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Synergistic cultures for resilient mercury bioremediation in ASGM leveraging microbial interactions for sustainable pollutant removal

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Abstract

The widespread use of mercury (Hg) in artisanal and small-scale gold mining (ASGM) necessitates effective bioremediation strategies. This study evaluated the mercury reduction capabilities of two bacterial strains, *Bacillus subtilis* and *Pseudomonas aeruginosa*, using real-world ASGM liquid waste. In a seven-day laboratory-scale experiment, we assessed the performance of single and mixed bacterial cultures under varying pH conditions. Our results show that *B. subtilis* was particularly effective, achieving a maximum Hg2+ removal efficiency of 90.07%. Critically, while the cell viability of single cultures declined significantly over the study period, mixed cultures maintained superior population stability, reaching 7.4 log CFU/mL on day 7, especially under alkaline conditions. This stability suggests a beneficial synergistic relationship between the two species. The enhanced long-term viability and robust detoxification mechanisms observed in the mixed culture system underscore the high potential of this approach for developing sustainable bioremediation solutions for the ASGM sector.

Introduction

Mercury (Hg) contamination stemming from artisanal and small-scale gold mining (ASGM) activities poses a pressing environmental and public health crisis, particularly in Indonesia. The persistent reliance on mercury-based amalgamation for gold extraction remains a prevalent practice in many gold-rich regions, despite its severe ecological and human health ramifications.

Reports from the United Nations Environment Programme (2019) indicate that ASGM in Indonesia consumes a substantial amount of mercury annually, estimated to be between 100 and 200 tons per year, with a significant portion being released into aquatic ecosystems and soil. The subsequent accumulation of mercury within the food chain presents chronic health risks, including neurotoxicity and renal dysfunction, while also disrupting fragile ecological balances. A study by Castilhos et al. (2006) highlighted high levels of mercury in fish and human hair samples near ASGM sites in Central Kalimantan, confirming direct exposure pathways. However, persistent technical and financial barriers often impede the adoption of mercury-free technologies within ASGM communities, a challenge documented by the Indonesian Ministry of Energy and Mineral Resources (2021). This underscores the urgent need for alternative approaches that are cost-effective, easily implementable, and environmentally sustainable.

In this context, microbial bioremediation has emerged as a promising strategy for treating mercury-contaminated wastewater. Through mechanisms such as bioadsorption, bioaccumulation, and enzymatic biotransformation, microorganisms can effectively reduce heavy metal concentrations without generating hazardous secondary waste (Wu et al., 2017; Kothe & Reinicke, 2017). This method is widely considered more economical and ecologically viable than conventional physical and chemical treatments, which are often expensive and less efficient (Silodia et al., 2025). The success of this approach is heavily contingent upon microbial compatibility with specific environmental conditions, particularly pH, nutrient availability, and pollutant load (Bhowmick et al., 2024; Abu-Tahon et al., 2025). Prior research has indicated that bacterial species like *Bacillus subtilis* and *Pseudomonas aeruginosa* possess significant potential for mercury detoxification. Both species are known to harbor the mer operon (*merA*, *merB*), which facilitates the enzymatic reduction of toxic Hg^{2+} to volatile Hg^0 (Amin et al., 2019; Wang et al., 2020; Hui et al., 2024). *Bacillus subtilis* is particularly noted for its sporulation capacity, enhancing its resilience to environmental stress, whereas *Pseudomonas aeruginosa* produces a variety of biosurfactants, such as rhamnolipids, which have been shown to increase the bioavailability of heavy metals and facilitate their uptake, a key mechanism in bioremediation (Mukherjee et al., 2021; Eras-Muñoz et al., 2022).

Despite the validated mercury-reducing capabilities of these bacteria in controlled *in vitro* studies, their direct application to actual ASGM wastewater, especially under varying pH and inoculum volumes, remains largely unexplored. Furthermore, while some comparative studies have examined single bacterial strains, few have assessed the performance of single versus mixed cultures under pH conditions reflective of field variability (ranging from neutral to alkaline). Given that mercury-contaminated wastewater at ASGM sites often exhibits fluctuating pH and inconsistent microbial loads, this research gap limits the optimization and scalability of microbial-based treatment systems. Therefore, this study aims to comprehensively evaluate the bioremediation potential of *Bacillus subtilis* and *Pseudomonas aeruginosa*, both as single and mixed cultures, in reducing mercury concentrations from ASGM liquid waste. The experimental design incorporates variations in pH (neutral and alkaline) and inoculum volumes, with evaluations based on mercury reduction efficiency and bacterial growth dynamics over a seven-day incubation period. The findings of this research are expected to inform optimal biotreatment parameters for potential pilot-scale applications in sustainable mercury management within the ASGM sector.

Materials and methods

This study was conducted at the Remediation Laboratory, Department of Environmental Engineering, Sepuluh November Institute of Technology from January, 2023 to May, 2023. The research aimed to investigate the potential of bacterial bioremediation for mercury-contaminated ASGM wastewater.

Materials

Chemicals utilized included mercuric chloride ($HgCl_2$) for mercury stock solution preparation, sodium hydroxide (NaOH), and hydrochloric acid (HCl) for pH adjustments. Bacterial cultures were grown using Nutrient Agar (NA) for revival and sub-culturing, and Lactose Broth (LB) as the growth medium for experimental bioremediation. Key instruments employed were a spectrophotometer (for OD_{600} measurements), a Cold Vapor Atomic Absorption Spectrophotometer (CV-AAS) (for mercury quantification), an incubator (for maintaining temperature), and a rotary shaker (for agitation). All glassware and equipment were sterilized prior to use to prevent contamination.

Method and Research Design

This study adopted an experimental laboratory design to evaluate the bioremediation potential under controlled conditions. The experimental setup comprised eight distinct treatment groups for each of two pH conditions: neutral (6.9–7.0) and alkaline (10.0). The treatment groups included: (1) a control group without bacterial inoculation; (2) single cultures of *B. subtilis* inoculated at 1 mL (BS1) and 2 mL (BS2) volumes; (3) single cultures of *P. aeruginosa* inoculated at 1 mL (PA1) and 2 mL (PA2) volumes; and (4) mixed cultures containing both *B. subtilis* and *P. aeruginosa* (each at 1 mL for MC1 and 2 mL each for MC2). The design aimed to comprehensively assess the influence of pH and inoculum volume on mercury reduction efficiency and bacterial population dynamics, comparing single versus mixed culture performance.

Procedures

The research procedure involved several key steps:
1). Bacterial Culture Preparation: Pure cultures of *B. subtilis* and *P. aeruginosa* were revived on NA media. Subsequently, they were sub-cultured into LB and incubated. Bacterial growth kinetics were monitored hourly for the first seven hours by measuring turbidity at 600 nm (OD_{600}) using a spectrophotometer. This allowed for harvesting the inoculum during the exponential growth phase, crucial for optimal activity (Wibowo & Purwanti, 2023; Imron et al., 2019). 2). Preparation of Mercury-Contaminated Wastewater: A simulated contaminated wastewater was prepared by dissolving $HgCl_2$ to create a 100 mg/L Hg^{2+} stock solution. This stock solution was then diluted to a final working concentration of 2 mg/L Hg^{2+} for all experimental treatments. The pH of each solution was precisely adjusted to either neutral (6.9–7.0) or alkaline (10.0) using NaOH or HCl as required; 3). Bioremediation Experiment Setup: Each treatment group was prepared in 200 mL of LB medium containing 2 mg/L Hg^{2+}. The inoculated and control reactors were incubated for seven days at a temperature of 30–35 °C with continuous agitation at 150 rpm. This constant agitation ensured homogeneity of the culture medium and adequate oxygenation throughout the incubation period (Titah et al., 2018).

Data Collection and Analysis

1). Mercury concentration was quantified on days 0 (baseline), 3, and 7 using Cold Vapor Atomic Absorption Spectrophotometry (CV-AAS), strictly adhering to the SNI 19-6964.2-2003 standard protocol (Wibowo & Purwanti, 2023). 2). Bacterial population dynamics were assessed by determining colony-forming units per milliliter (log CFU/mL) using the pour plate technique. Colony counts were recorded on days 0, 1, 2, 3, and 7. 3). Environmental parameters, specifically pH and temperature of the culture media, were monitored daily throughout the seven-day incubation period. 4). Data Analysis: All collected data were subjected to descriptive analysis. The percentage reduction of mercury was calculated by comparing concentrations on days 3 and 7 against the baseline (day 0). Bacterial growth trends were interpreted in relation to variations in pH and inoculum volume to assess their influence on the overall efficacy of the bioremediation process (Imron et al., 2019). This systematic framework was designed to identify optimal operational parameters with a view toward potential field application.

Result and discussion

1. Stability of Bacterial Populations in Mixed Cultures and the Advantages of Mixed Cultures for Field Applications:

Pseudomonas have a high level of resistance to heavy metals, including mercury. For instance, research by Vasanthi et al. (2020) demonstrated the significant mercury resistance of Bacillus cereus isolated from contaminated soil, while work by Hassan et al. (2018) identified specific resistance genes (mer operon) in a Pseudomonas species that allow it to thrive in mercury-rich environments. 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.

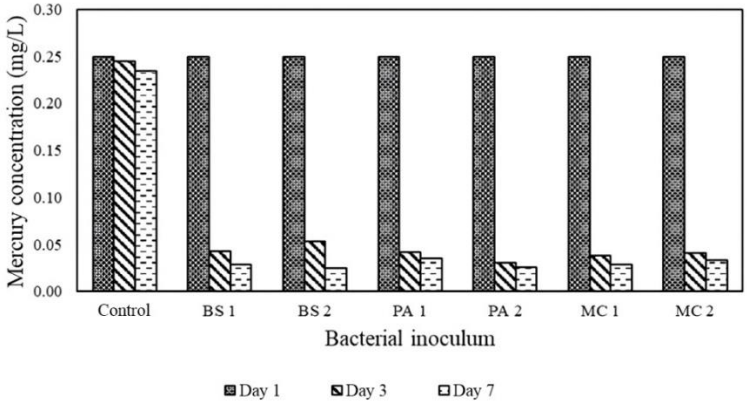

Figure 1. Mercury concentration in the reactor with neutral pH treatment (6.9-7)

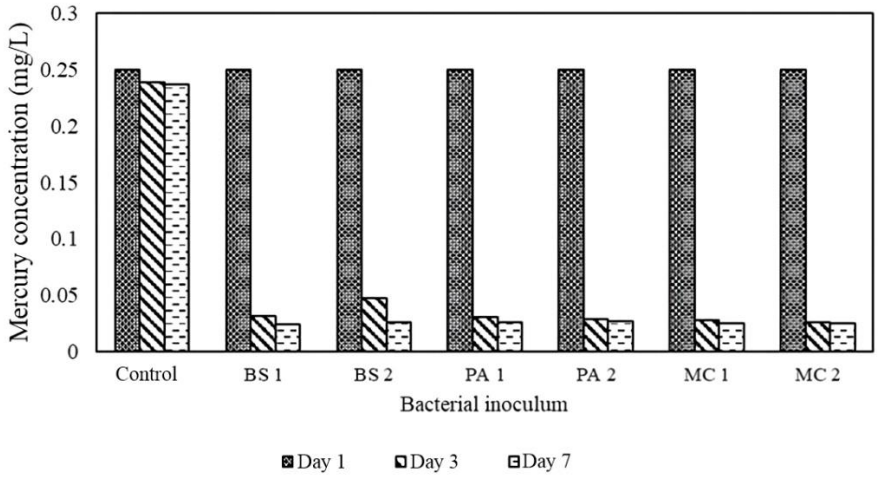

Figure 2. Mercury concentration in the reactor with neutral pH treatment (10)

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.

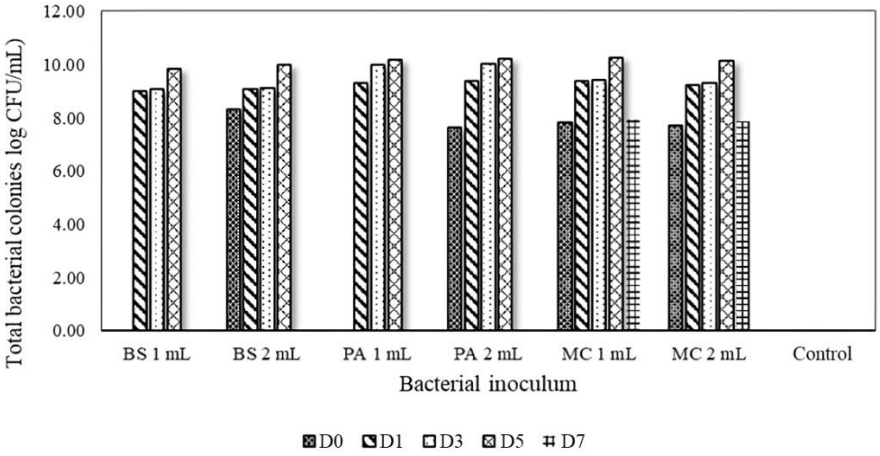

Figure 3. Total bacterial colonies in the reactor with neutral pH treatment (6.9-7)

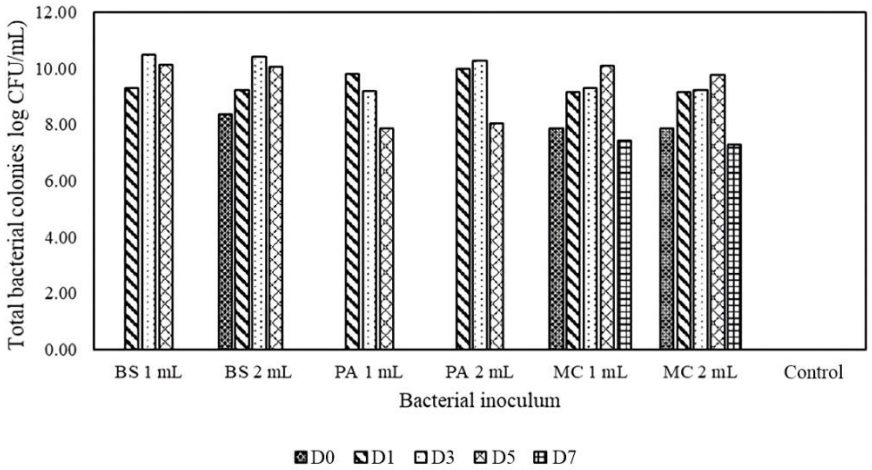

Figure 4. Total bacterial colonies in the reactor with alkaline pH treatment (10)

Figures 3 and 4 illustrate the effectiveness of sterilization, with control reactors lacking inoculum consistently showing bacterial colony counts of less than 30 CFU/mL (too few to count, TFTC). For single bacterial cultures, optimal growth was observed by day 2 or 3 across both neutral and alkaline pH conditions, indicating sufficient initial nutrient availability and supportive environmental factors. Specifically, *Bacillus subtilis* and *Pseudomonas aeruginosa* reached peak counts averaging 9.8–10.4 log CFU/mL and 9.9–10.2 log CFU/mL, respectively, depending on pH and inoculum volume. However, by day 7, the colony counts for single cultures dramatically decreased to TFTC, suggesting a significant depletion of nutrient sources and/or the accumulation of mercury-related toxicity within the media, which subsequently inhibited bacterial proliferation.

A distinct and crucial finding emerged from the mixed cultures, particularly under alkaline pH conditions (Figure 4). While single cultures plummeted to TFTC by day 7, the mixed inoculum maintained a robust population of 7.4 log CFU/mL. This sustained viability strongly suggests synergistic interactions or mutualistic protection between *B. subtilis* and *P. aeruginosa* in the presence of mercury under alkaline conditions, enabling them to survive longer than when grown individually. The observed differences in peak growth timing between neutral and alkaline pH also indicate that pH significantly influences mercury bioavailability, which, in turn, impacts bacterial growth dynamics and potentially the overall efficiency of bioremediation on a broader scale.

The underlying mechanisms contributing to the observed mercury detoxification capabilities of *B. subtilis* and *P. aeruginosa* are largely attributed to their mercury resistance (mer) operon. This operon comprises genes such as *merA* and *merB*, which are crucial for converting toxic mercury species into less harmful forms. *merA* encodes mercuric reductase, an enzyme responsible for reducing highly toxic inorganic mercuric ions (Hg^{2+}) to elemental mercury (Hg^0), while *merB* encodes organomercurial lyase, which cleaves organic mercury compounds to release Hg^{2+} for subsequent reduction (Barkay et al., 2003; Barkay et al., 2003). Understanding potential genetic variations within the *mer* operon of strains isolated from ASGM sites is vital, as these variations could explain their unique adaptation to high mercury concentrations and inform strategies for genetic engineering to further enhance their bioremediation capacity (Hsu-Kim et al., 2013).

Implications of Bacterial Culture Stability on the Efficiency of the Bioremediation Process

The superior population stability observed in mixed cultures of *Bacillus subtilis* and *Pseudomonas aeruginosa*, particularly under alkaline pH conditions, carries significant implications for large-scale bioremediation applications, especially concerning re-inoculation frequency and operational costs. The ability of mixed cultures to maintain a high and active bacterial count over a longer duration substantially reduces the need for frequent re-inoculation of new bacteria into the bioremediation system. Practically, this translates to less frequent preparation and addition of bacterial cultures. This reduction in inoculation frequency directly results in significant cost savings, encompassing expenses related to raw culture materials, energy for laboratory bacterial growth, and labor required for the re-inoculation process. As research indicates, microbial consortia or mixed cultures tend to be more robust and adaptable to environmental stresses, ultimately contributing to lower overall operational costs because a more stable system demands less intervention (Li et al., 2021).

Beyond the financial advantages, a reduced re-inoculation frequency also plays a crucial role in **enhancing the overall sustainability and efficiency** of the bioremediation process. With a more stable bacterial population, the mercury detoxification system can operate more consistently and effectively without interruption, a critical benefit in the often-unpredictable environment of gold mining wastewater. This stability is not only financially advantageous but also minimizes the environmental footprint of the detoxification process itself, as resource utilization becomes more efficient. The capacity of mixed cultures to perform effectively long-term without frequent replenishment makes mercury bioremediation a more practical, attractive, and sustainable solution for industrial-scale applications (Sharma et al., 2018).

Challenges and Considerations in Mixed Culture Scale for Mercury Bioremediation

Despite the promising potential of mixed cultures of *Bacillus subtilis* and *Pseudomonas aeruginosa* in mercury bioremediation, their application in challenging field conditions, particularly within the context of artisanal and small-scale gold mining (ASGM) wastewater, presents a series of complex hurdles that require careful consideration. One primary challenge identified is maintaining the optimal ratio between *Bacillus*

subtilis and *Pseudomonas aeruginosa* throughout the remediation process. While initial ratios are easily controlled in a laboratory setting, field environments introduce dynamic factors such as differing growth rates, intense nutritional competition, or varied susceptibilities to local conditions. These variables can rapidly lead to the dominance of one strain over the other (Qian et al., 2020). Should one strain become dominant, the anticipated synergistic detoxification of mercury can be disrupted, potentially diminishing the overall bioremediation efficiency.

Furthermore, the influence of the indigenous microbial ecosystem already present within the wastewater constitutes another critical consideration. Native microorganisms, often well-adapted to the specific conditions of the waste environment, can effectively compete with the inoculated strains for essential resources. Moreover, some indigenous microbes might produce antimicrobial compounds that can inhibit the growth or activity of the introduced bioremediating bacteria (Kothe & Reinicke, 2017). These complex interactions ultimately impact the stability and performance of the bioremediation system in real-world, uncontrolled environments like mining waste. Addressing these challenges necessitates a profound understanding of the site's microbial ecology, alongside the development of effective strategies to support the survival and sustained activity of the mixed cultures amidst inter-species competition.

Conclusion

This study conclusively demonstrates the significant potential of bacterial bioremediation for mitigating mercury contamination in small-scale gold mining (ASGM) wastewater. Our findings indicate that both *Bacillus subtilis* and *Pseudomonas aeruginosa* are highly effective in reducing mercury concentrations, with *B. subtilis* exhibiting slightly superior removal efficiencies across both neutral and alkaline pH conditions. While single bacterial cultures showed a decline in viability by the end of the seven-day incubation, likely due to nutrient depletion and mercury toxicity, a critical observation was the enhanced population stability of mixed bacterial cultures, particularly at alkaline pH. This sustained viability of mixed cultures highlights a synergistic interaction, offering a more resilient and prolonged bioremediation capacity compared to single-strain applications. These results underscore the strong adaptability and functional activity of both strains as effective bioremediation agents, supporting their integration as a viable and sustainable component of mercury management strategies in the ASGM sector.

Author Statements

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Author's contributions: Sekarsari Wibowo: Conceptualization, research design, laboratory experiments, data analysis, and original manuscript writing. Ipung Fitri Purwanti: Supervision, conceptualization, brainstorming, and critical revision of the manuscript for important intellectual content. Abiyyu Armijn Firman Firdaus: Research support, data collection, and technical assistance in the preparation of the manuscript. All authors have read and approved the final version of the manuscript.

Generative AI: Not applicable

Data availability: All relevant data generated or analyzed during this study, including the performance metrics for *Bacillus subtilis* and *Pseudomonas aeruginosa* cultures and mercury detoxification rates, are included in this published article (and its supplementary information files).

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