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# Protein Profile and Decolorization Potential of Copper-Resistant *Klebsiella pneumonia* CKJ 500 2.1.2 in Response to Textile Dyes and Copper

**Wahyu Irawati[1*], Reinhard Pinontoan[2], Jessica Marcellie[2], Andreas Valiant[2], Marcelia Sugata[2]**

[1]*Department of Biology, Faculty of Education, Universitas Pelita Harapan. Jl.M.H. Thamrin Boulevard 1100, Lippo Karawaci, Tangerang 15811, Banten, Indonesia*
[2]*Department of Biology, Faculty of Health Sciences, Universitas Pelita Harapan. Jl.M.H. Thamrin Boulevard 1100, Lippo Karawaci, Tangerang 15811, Banten, Indonesia*

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## ABSTRACT

Heavy metals and synthetic dyes are major environmental pollutants, particularly in industrial effluents. The development of effective and safe bioremediation strategies to mitigate their ecological impact is therefore critical. In this study, the copper-resistant bacterium *Klebsiella pneumoniae* CKJ 500 2.1.2 was investigated for its capacity to decolorize textile dyes—specifically malachite green—and its associated enzymatic activities were characterized. Bacterial resistance was assessed using Luria–Bertani agar containing varying concentrations of copper and dyes. Decolorization efficiency was evaluated spectrophotometrically, protein expression was analyzed using SDS-PAGE, and dye degradation products were identified using gas chromatography–mass spectrometry (GC-MS). The strain exhibited high tolerance to both copper and dyes, achieving 99.4% decolorization of malachite green and 81% for Congo red. The presence of copper inhibited the decolorization of most dyes, except malachite green and methylene blue. SDS-PAGE analysis identified three key enzymes: laccase (~60 kDa), manganese peroxidase (~39 kDa), and azoreductase (~22 kDa). GC-MS revealed both toxic and non-toxic degradation intermediates, indicating partial detoxification. These findings highlight the potential of *K. pneumoniae* CKJ 500 2.1.2 for bioremediation of dye-contaminated effluents. However, further research is required to elucidate the complete enzymatic pathways involved and to ensure environmentally safe dye degradation.

## 1. Introduction

The textile industry in Indonesia grew by 15.08% in 2019 (Ministry of Industry 2019), but this expansion has not been matched by proper waste management (Sutrisno 2022), leading to significant environmental pollution. Rivers such as the Cikijing in Bandung, the Citarum in West Java, and the Sumbernangka in East Java receive large volumes of untreated wastewater from textile activities, including dyeing, printing, and garment manufacturing (Irawati *et al.* 2025). The Citarum River, considered among the world's most polluted, is heavily contaminated with hazardous chemicals, dyes, and metals. Similarly, pollution in the Sumbernangka River has disrupted aquatic ecosystems (Pratiwi 2021). These cases underline the urgent need for better wastewater treatment and environmental monitoring.

Textile industry waste is broadly classified into liquid and solid waste. Liquid effluents from dyeing and finishing often contain heavy metals like lead, copper, and chromium. Solid waste includes fibers, sludge, and residual chemicals. Discharge of these untreated wastes poses serious risks to water quality. For instance, the Cikijing River showed high levels of Total Dissolved Solids (1597.33 mg/L) and Suspended Solids (97.40 mg/L), a low pH, and high conductivity (Putri & Afdal

*Corresponding Author
E-mail Address: wahyu.irawati@uph.edu

2023). While metal levels met legal limits, long-term accumulation may still harm ecosystems and human health.

Common textile dyes—Reactive Orange 16, Congo red, Reactive Black, Direct Red, methylene blue, and malachite green—are persistent and often toxic. These dyes can interact with copper via catalysis or complexation, affecting their degradation. For instance, copper oxide and $H_2O_2$ degrade Reactive Orange 16 (Abdullah & Wong 2010), and Ir–Cu nanoparticles degrade Congo red (Pooja & Goel 2021). Copper complexation can also reduce the color intensity of dyes (Giraud *et al.* 2018). Although less studied, dyes such as methylene blue and malachite green may exhibit interactions with metals similar to those of other dyes. These dyes pose health and ecological risks. Malachite green causes anemia in aquatic species (Jefri *et al.* 2024); methylene blue inhibits photosynthesis and has carcinogenic potential (Khan *et al.* 2022); and Congo red requires advanced treatments due to its stability (Utami *et al.* 2023). Limited local data on Reactive Orange 16, Reactive Black, and Direct Red suggest high resistance to degradation, underscoring the need for more sustainable treatment methods.

Bioremediation offers an eco-friendly, cost-effective approach to treating textile waste. Microorganisms adapted to metal-contaminated environments can degrade dyes via bioaccumulation and biosorption (Adolf *et al.* 2020). *Klebsiella pneumoniae* has shown dual resistance to synthetic dyes and heavy metals, making it a promising candidate for bioremediation (Sennaj *et al.* 2023). This bacterium produces enzymes like laccase, azoreductase, and manganese peroxidase, which break down dye structures (Khandare & Govindwar 2016). For example, *Klebsiella pneumoniae* MW815592 fully decolorized Reactive Yellow 145 at 200 ppm in 38 hours (Sennaj *et al.* 2023). Copper-resistant strains also express proteins such as CopA and CopB to survive under metal stress (Irawati *et al.* 2021).

This study investigates *Klebsiella pneumoniae* CKJ 500 2.1.2, isolated from the polluted Cikijing River, to assess its ability to decolorize various textile dyes in the presence of copper. Specifically, the research evaluates its resistance to both synthetic dyes and copper, determines decolorization efficiency, and analyzes its protein expression under pollutant exposure.

## 2. Materials and Methods

### 2.1. Resistance of *Klebsiella pneumoniae* CKJ 500 2.1.2 to Textile Dyes

Six textile dyes—Reactive Orange 16, Congo red, Reactive Black 5, Direct Red 80, methylene blue, and malachite green—were prepared at a stock concentration of 10,000 ppm for the resistance assay. The streak plate method was employed to assess dye tolerance. A single loopful of *Klebsiella pneumoniae* CKJ 500 2.1.2 was streaked onto sterile Luria–Bertani (LB) agar plates supplemented with each dye at a predetermined concentration. The inoculated plates were incubated at 37°C for 24 hours, after which bacterial growth was evaluated to determine resistance (Irawati *et al.* 2022).

### 2.2. Decolorization Assay of *Klebsiella pneumoniae* CKJ 500 2.1.2

*Klebsiella pneumoniae* CKJ 500 2.1.2 was cultivated in LB broth medium and incubated at 37°C in a shaker incubator (200 rpm) to obtain the starter culture. Bacterial growth was monitored spectrophotometrically at 600 nm until the optical density ($OD_{600}$) reached 0.8, using LB broth as the blank. A 1% (v/v) inoculum of this culture was added to 50 mL of LB broth supplemented with 100 ppm of each dye, with or without 0.1 mM $CuSO_4$. Cultures were incubated at 37°C in a shaker incubator at 200 rpm for 24 hours (Irawati *et al.* 2022). Following incubation, 1 mL of culture was centrifuged at 8,000 rpm for 5 minutes. The supernatant was collected, and its absorbance was measured at 300–900 nm using a spectrophotometer. LB broth served as the blank, and dye-only controls (without bacteria) were used for comparison. The percentage of dye decolorization was calculated using the following formula (Irawati *et al.* 2022):

$$\% \text{ decolorization} = \frac{\text{control absorbance} - \text{decolorization absorbance}}{\text{control absorbance}} \times 100\%$$

### 2.3. Protein Profiling of *Klebsiella pneumoniae* CKJ 500 2.1.2

A 1 mL sample was centrifuged at 8,000 rpm for 5 minutes. The supernatant was mixed with cold acetone (1:3 v/v) and incubated at –20°C overnight for protein precipitation. The mixture was centrifuged at 13,000

rpm for 10 minutes. The protein pellet was air-dried at room temperature for 30 minutes and resuspended in phosphate buffer. Proteins were separated by SDS-PAGE using a 12% resolving gel prepared from 13.75 mL of 1.5 M Tris-Cl (pH 8.8), 150 µL 10% SDS, 5.03 mL distilled water, and 6 mL 30% acrylamide. After degassing for 15 minutes, 75 µL 10% APS and 7.5 µL TEMED were added. The 4% stacking gel contained 10.98 mL 30% acrylamide, 3.78 mL 0.5 M Tris-HCl (pH 6.8), 9 mL distilled water, and 150 µL 10% SDS. After adding 1 mL of isopropanol to remove bubbles, the gel was polymerized for 15 minutes, then 75 µL of 10% APS and 7.5 µL of TEMED were added (Irawati *et al.* 2022). Electrophoresis was run at 50 V in 1× running buffer using a Bio-Rad system (Bio-Rad 2014).

### 2.4. Dye Degradation Analysis of *Klebsiella pneumoniae* CKJ 500 2.1.2

Five millilitres of supernatant from selected dye decolorization was extracted with 5 mL ethyl acetate and vortexed. The organic phase was separated, dried by rotary evaporation, and residual moisture removed with anhydrous sodium sulphate ($NaSO_4$). The dried extract was reconstituted in methanol for GC-MS analysis. The GC-MS used a capillary column with helium as the carrier gas at a constant flow rate of 1.0 mL/min, injected in splitless mode with an ionization energy of 70 eV. The oven was held at 80°C for 10 minutes, ramped to 280°C at 10°C/min, and held for 7 minutes. Metabolites were identified by comparing retention times and mass spectra with databases.

## 3. Results

### 3.1. Resistance of *Klebsiella pneumoniae* CKJ 500 2.1.2 to Textile Dyes

Resistance testing was performed by incorporating selected textile dyes into sterilized Luria-Bertani (LB) agar at defined concentrations. The agar plates were then inoculated with *Klebsiella pneumoniae* CKJ 500 2.1.2 using the streak plate method to assess bacterial growth in the presence of each dye. The results of the resistance tests for *Klebsiella pneumoniae* CKJ 500 2.1.2 against textile dyes on agar plates are presented in Figures 1 and 2.

Figure 1 illustrates bacterial growth on media supplemented with Reactive Orange 16, Congo red, and Direct Red 80 dyes. In contrast, Figure 2 shows growth on media containing the dyes Reactive Black, methylene blue, and malachite green. In each case,

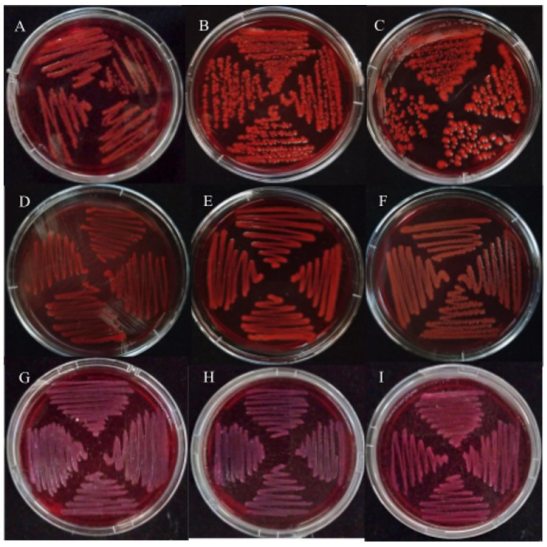



Figure 1. Growth of *Klebsiella pneumoniae* CKJ 500 2.1.2 on LB agar supplemented with Reactive Orange 16, Congo red, and Direct Red 80 dyes. (A-C) Bacterial growth on LB medium with reactive orange 16 at concentrations of (A) 4,000 ppm, (B) 5,000 ppm, and (C) 6,000 ppm. (D-F) Bacterial growth on LB medium with Congo red at concentrations of (D) 1,000 ppm, (E) 2,000 ppm, and (F) 3,000 ppm. (G-I) Bacterial growth on LB medium with Direct Red 80 at concentrations of (G) 1,000 ppm, (H) 2,000 ppm, and (I) 3,000 ppm. Each treatment was performed in quadruplicate and incubated for 24 hours at 37°C

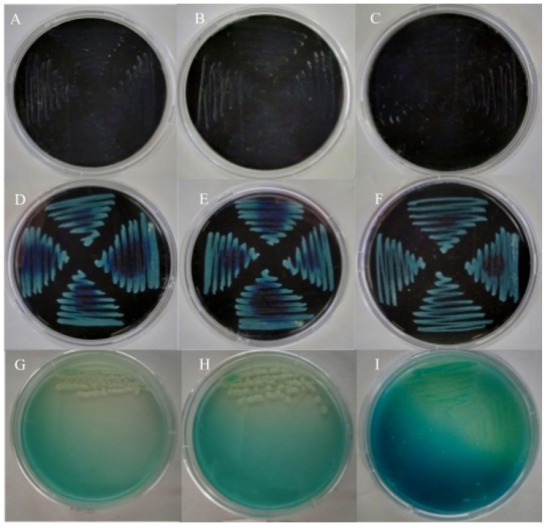



Figure 2. Growth of *Klebsiella pneumoniae* CKJ 500 2.1.2 on LB agar supplemented with Reactive Black 5, methylene blue, and malachite green dyes. (A-C) Bacterial growth on LB medium with Reactive Black 5 at concentrations of (A) 8,000 ppm, (B) 9,000 ppm, and (C) 10,000 ppm. (D-F) Bacterial growth on LB medium with methylene blue at concentrations of (D) 6,000 ppm, (E) 7,000 ppm, and (F) 8,000 ppm. (G-I) Bacterial growth on LB medium with malachite green at concentrations of (G) 700 ppm, (H) 800 ppm, and (I) 1,000 ppm. Each treatment was conducted in quadruplicate and incubated for 24 hours at 37°C

the bacteria were cultured on LB agar plates with increasing concentrations of the dye to assess their maximum tolerance. *Klebsiella pneumoniae* CKJ 500 2.1.2 exhibited robust growth on Congo red and Direct Red 80 at 1,000–3,000 ppm and tolerated Reactive Black 5 and methylene blue up to 10,000 ppm without inhibition. In contrast, growth was inhibited by Reactive Orange 16 and malachite green, though a clear zone of malachite green decolorization was observed on day 1. Table 1 summarizes the growth response of *Klebsiella pneumoniae* CKJ 500 2.1.2 to different concentrations of various textile dyes.

### 3.2. Decolorization of Dyes by *Klebsiella pneumoniae* CKJ 500 2.1.2

*Klebsiella pneumoniae* CKJ 500 2.1.2 decolorized 29.3% of Reactive Orange 16 within the first day of incubation (Figure 3), with no significant

Table 1. Growth of *Klebsiella pneumoniae* CKJ 500 2.1.2 to varying concentrations of different textile dyes

| Type of textile dye | Dye concentration (ppm) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1,000 | 2,000 | 3,000 | 4,000 | 5,000 | 6,000 | 7,000 | 8,000 | 9,000 | 10,000 |
| Reactive Orange 16 | v | v | v | v | v | v | - | - | - | - |
| Congo red | v | v | v | - | - | - | - | - | - | - |
| Reactive Black 5 | v | v | v | v | v | v | v | v | v | v |
| Direct Red 80 | v | v | v | - | - | - | - | - | - | - |
| Methylene blue | v | v | v | v | v | v | v | v | - | - |
| Malachite green | v | x | x | x | x | x | x | x | x | x |

v: bacterial growth observed, x: no bacterial growth, –: treatment not tested. The resistance test was conducted using the streak plate method on LB agar medium supplemented with various concentrations and types of textile dyes. Each treatment was performed in quadruplicate

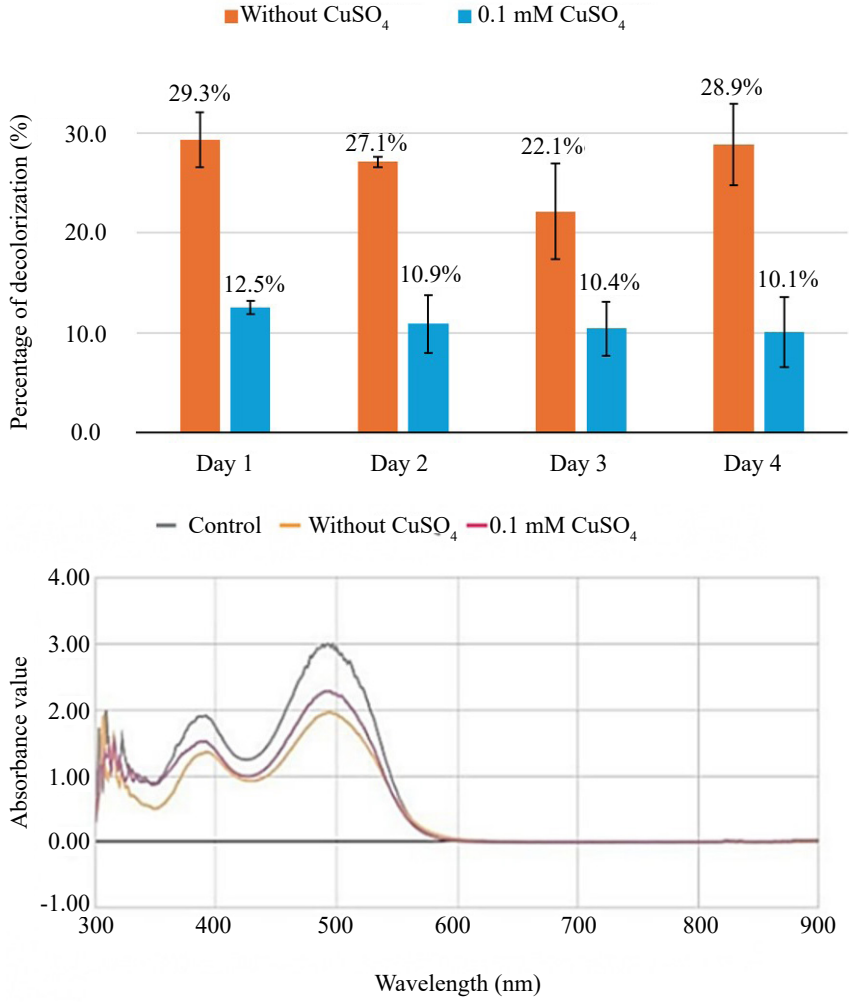



Figure 3. Spectrophotometric analysis of the decolorization potential of *Klebsiella pneumoniae* CKJ 500 2.1.2 on Reactive Orange 16 dye over time: (top) percentage of decolorization and (bottom) absorbance spectra at different wavelengths on the first day

increase over the following three days. To assess the effect of copper sulphate ($CuSO_4$), an additional experiment was conducted. In the presence of copper, decolorization decreased to 12.5%, likely due to copper's inhibitory effect on bacterial growth rather than its role as an enzymatic cofactor. For Congo red, *Klebsiella pneumoniae* CKJ 500 2.1.2 achieved 20.3% decolorization by day two in LB medium without copper, increasing to 72.9% by day three (Figure 4). With copper, decolorization was only 14.1% by day three, rising to 80.9% by day four. Despite the initial delay, the final decolorization levels were comparable in the presence (80.9%) and absence (81%) of copper.

*Klebsiella pneumoniae* CKJ 500 2.1.2 achieved a maximum decolorization of 27.6% for Reactive Black 5, which was reduced to 18.2% with the addition of copper (Figure 5). Decolorization decreased on day two but increased again by days three and four. The dye's initial absorbance at 597 nm was 3.19, which decreased to 2.31 in the absence of copper and to 2.61 in its presence, corresponding to 27.6% and 18.2% decolorization, respectively. For Direct Red 80, the strain showed low decolorization—10.7% without copper and 10.4% with 0.1 mM copper (Figure 6)—with no notable changes across the incubation period. Compared to other dyes, decolorization of Direct Red 80 remained minimal.

*Klebsiella pneumoniae* CKJ 500 2.1.2 decolorized methylene blue by 26.6% without copper, and by 51.1% with 0.1 mM copper (Figure 7), indicating enhanced decolorization in the presence of copper. No significant changes were observed over days two

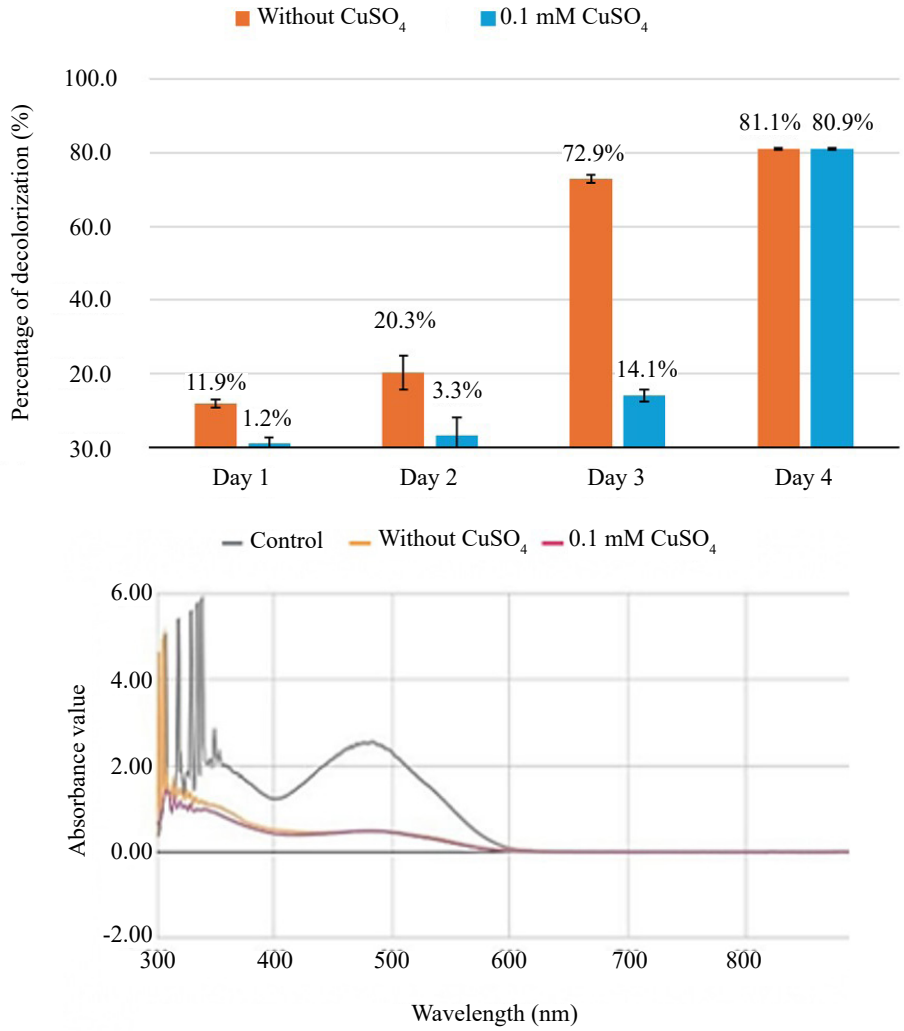



Figure 4. Spectrophotometric analysis of the decolorization potential of *Klebsiella pneumoniae* CKJ 500 2.1.2 on Congo red dye over time: (top) percentage of decolorization and (bottom) absorbance spectra at different wavelengths on the fourth day

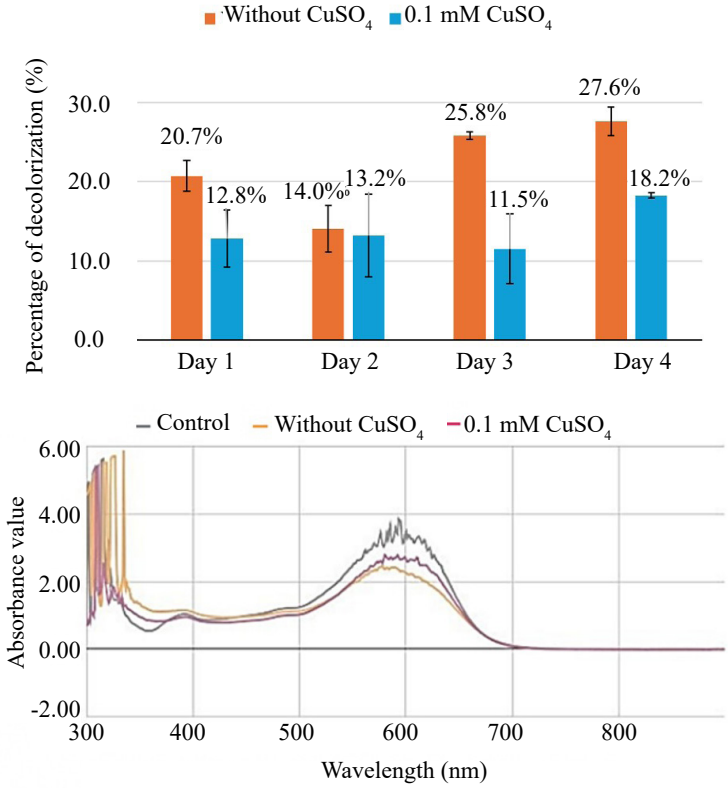



Figure 5. Spectrophotometric analysis of the decolorization potential of *Klebsiella pneumoniae* CKJ 500 2.1.2 on Reactive Black 5 dye over time: (top) percentage of decolorization and (bottom) absorbance spectra at different wavelengths on the fourth day

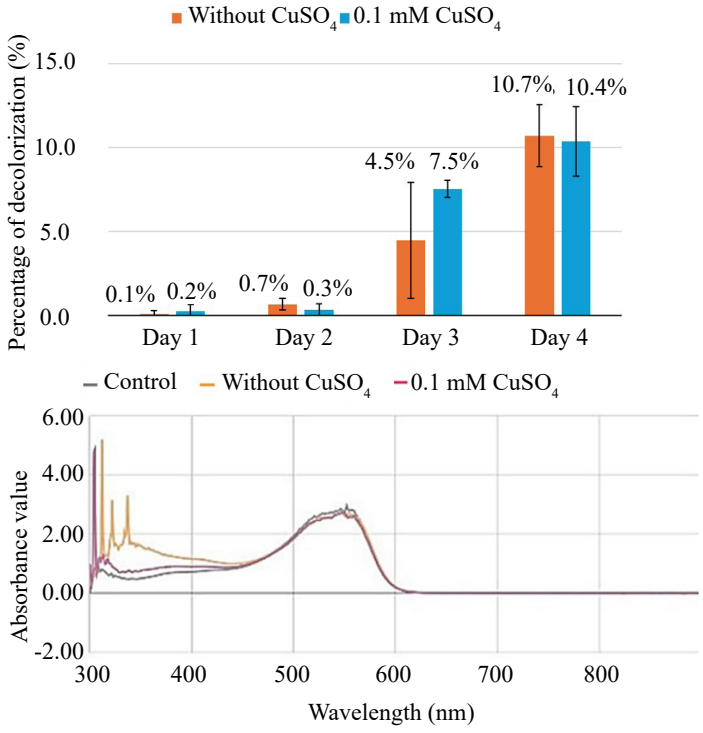



Figure 6. Spectrophotometric analysis of the decolorization potential of *Klebsiella pneumoniae* CKJ 500 2.1.2 on Direct Red 80 dye over time: (top) percentage of decolorization and (bottom) absorbance spectra at different wavelengths on the fourth day

to four. For malachite green, the strain showed rapid and efficient decolorization, reaching 97.5% without copper and 90% with copper on the first day (Figure 8). Decolorization remained consistently high throughout the incubation. By day four, maximum decolorization was observed—99.4% with copper and 99.3% without. Although copper initially reduced decolorization, a

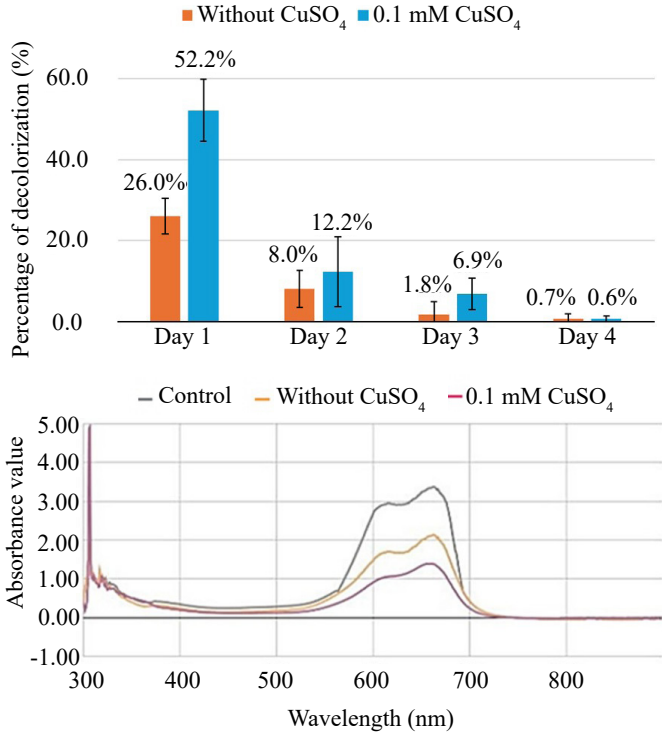



Figure 7. Spectrophotometric analysis of the decolorization potential of *Klebsiella pneumoniae* CKJ 500 2.1.2 on methylene blue dye over time: (top) percentage of decolorization and (bottom) absorbance spectra at different wavelengths on the first day

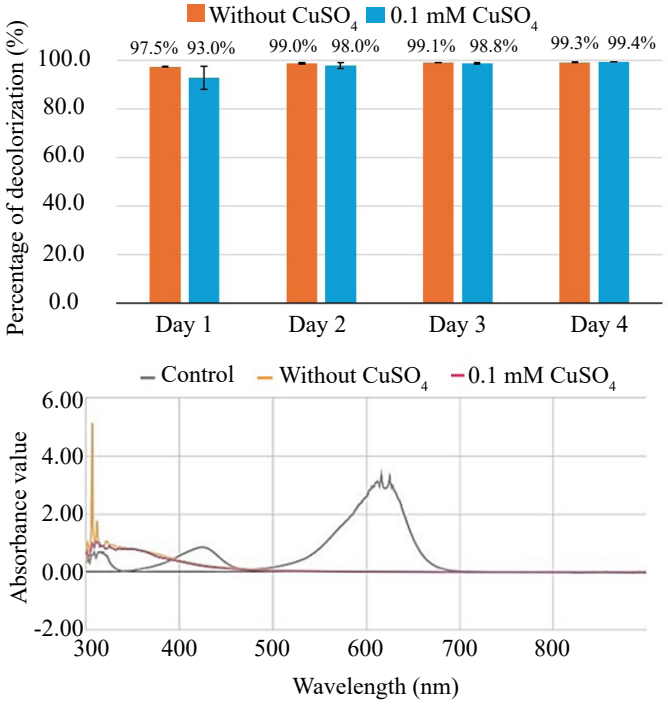



Figure 8. Spectrophotometric analysis of the decolorization potential of *Klebsiella pneumoniae* CKJ 500 2.1.2 on malachite green dye over time: (top) percentage of decolorization and (bottom) absorbance spectra at different wavelengths on the fourth day

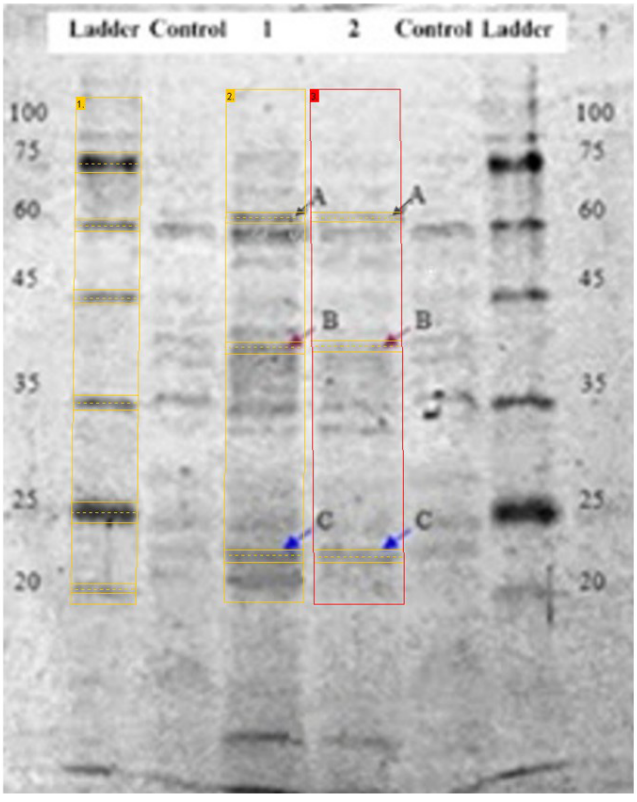
gradual increase over time suggests adaptive enzymatic activity.

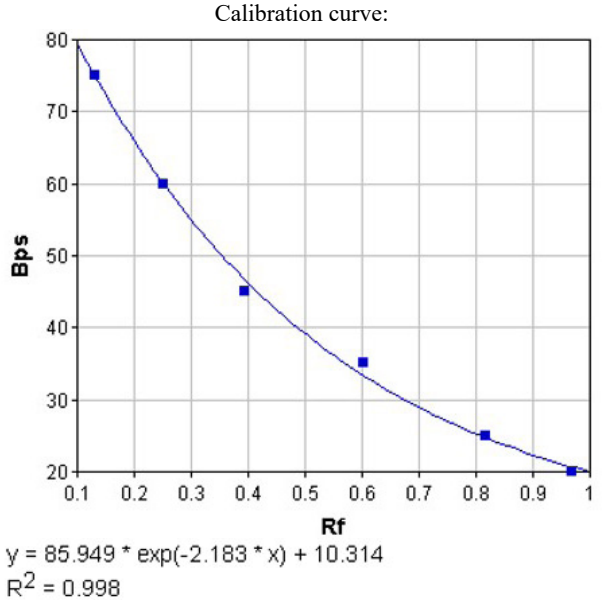
### 3.3. Effect of Malachite Green on Protein Profile of *Klebsiella pneumoniae* CKJ 500 2.1.2

SDS-PAGE analysis revealed three protein bands in samples treated with malachite green: Band A (approximately 60 kDa), Band B (39 kDa), and Band C (22 kDa) (Figure 9). These bands were prominent in samples exposed to malachite green but appeared faint under control conditions containing only $CuSO_4$ (Table 2).

### 3.4. Malachite Green Degradation by *Klebsiella pneumoniae* CKJ 500 2.1.2

Gas Chromatography–Mass Spectrometry (GC-MS) analysis was conducted by BMT Indonesia on supernatant samples from *Klebsiella pneumoniae* CKJ 500 2.1.2 cultures treated with 100 ppm malachite green dye. Figure 10 shows the GC-MS chromatogram




Ladder:

| Band # | Peak Rf | Peak value | Raw volume | Cal. volume | MW |
|---|---|---|---|---|---|
| 1 | 0.132 | 71 | 1672 | 1672 | 75 |
| 2 | 0.253 | 139 | 1635 | 1635 | 60 |
| 3 | 0.396 | 149 | 1868 | 1868 | 45 |
| 4 | 0.603 | 129 | 1920 | 1920 | 35 |
| 5 | 0.818 | 69 | 1585 | 1585 | 25 |
| 6 | 0.968 | 161 | 1333 | 1333 | 20 |

Lane 1

| Band # | Peak Rf | Peak value | Raw volume | Cal. volume | MW |
|---|---|---|---|---|---|
| A | 0.25 | 151 | 1438 | - | 60 |
| B | 0.503 | 149 | 1565 | - | 39 |
| C | 0.907 | 126 | 1329 | - | 22 |

Lane 2

| Band # | Peak Rf | Peak value | Raw volume | Cal. volume | MW |
|---|---|---|---|---|---|
| A | 0.249 | 185 | 1501 | - | 60 |
| B | 0.498 | 177 | 1659 | - | 39 |
| C | 0.905 | 156 | 1603 | - | 22 |

Figure 9. SDS-PAGE analysis of the supernatant protein profile of *Klebsiella pneumoniae* CKJ 500 2.1.2 with and without malachite green treatment. The analysis was performed using gel analyzer software

Table 2. Molecular weights and putative identities of protein bands in *Klebsiella pneumoniae* CKJ 500 2.1.2 with and without malachite green treatment

| Sample | Weight (kDa) | Suspected protein | Control | Suspected protein | |
|---|---|---|---|---|---|
| | | | | (-) $CuSO_4$ | (-) $CuSO_4$ |
| A | 60 | Laccase (multicopper oxidases) | Present | Thickened* | Thickened |
| B | 39 | Tyrosinase | Very thin | Thickened* | Thickened |
| C | 22 | Azoreductase | Present | Thickened* | Thickened |

Asterisks (*) indicate significantly increased (very thick) or decreased (very thin) protein band intensity. Protein profiles of the bacterial supernatant treated with malachite green were compared with the control ($CuSO_4$ only), highlighting differential protein expression in response to the dye.

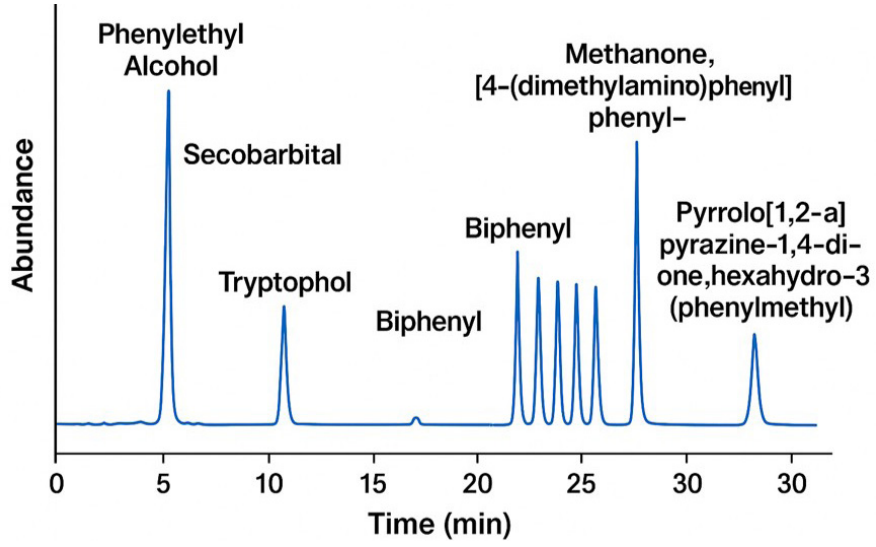



Figure 10. GC-MS analysis of degradation products from *Klebsiella pneumoniae* CKJ 500 2.1.2 cultures treated with 100 ppm malachite green

of degraded malachite green, revealing the formation of multiple degradation products that reflect complex chemical transformation pathways. Among the identified compounds are phenylethyl alcohol, secobarbital, tryptophol, biphenyl (with multiple peaks), [4-(dimethylamino)phenyl]phenyl-methanone, and pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl). These compounds indicate that malachite green undergoes both oxidative and reductive cleavage of its aromatic rings and side chains.

## 4. Discussion

In the present study, varying dye concentrations were used to determine the survival capabilities of *Klebsiella pneumoniae* CKJ 500 2.1.2. Bacteria isolated from contaminated environments often exhibit adaptive genetic changes that enhance survival under toxic conditions (Adolf *et al.* 2020). For instance, Irawati *et al.* (2023) demonstrated variability in methylene blue tolerance among *Escherichia coli* strains isolated from the Citarum River: *E. coli* CTR1000.1.1 tolerated concentrations up to 500 ppm, while *E. coli* CTR10003.1 was inhibited at 200 ppm. This highlights that even strains of the same species can differ in their resistance to environmental pollutants.

*Klebsiella pneumoniae* CKJ 500 2.1.2 growth was unaffected by Congo red, Direct Red 80, Reactive Black 5, and methylene blue, but was significantly inhibited by malachite green and Reactive Orange 16, likely due to their toxicity or structural complexity. Early malachite green decolorization (Figure 2) suggests enzymatic degradation, consistent with Li *et al.* (2019), who reported similar activity in *Klebsiella pneumoniae* WA-1. Moderate decolorization of Reactive Orange 16 (Figure 3), a mono-azo dye, by *Klebsiella pneumoniae* CKJ 500 2.1.2 aligns with degradation reported in related *Klebsiella* strains (Meerbergen *et al.* 2018). However, copper ($CuSO_4$) significantly reduced decolorization efficiency, likely by inhibiting bacterial growth rather than acting as a cofactor, illustrating its dual role as both an enzyme cofactor and a potential toxin.

During initial exposure to Congo red without copper, *Klebsiella pneumoniae* CKJ 500 2.1.2 showed low decolorization by day two (Figure 4), suggesting an adaptation phase involving metabolic and genetic adjustments (Dixit & Garg 2020). A sharp increase on day three indicated successful activation of dye-degradation pathways. In contrast, copper exposure extended the adaptation phase, with only 14.1% decolorization observed by day three. By day four, however, decolorization increased sharply to 80.9%, suggesting delayed enzyme synthesis. The duration of the adaptation phase can vary depending on environmental stressors such as heavy metals, which may inhibit or delay bacterial metabolic responses (Jay *et al.* 2005).

*Klebsiella pneumoniae* CKJ 500 2.1.2 showed limited ability to decolorize Reactive Black 5, with efficiency further reduced by copper exposure (Figure 5). This decline is likely due to the toxic effects of

heavy metals, which disrupt metabolism and enzyme activity (Giachino & Waldron 2020). Under metal stress, bacteria prioritize survival over the production of non-essential enzymes, such as those involved in dye degradation. The observed temporal pattern suggests enzyme involvement: decreased activity on day two may reflect inhibition. In contrast, increased decolorization on days three and four indicates inducible enzyme synthesis in response to prolonged dye exposure (Berg *et al.* 2015). Overall, the modest decolorization—especially under copper stress—highlights the challenges of degrading complex dyes in metal-contaminated environments.

Decolorization of Direct Red 80 showed no significant change (Figure 6), likely due to its high poly-azo content, including four azo bonds (NCBI 2023), which are more resistant to microbial degradation. In contrast, *Klebsiella pneumoniae* CKJ 500 2.1.2 demonstrated enhanced decolorization of methylene blue in the presence of 0.1 mM copper, nearly doubling efficiency compared to the no-copper condition (Figure 7). This suggests a copper-dependent enzymatic mechanism, consistent with copper's known role as both an inducer and cofactor for laccase enzymes (Duran-Sequeda *et al.* 2022). However, no further increase in decolorization was observed over 4 days, suggesting an early plateau, potentially due to the accumulation of intermediate products that may repolymerize or react with free radicals, thereby affecting final absorbance readings. This highlights the complexity of interpreting decolorization solely from absorbance measurements, and further biochemical analyses are needed to confirm enzyme pathways and the stability of intermediates.

*Klebsiella pneumoniae* CKJ 500 2.1.2 also exhibited rapid and efficient decolorization of malachite green, degrading nearly all of the dye within 24 hours, regardless of the presence of copper (Figure 8). This performance parallels that of *Klebsiella pneumoniae* WA-1, known for complete malachite green degradation (Li *et al.* 2019). As a simpler dye with a single azo bond, malachite green is generally easier to degrade, though its stability varies with substituents. These results suggest that *Klebsiella pneumoniae* CKJ 500 2.1.2 possesses robust enzymatic mechanisms for azo dye degradation, consistent with previous observations in related strains such as ST16.16/034 (Meerbergen *et al.* 2018).

Although copper slightly reduced malachite green decolorization on day one—likely due to an extended bacterial adaptation phase—it ultimately enhanced degradation, possibly by acting as a cofactor for laccase enzymes (Rolfe *et al.* 2011; Duran-Sequeda *et al.* 2022). In contrast, copper inhibited the decolorization of Reactive Orange 16, suggesting a stress response rather than enzymatic activation. These findings indicate that copper's impact on dye degradation depends on dye structure, enzyme specificity, and metal concentration. Klebsiella pneumoniae produces extracellular enzymes, such as laccase and azoreductase, which are key to the degradation of synthetic dyes (Dixit & Garg 2020). In this study, SDS-PAGE analysis revealed distinct protein bands potentially linked to dye degradation. Band A (~60 kDa) likely corresponds to laccase, consistent with reported bacterial laccases ranging from 55 to 66 kDa (Reiss *et al.* 2011; Rani *et al.* 2022). Band B (~39 kDa), prominent in malachite green-treated samples but faint in $CuSO_4$ controls, likely represents tyrosinase, a copper-dependent oxidase induced by dye exposure (Ahmed *et al.* 2021). Band C (~22 kDa) matches the molecular weight of azoreductase, consistent with the AzK gene product in *Klebsiella pneumoniae* (Mustafa *et al.* 2023), and showed increased intensity in treated samples, supporting its role in azo bond cleavage. The elevated presence of Bands A, B, and C in malachite green-treated samples suggests enzyme induction as part of an adaptive response, enabling the bacterium to degrade toxic dyes. These findings underscore inducible enzyme expression as a key mechanism in bacterial dye biodegradation and support the bioremediation potential of *Klebsiella pneumoniae* CKJ 500 2.1.2.

GC-MS analysis revealed several byproducts of malachite green degradation by *Klebsiella pneumoniae* CKJ 500 2.1.2, indicating diverse chemical transformations. Phenylethyl alcohol suggests oxidation of ethyl-substituted aromatics, while tryptophol and secobarbital point to deeper enzymatic or oxidative degradation (Xu & Tong 2017). Biphenyl indicates cleavage of the dye's central aromatic structure, a key detoxification step (Gajendiran *et al.* 2024). The detection of [4-(dimethylamino)phenyl] phenylmethanone, structurally related to aromatic amines, suggests incomplete mineralization and the potential persistence of bioactive intermediates (Song *et al.* 2020). These findings highlight the need to assess the ecological impact of degradation byproducts, some of which may retain biological activity or pose environmental risks if not fully mineralized.

In conclusion, *Klebsiella pneumoniae* CKJ 500 2.1.2 exhibited strong tolerance to textile dyes and copper, surviving up to 10,000 ppm Reactive Black

5, 8,000 ppm methylene blue, and 8 mM copper. It showed high decolorization efficiency, particularly for malachite green (96% in 24 h at 100 ppm), and also decolorized Congo red (83.22%) and Reactive Black 5 (33.23%) over four days. GC-MS analysis revealed both toxic and non-toxic degradation products, indicating partial detoxification. While the strain shows strong bioremediation potential, further studies are needed to evaluate the environmental impact of residual byproducts.

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## References

Abdullah, R., Wong, L.S., 2010. Decolorization of reactive orange 16 by $CuO/H_2O_2$ system. *Sains Malays.* 39, 587-591.

Adolf, W., Smith, J., Lee, M., 2020. Bioremediation of heavy metals: mechanisms and applications. *Environ. Microbiol. J.* 15, 123-135. https://doi.org/10.1016/emj.2020.04.005

Ahmed, A., Gill, K.R., Khan, H.A., Aman, S., Qureshi, J.A., Mehmood, R., 2021. Pharmacological and toxicological appraisal of promising enzyme-tyrosinase isolated from soil bacteria-an update. *J. Pharm. Res. Int.* 33, 273-288. https://doi.org/10.9734/jpri/2021/v33i28A31534

Berg, J.M., Tymoczko, J.L., Gatto, G.J., 2015. *Biokimia Stryer*, eighth ed. WH Freeman & Company, New York.

Bio-Rad Protocol 2014. Available at: https://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6201.pdf. [Date accessed: 7 July 2022]

Dixit, S., Garg, S., 2020. Biodegradation of environmentally hazardous azo dyes and aromatic amines using *Klebsiella pneumoniae. J. Environ. Eng.* 144, 7870.0001353. https://doi.org/10.1061/(ASCE)EE.1943-7870.0001353

Duran-Sequeda, D., Suspes, D., Maestre, E., Alfaro, M., Perez, G., Ramírez, L., Pisabarro, A.G., Sierra, R. 2022. Effect of nutritional factors and copper on regulation of laccase enzyme in *Pleurotus ostreatus. J. Fungi* 8, 7. https://doi.org/10.3390/jof8010007

Gajendiran, B., Abraham, J., Mishra, P., 2024. Biodegradation of methyl orange by Streptomyces albidoflavus with GC-MS identification of intermediates. *BMC Microbiol.* 24, 231.

Giachino, A., Waldron, K.J., 2020. Copper tolerance in bacteria requires the activation of multiple accessory pathways. *Mol. Microbiol.* 114, 377–390. https://doi.org/10.1111/mmi.14522

Giraud, L., Borde, C., Audebert, R., 2018. Interaction between Direct Red 81 dye and Cu(II) ions in aqueous solution. *Molecules.* 23, 242. https://doi.org/10.3390/molecules23020242

Irawati, W., Pinontoan, R., Mouretta, B., Yuwono, T., 2021. Potential of copper-resistant bacteria *Acinetobacter* sp. strain CN5 in decolorizing dyes. *Biodiversitas.* 23, 680-686. https://doi.org/10.13057/biodiv/d230212

Irawati, W., Timothy, M., Soentoro, S.E., Pinontoan, R., Yuwono, T., Lindarto, V., 2022. Indigenous multi-resistant bacteria to copper and dyes from Kapuas River, Indonesia. *Biodiversitas.* 23, 6661-6668. https://doi.org/10.13057/biodiv/d231265

Irawati, W., Susilowati, D.N., Sopiah, N., Lindarto, V., Pinontoan, R., Yuwono, T., 2023. Copper and dye-resistant bacteria from Citarum River, Indonesia. *Biodiversitas.* 24, 1215-1223. https://doi.org/10.13057/biodiv/d240263

Irawati, W., Pinontoan, R., Yuwono, T., Sofiana, I., Lindarto, V., Susilowati, D.N., 2025. Indigenous copper- and dye-resistant bacteria from Cikijing River. *J. Trop. Biodivers. Biotechnol.* 10, 1-12. https://doi.org/10.22146/jtbb.12527

Jay, J.M., Loessner, M.J., Golden, D.A., 2005. *Modern Food Microbiology.* Springer, New York.

Jefri, J., Fithri, N.A., Lesbani, A., 2024. Malachite green removal using Gambir-modified NiFe LDH. *Bull. Chem. React. Eng. Catal.* 19, 573-584. https://doi.org/10.9767/bcrec.20215

Khan, I., Saeed, K., Zekker, I., Zhang, B., Hendi, A.H., Ahmad, A., Ahmad, S., Zada, N., Ahmad, H., Shah, L.A., Shah, T., Khan, I., 2022. Review on methylene blue: its properties, uses, toxicity and photodegradation. *Water.* 14, 242. https://doi.org/10.3390/w14020242

Khandare, R.V., Govindwar, S.P., 2016. Microbial degradation of textile dyes: mechanisms and pathways. *Environ. Waste Manag.* 14, 399-429.

Li, C., Liu, D., Yang, X., Chen, S., Deng, J., Rong, H., Li, C., 2019. Biodegradation of malachite green by *Klebsiella pneumoniae* strain WA-1. *Environ. Prog. Sustain. Energy.* 39, e13346. https://doi.org/10.1002/ep.13346

Meerbergen, K., Willems, K.A., Dewil, R., Van Impe, J., Appels, L., Lievens, B., 2018. Isolation and screening of bacterial isolates from wastewater treatment plants to decolorize azo dyes. *J. Biosci. Bioeng.* 125, 448-456. https://doi.org/10.1016/j.jbiosc.2017.11.008

Ministry of Industry 2019. Available at https://www.kemenperin.go.id/artikel/21230. [Date accessed: 7 July 2022]

Mustafa, G., Zahid, M.T., Kurade, M.B., Patil, S.M., Shakoori, F.R., Shafiq, Z., Ihsan, S., Ahn, Y., Khan, A.A., Gacem, A., Jeon, B., 2023. Molecular characterization of azoreductase and its potential for the decolorization of Remazol Red R and Acid Blue 29. *Environmental Pollution.* 335, 122253. https://doi.org/10.1016/j.envpol.2023.122253

NCBI PubChem Compound Summary 2023. Available at https://pubchem.ncbi.nlm.nih.gov/compound/Direct-red-23. [Date accessed: 7 July 2023]

Pooja, Goel, M., 2021. Ir-Cu nanocomposite for congo red degradation. *J. Appl. Spectrosc.* 88, 858-864. https://doi.org/10.1134/S0023158421050050

Pratiwi, S.S.D., 2021. Analisis dampak pencemaran air sungai akibat industri. *J. Res. Educ. Chem.* 3, 122-142. https://doi.org/10.25299/jrec.2021.vol3(2).7774

Putri, D.A., Afdal, 2023. Water pollution profile of the Cikijing River. *J. Fis. Unand.* 12, 541-547. https://doi.org/10.25077/jfu.12.4.541-547.2023

Rani, A., Singh, R., Kumar, V., 2022. Extracellular laccase production by *Klebsiella pneumoniae* for synthetic dye degradation. *RSC Advances.* 12, 14378-14387. https://doi.org/10.1039/D2RA01204K

Reiss, R., Ihssen, J., Richter, M., Eichhorn, E., Schilling, B., Thöny-Meyer, L., 2011. Laccase versus laccase-like multi-copper oxidase: a comparative study of similar enzymes with diverse substrate spectra. *PLOS ONE.* 6, e20500. https://doi.org/10.1371/journal.pone.0020500

Rolfe, M.D., Rice, C.J., Lucchini, S., Pin, C., Thompson, A., Cameron, A.D.S., Alston, M., Stringer, M.F., Betts, R.P., Baranyi, J., Peck, M.W., Hinton, J.C.D., 2011. Lag phase prepares bacteria for exponential growth. *J. Bacteriol.* 194, 686-701. https://doi.org/10.1128/JB.06112-11

Sennaj, R., Lemriss, S., Souiri, A., Kabbaj, S.E.L., Chafik, A., Essamadi, A.K., Benali, T., Fassouane, A., Dari, K., Aassila, H., 2023. Eco-friendly degradation of reactive dyes by *Klebsiella pneumoniae. J. Microbiol. Methods.* 204, 106659. https://doi.org/10.1016/j.mimet.2022.106659

Song, J., Han, G., Wang, Y., Jiang, X., Zhao, D., Li, M., Yang, Z., Ma, Q., Parales, R. E., Ruan, Z., 2020. Pathway and kinetics of malachite green biodegradation by *Pseudomonas veronii*. *Sci. Rep.* 10, 4502. https://doi.org/10.1038/s41598-020-61442-z

Sutrisno, A., 2022. Textile industry in Indonesia amidst COVID-19. *Kybernol. J. Gov. Stud.* 2, 1-8. https://doi.org/10.26618/kjgs.v2i1.7853

Utami, N.P., Wardana, A.A., Sari, L.K., 2023. Fotodegradasi congo red menggunakan ZnO-arang aktif dan $H_2O_2$. *J. Kim.* 17, 12-20. https://doi.org/10.24843/JCHEM.2024.v18.i02.p05

Xu, J., Tong, R., 2017. An environmentally friendly protocol for oxidative halocyclization of tryptamine and tryptophol derivatives. *Green Chem.* 19, 2952-2956. https://doi.org/10.1039/C7GC01341H