Macrothink Institute™

Journal of Agricultural Studies
ISSN 2166-0379
2019, Vol. 7, No. 3

Antibiotic Resistance of *Azotobacter* Isolated from Mercury-Contaminated Area

Reginawanti Hindersah (corresponding author)

Faculty of Agriculture Universitas Padjadjaran, Sumedang 45363, West Java, Indonesia

Centre of Excellence “Maluku Corner” Universitas Padjadjaran

E-mail: reginawanti@unpad.ac.id

Gina Nurhabibah

Graduated from Agrotechnology Undergraduate Program

Faculty of Agriculture Universitas Padjadjaran, Sumedang 45363, West Java, Indonesia

E-mail: nurhabibahg@gmail.com

Priyanka Asmiran

Graduated from Soil Science Master Program, Faculty of Agriculture Universitas Padjadjaran

Jalan Raya Bandung Sumedang Km. 21 Jatinangor, Sumedang 45363, Indonesia

Phone/fax 022-7797316, E-mail: priyankaasmiran@gmail.com

Etty Pratiwi

Indonesian Soil Research Institute, Bogor 16114 Indonesia

E-mail: ettypratiwi@yahoo.com

Received: May 26, 2019 Accepted: July 22, 2019 Published: August 1, 2019

doi:10.5296/jas.v7i3.14834 URL: https://doi.org/10.5296/jas.v7i3.14834

Abstract

Nitrogen-fixing *Azotobacter* is a renewable source of biofertilizer for plant growth. Increased of antibiotic level in soil due to intensive used manure is believed to induce bacterial sensitivity to antaibiotic. An antibiotic sensitivity test has been carried out to study the inhibition effect of ampicillin, streptomycin, tetracycline and chloramphenicol on *Azotobacter* isolated from mercury-contaminated taling. The resistance test was performend by using disc plate method in Nitrogen-free Ashby’s agar with and without mercury. The results showed that the presence of 20 mg/L mercury in plate agar totally inhibited

Journal of Agricultural Studies
ISSN 2166-0379
2019, Vol. 7, No. 3

Azotobater growth. In the absence of mercury chloride, all isolates showed different sensitivity to antibiotics. Growth of *Azotobacter* buru1 was only inhibited by tetracycline. *Azotobacter* buru2 was susceptible to high and low concentration of tetracycline and streptomycin but they were resistance to low concentration of chloramphenicol as well as ampicillin; while *Azotobacter* bd3a were sensitive to all tested antibiotic. In conclusion, order of *Azotobacter* resistance to antibiotics in the absence of mercury was Bd3a<Buru2<Buru1. This research have not revealed the resistance of *Azotobacter* to antibiotic in the presence of mercury.

Keywords: antibiotic, gold-mine tailing, mercury, nitrogen-fixing bacteria

1. Introduction

Antibiotics are main chemotherapeutic agents for managing human and animal infectious diseases. Animal manure is believed contribute to increased antibiotic level in soil due to intensive used of antibiotic in animal husbandry. Antibiotics utilized in livestock production are excreted in the feces and therefore transferred to soil when manure is used as organic matter amendments. However, organic matter application in agriculture is necessary for returning nutrients to the soil, providing a satisfied growth medium and nutrient for microbes; and further increasing microbial population as well as quality and productivity of soil (Nakhro and Dkhar, 2010; Masciandaro et al., 2013; Faissal et al., 2017).

Tetracycline is the most resistant antibiotic detected at concentration in excess of 1 mg/kg in terrestrial environments (Massé et al., 2014; DeVries and Zhang, 2016). Antibiotics influence soil microbe metabolism and promote their antibiotic resistance in soil. Manure containing antibiotic enriched soil antibiotic and induced antibiotic resistance genes (Xie et al., 2018). In healthy soil, beneficial microbial communities are important organisms which affect among others the availability of nitrogen; the essential macro nutrients for plant growth and productivity. Microbial activity in soil reduce inert inorganic N_2 gasses through nitrogen fixation to NH_3 which further is converted to NH_4^+ and NH_3^- that uptake by plant roots (DeVries and Zhang, 2016).

Nitrogen fixing *Azotobacter* is widely used plant growth promoting rhizobacteria (PGPR) in agricultural practice to improve soil fertility and prevent soil degradation. Rhizobacteria *Azotobacter* is believed develop antibiotic resistance mechanisms which is useful to proliferte in antibiotic-contaminated soil. A gene-antibiotic cassette in *A. vinelandii algC* mutant JGG1 as well as the wild-type strain ATCC 9046 through blot analysis of total DNA was reported (Gaona et al., 2004). The genome of Gram Negative N-fixing *A. chroococcum* NCIMB 8003 (ATCC 4412) (Ac-8003) determined that they carry a variety of accessory genes e.g. antibiotic resistance genes (Robson et al., 2015).

Azotobacter also might be used in mercury-contaminated soil bioremediation due to their multiple resistance for certain heavy metal (Abo-Amer et al., 2013; Hindersah et al., 2017; Rizvi and Khan, 2018;). Plants in which rhizosphere colonized by heterotrophic *Azotobacter* enhanced their tolerance to heavy metal toxicity (Nanda and Abraham, 2011; Sobariu et al., 2017). Amalgamation process in gold mine significantly discharge Hg to soil; mercury

Journal of Agricultural Studies
ISSN 2166-0379
2019, Vol. 7, No. 3

concentrations remain elevated in soil and sediment near closed gold mine (Opiso et al., 2018). In Buru Island of Maluku Indonesia, agricultural field near closed gold mine was contaminated by Hg up to 35 mg/kg (Hindersah et al., 2018).

Bioremediation is an effective way to reduce available Hg concentration in soil. Application of metal detoxifying bacteria with plant-beneficial properties is a cost effective and environmental friendly metal bioremediation method (Shinwari et al., 2015). Since microbes are the agents for change mercury availability in soil, the application of organic matter enhance microbial density should be considered. In case of *Azotobacter* inoculation in bioremediation-bioaugmentation process, we need to apply the antibiotic resistance *Azotobacter* to ensure their proliferation and activity. The objective of this research was to study the inhibition effect of tetracycline, chloramphenicol, ampicillin and streptomycin on some *Azotobacter* isolate which is isolated from Hg-contaminated soil.

2. Material and Method

2.1 Azotobacter

Three isolates of *Azotobacter* were the collection of Soil Biology Laboratory, Department of Soil Science, Universitas Padjadjaran. *Azotobacter* buru1 and *Azotobacter* buru2 have been isolated from rhizosphere of wiregrass (Eleusin indica (L.) Gaertn) grown in mercury-contaminated tailing at closed gold mine of Botak Mountain in Buru District of Maluku Province. *Azotobacter* bd3a were isolated from gold mine tailing at the same area which contain 100 mg/kg of mercury. All pure cultures of bacteria were maintained in N-free Asbhy's slant at 4 ^{o}C. Liquid culture of each isolates were prepared in nitrogen-free Ashby's broth (10 g mannitol, 0.2 g KH_2PO_4, 0.2 g $MgSO_4.7H_2O$, 0.2 g NaCl, 0.1 g $CaCO_3$, 10 mg Na_2MoO_4, and 1 L distillated water). The media has been sterilized in autuclave fo 20 minutes at 121 ^{o}C before *Azotobacter* inoculation.

2.2 Growth Curve Determination

Curve growth of three *Azotobacter* isolates was determined by used of N-free Ashby's broth. A total of 1 mL of each isolates were poured into 100 mL of Ashby's broth; the cultures were incubated for eight days at 30 ^{o}C on gyratory shaker at 115 rotations per minute. Bacterial count carried out once a day on Ashby's agar after serial dilution. Plates were incubated for 48 hours at room temperature in order to count clear, convex and slimy *Azotobacter* colonies. Doubling time was calculated at logarithmic phase by using the formula of Widdel (2010).

2.3 Antibiotic Sensitivity Test

The disk diffusion susceptibility method was used for antibiotic sensitivity test (Jorgensen and Ferraro, 2009) which has been well standardized. Sensitivity test has been done to determine the resistance of *Azotobacter* on several concentration of tetracycline, chloramphenicol, ampicillin and streptomycin. Antibiotic solution of 10, 50, 100, 500 and 1,000 mg/L were prepared by diluting each antibiotic powder in sterilized distillated water.

One milliliter of liquid culture of each isolates was added to 100 mL nitrogen-free Ashby's broth in individual Erlenmeyer Flask. After three-day incubation, 1 mL of culture was spread

out on the surface of Ashby's agar contaminated with 20 mg/kg $HgCl_2$ and on Hg-free agar. The test was performed by dipping paper disc into antibiotic solution and placed onto a plate agar upon which single *Azotobacter* isolate was growing. Plates were incubated at 30°C for 72 h (based on their curve growth in first experiment) prior to inhibition zone determination around each antibiotic disk. Diameter of halo zone were measured to the nearest millimeter. The main process and sub-processes of the research was summarized in Fig 1.

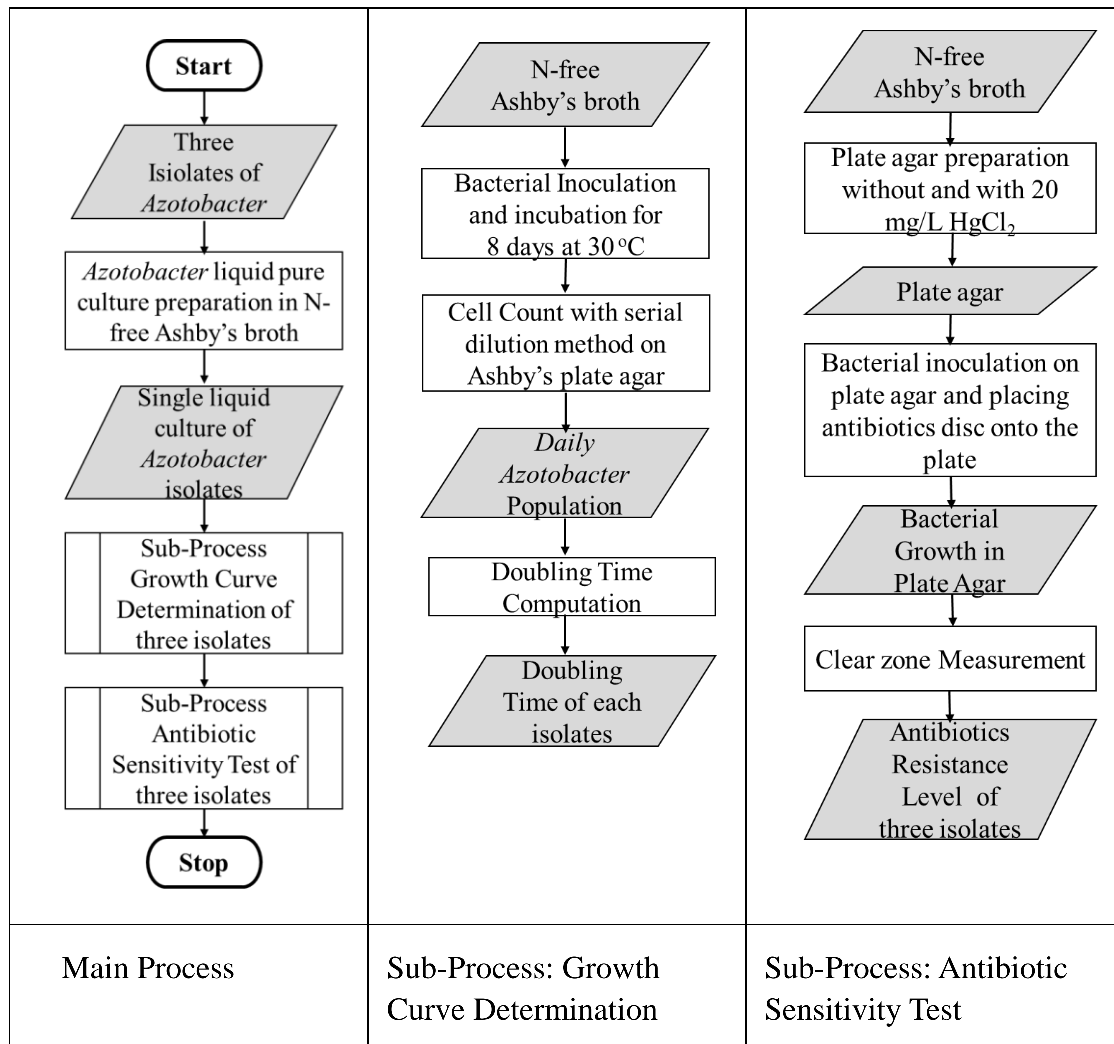

Figure 1. Research method on antibiotic sensitivity test of three isolates of *Azotobacter*

3. Results and Discussion

3.1 Growth Curve of Azotobacter

Based on the colony count on plate agar, bacterial population were reduced from day one to day two which showed that all isolated experienced lag phase (Fig 2). This phase is characterized by very little to no bacterial growth. Logarithmic phase was begun at day two and terminated at day 5. The calculation of doubling time (DT) showed that between day 2 and day 5, DT of buru2 is 4.6 h; higher than those of buru1 (3.9 h). These evidence proofed that cell proliferation of buru2 was slower than those of buru1. The slowest cell multiplication and higher DT was shown by isolates bd3a with DT of 4.9 hours. Doubling

time of *A. vinelandii* wild type strain was 4 h in liquid Burk's sucrose medium under diazotrophic conditions was 4 h. The DT was 3 h and 3.6 h in the media with 10 mM and 25 mM NH_4 acetate (Mus et al., 2017) reflecting the slower cell division of all isolates.

Bacterial growth curve and DT of each bacterial species or isolate are determined by all the factors that affect bacterial metabolisms and growth, mainly carbon source, nutritional composition, medium acidity, temperature, and aeration. All isolates took two days to adjust with the new environment before they enter logarithmic phase (Fig 2).

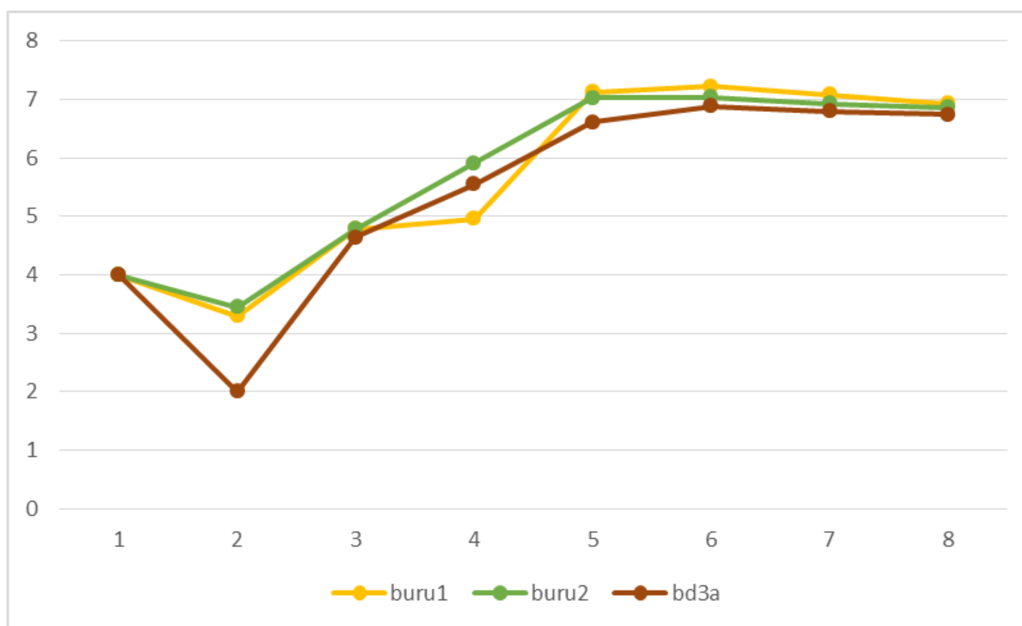

Figure 2. Growth curve of three isolates of *Azotobacter* on Nitrogen-free Ashby's agar

Population of *Azotobacter* bd3a was decline sharply during two-day adjustment compared to other isolates. There was no distinct difference in population of all isolate at the end of the experiment. Among the three isolates tested at the end of the incubation period, bd3a cell population was the lowest. However, growth in all three isolates was not significantly differ, at the end of the incubation the population of all isolates was around 10^7 cfu/mL. If the purpose of the production of a bacterial cell is to obtain inoculant with the highest density, then isolates buru1 was a better one. Based on curve growth, antibiotic sensitivity determination of three *Azotobacter* isolate was perform up to three days in liquid culture.

3.2 Antibiotic Resistance Profile of Azotobacter

Irrespective of generic antibiotic and *Azotobacter* isolates, *Azotobacter* colonies failed to grow on plates with Hg (Fig 3) which showed their susceptibility to 20 mg/kg of $HgCl_2$. Our previous study demonstrated the resistance of bd3a in Ashby's broth contaminated with 20 mg/L HgCl but buru1 and buru2 were only resistant to 15 mg/L HgCl (Hindersah et al., 2017). In the presence of Hg 20 mg/L on Ashby's agar, bacterial resistance to four generic antibiotics could not be evaluated. In Hg-free agar, bacterial colonies did not appear at 24 hours after incubation but the colonies were visible at day two (Fig 4). In general, inhibition zone depends

on antibiotic concentration resulting higher inhibition zone diameter around disk dipped in higher concentration of antibiotic (Fig 5).

At the second day, growth of bd3a has been inhibited by all concentrations of ampicillin and tetracycline but bd3a was resistant to either 10 mg/L streptomycin or 50 mg/L and 200 mg/kg chloramphenicol. Antibiotic resistance pattern of each *Azotobacter* isolate at day three in Hg-free media was largely depend on antibiotic and bacterial isolates used in this assay (Table 1 and Table 2). Halo zone in *Azotobacter* buru1 was only detected around 1,000 and 500 mg/L tetracycline disks (Table 1). Growth of buru2 was inhibited by higher concentration of tetracycline and ampicillin, and all concentration of streptomycin but they did not sensitive to low concentration of chloramphenicol; meanwhile inhibition zones were determined in most of antibiotic disk on plate agar in which *Azotobacter* Bd3a grow (Tabel 1 and Table 2).

There was no increased of clear zone diameter on the third day for isolate buru1, buru2 as well bd3a compared to those on the second day, but clear zone diameter around disk dipped in 1,000 and 500 mg/L tetracycline solution increased mostly doubled (Table 1) showing relatively high sensitivity of Buru2 on high concentration of tetracycline.

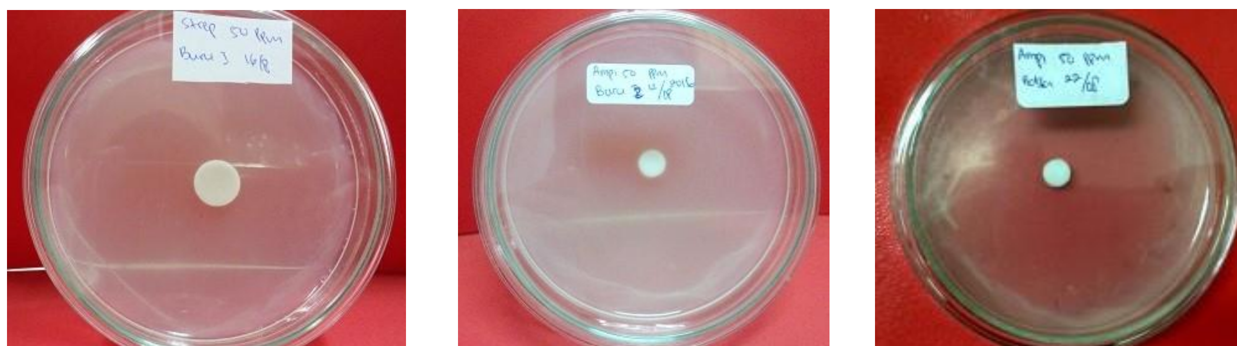

Figure 3. Growth failure of *Azotobacter* in Ashby's agar contaminated with mercury

Figure 4. Colony characteristics of *Azotobacter* Buru1, Buru2 and Bd3a at day two on Ashby's agar without mercury but with antibiotic; colonies of Buru1 and Bd3a were scattered on the plate but Buru2 showed rigorous colony growth on the plate

Figure 5. Inhibition zone around 500 mg/L streptomycin disk on *Azotobacter* Buru2 growth (left) was larger than around 50 mg/L streptomycin disk (right)

Table 1. Inhibition zone around tetracycline and chloramphenicol disk in which three isolates of *Azotobacter* did not grow on Hg-free Ashby's agar

Azotobacter Isolates	Day	Tetracycline (mg/L)						Chloramphenicol (mg/L)					
		1,000	500	200	100	50	10	1,000	500	200	100	50	10
		Inhibition zone (cm)											
Buru1	2	1.6	1.5	-	-	-	-	*	*	*	*	*	*
	3	1.6	1.5	-	-	-	-	-	-	-	-	-	-
Buru2	2	2.2	2.0	5.0	1.8	*	1.5	1.5	-	-	-	-	-
	3	5.0	5.0	5.0	1.8	*	1.5	1.5	-	-	-	-	-
Bd3a	2	0.6	0.3	0.1	0.1	0.1	0.1	1.0	0.7	-	0.3	-	0.1
	3	0.6	0.4	0.1	0.1	0.1	0.1	1.0	0.8	-	0.3	-	0.1

*No bacterial growth; - no inhibition on bacterial growth

Tabel 2. Inhibition zone around ampicillin and streptomycin disk in which three isolates of *Azotobacter* grown on Hg-free Ashby's agar

Isolates	Day	Ampicillin (mg/kg)						Streptomycin (mg/kg)					
		1,000	500	200	100	50	10	1,000	500	200	100	50	10
		Inhibition zone (cm)											
Buru1	2	*	*	*	*	*	*	*	*	*	*	*	*
	3	-	-	-	-	-	-	-	-	-	-	-	-
Buru 2	2	1.4	0.6	0.5	-	-	-	2.0	1.5	2.0	1.0	1.0	0.5
	3	1.4	0.6	0.5	-	-	-	2,2	2,0	1,5	1,6	1,3	1,0
Bd3a	2	1.2	1.2	0.5	0.4	0.3	0.1	1.2	0.3	0.3	0.2	0.1	-
	3	1.3	1.2	0.5	0.4	0.3	0.1	1.2	0.4	0.3	0.2	0.1	-

*No bacterial growth; - no inhibition on bacterial growth

Bacterial resistance to antibiotics is a natural ability to defend themselves against the harmful effects of antibiotics. Generally, bacterial resistance to antibiotics is performed through three mechanisms; i.e. mutations in porin, inactivation of antibiotics and changes in the active site where the formation of binding of antibiotics by bacteria (Delcour, 2009). The results of this

assay was similar to the ability of *A. chroococcum* to proliferate in the presence of antibiotic include ampicillin, chloramphenicol, streptomycin and tetracycline (Sindhu et al., 1989; Aung et al., 2016).

Antibiotic resistance of *Azotobacter* was reported elsewhere. The antibiotic resistance of *A. chroococcum* and *A. benjerinckii* were demonstrated by their growth in agar media with 3 % (w/v) of chloramphenicol (Aung et al., 2016) which verified that this prominent N-fixing PGPR might stand in antibiotic contaminated soil. *Azotobacter* was more resistance to antibiotics compared to other soil bacteria; *Azotobacter* count decreased only ten days after soil contamination with ampicilline and streptomycine and 100 days after contamination *Azotobacter* growth was recovered (Akimenko et al., 2017).

Azotobacter cell wall contain polysaccharides which is well known as exopolysaccharide (EPS); an outer structure of microbial cells associated the nitrogenase protection (Sabra et al., 2000; Prasad et al., 2014; Hindersah et al., 2017). Formation of EPS is also related to bacterial mechanisms avoid heavy metals toxicity through sequester positively charged heavy metal ions (Gupta and Diwan, 2016). For pathogenic bacteria, EPS is also related to their antibiotic sensitivity. Increased capsular EPS production during antibiotic exposure is regulated in response to antibiotic stress in opportunistic pathogen *Acinetobacter baumannii* (Geisinger and Isbe, 2015). The correlation between EPS and antibiotic resistance for *Azotobacter* has not studied intensively. All *Azotobacter* isolate in this experiment synthesize EPS in liquid culture but their EPS production related to antibiotic resistance has not been studied.

The three isolates showed different sensitivity to antibiotic in the absence of Mercury but 20 mg/L of HgCl was too high to maintain cell proliferation in Nitrogen-free Ashby's agar. Mercury might inhibit nitrogenase resulting lack of available nitrogen mainly nitrate; major macronutrient in cell formation and development. *Azotobacter* proliferation in the presence of mercury has been documented; *A. chrooccocum* isolated from wheat (*Triticum aestivum*) rhizospheric soil irrigated with industrial wastewater about 10 years had a highest minimum inhibitory concentration of 200 mg/L for Hg^{2+} (Aleem et al., 2003). More EPS production in the presence of Hg was reported for certain *Azotobacter* strain (Rasulov, 2013; Hindersah et al., 2017). Bacterial EPS is a prominent natural material to be integrated in bioremediation of metal-contaminated soil in order to reduce their toxic effect of even its low concentration on food chain.

Profile of antibiotic sensitivity will be an important trait for the selection of Gram negative *Azotobacter* isolates which might stand in antibiotic contaminated soil. For Gram negative bacteria, antibiotics changed enzyme activity and ability to metabolize different carbon sources, and altered microbial biomass (Cycoń et al., 2019). The majority of antibiotics are not completely metabolized in the bodies of livestock; animal manure amendment on agricultural farm causing antibiotics discharged on soil. Our results determined that *Azotobacter* isolated from Hg-contaminated area might have also an ability to proliferate in certain antibiotic contaminated environment which is will be important to overcome increased antibiotic problem in soil.

4. Conclusion

In Nitrogen-free Asbhy's broth, generation time from day 2 to day 5 of *Azotobacter* buru1, buru2 and bd3a were 3.9 h, 4.6 h and 4.9 h respectively reflecting their slow cell division. All isolates had two-day lag phase in Ashby's broth before entering logarithmic phase during another three days. Cell count of *Azotobacter* bd3a was decline sharply during lag phase compared two other isolates but no distinct difference in all isolate population at day eights.

The presence of 20 mg/L mercury chloride in Nitrogen-free Ashby's agar totally ceased *Azotobater* growth; bacterial colony did not grow on the surface of agar so that their resistance to antibiotic remain uncertain. In the absence of mercury chloride, two isolates showed multiple resistance at least to two kind of antibiotics. Growth of *Azotobacter* buru1 was only inhibited by tetracycline showing their resistance to Chloramphenicol, Ampicillin and Streptomycin. *Azotobacter* buru2 was susceptible to high and low concentration of either streptomycin or Tetracycline but resistance to low concentration of chloramphenicol and tetracycline. Susceptibility for all tested antibiotic was showed by *Azotobacter* bd3a. *Azotobacter* resistance profile to antibiotic was clearly demonstrated in agar media without Hg. In conclusion, order of resistance to antibiotics was bd3a<buru2<buru1.

Azotobacter plays in important role for making fertilization in plant production more efficient and moreover detoxifing heavy metal in contaminated agricultural area. *Azotobacter* are renewable biological agent that does not require high production costs. Bioremediation of heavy-metal contaminated soil by bioaugmentation method by versatile *Azotobacter* is easy, safe, and cost effective.

Acknowledgement

Authors thank to Directorate General of Higher Education Indonesia for providing Basic Research Fund of year 2016.

References

Abo-Amer, A. E., Abu-gharbia, M. A., Soltan, E. M., & Abd El-Raheem, W. M. (2014). Isolation and Molecular Characterization of Heavy Metal-Resistant *Azotobacter chroococcum* from Agricultural Soil and Their Potential Application in Bioremediation. *Geomicrobiology journal*, *31*(7), 551-561. https://doi.org/10.1080/01490451.2013.850561

Akimenko, Y. V., Kazeev, K. S., Kolesnikov, S. I., Myasnikova, M. A., & Minnikova, T. V. (2017). Assessing Resistance of the Microbial Community in Soils to Pollution with Antibiotics. *Asian Journal of Pharmaceutics,* *11*(4), S798-S804. https://doi.org/10.22377/ajp.v11i04.1715

Aleem, A., Isar, J., & Mali, A. (2003). Impact of long-Term Application of Industrial Wastewater on The Emergence of Resistance Traits in *Azotobacter chroococcum* isolated from Rhizospheric Soil. *Bioresource Technology, 86*(1), Abstract. https://doi.org/10.1016/S0960-8524(02)00134-7

Aung, A., Yu, S. S., & Sev, T. M. (2016). Development of Cellulolytic Nitrogen-Fixing Activity of *Azotobacter* spp. by Transposon Mutagenesis. *Journal of Scientific and Innovative Research*, *5*(4), 130-134. http://www.jsirjournal.com/Vol5_Issue4_06.pdf

Cycoń, M., Mrozik, A., & Piotrowska-Seget, Z. (2019). Antibiotics in the Soil Environment—Degradation and Their Impact on Microbial Activity and Diversity. *Frontier in Microbiology, 10*(Article 338). https://doi.org/10.3389/fmicb.2019.00338

Delcour, A. H. (2009). Membrane Permeability and Antibiotic Resistance. *Biochimica et Biophysica Acta*, *1794*(5). 808–816. https://doi.org/10.1016/j.bbapap.2008.11.005

DeVries, S. L., & Zhang, P. (2016). Antibiotics and the Terrestrial Nitrogen Cycle: A Review. *Current Pollution Reports*, *2*, 51–67. https://doi.org/10.1007/s40726-016-0027-3

Faissal, A., Ouazzani, N., Parrado, J. R., Dary, M., Manyani, H., Morgado, B. R., ... Mandi, L. (2017). Impact of fertilization by natural manure on the microbial quality of soil: Molecular approach. *Saudi Journal of Biological Science, 24*(6), 1437-1443. https://doi.org/10.1016/j.sjbs.2017.01.005

Gaona, G., Núñez, C., Goldberg, J. B., Linford, A. S., Nájera, R., Castañeda, M., Guzmán, J., Espín, G., & Soberón-Chávez, G. (2004). Characterization of The *Azotobacter vinelandii algC* Gene Involved in Alginate and Lipopolysaccharide Production. *FEMS Microbiology Letters*, *238*(1), 199-206. https://doi.org/10.1016/j.femsle.2004.07.044

Geisinger, E., & Isbe, R. R. (2015). Antibiotic Modulation of Capsular Exopolysaccharide and Virulence in *Acinetobacter baumannii. PLoS Pathogen, 11*(2), e1004691. https://doi.org/10.1371/journal.ppat.1004691

Gupta, P., & Diwan, B. (2016). Bacterial Exopolysaccharide Mediated Heavy Metal Removal: A Review on Biosynthesis, Mechanism and Remediation Strategies. *Biotechnology Reports,* https://doi.org/10.1016/j.btre.2016.12.006

Hindersah, R., Mulyani, O., & Osok, R. (2017). Proliferation and Exopolysaccharide Production of *Azotobacter* in the Presence of Mercury. *Biodiversity Journal*, *8*(1), 21–26 https://doi.org/10.15243/jdmlm.2018.052.1027

Hindersah, R., Risamasu, R., Kalay, A. M., Dewi, T., & Makatita, I. (2018). Mercury Contamination in Soil, Tailing and Plants on Agricultural Fields Near Closed Gold Mine in Buru Island, Maluku. *Journal of Degraded Mine and Land Management, 5*(1), 1027-1034. https://doi.org/10.15243/jdmlm.2018.052.1027

Jorgensen, J. H., & Ferraro, M. J. (2009). Antimicrobial Susceptibility Testing: A Review of General Principles and Contemporary Practices. *Clinical Infectious Diseases*, *49*(11), 1749-1755. https://doi.org/10.1086/647952

Masciandaro, G., Macci, C., Peruzzi, E., Ceccanti, B., & Doni, S. (2013). Organic Matter–Microorganism–Plant in Soil Bioremediation: A Synergic Approach. *Review on Environmental Science and Biotechnology*, *12*(4), 399-419. https://doi.org/10.1007/s11157-013-9313-3

Massé, D. I., Saady, N. M., & Gilbert, Y. (2014). Potential of Biological Processes to Eliminate Antibiotics in Livestock Manure: An Overview. *Animals*, *4*(2), 146-163. https://doi.org/10.3390/ani4020146

Mus, F., Tseng, A., Dixon, R., & Peters, J. W. (2017). Diazotrophic Growth Allows *Azotobacter vinelandii* to Overcome the Deleterious Effects of A glnE Deletion. *Applied and Environmental Microbiology*, *83*, e00808-17. https://doi.org/10.1128/AEM.00808-17

Nakho, D., & Dkhar, M. S. (2010). Impact of Organic and Inorganic Fertilizers on Microbial Populations and Biomass Carbon in Paddy Field Soil. *Journal of Agronomy*, *9*(3), 102-110. https://doi.org/10.3923/ja.2010.102.110

Nanda, S., & Abraham, J. (2011). Impact of Heavy Metals on The Rhizosphere Microflora of *Jatropha multifida* and Their Effective Remediation. *African Journal of Biotechnology*, *10*(56), 11948-11955. DOI:10.5897/AJB10.1588

Opiso, F. M., Aseneiro, J. P. J., & Banda, H. B. T. (2018). Solid-Phase Partitioning of Mercury in Artisanal Gold Mine Tailings from Selected Key Areas in Mindanao, Philippines, and Its Implications for Mercury Detoxification. *Waste Management & Research*, *36*(3), 269–276. https://doi.org/10.1177/0734242X17753534

Prasad, R. K., Gautam, R., & Behal, S. (2014). Exopolysachharide Secreting Bacteria: Potential for Useful Applications. *International Journal of Research*, *1*(1), 17-32. www.internationaljournalofresearch.com

Rasulov, B. A., Yili, A., & Aisa, H.A. (2011). Biosorption of Metal Ions by Exopolysaccharide Produced by *Azotobacter chroococcum* XU1. *Journal of Environmental Protection*, *4*(9), 989-993. https://doi.org/10.4236/jep.2013.49114

Rizvi, A., & Khan, M. S. (2018). Heavy Metal Induced Oxidative Damage and Root Morphology Alterations of Maize (*Zea mays* L.) Plants and Stress Mitigation by Metal Tolerant Nitrogen Fixing *Azotobacter chroococcum*. *Ecotoxicological Environmental Safety*, *157*, 9-20. https://doi.org/10.1016/j.ecoenv.2018.03.063

Robson, R. L., Jones, R., Robson, R. M., Schwartz, A., & Richardson, T. H. (2015). *Azotobacter Genomes: The Genome of Azotobacter chroococcum NCIMB 8003 (ATCC 4412).* PLoS ONE, 10(6), e0127997. https://doi.org/10.1371/journal.pone.0127997

Sabra, A., Zeng, P., Lonsdorf, H., & Deckwer, W. D. (2000). Effect of Oxygen on Formation and Structure of *Azotobacter vinelandii* Alginate and Its Role in Producing Nitrogenase. *Applied Environmental Microbiology, 66*(9), 4037-4044. https://doi.org/10.1128/AEM.66.9.4037-4044.2000

Shinwari, K. I., Shah, A., Afridi, M. I., Zeeshan, M., Hussain, H., Hussain, J., Ahmad, O., & Jamil, M. (2015). Application of Plant Growth Promoting Rhizobacteria in Bioremediation of Heavy Metal Polluted Soil. *Asian Journal of Multidisciplinary Studies, 3*(4), 179-185.

Sindhu, S. S., Grover, V., Narula, N., & Lakshminarayana, K. (1989). Occurrence of Multiple Antibiotic Resistance in Azotobacter chroococcum. Zentralblatt fur Mikrobiologie, *144*(2), 97-101. https://doi.org/10.1016/S0232-4393(89)80071-X

Sobariu, D. L., Fertu, D. I. T., Diaconu, M., Pavel, L. V., Hlihor, R. M., Drăgoi, E. N., Curteanu, S., Lenz, M., Corvini, P., & Gavrilescu, M. (2017). Rhizobacteria and Plant Symbiosis in Heavy Metal Uptake and Its Implications for Soil Bioremediation. *New Biotechnology*, *39*(Pt A), 125-134. https://doi.org/10.1016/j.nbt.2016.09.002

Widdel, F. (2010). Theory and Measurement of Bacterial Growth. [Online] file:///C:/Users/Reginawanti/Downloads/TheoryandMeasurementofBacterialGrowth.pdf (May 25, 2019).

Xie, W. Y., Shen, Q., & Zhao, F. J. (2008). Antibiotics and Antibiotic Resistance from Animal Manures to Soil: A review. *European Journal of Soil Science, 69*(1), 181–195. https://doi.org/10.1111/ejss.12494

Copyright Disclaimer

Copyright for this article is retained by the author(s), with first publication rights granted to the journal.

This is an open-access article distributed under the terms and conditions of the Creative Commons Attribution license (http://creativecommons.org/licenses/by/4.0/).