Indigenous bacterial bioremediation: Chromium-reducing capabilities of native strains from Halmahera

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Abstract. Indonesia, with its abundant natural resources, faces significant environmental degradation from open-pit mining, that lead to deforestation and heavy metal contamination, which pose dangers to humans and ecosystem. While bacterial bioremediation of heavy metals is well-documented, studies on chromium bioremediation using indigenous Indonesian bacteria remains scarce. This study characterizes chromium-reducing bacteria isolated from post-mining soil in Halmahera, Indonesia, and evaluates their potential for both bioremediation and reforestation of post-mining lands. Macroscopic analyses were used to determine the genus of the Halmahera bacterial isolates and their chromium reducing-capability was assessed using Diphenylcarbazide (DPC) method. Notably these isolates achieved a chromium reduction efficiency of up to 39%, slightly lower than 50% reduction by *Pseudomonas aeruginosa* in another study, as well as exhibiting phosphate solubility index of 0.66, which shows the bacteria potential to promote plant growth. Our findings demonstrate that these indigenous bacteria are promising candidates for sustainable bioremediation efforts, effectively reducing chromium contamination and simultaneously accelerating reforestation in post-mining areas. This dual action contributes to developing scalable, eco-friendly strategies to mitigate environmental damage caused by mining in Indonesia and similar post-mining environment globally, potentially paving the way for industrial applications in environmental restoration.

1 Introduction

Anthropogenic activities, especially agricultural and industrial processes such as electroplating, pigment production, stainless steel production, and leather industry have resulted in widespread heavy metal contamination that poses a significant threat to ecosystem

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and human health [1, 2]. One such heavy metal, chromium, particularly in its hexavalent form (Cr(VI)), stands out as highly concerning pollutant due to its high toxicity, mobility, carcinogenic, and mutagenic potential [3-5]. Classified as Group 1 human carcinogen by the International Agency for Research on Cancer (IARC), Cr(VI) demands urgent remediation efforts [6]. While conventional remediation methods such as chemical precipitation exist, it often prove costly, inefficient for large-scale remediation, and can also generate hazardous byproducts [7, 8]. Therefore, "green methods" such as bioremediation which utilize bacterial strains capable of reducing the toxic Cr(VI) to less toxic trivalent form Cr(III) are gaining considerable attention [8]. Understanding the capabilities of native bacteria to reduce hexavalent chromium has become very important for developing specific and efficient bioremediation strategies.

Global burden of heavy metal pollution, particularly chromium is substantial, with an estimated 75,000 tons of chromium waste generated annually from anthropogenic sources and around one-third of which is in hexavalent chromium form [9]. Cr(VI) can readily enter the food chain, causing severe health issues, including DNA damage, respiratory problems, and cancer in exposed populations [8, 10, 11]. Bacterial bioremediation which leverages the ability of certain bacterial species to reduce Cr(VI) to Cr(III) offers a promising, cost-effective, and environmentally friendly alternative to conventional methods [12, 13]. Notably, Indigenous bacterial strains from contaminated sites are especially valuable for bioremediation effort as they are often exhibits higher activity and tolerance, making them ideal agents for *in situ remediation* [14, 15].

Indonesia's rapid industrial growth, particularly in mining and manufacturing, has led to significant chromium contamination. For instance, chromium contamination has been reported in groundwater around the Pangkajene river in Sulawesi [16], as well as in soil in Pati, Central Java, and Bandung, West Java [17]. This contamination of Cr(VI) is a serious threat for Indonesia's rich biodiversity and public health particularly in area close to mining activities such as the Island of Halmahera, known for its extensive nickel mining operations [18]. Therefore, developing sustainable and effective chromium remediation strategies is crucial. While bioremediation offers a potentially sustainable and site-specific solution, the exploration and characterization of native chromium-reducing bacteria in Indonesia, especially in regions significantly impacted by mining like Halmahera remain largely unexplored. This knowledge gap hinders the development of effective bioremediation strategies tailored to Indonesia's environmental conditions that can be deployed with minimum side-effect due to the usage of indigenous bacteria. This study aims to isolate, identify, and characterize indigenous chromium-reducing bacteria collected in post-mining soil sample in Halmahera. The primary objectives of this research are: (1) to isolate and characterize indigenous chromium-reducing bacterial strains from post-mining soil in Halmahera, (2) to evaluate the Cr(VI) reduction capacity of these isolates, (3) evaluate plant-growth promoting activity of these isolates. Post-mining samples already collected from Halmahera will be used as source of the indigenous bacteria which will be isolated characterized through microscopic and macroscopic parameters. The chromium reduction capacity and plant-growth promoting activity will be tested using spectrophotometric assays. Methods and materials used in this paper will be further explained in section 2, while section 3 presents the results and discusses the characters of each isolate and their capability to reduce chromium and plant growth promoting activity. Summary of all the key findings, and outlines of future research directions will be presented in the last section.

2 Methods and materials

2.1 Bacteria characterization

The bacterial isolate used in this research was obtained from the microbiology laboratory stock culture, previously isolated from nickel mining site in Halmahera, North Maluku. This specific isolate was chosen for its potential chromium-reducing capabilities based on its origin in a chromium-contaminated environment. The strain was initially rejuvenated by cultivating it on Nutrient Agar (NA) medium, a standard growth medium for a wide range of bacteria, containing 5g/L peptone, 3g/L beef extract, 5g/L NaCl, and 15 g/L agar at 37°C for 24h. Pure cultures were obtained using the T-streak technique and individual colonies were then selected for subsequent characterization studies. All cultivation procedures were performed under aseptic conditions in a laminar flow cabinet to prevent contamination. Macroscopic characterization of the isolates was conducted by examining 24h old colonies grown on NA plates at 37°C. Colony characteristics including pigmentation, shape, margin, elevation, and surface texture were documented to aid in bacterial identification. For microscopic analysis, bacterial cells were subjected to Gram staining, a basic technique for differentiating bacterial species based on their cell wall properties. Smears were treated with 2-3 drops of crystal violet for (1 min), Gram's Iodine Mordant (1 min), 95% ethyl alcohol, and then safranin (45s), with thorough washing between each step. The stained preparations were then examined under a light microscope at 1000x magnification using oil immersion. Cell morphology and Gram reaction were recorded.

2.2 Diphenylcarbazide tests for chromium reducing

Bacterial cultures for chromium reduction assays were prepared by inoculating the isolate into Nutrient Broth (NB) medium, a nutrient-rich medium suitable for supporting bacterial growth, containing 5g/L peptone, 3g/L beef extract, and 5g/L NaCl. The preculture was then incubated in a shaker incubator at 37°C with continuous agitation at 150 rpm for 24 hours to ensure optimal growth and aeration. Bacteria in preculture was then subsequently cultured under identical conditions. 10 ml of cultured bacteria was moved to falcon tube and then 1 mL of supernatant was transferred to microtube to be centrifuged at 10.000 rpm at 10°C for 10 minutes. Chromium reducing capability was quantified using the 1,5-diphenylcarbazide (DPC) method, a standard colorimetric assay for detecting $Cr(VI)$ due to its sensitivity and specificity. In this method, $Cr(VI)$ will reacts with 1,5 DPC, forming a purple-coloured complex. 200 μL of cell-free supernatant was transferred to sterile microtubes and mixed with 400 μL of 11ppm DPC-Cr solution. The reaction mixture was allowed to stabilize at room temperatures for 10 minutes. The absorbance was then measured at 540 nm using a microplate reader. The chromium reduction efficiency was calculated and expressed as the percentage of $Cr(VI)$ reduced relative to the initial concentration.

2.3 Phosphate solubilization assays

Phosphate solubilization capability of bacterial isolates was evaluated through qualitative and quantitative assays. For qualitative assay, 1 ose of bacterial isolates were inoculated using sterile inoculation loop onto Pikovskaya's agar medium, a selective medium designed to detect phosphate-solubilizing bacteria, containing per liter: 10 g glucose, 5 g $Ca_3(PO_4)_2$, 0.5 g $(NH_4)_2SO_4$, 0.2 g KCl, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.0001 g $MnSO_4 \cdot H_2O$, 0.0001 g $FeSO_4 \cdot 7H_2O$, 0.5 g yeast extract, and 15 g agar. The Pikovskaya's agar plates were then incubated at room temperature for 7 days. Halo zone formation around bacterial colonies indicates positive

phosphate solubilization activity as the bacteria release organic acids that solubilize the insoluble phosphate in the medium. The ratio between diameter of halo zone and diameter of colonies represents the phosphate solubilization index, providing a semi-quantitave measure of phosphate solubilizing activity. Quantitative determination of phosphate solubilization was conducted in liquid Pikovskaya's medium. 1 ose of bacterial isolates were inoculated into 10 mL of sterile Pikovskaya's medium and incubated at 28°C with continuous agitation at 150 rpm for 7 days. The culture medium was then filtered through Whatman filter paper to remove cellular debris, followed by centrifugation at 10.000 rpm at 4°C for 15 minutes to obtain cell-free supernatant. The concentration of soluble phosphate was determined through colorimetric means at 400nm by mixing 1 mL of colouring reagents with 0.5 mL of hydrazine sulphate solution. The reaction mixture was incubated in water bath for 10 minutes, followed by cooling to room temperature for 30 minutes.

2.4 Data analysis

Chromium reduction efficiency was calculated as a percentage relative to the initial Cr(VI) concentration. Phosphate solubilization was quantified by measuring the concentration of soluble phosphate in the supernatant. Data were analysed using one-way ANOVA and all experiments were performed in triplicate.

3 Results and discussion

3.1 Bacteria characterization

Four bacteria isolate were successfully cultured from stock (Figure 1) and subsequently analysed to characterize them based on their colony morphology and microscopic characteristics. These characteristics can offer valuable insights into their potential for bioremediation. Table 1 showed differences in colony morphology for each isolate although some share common traits. The observed morphological variations such as distinct marginal patterns and surface textures in particular suggest potential differences in how these bacteria aggregate and the composition of the extracellular matrix (ECM) they produce [19, 20]. ECM plays a crucial role in bacterial survival and ecological adaptations by inferring resistance to antibiotics and environmental stresses [19, 21, 22]. For example, the rhizoid margins observed in FCC.3679 (KT) (Figure 1A) generally indicate spreading behaviour which suggest this particular strain is capable of producing biosurfactant compounds such as surfactin [23]. Surfactin or other surfactant compounds lowers surface tension, increasing substrate wettability, and promoting cell motility, thus increasing its capabilities for surface colonization [24, 25]. Biosurfactants are also important in bioremediation as they can enhance the bioavailability of pollutants, making it easier for bacteria to degrade [26]. The bright yellow coloration in this strain is also noteworthy as pigments in bacteria may act as protective compounds against UV and help to prevent damage due to oxidative stress [27-29]. This pigmentation may help bacteria to thrive in harsh environments like post-mining sites which contains harmful substances such as heavy metals that can cause oxidative stress. Filamentous margins on both FCC.3681 (Figure 1D) and FCC.3682 (Figure 1C) may indicate potential cellular differentiation and multicellular behaviour for nutrient acquisition or surface attachment [30-32] while curled margin pattern displayed by FCC.3679 (P) (Figure 1B) might indicate a specific growth patterns and cell wall modifications that influence how the bacteria interact with their environment [33]. Rough surface texture found only on FCC.3681 along with irregular and flat colony suggests altered cell-to-cell adhesion properties and possible modifications in extracellular polymeric substances (EPS) production

which is often associated with biofilm formation that can enhance bacterial survival and stress tolerance [34, 35]. Conversely, the smooth surface texture observed other isolates might indicate different EPS production pathways or cell wall composition. These morphological features taken collectively may suggest distinct evolutionary adaptations that influence each bacteria ecological fitness, pathogenicity, or biological applications.

Table 1. Colony morphologies of four bacterial isolate. Each isolate has its own unique characteristics with FCC.3679 (KT) being the most notable for its bright yellow pigmentation.

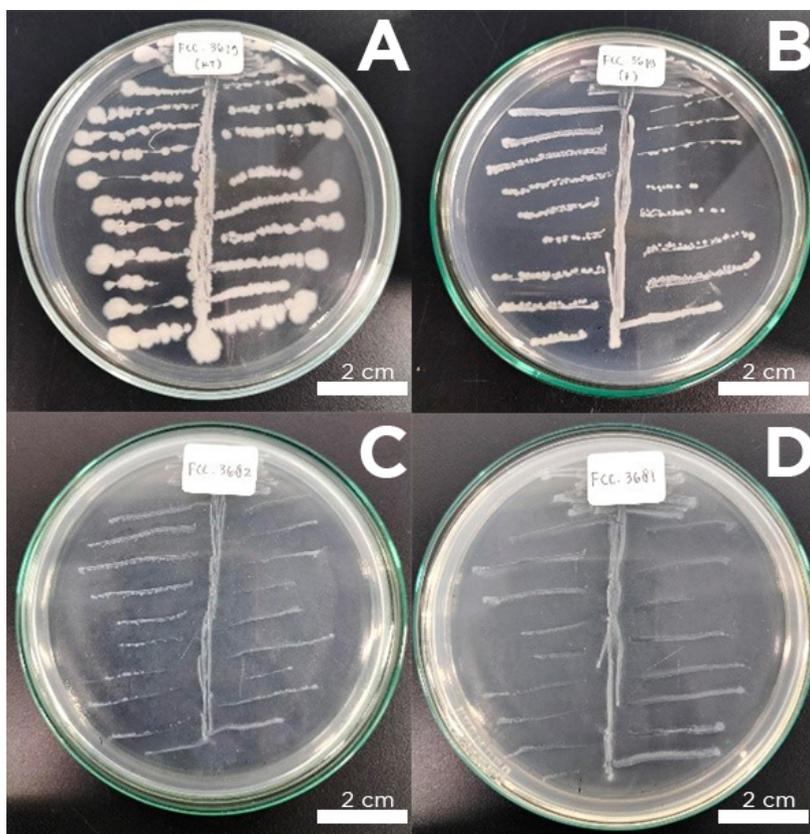| No.	Isolate Code	Pigmentation	Shape	Margin	Elevation	Texture
1.	FCC. 3679 (KT)	Bright Yellow	Circular	Rhizoid	Raised	Smooth
2.	FCC. 3679 (P)	White	Circular	Curled	Raised	Smooth
3.	FCC. 3681	White	Irregular	Filamentous	Flat	Rough
4.	FCC. 3682	White	Circular	Filamentous	Raised	Smooth

Fig. 1. Four bacterial isolates, (A) Isolate FCC.3679 (KT), (B) FCC.367 (P), (C) FCC.3682, (D) FCC.3681 was grown on NA media in 8 cm petri dish.
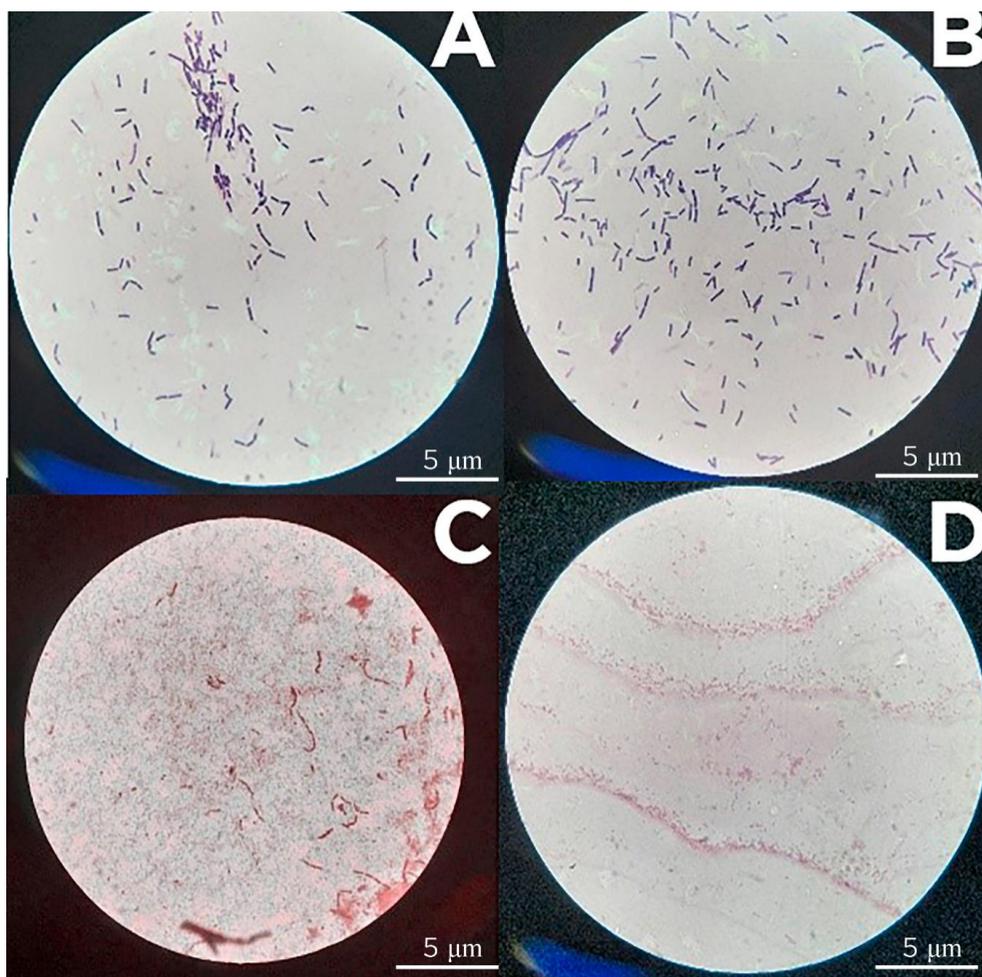
Fig. 2. Gram staining and microscopic examination. All isolates have rod-shaped cell (bacillary). (A) FCC.3679 (KT) and (B) FCC.3679 (P) have purple staining indicating positive Gram test, while (C) FCC.3682 and (D) FCC.3681 have red staining indicating negative Gram test. All isolate was grown on nutrient agar media for 24h.

Microscopic examination and Gram staining (Figure 2) revealed further characteristics at the cellular level. All four isolates exhibited bacillary morphology although with notable differences in cell wall composition. This shape is advantageous for nutrient uptake particularly in nutrient-limited environments and also aids in motility and biofilm formation due to its optimum surface area-to-volume ratio [36-39]. These features likely contribute to the isolates ability to colonize and persist in heavy metal-contaminated soil. Isolate FCC.3679 which further divided into KT and P variants exhibited Gram-positive characteristics (Fig 2A and 2B) which indicates thick peptidoglycan layer. In contrast, FCC.3681 (Fig 2D) and FCC.3682 (Figure 2C) displayed Gram-negative characteristics with pinkish coloration indicating thin peptidoglycan layer [40]. The Gram-negative isolates outer membrane, which contains lipopolysaccharides (LPS) might contribute to their distinctive filamentous margins and their resistance to toxic substances, further enhancing their adaptability in challenging environments [41, 42]. Based on the characteristics each isolates have and referring to *Bergey's manual of determinative bacteriology* FCC.3679 (KT) and FCC.3679 (P) show similarities with genus *Pantoea* and *Bacillus* respectively, while FCC.3681 and FCC.3682

share traits with genus *Stenotrophomonas* and *Proteus*. These genera often include species known for their bioremediation potential [43-46]. In summary, the observed morphological and microscopic characteristics indicate that these isolates possess traits that could be beneficial for bioremediation applications, such as enhanced stress tolerance, increasing bioavailability of the pollutant.

3.2 Chromium-reducing capabilities

All of the bacteria isolate in this study was sampled in post-mining soil, an environment often polluted by various heavy metals, including hexavalent chromium Cr(VI) that has toxic effect on living cells [47]. Bacteria have evolved various mechanisms to resist Cr(VI) toxicity through both extracellular and intracellular processes. These mechanisms can involve actively pumping chromate ions out of the cell via transporters or reducing Cr(VI) to the less toxic Cr(III). Bacteria can employ chromate efflux system facilitated by chromate transporters to actively extrude chromate ions from cytoplasm [48]. Reduction of Cr(VI) to the less toxic Cr(III) represents another detoxification strategy that occurs through both non-enzymatic and enzymatic pathways. Enzymatic reduction primarily mediated by oxidoreductases while non-enzymatic reduction occurs through interactions with cellular components that has reducing capabilities such as glutathione or ascorbate [49].
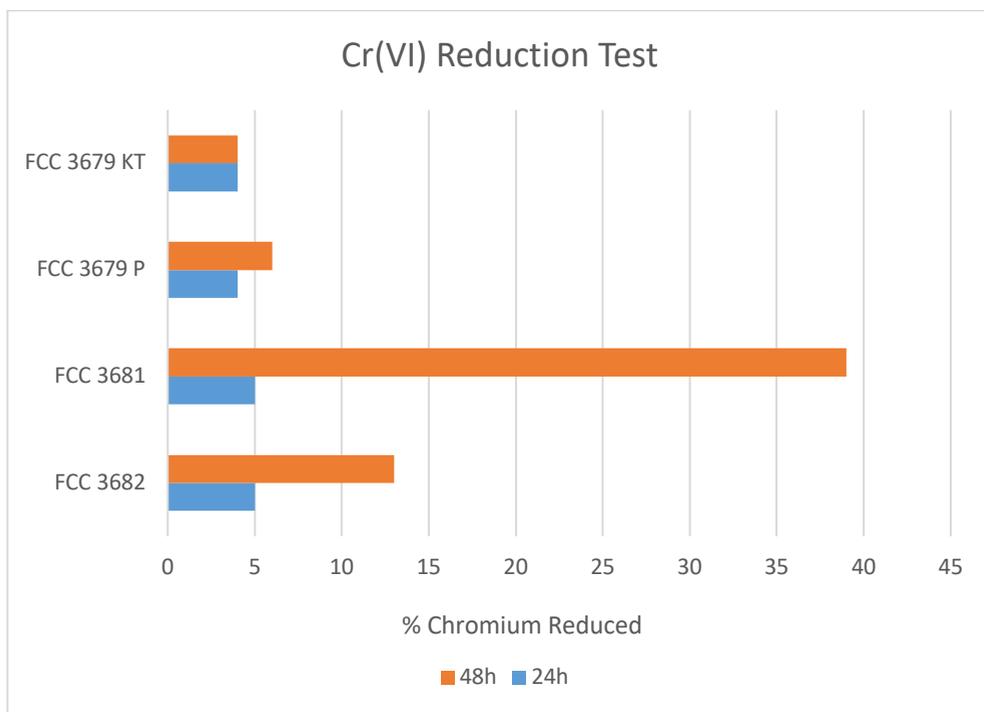

Fig. 3. Chromium reduction percentages of all isolates in 48 hours period with 24 hours interval. In the first 24 hours all isolate struggle to reduce chromium with the highest level of reduction were 5% by both FCC.3682 and FCC.3681. The next 24 hours shows dramatic increase for FCC.3681.

The chromium-reducing capabilities of the isolates were evaluated using diphenylcarbazide method with an initial Cr(VI) concentration of 11 mg/L. As shown in Figure 3, all isolates exhibited varying degrees of chromium reduction capacity over 48 hours incubation period. During the initial 24 hours, modest reduction rates were observed, with

only FCC.3681 and FCC.3682 showing slightly higher reduction (5%) compared to both FCC.3679 (KT) and FCC.3679 (P) which both reduced Cr(VI) by only 4%. However, during the subsequent 24-hour period, the reduction dynamics showed more variations, suggesting distinct chromium metabolic capabilities among the four isolates. Most notably, FCC 3681 exhibited the most significant improvement in reduction capability, achieving a remarkable 39% reduction after 48 hours, nearly an eight-fold increase from the initial 5% reduction during the first 24 hours. This substantial increase in reduction capacity suggests the possible activation of chromium reduction pathway that involves delayed expression of chromate reductases or the activation of secondary mechanisms triggered by prolonged chromium exposure. Reduction capabilities of the other isolates only show modest improvement in the case of FCC.3682, reaching 13% after 48 hours, and relatively unchanged in both FCC.3679 (P) with 6% reduction and FCC.3679 (KT) with 3% reduction. These differences in reduction capacity after 48 hours point to fundamentally different resistance strategies. Particularly noteworthy is the slight decline in reduction capacity of FCC.3679(KT) from 4% to 3% as it may indicate stress responses from the cell or depletion of reducing agents necessary for sustained reduction and ultimately cell survival.

The stark contrast between FCC.3681 robust capability and the subpar capability of the other isolates points to a difference in molecular mechanisms of these isolates for dealing with high chromium concentration in their immediate environment. The delayed but substantial increase in reduction efficiency showed by FCC.3681 may indicate the presence of resistance systems possibly by specialized chromate reductases or enhanced cellular protection mechanisms induced by prolonged exposure to chromium. While the 39% reduction observed in this study are moderate compared to previously reported chromium reducing bacteria such as *Pseudomonas putida* which can reduce 50% of Cr(VI) from starting concentration of 100 mg/L over 24h period [50] and *Bacillus subtilis* MNU16 which has shown chromium reducing capabilities up to 75% of Cr(VI) from starting concentrations of 50 mg/L [51]. Several factors could explain the comparatively lower reduction rates in these isolates, despite their origin in a post-mining site. One possibility is that these bacteria have already evolved to thrive in environments with higher hexavalent chromium concentrations. Consequently, the Cr(VI) concentrations used in this study might be suboptimal for inducing maximal chromate reductase activity in these isolates and due to their evolution, they may not need to reduce all the hexavalent chromium present when exposed to lower concentration. Another possibility at play is that these bacteria may preferentially utilize chromate efflux transporters over reductase enzymes to alleviate chromium toxicity at lower concentrations or when initially exposed to Cr(VI), potentially relying more heavily on reductase activity as chromium concentrations or exposure time increases. It is also worth noting that the effectiveness of bioremediation can be affected by various environmental factors such as pH, temperature, and nutrient availability. Optimization of these factors could potentially enhance the chromium reduction efficiency of these isolates.

Future research could investigate several ways to improve chromium reduction capabilities of these isolates including optimization of environmental conditions as well as Cr(VI) concentration and exposure time, investigating the potential co-culture with other chromium-reducing bacteria and exploring structural information of chromate reductase to open up the possibility of protein engineering approaches to enhance chromate reductase activity. Additionally, investigation into the specific mechanisms underlying the delayed chromium reducing activity in FCC.3681 could provide valuable insights for developing more effective bacterial bioremediation strategies.

3.3 Phosphate solubilizing

Phosphorus (P) is an essential element that served as vital component of macromolecules in all life forms. However, majority of phosphorus in soil exists in insoluble forms, rendering it inaccessible to plants and can significantly constrains plant biomass growth and yields [52]. Phosphate solubilization by soil bacteria is crucial for environmental sustainability and agricultural productivity as it converts insoluble phosphate into accessible, soluble phosphate that can be taken up and used by plants [53]. Many pathways used by bacteria to freed phosphate, generally through the production and secretion of organic acids which may chelate cationic partners of insoluble phosphates, thereby releasing soluble phosphate into the soil [53]. Additionally, some bacteria produces phosphatases, an enzyme capable of hydrolysing organic phosphorus, further increasing phosphate availability [54]. This ability of soil bacteria to solubilize phosphate has significant implications as it can potentially reducing the need for synthetic phosphate fertilizers which can degrade soil in long-term usage. Moreover, phosphate solubilizing bacteria in the context of soil remediation can play a vital role in restoring soil fertility and promoting vegetation, the latter became more important particularly in post-mining areas.
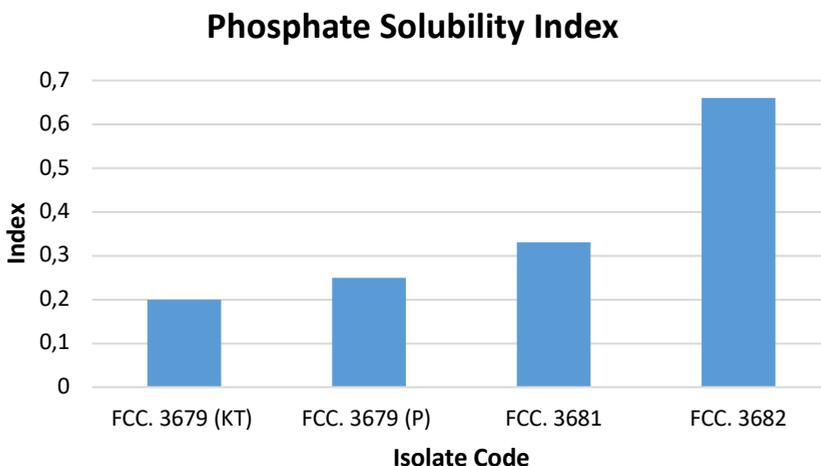
Phosphate Solubility Index

Fig. 4. All four isolates showed capability to solubilize phosphate based on formation of halo zone around the colonies grown in Pikovskaya's agar medium. Isolate FCC.3682 has the highest Phosphate index of 0.66.

The phosphate solubilization capabilities of four isolates (Figure 4) shows an interesting variation between the solubilization area calculated as phosphate solubilization index (PSI) and actual soluble phosphate production (Figure 5). FCC.3682 showed the highest index of 0.66 but interestingly it was FCC 3679 (P) that showed the highest soluble phosphate production at 3.90 ppm while FCC.3682 produced the least amount of soluble phosphate at 2.27 ppm. The discrepancy suggests that while PSI is useful as preliminary indicator of bacterium's ability to solubilize phosphate, the relationship between halo zone formation and the actual soluble phosphate is not always linear and may be influenced by various factors.

Several factors could explain this discrepancy, FCC.3682 might exhibits aggressive initial solubilization that cannot be sustained over time, while FCC.3679 may possess more efficient long-term solubilization mechanisms. The inability of FCC.3682 to sustain production of soluble phosphate is likely due to significant energy expenditure needed in initial solubilization. Higher initial solubilization might also trigger stronger negative feedback

mechanisms that stopped the reaction prematurely, while isolates with lower index might experience less inhibition and thus able to consistently produce soluble phosphate. Another thing that may explain the discrepancy is that different isolates used in this research may preferentially solubilize different forms of inorganic phosphate and because of that the phosphate solubilize index assay might not fully reflect the diversity of phosphate sources that can be utilized by the bacteria [55].
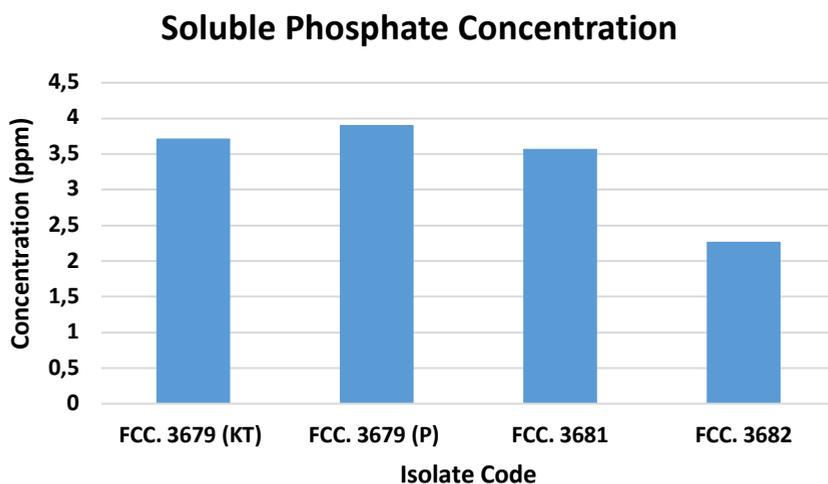

Fig. 5 All four isolates are capable of producing soluble phosphate in Pikovskaya's broth medium. Interestingly FCC.3682 which has the highest PSI produces the least amount of soluble phosphate in this test

Nonetheless, despite the discrepancy, this assay showed that all of the isolates are capable of producing soluble phosphate. This trait along with their ability to reduce chromium indicates potential applications in developing strong plant-microbe collaborations for contaminated soil remediation. The concurrent production of plant growth-promoting compounds under heavy metal stress could enhance plant growth by increasing tolerance to heavy metal toxicity and oxidative stress, promoting biofilm formation, and modifying the microenvironment, ultimately contributing to enhanced phytoremediation efficiency [56]. Furthermore, the observed characteristics also indicate potential applications in developing site-specific remediation strategies that account for soil characteristics, contamination patterns, and environmental variables. For example, in areas with low phosphate availability, deploying these isolates could simultaneously address heavy metal contamination and improve soil fertility, which promotes plant growth and ecosystem restoration.

Further research is needed particularly in the molecular characterization of chromium reduction mechanisms, the optimization of plant-bacteria combinations, and the possibility of developing multi-strain consortia for enhanced remediation efficiency. Understanding the specific mechanisms behind the observed discrepancies between PSI and soluble phosphate production will also be crucial for selecting and optimizing bacterial strains for specific bioremediation and agricultural applications.

4 Conclusion

This study demonstrates the potential of Indigenous bacteria from the genera *Proteus, Pantoea, Stenotrophomonas,* and *Bacillus*, isolated from post-mining soil in Halmahera,

North Maluku as effective agents for chromium bioremediation. These isolates exhibited promising capacity to reduce hexavalent chromium by up to 39%. Furthermore, the bacteria capability to solubilize phosphate suggest a potential synergistic role in chromium phytoremediation strategies by enhancing both phosphate and chromium bioavailability while simultaneously reducing toxic effects of chromium and potentially alleviate oxidative stress in plants due to heavy metal which could promote healthier and might even enhance plant biomass growth, thereby accelerating chromium removal from contaminated soil. This dual action also offers the advantage of cost-effectiveness and environmental sustainability over conventional remediation methods. These findings highlight the value of exploring and harnessing indigenous microbial communities for developing sustainable bioremediation strategies in post-mining environments. To further advance this research, future studies should focus on evaluating the efficacy of these isolates under field conditions and exploring the potential of microbial genetic engineering to enhance their chromium reduction and plant growth-promoting capabilities. Additionally, in-depth investigations are needed to elucidate the specific mechanisms of chromium reduction and phosphate solubilization by these bacterial genera to optimize their application for industrial-scale bioremediation efforts.

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