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Exploration of indigenous copper and dye-resistant bacteria isolated from Citarum River, West Java, Indonesia

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Abstract. *Irawati W, Susilowati DN, Sofiana I, Lindarto V, Pinontoan R, Yuwono T. 2023. Exploration of indigenous copper and dye-resistant bacteria isolated from Citarum River, West Java, Indonesia. Biodiversitas 24: 1215-1223.* Bacterial bioremediation utilizing indigenous bacteria has been reported as an effective, economical, and eco-friendly solution to marine contamination. However, study on the use of dye and copper-resistant bacteria has not been done much. The study aimed to isolate copper and dye-resistant bacteria, determining copper and dye resistance and decolorization abilities. Copper and dye-resistant bacteria were isolated from the Citarum River, West Java, Indonesia. Bacterial isolates were identified based on 16S rDNA gene analysis. Copper resistance was determined by measuring the minimum inhibitory concentration (MIC) of $CuSO_4$. Dye resistance was observed by growing the bacterial isolates on a medium containing 100-500 ppm of various dyes. The dye decolorization was analyzed by monitoring the absorbance of each dye using a spectrophotometer. Fifty-four of copper indigenous resistant bacteria have been isolated. Nine bacterial isolates that showed high resistance to copper and dye with the MIC of 11 mM $CuSO_4$ were identified as *Siccibacter colletis, Acinetobacter baumannii, Lysinibacillus fusiformis, Bacillus cereus,* and *Escherichia coli.* The highest multi-resistant bacterium was *Bacillus cereus* CTR 200 3.2 with decolorization rates of 93.04%, 61.9%, and 87.43% on 100 ppm methylene blue, malachite green, and basic fuchsine dye, respectively. However, adding 5 mM $CuSO_4$ reduced those decolorization rates to 39.39%, 10.48%, and 7.39%, respectively.

Keywords: Bacteria, basic fuchsine, copper, decolorization, dye, indigenous, malachite green, methylene blue, multi-resistant

INTRODUCTION

Citarum River, the most polluted river in the world, stretches 270 km across West Java, Indonesia. Surrounded by >2,000 textile factories, an estimated 20,000 tons of waste and 340,000 tons of wastewater are unethically discharged into the streams of Citarum daily. Textile effluent contains non-degradable compounds such as synthetic chemicals and toxic heavy metals (Senthil Kumar and Saravanan 2017). Industrial effluents containing a multitude of heavy metals and synthetic dyes are commonly released into surrounding bodies of water, permanently altering marine environments. Heavy metals with a density of over 5 g/cm^3 are majorly generated as waste by chemical manufacturing, metallurgical, mining, battery, petroleum, and pharmaceutical industries (Verma et al. 2021). Atmospheric deposition of heavy metals is followed by infiltration on food chain networks. Those infiltrations lead to bioaccumulation and biomagnification at higher trophic levels (Ali and Khan 2019).

Synthetic dyes extensively used by textile, tannery, cosmetics, pharmaceutics, paper, printing, and plastic manufacturers may also constitute industrial effluent. Those waste ingredients pose as micropollutants threatening marine sustainability. Industrial sectors favor a variety of synthetic dyes such as methylene blue, malachite green, Congo red, and basic fuchsine due to commercial popularity and its high affinity to bind with cellulosic fiber (Irawati et al. 2022; Karim et al. 2018). They were designed as long-lasting and highly stable colorants that do not fade on light, water, or heat exposure. Approximately 15-50% of dyes released into post-production wastewater circles coasts for an extended period without undergoing natural degradation (Lellis et al. 2019; Tkaczyk et al. 2020). As a result, relatively small quantities of dye (10-50 mg/L) are highly visible in water resources (Rani et al. 2014). Aquatic organisms, especially plants, will experience inhibited growth due to reduced light penetration and dissolved oxygen levels. At the same time, animals risk developing mutagenic and carcinogenic health effects, which may be incorporated into the food web and eventually harm humans (Abe et al. 2018; Khan and Malik 2018).

Copper is one of the materials used to make various synthetic dyes; thus, textile effluent has been reported to contain both copper and dyes (Arora et al. 2017). Copper is

considered one of the most ubiquitous trace elements essential for all living organisms, but direct and chronic exposure can significantly reduce marine biodiversity and impact human health (Hamid et al. 2022; Wang et al. 2019). For instance, copper toxicity could limit cell defense mechanisms against oxygen-free radicals, inducing cell mutagenesis and carcinogenesis and leading to various cancers (Lobo et al. 2010). Furthermore, copper ingestion tends to accumulate in the liver and brain, leading to organ function abnormalities and/or damage, resulting in death. Copper toxicity may also damage the central nervous system, leading to neurodegenerative disorders or behavioral problems following childhood exposure (Kardos et al. 2018).

Bacterial bioremediation, a natural and economical process that utilizes bacterial isolates comprised of functional derivatives, could be the solution to copper and dye contamination. Selected bacterial isolates could be cultivated as bioremediation agents for performing resistance mechanisms. For example, biosorption, bioaccumulation, and decolorization to minimize the toxicity of anthropogenic contaminants on copper and dye. Indigenous bacterial species that inhabit contaminated sites can develop resistance mechanisms as an adaptive response to cellular stress. Microbial morphology, physiology, and metabolic mechanisms enable bacteria to develop resistance mechanisms despite direct and long-term exposure. For example, highly copper-resistant bacterial isolates conduct biosorption by binding copper ions onto the cell wall. The copper is then transported across the cell membrane before proceeding with bioaccumulation. The excess copper ions are intracellularly stored within the membrane fraction and inside the cytoplasm (Irawati et al. 2022). Dye-resistant bacterial isolates can decolorize dyes by degrading chemical dye structures as initiated by oxidative and reductive enzymes secreted during metabolism (Jadhav et al. 2016).

Previous studies demonstrate that indigenous bacteria can be effectively employed as bioremediation agents as they easily adapt to contaminated habitats in which they were isolated. Various bacterial genera, including Acinetobacter, Bacillus, Cupriavidus, Enterobacter, and Klebsiella, isolated from copper-contaminated sites in Indonesia can be successfully cultivated to resist copper (Irawati et al. 2022; Irawati and Tahya 2021; Jadhav et al. 2016; Trihadiningrum et al. 2014). Genera Acinetobacter, Bacillus, Pseudomonas, and Zooglea isolated from dye-contaminated resources in Indonesia have also been proven to resist multiple dye variants (Irawati et al. 2022). However, only a limited quantity of studies has investigated the ability of indigenous bacteria to perform resistance mechanisms in the presence of both copper and dyes. Therefore, this study aims to: (1) isolate and identify copper and dye-resistant bacteria from Citarum River, Indonesia, (2) determine the resistance of bacteria exposed to copper and dyes commonly used in the textile industry, and (3) measure the ability of chosen bacterial isolates to decolorize selected dyes.

MATERIALS AND METHODS

Wastewater Sampling

Bacteria-containing wastewater was obtained from the Citarum River, West Java, Indonesia, and stored in a plastic canister at 27ºC. First, wastewater sampling was conducted by thoroughly shaking the plastic canister to homogenize the liquid beforehand. Next, approximately 100 ml of the wastewater was procured in a centrifuge tube and labeled as an original solution. Wastewater samples were dissolved into liquid LB medium at a dilution factor of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}. Approximately 100 µl of each diluted suspension was plated onto solid LB medium, then incubated at 37ºC for 24 hours until a mixed culture was formed. Distinct bacterial colonies were repeatedly inoculated onto a solid medium until a pure culture for 54 bacterial isolates was attained (Irawati et al. 2022).

Growth Media Preparation

Growth media were prepared by adding 2 g Luria Bertani broth containing 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, and 0.1 g/L glucose into 1L of dH_2O. Solid media were formulated by adding 2 g American Bacteriological Agar. The LB medium mixture was sterilized in an autoclave at 121ºC and 1 atm for 15 minutes. A 1 M stock solution of $CuSO_4$ was prepared and filter-sterilized using a 0.2 µm syringe filter (Merck Millipore) to produce copper-supplemented media containing 0, 2, 3, 4, 5, 6, 7, 8, 9, and 10 mM $CuSO_4$. A stock solution of 10,000 ppm methylene blue, malachite green, congo red, and basic fuchsine dye was also prepared to create dye-supplemented media containing 50, 100, 200, 300, 400, 500, 600, 700, and 800 ppm. Supplemented media containing various concentrations of copper, dye, and copper and dye (5 mM $CuSO_4$ alongside 50, 100, and 200 ppm each dye) (Irawati et al. 2022).

Copper-resistance test

Bacterial copper resistance was analyzed on agar LB medium supplemented with 0, 2, 3, 4, 5, 6, 7, 8, 9, and 10 mM of $CuSO_4$. Each isolate was inoculated onto media using the four-quadrant streaking method, then incubated at 37ºC for 24 hours. The growth analysis was performed through manual observation based on the presence of bacterial colonies. Minimum Inhibitory Concentration (MIC) values for each isolate were determined based on the highest concentration of copper isolate that was not successfully cultivated. Bacterial isolates with the highest MIC were then selected for further experiments (Irawati et al. 2016).

Morphological and molecular characterization

The selected bacterial isolates were morphologically characterized through qualitative observation. Then, they are molecularly identified based on their 16S rDNA gene. The 16sRDNA amplification was performed on isolates using 5 units/µL initial concentration and 1.25 units/25 L final enzyme concentration of Platinum® Tag DNA Polymerase (Invitrogen no. cat. 10966-018) as the selected polymerase. Amplification results were then sequenced and

analyzed using the ProSeq program. The 16S rDNA sequence alignment of the isolates was also compared to provided sequences in the gene bank data using the Basic Local Alignment Search Tool (BLAST) for isolate identification and percentage of similarity determination. Phylogenetic studies followed by analyzing the base sequence of 16S rDNA genes of each bacterial isolated using Neighbour Joining 1000x Bootstrap method using *Clostridium botulinum* as the outgroup in CLUSTAL X. The results were then displayed in a dendrogram using the TreeView program (Irawati et al. 2022).

Dye-resistance test and decolorization analysis

Solid LB medium supplemented with 50, 100, 200, 300, 400, 500, 600, 700, and 800 ppm methylene blue, malachite green, congo red, and basic fuchsine dye were used for bacterial cultivation. First, a loopful of bacteria was inoculated onto media using the four-quadrant streaking method. Growth analysis was then performed through manual observation based on forming clear zones around bacterial colonies (Irawati et al. 2019).

Bacteria were inoculated into a 50 ppm dye-supplemented liquid LB medium and incubated at 37°C for 24 hours. One mL of bacteria culture was drawn for liquid culture, then centrifuged at 12,000 rpm for 5 minutes for OD600 measurement every 3 hours. Quantification of the remaining dye concentration in the medium was conducted using spectrophotometry at 300-900 nm. Decolorization measurement was performed in triplicates. The following formula was used to calculate the percentage of decolorization (Chen et al. 2003):

$$\%\ \text{Decolorization} = \frac{\text{absorbance of control} - \text{absorbance of treated sample}}{\text{absorbance of control}} \times 100\%$$

Multi-resistance test

Solid LB medium supplemented with a 5 mM $CuSO_4$ and 50, 100, and 200 ppm methylene blue, malachite green, congo red, and basic fuchsine dye was used for bacterial cultivation. First, a loopful of bacteria was inoculated onto media using the four-quadrant streaking method. Growth analysis was then performed through manual observation based on the growth of bacteria and the formation of clear zones around bacterial colonies.

RESULTS AND DISCUSSION

Isolation of Copper-resistant Bacteria

Fifty-four bacterial isolates from Citarum River, Indonesia, grew on media supplemented with various copper concentrations ranging from 0-10 mM of CuSO4. The number of bacterial colonies that grew on a medium containing 10 mM $CuSO_4$ was almost the same as on a medium containing 2 mM $CuSO_4$. A high concentration of $CuSO_4$ did not significantly affect the number of colony types, indicating that the bacteria growing in the Citarum river were developing copper-resistance mechanisms during stress exposure (Table 1).

The copper resistance test showed nine high copper-resistant bacteria isolates from Citarum River with the MIC of 11 mM $CuSO_4$ (Table 2). The nine isolates were coded with CTR 200 1.1, CTR 200 1.2, CTR 200 2.1, CTR 200 3.2, CTR 1000 1.1, CTR 1000 2.1, CTR 1000 2.2, CTR 1000 3.1, and CTR 1000 3.2 will be used for further investigation.

Characterization of copper-resistant bacteria

The results of morphological characteristics showed that the most copper-resistant bacteria isolated from the Citarum River were Gram-negative bacteria. All shapes of the bacterial cell were basil. All bacterial colonies were circular and entire in shape with a flat surface, except for isolate CTR1000 2.1, which was undulate. Colonies of isolates CTR 200 1.1, CTR 1000 1.1, CTR 1000 2.1, and CTR 1000 3.2 were ivory, while isolates CTR 200 1.2, CTR 200 2.1, CTR 200 3.2, and CTR 1000 2.2 were cream. Only CTR 1000 3.1 isolate was white (Table 3).

Molecular characterization based on 16s rDNA sequence identified five distinct bacterial species from nine copper-resistant bacteria. Isolates CTR 1000 1.1, CTR 1000 2.1, 1000 3.1, and 1000 3.2 were identified as *Escherichia coli* (100% similarity), CTR 200 3.2 and CTR 1000 2.2 as *Bacillus cereus* (100% similarity), CTR 200 1.1 as *Siccibacter colletis* (100% similarity), CTR 200 1.2 as *Acinetobacter baumannii* (100% similarity), and CTR 200 2.1 as *Lysinibacillus fusiformis* (100% similarity) (Table 4).

Table 1. Purification results of bacteria isolated from Citarum River cultivated on media supplemented with 0-10 mM of $CuSO_4$

$CuSO_4$ conc. (mM)	Number of colony type	Isolate code
0	5	CTR 0.1.1, CTR 0.1.2, CTR 0.2.1, CTR 0.3.1, CTR 0.3.2
2	6	CTR 200 1.1, CTR 200 1.2, CTR 200 2.1, CTR 200 2.2, CTR 200 3.1, CTR 200 3.2,
3	4	CTR 300 1.1, CTR 300 2.1, CTR 300 2.2, CTR 300 3.2
4	7	CTR 400 1.1, CTR 400 1.2, CTR 400 2.1, CTR 400 2.2, CTR 400 3.1, CTR 400 3.2, CTR 400 3.2.2
5	6	CTR 500 1.1, CTR 500 1.2, CTR 500 2.1, CTR 500 2.2, CTR 500 3.1, CTR 500 3.2
6	2	CTR 600 2.1, CTR 600 3.2
7	6	CTR 700 1.1, CTR 700 1.2, CTR 700 2.1, CTR 700 2.2, CTR 700 3.1, CTR 700 3.2,
8	5	CTR 800 1.2, CTR 800 2.1, CTR 800 2.2, CTR 800 3.1, CTR 800 3.2
9	7	CTR 900 1.1, CTR 900 1.2, CTR 900 2.1.1, CTR 900 2.1, CTR 900 2.2, CTR 900 3.1, CTR 900 3.2
10	6	CTR 1000 1.1, CTR 1000 1.2, CTR 1000 2.1, CTR 1000 2.2, CTR 1000 3.1, CTR 1000 3.2
∑ Bacterial isolates	54	

Table 2. Copper-resistance test of Citarum isolates grown on media supplemented with 1-10 mM of $CuSO_4$

Isolate code	Resistance test											MIC
	1	2	3	4	5	6	7	8	9	10	11	(mM)
CTR 0 1.1	+	+	+	+	+	-	-	-	-	-	-	6
CTR 0 1.2	-	-	-	-	-	-	-	-	-	-	-	1
CTR 0 2.1	+	+	+	+	+	-	-	-	-	-	-	6
CTR 0 3.1	+	+	+	+	+	-	-	-	-	-	-	6
CTR 0 3.2	+	+	+	+	+	-	-	-	-	-	-	6
CTR 200 1.1	+	+	+	+	+	+	+	+	+	+	-	11
CTR 200 1.2	+	+	+	+	+	+	+	+	+	+	-	11
CTR 200 2.1	+	+	+	+	+	+	+	+	+	+	-	11
CTR 200 2.2	+	+	+	+	+	-	-	-	-	-	-	6
CTR 200 3.1	+	+	+	+	+	+	+	+	+	+	-	10
CTR 200 3.2	+	+	+	+	+	+	+	+	+	+	-	11
CTR 300 1.1	+	+	+	-	-	-	-	-	-	-	-	4
CTR 300 2.1	+	+	+	-	-	-	-	-	-	-	-	4
CTR 300 2.2	+	+	+	-	-	-	-	-	-	-	-	4
CTR 300 3.2	+	+	+	-	-	-	-	-	-	-	-	4
CTR 400 1.1	+	+	+	+	-	-	-	-	-	-	-	5
CTR 400 1.2	+	+	+	+	-	-	-	-	-	-	-	5
CTR 400 2.1	+	+	+	+	-	-	-	-	-	-	-	5
CTR 400 2.2	+	+	+	+	-	-	-	-	-	-	-	5
CTR 400 3.1	+	+	+	+	-	-	-	-	-	-	-	5
CTR 400 3.2	+	+	+	+	-	-	-	-	-	-	-	5
CTR 400 3.2.2	+	+	+	+	-	-	-	-	-	-	-	5
CTR 500 1.1	+	+	+	+	+	-	-	-	-	-	-	6
CTR 500 1.2	+	+	+	+	+	-	-	-	-	-	-	6
CTR 500 2.1	+	+	+	+	+	-	-	-	-	-	-	6
CTR 500 2.2	+	+	+	+	+	-	-	-	-	-	-	6
CTR 500 3.1	+	+	+	+	+	-	-	-	-	-	-	6
CTR 500 3.2	+	+	+	+	+	-	-	-	-	-	-	6
CTR 600 2.1	+	+	+	+	+	+	+	+	+	-	-	10
CTR 600 3.2	+	+	+	+	+	+	+	+	+	-	-	10
CTR 700 1.1	+	+	+	+	+	+	+	+	+	-	-	10
CTR 700 1.2	+	+	+	+	+	+	+	+	+	-	-	10
CTR 700 2.1	+	+	+	+	+	+	+	+	+	-	-	10
CTR 700 2.2	+	+	+	+	+	+	+	+	+	-	-	10
CTR 700 3.1	+	+	+	+	+	+	+	+	+	-	-	10
CTR 700 3.2	+	+	+	+	+	+	+	+	+	-	-	10
CTR 800 1.2	+	+	+	+	+	+	+	+	+	-	-	10
CTR 800 2.1	+	+	+	+	+	+	+	+	+	-	-	10
CTR 800 2.2	+	+	+	+	+	+	+	+	+	-	-	10
CTR 800 3.1	+	+	+	+	+	+	+	+	+	-	-	10
CTR 800 3.2	+	+	+	+	+	+	+	+	+	-	-	10
CTR 900 1.1	+	+	+	+	+	+	+	+	+	-	-	10
CTR 900 1.2	+	+	+	+	+	+	+	+	+	-	-	10
CTR 900 2.1.1	+	+	+	+	+	+	+	+	+	-	-	10
CTR 900 2.1	+	+	+	+	+	+	+	+	+	-	-	10
CTR 900 2.2	+	+	+	+	+	+	+	+	+	-	-	10
CTR 900 3.1	+	+	+	+	+	+	+	+	+	-	-	10
CTR 900 3.2	+	+	+	+	+	+	+	+	+	-	-	10
CTR 1000 1.1	+	+	+	+	+	+	+	+	+	+	-	11
CTR 1000 1.2	+	+	+	+	+	+	+	+	+	-	-	10
CTR 1000 2.1	+	+	+	+	+	+	+	+	+	+	-	11
CTR 1000 2.2	+	+	+	+	+	+	+	+	+	+	-	11
CTR 1000 3.1	+	+	+	+	+	+	+	+	+	+	-	11
CTR 1000 3.2	+	+	+	+	+	+	+	+	+	+	-	11

Note: (+) represents growth, and (-) represents no growth.

Table 3. Morphological characterization of chosen copper-resistant bacterial isolates

Isolate code	Shape	Color	Edge	Surface	Cell shape	Gram
CTR 200 1.1	Circular	Ivory	Entire	Flat	Basil	-
CTR 200 1.2	Circular	Cream	Entire	Flat	Basil	-
CTR 200 2.1	Circular	Cream	Entire	Flat	Basil	+
CTR 200 3.2	Circular	Cream	Entire	Flat	Basil	+
CTR 1000 1.1	Circular	Ivory	Entire	Flat	Basil	-
CTR 1000 2.1	Circular	Ivory	Undulate	Flat	Basil	-
CTR 1000 2.2	Circular	Cream	Entire	Flat	Basil	+
CTR 1000 3.1	Circular	White	Entire	Flat	Basil	-
CTR 1000 3.2	Circular	Ivory	Entire	Flat	Basil	-

Bacterial dye and copper resistance

The test on copper and dye resistance showed that *Siccibacter colletis* CTR 200 1.1 was only resistant to 500 ppm methylene blue without being supplemented with copper. *Acinetobacter baumannii* 200.1.2 was resistant to 50 ppm methylene blue and 50 ppm basic fuchsine without being supplemented with 5 mM $CuSO_4$. *Lysinibacillus fusiformis* CTR 200 2.1 could not grow on a medium containing any dyes supplemented with copper. *Bacillus cereus* CTR 200 3.2 was resistant to 50 ppm methylene blue, 50 ppm basic fuchsine, and 500 ppm malachite green supplemented with 5 mM $CuSO_4$. *Escherichia coli* CTR 1000 1.1 was resistant to 700 methylene blue and 600 ppm congo red supplemented with 5 mM $CuSO_4$. *Bacillus cereus* CTR 1000 2.1 was resistant to 50 ppm congo red without copper. *Bacillus cereus* 1000 2.2 was resistant to 200 ppm malachite green without copper. *Escherichia coli* CTR 1000 3.1 was resistant to 200 ppm methylene blue, 200 ppm basic fuchsine, and 500 ppm congo red supplemented with 5 mM $CuSO_4$, respectively. *Escherichia coli* CTR 1000 3.2 was resistant to 500 ppm malachite green without copper and 200 ppm basic fuchsine supplemented with 5 mM $CuSO_4$. It was concluded that from nine copper-resistant bacteria, only *Acinetobacter baumannii* CTR 200 1.2, *Bacillus cereus* CTR 200 3.2, *Escherichia coli* CTR 1000 1.1, *Escherichia coli* CTR 1000 3.1, *Escherichia coli* CTR 1000 3.2 were multi-resistant to both copper and dye (Table 5).

Bacterial dye decolorization

The distinct, clear zones were formed around the colonies of *Bacillus cereus* CTR 200 3.2 on 50 ppm methylene blue, 50 ppm basic fuchsine, and 500 ppm malachite green. *Bacillus cereus* CTR 1000 2.2 also formed a clear zone when grown on a medium containing 50 ppm congo red, 200 ppm malachite green, and 200 ppm basic fuchsine. Meanwhile, *Escherichia coli* CTR 1000 1.1, *Escherichia coli* CTR 1000 3.2, and *Siccibacter colletis* CTR 200 1.1 formed clear zone in medium containing 200 ppm, 500 ppm methylene blue, and 500 ppm basic fuchsine dye, respectively (Figure 1).

The multi-resistant test in the medium containing the higher concentration of dyes showed that *Bacillus cereus, Escherichia coli, and Acinetobacter baumannii* isolates from Citarum River were the three highest multi-resistant bacteria. That result shows bacteria resisted in a medium containing 5 mM $CuSO_4$ and could decolorize 200-700 ppm dyes (Figures 2 and 3).

Table 4. Molecular characterization of chosen copper-resistant bacterial isolates based on 16S rDNA

Isolate code	The closest taxon to BLAST results on NCBI	Similarity (%)
CTR 200 1.1	*Siccibacter colletis*	100
CTR 200 1.2	*Acinetobacter baumannii*	100
CTR 200 2.1	*Lysinibacillus fusiformis*	100
CTR 200 3.2	*Bacillus cereus*	100
CTR 1000 1.1	*Escherichia coli*	100
CTR 1000 2.1	*Escherichia coli*	100
CTR 1000 2.2	*Bacillus cereus*	100
CTR 1000 3.1	*Escherichia coli*	100
CTR 1000 3.2	*Escherichia coli*	100

Table 5. The ability of Citarum isolates to grow on a medium supplemented with dyes and copper

Isolate code	Species	Maximum tolerable concentration	
		Dyes	$CuSO_4$
CTR 200 1.1	*Siccibacter colletis*	500 ppm MG	0 mM $CuSO_4$
CTR 200 1.2	*Acinetobacter baumannii*	50 ppm MB	0 mM $CuSO_4$
		50 ppm BF	0 mM $CuSO_4$
		500 ppm BF	5 mM $CuSO_4$
CTR 200 2.1	*Lysinibacillus fusiformis*	0 ppm	0 mM $CuSO_4$
CTR 200 3.2	*Bacillus cereus*	50 ppm MB,	5 mM $CuSO_4$
		50 ppm BF	5 mM $CuSO_4$
		500 ppm MG	5 mM $CuSO_4$
CTR 1000 1.1	*Escherichia coli*	700 ppm MB	5 mM $CuSO_4$
		600 ppm CR	5 mM $CuSO_4$
CTR 1000 2.1	*Bacillus cereus*	50 ppm CR	0 mM $CuSO_4$
CTR 1000 2.2	*Bacillus cereus*	200 ppm MG	0 mM $CuSO_4$
CTR 1000 3.1	*Escherichia coli*	200 ppm MB	5 mM $CuSO_4$
		200 ppm BF	5 mM $CuSO_4$
		500 ppm CR	5 mM $CuSO_4$
CTR 1000 3.2	*Escherichia coli*	500 ppm MG	0 mM $CuSO_4$
		200 ppm BF	5 mM $CuSO_4$

Note: MB: Methylene Blue, MG: Malachite Green, BF: Basic Fuchsine, CR: Congo Red.

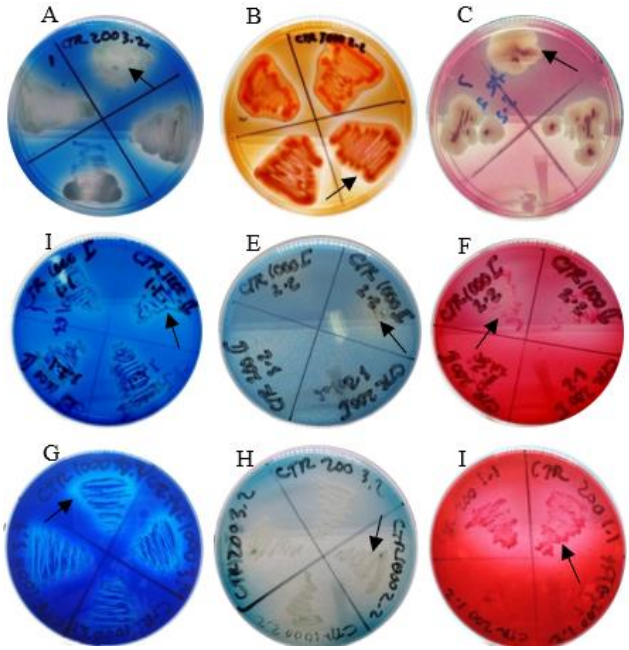

Figure 1. Decolorization ability of bacterial isolates. 50 ppm **(A)** *Bacillus cereus* CTR 200 3.2 on methylene blue **(B)** *Bacillus cereus* CTR 1000 2.2 on Congo red, **(C)** *Bacillus cereus* CTR 200 3.2 on basic fuchsine 200 ppm: (D) *Escherichia coli* CTR 1000 1.1 on methylene blue, (E) *Bacillus cereus* CTR 1000 2.2 on the malachite green, (F) *Bacillus cereus* CTR 1000 2.2 on basic fuchsine, 500 ppm: (G) *Escherichia coli* CTR 1000 2.2 on methylene blue (H) *Bacillus cereus* CTR 200 3.2 on the malachite green, (I) *Siccibacter colletis* CTR 200 1.1 on basic fuchsine dye. Arrow represents a clear zone

The *Bacillus cereus* CTR 200 3.2 is a copper-resistant bacteria with a high ability to decolorize methylene blue, malachite green, and basic fuchsine. That suggests the bacterium has complementary chemical, physical, and physiological features that allow it to develop multi-resistance to copper and dyes. The *Bacillus cereus* CTR 200 3.2 managed to decolorize methylene blue, malachite green, and basic fuchsine medium. Albeit, the percentage decreased from 93.04% to 39.39%, 61.9% to 10.48%, and 87.43% to 7.39%, respectively, in the presence of 5 mM $CuSO_4$ (Figure 4).

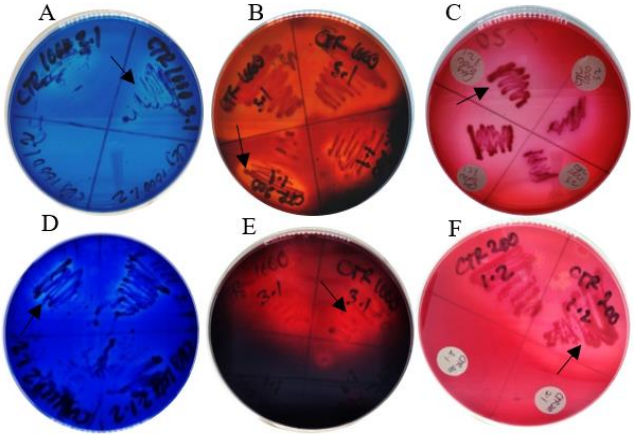

Figure 2. Multi-resistance bacterial isolates to 5 mM copper and 200-500 ppm dyes. (A) *Escherichia coli* CTR 1000 3.1 on 200 ppm methylene blue, (B) *Escherichia coli* CTR 1000 3.1 on 200 ppm Congo red, (C) *Escherichia coli* CTR 1000 3.2 on 200 ppm basic fuchsine supplemented with 5 mM $CuSO_4$. 500 ppm: (D) *Escherichia coli* CTR 1000 1.1 on 500 ppm methylene blue, (E) *Escherichia coli* CTR 1000 3.1 on 500 ppm Congo red, and (F) *Acinetobacter baumannii* CTR 200 1.2 on 500 ppm basic fuchsine supplemented with 5 mM $CuSO_4$. Arrow represents a clear zone

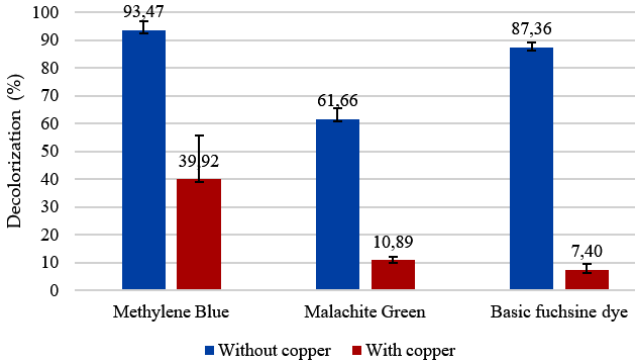

Figure 4. The decolorization ability of the *Bacillus cereus* CTR 200 3.2 on methylene blue, malachite green, and basic fuchsine dye, which was grown on media supplemented with 5 mM of $CuSO_4$

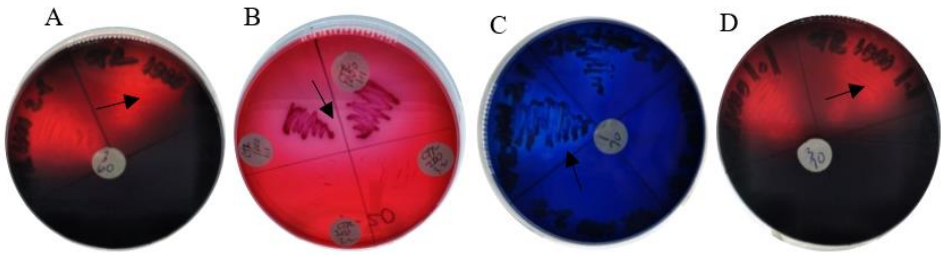

Figure 3. Multi-resistance bacterial isolates to 5 mM copper and 600-700 ppm dyes. (A) *Bacillus cereus* CTR 1000 2.1 on 5 mM $CuSO_4$ + 600 ppm Congo red (B) *Escherichia coli* CTR 1000 1.1 on 600 ppm basic fuchsine, (C) *Bacillus cereus* CTR 1000 2.1 on 700 ppm methylene blue, (D) *Escherichia coli* on 700 ppm congo red dye. Arrow represents a clear zone

Discussion

Copper is required as a micronutrient and/or cofactor for normal cellular activity and growth in living organisms at low concentrations, but it is toxic at high concentrations (Shamim 2018). Copper toxicity damages the cell membrane and DNA due to altered nucleic acid structure and interferes with normal cellular activity due to altered enzyme specificity (Afzal et al. 2017). Bacteria develop an adaptive homeostasis response to copper toxicity by maintaining sufficient intracellular concentrations of copper as a micronutrient and/or cofactor while simultaneously evading and/or repairing cell damage caused by copper (Rowland and Niederweis 2013). Fifty-four bacteria isolate from Citarum River were the nine highest copper-resistant bacteria with MIC value up to 11 mM $CuSO_4$. Based on a previous study on copper-resistant bacteria, these bacterial isolates were categorized as highly resistant to copper. It might be because the Citarum River was contaminated by a high concentration of copper over a long period, so the bacterial community adapted to copper toxic conditions. Similar studies were done in Indonesian report that indigenous bacteria isolated from Cikapundung River, West Java, and Cisadane River, Banten had MIC values of 6-8 mM and from the eastern coast of Surabaya, 9-11 mM (Nurlaila et al. 2021); also industrial sewage in Kemisan River, Banten, 10 mM of $CuSO_4$ (Irawati et al. 2017). Six bacterial species isolated from copper mine tailings in Xinjiang, China, also showed a wide MIC range of 1-11 mM $CuSO_4$ (Kumari et al. 2015).

Most of the bacterial isolates observed from Citarum River were Gram-negative bacteria. Biswas et al. (2021) suggest that Gram-negative bacteria avoid toxic heavy metal ion accumulation by utilizing innate efflux pump systems. Instead of further accumulating, Gram-negative specializes in removing already-accumulated heavy metal ions to sustain growth and development in a highly toxic environment. Therefore, it could be suggested that Gram-negative bacteria are naturally equipped with an advantage to thrive even in copper-contaminated environments. Nine highly copper-resistant bacterial isolates are found, i.e., *Siccibacter colletis* CTR 200 1.1, *Acinetobacter baumannii* CTR 200 1.2, *Lysinibacillus fusiformis* CTR 200 2.1, *Bacillus cereus* CTR 200 3.2, *Escherichia coli* CTR 1000 1.1, *Escherichia coli* CTR 1000 2.1, *Escherichia coli* CTR 1000 2.2, *Escherichia coli* CTR 1000 3.1, *Escherichia coli* CTR 1000 3.2.

The three bacterial isolates that have the most potential in multi-resistance to copper and several dyes and decolorize them were *Bacillus cereus*, *Escherichia coli*, and *Acinetobacter baumannii*. These three bacteria can grow at 5 mM $CuSO_4$ and decolorize 200-700 ppm dye. Decolorization can be defined as the process of removing dyes from stained specimens. Dye decolorization occurs through adsorption or biodegradation. That process is evidenced by clear zones formed around dye-adsorbing bacterial colonies grown on dye-supplemented media (Mahbub et al. 2012). *Bacillus cereus* is equipped with favorable enzymes that help bacteria perform decolorization. For example, *B. cereus* strains produce crude protease to remove benzene rings from the chemical structure of malachite green dye (Wanyonyi et al. 2017). At the same time, *E. coli* decolorize dyes through metabolic degradation (Cerboneschi et al. 2015).

Bacillus cereus CTR 200 3.2 was able to grow in a medium containing 5 mM $CuSO_4$ and 200 ppm methylene blue, malachite green, and basic fuchsine medium. The bacterium decolorized the dye at 93.04%, 61.9%, and 87.43%, respectively. Adding 5 mM $CuSO_4$ reduced decolorization ability to 39.39%, 10.48%, and 7.39%, respectively. The finding of copper multi-resistant bacteria and dyes that can decolorize some dyes was a novelty in this study and hopefully can be applied in treating textile industry waste containing copper. Only a limited number of studies have investigated the multi-resistance of indigenous bacteria on copper and dye. For instance, *Burkholderia cepacia* sp. IrV1 isolated from laboratory waste effluent in Indonesia showed resistance to 7 mM copper and 100 ppm of methylene blue and basic fuchsine dye (Irawati et al. 2022). High copper concentrations of 5 mM and above inhibit enzymatic processes due to their toxicity (Murugesan et al. 2009). A study reported a similar occurrence where the *Ochrobactrum pseudogrignonense* strain previously capable of decolorizing 400 ppm malachite green in the absence of copper only decolorized up to 100 ppm in the presence of 10 and 20 mM $CuSO_4$. Thus, it can be concluded that copper toxicity adversely affects the process of decolorization. The *Ochrobactrum pseudogrignonense* acquired from copper mine wastewater in Balaghat, India, could resist up to 20 mM copper and decolorized 100 ppm malachite green dye (Chaturvedi and Verma 2015). *Acinetobacter* sp. CN5 obtained from the Cikapundung River in Indonesia also resisted up to 7 mM copper and decolorized 57.64% of methylene blue and 91.37% of basic fuchsine, respectively (Irawati et al. 2022).

Al-Sulami and Jaafar (Jaafar et al. 2015) suggest that bacteria have physiological and genetic properties that allow specific species to accumulate heavy metals and simultaneously remove synthetic dyes (Jaafar et al. 2015). For example, bacterial isolates from the Citarum River were successfully cultivated on media containing various dye types due to the development of enzyme-mediated resistance mechanisms (Ramzan et al. 2022). Bacteria isolates from contaminated sites tend to develop dye-resistance mechanisms by modifying biochemical properties to catalyze enzyme reactions. That reaction leads to catabolic activity, such as the degradation and detoxification of synthetic dyes. That process is an adaptive response to protect cellular components from direct and prolonged dye exposure (Jaafar et al. 2015; Hsueh et al. 2017). Jayapal et al. (2018) suggested that dye biodegradation occurs through two main phases: disintegration and mineralization. First, bacteria secrete extracellular reductive enzymes, including azo reductase, triphenylmethane reductase, or oxidative enzymes (hydrogenase, laccase, and peroxidase). Next, that process could help disintegrate the complex chemical structure of dyes (Jadhav et al. 2016), then mineralize into simpler and less toxic compounds (Jamee and Siddique 2019).

In conclusion, fifty-four bacterial isolates were retrieved from the Citarum River, West Java, Indonesia.

Nine highly copper-resistant bacterial isolates were found, i.e., *Siccibacter colletis* CTR 200 1.1, *Acinetobacter baumannii* CTR 200 1.2, *Lysinibacillus fusiformis* CTR 200 2.1, *Bacillus cereus* CTR 200 3.2, *Escherichia coli* CTR 1000 1.1, *Escherichia coli* CTR 1000 2.1, *Escherichia coli* CTR 1000 2.2, *Escherichia coli* CTR 1000 3.1, *Escherichia coli* CTR 1000 3.2. with MIC of 11 mM of $CuSO_4$. All bacterial isolates have multi-resistance to copper and some dyes, i.e., methylene blue, malachite green, basic fuchsine, and Congo red, up to 700 ppm. *Bacillus cereus* CTR 200 3.2 showed high decolorization rates of 93.04%, 61.9%, and 87.43% on methylene blue, malachite green, and basic fuchsine dye, respectively. However, due to copper toxicity, the decolorization rates were reduced to 39.39%, 10.48%, and 7.39% on enriched medium supplemented with 5 mM $CuSO_4$. Each bacteria from the Citarum River, excluding *Lysinibacillus fusiformis* CTR 200 2.1, were equipped with high multi-resistance to copper and dye, carrying the great potential to be utilized in future bioremediation processes.

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