

## Exploration *smtAB* Gene of Lead (Pb) Resistance in Isolate Bacteria from Lapindo Mud

Diah Arifiani<sup>1</sup>, Kholifah Holil, Umaiyatus Syarifah<sup>3</sup>

Department of Biology Faculty of Science and Technology  
UIN Maulana Malik Ibrahim Malang  
Jl. No. Gajayana 50 Malang  
E-mail: arifiani497@gmail.com

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### ABSTRACT

Indigenous bacteria are bacteria that have the ability to reduce harmful compounds such as heavy metals. Indigenous bacteria isolated from Lapindo mud are known to have lead capability. This ability is due to the presence of a resistant gene possessed by the bacteria. One of these genes is the *smtAB* gene that works to produce heavy metal-binding proteins, namely *metallothionein*. Each bacterium has a different heavy metal resistance gene. Therefore this study aims to determine the species of bacteria that have genes *smtAB* in bacterial isolates obtained from Lapindo Mud. The type of research conducted is descriptive qualitative. In this study, there are several stages, plasmid DNA isolation by alkaline lysis method, *smtAB* gene amplification and sequence confirmation by amplicon sequencing results. PCR amplification results of subsequent visits similarity (similarity) sequence gene sequencing results with the data *smtAB* from GenBank and processed using Bioedit. Based on the results of DNA plasmid amplification *Bacillus subtilis* isolated from Lumpur Lapindo showed that the bacterial isolates contained genes *smtAB*. While data sequencing results showed that *Bacillus subtilis* has similarity with *Synechococcus* sp. IU 625 *metallothionein smtAB* gene complete *cds* value 0.5.

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### Corresponding Author:

Diah Arifiani<sup>1</sup>,

Department of Biology Faculty of Science and Technology

UIN Maulana Malik Ibrahim Malang

Jl. No. Gajayana 50 Malang

Email: [arifiani497@gmail.com](mailto:arifiani497@gmail.com)

## 1. INTRODUCTION

The most environmental problem is pollution caused by heavy metals as a result of increased industrial (Peter *et al*, 2014, Badjoeri and Zarkasyi, 2010). This causes the accumulation of waste to be higher. However, since the environment has limited degradation capacity, this can cause the environment to be unable to handle the accumulation (Arinda, 2012).

One of heavy metal pollutants is lead (Pb). Pb can cause harmful effects on humans (Hibrianti, 2013), plants (Sharma and Dubey, 2005) and animals (Byrne and Gill, 2011). To reduce the negative effects caused by heavy metals used biotechnology remediation or better known as bioremediation.

Bioremediation is a technique to reduce or break down harmful compounds in the environment by utilizing microorganisms (Soleimani and Jaber, 2014) such as molds, yeasts, and bacteria (Chatterjee *et al*, 2012). There are many types of bacteria that have the potential for bioremediations, one of them is *indigenous* bacteria. *Indigenous* bacteria could be isolated from the polluted environment of heavy metals like Lapindo Mud. Lapindo Mud bacterial isolates potentially reducing the Pb is *Acinetobacter baumannii*, *Bacillus subtilis* and *Pseudomonas pseudomallei* *Brevibacillus laterosporus* (Rohmah, 2016). The bacteria are able to survive on heavy metal contaminated environment for their resistant genes.

One of resistance gene is *smtAB* that involved in the production of lead-binding protein (*metallothionein*) that helps protect cells through regulation of cell apoptosis process induced by heavy metals (Shimoda *et al*, 2003). This study aims to determine the species of bacteria from the Lapindo Mud that has *smtAB* gene.

## 2. RESEARCH METHOD

This research was conducted in December 2016 until January 2017 in laboratory of microbiology and laboratory of Genetics and Molecular Biology Department of Biology Faculty of Science and Technology of State Islamic University Maulana Malik Ibrahim Malang.

The tools used are waterbath shaker, autoclave, micropipette, vortex, centrifuge, tube eppendorf, PCR machine (thermal cycler) series BioRad, incubator, NanoDrop, LAF (Laminar Air Flow), the device electrophoresis brands BioRad, UV machine transilluminator (gel Doc) series of molecular BioRad gel Doc TM Image Xr imaging system and Quantity One software.

The materials used are isolates Lumpur Lapindo has been known as a species of *Acinetobacter baumannii*, *Bacillus subtilis*, *Brevibacillus laterosporus*, and *Pseudomonas pseudomallei*, Luria Bertani broth (LB broth), buffer TE (Tris-EDTA), 70% ethanol, ethanol 96%, Glucose/Tris/ EDTA (GTE), SDS/NaOH, potassium acetate 5M, RNase 10mg/mL, agarose gel, TBE (Tris Boric EDTA), EtBr (Ethidium bromide), loading dye, deionized water, Master Mix PCR brand Promega (Taq polymerase, dNTPs, PCR buffer), smt1 primer (5'CGT CGA GAT GAC TGC AGA AG 3') and smt2 (5'CGA GAT GAT GGG CGT TTT AA 3'), marker (10kB DNA Ladder fast2), the isolated DNA (template), aquabidest, distilled water, and ice cubes.

### A. Making Media Rejuvenation and Bacteria Culture

The medium used for bacteria rejuvenation is Nutrient Agar (NA) while using a bacterial culture media LB medium (Luria Bertani) broth. NA media of 5 g is dissolved in 250 mL of aquades in a 500 mL beaker glass and heated over a hotplate until the medium is completely dissolved.

The medium used for bacterial culture is the Luria Bertani Broth (LB broth) medium. Media LB 5 g was dissolved in 200 mL of aquades in a 250 mL erlenmeyer tube. The dissolved media is characterized by a medium that has been turned clear. Before the media used to culture the bacteria, media sterilized using an autoclave at 121°C, 1 atm pressure for 15 minutes.

### B. Bacterial Rejuvenation

Media Nutrient Agar (NA) that had been prepared previously placed in the Laminar Air Flow (LAF). Then, prepared isolates *Acinetobacter baumannii*, *Bacillus subtilis*, *Brevibacillus laterosporus*, and *Pseudomonas pseudomallei*. Rejuvenation of bacteria is done by taking in aseptic 1 ose bacteria and *streak* on the media NA slant. Isolates were then incubated at 37°C.

### C. Preparation of Bacterial Culture

For culturing bacteria *Acinetobacter baumannii*, *Bacillus subtilis* and *Pseudomonas pseudomallei* *Brevibacillus laterosporus* in LB liquid medium and grown for 24 hours at 37°C is agitated at a speed of 150-175 rpm in a water bath shaker. After 24 hours of bacterial cell growth seen in culture with cloudy indications of media without the odor and mucus in the media. After that isolates are ready to be isolated DNA.

### D. Isolation of Plasmid DNA

The first step in this method is bacterial isolates inoculated on 40 mL of sterile LB medium and grown for 24 hours. As much as 2 ml bacterial suspension was precipitated by using mikrosentrifus speed of 13000 rpm for 20 seconds at a temperature of 4°C. After centrifugation, the supernatant was discarded and the pellet taken. Then, 200µL of GTE solution was added to the pellet and incubated for five minutes at room temperature. After incubation, 200 µL of NaOH / SDS solution was added and the solution mixed over ice for 5 min. The next step, the solution mixture was added 150µL potassium acetate and divortex at 7000 for 2 seconds, then put on ice for five minutes. Subsequently, the solution was centrifuged for 3 minutes at 13000 rpm at 4°C. Then, the supernatant was transferred to another tube and added 0.8 mL of 96% ethanol, the solution was allowed to stand for 2 minutes at room temperature for nucleic acid precipitation. Subsequently, the solution was centrifuged for 1 min at room temperature to obtain precipitated DNA and RNA. After that, the pellets are DNA and RNA deposits rinsed with 1 mL of ethanol 70% and waited until dry. Suspended pellet with 50µL TE buffer. To remove RNA contamination 2µL RNase solution 10mg/mL was used. The result of isolation is plasmid DNA (Ausubel, 2003).

### E. DNA Concentration Measurements

Quantitative testing of isolated genomic DNA was measured by nanodrop. The isolated plasmid DNA samples from each isolate were taken as 1 µl and fed into the nanodrop tool. Measurements were made using absorbance values at 260 nm and 280 nm wavelengths.

### F. DNA Amplification (PCR)

*SmtAB* gene amplification in this study refers to the amplification process carried out by, Shamim and Dubey (2012) that have been modified. DNA template with a concentration of 50ng/mL, 2 mL PCR Nuclease-free water, 10 uL of Promega PCR mix brands (PCR reaction buffer, 1- TM Taq DNA polymerase (5U / mL), dNTPs (2.5 mm)), and primary smt1 and smt2 each 1 µL (10 µM / liter), Primer used is Smt1 (5'CGG GAT CGG TGC AGA GAC AG 3') and smt2 (5'GAT CGA GGG CGT TTT GAT AA 3'). The amplification process was carried out for 35 cycles, each cycle consisting of denaturation stage at 94°C for 1 minute, annealing at 53°C for 1 minute, and

extension at 72°C for 2 minutes, final extension stage at 72°C for 5 minutes. The results obtained were then visualized with 2% agarose gel electrophoresis.

#### G. Electrophoresis

Electrophoresis stage in this research was conducted for qualitative DNA test and visualization of PCR result. The agarose gel was prepared with a concentration of 1.5% in the TBE 1X buffer for the qualitative test. Samples of the isolated for electrophoresis and *dye loading* volume (3: 1) and electrophoresis at a voltage of 70 volts for 30 minutes. The electrophoresis step for visualization of PCR results was in accordance with the qualitative DNA steps with 2% agarose gel in TBE 1X buffer and electrophoresis at 75 volts for 90 min. The electrophoresis gel was observed with UV light (UV transilluminator), the emerging DNA fragments documented by applying on Quantity One.

### 3. RESULTS AND ANALYSIS

#### a. Test The quantity of DNA

DNA quantity test is done to know the concentration and value of DNA purity. If the absorbance value is less than 1.8 then the DNA is contaminated with proteins, residual materials or sample metabolites. If the absorbance value is more than 2.00 then the DNA is contaminated with RNA (Sambrook, 2001). Quantity test results are presented in table 1.

Table 1. Test quantity of DNA Plasmid

No.	Nama Isolat	Ulangan	Kemurnian (260/280)	Konsentrasi (ng/uL)	Kesimpulan*
1.	<i>Acinetobacter baumannii</i>	1	1,90	260,83	Murni
		2	1,90	233,48	Murni
		3	1,94	1472,63	Murni
2.	<i>Bacillus subtilis</i>	1	1,89	2448,73	Murni
		2	2,11	2232,73	Kontaminasi RNA
		3	1,89	2162,96	Murni
3.	<i>Brevibacillus laterosporus</i>	1	2,09	1609,9	Kontaminasi RNA
		2	1,83	2109,2	Murni
		3	1,77	2930,03	Kontaminasi Protein
4.	<i>Pseudomonas pseudomallei</i>	1	1,94	792,59	Murni
		2	1,91	732,88	Murni
		3	2,06	277,8	Kontaminasi RNA

Note: \* Based on the comparison of the absorbance wavelength 260/280 nm (Sambrook, 2001).

The quantity test results showed that 25% (3 of 24 plasmid DNA samples) isolated were still contaminated with both protein and RNA. From the above table it can be seen that the bacteria *Bacillus subtilis* and *Brevibacillus laterosporus* have DNA concentrations were relatively high compared with the two other bacteria. The amount of DNA concentration obtained can be affected by the cell lysis process, where if the lysis process goes accordingly, then the amount of DNA obtained will be greater. Based on the characteristics, Ulrich and Hughes (2001) stated that the gram-negative bacteria more susceptible to lysis of cells for DNA isolation process compared to gram-positive bacteria for their peptidoglycan more on gram-positive bacteria. This is not in accordance with the results of research that has been done, isolates of *Bacillus subtilis* and *Brevibacillus laterosporus* is a gram-positive bacteria but have a high DNA concentrations. This may occur due to gram-negative bacteria is easy to settle (be pelleted) during centrifugation of the bacterial suspension, thus, difficult homogeneous sediment bacteria when mixed with lysis buffer and lysis process to be not optimal. According to Komalasari (2009), the amount of DNA concentrations obtained is influenced by two factors: the extraction rate at the time of DNA isolation and the addition of lysis buffer composition. The extraction velocity factor is the most influential factor because at the cell lysis stage and the precipitation of supernatant taking should be done per sample so that some samples occur of DNA precipitation.

#### b. Quality Test DNA

After the DNA quantity test, followed by quality test using gel electrophoresis. Qualitative test results are presented in Figure 1.

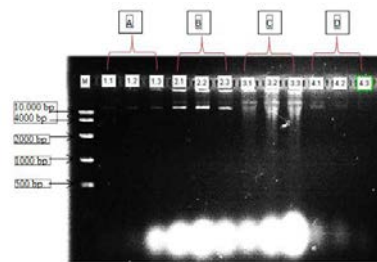


Figure 1. Qualitative test by gel electrophoresis. a. *A.baumannii*, b. *B. subtilis*, c. *B.laterosporus*, d. *P.pseudomallei*.

Quality test results showed that based on the value of the concentration of DNA, the results obtained from the test in accordance with the quantity of test bacteria *Bacillus subtilis* which has a thick and DNA bands showed DNA concentrations were high compared to other bacterial isolates. While the visualization of bacteria *Brevibacillus laterosporus* has a ribbon spread. Irmawati (2003) suggests that thick, collected and non-diffuse DNA bands show high concentrations and total DNA isolated under intact conditions and visible spread of DNA bands indicating a bond between the broken DNA molecules during the isolation process.

#### c. Gene amplification *smtAB*

After testing the quantity and quality, further DNA amplified with gene *smtAB*. The amplification results are presented in Figure 2.

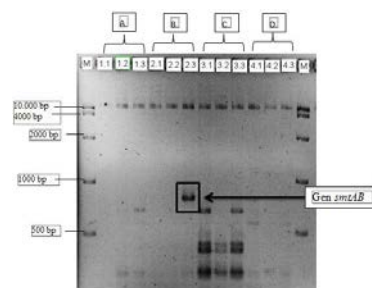


Figure 2. The results of the gene amplification *smtAB*. A. *A.baumannii*, b. *B. subtilis*, c. *B.laterosporus*, d. *P.pseudomallei*.

Figure 2 shows that there are two primary isolates amplifying the genes *smtAB*, but the identified genes are *Bacillus subtilis smtAB* with a size of 800 bp gene. Other un-amplified bacterial isolates may have other resistant genes. According Marche *et al.* (2017), bacteria *Brevibacillus laterosporus* have CpbA and CpbB genes that produce proteins that protect the body casing, *A.baumannii* have resistant mechanism in detoxification and effluks (Williams *et al.*, 2010) and *P.pseudomallei* using biofilms in defending themselves from exposure to metals Weight (Vorachit, 1993).

#### d. Sequencing

The amplification results are confirmed by using sequencing. Based on the results of sequencing the bacterium *Bacillus subtilis* has a length of 702 bp gene sequences were then compared with data *smtAB* of some bacterial genes in the NCBI GenBank. The results are presented in a tree filogenik *alignment* in Figure 3.



Figure 3. Phylogeny tree of *alignment* results *smtAB* gene.

Based on Figure 3 is known that the bacterium *Bacillus subtilis* has a closeness with *Synechococcus* sp. 625 IU complete *cds SMTA* and *smtB* the closeness score of 0.5.

#### 4. CONCLUSION

Based on research that has been done, the bacteria have a gene *smtAB* based on the amplification and sequencing are *Bacillus subtilis* and have similarities with the *Synechococcus sp. 625 IU* complete cds SMTA and *smtB* based on the alignment.

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