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Industrial Sludge Active Bacteria Potency Test of PT Surabaya Industrial Estate Rungkut (SIER) as a Heavy Metal Bioremediator and Biofertilizer

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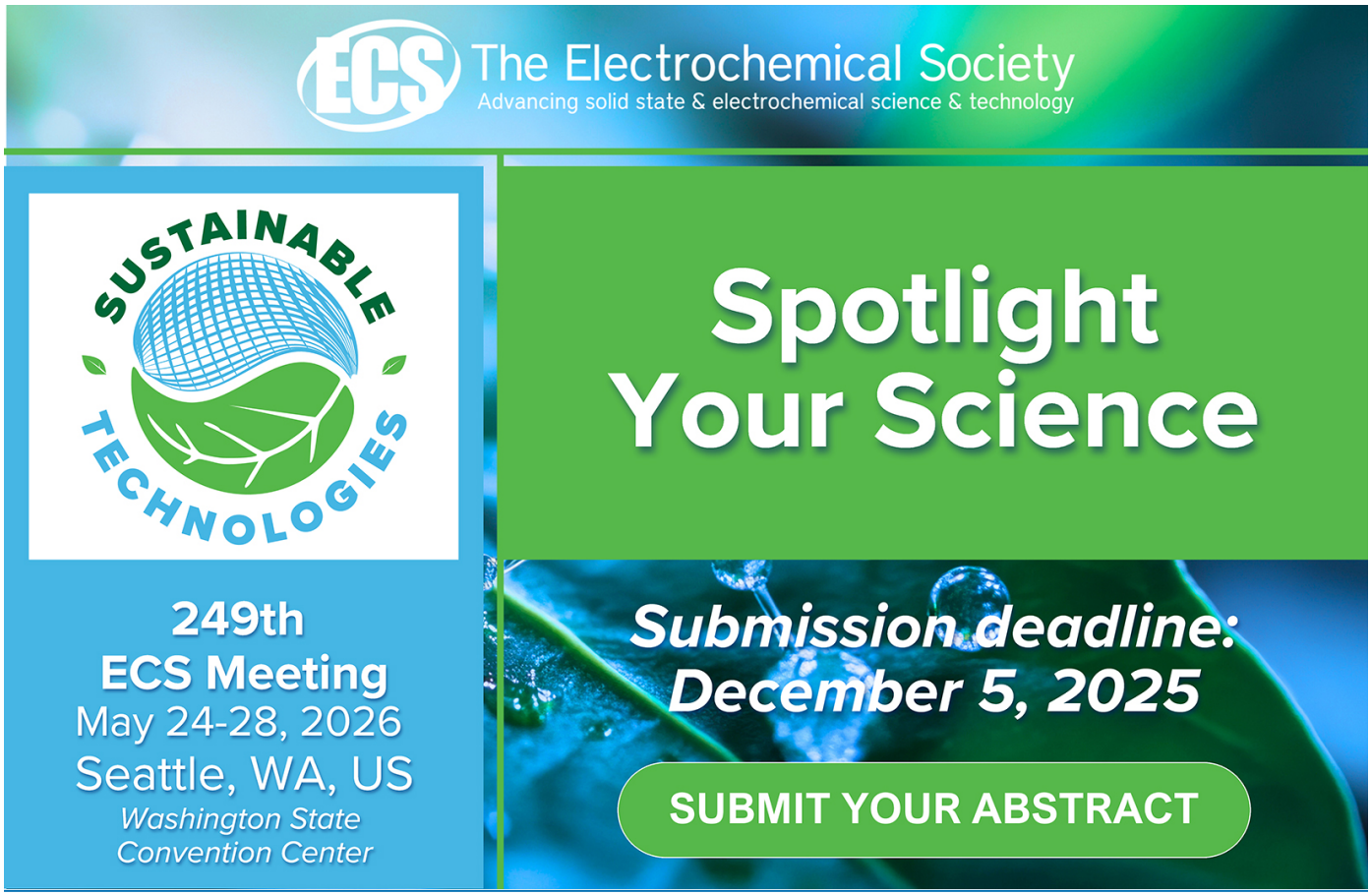

Industrial Sludge Active Bacteria Potency Test of PT Surabaya Industrial Estate Rungkut (SIER) as a Heavy Metal Bioremediator and Biofertilizer

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Abstract. Heavy metal contamination is a crucial problem to solve because it leads to poisoning, phytotoxicity and soil fertility reduction. Microbial assisted bioremediation is proposed to solve the problem due to their ability to adapt and mitigate heavy metal adverse effect. This study was aimed to determine the diversity of bacterial colonies, the level of tolerance of bacterial isolates, and the ability of bacterial isolates to produce plant growth hormone and secondary metabolites. Heavy metal tolerance tests were carried out on 6 bacterial isolates, namely S_1aB_1, S_1aB_2, S_1bB_4, S_2aB_5, S_3aB_7, and S_4bB_{11}. S_4bB_{11} was found as the isolate with the highest growth pattern based on spectrophotometry optical density (OD) when the isolate grown in Pb and Hg supplemented environment. Further GC-MS analysis on S_4bB_{11} detected several metabolites that play a role in heavy metal bioremediation namely α-Ketoglutaric acid, diaminopimelic acid and mannose. In addition, the presence of growth hormones (3indole acetic acid and kinetin) were also detected. Thus, the bacteria was predicted to have dual functions, both as industrial waste bioremediator and biofertilizer.

1. Introduction

Heavy metal contamination is a major problem in agricultural soils that often happens because of the presence of industrial activities nearby. Some of them are Pb, Cr, Cu, Ni, Zn, Cd and Hg [1]. According to Gupta [2], irrigated agricultural land with heavy metal contaminants in a certain period of time causes a decrease in land fertility. This is because heavy metals can reduce soil microbial activity, decrease soil capacity, and inhibit the process of soil mineralization [3]. Long-term contamination by heavy metals also causes a buildup of heavy metals in the soil which at a certain level can cause phytotoxicity [4].

One of the methods to overcome the problem of heavy metal pollution is bioremediation utilizing microbes. Some of the microbial mechanisms adapt to the metal-contaminated environment, such as microbial precipitation of insoluble metal salts, conversion of metals into an energy source, immobilization of metals into cell walls, changing the permeability of microbial cell membranes to metals, synthesis of chelating agents, and reducing metals into non-toxic forms. The ability of these microbes which are used in the metal detoxification process is known as bioremediation [5].

Bioremediation is the application of the principles of biological processes for the treatment of soil, groundwater, and active sludge contaminated by toxic and hazardous chemical wastes [6].

Bioremediation is a process in which harmful organic materials are degraded biologically by microorganisms into other compounds such as methane, CO_2, water, organic salts, biomass and by-products which are simpler than the original compounds [7]. This process is based on the carbon cycle, namely by recycling the form of organic and inorganic compounds through oxidation and reduction interactions (chemical redox reactions). Bioremediation can be carried out directly in a polluted environment (in-situ) by using biota or microflora in that environment or outside the polluted environment (ex-situ) by using inoculants that can degrade organic contaminants in the area. One form of ex-situ bioremediation is by inoculating a bacterial culture in a contaminated medium that is called as bioaugmentation [7].

Based on the principle above, the authors conducted research on the potential of bacteria contained in industrial waste to bioremediate environments contaminated by heavy metals as well as the ability of bacteria to produce phytohormones that can be used by plants in the form of biofertilizers. The results of this study are expected to be able to identify and produce bacterial isolates that are tolerant of heavy metals so that they can assist in bioremediation efforts for environments contaminated by heavy metals and these bacteria can become biological agents in soil fertility by producing phytohormones that promote the growth of production plants in the environment.

2. Materials and methods

2.1. Preparation of bacterial isolates

Six selected bacterial isolates were used in this study, namely S_1aB_1, S_1aB_2, S_1bB_4, S_2aB_5, S_3aB_7, and S_4bB_{11}. They are isolated from previous study as plastic waste tolerant bacterial isolates obtained from industrial waste sludge of PT SIER. The isolates were maintained in LB medium for daily use according to Putra [8].

2.2. Assay of heavy metal tolerance

The isolates were evaluated for their tolerance against two kinds of heavy metals (Hg^{2+} and Pb^{2+}). Each isolate were grown in M63 broth (100 mM KH_2PO_4, 75 mM KOH, 15 mM $(NH_4)_2SO_4$, 0.16 mM $MgSO_4$, 3.9 µM $FeSO_4$ and D-glucose 10 mM) supplemented with 100 ppm of each heavy metal [9]. The isolates were then incubated for 24 h under shaking (125 rpm) at 36 ^oC. Samples were taken every 2 h and bacterial growth was measured by observing optical density (OD) at λ 420 nm, using Shimadzu UV-1601PC spectrophotometer. The measured OD value is proportional to the number of cells present in the culture tested in a spectrophotometer [10]. Growth pattern of the isolates were transformed and projected using regression analysis so that the growth trend line can be obtained. Any isolates with stable growth pattern in both heavy metal treatments were chosen for further analysis.

2.3. Assay of phytohormone and other extracellular metabolite products

The chosen isolates were predicted to synthesize phytohormones (e.g. 3-indole acetic acid) and other extracellular metabolites related to heavy metal resistance. These were investigated by growing the isolates with the presence of heavy metal. The isolates were grown in M63 broth with tryptophan and Hg^{2+} as treatment; and M63 broth with tryptophan only as control. Both tryptophan and Hg^{2+} were set at 100 ppm. The presence of any phytohormones and extracellular metabolites produced were investigated using gas chromatography-mass spectrophotometry (GC-MS) procedure according to Bolten [11].

3. Results and dicussion

3.1. Heavy metal tolerance test

Figure 1 showed that not all bacterial isolates were able to survive under the exposure of 100 ppm Hg^{2+}. Among all, only S4bB11 and S1bB4 that showed stable growth pattern based on the trendline graph generated from regression analysis. Meanwhile, the growth of other isolates were suppressed in the presence of Hg^{2+}. S_4bB_{11} was found to be the isolate with the highest growth pattern. It peaked at

OD of 1.385 followed by S_1bB_4 with OD of 1.287. Since both S_4bB_{11} and S_1bB_4 had high regression coefficient at 91 and 93 % (Table 1), therefore, the use of regression analysis procedure was valid to represent the growth pattern of the bacteria.

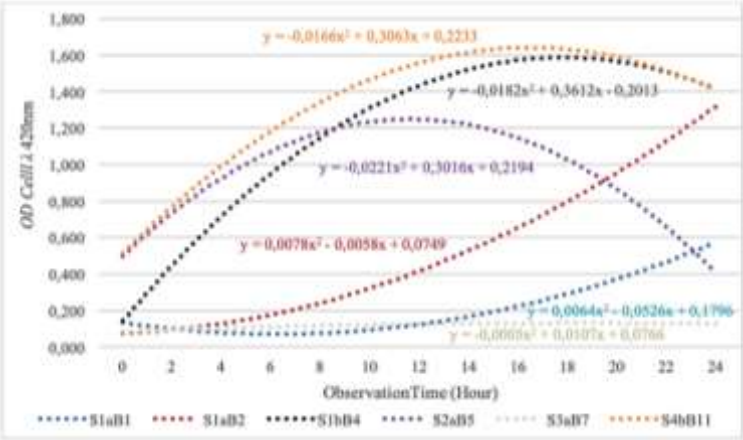


Figure 1. Trendline graph of six isolates growth pattern in 100 ppm Hg^{2+} (mercury).

Table 1. Regression analysis of bacterial growth trendline in 100 ppm Hg (mercury).

Isolate	Regression equation	R^2 (%)	X_{max} (hours)	Y_{max}
S_1aB_1	$y = 0.0064x^2 - 0.0526x + 0.1796$	93	24	0.577
S_1aB_2	$y = 0.0078x^2 - 0.0058x + 0.0749$	96	24	1.318
S_1bB_4	$y = -0.0182x^2 + 0.3612x - 0.2013$	93	17.5	1.591
S_2aB_5	$y = -0.0221x^2 + 0.3016x + 0.2194$	58	11.38	1.029
S_3aB_7	$y = -0.0005x^2 + 0.0107x + 0.0766$	7	19.24	0.057
S_4bB_{11}	$y = -0.0166x^2 + 0.3063x + 0.2233$	91	16.26	1.413

Another similar result was also found in Pb^{2+} heavy metal test (Figure 2). As in previous Hg^{2+} heavy metal test, both S_4bB_{11} and S_1bB_4 showed stable growth pattern in Pb^{2+} supplemented environment. However, this time S_1bB_4 was the isolate with the highest growth. It peaked at OD of 1.743 at 15.26 h with regression coefficient percentage 80% (Table 2). Meanwhile, other isolates displayed a poor projection of growth patterns where within 24 hours and were unable to reach the stationary phase. It seemed that the four other isolates (S_1aB_1, S_1aB_2, S_2aB_5 and S_3aB_7) were supressed in the presence of Pb^{2+} compared to S_1bB_4 and S_4bB_{11} which had better resistance to the toxicity of Pb^{2+}.

The results from the heavy metal tolerance test (Hg and Pb) showed that the ability of bacterial growth in each type of heavy metal was different. Based on the growth pattern and regression analysis, S_4bB_{11} isolate had a stable ability in the heavy metal tolerance test, especially on Hg. This implied that the bacterial isolates were able to bioremediate Hg. According to Priyadarshanee and Das [12], bioremediation occurs due to enzyme activity produced by microorganisms modifying toxic pollutants such as heavy metals by changing the chemical structure of these pollutants (biotransformation). The mechanism carried out by bacteria degrades toxic pollutants, changing the structure of complex pollutants to become less complex and ends up becoming non-toxic metabolites or enzymes. This occured due to the adaptation process carried out by the cells to protect themselves from toxic heavy metals.

One of the bacterial mechanisms carried out to protect themselves is by producing extracellular polysaccharides. This capability is mostly present in gram-negative bacteria. Moreover, gram-negative bacteria tend to have better heavy metal resistance compared to gram-positive bacteria because the cell wall structure of gram-negative is more complex and has the better ability to bind and immobilize metal ions, including heavy metal [12]. The results showed that S_1bB_4 and S_4bB_{11} isolates had better adaptability to both Hg and Pb heavy metal treatments in the tolerance test, which previous study showed that these two isolates belong to gram-negative bacteria [8]. The results were in accordance with the previous work of Priyadarshanee and Das [12] and Beveridge [13] which stated that gram-negative bacteria have a better heavy metal tolerance level than of gram-positive because their thicker cells walls, in a range of 15-80 nm.

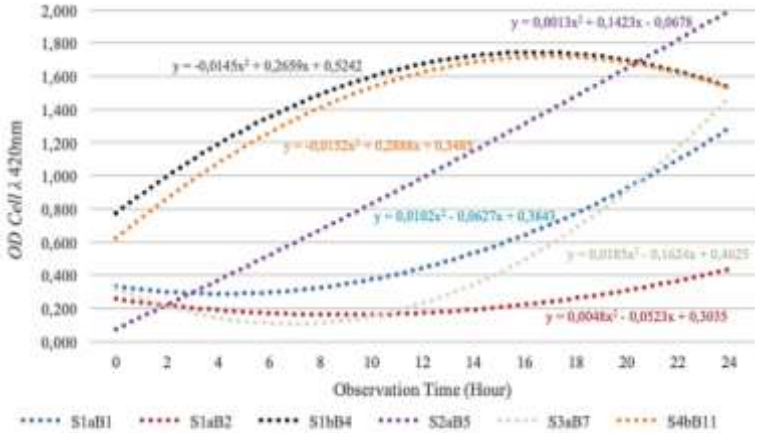


Figure 2. Trendline graph of six isolates growth pattern in 100 ppm Pb^{2+} (lead).

Table 2. Regression analysis of bacterial growth trendline in 100 ppm Pb (lead).

Isolate	Regression equation	R^2 (%)	X_{max} (hours)	Y_{max}
S_1aB_1	$y = 0.0102x^2 - 0.0627x + 0.3843$	93	24	1.293
S_1aB_2	$y = 0.0048x^2 - 0.0523x + 0.3035$	83	24	0.435
S_1bB_4	$y = -0.0145x^2 + 0.2659x + 0.5242$	80	15.26	1.743
S_2aB_5	$y = 0.0013x^2 + 0.1423x - 0.0678$	93	24	2.002
S_3aB_7	$y = 0.0185x^2 - 0.1624x + 0.4625$	97	24	1.478
S_4bB_{11}	$y = -0.0152x^2 + 0.2888x + 0.3485$	83	16.8	1.720

3.2. Analysis of specific metabolites as bioremediator of heavy metal Hg

Isolates S_4bB_{11} was chosen to be analyzed further for the possibility of producing Hg bioremediator related metabolites. Metabolite analysis was facilitated by GC-MS using 2 treatments, namely S_4bB_{11} + Tryptophan as control and S_4bB_{11} + Tryptophan + 100 ppm Hg as treatment. The results of the chromatogram showed as many as 55 and 53 types of compounds were detected on the control and the treatment, respectively. The compounds were classified into 5 categories, namely phytohormones, amino acids, carbohydrates, fatty acids, and other metabolites as showed in Figure 3.

Among all metabolites detected from GC-MS analysis, some of them which might be related to heavy metal bioremediation mechanism were showed in Table 3. There was quite significant difference in the metabolic system of S_4bB_{11} isolate when this isolates were grown on M63 medium +

tryptophan with (as treatment) and without (as control) supplementation of 100ppm Hg as a form of heavy metal stress. The isolate grown in the control medium was found to be oriented towards producing metabolites that facilitate the conversion of compounds in tryptophan and M63 medium into more carbon sources. This was showed by the production of arabinose and xanthine which were related to the increase of stachyose production. According to Rusmono [14], successfully hydrolyzed stachyose would produce 2 molecules of galactose, 1 molecule of fructose, and 1 molecule of glucose.

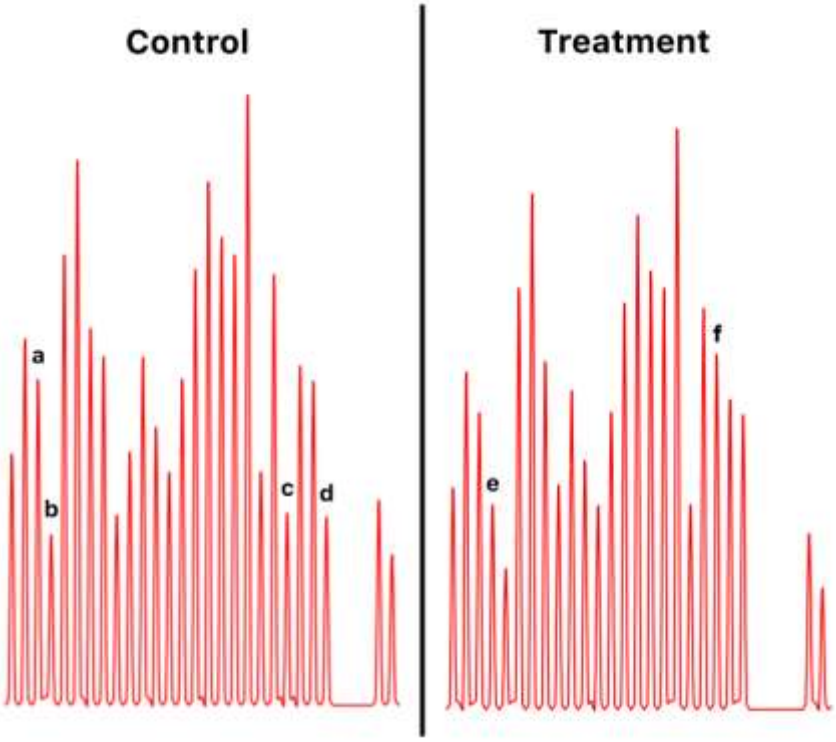


Figure 3. GC-MS chromatogram of S_4bB_{11} detected several different metabolites when the isolate grown in control compared to treatment with 100 ppm of Hg. As showed in control: a. Arabinose; b. Xanthine; c. Citric acid; d. 2,4-diacetylphloroglucinol; and in treatment: e. α-ketoglutaric acid; f. Diaminopimelic acid.

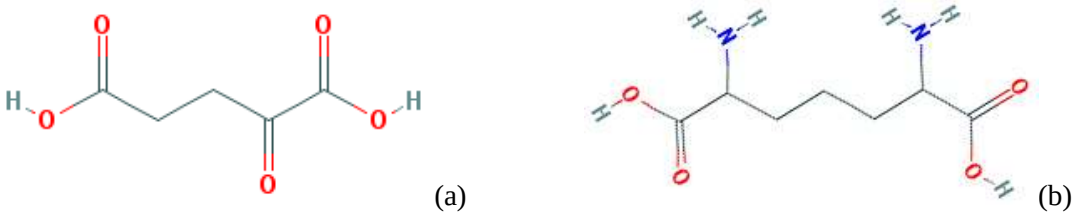
However, this metabolic orientation changed when S_4bB_{11} isolate was exposed to double stress conditions, which are environmental stress (M63 media) and heavy metal stress (100 ppm Hg). The S_4bB_{11} isolate shifted its orientation to produce metabolites that facilitated the cells to protect themselves from high levels of Hg toxicity. The mechanisms undertook were deactivation of the production process of 4 metabolites, namely arabinose, xanthine, citric acid, and 2,4-diacetylphloroglucinol. Moreover, 2 new metabolites were produced instead by S_4bB_{11} i.e. α-ketoglutaric acid and diaminopimelic acid. Both metabolites had an active role in the formation of thicker and more stable peptidoglycan cell walls in an effort to protect themselves from the toxic Hg. It is in line with the opinion of Kim [15] that diaminopimelic acid has a role in the preparation of peptidoglycan cell walls. Meanwhile, α-ketoglutaric acid specifically reduces protein catabolism and increases protein synthesis. This argument was supported by an increase in the production of mannose by the isolate. Mannose is a high-type oligosaccharide that has an important role in controlling the quality of several intracellular proteins, and functions as an important constituents of the innate immune system on microbial surfaces [16]. This serial abilities is a form of bacterial response to the heavy metal stress Hg by changing the permeability of microbial cell membranes to facilitates the immobilizion of heavy metal on the cell walls [17].

The molecular structures of α-ketoglutaric acid and Diaminopimelic acid were showed in Figure 4. It was showed that both have O-H chains which act as a potential chelating agent that can bind Hg^{2+} ions. According to Tamzil [18], chelation can occur at two or more coordinate positions of two or more of the same donors. In such fashion, they convert Hg^{2+} into organo-metallic compounds that have high stability. Those characters of S_4bB_{11} seemed to facilitate the isolate to deals with the stress of heavy metal (Hg), particularly by producing metabolites that act as a potential chelating agent and reducing the heavy metal to a non-toxic form.

Table 3. Specific metabolites of S4bB11 bacteria which act as bioremediators of heavy metal Hg.

Compound	Composition (%)	Sample		Remarks
		Control	Treatment	
Arabinose	1.96	+	-	Selectively inhibit sucrase activity and suppress plasma glucose increase
Xanthine	1.07	+	-	Intermediate degradation of adenosine mono-phosphate to uric acid
Citric acid	1.08	+	-	Anti-coagulant agent (calcium chelating)
2,4-diacetylphloroglucinol	1.06	+	-	Anti-fungal agent
α-ketoglutaric acid	1.30	-	+	It binds to nitrogen, stimulates protein synthesis, and inhibits protein degradation.
Diaminopimelic acid	2.27	-	+	The constituent of peptidoglycan cell walls.
Stachyose[a]	3.52	+	+	Raffinose derived metabolites.
Mannose[b]	3.71	+	+	Controllers of intracellular protein quality, an important constituent of the microbial immune system.

[a] indicates the metabolite with the highest composition in the control sample.
[b] indicates the metabolite with the highest composition in the treatment sample.



(a) (b)

Figure 4. Molecular structures of (a) α-ketoglutaric acid, and (b) diaminopimelic acid as generated by Shimadzu GC-MS software.

3.3 Analysis of plant growth hormone metabolites

Four groups of plant growth hormones (phytohormones) have been identified based on GC-MS assay; though, not all compounds were the complete phytohormone forms (Table 4). Two compounds detected were the phytohormones precursors in S_4bB_{11} isolate, namely N6-benzyladenine 9 glucoside and kinetin glucoside. Meanwhile, the complete phytohormone that have been successfully synthesized and have the potential to promote plant growth were 3-indole acetic acid and kinetin (cytokinin).

Table 4. Results of phytohormone detected based on the chromatogram library obtained from GC-MS analysis.

Compound	Composition (%)		Remarks
	Control	Treatment	
3-indole acetic acid	1.83	1.90	Phytohormone
Kinetin	1.15	1.12	Phytohormone
N6-benzyladenine 9 glucoside	1.30	1.29	Precursor
Kinetin glucoside	1.18	1.15	Precursor

3-indole acetic acid (IAA) is a phytohormone belongs to auxin group. This phytohormone is naturally produced by plants; though, some bacteria can also produce IAA [19]. Auxin plays an active role in plant vegetative growth by assisting root development, initiating in abscission process, inhibiting the growth of side shoots, and being involved in the formation of xylem and phloem transport networks [20, 21]. Another type of phytohormone detected from S_4bB_{11} isolate was kinetin, a phytohormone belongs to cytokinin group. Kinetin has the ability to initiate cytokinesis processes in plant cells and involved in morphogenesis. In the process of plant growth, regarding to the phase, the interaction of cytokinin altogether with auxin influenced on plant tissue differentiation [22, 23].

4. Conclusion

Among six bacterial isolates investigated in this study, the isolate of S_4bB_{11} showed the best performance in heavy metal stress adaptation and resistance as displayed from the stable growth pattern in both Hg and Pb supplemented environment. Further GC-MS analysis on S_4bB_{11} detected several metabolites that play a role in the bioremediation process of heavy metal stress, particularly α-ketoglutaric acid, diaminopimelic acid, and mannose. In addition, the presence of phytohormones 3-indole acetic acid (IAA) and kinetin which belong to phytohormone group of auxins and cytokinin, respectively; were also detected. These results suggested that the bacterial isolates have dual function as a bioremediation agent and a potential biofertilizer for to apply for promoting plant growth.

5. References

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