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Indigenous river bacteria from West Java, Indonesia with copper tolerance and malachite green decolorization potential

WAHYU IRAWATI[1,♥], IMMANUELLA GRACE KRISTINA[2], REINHARD PINONTOAN[2], MARCELIA SUGATA[2,♥♥]

[1]Department of Biology Education, Faculty of Education, Universitas Pelita Harapan. Jl. M. H. Thamrin Boulevard 1100, Tangerang 15811, Banten, Indonesia. Tel./fax.: +62-21-5460901, ♥email: wahyu.irawati@uph.edu

[2]Department of Biology, Faculty of Health Sciences, Universitas Pelita Harapan. Jl. M. H. Thamrin Boulevard 1100, Tangerang 15811, Banten, Indonesia. Tel./fax.: +62-21-5460901, ♥♥email: marcelia.sugata@lecturer.uph.edu

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Abstract. *Irawati W, Kristina IG, Pinontoan R, Sugata M. 2026. Indigenous river bacteria from West Java, Indonesia with copper tolerance and malachite green decolorization potential. Biodiversitas 27 (2): d270222. https://doi.org/10.13057/biodiv/d270222*. River ecosystems are important reservoirs of freshwater biodiversity, but are increasingly threatened by textile-derived dyes and heavy metals. These contaminants impose chronic chemical stress, so selecting pollution-adapted indigenous bacteria represents a valuable biological resource for sustainable bioremediation. Three indigenous bacterial isolates from textile-polluted rivers in West Java, Indonesia—*Lysinibacillus capsici* CKJ 1000 1.1, *Bacillus proteolyticus* CKJ 1000 2.2, and *Bacillus cereus* CTR 200 1.1—were evaluated for copper (Cu) tolerance, while Cu biosorption in LB broth (2% inoculum, OD600 = 0.8, 48 h, 37°C, 150 rpm) was quantified by atomic absorption spectrophotometry. MG resistance and decolorization were examined on MG-supplemented agar (0, 10, 20, 50, and 100 ppm) and in liquid LB (10 or 20 ppm; 48 or 96 h) using UV-Vis spectrophotometry. Putative MG transformation products were further profiled using LC-HRMS. *L. capsici* exhibited reduced growth at ≥8 mM, whereas *B. cereus* and *B. proteolyticus* demonstrated higher tolerance, maintaining growth even at 10 mM $CuSO_4$. Copper biosorption was the highest in *L. capsici* (13.71% at 4 mM $CuSO_4$), followed by *B. cereus* (10.8%) and *B. proteolyticus* (9.10%). MG decolorization showed clear strain-dependent variation; at 10 ppm MG after 48 h, *L. capsici* achieved the highest decolorization efficiency (92.50%), followed by *B. cereus* (68.68%) and *B. proteolyticus* (47.62%). This study provides the first quantitative comparison of copper biosorption and MG decolorization under standardized conditions for these indigenous riverine isolates, coupled with LC-HRMS profiling of MG transformation products for the top-performing strain. Putative transformation products, including aniline and benzophenone derivatives, were detected; however, ecotoxicity was not assessed, and the environmental safety of the resulting metabolites cannot be concluded. These findings highlight pollution-adapted freshwater bacteria as promising biological resources for integrated metal-dye bioremediation, pending further toxicological validation.

Keywords: Biodegradation, Cikijing, Citarum, dye degradation products, multi-resistant

INTRODUCTION

River ecosystems are important reservoirs of freshwater biodiversity that host complex microbial communities responsible for biogeochemical cycling and ecosystem stability. However, rapid industrialization—particularly textile manufacturing—has imposed substantial chemical pressure on many river systems through the discharge of untreated or insufficiently treated wastewater. Textile industries are major contributors to aquatic pollution through intensive use of synthetic dyes and heavy metals, underscoring the need to understand ecological responses in impacted freshwater environments (Filho et al. 2022).

Among textile dyes, Malachite Green (MG) is one of the most problematic contaminants. MG is a cationic triphenylmethane dye widely used for its intense colour intensity, stability, and low cost. Despite these advantages, MG is environmentally persistent and exhibits severe toxicological effects (Jalandhar et al. 2025). It resists natural degradation and can persist in aquatic ecosystems for extended periods, causing mutagenic, carcinogenic, teratogenic, and cytotoxic effects, including oxidative stress, DNA damage, mitochondrial dysfunction, and endocrine disruption (Yadav and Qanungo 2023). Even at concentrations less than 1 mg L^{-1}, MG is toxic to algae, fish, molluscs, and crustaceans (Sharma and Chadha 2023). Due to its high solubility and strong affinity for sediments, MG can be transported over long distances and accumulate in aquatic food webs, making it a persistent ecological stressor.

Textile wastewater also contains heavy metals introduced during dye fixation, fabric treatment, and equipment maintenance. Copper (Cu), lead (Pb), and zinc (Zn) are commonly detected (Sani et al. 2018), with Cu being of particular concern due to its widespread use and toxicity at relatively low concentrations. In Indonesian rivers affected by textile effluents, Cu concentrations range from 0.1 to 2.5 mg L^{-1}, exceeding the national surface water quality limit of 0.02 mg L^{-1} (Irawati et al. 2016). Chronic Cu exposure disrupts metabolism, induces oxidative stress, damages aquatic organisms, and poses risks to human health via contaminated water and food chains (Wang et al. 2024).

The frequent co-occurrence of MG and Cu in textile wastewater creates intense selective pressures that shape microbial community structure and function. Conventional

treatment approaches—including coagulation-flocculation, chemical precipitation, advanced oxidation, and membrane filtration—can reduce contaminant loads but are often costly and energy-intensive, and they generate secondary hazardous sludge. These limitations have driven interest in microbial processes capable of transforming or immobilizing pollutants through enzymatic degradation, redox reactions, and biosorption (Pertile et al. 2020). Such microbial activities represent functional traits reflecting adaptation to long-term chemical stress.

Many bacteria possess mechanisms that enable them to survive under dye or heavy-metal stress. Dye decolorization is commonly mediated by enzymes such as laccases, lignin peroxidases, tyrosinases, and reductases (Khandare and Govindwar 2016; Valerie et al. 2018), while copper tolerance involves efflux systems, enzymatic detoxification, intracellular sequestration, and biosorption onto cell wall components (Das et al. 2016). However, most studies focus on single functional traits, report qualitative outcomes, or rely solely on color removal without profiling transformation products, making it challenging to distinguish chemical modification from simple adsorption. Consequently, strain-level functional diversity of indigenous freshwater bacteria from chronically polluted rivers remains poorly documented.

Indigenous cultivable bacteria from textile-impacted rivers represent localized freshwater microbial diversity and functional adaptation to chronic chemical stress. Rivers in West Java, Indonesia, exposed to long-term textile effluent discharge, have yielded bacterial isolates resistant to both dyes and heavy metals. Previous studies identified *Lysinibacillus capsici* CKJ 1000 1.1 and *Bacillus proteolyticus* CKJ 1000 2.2 from the Cikijing River (Irawati et al. 2025) and *Bacillus cereus* CTR 200 1.1 from the Citarum River (Irawati et al. 2023b) as resistant bacterial isolates. These isolates have been taxonomically identified and preserved as cultivable strains; however, their comparative tolerance traits, quantitative biosorption capacity, and MG transformation profiles have not yet been systematically evaluated.

Therefore, this study investigates indigenous bacterial isolates from textile-polluted rivers in West Java by quantitatively evaluating their dual functional traits—copper biosorption and malachite green decolorization—and by profiling putative MG transformation products using LC-HRMS. The study documents strain-level functional diversity within freshwater microbial biodiversity and explores stress-adapted indigenous bacteria as potential biological resources.

MATERIALS AND METHODS

Bacterial isolates and medium preparation

The bacterial isolates used in this study—*L. capsici* CKJ 1000 1.1 (GenBank accession number: CP185952) and *B. proteolyticus* CKJ 1000 2.2 from the Cikijing River, and *Bacillus cereus* CTR 200 1.1 (GenBank accession number: JBNGBZ01) from the Citarum River, West Java, Indonesia—were previously identified and taxonomically confirmed by 16S rRNA gene sequencing. The 16S rRNA gene sequence of *B. proteolyticus* CKJ 1000 2.2 has not yet been deposited in a public database. Simple biochemical characterization was also performed to support the identification (Irawati et al. 2023b; Irawati et al. 2025). The bacteria were cultured in Luria-Bertani (LB) medium containing tryptone (10 g/L), yeast extract (5 g/L), NaCl (10 g/L) at pH 7.5±0.2. For a solid medium, 2% (w/v) agar was added. All liquid experiments were carried out in Erlenmeyer flasks. Media were sterilized by autoclaving at 121°C and 1 atm for 15 minutes.

Stock solutions of 1 M $CuSO_4$ and 10,000 ppm Malachite Green (MG) were prepared and added to the selective media to achieve final concentrations of 5-10 mM $CuSO_4$ and 20, 50, or 100 ppm MG, respectively (Irawati et al. 2022a).

Copper tolerance and biosorption assay

A plate-based copper tolerance assay was conducted to assess bacterial growth under increasing copper concentrations. LB agar plates supplemented with $CuSO_4$ (5-10 mM) were prepared, and each isolate was streaked and incubated at 37°C for 24 h. Copper tolerance was defined strictly based on growth intensity, assessed qualitatively by colony size and density, and recorded at the highest $CuSO_4$ concentration at which visible growth was observed. Pigmentation changes, when present, were noted only as qualitative observations and were not interpreted as evidence of copper absorption. Isolates showing visible growth at the highest copper concentrations were selected for subsequent biosorption analysis (Irawati et al. 2016).

The biosorption assay was conducted in LB broth supplemented with $CuSO_4$ at the designated concentrations. A 2% (v/v) inoculum from each bacterial starter culture (OD_{600} = 0.8) was added to 50 mL of LB broth and incubated in a shaking incubator at 150 rpm and 37°C for 48 h. After incubation, the cultures were centrifuged at 5,000×g for 15 min. The resulting supernatants were digested with nitric acid (HNO_3) and heated on a hot plate. The control consisted of the LB broth containing the same concentration of $CuSO_4$ without bacterial inoculation. An abiotic control was included to assess possible precipitation and adsorption in the absence of cells. Copper concentrations were quantified using an Atomic Absorption Spectrophotometer (AAS) (Irawati et al. 2020). The biosorption experiments were performed in biological triplicate (n = 3).

Dye resistance and decolorization assay

Bacterial isolate resistance to Malachite Green (MG) was qualitatively assessed by streaking them onto LB agar plates supplemented with 0, 10, 20, 50, or 100 ppm MG. Plates were incubated at 37°C, and bacterial growth and decolorization zones were recorded after 24 h for plates containing 10, 20, or 50 ppm MG. Plates containing 100 ppm MG were observed after 4 days of incubation.

Quantitative liquid-phase decolorization assays were performed by inoculating bacterial cultures into LB broth containing 10 or 20 ppm MG and incubating in a shaking incubator at 37°C for 48 or 96 h. The assay was conducted in technical triplicate. After incubation, samples were centrifuged at 5,000 rpm for 10 min to remove bacterial

cells. The absorbance of the supernatant was measured using a UV-Vis spectrophotometer (Biobase) across the wavelength range of 300-900 nm. The control ($A_{control}$) consisted of uninoculated LB broth containing MG, incubated under the same conditions to determine any abiotic degradation. Decolorization efficiency was determined by monitoring the decrease in absorbance at 618 nm and calculated using the following equation (Irawati et al. 2022a).

$$\%_{Decolorization} = \frac{A^{618}_{control} - A^{618}_{treatment}}{A^{618}_{control}} \times 100\%$$

Where:

$A^{618}{}_{control}$: Absorbance of the control

$A^{618}{}_{treatment}$: Absorbance of the test sample at time t

Degradation products were analyzed by liquid chromatography-high-resolution mass spectrometry (LC-HRMS) using a Thermo Scientific™ Vanquish™ UHPLC system equipped with a binary pump and coupled to a Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap™ high-resolution mass spectrometer. Samples obtained from malachite green (MG; 10 ppm) decolorization assays were subjected to LC-HRMS analysis. Chromatographic separation was performed on a Thermo Scientific™ Accucore™ Phenyl-Hexyl column (100 mm × 2.1 mm i.d., 2.6 µm particle size) maintained at 40°C. The mobile phases consisted of MS-grade water containing 0.1% (v/v) formic acid (eluent A) and MS-grade methanol containing 0.1% (v/v) formic acid (eluent B), delivered at a flow rate of 0.3 mL min^{-1}. The gradient program was as follows: 95% A / 5% B at 0.01 min, linearly ramped to 10% A / 90% B at 16.0 min, held until 20.0 min, and returned to initial conditions (95% A / 5% B) at 25.0 min. The injection volume was 3 µL. Mass spectrometric detection was performed using Electrospray Ionization (ESI) operated in both positive and negative ion modes. Nitrogen was used as the sheath gas (32 arbitrary units), auxiliary gas (8 arbitrary units), and sweep gas (4 arbitrary units). The spray voltage was set at 3.30 kV, with a capillary temperature of 320°C and an auxiliary gas heater temperature of 30°C. Full-scan MS data were acquired over an m/z range of 66.7-1000 at a resolution of 70,000, while data-dependent MS/MS (dd-MS^2) spectra were acquired at a resolution of 17,600.

Statistical analysis

Experiments performed in biological triplicate (n = 3) were analyzed statistically using one-way ANOVA followed by Tukey's post hoc test to determine significant differences between treatments. Data are presented as mean±Standard Deviation (SD). A p-value<0.05 was considered statistically significant. All analyses were performed using SPSS version 22.

RESULTS AND DISCUSSION

Copper tolerance

Copper tolerance of the three indigenous bacterial isolates recovered from textile-polluted rivers in West Java, Indonesia (the Cikijing and Citarum Rivers) was evaluated qualitatively on LB agar supplemented with increasing concentrations of $CuSO_4$. All isolates exhibited visible growth under copper exposure, although the maximum tolerated concentration differed among strains (Table 1). *B. proteolyticus* CKJ 1000 2.2 and *B. cereus* CTR 200 1.1 maintained visible colony formation across all tested concentrations (5-10 mM $CuSO_4$), although growth intensity decreased at higher concentrations. In contrast, *L. capsici* CKJ 1000 1.1 exhibited moderate growth up to 7 mM $CuSO_4$ and weak growth at 8-10 mM. At concentrations exceeding these thresholds, growth was strongly inhibited or absent. In addition to growth inhibition patterns, visible pigmentation changes in the agar medium were observed as copper concentration increased. The medium shifted from its original greenish appearance to progressively darker brownish hues at higher copper levels, particularly around actively growing colonies.

Copper is an essential micronutrient that serves as a cofactor for numerous enzymes involved in respiration, oxidative stress defense, and electron transfer (Koh et al. 2017). However, excess copper is highly toxic by catalyzing the formation of reactive oxygen species, disrupting protein structure, inhibiting enzymatic activity, and inducing DNA damage (Ladomersky and Petris 2015). The ability of the indigenous isolates to grow under elevated copper concentrations reflects adaptive traits of native riverine microbial communities exposed to chronic metal stress (Irawati et al. 2020). Differences in copper tolerance among isolates likely reflect strain-specific variations in copper homeostasis systems, including efflux transporters, intracellular sequestration, and cell wall binding capacity. Pigmentation changes in the medium under copper exposure have been reported previously (Irawati et al. 2021) and were treated strictly as qualitative observations rather than as quantitative indicators of copper uptake. Quantitative copper removal by the isolates is measured in the subsequent biosorption assay.

Copper biosorption

Based on the qualitative tolerance assay, copper biosorption experiments were conducted at lower $CuSO_4$ concentrations of 3 mM and 4 mM in LB broth, corresponding to initial copper concentrations of 130.5 mg L^{-1} and 169.9 mg L^{-1}, respectively, as determined by Atomic Absorption Spectroscopy (AAS). After 48 h of incubation, residual copper concentrations in culture supernatants were measured, and percent removal was calculated (Figure 1). All three indigenous riverine bacterial isolates demonstrated measurable copper removal under both conditions. *L. capsici* CKJ 1000 1.1 exhibited the highest removal efficiencies, with approximately 13.33% removal at 3 mM and 13.71% at 4 mM $CuSO_4$. *B. proteolyticus* CKJ 1000 2.2 and *B. cereus* CTR 200 1.1 showed slightly lower removal efficiencies. However, there were no significant differences among isolates or between copper concentrations (p = 0.3342).

Table 1. Copper tolerance of indigenous bacterial isolates from rivers in West Java was evaluated on LB agar supplemented with varying concentrations of $CuSO_4$ after incubation at 37°C for 24 h

Bacterial isolates	5 mM $CuSO_4$	6 mM $CuSO_4$	7 mM $CuSO_4$	8 mM $CuSO_4$	9 mM $CuSO_4$	10 mM $CuSO_4$
Control	-	-	-	-	-	-
Lysinibacillus capsici CKJ 1000 1.1	++	++	++	+	+	+
Bacillus proteolyticus CKJ 1000 2.2	+++	+++	+++	++	++	+
Bacillus cereus CTR 200 1.1	+++	+++	+++	+++	++	+

Note: Copper tolerance was evaluated based on growth intensity (colony size and density). The scoring rubric was defined as follows: +: Weak growth, ++: Moderate growth, +++: Strong growth, -: No visible growth

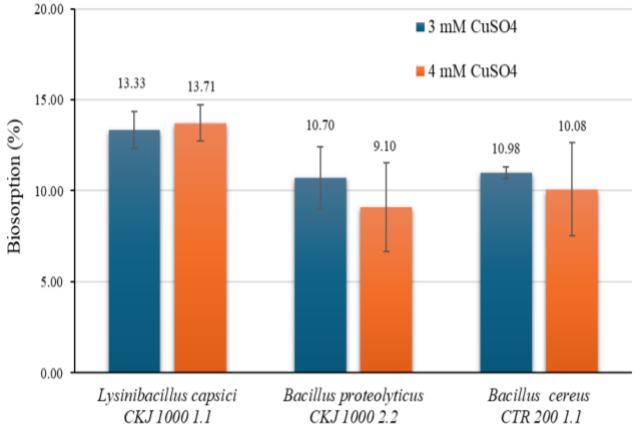

Figure 1. Copper biosorption by bacterial isolates after 48 h of incubation in LB broth supplemented with $CuSO_4$. Residual Cu concentrations in culture supernatants were quantified by Atomic Absorption Spectroscopy (AAS). Values represent net Cu removal per culture and were not normalized to dry cell mass. Data are presented as mean±SD of three biological replicates (n = 3). Statistical analysis was performed using one-way ANOVA ($p = 0.3342$)

The copper concentrations used in this study fall within the range reported for textile-impacted and industrially contaminated waters, which may contain copper levels ranging from approximately 2.5 mg L^{-1} to over 10,000 mg L^{-1} depending on industrial activity and treatment status (Liu et al. 2024). In Indonesia, copper concentrations of 0.1-2.5 mg L^{-1} have been reported in rivers impacted by textile industry discharge, far exceeding the national surface water quality limit of 0.02 mg L^{-1} (Irawati et al. 2016). The use of 3-4 mM $CuSO_4$ represents a compromise between environmental relevance and maintaining microbial viability under controlled laboratory conditions. The ability of these indigenous isolates to remove copper under such conditions reflects functional traits of cultivable bacterial components adapted to metal-stressed river ecosystems. Comparable tolerance has been reported for another Indonesian river isolate, *Acinetobacter* sp. IrC2, which can grow in medium containing up to 4 mM $CuSO_4$ (Irawati et al. 2021).

The relatively higher performance of *L. capsici* CKJ 1000 1.1 may be attributed to a combination of structural and physiological traits. As Gram-positive bacteria, *Lysinibacillus* and *Bacillus* possess thick peptidoglycan layers enriched in negatively charged functional groups—carboxyl, hydroxyl, and amino groups—that promote passive metal adsorption (Dhanwal et al. 2018; Biswas et al. 2021). Beyond passive adsorption, copper tolerance and biosorption may be enhanced by active cellular responses.

These include the production of Extracellular Polymeric Substances (EPS), which provide additional binding sites for metal ions, and the operation of copper efflux systems such as CopA, which reduce intracellular toxicity (Chihomvu et al. 2015; Alotaibi et al. 2021; Wróbel et al. 2023). These biosorption traits represent ecological adaptations of native riverine bacteria to chronic metal exposure rather than outcomes of optimized engineering conditions.

The copper removal efficiencies observed in this work are lower than those reported in the previous study under optimized conditions. Soil-derived *B. cereus* strain CU4A removed approximately 87% of Cu^{2+} from solution, with functional groups on the bacterial surface confirmed as the primary binding sites using FTIR and SEM-EDS analysis (Dhanwal et al. 2018). Similarly, *B. subtilis* has demonstrated copper removal efficiency approaching 89% under optimized pH and biomass conditions (Rocco et al. 2024), while EPS isolated from *Bacillus* sp. MBFF19 exhibited adsorption capacities of 89.6 mg Cu g^{-1} EPS (Gupta and Diwan 2016). Nevertheless, direct quantitative comparison with previously reported high removal efficiencies should be interpreted cautiously, as experimental normalization parameters, including initial metal concentration, biomass loading, contact time, pH, temperature, and medium composition, strongly influence biosorption values.

Dye resistance

The response of three indigenous riverine bacterial isolates to Malachite Green (MG) was also evaluated. Bacterial growth on MG-supplemented agar showed that all isolates survived and proliferated across a range of MG concentrations, although growth intensity decreased as dye concentration increased (Table 2). Among the isolates, *L. capsici* CKJ 1000 1.1 consistently exhibited the strongest growth across all tested MG concentrations. Clear or faded zones surrounding colonies indicated active MG decolorization. *L. capsici* CKJ 1000 1.1 produced clear zones within 24 h at 10, 20, and 50 ppm MG, whereas decolorization at 100 ppm required approximately four days to become visible. At lower MG concentrations (10-20 ppm), growth remained robust, while growth was visibly reduced at 50 and 100 ppm.

Malachite Green (MG) is a persistent triphenylmethane dye commonly detected in water impacted by textiles and is highly toxic to aquatic organisms. High MG concentrations disrupt membrane integrity, inhibit cell division, and induce oxidative stress, leading to reduced growth or cell death (Nassar et al. 2024; Bibi et al. 2025). The ability of all three indigenous isolates to grow in the presence of MG reflects functional tolerance traits of cultivable bacteria originating from dye-impacted river environments. The better growth and earlier decolorization observed in *L. capsici* CKJ 1000 1.1 are consistent with a higher adaptive capacity relative to the other tested isolates. The delayed appearance of clear zones at higher MG concentrations indicates increased physiological stress before the onset of observable dye transformation. Collectively, these findings showed strain-dependent functional traits associated with MG tolerance and transformation among indigenous river isolates and indicate that decolorization capacity varies among native bacterial strains adapted to polluted aquatic ecosystems.

Dye decolorization

Quantitative decolorization analysis showed a progressive decrease in Malachite Green (MG) absorbance at 618 nm, confirming effective degradation of the dye by the bacterial isolates (Figure 2). The untreated MG (control) exhibited the highest absorbance, whereas spectra obtained after bacterial treatment showed markedly reduced peak intensities, indicating increasing levels of decolorization. Minor alterations in peak shape and the appearance of low-wavelength absorbance features suggest the formation of intermediate compounds during reductive or oxidative transformation of MG.

The reduction in MG absorbance at 618 nm, together with the emergence of minor spectral features at lower wavelengths, indicates that decolorization was primarily due to chemical transformation rather than simple adsorption. Similar spectral patterns have been reported during bacterial MG degradation, in which intermediates such as leucomalachite green, benzophenone derivatives, *N, N*-dimethylaniline, and other aromatic fragments are formed (Song et al. 2020; El-Bendary et al. 2023).

The decolorization of the three indigenous river isolates, i.e., *L. capsici* CKJ 1000 1.1, *B. proteolyticus* CKJ 1000 2.2, and *B. cereus* CTR 200 1.1, was evaluated at MG concentrations of 10 and 20 ppm over incubation periods of 48 and 96 h (Figure 2). *L. capsici* CKJ 1000 1.1 exhibited the highest decolorization efficiency (92.50%) at 10 ppm after 48 h incubation, indicating rapid dye removal under lower concentration and shorter exposure. At 20 ppm after 48 h, *B. cereus* CTR 200 1.1 showed superior decolorization (95.78%), while *L. capsici* CKJ 1000 1.1 and *B. proteolyticus* CKJ 1000 2.2 achieved decolorization rates of 76.52% and 75.21%, respectively.

Higher decolorization was consistently observed at 48 h than at 96 h, suggesting that MG transformation was more effective during the early incubation. Reduced efficiencies at prolonged exposure may reflect nutrient depletion, decreased metabolic activity, or accumulation of inhibitory transformation products, although these factors were not directly examined. Similar time-dependent declines have been reported in other dye biodegradation studies, highlighting the importance of optimizing incubation duration.

Strain-specific differences in decolorization likely reflect variations in physiological and biochemical capacities among the indigenous isolates. Bacterial dye removal commonly involves surface biosorption and enzymatic biodegradation mediated by reductases and oxidases (Moyo et al. 2022). The comparatively strong performance of *Lysinibacillus* species, particularly *L. capsici* CKJ 1000 1.1, has been associated with enhanced production of Extracellular Polymeric Substances (EPS), which provide additional adsorption sites, protect cells from chemical stress, and facilitate stable biofilm formation. EPS-mediated protection may further support the activity of intracellular or membrane-associated reductases and oxidases that cleave the chromophore into smaller aromatic intermediates (Marmion et al. 2022).

Table 2. The growth of indigenous bacterial isolates from rivers in West Java and decoloration zones on Malachite Green (MG) agar at varying concentrations after 24 h (10-50 ppm) or 4 days (100 ppm)

Concentrations	Control (only malachite green)	*Lysinibacillus capsici* CKJ 1000 1.1	*Bacillus proteolyticus* CKJ 1000 2.2	*Bacillus cereus* CTR 200 1.1
10 ppm	-	+++	++	++
20 ppm	-	+++	++	++
50 ppm	-	+	+	+
100 ppm	-	+	+	+

Note: Dye resistance was evaluated based on growth intensity (colony size and density). The scoring rubric was defined as follows: +: Weak growth, ++: Moderate growth, +++: Strong growth, -: No visible growth

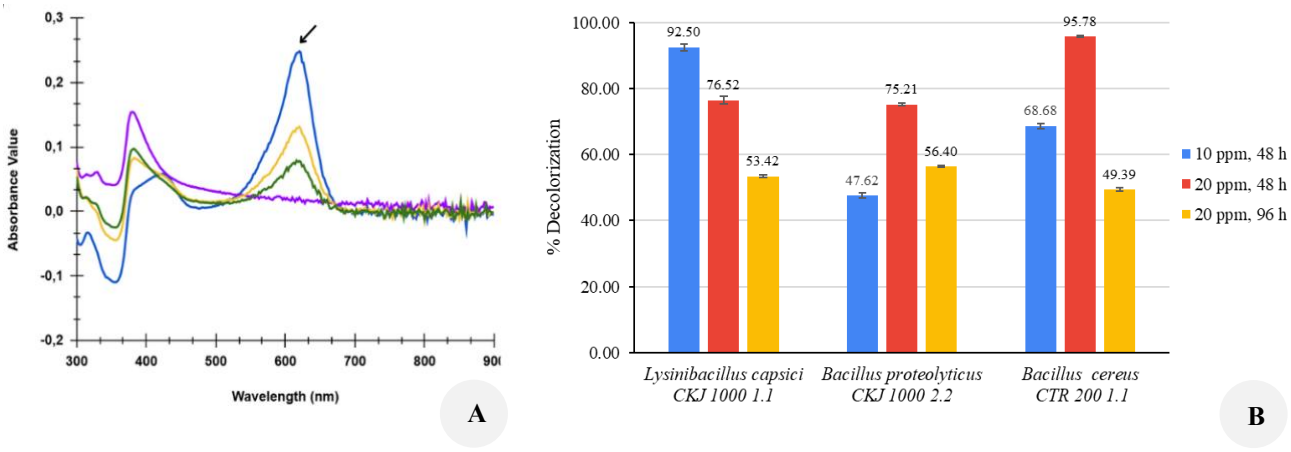

Figure 2. Bacterial cultures grown in LB medium containing 10 ppm Malachite Green (MG), incubated for 48 h, were centrifuged at 5,000 rpm for 10 min to remove bacterial cells, and the optical density of the supernatant was analyzed. A. UV-Vis absorbance spectrum (300-900 nm) of the supernatants. Colors indicate: Control (MG only): Blue, CKJ 1000 2.2: Yellow, CTR 200 1.1: Green, CKJ 1000 1.1: Purple. The arrow marks the characteristic of the MG absorbance peak at 618 nm. B. Percentage decolorization calculated from absorbance at 618 nm, presented as mean±SD of three technical replicates (n = 3). Error bars reflect variation among technical replicates only, no statistical tests were performed

The high decolorization capacity of *L. capsici* CKJ 1000 1.1 is consistent with previous reports on *Lysinibacillus* spp. showing strong MG removal (Miyar et al. 2021; Vijaylakshmi et al. 2023). In contrast, *Bacillus* species generally exhibit moderate removal efficiency (Shah et al. 2013; Kaushik and Seth 2021), consistent with the performance of *B. cereus* CTR 200 1.1 and *B. proteolyticus* CKJ 1000 2.2 in this study. Collectively, these results document strain-dependent functional diversity in MG transformation among indigenous river bacteria and highlight *L. capsici* CKJ 1000 1.1 as a notable local microbial resource with strong dye-transformation capacity under laboratory conditions.

Positioning indigenous river isolates within Indonesian freshwater microbial biodiversity

Although the present study focuses on three bacterial isolates, their relevance to biodiversity-oriented research lies in their status as indigenous microbial resources selectively enriched by chronic chemical stress in polluted freshwater ecosystems. River systems receiving industrial and urban effluents act as ecological filters, shaping distinct microbial assemblages that favor adaptive traits related to heavy-metal tolerance, xenobiotic transformation, and metabolic versatility.

The isolates originate from two major Indonesian river systems (the Cikijing and Citarum Rivers, West Java) that have experienced long-term exposure to textile-associated dyes and metal contaminants. Taxonomically, these isolates belong to the genera *Lysinibacillus* and *Bacillus*, which are frequently reported from Indonesian rivers but still underrepresented at the strain level with publicly available sequence data. The provision of accession numbers in this study contributes to the documented phylogenetic and functional diversity of freshwater bacteria from Indonesia.

To contextualize these isolates within national freshwater microbial biodiversity, Table 3 compares the present strains with previously reported pollutant-tolerant bacteria isolated from Indonesian aquatic environments, including rivers and lakes from West Java, Kalimantan, Sulawesi, Maluku, and Surabaya. The comparison reveals taxonomic recurrence across geographically distinct systems and functional convergence toward pollution-associated adaptive traits, including tolerance to copper or lead, dye decolorization, and the transformation of organic pollutants. Importantly, not all reported isolates share identical resistance profiles, reflecting both local contamination histories and functional diversification among indigenous bacterial communities.

Within this framework, functional traits such as metal tolerance (Cu or Pb) and dye decolorization are interpreted not solely as bioremediation capacities, but as ecological adaptations that contribute to microbial persistence and diversity in contaminated freshwater habitats. Following this biodiversity-based positioning, chemical evidence for malachite green transformation was further examined using LC-HRMS to support functional differentiation at the strain level.

Table 3. Comparative positioning of indigenous pollutant-tolerant bacteria from Indonesian freshwater environments

Bacterial isolate(s)	Source	Reported stress tolerance / functional trait	Reference
Lysinibacillus capsici CKJ 1000 1.1 (accession number: CP185952)	Cikijing River, West Java	Copper tolerance, Malachite Green (MG) decolorization, metabolic transformation products	This study, Irawati et al. (2025)
Bacillus proteolyticus CKJ 1000 2.2	Cikijing River, West Java	Copper tolerance, MG decolorization	This study, Irawati et al. (2025)
Bacillus cereus CTR 200 1.1 (accession number: JBNGBZ01)	Citarum River, West Java	Copper tolerance, MG decolorization	This study, Irawati et al. (2023b)
Serratia nematodiphila Suk13	Sukolilo River, Surabaya	Copper tolerance, multi-dye tolerance and decolorization (MG, MB, CR, MO, RB, DY, BF, RO, DO, RR, WY, WR)	Irawati et al. (2024)
Klebsiella grimontii	Cisadane River, Tangerang	Copper tolerance, decolorization of 12 textile dyes	Irawati et al. (2023a)
Acinetobacter sp. IrC1 (accession number: JX009133)	Wastewater treatment plant, Surabaya	Copper tolerance, multi-dye decolorization	Irawati et al. (2023c)
Enterobacter hormaechei KIMS8, *Enterobacter cloacae* KIMS10	Kapuas River, Kalimantan	Copper tolerance, methylene blue and reactive black decolorization	Irawati et al. (2022b)
Pantoea agglomerans, *Shigella flexneri*	Cisadane River, Tangerang	Copper tolerance, dye tolerance, and decolorization	Irawati et al. (2022c)
Aeromonas sp., *Bacillus* sp., *Pseudomonas* sp.	Way Tomu River, Ambon	Organic-pollutant biodegradation, tolerance to polluted river conditions	Ratu et al. (2023)
Comamonas testosteroni	Tempe Lake, South Sulawesi	Lead (Pb) reduction, heavy-metal tolerance	Yani et al. (2020)

Note: Reported stress tolerance and functional traits are presented as described in the respective studies and may reflect growth tolerance, biosorption, enzymatic decolorization, or metabolic transformation rather than genetically resolved resistance mechanisms

Analysis of malachite green degradation products

To further examine MG biodegradation, metabolic products formed during decolorization by *L. capsici* CKJ 1000 1.1 were analyzed using LC-HRMS. Samples were collected from cultures achieving 92.50% decolorization at 10 ppm MG. The LC-HRMS chromatogram revealed multiple distinct peaks corresponding to putative MG transformation products (Figure 3). Identification of compounds based on LC-HRMS was carried out using observed m/z values, retention times, and comparisons with previously reported MG degradation pathways (Song et al. 2020; Miyar et al. 2021; El-Bendary et al. 2023; Liaqat et al. 2023; Vijaylakshmi et al. 2023). Compound identities were inferred from accurate mass and literature comparison only; neither authentic reference standards nor MS/MS spectral library confirmation was employed. Based on the Metabolomics Standards Initiative (MSI) criteria, the reported features are classified as Level 4 identifications, relying on accurate mass and literature comparison without structural confirmation. Consequently, no toxicological attributes were assigned to the compounds listed in Table 3, and the annotations should be regarded as tentative.

The LC-HRMS chromatogram (Figure 3) revealed several peaks corresponding to MG transformation products, indicating chemical transformation of the parent dye rather than simple adsorption. MG-related ions, including leucomalachite green and demethylated derivatives, were detected at low relative abundances (<0.01% area), consistent with residual parent compound and transient intermediates, and indicating substantial depletion of MG under the tested conditions. A wide range of compound masses was observed, consistent with previously reported MG degradation intermediates. Low-mass ions (m/z 119-132) likely correspond to small aromatic fragments formed via demethylation and chromophore cleavage, while mid-range ions (m/z 268-309) are consistent with partially degraded benzophenone- or carbinol-type structures. A higher-mass ion (m/z 354.9900) may represent a transient oxidized or hydroxylated MG derivative (Song et al. 2020; El-Bendary et al. 2023).

The suspected transformation products are summarized in Table 4. Compounds present at relatively higher abundances (≥0.01% peak area) included benzaldehyde, benzophenone, 4-dimethylaminophenol, aniline, and bis(4-aminophenyl)methanone, suggesting cleavage of the triphenylmethane structure and the formation of lower-molecular-weight aromatic metabolites. In addition, several MG-related intermediates, such as leucomalachite green and desmethylated MG derivatives, along with diaryl ketones and aromatic hydrocarbons, were detected at trace levels (<0.01% peak area), indicating minor or transient transformation pathways.

Collectively, the detection of both relatively abundant aromatic fragments and trace-level MG intermediates supports stepwise MG transformation involving reduction, demethylation, and cleavage of the chromophore, as reported in previous bacterial degradation studies (Miyar et al. 2021; Liaqat et al. 2023). These LC-HRMS findings align with the UV-Vis spectral changes observed earlier and support active structural transformation of MG by *L. capsici* CKJ 1000 1.1 under the conditions tested. However, the precise enzymatic mechanisms cannot be resolved from this study.

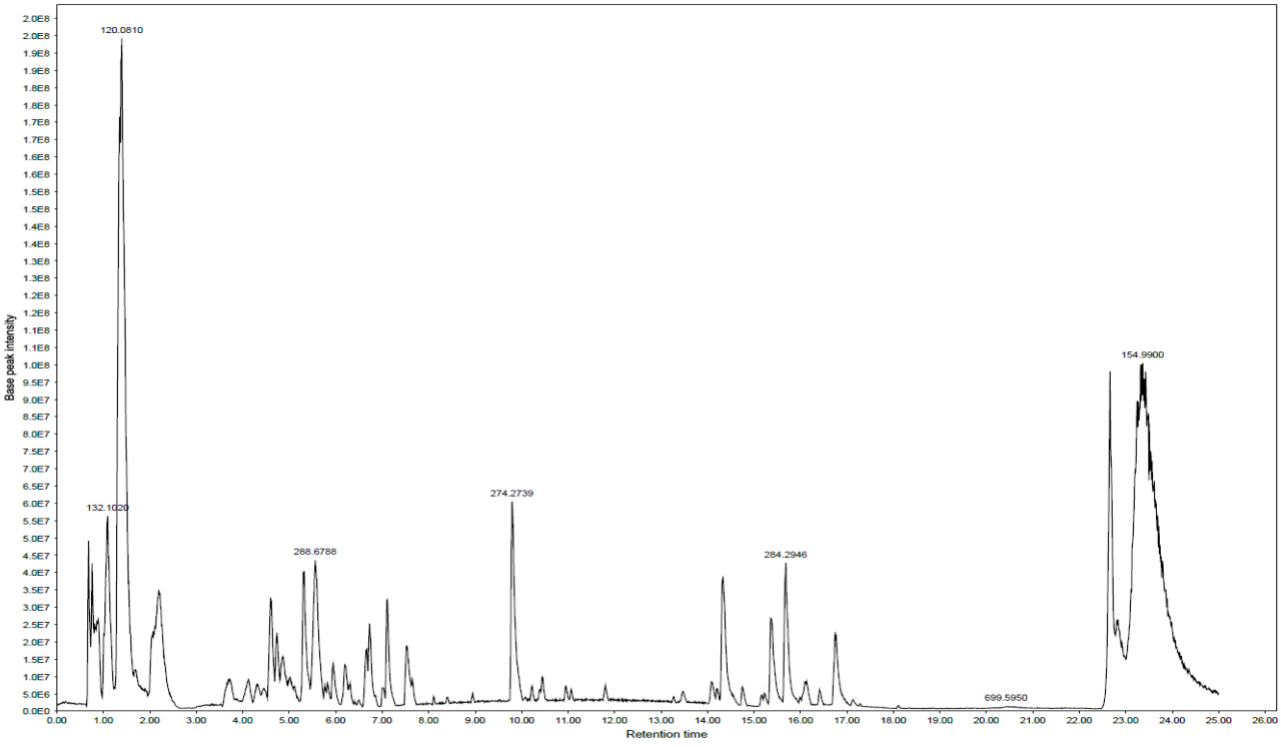

Figure 3. LC-HRMS chromatograms of compounds from Malachite Green (MG) degradation by *Lysinibacillus capsici* CKJ 1000 1.1 after 48 h incubation in LB medium containing 10 ppm MG

Table 4. Mass spectrometry analysis of compounds resulting from malachite green (10 ppm) degradation by *Lysinibacillus capsici* CKJ 1000 1.1 after 48 h of incubation

Compound class	Putative compound	Adduct	Formula	Mass	RT (min)	Area	%Area
Malachite green derivatives	Desmalachite green	[M]+	$C_{22}H_{23}N_2$	315.1854	9.79	74979	0.006*
Benzophenone derivatives	Benzophenone	[M+H]+	$C_{13}H_{10}O$	183.0809	10.88	219243	0.018
	Bis(4-aminophenyl) methanone	[M]+	$C_{13}H_{12}N_2O$	212.0936	13.28	50211	0.004
Aniline derivatives	Aniline	[M+H]+	C_6H_7N	94.0655	15.39	158179	0.013
Aminated phenol	4-dimethylaminophenol	[M+H]+	$C_8H_{11}NO$	138.0915	1.28	203888	0.016
Benzaldehyde derivatives	Benzaldehyde	[M+H]+	C_7H_6O	107.0494	1.39	425719	0.034

Note: *Lysinibacillus capsici* CKJ 1000 1.1 was incubated for 48 hours in LB containing 10 ppm MG. Culture supernatants were analyzed using Liquid Chromatography-High Resolution Mass Spectrometry (LC-HRMS) to determine dye degradation products. The table reports chemical formula, observed mass (Found Mass), retention time (RT, min), chromatographic peak area, and relative peak area (%Area). Only metabolites with %Area ≥0.01% are included. *Detected but below 0.01% area; retained only where structurally relevant to malachite green transformation.

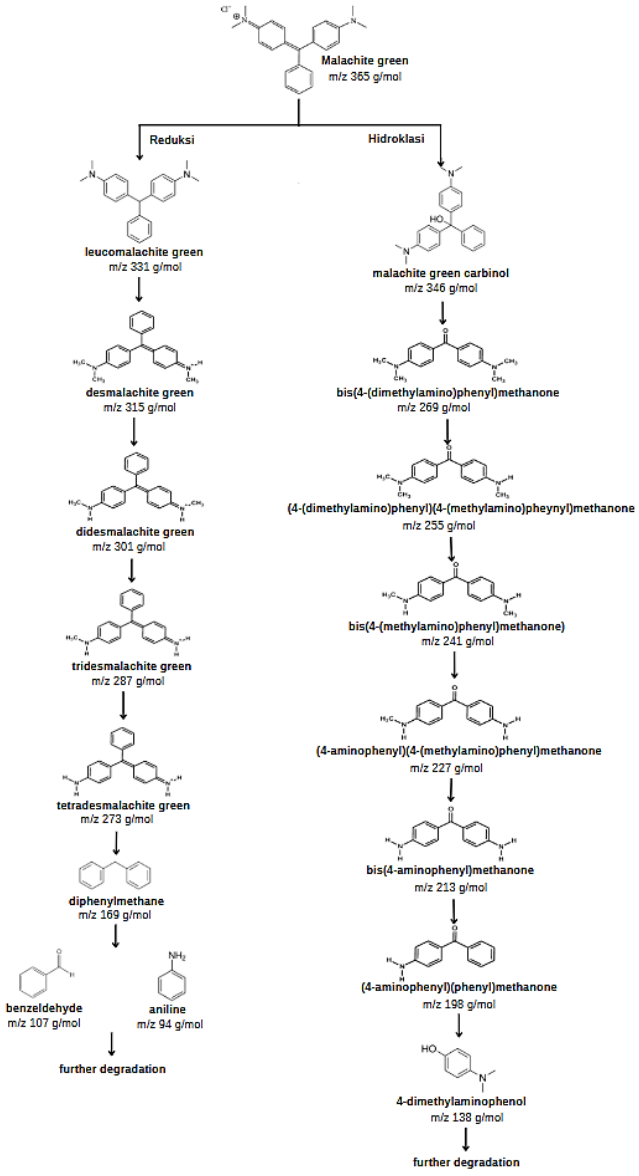

Figure 4. Proposed pathway of malachite green degradation adapted from previously reported pathways (Song et al. 2020; El-Bendary et al. 2023). The pathway is literature-derived and has been modified only to reflect degradation products detected in this study by LC-HRMS, individual transformation steps were not experimentally verified

Figure 4 illustrates a putative degradation pathway constructed based on the detected mass features and comparison with reported pathways. It might involve initial reduction of MG to leucomalachite green, followed by sequential N-demethylation, cleavage of the triphenylmethane backbone, and formation of lower-molecular-weight aromatic compounds (El-Bendary et al. 2023; Bibi et al. 2025). Two major transformation routes—reductive and hydrolytic—appear to operate in parallel, producing benzophenone derivatives, aromatic amines, and phenolic fragments that are more readily mineralized by microbes.

Although several intermediate compounds, including aromatic amines and benzophenone derivatives, are known to possess mutagenic activity or organ-specific toxicities, their reduced structural complexity may enhance susceptibility to further biodegradation (Stammati et al. 2005; Wang et al. 2012; Mohammed et al. 2020; Sharma and Chadha 2023). Additional downstream products, such as phenol and benzaldehyde, may still pose environmental risks (Andersen 2006; Ballantyne et al. 2007; NCBI 2025). Therefore, the metabolic transformation of malachite green does not necessarily imply detoxification. The toxicity of intermediate and downstream products was not evaluated in this study, and additional ecotoxicological assessments (e.g., *Daphnia magna*, Microtox, or fish cell-based assays) are required to confirm whether the treated effluent exhibits reduced environmental risk.

Study limitations and future directions

Despite the promising laboratory results, several limitations should be considered when interpreting this study. All copper biosorption and Malachite Green (MG) decolorization assays were conducted in rich, unbuffered LB medium at 37°C to ensure reproducible bacterial growth. These conditions support experimental consistency; however, they do not fully reflect environmental settings, where temperatures typically range from 25-30°C and pH, ionic strength, and nutrient availability fluctuate considerably. Consequently, the observed performance may differ between natural and engineered treatment systems. Future studies should incorporate environmentally relevant, buffered media, broader temperature and pH ranges, and report physicochemical parameters such as pH, ionic strength, and free Cu^{2+} concentrations.

Copper biosorption was expressed solely as percentage removal. It was not normalized to biomass concentration or Extracellular Polymeric Substance (EPS) content, limiting direct comparison with literature values reported on a mass basis. Biomass-related parameters, including dry weight and EPS yield, were not quantified, and mechanistic interpretations regarding EPS production, metal-resistance gene expression, and copper localization remain inferential. Additionally, the relatively modest Cu removal efficiency observed under the present conditions indicates that substantial optimization—such as adjusting biomass density, pH, contact time, and reactor configuration—would be necessary before any practical application.

The analyses of MG decolorization and LC-HRMS used centrifuged but unfiltered culture supernatants; however, blank corrections were applied; however, residual cell debris or soluble microbial products may have contributed to background absorbance and tentative metabolite assignments. LC-HRMS compound identifications were indicative (MSI Level 4), and no genomic, enzymatic, or ecotoxicological assays were conducted to confirm degradation pathways or environmental safety. As noted in the Analysis of Malachite Green Degradation Products subsection, some MG transformation intermediates may retain residual toxicity, and the safety of resulting metabolites remains uncertain. Furthermore, several experiments relied on technical rather than biological replicates, limiting the robustness and generalizability of the findings.

Overall, future work should focus on using environmentally relevant media, normalizing adsorption metrics to biomass and EPS, employing biological replication, conducting genomic and enzymatic analyses, performing comprehensive metabolite profiling, and including ecotoxicological assessments. These efforts will be essential for translating the demonstrated laboratory potential of the bacterial isolates into reliable, safe, and ecologically meaningful bioremediation applications.

In conclusion, *L. capsici* CKJ 1000 1.1 demonstrated copper tolerance up to 7 mM $CuSO_4$ and achieved the highest Malachite Green (MG) decolorization, reaching 92.50% within two days at 10 ppm, surpassing *B. cereus* (68.68%) and *B. proteolyticus* (47.62%). Cu biosorption efficiencies were 13.33% at 3 mM and 13.71% at 4 mM $CuSO_4$. MG transformation produced multiple low-molecular-weight intermediates, some of which may retain environmental hazards; therefore, transformation does not necessarily imply detoxification. The toxicity of the treated effluent was not assessed, and additional ecotoxicological studies, process optimization, and scaling experiments are required before practical bioremediation application. Beyond functional performance, the three indigenous bacterial isolates from polluted rivers in Indonesia represent accession-verifiable, cultivable microbial resources, providing baseline biodiversity data on native bacteria adapted to chemical stress. By documenting and characterizing these strains, complete with accession information, this study contributes to regional freshwater microbial diversity inventories. It establishes reference points for understanding the distribution, functional potential, and adaptive traits of native bacteria in contaminated aquatic ecosystems. Collectively, these findings provide a reference framework for future ecological, microbiological, and applied studies on microbial diversity, functional traits, and bioremediation potential in co-contaminated freshwater systems.

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