VOLATILIZATION MERCURY (II) INTO MERCURY (0) USING CRUDE ENZYME PRODUCED BY A MERCURY–RESISTANT AZOTOBACTERIA

Enny Zulaika[1]*, Anjar Lulu Sakinah[1], Kusnul Khotimah[1], Nengah Dwianita Kuswytasari[1], Nurhidayatul Alami[1], Nita Citrasari[2], Langkah Sembiring[3], Maya Shovitri[1]

*[1]Department of Biology, Faculty of Mathematics and Natural Sciences, ITS Surabaya, **INDONESIA***
*[2]Faculty of Science and Technology, Airlangga University, **INDONESIA***
*[3]Faculty of Biology, Gadjah Mada University, Yogyakarta, **INDONESIA***

*Corresponding author: enny@bio.its.ac.id

ABSTRACT

Mercury was poisonous and hazardous heavy metal, even one genus bacteria of Azotobacter was able to grow in a mercury contaminated habitat. This research aim was to select potential mercury resistant Azotobacter which was able to produce an extracellular mercury reductase for reducing toxic ion Hg^{2+} into a volatile less toxic ion Hg^0. The isolates were A1a, A5 and A9 from an urban farming soil in ITS. Their viability under mercury stress was tested individually in an Azotobacter-selective agar each containing 0.1; and 5 mg/L $HgCl_2$. The 24-hours bacterial growth was spectrophotometrically determined at λ600 nm each hour. The crude extracellular mercury reductase produced under 5mg/L dan 10mg/L $HgCl_2$ stress was then extracted followed general method. The enzyme activity was spectrophotometrically measured at 340 nm to detect soluble oxidized NADH in a defined medium for a mercury reductase assay after 12 hours incubation. The viability test showed that those 3 isolates had a similar growth curve pattern; all of them were growing under 0.1; and 5 mg/L $HgCl_2$, even after 12 hours incubation time they were start dying. Anyhow isolate A1a was the slowest growing Azotobacter under particular mercury stress. Enzyme activity tended to decrease over time; isolates A5 and A9 showed a greater enzyme activity than isolate A1a. Under 5mg/L $HgCl_2$ stress, after 30, 60 and 120 minutes, isolate A5 and A9 produced 2.50U, 1.15U and 0.60U enzyme respectively, while under 10mg/L $HgCl_2$ they were 2.70U, 1.44U and 0.7U.

***Keywords**: Azotobacter, $HgCl_2$, mercuric reductase, reduction*

INTRODUCTION

Mercury is a heavy metal with an unclear biological benefit, but it can negatively interfere bacterial metabolism in a very low concentration, since it is

able to form a harmful complex with other compounds inside organism (Drott *et al*., 2008) and it is not a degradable metal. Mercury contamination may occur in soil and water due to many causes, ex. gold mining, chemical industry, anthropogenic residues (Li et al, 2009). The form of ionic mercury (Hg^{2+}) is the most toxic state of mercury that can be accumulated in keedney, while if in form of methylmercury (CH_3Hg) it could be in brain (Chojnacka, 2010). Therefor Indonesia government has a restricted rule to protect environment from human commercial activity; acceptable mercury concentration in environment is 0.001 mg/L threshold.

In general ion mercury (Hg^{2+}) could inhibit bacterial growth at 5µg/L (Boening, 2000), but there is also mercury resistant bacteria which can survive under mercury stress (Brown *et al*., 2002). Zulaika *et al.* (2012) isolated *Bacillus, Staphylococcus*, and *Azotobacter* from Kalimas-Surabaya River that could still grow under a 10mg/L mercury stress. Khotimah dan Zulaika (2013) also reported that Azotobacter isolated from eco-urban farming ITS-Surabaya area was resistant to mercury up to 5 mg/L. Those isolates were very interested to be explored due to their resistance.

It has been reported that *mer*-operon played an important in mercury resistance mechanism; this operon produce a mercury reductase reducing toxic ion Hg^{2+} into a volatile Hg^0 with NADH or NADPH as an electron donor (Brown *et al.,* 2002) The volatile Hg^0 was then secreted out by bacteria to environment (Kiyono & Pan-Hou, 2006). A species of *Hydrogenivirga* sp. 379 128 R1-1 was able to reduce 150 µmol Hg^{2+} per 10^6cells/hour into Hg^0 (Freedman *et al*., 2012). Zulaika & Sembiring (2013) also reported tha *Bacillus* S1 was able to reduce 1.48 mg/L $Hg2^+$ per 10^9cells/minutes with 0.18% efficiency/minutes.

MATERIALS AND METHODS

Isolates

The isolate *Azotobacter* A1a, A5 and A9 was internal lab bacterial collection from an eco-urban farming Institut Teknologi Sepuluh Nopember (ITS), Surabaya-Indonesia. They were resistant to a 5 mg/L $HgCl_2$ (Sakinah & Zulaika, 2013).

Viability of *Azotobacter* in a $HgCl_2$ stress

Viability test was determining growth curve of isolate A1a, A5 and A9 in a 0.1 dan 5 mg/L $HgCl_2$ containing NB medium. One ose pure isolate *Azotobacter* was cultured in a 20 mL NB medium and incubated with shaking 100 rpm for 24 hours in room temperature. The culture was then transferred into 180 mL fresh NB medium containing 0.1 mg/L and 5 mg/L $HgCl_2$ separately. The growths were spectrophotometrically measured at $\lambda 600$ nm each hour for 24 hours at room temperature. The growth control was bacterial growth pattern in a NB medium without mercury.

Enzyme extraction

About 3 ml of a 12 hours pure *Azotobacter* culture was retransferred into 30 ml fresh NB medium containing 0.1 mg/L $HgCl_2$ and incubated for 12 hour at room

temperature until cells reached 10^9cells/mL. Bacteria were then harvested by do a 12,000 rpm centrifugation for 10 minutes at 4°C. Cell pellet was suspended in a 30 mL Phosphate Buffer Saline (PBS, pH±7) and destructed by sonication (600 watt, amplitude 50%) for 60 seconds at 4°C temperature. Destructed cell was then centrifuged again at 12,000 rpm for 30 minutes. The supernatant was the crude extract enzyme collected carefully into dark tube (Ogunseitan, 1998).

Mercuric reductase-enzyme

The crude extract enzyme was then added into a Mercury Reduktase Assay (MRA) solution with ratio 1:1, then 5 mg/L and 10 mg/L $HgCl_2$ separately added into it. A MRA solution were 50 mM PBS pH ±7; 0.5 mM EDTA; 0.1% (v/v) β-mercaptoethanol, 100 µM NADH, 0.2 mM $MgSO_4$ and $HgCl_2$ (Ogunseitan, 1998). The mixtures were incubated for 30, 60, and 120 minutes in dark room at room temperature. Spectrophotometer $\lambda\ 340\ \mathrm{nm}$ was used to measured enzyme activity (Takeuchi *et al*., 1999). Control was a MRA solution without crude extract enzyme.

One unit mercuric reductase activity was defined as a number of oxidized μM NADH per cell per minute (Ghosh *et al*., 1999) and reduced mg/L Hg^2 per minutes (Zeroual *et al*., 2003). The number of oxidized µM NADH was calculated based on a concentration standard curve; with a linier regression $y = ax + b$; $R^2 \geq 0.8$.

The number of reduced Hg^{2+} was calculated with equation (1) and (2)

(X µM NADH/1000000)*271.59*1000 (1)
X = oxidized NADH
1000000 = conversion M become µM
271.59 = relative molecular weight $HgCl_2$
1000 = conversion gram become mg

(Ar Hg/Mr $HgCl_2$)* mg/L reduced $HgCl_2$ (2)
Ar = Atom weight of Hg (200.59)
Mr = Molecular weight of $HgCl_2$ (271.59)

RESULT AND DISCUSSION

Viability *Azotobacter* toward $HgCl_2$

Isolates *Azotobacter* A1a, A5 and A9 from eco urban farming ITS were resistant to 5 mg/L $HgCl_2$ (Sakinah & Zulaika, 2013). Viability test was supposed to detect their growth under a mercury stress. Figure 1 showed that all three isolates had a similar pattern of cell growth curve under mercury stress; even they were more resistant in a 0.1 mg/L $HgCl_2$ stress rather than 5 mg/L $HgCl_2$. The lowest growth was significantly influenced by 5 mg/L $HgCl_2$ stress; this clearly showed by isolate A1a in which it fast decreased after 12 hours (**Figure1**). This indicated that all three isolate still kept doing binary regeneration even mercury slowing down the degeneration speed.

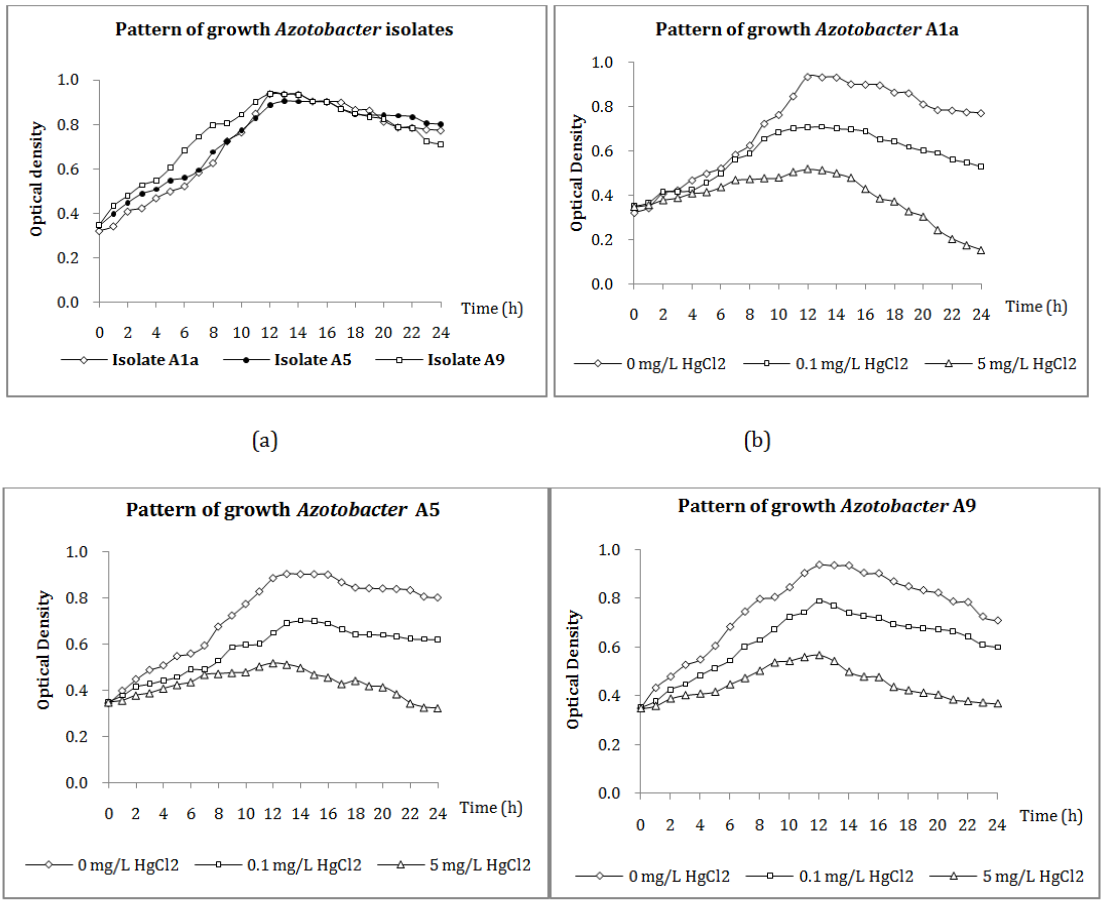

Figure 1. *Viability of isolate Azotobacter in NB medium. (a) without-$HgCl_2$ and (b,c,d) with $HgCl_2$*

It could be as preliminary suggestion that *Azotobacter* isolates has *mer* operon, but each with a unique gene expression regulation. Further molecular investigation should address this preliminary suggestion. *Mer*-operon consisted of many genes which were responsible for mercury reduction, for instance *mer*A gene coded mercuric reductase for reducing toxic Hg^{2+} into volatile Hg^0 (Takeuchi & Sugio, 2006), and a gene for mercury lyase which degraded methylmercury (CH_3Hg) to Hg^{2+} (Barkay *et al*., 2003). Different *mer* operon may influence different mercury resistance among isolates, as each species had unique *mer* operon composition. The regulation of gene expression of *mer* operon gene was also playing an important role as well for resistance in each bacteria. Many factors should be considered for gene regulation (Iyer *et al*., 2005).

Mercuric reductase activity

Figure 2 showed their mercury reductase activity; it was also decreasing over incubation time, for instance isolate A5 the highest one, under a 5 mg/L $HgCl_2$

mercury stress, after 30, 60 and 120 minute of incubation time reduced Hg^{2+} into Hg^0 with mercury reductase activity of $2.55*10^{-3}$U, $1.17*10^{-3}$U and $0.61*10^{-3}$U per 10^9 cells per minute, respectively. This enzymatic activity remained equal even isolate A5 was threated under a 10 mg/L $HgCl_2$ mercury stress. Isolate A1a showed the lowest mercury reductase activity. Decreasing enzyme activity over incubation time may due to depleting NADH as electron donor (**Figure 3**), since 100 µM NADH was the sole added electron donor in this reaction. NADH might play as electron donor or coenzyme for mercury reductase to reduce and volatilized Hg^{2+} into Hg^0 (Zeroual *et al.*, 2003). Takeuchi and Sugio (2006) mentioned that activity of mercury reductase was really NADPH/NADP dependent as it played as an electron donor. Nicotinamide group in reducing form can absorb light at 340 nm wavelength in volatility ion Hg^0 (Murray, 1996).

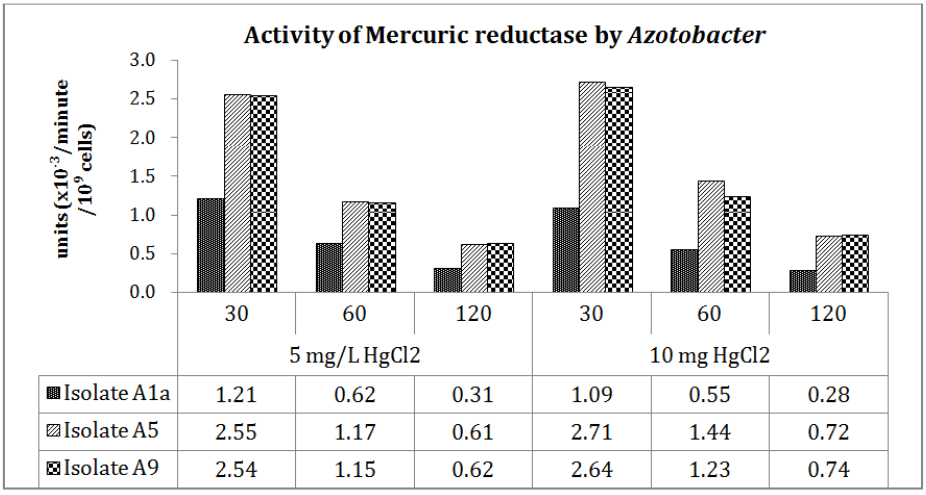

Figure 2. *Mercuric reductase activity in isolate Azotobacter*

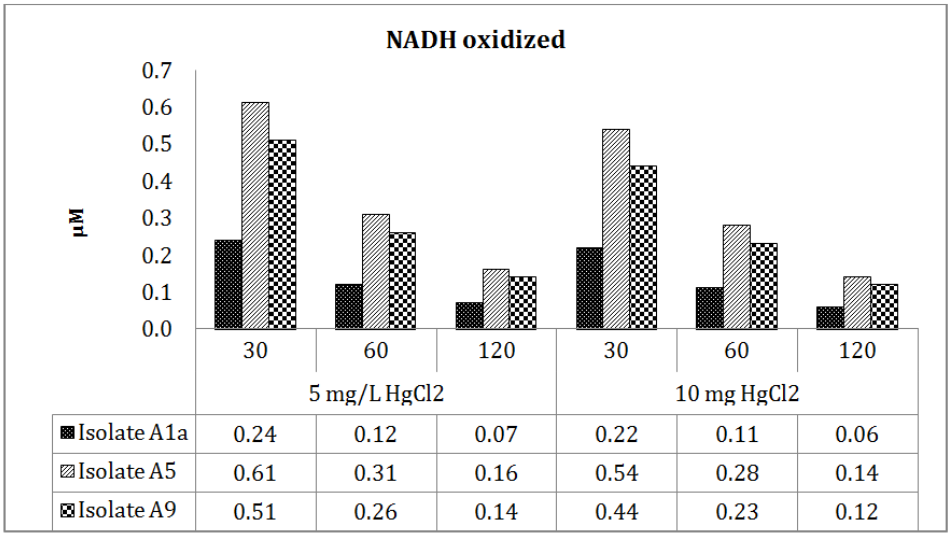

Figure 3. *Oxidized- NADH in isolate Azotobacter*

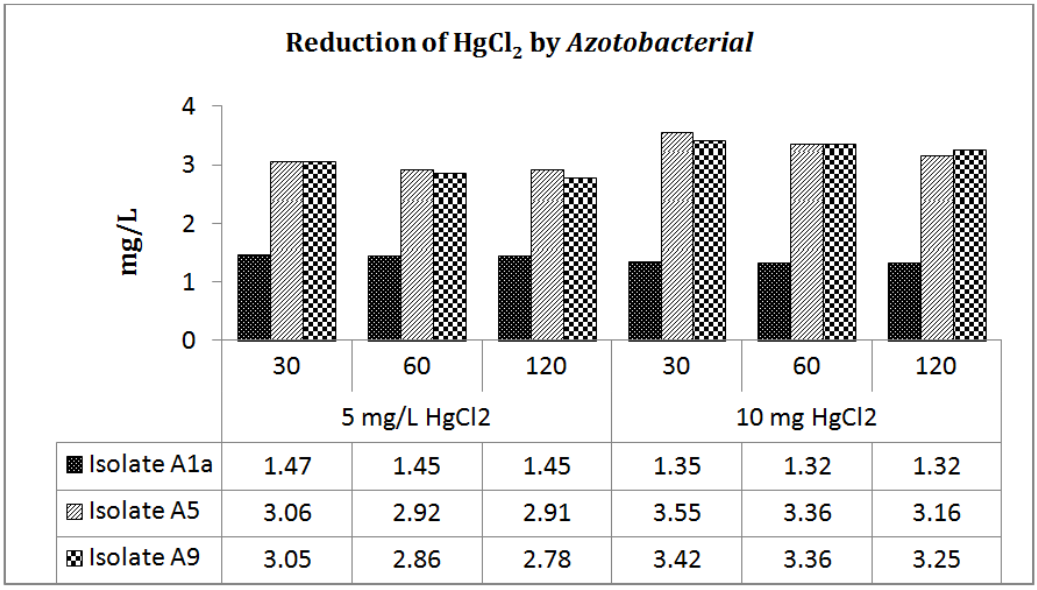

Figure 4. *HgCl2 reduction*

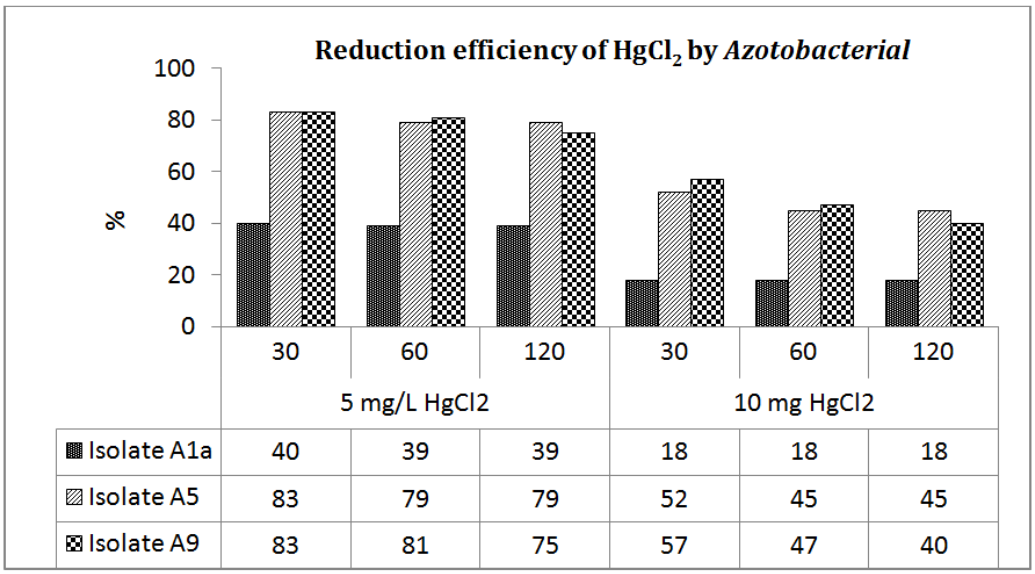

Figure 5. *HgCl2 reduction efficiency*

Complementary data showed in **Figure 4**. It was clearly seen that NADH oxidation was followed by Hg^{2+} reduction, as reduced Hg^{2+} was detected over time incubation; even no increasing reduced Hg^{2+} concentration over time. Based on Figure 4, isolate A5 and A9 seemly had the same reduction ability under 5 mg/L and 10 mg/L $HgCl_2$. But **Figure 5** indicated reduction was more efficient if under 5 mg/L $HgCl_2$. This opened other investigation to explore molecular study in protein expression.

CONCLUSION

Isolate *Azotobacter* A5 and A9 were excellent mercury-resistant bacteria that do volatilization by reducing toxic ion Hg^{2+} to a less toxic volatile ion Hg^0 in 30 to 120 minutes with maximum mercury reductase activity of $2.55*10^{-3}$U per 10^9 cells per minute. Those isolates were potential bioremediation agent to rehabilitate mercury contaminated field.

ACKNOWLEDGEMENTS

The research was supported by Institut Teknologi Sepuluh Nopember (ITS)-Surabaya, Indonesia, with Contract Number: 003246.256/IT2.11/PN.08/2015.

REFERENCES

1) Barkey, T., Miller, S.M. & Summer, A.O. 2003. Bacterial Mercury Resistance from Atoms to Ecosystem, *FEMS Microbiology Review,* 27(2-3): 355-384.
2) Boening, D.W. 2000. Ecological Effects, Transport and Fate of Mercury: A General Review. *Chemosphere,* 40: 1335-1351.
3) Brown, N., Shih, Y., Leang, C., Glendinning, K., Hobman, J. & Wilson, J. 2002. Mercury Transport and Resistance. International Biometals Symposium. *Biometals,* 715 - 718.
4) Chojnacka, K. 2010. Biosorption and Bioaccumulation: The Prospects for Practical Applications. *Environment International*, 36: 299-307.
5) Drott, A., Lambertsson, L., Björn, E. & Skyllberg, U. 2008. Potential demethylation rate determinations in relation to concentrations of MeHg, Hg and pore water speciation of MeHg in contaminated sediments. *Marine Chemistry*, 112: 93-101.
6) Freedman, Z., Zhu, C. & Barkay, T. 2012. Mercury Resistance, Mercuric Reductase Activities and Expression Among Chemotrophic Thermophilic Aquificae. *Applied Environmental Microbiology*, 10: 1-34.
7) Ghosh, S., Sadhukhan, P. C., Chaudhuri, J., Ghosh, D. K. & Mandal, A. 1999. Purification and Properties of Mercuric Reductase from *Azotobacter chroococcum*. *Journal of Applied Microbiology*, 86: 7–12.
8) Iyer, A., Mody, K. & Jha, B. 2005. Biosorption of Heavy Metals by a Marine Bacterium. *Marine Pollution Bulletin*, 50: 340-343.
9) Khotimah, K. & Zulaika, E. 2013. *Azotobacter* Sebagai Bioakumulator Merkuri. Jurnal Sains dan Senin POMITS, 2(1): 1-5, ISSN: 2337-3539 (2301-9271 Print)
10) Kiyono, M. & Pan-Hou, H. 2006. Genetic Enginering of Bacteria for Environmental Remediation of Mercury. *Journal of Health Science,* 52 (3): 199-204.
11) Li, P., Feng, X.B., Qiu, G.L., Shang, L.H. & Li, G.Z. 2009. Mercury Pollution in Asia: A Review of the Contaminated Sites. *Journal of Hazardous Materials*, 168: 591-601.

12) Murray, M. 1996. *The Encyclopaedia of Nutritional Supplements*. P. 44-53.
13) Ogunseitan, O. A. 1998. Protein Method for Investigating Mercuric Reductase Gene Expression in Aquatic Environments. *Applied Environmental Microbiology*, 64(2): 695-702.
14) Sakinah, A. S. & Zulaika, E. 2013. Resistensi *Azotobacter* terhadap $HgCl_2$ yang Berpotensi Menghasilkan Enzim Merkuri. Jurnal Sains dan Seni POMITS, 3(2). ISSN: 2337-3520 (2301-9271 Print)
15) Takeuchi, F., Iwahori, K. Maeda, T. Kamimura, K. & Sugio. T. 1999. Isolation and Some Properties of the Ferrooxidans Strains with Differing Levels of Mercury Resistance from Natural Environments. *Journal of Bioscience and Bioenginnering,* 88(4): 387-392.
16) Takeuchi, F & Sugio, T. 2006. Volatilization and Recovery of Mercury from Mercury-polluted Soil and Wastewater Using Mercury-resistant *Acidithiobacillus ferrooxidans* strain SUG 2-2 and MON-1. *Environmental Sciences,* 13 (6): 305-316.
17) Zeroual, Y. A., Moutaouakkil, F.Z., Dzairi, M., Talbi, P.U., Chung, K. L. & Blaghen, M. 2003. Purification and Characterization of Cytosolic Mercuric Reductase from *Klebsiella pneumonia*. *Annals of Microbiology,* 53: 149-160.
18) Zulaika, E. & Sembiring, L. 2013. Indigenous Mercury Resistant Bacterial Isolates Belong To The Genus Bacillus From Kalimas Surabaya As A Potential Mercury Bioreducer. *Journal of Applied Environmental and Biological Sciences,* 4(1): 72-76.