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The Potential of Bacillus subtilis and Pseudomonas aeruginosa in Reducing Hg^{2+} Levels in Small-Scale Gold Mining Liquid Waste in Indonesia

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Abstract
Mercury contamination resulting from artisanal and small-scale gold mining (ASGM) poses a significant threat to both environmental and human health. This study evaluates the effectiveness of two bacterial strains, Bacillus subtilis and Pseudomonas aeruginosa, in reducing mercury (Hg^{2+}) concentrations in ASGM liquid waste. Methods: Experiments were conducted over seven days under controlled conditions, with two pH settings (neutral and alkaline), by monitoring total mercury reduction and bacterial growth (CFU/mL). Result and discussion: Results indicated that B. subtilis achieved the highest mercury removal efficiency—up to 90.07% at neutral pH and 89.51% at alkaline pH. P. aeruginosa also showed high efficacy, though slightly lower in comparison. Colony counts peaked on day two but declined by day seven, likely due to nutrient depletion and mercury toxicity, particularly at neutral pH. In contrast, control reactors without bacterial inoculation showed negligible changes in Hg concentration and no significant colony development. Conclusion: Despite the decline in bacterial counts over time, both strains remained functionally active, demonstrating strong adaptability and potential as effective bioremediation agents for mercury-contaminated wastewater. These findings support the integration of bacterial bioremediation as a viable component of sustainable mercury management in ASGM sectors.
Keywords: *artisanal gold mining, bacillus subtilis, bioremediation, mercury, pseudomonas aeruginosa*

Abstrak
Kontaminasi merkuri yang diakibatkan oleh penambangan emas skala kecil dan artisanal (ASGM) menimbulkan ancaman yang signifikan terhadap kesehatan lingkungan dan manusia. Studi ini mengevaluasi efektivitas dua strain bakteri, Bacillus subtilis dan Pseudomonas aeruginosa, dalam mengurangi konsentrasi merkuri (Hg^{2+}) dalam limbah cair ASGM. Eksperimen dilakukan selama tujuh hari dalam kondisi terkendali, dengan dua pengaturan pH (netral dan basa), dengan memantau total pengurangan merkuri dan pertumbuhan bakteri (CFU/Ml. Hasil menunjukkan bahwa B. subtilis mencapai efisiensi penghilangan merkuri tertinggi—hingga 90,07% pada pH netral dan 89,51% pada pH basa. P. aeruginosa juga menunjukkan kemanjuran yang tinggi, meskipun sedikit lebih rendah sebagai perbandingan. Jumlah koloni mencapai puncaknya pada hari kedua tetapi menurun pada hari ketujuh, kemungkinan karena penipisan nutrisi dan keracunan merkuri, khususnya pada pH netral. Sebaliknya, reaktor kontrol tanpa inokulasi bakteri menunjukkan perubahan yang dapat diabaikan dalam konsentrasi Hg dan tidak ada perkembangan koloni yang signifikan. Kesimpulan: Meskipun terjadi penurunan jumlah bakteri dari waktu ke waktu, kedua galur tetap aktif secara fungsional, menunjukkan kemampuan beradaptasi yang kuat dan potensi sebagai agen bioremediasi yang efektif untuk air limbah yang terkontaminasi merkuri. Temuan ini mendukung integrasi bioremediasi bakteri sebagai komponen yang layak dari pengelolaan merkuri berkelanjutan di sektor ASGM.
Kata Kunci: *bacillus subtilis, bioremediasi, merkuri, penambangan emas tradisional, pseudomonas aeruginosa*

1. Introduction

Small-scale gold mining (ASGM) has become the primary source of livelihood for many communities across gold-rich regions in Indonesia. However, mercury (Hg)-based amalgamation remains the dominant extraction technique, leading to severe environmental and public health consequences [1] [2] [3]. According to the [4] report, ASGM in Indonesia consumes approximately 1,727.5 tons of mercury

annually, with an estimated 34.5 tons released into rivers, soils, and aquatic ecosystems. Mercury accumulation in the food chain presents chronic health risks, including neurotoxicity and renal dysfunction, while also disrupting ecological balance [5] [6] [7].

At the national level, Indonesia ratified the Minamata Convention through Law No. 11/2017 and implemented the National Action Plan for Mercury Reduction and Elimination (RAN-PPM) under Presidential Regulation No. 21/2019 [8]. Although these regulatory frameworks demonstrate strong policy commitment, technical and financial barriers continue to hinder the adoption of mercury-free technologies in ASGM communities. As a result, there is a growing need for alternative approaches that are low-cost, easy to implement, and environmentally sustainable [9].

Microbial bioremediation has emerged as a promising strategy for the treatment of mercury-contaminated wastewater. Through mechanisms such as bioadsorption, bioaccumulation, and enzymatic biotransformation, microbes can effectively reduce heavy metal concentrations without producing hazardous secondary waste [10] [11]. Previous studies suggest that bioremediation is more economical and ecologically viable than conventional physical and chemical treatments [12]. However, the success of this approach depends greatly on microbial compatibility with environmental conditions, particularly pH, nutrient availability, and pollutant load [13] [14] [15] [16] [17].

In this context, two indigenous bacterial species—Bacillus subtilis and Pseudomonas aeruginosa—have demonstrated strong potential in mercury detoxification. Both harbor the mer operon (merA, merB), which enables enzymatic reduction of toxic Hg^{2+} to volatile Hg^0 [18] [19] [20]. B. subtilis is known for its sporulation capacity, enhancing environmental resilience, whereas P. aeruginosa produces biosurfactants that increase metal bioavailability and uptake. While the mercury-reducing capabilities of these bacteria have been validated in controlled in vitro studies, their application to actual ASGM wastewater—particularly under varying pH and inoculum volumes—remains underexplored.

Moreover, although some comparative studies have examined single bacterial strains, very few have assessed the performance of single versus mixed cultures under pH conditions reflective of field variability (neutral to alkaline). Given that mercury-contaminated wastewater in ASGM sites often shows fluctuating pH and inconsistent microbial load, this gap limits the optimization and scalability of microbial-based treatment systems.

To address this research gap, the present study investigates the bioremediation potential of B. subtilis and P. aeruginosa, single colony and in mix culture, in reducing mercury concentrations from ASGM liquid waste. The experimental design includes pH variations (7 and 10) and inoculum volumes (1 mL and 2 mL), with evaluations based on mercury reduction efficiency (via Cold Vapor AAS) and bacterial growth dynamics (log CFU/mL and optical density) over a 7-day incubation period. The findings aim to inform optimal biotreatment parameters for potential pilot-scale applications in mercury management within the ASGM sector.

2. Material and Methods

This study commenced with the revival of Bacillus subtilis and Pseudomonas aeruginosa on Nutrient Agar (NA) media, followed by sub-culturing into Lactose Broth (LB) to determine their growth kinetics, as adapted from [8]. Bacterial growth was monitored by measuring turbidity at 600 nm (OD_{600}) using a spectrophotometer at hourly intervals for the first seven hours of incubation. This step ensured that the bacterial inoculum was harvested during the exponential growth phase, which is critical for optimal bioremediation activity [21] [8].

Simulated contaminated wastewater was prepared by dissolving $HgCl_2$ to create a 100 mg/L stock solution of mercury (Hg^{2+}), which was then diluted to a working concentration of 2 mg/L. The pH of the solution was adjusted to two target values: neutral (6.9–7.0) and alkaline (10.0), using NaOH or HCl, to assess the influence of pH on bacterial performance in mercury bioremediation.

The experimental setup comprised eight treatment groups under each pH condition. These included: (1) a control group without bacterial inoculation, (2) B. subtilis inoculated at 1 mL (BS1) and 2 mL (BS2), (3) P. aeruginosa inoculated at 1 mL (PA1) and 2 mL (PA2), and (4) a mixed culture of both bacteria at 1 mL each (MC1) and 2 mL each (MC2). Each treatment was conducted in 200 mL of LB medium containing 2 mg/L of Hg^{2+} and incubated for seven days at 30–35 °C with constant agitation at 150 rpm to maintain homogeneity and adequate oxygenation [22].

Mercury concentrations were quantified on days 0 (baseline), 3, and 7 using Cold Vapor Atomic Absorption Spectrophotometry (CV-AAS), following the SNI 19-6964.2-2003 standard protocol [8]. Bacterial population dynamics were assessed using the pour plate technique and expressed as colony-forming units per milliliter (log CFU/mL). Colony counts were recorded on days 0, 1, 2, 3, and 7.

Additionally, pH and temperature of the culture media were monitored daily throughout the incubation period.

Data were analyzed descriptively. The percentage reduction of mercury was calculated by comparing concentrations on days 3 and 7 to the baseline (day 0). Bacterial growth trends were interpreted in relation to pH and inoculum volume, to evaluate the influence of environmental variables on the efficacy of the bioremediation process [21]. This methodological framework was designed to determine optimal operational parameters for potential field application.

3. Results and Discussion

Bioremediation of Mercury by Bacteria

Mercury metal (Hg) bioremediation test was conducted for seven days to evaluate the effectiveness of B. subtilis and P. aeruginosa in reducing mercury concentration. Parameters measured included mercury levels and total number of bacteria. Mercury levels were measured on days 0, 3, and 7 using the cold vapor atomic absorption spectrophotometry method. Meanwhile, the number of bacteria was counted on days 0, 1, 2, 3, and 7. The seventh day was chosen as the end point of observation to obtain comprehensive information on the effectiveness of biodegradation and the optimal time for bacterial growth in single and mixed inoculum cultures.

Mercury Removal

This study aims to evaluate the effect of inoculum variations (B. subtilis, P. aeruginosa, and a mixture of both), inoculum volume variations, and initial pH conditions (neutral and alkaline) in mercury bioremediation. The results showed that the addition of inoculum significantly increased the efficiency of mercury removal compared to the control without inoculum. The initial mercury concentration (0.25 mg/L) decreased significantly since day 3, indicating that the bacteria began to actively absorb or convert mercury after the initial adaptation period.

At neutral pH (Figure 1), the mercury removal efficiency reached 88.46% and 90.07% with the addition of 1 mL and 2 mL of B. subtilis inoculum. For P. aeruginosa inoculum, the efficiencies were 86% and 89.85%, respectively, while the mixed inoculum showed efficiencies of 88.66% and 86.78%. In comparison, the control reactor only experienced a mercury reduction of 6.30%, confirming the significant role of bacteria in the bioremediation process.

At alkaline pH (Figure 2), the mercury removal efficiencies were relatively similar to neutral pH, namely 90.07% and 89.51% for B. subtilis; 89.37% and 89.29% for P. aeruginosa; and 89.98% and 89.96% for the mixed inoculum. The similar efficiencies between neutral and alkaline pH indicate that both bacteria are able to survive and remain effective under different pH conditions, which is relevant for applications in various polluted environments.

The effectiveness of this bioremediation is associated with the physiological characteristics of B. subtilis bacteria, as Gram-positive bacteria, have thick cell walls composed of peptidoglycan, providing better mechanical resistance to environmental stress, including the presence of heavy metals [18] [19]. In addition, this structure allows the adsorption and complexation of mercury, which accelerates the detoxification process. Meanwhile, P. aeruginosa, which is a Gram-negative bacteria, is known to have a resistance mechanism based on the mer operon system that facilitates the reduction of Hg^{2+} to volatile Hg^0 through the enzyme mercury reductase (merA) [18] [19] [20]. This mechanism allows bacteria to survive in contaminated environments and remain active in bioremediation. Several previous studies have also confirmed that bacteria from the genus Bacillus and Pseudomonas have a high level of resistance to heavy metals [23]. This finding strengthens the suspicion that these two bacteria can be optimized as efficient and sustainable bioremediation agents for handling mercury pollution on a wider scale.

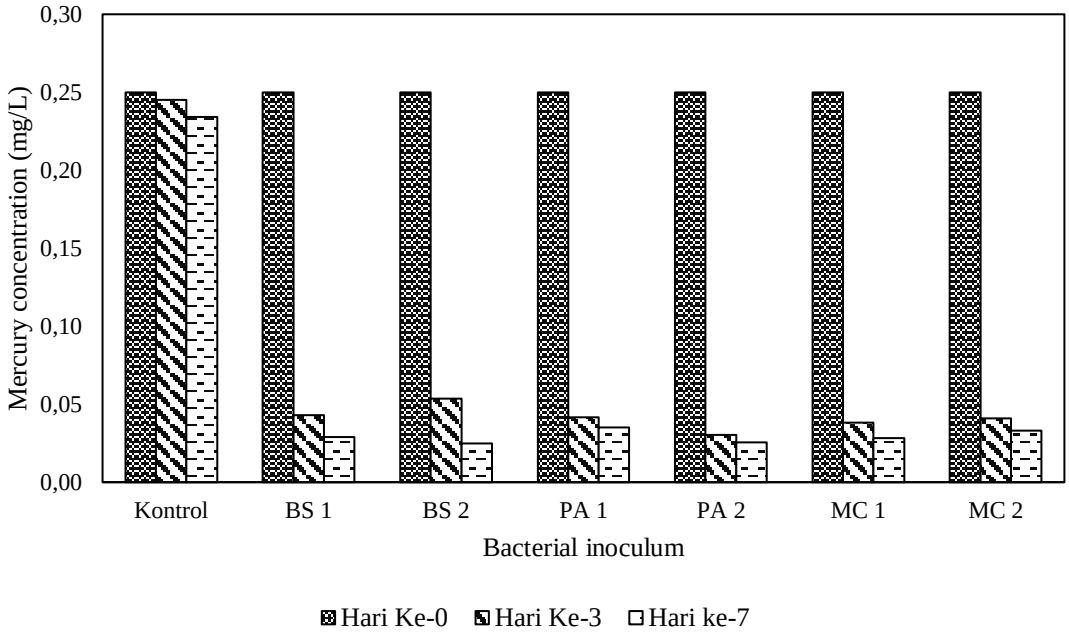

Figure 1. Mercury concentration in the reactor with neutral pH treatment (6.9-7)

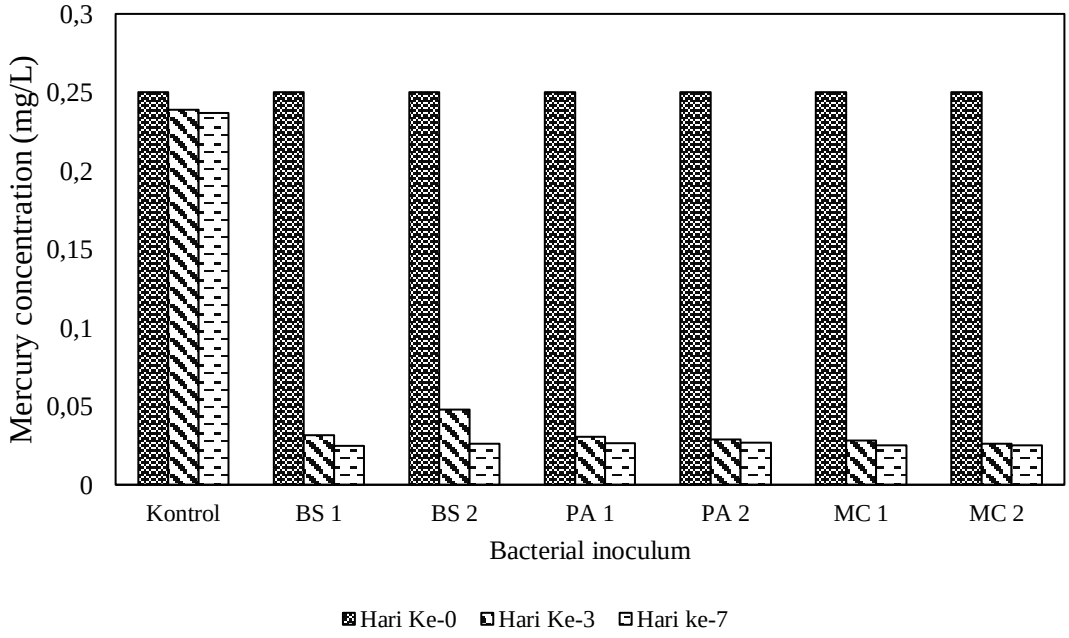

Figure 2. Mercury concentration in the reactor with neutral pH treatment (10)

Number of Bacterial Colonial Counts in The Biotreatment Process

This study used *B. subtilis* and *P. aeruginosa* with two culture methods: single inoculum and mixed culture). The number of bacterial colonies was calculated using the Total Plate Count (TPC) method with a range of 30-300 CFU/mL to ensure statistical validity. Measurements were taken on days 0, 1, 2, 3, and 7, and the results were converted to log CFU/mL for more accurate bacterial growth analysis.

Figure 3 and **Figure 4** show that the control reactor without inoculum has a bacterial colony count of <30 CFU/mL (too few to count, TFTC), indicating the effectiveness of sterilization in preventing contamination. Sterilization control is carried out by keeping the incubator closed, using laminar air flow (LAF) which is sterilized with UV for 20 minutes before use, and ensure sterilization of glassware and reactor cover.

In the neutral pH reactor (**Figure 3**), 1 mL and 2 mL *B. subtilis* inoculum produced a total colony count of 0 (TFTC) and 8.30 log CFU/mL, while *P. aeruginosa* inoculum produced 0 (TFTC) and 7.63 log CFU/mL. The peak growth occurred on day 3, with an average of 9.8 log CFU/mL for *B. subtilis* and 10.2

CFU/mL for *P. aeruginosa*, indicating sufficient nutrient availability and supportive environmental conditions [24]. However, on day 7 the number of colonies decreased drastically to TFTC, indicating depletion of nutrient sources in the media, which inhibited bacterial growth.

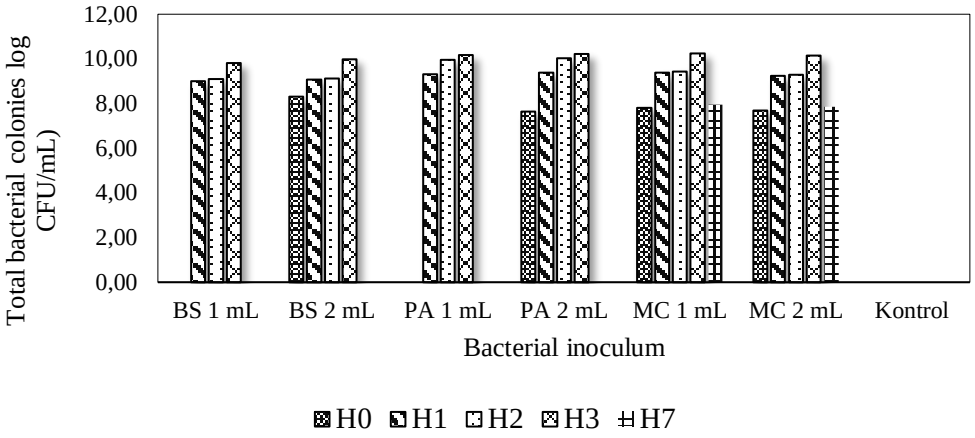

Figure 3. Total bacterial colonies in the reactor with neutral pH treatment (6.9-7)

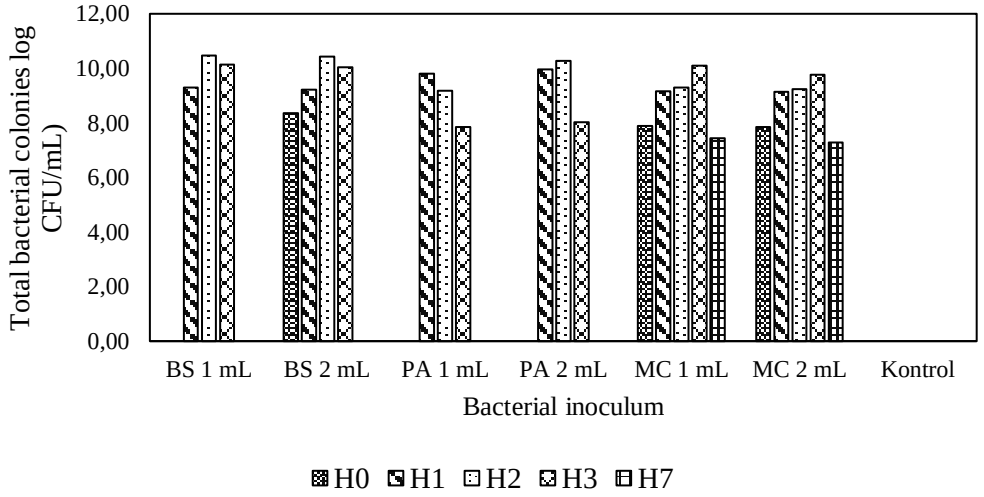

Figure 4. Total bacterial colonies in the reactor with alkaline pH treatment (10)

In the alkaline pH reactor (**Figure 4**), a similar growth pattern was observed. Day 0 showed TFTC in *P. aeruginosa* inoculum, while *B. subtilis* inoculum produced 8.3 log CFU/mL. The peak growth occurred earlier, namely on day 2, with a colony count of 10.4 log CFU/mL (*B. subtilis*) and 9.9 log CFU/mL (*P. aeruginosa*). The mixed inoculum showed a peak on day 3 with 9.9 log CFU/mL, indicating the potential for positive interactions between species in maintaining the population longer than single inoculum. On day 7, the mixed inoculum still had a colony count of 7.4 log CFU/mL, higher than the single inoculum which dropped to TFTC, indicating the possibility of synergy or mutualistic protection between species under alkaline pH conditions [24].

These results confirm that bacterial growth patterns are influenced by nutrient availability, microbial interactions, and environmental conditions. The drastic decrease in the number of colonies on day 7 could be attributed to the full consumption of nutrient sources and possible accumulation of mercury in the media, which can be toxic to bacteria. Furthermore, the difference in peak growth time between neutral and alkaline pH suggests that pH can affect the bioavailability of mercury, which may impact the efficiency of bioremediation on a broader scale.

4. Conclusion

This study demonstrates that Bacillus subtilis exhibits greater efficacy than Pseudomonas aeruginosa in reducing mercury concentrations in small-scale gold mining (ASGM) wastewater. Both bacterial strains were capable of achieving significant mercury removal efficiencies (>86%) across pH conditions ranging

from neutral (pH 7) to alkaline (pH 10), with the highest performance observed for B. subtilis at an inoculum volume of 2 mL. The growth kinetics of both bacteria showed optimal activity between days 2 and 3, followed by a decline on day 7, likely due to nutrient depletion in the culture medium. These results suggest that adjusting inoculum volume and pH conditions plays a critical role in enhancing bioremediation efficiency.

Furthermore, the use of mixed bacterial cultures was shown to prolong the bacterial survival phase compared to single cultures, potentially contributing to the sustainability of the bioremediation process. These findings provide a solid scientific foundation for advancing bacteria-based bioremediation as a viable, cost-effective, and environmentally friendly strategy for mercury detoxification in the ASGM sector.

Future studies are recommended to investigate the bioremediation potential of other indigenous bacterial strains with broader heavy metal detoxification capabilities. This could support the development of scalable and adaptable biological treatment systems for various types of industrial wastewater beyond mercury-contaminated effluents.

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