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Proliferation and exopolysaccharide production of *Azotobacter* in the presence of mercury

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ABSTRACT Bioremediation is a cheap, easy and effective method to improve the quality of heavy metal-contaminated agricultural land. Plant Growth Promoting Rhizobacteria recently has been proposed to be used in bioremediation of heavy metal. Rhizosphere-inhabitant *Azotobacter* produce exopolysaccharide (EPS) as a mechanism to avoid heavy metal poisoning; and in other hand EPS mobilize heavy metals in soil. The objective of this study was to get an information about growth and exopolysaccharide production profile of *Azotobacter* in media with and without mercury chloride. The bacteria were isolated from mercury-contaminated tailing at gold mining area in Maluku Province; and cultured in liquid medium containing 5, 10, 15 and 20 mg/L of $HgCl_2$. Cultures were incubated for 4 days at 115 rpm on gyratory shaker at room temperature. The results showed that all three isolates of *Azotobacter* enabled to grow in media with lower level of $HgCl_2$ but *Azotobacter* Buru-1 and Buru-2 did not grow on media with 20 mg/L of $HgCl_2$. *Azotobacter* bd3a was able to grow on media with 20 mg/L of $HgCl_2$ although the cell density was lower than that of control and lower level of mercury. The presence of mercury affected and generally suppressed the production of EPS; but the effect depend on the isolates. *Azotobacter* Buru-2 produced more EPS at 2 and 4 days after incubation in the presence of 20 mg/L of $HgCl_2$.

KEY WORDS Agricultural land; *Azotobacter*; Bioremediation; Mercury.

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INTRODUCTION

Illegal gold mining at Buru Island in Maluku Province, Indonesia produced million ton of tailing with low soil fertility and containing mercury since gold extraction was done with amalgamation process. Currently mercury-contaminated tailings was disposed improperly at gold mining site and agricultural areas. The relatively cheap, easy and effective way to reduce levels of mercury in agricultural land is bioremediation. The best way to reduce levels of heavy metals is increased mobility and availability of heavy metals in order to be more easily uptaken by plant accumulators of heavy metals. Recently Plant Growth Promotion Rhizobacteria (PGPR) has been developed as a bioremediation inoculant. *Azotobacter* is heterotrophic aerobic PGPR which are responsible for plant growth through non-symbiotic nitrogen fixation and phytohormone production.

One of EPS-producing microbes that potentially may be developed for the bioremediation of mercury-contaminated soil through the mechanisms of absorption is *Azotobacter*. Exopolysccharide (EPS),

an outer cell structures associated with the cell wall, is an important substance and has been known to have a real effect on the adsorption of metal (Prasad et al., 2014). The capacity of *Azotobacter* in synthesizing EPS has been widely reported (Vermani et al., 1997; Hindersah & Sudirja, 2010; Gauri et al., 2012). Exopolysaccharide is an extracellular polymer that can control mobilization of heavy metals (Chen et al., 1995; Hindersah et al., 2007; Micheletti et al., 2008). Exopolysaccharide production was mainly determined by the isolates and the presence of carbon and nitrogen available (Vermani et al., 1997; Hindersah & Sudirja, 2010). However, the production of EPS can be inhibited or induced by the presence of heavy metals such as although *Azotobacter* has been reported as soil bacteria that is resistant to mercury (François et al., 2011).

In the process to screening potent PGPR to remediate mercury-contaminated soil in Buru Island, some *Azotobacter* isolates have been isolated from tailing disposed on productive soil around gold mining area in Gunung Botak, Buru Regency. The bare tailings contain 10-306 mg/kg of total mercury deposited during three years mining from 2012–2105. The average total mercury concentration in agricultural land nearby mining area was 0.1–5 mg/kg which is normal for the soil formed for parent material containing no cinnabar, an more of mercury. This research was conducted to obtain the growth and EPS production profile of *Azotobacter* in the liquid media in the presence of mercury chloride. In the future, this *Azotobacter* will be used as bioremediation agent to decrease either total or available mercury level in tailing disposed on productive paddy soil.

MATERIAL AND METHODS

The study was conducted from May to June 2016 in Soil Biology Laboratory, Faculty of Agriculture, Universitas Padjadjaran. Source of isolates were mercury-contaminated tailings in the gold-mining area at District of Wamsait, Buru Regency, Maluku Priovince. *Azotobacter* isolates Buru-1 and Buru-2 isolated from the tailings contain 10 mg/kg of total mercury while *Azotobacter* bd3a was isolated from tailings containing 306 mg/kg of total mercury.

Three isolates of *Azotobacter* each were grown in a liquid medium described by Vermani et al. (1997) which contains 10 g sucrose, 1.0 g KH_2PO_4, 1.0 g $MgSO_4.7H_2O$; 0.5 g NaCl; 0.1 g $CaCO_3$; 0.1 g $NaNO_3$; 0.1 g $FeSO_4$; 10 mg Na_2MoO_4; 15 g agar; 1 L aquadest at pH 7, without and with 0, 5, 10, 15, and 20 mg/L of L $HgCl_2$. As many as 1% of pure cultures of *Azotobacter* on N-free media at a density of 108 cfu/mL was added into 25 mL of Vermani's liquid medium in the 100 mL erlenmeyer. Incubation was done on gyratory shaker with 115 rpm at room temperature for three days. Cell density of *Azotobacter* was measured every day for three consecutive days. Exopolysaccharide concentration in culture were analyzed at 2 and 4 days after incubation according to the method described by Vermani et al. (1997) modified by Hindersah & Sudirja (2010).

Bacterial cell density was determined by the Dilution Plate Method on Vermani's agar medium. The plates were incubated for 48 hours before the clear, convex and slimy *Azotobacter*'s colony were count. Exopolysaccharide production was determined by gravimetric, a total of 20 ml bacterial culture was centrifuged 9,000 rpm at 4 ^{0}C for 20 minutes; 10 mL of the supernatant was added with 20 mL of cold acetone and left overnight at 4 ^{0}C prior to centrifugation at 9,000 rpm at 4 ^{0}C for 20 minutes. Exopolysaccharide in the bottom of the tube was transferred to a Whatman no. 1 filter paper and heated at 35 ^{0}C for 1 hour and placed into a desiccator for 20 minutes before it was weighed. Acidity measurement was done by using potentiometer at room temperature; by dipping the electrode in bacterial liquid culture until a constant pH value.

RESULTS AND DISCUSSION

Viability of Azotobacter *in liquid media in the presence of mercury*

In this experiment, three isolates were cultured on liquid media both with and without mercury. The concentration of $HgCl_2$ were 5,10,15 and 20 mg/L equivalent to 3.7; 7.4; 11.1 and 14.8 mg/L mercury. All three isolates demonstrated the ability to live and proliferate on mercury exposure conditions expect Buru1 and Buru2 in higher mercury concentration (Table 1).

Azotobacter Isolates	$HgCl_2$ (mg/L)	Cell density (CFU)		
		Day 1	Day 2	Day 3
Buru 1	Control	55,000	820,000	11,900,000
	5	44,000	730,000	10,800,000
	10	22,300	410,000	4,500,000
	15	8,100	110,000	1,230,000
	20	0	0	0
Buru 2	Control	101,000	1,460,000	18,100,000
	5	98,000	1,330,000	17,200,000
	10	11,000	152,000	1,900,000
	15	4,200	67,000	700,000
	20	0	0	0
Bd3a	Control	98,000	1,480,000	17,900,000
	5	97,000	1,120,000	11,100,000
	10	77,000	960,000	10,300,000
	15	49,000	138,000	18,200,000
	20	28,000	350,000	480,000

Table 1. Effect of mercury chloride *Azotobacter* cell density in liquid culture after three days incubation with some level of mercury.

There was an increase of cell density from day one to day three after incubation. Comparing to control treatment, a clear decline in population was shown by all isolate cultured in medium with 15 mg/L and 20 mg/kg of mercury; isolate Buru-1 and Buru-2 did not grow in media with 20 mg/L of mercury. Decrease in mercury level caused decline in cell density especially in day three after incubation, explained that mercury has interfered with the metabolic system of *Azotobacter* especially Buru-1 and Buru-2. Isolates bd3a was more resistant to $HgCl_2$ at concentration of 15 and 20 mg/kg compared to another isolate. Cell of *Azotobacter* bd3a proliferated in liquid media with higher level of mercury although slower than cell proliferation in lower level of mercury and control treatment (Table 1).

Soil polluted by elevated levels of heavy metals caused negative effect on the activity of microbe and their diversity. According to Robinson & Tuovinen (1984), mercury resistant bacteria can be isolated not only from the location of mercury contaminated soil but also of sediment, waste oil and clinic or hospital. Bacterial resistance to mercury and mercury-containing organic material is determined by a plasmid, a small circular DNA molecules, which also encodes resistance to heavy metals and antibiotics. For example Actinobacteria is one of the bacteria that can reduce Hg (2+) to Hg (0) facilitated by the mercuric reductase (MERA); and plays an important role in biogeochemical cycles mercury in temperate environments (Møller et al., 2014).

Azotobacter resistance on mercury is also mediated by activity of mercury reductase and organomercury lyase which decreases the toxicity of mercury. *Azotobacter* can extend the phase lag with the presence of 10–50 mol/L of $HgCl_2$ and Nitrogen fixation capacity slightly inhibited when the bacteria were incubated with 10 mol/L of $HgCl_2$(Ghosh et al., 1996). Resistant *Pseudomonas*, *Cronobacter* and *Bacillus* bacteria detoxified mercury up to 95%; it was shown that *Cronobacter* species are the most efficient in eliminating mercury in NFB medium (Rafique et al., 2015).

Exopolysaccharide Profile

Production of exopolysaccharide (EPS) by three isolate of *Azotobacter* in Vermani's media with or

without mercury is shown in Fig 2. The presence of mercury influenced and generally suppressed production of EPS; but the effect depends on *Azotobacter* isolates. On day two, in general, the presence of mercury increased the concentration of EPS, but at 4 days after incubation there was a decrease in the production of EPS.

In general, EPS production of buru-1 and bd3a were significantly low compared to that of control, especially on day four. Two days after incubation the production of EPS of buru-2 with 20 mg/L of mercury was approximately 2-fold compared to control (Fig. 1). However at 4 days after incubation, the production of EPS of buru-2 decreased up to 50% in the presence of 20 mg/L of $HgCl_2$ (Fig. 2).

The presence of mercury in the media induced the production of EPS since bacteria develop mechanisms to avoid heavy metal poisoning. Exopolysaccharide can adsorb heavy metals before it is entering the system of metabolism. Mercury exposure for 4 days substantially reduces the concentration of EPS compared to the EPS production of *Azotobacter* in mercury-free medium (Fig 1).

The effect of Mercury on the synthesis of EPS by *Azotobacter* is reported elsewhere. EPS is formed to withstand drought, environmental stress, and especially to protect nitrogenase from oxygen (Sabra et al., 2000). Gupta & Diwan (2016) described that the establishment of bacterial EPS on the cell surface was to avoid heavy metal toxicity. Exopolysaccharide is one of the outer structure of prokaryotic and eukaryotic microbial cell; in the form of capsules or secreted as mucus that is not strongly attached on the cell surface (Prasad et al., 2014). Exopolysaccharide is a ligand that binds to metals through hydroxyl and carboxyl (Chen et al., 1995; Janecka et al., 2002) to facilitate the mobilization of heavy metals that can be absorbed by plants.

François et al. (2011) have successfully identified a bacterial EPS production in sludge and water

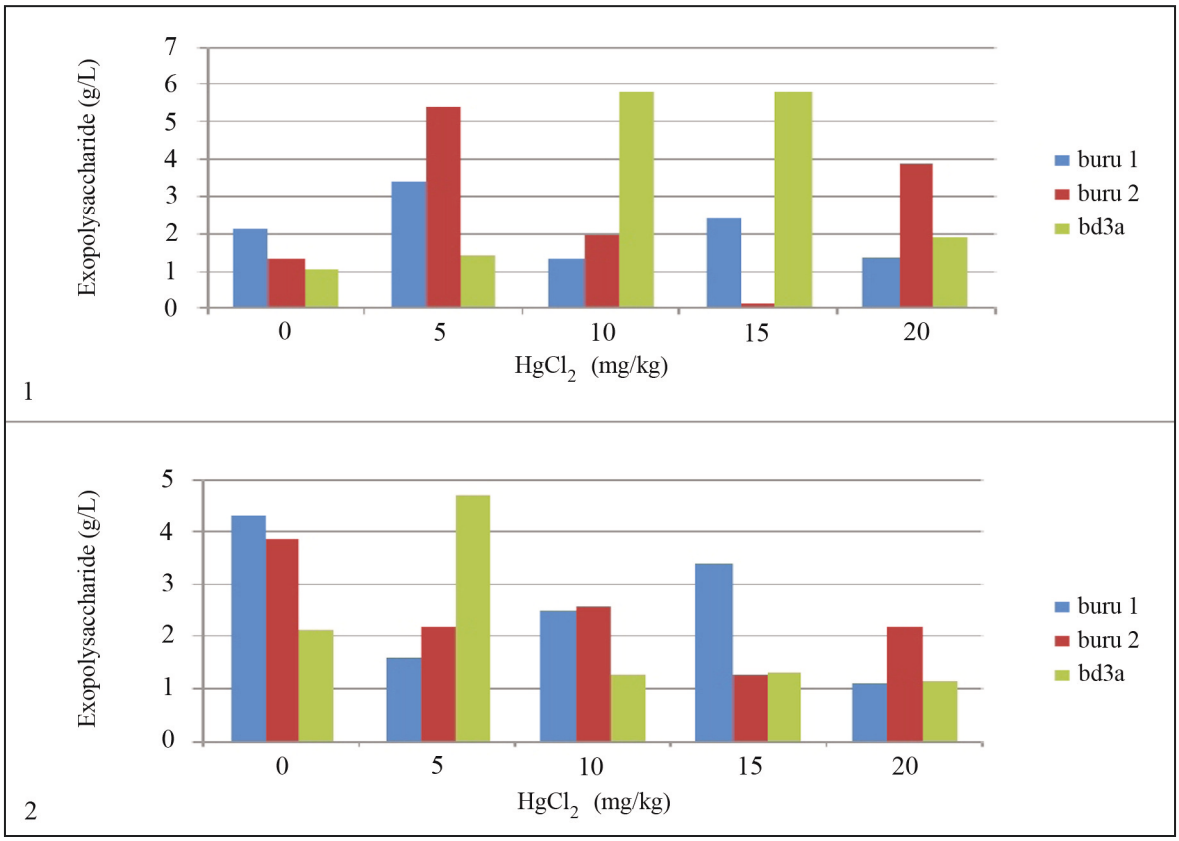

Figure 1. Effect of mercury on the Exopolisaccharide of three isolates of *Azotobacter* in liquid media with and without $HgCl_2$ on two (a) and four (b) days after incubation.

that can proliferate in media with some levels of mercury. Depending on the species, mercury can be extracted from biomass after cultured on media with mercury, showing that bacteria adsorb mercury. Exopolysachharide synthesis is also a natural mechanisms associated with antibiotics (François et al., 2011). For soil microbes, Microbial EPS is one of important substances which has a significant role in facilitating the improvement of soil pores, increasing the root adhering soil and also nutrient uptake especially of nitrogen (Alami et al., 2000). Such mechanism would be an important reason to use *Azotobacter* in bioremediation. *Azotobacter* might have a dual role; first as PGPR that promote growth through nitrogen fixation and phytohormone production and second as bioremediation agents through the production of EPS.

CONCLUSIONS

The results showed that all three isolates of *Azotobacter* were able to proliferate in liquid culture contaminated with mercury. *Azotobacter* was able to grow in media with $HgCl_2$ up to 20 mg/L and the production of EPS depends on isolates and mercury level in liquid media. Isolate of bd3a showed a declined growth in media with 20 mg/L of $HgCl_2$. The presence of mercury affected and generally suppressed the production of EPS; but the effect depends on the isolates. *Azotobacter* buru-2 was the most efficient un EPS producing on day two and four in the medium with 20 mg/L of $HgCl_2$.

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