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# Characteristics of rhizobacteria in potential hyperaccumulator vegetation and their resistance to gold mine tailing stress

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**Abstract:** The use of local bacteria is preferred in bioleaching as an environmental-friendly alternative technology in gold mining. In a preliminary study, rhizobacteria were isolated and cultured from three types of hyperaccumulator vegetation from the Ratatotok gold mine, Indonesia, namely *Pteris vittata* L., *Syzygium aromaticum* L., and *Swietenia mahagoni* Jacq. These rhizobacteria still need to be characterised and identified. This study is aimed to cover bacterial phenotypic characterisation, assessment of bacteria resistance to tailing, and identification of bacterial strains the exhibit the highest resistance to tailings. The assessment was carried out across a spectrum of tailing concentrations, selecting the three most robust strains for molecular identification. The process involved genotypic characterisation to determine the species name by analysing the 16S rRNA gene. The results reveal that the phenotypic characteristics of the bacteria isolates vary, but all of them are the indole acetic acid (IAA) hormone producers. The highest IAA producer is the isolate from the rhizosphere of *S. aromaticum*. Based on the genotypic characterisation test, three most resistant isolates to tailing stress are the following strains *Pseudomonas aeruginosa* (RTKP1) and *Stenotrophomonas geniculata* (RTKP2), both from the rhizosphere of *P. vittata*; as well as *Bacillus cereus* (RTKS) from the rhizosphere of *S. aromaticum*. These three strains need to be further tested for their bioleaching capability to recover gold from tailings. Additionally, this study recommends that gold recovery using biological agents can combine the role of hyperaccumulator plants in phytomining and rhizobacteria in bioleaching.

**Keywords:** gold-mine, hyperaccumulator-vegetation, rhizobacteria, resistance, tailing

## INTRODUCTION

Indonesia ranks 12$^{th}$ out of the top 20 gold-producing countries in the world (USGS, 2022), so gold has become an important commodity for the country. The Ratatotok District, Southeast Minahasa Regency, North Sulawesi Province, Indonesia, is a gold-producing area and still exhibits high gold content, varying from 0.012 to 2.41 $g \cdot Mg^{-1}$ (Azzaman, Idrus and Titisari, 2021). The authors conducted a preliminary study at the Ratatotok mine in 2022 to obtain information regarding the type of gold deposits, concentration of gold in the soil, and the types of potential hyperaccumulator vegetation at the mine site, as well as to isolate

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rhizobacteria (bacteria that live in the rhizosphere of potential hyperaccumulator vegetation). The preliminary study provided information about the type of gold deposit in Ratatotok, which is refractory and stored in sulphide ore, so the gold particles become invisible (Aminatun, Rakhmawati and Atun, 2022).

The term "invisible gold" refers to forms of gold that are difficult to extract. In general, the extraction of such gold involves cyanide acid, a toxic compound that poses a significant environmental threat and potential for ecological disaster (Gani, Abidjulu and Wuntu, 2017; Muyassaroh and Salami, 2018). Bioleaching, which uses bacteria for gold extraction, offers an environmentally friendly alternative. A previous study examined bioleaching using the iron-oxidising bacteria of *Acidithiobacillus ferrooxidans* (Brinza *et al.*, 2021). However, the induction of exogenous or exotic bacteria in the bioleaching process caused competition with local bacteria, thereby resulting in negative effects (Phyo *et al.*, 2020). They influenced the function of local ecosystems due to changes in local microorganism populations, and the level of biogeochemical cycles (Moore *et al.*, 2022). Therefore, the use of local bacteria is favoured. There is a crucial need to explore local bacteria, especially rhizobacteria in accumulator vegetation resilient to metal stress.

The preliminary study conducted by the authors in 2022 found that three types of potential hyperaccumulator plants had a high importance index and moderate bioaccumulation capability (*BCF*[1] > 0.1–1.0), namely *Pteris vitata* L., *Syzygium aromaticum* L., and *Swietenia mahagoni* Jacq., while other types had low bioaccumulation (*BCF* < 0.1) (Aminatun, Rakhmawati and Atun, 2022). A *BCF* value between 0.1 and 1.0 is considered moderate (Yoon *et al.*, 2006; Herlina *et al.*, 2020; Kurniawan, Hamim and Satya, 2022). The ability of vegetation to resist metal stress is possible due to the presence of rhizosphere bacteria colonies that live in plant roots and produce secondary metabolite compounds such as indole acetic acid (IAA) or other growth triggers as well as siderophore compounds. Thus, they can chelate metals in the soil and transport them into their cells (Govarthanan *et al.*, 2016). A previous study (Pramono *et al.*, 2012) found that bacterial isolates were tolerant to 15 ppm of Cr heavy metal stress, producing organic acid compounds playing an important role in the Cr phytoextraction mechanism in the corn. Furthermore, the preliminary study by the authors in 2022 succeeded in isolating bacterial colonies from the rhizosphere of the three potential hyperaccumulator vegetation, namely $39{\cdot}10^{15}$ from the rhizosphere of *Syzygium aromaticum* L., and $61{\cdot}10^{14}$ $CFU{\cdot}g^{-1}$ from the rhizosphere of *Swietenia mahagoni* Jacq., and these bacterial colonies are thought to influence the phytoextraction and bioaccumulation processes of gold in the three vegetation biomasses (Aminatun, Rakhmawati and Atun, 2022), so they have the potential to act as bioleaching agents. Research on gold bioleaching utilizing local bacteria, especially from the Ratatotok gold mine in North Sulawesi Province, Indonesia, has never been carried out. However, before testing its bioleaching potential, the bacterial colonies need to be characterised and tested for their resistance to metal stress in tailings. Therefore, the objectives of this study include (a) characterise bacterial isolate populations from the rhizosphere of three types of potential hyperaccumulator vegetation at the Ratatotok gold mine, (b) test for the resistance of the bacterial isolate population to gold mining tailing stress, and (c) identify bacterial strains that were most resistant to tailing stress.

[1] Bioconcentration factor (*BCF*) = the ability of plants for elemental accumulation from the substrate (Wu *et al.*, 2011 as cited in Mishra and Pandey, 2019).

## MATERIALS AND METHODS

### GENERAL INFORMATION

As stated in the introduction, prior to field exploration in 2022, culminating in the isolation and culture of bacterial populations from the rhizosphere of three potential hyperaccumulator plants at the Ratatotok gold mine. These plants include *Pteris vittata* L., *Syzygium aromaticum* L., and *Swietenia mahagoni* Jacq. Specifically, four bacterial isolates labeled A, B, C, and D were derived from *S. mahagoni;* eight isolates, E to L from *P. vittate*, and four isolates, M, O, P, and Q from *S. aromaticum* (Aminatun, Rakhmawati and Atun, 2022). Furthermore, the study included the following stages: (a) phenotypic characterisation of bacteria, (b) bacterial resistance test to tailings from the Ratatotok gold mine site, North Sulawesi Province, Indonesia, and (c) identification of bacterial strains most resistant to tailings. The methodologies employed in these stages are described below.

### PHENOTYPIC CHARACTERISATION OF BACTERIA

This phenotypic characterisation included Gram staining, bacterial spore staining, observation of colony morphology, observation of morphology on agar slant, growth test on liquid media, oxygen demand test, carbohydrate fermentation tests using glucose, maltose, sucrose, galactose, and lactose, amylum hydrolysis test, gelatin hydrolysis test, catalase test, citrate test, $H_2S$ production test, indole production test, motility test, temperature effect test, pH effect test, MR-VP (Methyl Red-Voges Proskauer) test, NaCl stress effect test, and indole acetic acid (IAA) production test.

The Gram staining was carried out by taking sterile distilled water and dripping it on a glass slide, followed by taking one loop of sample culture aseptically. The bacterial culture and sterile distilled water were then fixed using a Bunsen flame. Subsequently, it was dripped with crystal violet dye, left for 30 s, and washed with running water until the water flow appeared clear. After that, it was dripped with Lugol, left for 10 s, and washed again with running water until the water flow was clear. Next, it was dripped with 95% ethyl alcohol, left for 10–20 s, and washed with running water until the water flow was clear again. Then, it was dripped with safranin, left for 30 s, and washed with running water until the water flow appeared clear. Furthermore, the glass slide was air-dried and then observed under a microscope. If the colour of the bacteria was red, the bacteria were classified as gram-negative. If the colour of the bacteria was purple, the bacteria were classified as gram-positive. Moreover, the shape and arrangement of the bacterial cells were also observed.

The bacterial spore staining (endospores) was carried out by dripping sterile distilled water onto a glass slide and then taking one loop of sample culture aseptically. The bacterial culture and sterile distilled water were then fixed using a Bunsen flame. A piece of filter paper was placed to cover the smear. The malachite green paint was dripped onto the filter paper and then

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evaporated for 5 minutes over a bath. The malachite green paint was added when the filter paper started to dry. After evaporation, the filter paper was carefully taken out and then the slide was washed with running water until no more paint came off the slide. The bacterial smear was then dripped with safranin and left for 30 s. In the next step, the slide was washed with running water until the water flow was clear and then air-dried. The slide was then observed under a microscope. Bacterial endospores were green, while vegetative cell nuclei was red.

The observation of the colony morphology was carried out by growing bacterial colonies on a nutrient agar plate medium aseptically. One loop of bacterial culture was inoculated by placing it in the middle of the nutrient agar plate and then incubated at 37°C for 48 h. The growing bacterial colonies were then observed for shape, colour, elevation, edge of the colony, and the colony configuration. Endospores were expected to be formed by several genera of bacteria due to the lack of essential nutrients and water.

The morphological observation on the agar slant was carried out by growing bacterial colonies on a nutrient agar slant medium aseptically. One loop of bacterial culture was inoculated by streaking it straight in the middle of the nutrient agar slant medium, then incubated at 37°C for 48 h. The growing bacterial colonies were then observed for their growth characteristics.

The bacterial growth test in liquid media was carried out by inoculating one loop of bacterial culture in a nutrient broth (NB) medium in a test tube aseptically and then incubating it for 24 h. Furthermore, the characteristics of bacterial growth in the medium were observed, including uniform line turbidity, flocculant growth, pellicle, and sedimentation.

The bacterial oxygen demand test was carried out by inoculating one loop of bacterial culture in a nutrient broth (NB) medium in a test tube aseptically and then incubating it for 24 h. Next, the characteristics of bacterial growth in the medium were observed. Overall bacterial growth in the medium indicated that the bacteria were facultative anaerobes. Bacterial growth forming a ring only at the top of the medium indicated that the bacteria were aerobic, while bacterial growth only at the bottom of the medium indicated that the bacteria were anaerobic.

The carbohydrate fermentation test (using glucose, maltose, sucrose, galactose, and lactose) was carried out by growing bacteria in a carbohydrate medium (NB + 0.5% glucose/maltose/sucrose/galactose/lactose) which are given pH indicator (phenol red) in Durham tube. One loop of bacterial culture was inoculated in a test tube containing a growth medium, to which carbohydrates had been added aseptically, and then incubated for 24 h at room temperature. Microbes could ferment certain carbohydrates, resulting in an acidic pH and gas. The presence of this acid was indicated by a change in medium colour to orange/yellow, while gas production was detected by the formation of bubbles that were collected in Durham tube. The enzymatic reactions in this test were related to respiration and fermentation.

The amylum hydrolysis test was carried out by inoculating one loop of bacterial culture on a Starch Agar plate medium using one point and then incubating it for 24–48 h at room temperature. The bacterial colonies that grew were then dripped with iodine. If bacteria could hydrolyse amylum, a clear zone would form around the bacterial colony.

The gelatin hydrolysis test was carried out by inoculating one loop of bacterial culture on a nutrient gelatin medium by sticking it into the medium and then incubating it for 24–48 h at room temperature. The incubated medium was then put into the refrigerator. If the bacteria could hydrolyse gelatin, the medium would remain liquid even if they had been cooled in the refrigerator. The gelatin nutrient medium was semisolid at room temperature, liquid at 37°C or higher, and solid at low temperatures.

The catalase test was carried out by spreading thinly one loop of bacterial culture on a glass slide, which had been cleaned with alcohol, and then drying it over a Bunsen flame. The bacterial isolate was then dripped with 3% hydrogen peroxide. Positive results were indicated by the formation of bubbles on the bacterial smear.

The citrate test was carried out by inoculating one loop of bacterial culture in a Simmons citrate growth medium which had bromthymol blue indicator added by stabbing and scribbling, and then incubating it for 24–48 h at room temperature. Positive results were indicated by a change in the growth medium colour from green to blue due to the alkaline nature of the medium.

The test for $H_2S$ production was carried out by inoculating one loop of bacterial culture into a tube of sulphide indole motility (SIM) medium. The tube was then incubated at room temperature for 24–48 h. The $H_2S$ production is detected by the formation of black precipitates originating from iron(II) sulphide (FeS) formation, which occurs when $H_2S$ produced by bacteria reacts with iron (II) sulphate ($FeSO_4$).

The indole production test was carried out by inoculating one loop of bacterial culture in a SIM medium by stabbing and then incubating it for 24–48 h. After incubation, one drop of Kovac reagent was added. A positive result in the indole test is marked by the appearance of a red ring in the medium. The test is used to determine the presence of the tryptophane enzyme produced by bacteria.

The motility test was carried out by inoculating one loop of bacterial culture into a SIM and nutrient agar medium by stabbing it upright and then incubating it for 24 h at room temperature. The presence of motile bacteria is marked by changes in the medium which becomes cloudy in the puncture area. The test is used to determine the movement of flagellated bacteria.

The temperature effect test was carried out using three temperature levels, namely refrigerator temperature (–4°C), room temperature (25°C), and oven temperature (50°C). The effect of temperature on the growth of bacterial isolates was tested by inoculating one loop of bacterial culture in a NB medium. Then, it was incubated at three temperature levels for 24–48 h. The presence of bacteria that could grow in each treatment was indicated by changes in turbidity in the medium.

The pH effect test was carried out using three pH levels, namely pH 4, 7, and 9. One loop of bacterial culture was inoculated on a NB medium with various pH levels (4, 7, and 9). Then, it was incubated for 24–48 h at room temperature. The presence of bacteria that could grow in each treatment was indicated by changes in turbidity of the medium.

The MR-VP test was carried out by inoculating one loop of bacterial culture in an MR-VP medium in a test tube aseptically. It was then incubated for 24 h. The methyl red (MR) test was performed by adding 5 drops of methyl red and a change in medium colour was observed. The result of the test was positive when the medium colour would change to red. Then, for the Voges–Proskauer (VP) test, 6 drops of alpha naphthol and

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2 drops of KOH 40% were added, and a change in medium colour was observed. The result of the test was positive when the medium colour would change to red.

The NaCl stress effect test was performed using NaCl concentrations of 5 and 10%. One loop of bacterial culture was inoculated in a NB medium with variations of NaCl concentrations of 5 and 10%. Then, it was incubated for 24–48 h at room temperature. Next, a change in medium turbidity was observed. If bacteria grew, the medium would become increasingly cloudy. If the medium remained clear, the bacteria were not growing.

A qualitative and quantitative IAA production test was carried out. A qualitative IAA production test was carried out by inoculating bacteria on a nutrient agar plate + L-tryptophan (100 mg·dm$^{-3}$) medium. Then, it was incubated at room temperature for 48 h. Next, Salkowski's reagent was dripped on the isolate that had grown evenly, and it was incubated in the dark for 30 min. Positive results were indicated by a change in colony colour to red or pink.

A quantitative IAA production test was carried out by inoculating bacteria on a nutrient broth + L-tryptophan (100 mg·dm$^{-3}$) medium. Then, it was incubated for 72 h with a shaker at 120 rpm at room temperature. The bacterial suspension was taken and centrifuged at 3000 rpm for 30 minutes until supernatant and pellets were formed. Then, 1 cm$^3$ of the supernatant was added together with 2 cm$^3$ of Salkowski's reagent to a test tube. Subsequently, it was incubated in the dark for 30 min. Absorbance was measured using a spectrophotometer with a wavelength of 530 nm with a sterile NB 7 blank. The absorbance values were compared with the IAA standard curve that had been created to determine amounts of IAA produced by the bacteria. The IAA standard curve was prepared with synthetic IAA (10, 20, 30, 40, 50, and 60 ppm) + distilled water + Salkowski's reagent. Then, it was incubated in the dark for 30 min and the absorbance was measured using a spectrophotometer with a wavelength of 530 nm.

## BACTERIAL RESISTANCE TEST TO GOLD MINE TAILINGS

A test for bacterial resistance to tailings was carried out by making minimal salt media (MSM) with tailing concentrations of 30, 40, 50, and 60%. Then, the bacteria were inoculated by adding 10% bacterial suspension to the resistance test medium. The initial optical density (*OD*) of bacteria was measured using a spectrophotometer with a wavelength of 600 nm. Then, the bacteria were incubated for 24 h using an incubator shaker at 120 rpm at room temperature. After that, the final *OD* of the bacteria was measured using a spectrophotometer with a wavelength of 600 nm. If the *OD* of the bacteria increased, the bacteria could grow well.

Following the resistance test, tailings (spent ore) from the Ratatotok gold mine, North Sulawesi Province, Indonesia, were used to develop a tailing sampling map as shown in Figure 1.

Before its use for the resistance test, the tailings were turned into a pulp and tested for Au content at the mining company laboratory (PT Sumber Energi Jaya), in Ratatotok, North Sulawesi Province, Indonesia, using the fire assay method. The test result showed that the average Au content was 0.25 ppm.

## IDENTIFICATION OF THE BACTERIAL STRAINS MOST RESISTANT TO TAILINGS

Three bacterial isolates with the highest resistance to tailings were then identified at the PT Genetica Science Indonesia Laboratory, Tangerang, Banten, Indonesia by carrying out

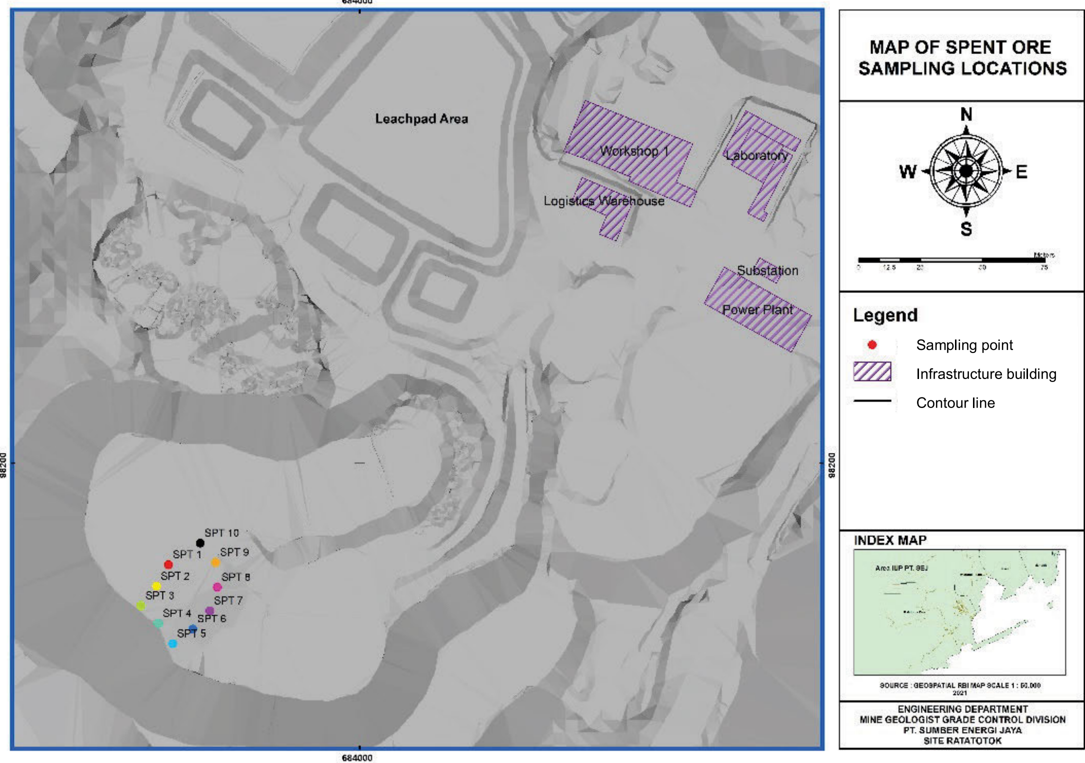


**Fig. 1.** Map of tailing (spent ore) sampling at the Ratatotok gold mining site, North Sulawesi, Indonesia; SPT 1–SPT 10 = sampling points of tailing; source: PT Sumber Energi Jaya (2023)

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genotypic characterisation to determine the species name based on the 16S rRNA gene. The method for DNA extraction was used with the Quick DNA™ Bacterial Miniprep Kit (Zymo Research, D6005). The study used universal primers 27F (5' AGAGTTT-GATCCTGGCTCAG 3') and 1493R (5' GGTTACCTTGTTAC-GACTT 3'). DNA amplification was conducted using MyTaq HS Red Mix (Bioline, BIO 25047). Purification of the resulting DNA was carried out using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research, D4008). After the 16S rRNA nucleotide sequence was determined, the basic local alignment search tool (BLAST) analysis was conducted to find sequence similarities in the existing database in the NCBI Genbank. A phylogenetic tree depicting the relationships between isolates was developed using the neighbour joining method with the molecular evolutionary genetics analysis (MEGA) X program (Kumar *et al.*, 2018).

The general identification does not determine specific bacterial genes as it generally uses the 16S gene marker. The length of the 16S gene sequence is approximately 1,550 bp and consists of conserved regions. Advantages of using 16S for identification include the ability to identify bacteria that cannot be cultured, high level of accuracy, and the time required is relatively short. The neighbour joining method is used for phylogenetic analysis because it can be applied for various needs, for instance creating a phylogenetic tree of the entire genomes. A distance matrix is created based on how many genes differ between species, and it is used to create a neighbour joining method tree. Usually, such trees are much more accurate than maximum likelihood method trees based on selected gene alignments (Janda and Abbott, 2007).

## RESULTS

### PHENOTYPIC CHARACTERISTICS OF RHIZOBACTERIA ISOLATES FROM RATATOTOK

The phenotypic characteristics of the bacterial isolates are shown in Table S1.

The results of observations of bacterial colony morphology are shown in Table 1.

Based on qualitative tests (Tab. S1), isolates of D, E, M, O, P, and Q were positive for producing the IAA hormone as indicated by a change in the colony colour to pink after being dripped with Salkowski's reagent. However, based on quantitative tests, all isolates were capable of producing the IAA hormone, but some isolates resulted in IAA hormone in small quantities so they were not visible in qualitative tests. The results of the quantitative test for IAA production are shown in Table 2.

### RESISTANCE OF BACTERIAL ISOLATES TO GOLD MINING TAILINGS

The results of the resistance test of bacterial isolates are presented in Table 3.

Based on the resistance test of bacterial isolates to gold mine tailings, the E, L, O, and P isolates were the most resistant as they exhibited stable growth patterns at various tailing concentrations and had the best growth capability at the highest tailing concentration. Bacterial growth was marked by an increase in optical density (absorbance) values. Isolates E and L came from the rhizosphere of *Pteris vittata*, while isolates O and P from the

**Table 1.** Bacterial colony morphology

| Isolate code | Shape | Colour | Elevation | Edge of the colony | Configuration |
|---|---|---|---|---|---|
| A | irregular | white | convex | lobate | wrinkled |
| B | irregular | yellowish white | convex | undulate | round |
| C | irregular | yellowish white | convex | undulate | round |
| D | irregular | white | convex | lobate | spreading |
| E | irregular | greenish white | convex | undulate | round and scalloped |
| F | irregular | greenish white | convex | undulate | round and scalloped |
| G | circular | white | convex | entire | concentric |
| H | irregular | brownish white | convex | undulate | concentric |
| I | circular | yellowish white | convex | undulate | round and scalloped |
| J | circular | white | convex | entire | concentric |
| K | irregular | yellowish white | convex | lobate | irregular and spreading |
| L | circular | yellowish white | convex | entire | round |
| M | irregular | brownish white | convex | lobate | concentric |
| O | irregular | white | convex | entire | concentric |
| P | irregular | white | convex | entire | concentric |
| Q | irregular | white | convex | entire | irregular and spreading |

Explanations: isolates codes as in Tab. S1.
Source: own study.

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**Table 2.** Quantitative test for IAA production

| Isolate code | IAA production (ppm) |
|---|---|
| A | 12,477 |
| B | 13,649 |
| C | 13,198 |
| D | 13,018 |
| E | 21,486 |
| F | 22,928 |
| G | 12,027 |
| H | 8,784 |
| I | 8,874 |
| J | 15,360 |
| K | 6,351 |
| L | 13,559 |
| M | 9,685 |
| O | 34,640 |
| P | 35,450 |
| Q | 37,162 |

Explanations: isolates codes as in Tab. S1.
Source: own study.

rhizosphere of *Syzygium aromaticum*. Based on these results, the *Pteris vittata* and *Syzygium aromaticum* plants have the potential as phytomining agents with the aid of bacteria around their roots (rhizobacteria).

### IDENTIFICATION OF BACTERIAL STRAINS MOST RESISTANT TO TAILINGS

The results of resistance tests to tailings stress were used as a basis for selecting bacterial strains that would be used as bioleaching agents in the next stage of this study. Three strains were selected to test the bioleaching ability of gold, namely isolates E, L, and P. Therefore, isolates E, L, and P were then identified molecularly. The results showed that isolate E was identified as *Pseudomonas aeruginosa* and its given strain name was RTKP1, isolate L was identified as *Stenotrophomonas geniculata* and its given strain name was RTKP2, and isolate P was identified as *Bacillus cereus* and its given strain name was RTKS. The phylogenetic tree is presented in Figure 2.

## DISCUSSION

The results of phenotypic analysis showed that the characteristics of the sixteen bacterial isolates varied (Tabs. S1 and 1), but all of them shared the same characteristics as producers of the IAA hormone. The highest IAA production was found in colonies O,

**Table 3.** Bacterial resistance tests at various tailing concentrations

| Isolate code | Optical density measurement | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 30% | | | 40% | | | 50% | | | | 60% | |
| | hour 0 | hour 24 | growth | hour 0 | hour 24 | growth | hour 0 | hour 24 | growth | hour 0 | hour 24 | growth |
| A | 0.370 | 0.816 | 0.446 | 0.637 | 0.658 | 0.021 | 0.322 | 0.401 | 0.079 | 0.531 | 0.846 | 0.315 |
| B | 0.394 | 0.863 | 0.469 | 0.427 | 0.845 | 0.418 | 0.482 | 0.817 | 0.335 | 0.659 | 1.142 | 0.483 |
| C | 0.406 | 0.578 | 0.172 | 0.618 | 0.824 | 0.206 | 0.706 | 1.015 | 0.309 | 0.742 | 1.072 | 0.330 |
| D | 0.207 | 0.402 | 0.195 | 0.906 | 1.833 | 0.927 | 0.970 | 1.778 | 0.808 | 1.102 | 1.452 | 0.350 |
| E | 0.663 | 0.674 | 0.011 | 0.450 | 1.436 | 0.986 | 0.294 | 1.494 | 1.200 | 0.226 | 0.814 | 0.588 |
| F | 0.270 | 0.577 | 0.307 | 0.226 | 0.692 | 0.466 | 0.280 | 0.857 | 0.577 | 0.246 | 0.779 | 0.533 |
| G | 0.406 | 0.886 | 0.480 | 0.449 | 0.646 | 0.197 | 0.483 | 0.707 | 0.224 | 0.476 | 1.015 | 0.539 |
| H | 0.692 | 0.976 | 0.284 | 0.846 | 0.949 | 0.103 | 0.753 | 1.003 | 0.250 | 0.671 | 1.018 | 0.347 |
| I | 0.588 | 0.892 | 0.304 | 0.916 | 0.984 | 0.068 | 0.661 | 0.997 | 0.336 | 0.658 | 1.072 | 0.414 |
| J | 0.729 | 0.818 | 0.089 | 0.621 | 0.985 | 0.364 | 0.661 | 1.061 | 0.400 | 0.259 | 0.654 | 0.395 |
| K | 0.719 | 0.758 | 0.039 | 0.208 | 0.659 | 0.451 | 0.211 | 0.783 | 0.572 | 0.175 | 0.507 | 0.332 |
| L | 0.326 | 0.835 | 0.509 | 0.364 | 0.482 | 0.118 | 0.360 | 0.569 | 0.209 | 0.411 | 1.005 | 0.594 |
| M | 0.354 | 0.643 | 0.289 | 0.853 | 1.102 | 0.249 | 0.898 | 0.909 | 0.011 | 0.342 | 0.761 | 0.419 |
| 0 | 0.638 | 1.075 | 0.437 | 0.458 | 1.037 | 0.579 | 0.382 | 1.379 | 0.997 | 0.425 | 1.093 | 0.668 |
| P | 0.291 | 1.169 | 0.878 | 0.236 | 1.192 | 0.956 | 0.495 | 1.226 | 0.731 | 0.281 | 1.182 | 0.901 |
| Q | 0.303 | 0.784 | 0.481 | 0.341 | 0.805 | 0.464 | 0.388 | 0.947 | 0.559 | 0.576 | 1.054 | 0.478 |

Explanations: isolates codes as in Tab. S1.
Source: own study.

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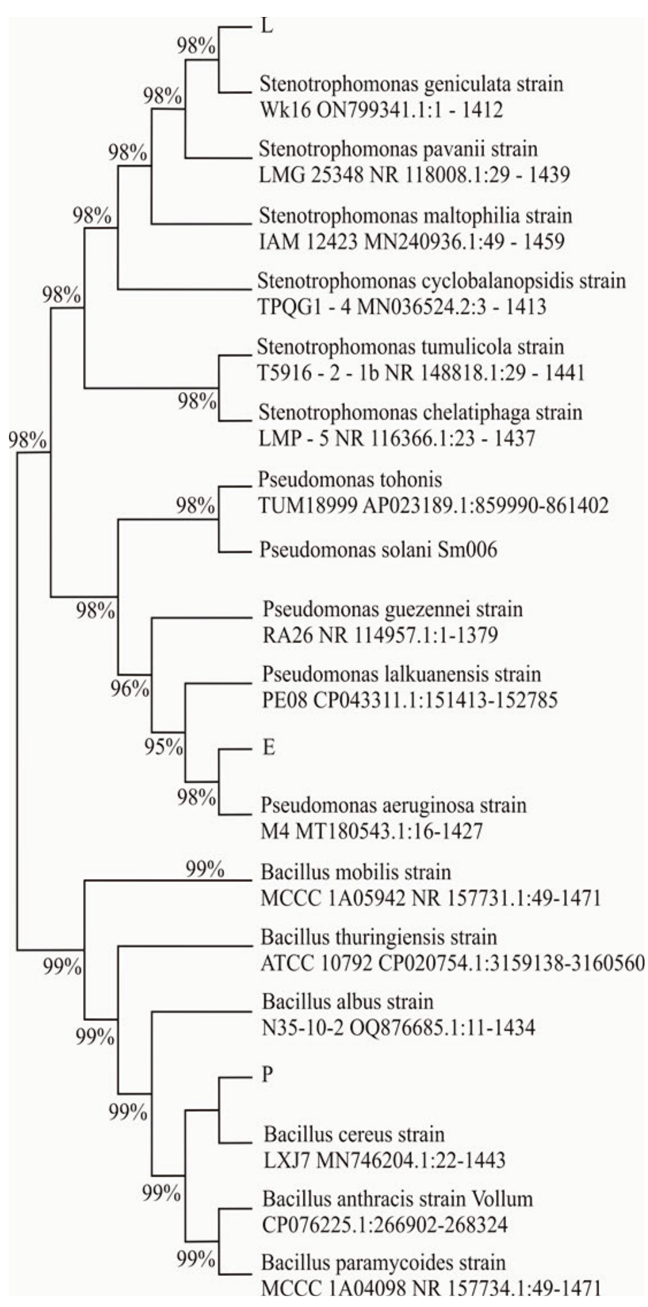


**Fig. 2.** The phylogenetic tree of the three resistant strains and other strains based on 16S rRNA gene analysis; source: own study

P, and Q which were isolates from the rhizosphere of *S. aromaticum* plants (Tab. 2). The preliminary study (Aminatun, Rakhmawati and Atun, 2022) also reported that the bacterial isolate from the rhizosphere of *S. aromaticum* resulted in higher amounts ($448 \cdot 10^{16}$ CFU·g$^{-1}$) compared to the bacterial isolates from the rhizosphere of *P. vittata* and *S. mahagoni*. In a similar vein, a previous study by Ishak, Ardyati and Aini (2018) found 110 bacterial isolates from the rhizosphere of *Z. aromaticum*, which were IAA producers.

The IAA is a plant growth hormone, so the bacteria that produce plant growth hormone are usually called plant growth promoting rhizobacteria (PGPR). The PGPR can mobilise metals and stimulate plant growth and development to increase the amount of metal extracted by plants with higher phytoextraction efficiency (Jalali and Lebeau, 2021). This has been confirmed by Mesa-Marín *et al.* (2018) who stated that hyperaccumulator plants associated with the PGPR could increase biomass and metal accumulation in roots. The previous study reported that experiments with isolated rhizobacteria from gold mine sites increased aboveground plant biomass (Nadeau, Laur and Khasa, 2018). Research on the rhizobacteria diversity in hyperaccumulator plants, non-hyperaccumulator plants, and non-vegetated soil reveal higher bacterial densities on the roots of hyperaccumulator plants. Most of these bacteria are resistant bacteria and produce IAA (Álvarez-López *et al.*, 2015).

Rhizobacteria also produce siderophores which react with chelate metals from the soil so the metals can be absorbed by plants, especially roots and leaves. Thereby, they increase the plant biomass. The resistance of rhizobacteria to various metals combined with the production of the IAA hormones and gibberellins can stimulate the growth and development of plants (Liu *et al.*, 2015; Pazos-Rojas *et al.*, 2018; Kamaruzzaman *et al.*, 2019; Manzoor *et al.*, 2019).

Based on the resistance test, isolate P from the rhizosphere of the *S. aromaticum* showed the highest resistance of all isolates. Isolate P exhibited a stable growth, had the best growth at the highest tailings concentration, and had the highest optical density increase at the highest tailing concentration (Tab. 3). The reason was that isolate P had higher capability to produce IAA compared to other isolates (Tab. 2).

The results of molecular identification showed that isolate E was *Pseudomonas aeruginosa* strain RTKP1, which came from the rhizosphere of *P. vittata*, isolate L was *Stenotrophomonas geniculata* strain RTKP2, which came from the rhizosphere of *P. vittata*, and isolate P was *Bacillus cereus* strain RTKS, which came from the rhizosphere of *S. aromaticum* (Fig. 2). Isolate showed 99.72% similarity to *Pseudomonas aeruginosa* M4 contained in samples taken from a food waste treatment plant. Isolate L showed similarity of 99.86% to *Stenotrophomonas geniculata* strain IAE94 of unknown origin, and isolate P had a similarity of 99.72% to *Bacillus cereus* strain LXJ7 isolated from the coastal Dinoflagellate bloom. Sequence ID from the NCBI Genbank for *Bacillus cereus* strain RTKS is OR905558 (NCBI, no date a). For *Stenotrophomonas geniculata* strain RTKP2 it is OR905565 (NCBI, no date c) and for *Pseudomonas aeruginosa* strain RTKP1 OR905567 (NCBI, no date c). In this study, the identification results for *B. cereus* from the rhizosphere of *S. aromaticum* were in line with a previous study by Dwimartina, Joko and Arwiyanto (2021) who stated that the dominant bacterial colony isolated from the rhizosphere of healthy clove plants (*S. aromaticum*) had an irregular pale white colony. Moreover, it was not slimy and had similar characteristics to *Bacillus* spp.

*B. cereus* is a gold-resistant bacterium (Zulaika *et al.*, 2019). It has been successfully isolated from gold mining sites in Brazil (Aguilar *et al.*, 2020). *B. cereus* is a spore-forming gram-positive bacterium that is commonly found in polluted environments. Its ability to form spores helps it to survive in hostile environments (Babalola, Aremu and Ayangbenro, 2019).

*B. cereus* is also an indicator of the gold biogeochemistry in soil. The frequency of *B. cereus* spores is higher in areas with high gold concentrations because it can dissolve gold which then stimulates the formation of spores. Therefore, *B. cereus* spores are confirmed to be present in soil at gold mining areas (Lao *et al.*, 2020). Because of its resistance, *B. cereus* is a potential bioleaching

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agent for various metals, such as iron (Jun *et al.*, 2020), gold (El-Sayed *et al.*, 2021), and arsenic (Cabrales-González *et al.*, 2022). *B. cereus* can also help hyperaccumulator plants in the removal and bioaccumulation of metals in bioleaching (Aurangzeb *et al.*, 2020).

Two other resistant isolates were *Pseudomonas aeruginosa* (strain RTKP1) and *Stenotrophomonas geniculata* (strain RTKP2). *P. aeruginosa* and *S. geniculata* are often referred to as the PGPR, as they stimulate plant growth (Babalola, Aremu and Ayangbenro, 2019; Khezrinejad, Khodakaramian and Shahryari, 2019). However, *P. aeruginosa* is more often referred to as a gold bioleaching agent because it is cyanogenic. Hence, it can produce hydrocyanide acid which can dissolve gold from minerals so it can extract and recover up to 70% of gold (Liang, Li and Ma, 2014; Natarajan and Ting, 2015; Suja *et al.*, 2018; Jorjani and Sabzkoohi, 2022).

## CONCLUSIONS

The phenotypic analysis shows that the characteristics of the sixteen bacterial isolates vary. However, all of them are indole acetic acid (IAA) hormone producers. The highest IAA production (37,162 ppm) was found in bacterial colonies which were isolates from the rhizosphere of *S. aromaticum*. Based on resistance tests, isolate P from the rhizosphere of the *S. aromaticum* had higher resistance than other isolates, which showed the best growth (increase in OD after 24 hours was 0.901) at the highest tailing concentration (60%). This finding could be attributed to its ability to produce IAA which was higher than many other isolates. Based on the genotypic characterisation test, three most resistant isolates to tailing stress were *Pseudomonas aeruginosa* strain RTKP1 and *Stenotrophomonas geniculata* strain RTKP2. They originated from the rhizosphere of *P. vittata*; whereas *Bacillus cereus* strain RTKS from the rhizosphere of *S. aromaticum*. These three bacterial strains need to be further tested for their bioleaching ability to recover gold from tailings. In addition, it is recommended to study gold recovery using biological agents that can combine the role of hyperaccumulator plants in phytomining and the role of rhizobacteria in bioleaching.

## SUPPLEMENTARY MATERIAL

Supplementary material to this article can be found online at: https://www.jwld.pl/files/Supplementary_material_Aminatun.pdf

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## CONFLICT OF INTERESTS

The authors declare no conflict of interests.

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