Isolation of Mercury-Resistant Endophytic and Rhizosphere Microorganisms from Grasses in Abandoned Gold Mining Area

Isolasi Mikroorganisme Endofit dan Rhizosfer Resisten Merkuri dari Rumput di Areal Bekas Tambang Emas

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ABSTRACT

There were about 900 hotspots of artisanal and small scale gold mining (ASGM) in Indonesia that recovered gold through amalgamation and cyanidation techniques. Amalgamation technique causes mercury (Hg) pollution to the soil. This study was a preliminary study that aimed to isolate Hg-resistant endophytic and rhizosphere microorganisms from pioneer grasses in the Hg-polluted soil. The most potential microorganism will be used for Hg phytoremediation in the future study. Pioneer grasses were collected from the abandoned gold mining area in Central Lombok Regency, West Nusa Tenggara. Total microorganisms were counted using Colony Forming Unit (CFU) or Standard Plate Count. The microorganism colony was characterized based on morphological characteristics. Hg-resistant endophytic and rhizosphere microorganisms were successfully isolated from pioneer grass (Cynodon dactylon and Eleusine indica) in the study site. The colonies of rhizosphere microorganisms were diverse morphologically compared to endophytic microorganisms based on the number of isolated microorganisms, 20 isolates and 17 isolates, respectively. The density of rhizosphere microorganisms was higher (96%) than endophytic microorganisms (4%). The density of rhizosphere bacteria and fungi were $4 \times 10^3$ and $2 \times 10^3$ CFU g$^{-1}$, respectively. However, the density of endophytic bacteria and fungi were only $2 \times 10^3$ and $1 \times 10^3$ CFU g$^{-1}$, respectively.

Keywords: endophytic microorganism, Hg-resistant, microorganism density, rhizosphere microorganism

ABSTRAK

Terdapat sekitar 900 titik pertambangan emas skala kecil (PESK) di Indonesia yang memperoleh emas melalui teknik amalgamasi dan sianidasi. Teknik amalgamasi menyebabkan pencemaran merkuri (Hg) di tanah. Penelitian ini merupakan penelitian pendahuluan (preliminary study) yang bertujuan untuk mengisolasi mikroorganisme endofit dan rhizosfer resisten Hg dari rumput pionir yang tumbuh di tanah yang tercemar Hg. Mikroorganisme endofit dan rhizosfer resisten Hg yang berhasil diisolasi berbasis penelitian selanjutnya. Sampel rumput pionir diambil dari lahan pertanian bekas kawasan pertambangan emas dengan teknik amalgamasi di Desa Bonjeruk, Kecamatan Jonggat, Kabupaten Lombok Tengah, Nusa Tenggara Barat. Total mikroorganisme dihitung menggunakan Colony Forming Unit (CFU) atau Standard Plate Count. Koloni mikroorganisme di karakterisasi berdasarkan ciri morfologi. Mikroorganisme endofit dan rizosfer yang resisten Hg berhasil diisolasi dari rumput pionir (Cynodon dactylon dan Eleusine indica) di lokasi penelitian. Koloni mikroorganisme rizosfer sangat beragam secara morfologi dibandingkan dengan mikroorganisme endofit berdasarkan jumlah mikroorganisme terisolasi, berturut-turut 20 isolat dan 17 isolat. Kepadatan mikroorganisme rizosfer lebih tinggi (96%) dibandingkan mikroorganisme endofit (4%). Kepadatan bakteri dan jamur rizosfer masing-masing adalah $4 \times 10^3$ dan $2 \times 10^3$ CFU g$^{-1}$ sedangkan kepadatan bakteri endofit dan jamur masing-masing hanya $2 \times 10^3$ dan $1 \times 10^3$ CFU g$^{-1}$.

Kata kunci: mikroorganisme, mikroorganisme endofit, mikroorganisme rizosfer, resisten Hg

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INTRODUCTION

Indonesia lies in the 7th ranked of gold-producing countries in 2017, with total production is 154.3 tonnes of gold per year (‘O’Connell et al., 2018). Indonesia’s gold production is supplied by large scale, medium scale, and artisanal and small scale gold mining (ASGM). There are about 900 identified hotspots of ASGM activity in Indonesia and they are found in 32 provinces and 197 cities/regencies all over Indonesia (BaliFokus Foundation, 2012). Artisanal and small-scale gold mining recovers gold through amalgamation and cyanidation techniques (Handayanto et al., 2014). Amalgamation technique (adding mercury to extract gold from the ore) causes mercury (Hg) pollution; the tailing (solid waste) that contains Hg might pollute soil and water (Gonçalves et al., 2017).

Hg pollution in the soil is harmful to humans and the environment (Marrugo-Negrete et al., 2016). Hg in the soil is persistent (Fan et al., 2018). Mercury concentration in the soil of an ASGM, with amalgamation technique, location in Lombok (Sekarbela, near Mataram city) is approximately 741-7,874 mg kg$^{-1}$ (Krisnayanti et al., 2012). This concentration exceeds the minimum concentration of Hg in solid waste based on Indonesian Government Regulation (2014), which is 0.3 mg kg$^{-1}$. Also, it exceeds the maximum concentration of Hg in the soil for agricultural land based on the Canadian Council of Ministers Environment (1999), which is 12 mg kg$^{-1}$. The high Hg concentration in the soil is a major limiting factor for plant growth (Hodson, 2012). The primary symptoms of Hg toxicity in maize are chlorosis (Muddarisna et al., 2013). Plants generally exhibit symptoms of heavy metal toxicity when it is exposed to a high concentration in the soil, except for hyperaccumulator plants that are resistant and able to accumulate heavy metals (Idris et al., 2004).

Some pioneer grass in gold-mine polluted areas such as Digitaria radicosa, Paspalum conjugatum, Cyperus kyllingia, Cynodon dactylon, and Eleusine indica exhibit Hg resistant (Hidayati et al., 2009; Muddarisna et al., 2013). The ability of plants to survive in heavy metal polluted soil is inseparable from plant association with beneficial microorganisms, either within plant body or rhizosphere niche (Thijs et al., 2017). These microorganisms produce plant growth-promoting substances such as indole acetic acid (IAA) and siderophore that help plants to survive in harsh conditions (Rajkumar et al., 2010; Tirry et al., 2018). Microorganisms that are isolated from heavy metal polluted soil also exhibit heavy metal resistance (Lodewyckx et al., 2002). This study aimed to isolate Hg-resistant endophytic and rhizosphere microorganisms (bacteria and fungi) from pioneer grasses in the abandoned gold mining area.

MATERIALS AND METHODS

Sampling Site

Pioneer grasses were collected from the abandoned gold mining area in Bonjeruk Village, Jonggat Sub-Regency, Central Lombok Regency, West Nusa Tenggara, Indonesia (8° 24’ - 8° 57’ S and 116° 05’ - 116° 24’ E, Figure 1). Grass samples were collected in April 2019. Mercury concentration in the soil was 41.37 mg kg$^{-1}$ (Ustiatik et al., 2020). Grass samples in the same length size were collected in triplicates (grass length 15-20 cm). Grass species were Cynodon dactylon and Eleusine indica. Soil in the rhizosphere of these grasses was also collected for rhizosphere microorganism isolation. Grass and soil samples were kept in the polyethylene plastics bag and stored in the cooling box for laboratory analysis.

Figure 1. Sampling site of Hg-resistant rhizosphere and endophytic microorganism from local grasses (Cynodon dactylon and Eleusine indica) in abandoned gold mining area, Central Lombok Regency, West Nusa Tenggara
**Endophytic Microorganism Isolation**

Grass samples were washed with running tap water to remove all the debris. Grass samples were surface sterilized with 70% ethanol for 3 minutes, sodium hypochlorite (NaClO) 2.5% for 5 minutes, and rinsed several times with sterile deionized water (Xu et al., 2014; Qian et al., 2018). The last rinsed water was cultured in the nutrient agar (OXOID CM0003B) to confirm the success of surface sterilization (Anjum and Chandra, 2015). Five grams of each grass samples were mashed with sterile mortar and pestle in the Laminar Air Flow (LAF). The mashed sample was suspended with 45 mL of sodium chloride (NaCl) solution 0.86% (v/v) and mixed it with Vortex. One milliliter of aliquot in 10 microliters of aliquot in 10 mL of NaCl to make ten folds of serial dilution (up to 10⁷). One hundred microliters of aliquot in each serial dilution was cultured in Nutrient Agar (NA) for bacteria isolation and Potato Dextrose Agar (OXOID CM0139B) for fungi isolation. The cultured media were added with 10 mg L⁻¹ of mercury chloride (HgCl₂) for Hg-resistant microorganism isolation (Xu et al., 2014; Anjum and Chandra, 2015; Chasanah et al., 2018). The inoculated media were incubated at 28 °C (room temperature) for 48 hours for microorganism enumeration and characterization (Nemati et al., 2016).

**Rhizosphere Microorganism Isolation**

Soil rhizosphere samples were cleaned from roots and all debris. Five grams of soil sample were suspended in 45 mL NaCl solution 0.86% (v/v) then mixed it with Vortex. One milliliter of aliquot was diluted in 9 mL of NaCl to make ten folds of serial dilution (up to 10⁷). One hundred microliters of aliquot in 10⁴, 10⁵, and 10⁶ serial dilution were cultured in NA for bacteria isolation and PDA for fungi isolation. The cultured media were added with 10 mg L⁻¹ of mercury chloride (HgCl₂) for Hg-resistant microorganism isolation (Xu et al., 2014; Anjum and Chandra, 2015; Chasanah et al., 2018). The inoculated media were incubated at room temperature (28 °C) for 48 hours for microorganism characterization and enumeration (Nemati et al., 2016).

**Microbial Enumeration and Characterization**

Total microorganisms were counted using Colony Forming Unit (CFU) or Standard Plate Count (Nemati et al., 2016). Microorganism colony diversity was characterized based on morphological characteristics such as colony size, form, margin, chromogenesis (pigmentation), elevation, and texture. A colony was determined as a single distinct colony when it was different among other colonies on the same Petri dish.

**Data Analysis**

Data were analyzed using One Way ANOVA at 5% significant level using Genstat software. The relationships among treatment grouping were determined with Tukey’s test at 5% significance level.
study by Krisnayanti et al. (2012). After eight years, Hg concentration in the abandoned gold mining area decreased. This location is currently used as agricultural land and fish ponds. Some grass species vegetated in this area, such as Cynodon dactylon and Eleusine indica. These grasses were pioneer vegetation in the study site.

Plants’ ability to grow in polluted areas cannot be separated from beneficial microorganisms and plants’ interaction. These microorganisms reside within the plant’s body or in the rhizosphere (Thijs et al., 2017). These microorganisms help plants survive in heavy metal stress conditions by producing plant growth hormones such as IAA, siderophore production, and nitrogen fixation (Rajkumar et al., 2009; Montalban et al., 2016).

Mercury concentration in the study site (41.37 mg kg⁻¹) exceeded Hg’s permissible concentration in the soil based on Indonesian Government Regulation (2014). It was also exceeded the minimum recommended level for agricultural land (Canadian Council of Ministers Environment, 1999; Ustiatik et al., 2020). Hg concentration in the previous study by Ustiatik et al. (2020) was 17 times lower than the study by Krisnayanti et al. (2012). After eight years, Hg concentration in the abandoned gold mining area decreased. This location is currently used as agricultural land and fish ponds. Some grass species vegetated in this area, such as Cynodon dactylon and Eleusine indica. These grasses were pioneer vegetation in the study site.

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| Isolate code | Size (mm) | Form | Margin | Chromogenesis | Elevation | Texture |
|--------------|-----------|------|--------|---------------|-----------|---------|
| BA           | 6.0       | Circular | Undulate | Cream | Convex | Smooth |
| BB           | 11.0      | Circular | Undulate | Orange | Umbonate | Smooth |
| BC           | 5.0       | Circular | Undulate | Orange | Convex | Smooth |
| BD           | 10.0      | Circular | Entire | Cream | Umbonate | Smooth |
| BE           | 8.0       | Circular | Undulate | Cream | Flat | Dry |
| BF           | 6.5       | Circular | Lobate | Cream | Umbonate | Rough |
| BG           | 13.25     | Irregular | Lobate | Cream | Umbonate | Rough |
| GA           | 3.5       | Circular | Lobate | Cream | Flat | Dry |
| GB           | 14.0      | Circular | Entire | Cream | Flat | Contain concentric rings |
| GC           | 31.5      | Rhizoid | Lobate | Cream | Flat | Dry |
| GD           | 14.0      | Circular | Entire | Cream | Flat | Contain concentric rings |
| GE           | 14.0      | Circular | Entire | Cream | Flat | Contain concentric rings |
| GF           | 31.5      | Rhizoid | Lobate | Cream | Flat | Dry |

Note: B = Cynodon dactylon; G = Eleusine indica

Figure 3. Endophytic bacteria colonies (A) and rhizosphere bacteria colonies (B)
Table 2. Colony morphology characterization of isolated endophytic fungi from local grasses

| Isolate code | Size (mm) | Form    | Margin | Pigmentation Front | Pigmentation Back | Elevation |
|--------------|-----------|---------|--------|--------------------|-------------------|-----------|
| B1           | 47.75     | Filamentous | Lobate | Black-greenish     | Grey (center) White (margin) | Raised    |
| B2           | 48.00     | Irregular | Undulate | White (margin) Goldish-yellow (center) | White-yellowish (margin) Goldish-yellow (center) | Raised    |
| G1           | 4.25      | Rhizoid  | Lobate | White              | White-yellowish   | Umbonate  |
| G2           | 4.25      | Rhizoid  | Lobate | White              | White-yellowish   | Umbonate  |

Note: B = *Cynodon dactylon*; G = *Eleusine indica*

Table 3. Colony morphology characterization of isolated rhizosphere bacteria from the rootzone of local grasses

| Isolate | Size (mm) | Form    | Margin | Chromogenesis | Elevation       | Texture          |
|---------|-----------|---------|--------|---------------|-----------------|------------------|
| RHI A   | 10.5      | Circular | Entire | Yellow        | Raised          | Contain concentric rings radial |
| RHI B   | 6.0       | Circular | Entire | Cream         | Raised          | Smooth and shiny  |
| RHI C   | 13.25     | Rhizoid  | Lobate | Cream         | Crateriform     | Contain concentric rings |
| RHI D   | 1.5       | Circular | Entire | Yellowish     | Convex          | Shiny            |
| RHI E   | 11.0      | Circular | Lobate | Cream         | Umbonate        | Smooth and shiny  |
| RHI F   | 14.0      | Irregular| Lobate | Cream         | Raised          | Contain concentric rings |
| RHI G   | 5.5       | Irregular| Entire | Cream         | Flat            | Dry              |
| RHI H   | 3.5       | Irregular| Entire | Yellowish     | Umbonate        | Smooth and shiny  |

Table 4. Colony morphology characterization of isolated rhizosphere fungi from the rootzone of local grasses

| Isolate | Size (mm) | Form    | Margin | Pigmentation Front | Pigmentation Back | Elevation |
|---------|-----------|---------|--------|--------------------|-------------------|-----------|
| RHI 1   | 32.5      | Irregular| Undulate | Yellow            | Yellow            | Raised    |
| RHI 2   | 40.0      | Rhizoid  | Filamentous | Gray            | Black             | Flat      |
| RHI 3   | 33.0      | Circular | Filamentous | Gray            | White-pinkish     | Flat      |
| RHI 4   | 18.0      | Irregular| Filamentous | White          | White-yellowish   | Flat      |
| RHI 5   | 42.5      | Irregular| Lobate   | White-Yellowish  | White-yellowish   | Umbonate  |
| RHI 6   | 15.0      | Circular | Lobate   | White            | White-brownish    | Umbonate  |
| RHI 7   | 20.0      | Circular | Undulate | Green            | Green-brownish    | Umbonate  |
| RHI 8   | 19.0      | Circular | Entire   | Green            | Black             | Raised    |
| RHI 9   | 8.5       | Circular | Filamentous | White          | Brown             | Umbonate  |
| RHI 10  | 9.5       | Circular | Lobate   | White            | White-yellowish   | Umbonate  |
| RHI 11  | 52.0      | Circular | Undulate | Pink-goldish     | White-brownish    | Crateriform |
| RHI 12  | 35.5      | Circular | Filamentous | Green-whitish   | Green-blackish    | Umbonate  |
CONCLUSION

The colonies of rhizosphere microorganisms were morphologically more diverse than the colonies of endophytic microorganisms. The density of rhizosphere microorganisms in the study site was higher than endophytic microorganisms. The density of rhizosphere bacteria and fungi were $47 \times 10^3$ and $2 \times 10^3$ CFU g$^{-1}$, respectively. However, the density of endophytic bacteria and fungi were only $2 \times 10^3$ and $1 \times 10^3$ CFU g$^{-1}$, respectively.

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