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Indigenous Mercury Resistant Bacterial Isolates Belong To The Genus *Bacillus* From Kalimas Surabaya As A Potential Mercury Bioreducer

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ABSTRACT

Three potential mercury resistant isolates (11 mg/L $HgCl_2$) belong to the genus *Bacillus*, namely *Bacillus* sp. S1, SS19, and DA11 obtained from Kalimas Surabaya were studied. Bacterial isolates were inoculated into nutrient broth containing 10, 15, and 15 mg/L $HgCl_2$, and incubated for 24 h at room temperature. Hg^{2+} concentration determination was prepared according to standard procedure of US Environmental Protection Agency (EPA) 200.7, and measured by Inductively Coupled Plasma Atomic Emission Spectrometry(ICP). The amount of Hg^{2+} reduction by the isolates was expressed as percentage of reduction from the original concentration. Results of the study showed that the highest capacity of the isolates to reduce $HgCl_2$ were different, namely, the isolates DA11 and S1 could reduce 74.5% and 63.6% at the original concentration of 10 mg/L, respectively, whrereas the isolate SS19 could only reduce 53.6% at the original concentration of 5 mg/L. Therefore, in terms of the capacity of bioreduction, the indigenous bacterial isolate (DA11) belong to the genus *Bacillus*could be used as potential mercury bioreducer.

KEYWORDS: Kalimas-Surabaya, *Bacillus*, mercury resistant, bioreducer

INTRODUCTION

Mercury is a toxic and hazardous heavy metal despite in a very low concentration [1]. Kalimas is one among the river in Surabaya Indonesia used to be raw material of drinking water. Government Act No. 82/2001 detetermined that mercury concentration in water is 0.001 mg/L, however, the concentration of mecury in the sediment of middle part of Kalimas river was found to be 0.105 mg/L [2] and the concentration of mercury in the sediment from downstream of the Kalimas river even reached 6.3 mg/L [3]. Such concentration of mercury was much more higher than the threshold value requires by the Government of the Republic of Indonesia. Therefore, the problem of mercury contamination of drinking water raw material source needs serious attention.

Bacteria are microorganisms which may be resistant to mercury and such group of bacteria is called mercury resistant bacteria [4]. The mercury resistant bacteria has capacity to adapt phisiologically and genetically [5] by means of *mer-operon*, a gene bearing by plasmids [6]. The *mer-operon* gene coding for mercury reductase is responsible to reduce Hg^{2+} to volatile Hg^0 [7]. The isolates of indigenous mercury resistant bacteria (DA11, S1, SS19) belong the genus *Bacillus* obtained from Kalimas River at Surabaya [8] are very potential to be used as mercury bioreducer due to their high resistancy to mercury of 11 mg/L $HgCl_2$ [9].

Mercury resistant bacteria was reported to be potential candidate for bioremediation agent [10] such as strain member of the genus *Bacillus* isolated from the Gulf of Mexico found to be able to reduce mercury up to 68% [11]. Subsequently, other isolates of *B. cereus* JUBT1 was reported to be able to eliminate almost all Hg^{2+} from the growth media [12]. In this study, potential bioreducer of indigenous mercury resistant bacteria belong to the genus *Bacillus* obtained from Kalimas Surabaya is reported.

MATERIALS AND METHODS

Determination of growth curve

One mL of a 12 hours old culture of the *Bacillus* sp. isolates (S1, SS19,DA11) and reference strain of *B. cereus* ATCC1178, containing 10^8 cell/mL were inoculated into a 50 mL of Nutrient Broth containing 1 mg/mL $HgCl_2$, incubated at room temperature for 24 hours on rotary shaker with speed at 100 rpm. Then, 5 mL of each strain culture was inoculated into 45 ml Nutrient Broth containing 1 mg/mL $HgCl_2$ and incubated at room temperature for 24 hours on rotary shaker with speed at 100 rpm. The growth curve of each strain was drawn based on OD_{600} measured spectrophotometrically at one hour interval time during the growth by using UV pectrophotometer Boeco S-22[13]. The age of culture to be used in the experiment of mercury bioreduction was based on mid point between the early exponential phase and the late exponential phase of each strain.

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Bioreduction of Hg^{2+} ions

The bioreduction of Hg^{2+} ions was parepared according to Colak *et al*., 2011 [14] and Hg^{2+} concentration was measusred according to standard procedure of US Environmental Protection Agency (EPA) 200.7 [15]. One mL of broth bacterial culture of 12 hours old containing 10^8 cell/mL was used to inoculate 50 mL nutrient broth medium containing of 5, 10, and 15 mg/L $HgCL_2$, and the same medium without innoculation was used as a control. The culture were incubated at room temperature for 24 hours then centrifuged at 4000 rpm for 30 minutes. Supernatant was then placed into a test tube, mixed with 0.5 mL concentrated HNO_3 and heated at $\leq 80^oC$ for ± 1 hour [15]. Measurement of Hg^{2+} concentration was carried out by Inductively Coupled Plasma Atomic Emission Spectrometry(ICP). The bioreduction capacity of the bacterial isolate was expressed as the difference between original and final concentration of Hg^{2+} according to the following formula of $c = a\text{-}b$; where c is Hg^{2+} bioreduction of the bacterial isolate, a is the original concentration of Hg^{2+} in the control experiment (uninoculated medium), b is the final concentration of Hg^{2+} in the treatment experiment (inoculated medium). The bioreduction efficiency of the bacterial isolate was determined by a formula of : $(c/a)100\%$.

Determination of cell viability

Viability of bacterial cell exposed to $HgCl_2$ was checked by density determination after 24 hours of exposition time. The bacterial density was determined directly by hemocytometer Neubauer and expressed as cells/mL as well as by viable count using pour plate method and expressed colony forming unit(CFU)/mL [16].

RESULTS AND DISCUSSION

Determination of growth curve

The growth curve (Figure 1) was used to determine the mid point between the early and the late exponential phase. As shown by Figure 1 all isolates indicated the same pattern of growth curve. The early exponential phase were at about 3-5 hours after incubation, and the late exponential phase were at 19 hours after incubation. Therefore, the mid point between early and late exponential phase for all isolates were concluded at about 11-13 hours after incubation. Based on the results, all experiment with the isolates were using the 12 hours old culture.

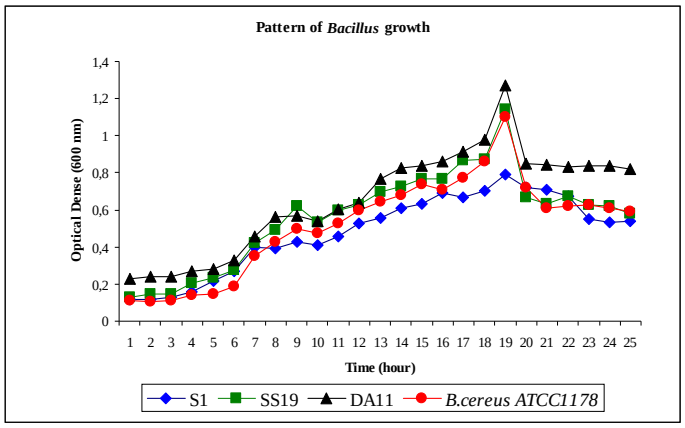


Figure 1. Growth curve of bacterial strains grown on Nutrient Broth containing 1 mg/mL $HgCl_2$ and incubated at room temperature.

Bioreduction of Hg^{2+} ions

The real concentration of $HgCl_2$ for theoritical concentration of 5, 10, and 15 mg/L after determining by ICP was 4.35, 7.34, and 10.51 mg/L, respectively. Therefore, those values were used as the initial concentration for each treatment. The results of mercury bioreduction is shown in Table 1.

Based on statistical analysis (*analysis of variant*), it was found that mercury concentration influenced significantly the eficiency of mercury bioreduction. However, the mercury concentration did not infulence the bioreduction capacity of each bacterial strain.

Table 1. Bioreduction of Hg^{2+} by *Bacillus* sp. isolates and reference strain of *B. cereus*

$HgCl_2$ (mg/L)	Hg^{2+} (mg/L) (a)	Strain code of *Bacillus*	Residual concentration of Hg^{2+} (mg/L)(b)	Bioreduction of Hg^{2+}(mg/L) (c) = (a-b)	* Bioreduction efficiency ofHg^{2+} (%) (d) = (c/a x 100%)
5	4.354	ATCC1178	1.0483 ± 0.12	3.3057 ± 0.14	75.9^{a} ± 3.10
		S1	1.7143 ± 0.84	2.6397 ± 1.18	60.6^{c} ± 2.70
		SS19	2.0203 ± 0.03	2.3337 ± 0.07	53.6^{d} ± 1.50
		DA11	1.3270 ± 0.06	3.0270 ± 0.15	69.5^{b}± 3.50
10	7.343	ATCC1178	3.7152 ± 2.78	3.6278 ± 3.33	49.4^{c} ± 4.54
		S1	2.6720 ± 0.49	4.6710 ± 0.52	63.6^{b}± 7.10
		SS19	5.0224 ± 1.24	2.3206 ± 1.98	31.6^{d} ± 2.70
		DA11	1.8760 ± 1.27	5.4670 ± 0.05	74.5^{a} ± 0,70
15	10.514	ATCC1178	6.4488 ± 0.66	4.0652 ± 0.80	38.7^{a} ± 7.60
		S1	6.8622 ± 0.81	3.6518 ± 2.17	34.7^{b}± 2.06
		SS19	8.5565 ± 1.27	1.9575 ± 1.86	18.6^{c} ± 1.17
		DA11	9.1703 ± 0.22	1.3437 ± 0.59	12.8^{d} ± 5.59

*** $HgCl_2$ concentration significantly influence the bioreduction efficiency of Hg^{2+} but did not influence the bioreduction capacity of each strain. Number with different letter indicating the significant difference among treatments ($p < 0,05$; a > b > c > d)**

At the concentration of 5 mg/L, all strains showed the efficiency of greater than 50%. The highest efficiency of mercury bioreduction was by *B. cereus* ATCC1178 (79.5%) then followed by *Bacillus* sp. DA11 (69.5%), S1(60.6%), and SS19 (53.6%). The reference strain of *B. cereus* ATCC1178 and *Bacillus* sp. SS19 were more efficient at the concentration of 5 mg/L than that of 10 mg/L, but *Bacillus* sp. DA11 and S1 were more efficient at the concentration of 10 mg/L. However, all of the strains were less efficient at the concentration of 15 mg/L since the efficiency of bioreduction were les than 40%.

The results is in accordance with the finding of Chojnacka, 2010 [17] that mercury resistant-bacteria could be reduced and bioaccumulate mercury. According to Aksu and Dönmez, 2000 [18], the process of bioaccumulation is carried out in two stages. In the fisrt stage, mercury is biosorbed relativley in a very short time, and subsequently in the second stage, the mercury is bioaccumulated more slowly due to the macanism of active transport. Therefore, the capacity of mercury resistant-bacteria to biosorp and bioaccumulate mercury could be used to lower mercury concentration.

Based on the fact that the isolates of *Bacillus* sp. (DA11, S1, SS19) obatained from Kalimas Surabaya was found to be resistant to 11 mg/L of $HgCl_2$ [9], it could be suggested that the isolates may have *mer operon* genes which has been known as a mercury resistancy mechanism among bacteria [10]. The results of this study clearly support several other findings such as *Bacillus* strain isolated from tropical estuary habitat in Mexico could reduce mercury as much as 68% [11], *B. megatrium* strain isolated from Cisadane-Banten, Indonesia could accumulate mercury as much as 90% [19], and *B. cereus* JUBT1 isolated from chlor-alkali industrial mud could completely eliminate mercury from its growing medium [12]. It has also been foud that indigenous bacteria isolated from mercury-contaminated habitat is more effective to lower and to eliminate mercury and therefore it is very potential to be used as bioremediation agent[10,20]. It has been clear that *mer operon* is reponsible for bioreduction of ionic mercury (Hg^{2+}) to elemental mercury (Hg^{0}) by bacteria via mechanism of enzimatic reaction and ligand binding [6]. However, other mechanism of mercury biosorbtion also exists, namely the mechanism involves exopolysaccharide (EPS) on bacterial cell wall [21]. It is reported that EPS on bacterial cell wall could act as a chelating agent for heavy metal. Subsequently, it has been known that metal ion interaction with cell wall in gram positive bacteria, especially strains member of *Bacillus*, involve carboxyl group of peptidoglycan and or phosphory group of secondary polymer of teichoic acid and teichuronic acid [22].

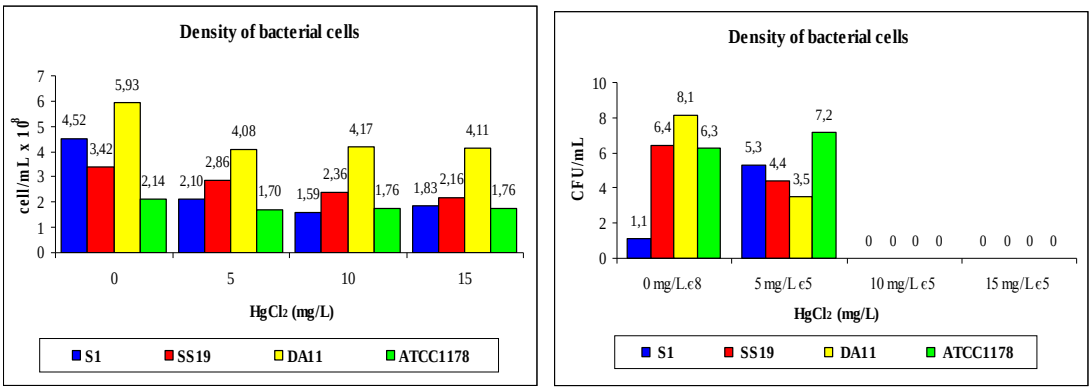
Cell viability

Cell viability was expressed as cell density measured by direct count of hemocytometer (cell/mL) and viable count of pour plate method (CFU/mL). The results of the experiment is shown in Table 2. At the concentration of 5 mg/L $HgCl_2$, cell density of *Bacillus* sp. DA11 was 4.1 x 10^8 cell/mL and 3.5 x10^5 CFU/mL.

Table 2. Bacterial cell viability during the exposition to various concentration mercury for 24 hours

$HgCl_2$ (mg/L)	Cell density	*Bacillus* sp.			*B. cereus* ATCC1178
		S1	SS19	DA11	
0	Σ cell x10^8/ml	4.5 ± 0.3	3.4 ± 0.4	5.9 ± 0.7	2.1 ± 0.4
	CFU x10^8/ml	1.1 ± 0.1	6.4 ± 0.3	8.1 ± 1.1	6.3 ± 0.7
5	Σ cell x10^8/ml	2.1 ± 0.2	2.8 ± 0.3	4.1 ± 0.2	1.7 ± 0.7
	CFU x10^5/ml	5.3 ± 0.8	4.4 ± 0.6	3.5 ± 0.4	7.2 ± 0.8
10	Σ cell x10^8/ml	1.6 ± 0.2	2.4 ± 0.3	4.2 ± 0.1	1.8 ± 0.03
	CFU x10^5/ml	0	0	0	0
15	Σ cell x10^8/ml	1.8 ± 0.1	2.2 ± 0.3	4.1 ± 0.01	1.8 ± 0.1
	CFU x10^5/ml	0	0	0	0

However, cell density of *Bacillus* sp. S1 by direct count was lower and only 2.1 x10^8 cell/mL although the density by viable count was higher (5.3 x10^5 CFU/mL) than the density of *Bacillus* sp. DA11. It can be concluded that cell viability decreased along with the increase of mercury concentration (**Figure 2**).



(A) Direct count (cell/ml) (B) Viable count (CFU/ml)

Figure 2. Density of bacterial cell after incubated for 24 hours

However, it is the fact that all the isolates could survive at the concentration of 5 mg/L $HgCl_2$ although they could not grow at the concentration of 10 and 15 mg/L $HgCl_2$ even after incubation of 3 days. On the basis of total count it showed that the isolate DA11 was the highest density, and followed by SS19, S1 and ATCC1178. However in viable count it was very clear that the most viable was *B. cereus* ATCC1178, followed by *Bacillus* sp. S1, SS19, and DA11. Therefore, among the test isolates, in terms of viability or survival rate, it could be seen that *Bacillus* sp. S1 is the best candidate for mercury bioremediation agent in comparison with DA11 and SS19 although its efficiency to bioreduce mercury (60.6%) was a bit lower than the isolate of DA11 (69.5%). It is interesting that the possibility of the three indigenous strains of *Bacillus* coul be combined together as a bioremediation agent in order to achieve the most optimum capacity to eliminate mercury from mercury contaminated waste.

CONCLUSION

From the results of the experimet it could be concluded that at the concentration of 5 mg/L all the test isolates are very potential to used as mercury bioremediation agent due to their capacity to bioreduce and bioaccumulate mercury of more than 50%. Among the three isolates tested at 5 mgL of $HgCl_2$, it was found that *Bacillus* sp. S1 was the best survivor after exposistion of 24 hours. However, it is interesting to study further the possibility of combination of the three indigenous isolate in order to develop the most potential mercury bioremediation agent from indigenous bacteria.

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