	306.0
4	37.7	5.0	296.7
5	38.0	6.0	264.3
6	45.7	47.3	314.0
7	50.3	41.3	3836.0
14	71.0	81.7	23,418.7
21	74.7	81.7	23,462.0
28	51.3	14.0	94.7

to Points A and B, the colonies isolated from the soils in Point C are present in a higher number.
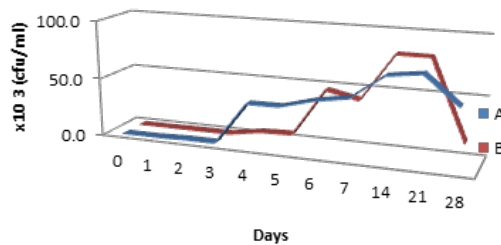

Figure 1. The population size of the soil bacteria isolated from Point A (t>10 years) and Point B (5<t<10 years) in the NA media during the 28-day incubation period
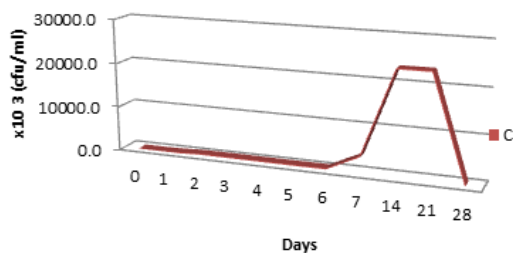

Figure 2. The population size of the soil bacteria isolated from Point C (t<5 years) in the NA media during the 28-day incubation period

The bacteria in the SBS media had a slow start of growth, and the population size was smaller than the one in the NA media (Table 3 and Figure 3). Energy and carbon sources (or electron acceptors) for microbial metabolism was available in the NA media, but they only existed at a limited amount in the SBS media. Carbon functions not only as an electron donor in cellular metabolism but also as a constitutive element of cells (Groudev et al., 2010). Since the soil bacteria in Point C had higher C content and C/N ratio in their natural environment, their colonies experienced difficulty in adapting to low nutrients, became undeveloped, and survived only until Day 21 in the SBS media. On the contrary, the colonies isolated from Points A and B had lower natural C content and C/N ratio.

Consequently, they had relatively better adaptability in the SBS media than the bacterial colonies isolated from Point C. The population of the soil bacteria in Points A and B grew until Day 28, and it was estimated to grow in prolonged incubation time continuously. The bacteria in Points A had the best adaptability among the sampling points in this research. This condition shows that, without the presence of pollutants in the soils, length of time and adaptability to extreme (i.e., nutrient-poor) environments from which the bacteria originate are the two factors that shape their adaptability in the media. Bac-

Table 3. The Size of the Soil Bacterial Population in the SBS Media

Incubation Periods (days)	HgCl$_2$ Concentrations (mg/L)	Number of Colonies (x10^3)(CFU/ml)		
		A(t>10 years)	B(5<t<10 years)	C(t<5 years)
1	0	0.0	0.0	0.0
	1	0.0	0.0	0.0
	5	0.0	0.0	0.0
	10	0.0	0.0	0.0
7	0	9.6	0.3	0.3
	1	5.8	6.7	3.4
	5	0.0	0.0	0.0
	10	0.0	0.0	0.0
14	0	20.9	11.6	0.5
	1	11.4	19.0	10.2
	5	3.7	0.1	0.4
	10	0.0	0.0	0.0
21	0	21.2	9.0	2.2
	1	15.9	9.0	17.5
	5	7.1	0.1	0.4
	10	0.0	0.0	0.0
28	0	30.4	12.6	0.8
	1	9.6	5.6	11.5
	5	3.7	0.1	0.0
	10	0.0	0.0	0.0

teria originating from the local environment can easily adapt to a polluted one (Rodrigues et al., 2015)acrylic coupons with a thin layer of oil on the surface were incubated in the coastal water of Trindade Island, Brazil, for 60 days. The microorganisms adhered to the coupons were isolated using enrichment medium with hexadecane and naphthalene as the sole carbon and energy source. A total of 15 bacterial isolates were obtained, and the ability of these isolates to use different hydrocarbons as the source of carbon and energy was investigated. None of the isolates produced biosurfactants under our experimental conditions. Subsequently, identification methods such as partial sequencing of the 16S rRNA gene and analysis of fatty acids (MIDI. Wingfield et al. (2011) explain that some microorganisms have genetic equipment that can overcome metals and survive in extreme conditions. Although microorganisms are isolated from extreme environments, most of them can grow optimally within a narrow range (Vidali, 2001).
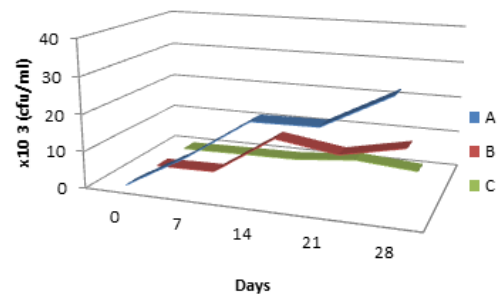

Figure 3. The population size of the soil bacteria isolated from Point A (t>10years), Point B (5<t<10 years), and Point C (t<5 years) in the SBS media without the addition of HgCl$_2$

Based on Figure 4 and Figure 5, the mercury concentration in the HgCl$_2$-enriched SBS media greatly influenced the size of the bacterial population. The bacteria in the media added with smaller concentrations of HgCl$_2$ formed higher population size. They grew slowly only until the

addition of 5 mg/L HgCl$_2$ and started to grow on Day 14 at different rates in each age group of mines. This finding is in line with the theory proposed by (Ghorbani et al., 2002), which states that the effects of heavy metal content on microbial growth and life are different from one concentration to another.
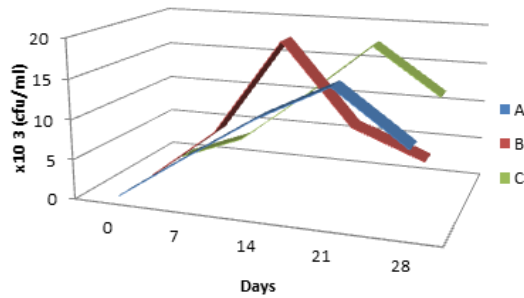

Figure 4. The population size of the soil bacteria isolated from Point A (t>10 years), Point B (5<t<10 years), and Point C (t<5 years) in SBS media enriched with 1 mg/L HgCl$_2$
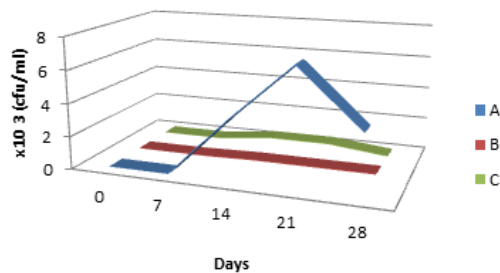

Figure 5. The population size of the soil bacteria isolated from Point A (t>10 years), Point B (5<t<10 years), and Point C (t<5 years) in SBS media enriched with 5 mg/L HgCl$_2$

In the SBS media enriched with 1 mg/L HgCl$_2$, the population sizes of all age groups of mines had a relatively similar figure (indicating comparable adaptability) possibly because the natural mercury concentration in the soil was close to 1 mg/L. At this concentration, the population in Points A and C increased from Day 0 to Day 21 then continuously decreased until Day 28, whereas the one in Point B increased from Day 0 to Day 14 then progressively decreased until the end of the incubation period on Day 28. This condition shows that when HgCl$_2$ remains at a concentration of 1 mg/L, it does not exhibit any significant effects on the bacterial growth and population size in the three age groups of mines, except for the different response of the bacterial population in Point B.

In the SBS media enriched with 5 mg/L

HgCl$_2$, a large bacterial population was only found in Point A with an increase from Day 7 to Day 21, while the bacterial growth in Points B and C was relatively slower. The different growth rates indicated that the bacteria in older mines had better adaptability to greater pollutant loads (i.e., higher mercury concentration). When bacteria can grow to certain population size, they are considered adaptive and resistant to polluted land, which means that their surface protein production can successfully bind heavy metals (Hardiani et al., 2011). In this research, large pollutant loads tended to delay the bacterial growth (i.e., started on Day 7) because bacteria needed time to adapt to the highly contaminated environment and, at the same time, prepared themselves for growth. Bacteria from the local environment can live in a polluted one for a long time, representing their ability to adapt (Rodrigues et al., 2015)acrylic coupons with a thin layer of oil on the surface were incubated in the coastal water of Trindade Island, Brazil, for 60 days. The microorganisms adhered to the coupons were isolated using enrichment medium with hexadecane and naphthalene as the sole carbon and energy source. A total of 15 bacterial isolates were obtained, and the ability of these isolates to use different hydrocarbons as the source of carbon and energy was investigated. None of the isolates produced biosurfactants under our experimental conditions. Subsequently, identification methods such as partial sequencing of the 16S rRNA gene and analysis of fatty acids (MIDI.

When exposed to 10 mg/L HgCl$_2$, no colonies could grow until the end of the incubation period. All bacterial populations could not adapt to the toxicity of mercury at a concentration of >5 mg/L, i.e., the highest mercury concentration that still allowed the growth of mercury-tolerant bacteria in the study area. This finding is different compared to the result of study by Anyanwu et al. (2011), which reported that the bacterial growth in mercury-contaminated soils is suppressed when the mercury remains at 300 mg/L and 500 mg/L during the 28-day incubation period. The underlying factor is the adaptability of soil microorganisms, which differs from one to another and depends on the concentration of heavy metals that can affect the microorganism growth and life (Ghorbani et al., 2002).

The treatments involving the addition of HgCl$_2$ as much as and slightly above its natural concentration (i.e., 0.1-0.5 mg/L and 1 mg/L, respectively) to the inoculum-enriched media aimed to provide an overview of microbial sensitivity to metal toxicity, as evident in the microbial

Table 4. The Mean Total Mercury Concentrations in Media from Day 0 to Day 7

Treatments	Total Mercury Concentrations (mg/L)							
	0	1	2	3	4	5	6	7
Control	0.007	0.001	0.006	0.002	0.005	0.011	0.011	0.004
0.1 mg/L	0.104	0.023	0.103	0.095	0.112	0.125	0.084	0.196
0.5 mg/L	0.478	0.125	0.131	0.084	0.378	0.372	0.490	0.151
1 mg/L	1.505	0.217	0.131	0.373	0.151	0.240	0.209	0.554

metal transformation activity after seven (7) days of incubation (Table 4). In this research, the addition of $HgCl_2$ in small concentrations yielded a constant total mercury concentration from Day 1 to Day 7. However, when added at greater concentrations, (0.5 mg/L and 1 mg/L $HgCl_2$) reduced the total mercury concentration during the measurement period.

Microbial metal transformation depends on how much interference the metal gives to microorganisms. Anyanwu et al. (2011) affirmed the direct correlation between metal concentration and microorganism growth. Furthermore, the information at which concentration the pollutant in the study area can stimulate bacteria to transform metals is important to identify. Accordingly, this research started the test with the known range of mercury concentration in the study area. In nature, the measured mercury concentration was between 0.1 and 0.5 mg/L. In the media enriched with a low concentration of $HgCl_2$ (0.1 mg/L; <0.5 mg/L), the measured total mercury concentration from Day 1 to Day 7 was static (Figure 6). This finding provides information that the biotransformation process of mercury occurs very slowly when the mercury concentration is low. In other words, when mercury is added at concentrations similar to its natural presence in the environment, it does not generate enough toxicity to increase the sensitivity of bacteria to performing biotransformation. Accordingly, at 0.1-0.5 mg/L, mercury does not appear to hinder microbial growth, but it allows microorganisms to survive instead (Figure 7). The presence of metal compounds at low concentrations is needed to maintain the productivity of coenzymes and the potential of cell membranes. It also functions as an ionic regulator in the environment.

On the contrary, in the media added with a high concentration of $HgCl_2$ (1 mg/L; >0.5 mg/L), the biotransformation process occurred relatively faster and reduced the total mercury by up to 60%. Using a variety of mercury concentrations, Santi & Goenadi (2009) affirmed that the use of 5 mg/L mercury can reduce approximately 53.3% of mercury content in the soil. Rayhan et al. (2016) prove that bacteria are resistant to mercury with EC50 values of 4.5 mg/L and 44.15 mg/L. According to their toxicity study, 79% mercury was volatilized in the culture solutions after six (6) days, and a small portion of mercury was accumulated in the cells.
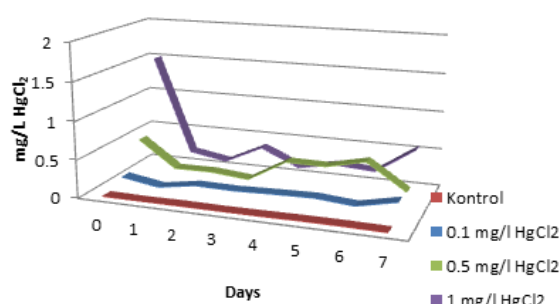

Figure 6. The mercury removal in the control treatment (without the addition of $HgCl_2$) and treatments with different levels of $HgCl_2$ (i.e., 0.1 mg/L, 0.5 mg/L, and 1 mg/L) from Day 0 to Day 7.

Figure 7. The bacterial population in the control treatment (without the addition of $HgCl_2$) and treatments with different levels of $HgCl_2$ (i.e., 0.1 mg/L, 0.5 mg/L, and 1 mg/L) from Day 0 to Day 7.
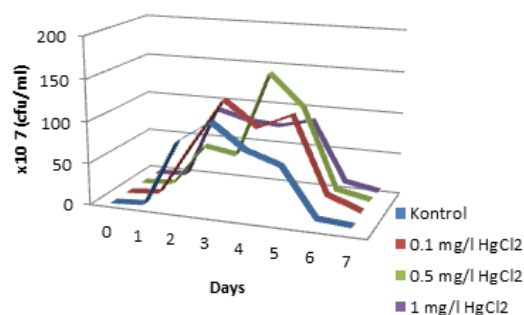
In general, when $HgCl_2$ was added at concentrations similar to the upper and lower limits of its natural presence (i.e., 0.1 mg/L and 0.5 mg/L, respectively), the size of the bacterial population grown in the media was larger than in the addition of 1 mg/L $HgCl_2$ (Table 5).

When added at concentrations of up to 1 mg/L, mercury increases bacterial sensitivity to

Table 5. The Mean Size of Bacterial Population from Day 0 to Day 7

Treatments	Number of Colonies in the i-th Day							
	0	1	2	3	4	5	6	7
	x 10^7	x 10^7	x 10^7	x 10^7	x 10^7	x 10^7	x 10^7	x 10^7
Control	0.02	2.86	81.50	108.50	79.50	66.00	9.13	5.95
0.1 mg/L	0.02	3.59	65.75	124.75	95.50	113.50	24.00	8.95
0.5 mg/L	0.02	3.10	54.25	47.50	150.75	113.50	17.30	9.20
1 mg/L	0.02	2.34	91.00	80.25	77.00	87.25	14.55	7.13

its toxicity. When microorganisms begin to feel threatened and are forced to defend themselves from the toxicity of metals, they prepare their genetic equipment (Wingfield et al., 2011) and transform the metals in their environment. Pepi et al. (2011) explain that the minimum concentration of Hg^{2+} that can hamper the growth of mercury-resistant bacterial strains is between 0.05 mg/L and 0.1 mg/L and, therefore, this range of concentration becomes the indication that the genera are resistant to a certain level of mercury. This research found that the presence of pollutants in relatively large quantities (1 mg/L) did not prevent bacteria from growing although their population growth from Day 0 to Day 7 was not as large as when $HgCl_2$ was added at smaller concentrations (0.1-0.5 mg/L). The number of colonies was 150.75 x10^7, close to the number of colonies identified in Santi & Goenadi (2009), i.e., 10^8 after seven (7) days of incubation.

The bacterial morphology test results showed two shapes of cells, *cocci* (spherical) and *bacilli* (rod-shaped). Based on the Gram staining, all of them were Gram-negative bacteria.

Table 6. The Cell Morphology Test Results of Mercury-Resistant Bacteria

Colors	Shapes	Gram Staining Results
White	Spherical	Negative
White	Rod-shaped	Negative
Orange	Spherical	Negative
Yellow	Rod-shaped	Negative

Based on the microscopic observations of the cells, the entire colony in this research was red and Gram-negative. These results are in line with Nofiani & Gusrizal (2004), which examine the narrow spectrum of the mercury-resistant bacteria in former PETI (i.e., unlicensed gold mining) at the same location. Gram-negative bacteria have a higher tolerance to metals than the Gram-positive because they have cell walls with a more complex structure that can bind and immo-

bilize metallic ions, including Hg^{2+} (Amaral et al., 2014). Ahmad et al. (2005) suggest that the toxic effects of heavy metals can be blocked by the extracellular polysaccharides produced by cells.

The research showed that lower mercury concentrations in nature reduced the natural ability of bacteria to transform pollutants. Acting as preliminary data, this information proves that mercury bioremediation in mining land with relatively low mercury concentration requires biostimulant. In other words, the indigenous soil bacteria can transform mercury after receiving biostimulants, such as nutrients and aeration, which until now has never been performed in situ in Indonesia. These findings can assist researchers to develop a technological approach to control mercury pollution in former traditional gold mines in an environmentally friendly manner using indigenous soil bacteria.

CONCLUSION

Soil bacteria, particularly in old mining sites (t>10 years), have the potential to transform mercury that is present with higher concentrations than its natural level in soils (<5 mg/l). There is a relationship between the natural concentration of mercury and the ability of Hg-tolerant bacteria to transform mercury in the media. When exposed to mercury at the same concentration as its natural presence (0.1-0.5 mg/L), the bacteria are not stimulated. Accordingly, the transformation process runs very slowly and has low performance. Conversely, adding mercury at higher concentrations (1 mg/L) can stimulate bacterial activity to transform this metal with a better level of performance.

ACKNOWLEDGMENT

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