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Research Article

Exploration the Potency of Copper and Dyes Multi-Resistant of Indigenous Bacteria Isolated from Cikijing River, West Java

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ABSTRACT

Various types of textile dye have been reported to contaminate the Cikijing River, West Java, Indonesia due to its location within the industrial region of Rancaekek District. It has been understood that certain bacterial species develop copper resistance and dye decolourisation as a mechanism of stress adaptation. The study aims at isolating and characterising copper and dye resistance as well as decolourisation ability of bacteria isolated from the Cikijing River. Copper-resistant bacteria were isolated using a series dilution method on Luria Bertani media supplemented with the addition of 1-10 mM $CuSO_4$. Purified bacterial isolates were then tested for copper resistance onto LB agar medium supplemented with $CuSO_4$ concentrations ranging from 0 mM to 20 mM and decolourisation of various dyes. A total of 59 copper-resistant bacteria were successfully isolated, nine of them showed the highest copper resistance with a MIC value from 11 mM up to 16 mM $CuSO_4$ and resistance to 4 types of dyes up to 700 ppm. The 16S rDNA analysis showed that the nine isolates were *Klebsiella* sp., *Klebsiella pneumoniae*, *Lysinibacillus boronitolerans*, *Lysinibacillus fusiformis*, *Bacillus proteoliticus*, *Pseudomonas stutzeri*, *Klebsiella variicola*, *Citrobacter freundii*, and *Klebsiella variicola*. Out of nine isolates, five were found resistant to 5 mM $CuSO_4$ and decolourise Methylene Blue, Congo Red, and Basic Fuchsine dyes at a maximum concentration of 700 ppm.

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INTRODUCTION

Cikijing River is a densely populated industrial area located in the Rancaekek District of West Java, Indonesia. Categorised as a tributary of Citarik and Citarum Rivers that flow through 13 cities, the Cikijing River is an important water source for residential and industrial regions, agriculture irrigation, and hydroelectric turbines (Cavelle 2013; Pantjawati et al. 2020; Riyadi et al. 2020). However, the Cijiking River has an alarmingly high level of pollution due to rapid population growth and anthropogenic activities such as farming and product manufacturing (Prananda et al. 2017). Since Rancaekek District is the main centre for textile industries, Cikijing River is known to be contaminated with a variety of heavy metals and synthetic dyes on the sediments (Fadhilah et al. 2018). Deposition and accumulation of heavy metals in river sediments may infiltrate the aquatic food chain, potentially leading to bioaccumulation and biomagnification. Heavy metals that are toxic may also disrupt the growth and survival rate of terrestrial plants. Several toxic heavy metals that have been found circulating Cikijing River include cadmium, chromium, copper, zinc, mercury, arsenic, and lead (Septiono et al. 2015). Cikijing River contains a significantly high copper concentration (0.0233 ppm) that surpasses the international limit (0.014 ppm) (Mahardika & Salami 2012). As a transition metal, copper is an essential micronutrient and cofactor for all living organisms at low concentrations, but detrimental at high concentrations (Zeng & Han 2020). Copper waste is commonly generated through industrial, agricultural, and aquacultural activities (Argudín et al. 2019). Due to its non-biodegradability, copper is a human and environmental health hazard. Extended exposure to copper may result in allergic rhinitis, hyper lacrimation, hypersalivation, and photophobia, while chronic exposure leads to Wilson's Disease which is characterised by a Kayser-Fleischer ring caused by copper accumulation inside the cornea. Nervous and gastrointestinal (especially liver and kidney) disorders may also result from copper toxicity (Karim et al. 2018).

Synthetic dye is a toxic and reactive compound that creates environmental imbalances. Textile, paper printing, colour photography, pharmaceutical, cosmetic, and food industries are among the most frequent synthetic dye users (Tkaczyk et al. 2020). Dye-contaminated wastewater is difficult to degrade and detoxify due to the complex chemical structure of dyes (Lu et al. 2009). The complexity of dyes is determined because of their diverse functional group and aromatic system, dyes typically contain multiple group functions that contribute to their chemical behaviour and interaction with substrates, while the aromatic system gives dye structure the ability to decolonize electrons which make the structure stable. Dye-covered water surfaces prevent sunlight from entering, causing the increase of biochemical oxygen demand, decreasing photosynthetic activity, and disrupting aquatic biota growth.

Cikijing River needs to be immediately treated to minimise its level of copper and dye contamination. Conventional physical and chemical treatment methods are ineffective as they are high costs and produce secondary waste (Ayangbenro & Babalola 2017). In contrast, bioremediation is an efficient, cost-effective, and eco-friendly biological method that utilises microorganisms to remove environmental contaminants (Palanivel et al. 2020). Bacteria are pervasive microorganisms that can be found in almost any setting, including copper- and dye-contaminated areas (Cocconcelli & Fontana 2014). Certain bacterial species are equipped with morphologically and metabolically advantageous features that allow them to develop copper resistance and dye decolourisation mechanisms as an adaptive response to cellular stress. Previous studies demonstrate that various types of bacterial species, such as *Siccibacter colletis*, *Acinetobacter baumanii*, *Bacillus cereus*, and *Escherichia coli* isolated

from Citarum River in Indonesia are capable of tolerating copper and dye toxicity (Irawati et al. 2023). Therefore, an investigation to discover bacterial species capable of resisting copper and dye, as well as decolorizing dye, is imperative. Accordingly, this study was aimed at: 1) Identifying of multi-dye resistant bacteria, 2) measuring the copper resistance of selected isolates, and 3) analysing the dye-resistance and dye-degrading abilities of selected isolates on 4 types of commonly used textile dye.

MATERIALS AND METHODS

Water Sampling

Water samples were obtained from Cikijing River in West Java, Indonesia, around the industrial area of PT. Kahatex. Random sampling was performed at three different location points close to the textile factory waste disposal site. The first sample was taken from under a metal bridge, the second sample was approximately 10 meters from the first sampling scene, and the third sample was approximately 5 meters from the second sampling location. Samples were stored inside a sterile bottle before being brought to laboratory for further research.

Bacterial isolation and purification

Copper-resistant bacteria were isolated by cultivating water sampling on Luria Bertani (LB) Agar supplemented with $CuSO_4$ with serial dilution. The stock of 1M $CuSO_4$ was added to an autoclaved medium with various concentrations. Approximately 100 µl of each water sample was spread onto LB agar (25 g L^{-1}) supplemented with the addition of 1-10 mM $CuSO_4$ and was incubated at 37 °C for 48 hours. Each sampling was done in duplicate, with a non-copper-supplemented medium prepared as a negative control.

Bacterial colonies that appeared after the incubation with unique morphological characteristics were purified, then sub-cultured on LB agar medium supplemented with the same $CuSO_4$ concentrations used during the initial culture. Cultures were incubated at 37 °C for 48 hours before undergoing next round of purification for preservation.

Copper-resistance determination assay

Minimum Inhibitory Concentration (MIC) was determined by streaking one full loop of bacterial isolate onto LB agar medium supplemented with $CuSO_4$ concentrations ranging from 0 mM to 20 mM (Irawati et al. 2023). Each assay was repeated four times and incubated at 37 °C for 48 hours. The highest copper concentrations with no observed bacterial growth were noted as the MIC of each isolate. Bacterial isolates that grew on the highest $CuSO_4$ concentration were selected for further investigation.

Dye resistance assay

Bacterial isolates were inoculated onto LB agar medium supplemented with various concentrations of textile dye. Twelve textile dye variants were used for the dye-resistance and decolourisation assay, namely Methylene Blue (MB), Malachite Green (MG), Congo Red (CR), Mordant Orange (MO), Reactive Black (RB), Direct Yellow (DY), Basic Fuchsine (BF), Reactive Orange (RO), Disperse Orange (DO), Remasol (R), Wantex Red (WR), and Wantex Yellow (WY). The stock solution of 10,000 ppm dyes was diluted in sterile aquadest and was sterilised with a filter membrane of 0.20 µM. Each dye was added to the autoclaved LB medium up to the appropriate concentration. The dye concentration used for dye resistance and decolourisation essays ranged from 100-1000 ppm (Irawati et al. 2023). Each assay was done in quadruple and incubated at 37 °C for 48 hours. The highest dye concentrations with no observed bacterial growth were noted as the MIC of each isolate. Bacterial

isolates that grew on the highest dye concentration were selected for further investigation. Decolourisation was observed based on the formation of clear zones around bacterial colonies.

Copper and dye multi-resistance assay

Copper and dye multi-resistance of the bacterial isolates were determined based on growth observations. Bacterial isolates were inoculated onto LB agar medium supplemented with 5 mM of $CuSO_4$ and either 200 ppm, 500 ppm, 600 ppm, or 700 ppm of Methylene Blue (MB), Malachite Green (MG), Congo Red (CR), or Basic Fuchsine (BF) dye (Irawati et al. 2023). Bacterial growth was observed after an incubation period of 37 °C for 48 hours.

Molecular identification of copper and dye-resistant bacteria

Selected bacterial isolates were first inoculated into Nutrient Broth agar medium (13 g L^{-1}), and then isolated using the TIANamp Genomic DNA Kit (Tiangen). DNA concentration and purity were confirmed using a Nanodrop™ 2000 Spectrophotometer (Thermo Fischer Scientific). Bacterial 16S rDNA amplification was performed by Polymerase Chain Reaction with a master mix of 25 µl volume consisted of the following: 12.5 µl of GoTaq® Green (Promega), 1 µl of forward primer (5'-CGCCTGTTTAACAAAAACAT-3'), 1 µl of reverse primer (5'CCGGTCTGAACCAGATCATGT-3'), 2 µl of DNA template, and 8.5 µl of nuclease-free water. Visualization of PCR product was done using gel electrophoresis. Results of the electrophoresis were observed under a UV transilluminator. DNA sequencing results were edited using the ChromasPro 2.6.2 (Technelysium) followed by homology search using the Basic Local Alignment Search Tool (BLAST) on http://www.ncbi.nlm.nih.gov.

RESULTS AND DISCUSSION

Copper-resistant bacteria isolated from Cikijing River

Fifty-nine copper-resistant bacteria have been successfully isolated from Cikijing River. Table 1 shows that all copper-resistant bacteria isolated have copper resistance with the MIC values from 1 until 16 mM $CuSO_4$. Irawati et al. (2020) reported that bacteria with a MIC value of more than 4.7 mM were categorized as very resistant. Among the 59 copper-resistant isolates, 58 isolates showed the highest MIC value of 6-16 mM and only 1 isolate CKJ 0.3.1 which has a MIC value of 1 mM, therefore it was concluded that most of the bacterial isolates had high resistance to copper. Four bacterial isolates (CKJ 300 1.2, CKJ 300 3.1, CKJ 500 2.1.2, and CKJ 500 2.2) had higher copper resistance than the previous studies on copper-resistant bacteria isolated from copper-polluted areas in Indonesia. Previously, it was reported that the MICs of bacterial isolates from Cikapundung and Cisadane River in West Java were up to 6-8 mM (Nurlaila et al. 2020), Kapuas River in Central Kalimantan up to 7 mM (Irawati et al. 2022), Citarum River in West Java up to 10 mM (Irawati et al. 2023), and Kemisan River in Banten Province tolerated up to 10 mM (Irawati et al. 2017).

Resistance to copper and dyes

Nine selected bacterial isolates demonstrated varying resistance to the four types of dyes, with some isolates showing resistance up to a maximum concentration of 700 ppm. These isolates are shown in Table 2.

Multi-resistance testing was conducted on selected copper-resistant bacteria previously cultured on LB agar media supplemented with 5 mM of $CuSO_4$ and various dyes (MB, MG, CR, and BF) with concentrations ranging from 200 ppm to 700 ppm. Out of the nine selected bacterial isolates, five isolates (CKJ 300 1.2, CKJ 500 2.1.2, CKJ 1000 2.2, CKJ 1000 3.1.1, and CKJ

Table 1. Determination results of copper-resistant bacterial isolates from Cikijing River.

No.	MIC value (mM)	Isolate Codes
1.	1	CKJ 0 3.1
2.	6	CKJ 0 1.1; CKJ 0 2.1; CKJ 0 2.2; CKJ 0 3.2; CKJ 200 1.2.2; CKJ 200 3.1; CKJ 400 1.2; CKJ 500 1.2; CKJ 500 2.1; CKJ 500 3.1
3.	7	CKJ 600 2.1; CKJ 600 3.1
4.	8	CKJ 700 1.1; CKJ 700 1.2; CKJ 700 2.1; CKJ 700 3.1; CKJ 700 3.2
5.	9	CKJ 200 2.2; CKJ 400 3.2; CKJ 800 1.1; CKJ 800 1.2; CKJ 800 3.1; CKJ 800 3.2
6.	10	CKJ 400 2.1; CKJ 900 1.1; CKJ 900 1.1.2; CKJ 900 1.2; CKJ 900 1.2.1; CKJ 900 2.1; CKJ 900 2.1.1; CKJ 900 2.2; CKJ 900 2.2.1; CKJ 900 3.1; CKJ 900 3.2
7.	11	CKJ 200 1.1; CKJ 200 1.2; CKJ 200 2.1; CKJ 200 3.2; CKJ 300 2.2; CKJ 300 3.2; CKJ 400 2.2; CKJ 500 1.1; CKJ 500 3.2; CKJ 1000 1.1; CKJ 1000 1.1.1; CKJ 1000 1.2; CKJ 1000 2.1; CKJ 1000 2.1.1; CKJ 1000 2.2; CKJ 1000 2.2.1; CKJ 1000 3.1; CKJ 1000 3.1.1; CKJ 1000 3.2; CKJ 1000 3.2.1
8.	16	CKJ 300 1.2; CKJ 300 3.1; CKJ 500 2.1.2; CKJ 500 2.2

1000 3.2.1) were found to be resistant to 5 mM $CuSO_4$ and capable of decolorizing dyes (MB, CR, and BF) at the highest concentration of 700 ppm.

Table 2. Resistance of bacterial isolates to copper and dyes.

No.	Isolate Codes	Maximum concentration of Dye Type (ppm)			
		MB	CR	BF	MG
1.	CKJ 300 1.2	700	700	700	-
2.	CKJ 500 2.1.2	700	700	700	-
3.	CKJ 500 2.2	700	500	500	-
4.	CKJ 1000 1.1	700	500	500	-
5.	CKJ 1000 1.2	-	200	-	-
6	CKJ 1000 2.2	700	700	700	-
7.	CKJ 1000 3.1	500	-	-	-
8.	CKJ 1000 3.1.1	700	700	700	500
9.	CKJ 1000 3.2.1	700	700	700	500

Although research on bacteria with multi-resistance to copper and various dyes remains limited, numerous studies have investigated bacteria resistant to dyes. Ren et al. (2006) found that *Aeromonas hydrophila* isolated from textile printing activated sludge in Guangzhou, China, displayed resistance to 50 mg L^{-1} of malachite green, basic fuchsine, and reactive black. Additionally, An et al. (2002) showed that *Citrobacter* sp. obtained from soil near a textile dyeing industrial effluent treatment plant in Korea exhibited resistance to both basic fuchsine and congo red dye. Bacterial isolates can be resistant to copper by accumulating copper into cells (Irawati el al. 2020; Irawati et al. 2021a, 2021b).

Decolourisation at high dye concentrations appeared to be less distinct compared to low concentrations due to different levels of toxicity (Jamee & Siddique 2019). Furthermore, each bacteria have different ability to adapt the toxicity for each dye. The result of each bacterium on 3 dyes can be seen in Figure 1.

Decolourisation is defined as the process of removing dyes from stained specimens through adsorption or degradation (Victor et al. 2020). Bacteria-mediated dye decolourisation is determined by several factors, including but not limited to dye structure, dye concentration, and bacterial metabolism. Decolorization mainly occurs due to the synthesis and collaboration of extracellular enzymes such as azoreductase, laccase, lignin peroxidase, and protease

(Misal et al. 2011). Specific enzymes cleave specific bonds and chromophore centres, contributing to the overall decolorization process (Jamee & Siddique 2019). Azoreductase cleaves azo bonds (–N=N–) under anaerobic conditions (Saratale et al. 2009). Laccase breaks up nitro functional groups $N(CH_3)_2$ (Zucca et al. 2015).

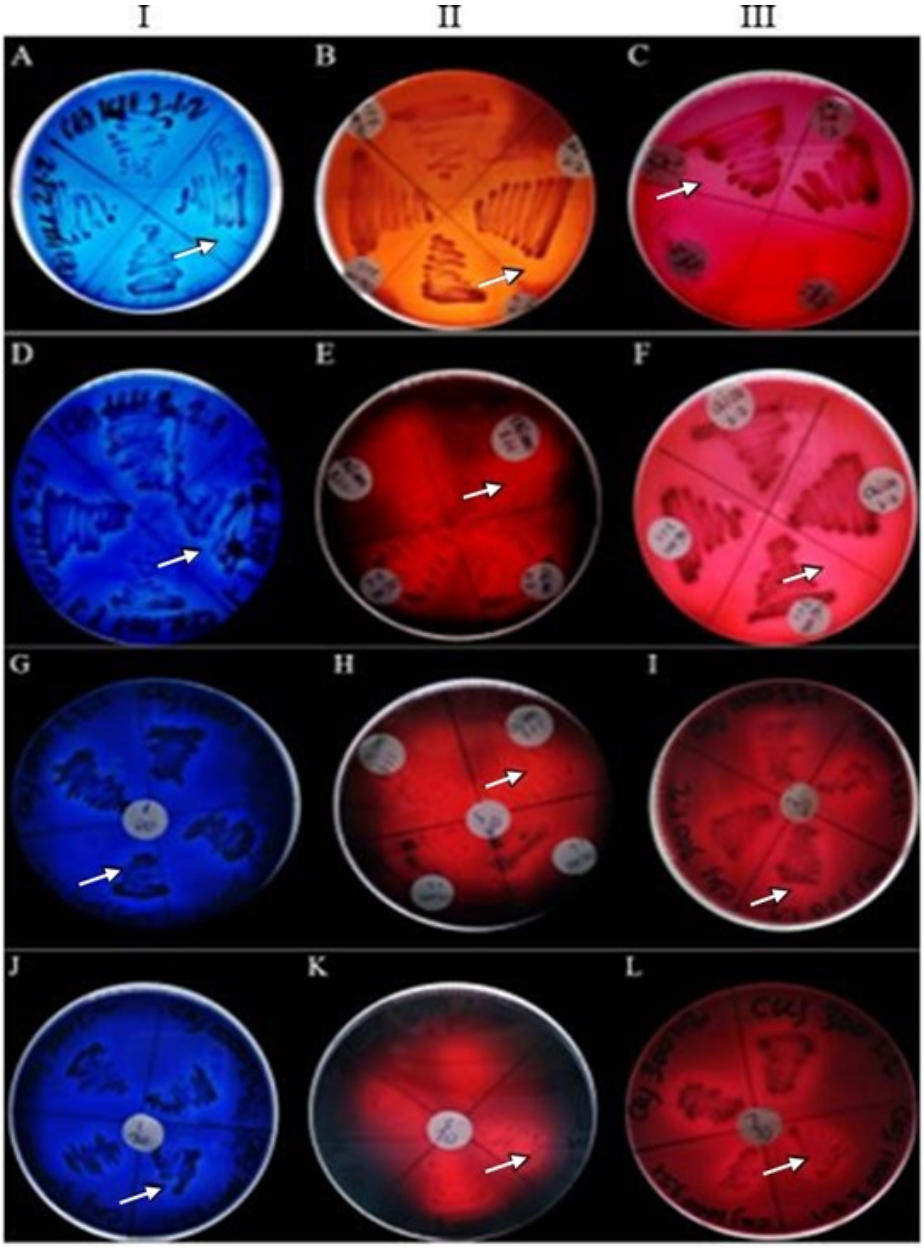

Figure 1. Decolourisation of copper and dye-resistant bacteria isolated from the Cikijing River on LB agar media supplemented with 5 mM $CuSO_4$ and 200-700 ppm of dyes: I. Methylene Blue, II. Congo Red, III. Basic Fuchsine. A. Isolate 2.1.2; B. Isolate 2.2; C. Isolate 1.2; D. Isolate 3.2.1; E. Isolate 3.1.1; F. Isolate 2.2 G. Isolate 3.2.1; H. Isolate 3.2.1; I. Isolate 1.2; J. Isolate 1.2, 3; K. Isolate 2.1; L. Isolate 2.2. Arrows show a clear zone surrounding the bacterial colonies.

Excluding CKJ 1000 1.2, each of the nine bacterial isolates demonstrated high resistance to at least one type of dye. Excluding CKJ 1000 3.1, eight isolates successfully decolorized at least one of the three selected dyes. However, only two out of nine isolates, CKJ 1000 3.1.1 and CKJ 1000 3.2.1, were capable of decolorizing dye including MG. According to Junqueira et al. (2010), MG is a triphenylmethane dye that exerts photodynamic antimicrobial effects on a variety of bacterial species. It is plausible that CKJ 1000 3.1.1 and CKJ 1000 3.2.1 were the only non-affected bacterial isolates in the selection.

Interestingly, CKJ 1000 1.2 was incapable of decolourising any type of dye in the absence of copper but successfully decolorized CR dye in the presence of copper. In contrast, CKJ 1000 3.1 was capable of decolorizing MB dye in the absence of copper but unable to decolorize any type of dye in the presence of copper. The first phenomenon supports the theory that copper is a cofactor for enzymatic reactions, while the second confirms that copper disrupts metabolic activity (Knop et al. 2017; Xue et al. 2023).

Identification of copper-resistance bacterial isolates

Based on morphological and molecular characterization (Table 3), four bacterial isolates belong to the *Klebsiella* genus. *Klebsiella* is a Gram-negative bacterium with a rod-shaped cell. Based on the determination data in this study, *Klebsiella* has the highest MIC value, which is 16 mM, except for CKJ 1000 3.1.1 which is 11 mM (Table 1). Bacteria belonging to this genus include *Klebsiella* sp. (CKJ 300 1.2) with 100 % sequence homology, *K. pneumoniae* (CKJ 500 2.1.2) with sequence homology above 98 %, *K. pneumoniae* (CKJ 500 2.2) with sequence homology above 98 %, and *K. variicola* (CKJ 1000 3.1.1) with sequence homology above 99 % (Table 3). The phylogenetic analysis indicated that CKJ 500 2.1.2 and CKJ 500 2.2 isolates are clustered into coherent groups with *K.pneumoniae* (Figure 2). These bacteria are common opportunistic pathogens for humans and animals as well as resident or temporary flora (especially in the digestive tract). Zulfiqar and Shakoori (2012) previously isolated *K. pneumoniae* that was resistant to copper with a range of MIC values of 5-6 mM. Furthermore, Mustafa et al. (2021) successfully cultivated *Klebsiella* that decolourised around 96 % of 200 ppm Disperse Blue dye within 24 hours.

Two bacterial isolates belong to the *Lysinibacillus* genus, a Gram-positive bacterium with a rod-shaped cell. Bacteria belonging to this genus include *L. boronitolerans* (CKJ 1000 1.1) with a sequence homology above 99 %, *L. fusiformis* (CKJ 1000 1.2) with 100 % sequence homology (Table 3). The phylogenetic analysis showed that CKJ 1000 1.1 and CKJ 1000 1.2 isolates are clustered into coherent groups with *L. boronitolerans* and *L. fusiformis*, respectively (Figure 2). *Lysinibacillus* has been known to have the potential as a heavy metal biosorption agent (Mathivanan et al. 2016). *Lysinibacillus* can decolourise azo dyes up to 96 % with the help of azoreductase, laccase, lignin, and peroxidase enzymes (Sari & Simarani 2019).

Isolate CKJ 1000 2.2 was identified as *B. proteoliticus* with 100 % sequence homology (Table 3) and based on phylogenetic analysis indicated the same coherent group with *B. thuringiensis* and *B. proteoliticus* (Figure 2). This bacterium is a Gram-positive bacterium with a rod-shaped cell. The MIC value for this bacterium is 11 mM. Research on *B. proteoliticus* as a copper bioaccumulation agent has been well known. According to Islam et al. (2020), *B. proteoliticus* was found in polluted environments and can also be a copper biosorption agent. *Bacillus* is effective in degrading azo dyes with the help of extracellular enzymes such as azoreductase and ligninase (Wu et al. 2022).

Isolate CKJ 1000 3.1 was identified as *P. stutzeri* with a sequence homology above 99 % (Table 3) and also confirmed by the results of phylogenetic analysis (Figure 2). This bacterium is a Gram-negative bacterium with a rod-shaped cell. The MIC value of the isolate was 11 mM (Table 1). According to Palanivel et al. (2020), *P. stutzeri* is a potential agent for copper bioremediation. *Pseudomonas* has also been reported to degrade up to 80 % of crystal violet dye at a concentration of 60 µM.

Isolate CKJ 1000 3.2.1 was identified as *C. freundii* with a sequence homology above 97 % (Table 3) and also confirmed with phylogenetic analysis that CKJ 1000 3.2.1 are in the same group as *C. freundii* (Figure 2). This bacterium is a Gram-negative bacterium with a rod-shaped cell. The MIC value

of the isolate was 11 mM (Table 1). This bacterium has great potential for the treatment of industrial waste containing copper under aerobic and anaerobic conditions (Wang et al. 2013). Benhalima et al. (2019) reported that *C. freundii* is a copper-resistant bacterium with an MIC value of 10 mM. *Citrobacter* is known to remove dyes through enzymatic degradation mechanisms (An et al. 2002).

For further research, Copper and dyes multi-resistance bacterial isolates will be determined for the ability to decolourise of dyes and to reduce copper concentration. The bacterial isolates which have multi resistance to copper and dyes will be applied in wastewater treatment plant using bioreactor.

CONCLUSION

Nine isolates from 59 copper resistant bacteria isolated from Cikijing River, that are *Klebsiella pneumoniae*, *Lysinibacillus boronitolerans*, *Lysinibacillus fusiformis*, *Bacillus proteoliticus*, *Pseudomonas stutzeri*, *Klebsiella variicola*, and *Citrobacter freundii*. Out of the nine selected bacterial isolates, five isolates (CKJ 300 1.2, CKJ 500 2.1.2, CKJ 1000 2.2, CKJ 1000 3.1.1, and CKJ 1000 3.2.1) were found to be resistant to 5 mM $CuSO_4$ and capable of decolorizing dyes (Methylene Blue, Congo Red, and Basic Fuchsine) at the highest concentration of 700 ppm. The selected bacterial isolates from the Cikijing River exhibit great potential to be further developed as bioremediation agents employed in biological processes for wastewater treatment.

AUTHORS CONTRIBUTION

W.I. and D.N.S designed the research and supervised all the processes. R.P. and T.Y designed the IS collected and analysed the data, and VL wrote the manuscript.

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CONFLICT OF INTEREST

Please state any conflict of interest regarding the research or the research funding.

Table 3. Top homology search of copper- and dye-resistant bacterial isolates based on 16S rDNA.

Isolate code	The closes taxon to BLAST results on NCBI	Max score	Query coverage (%)	Accession	Sequence Similarity (%)
CKJ 300 1.2	*Klebsiella* sp.	1410	100	HM462447.1	100
CKJ 500 2.1.2	*Klebsiella pneumoniae*	1400	100	LC455961.1	98.85
CKJ 500 2.2	*Klebsiella pneumoniae*	1351	100	LC455961.1	98.94
CKJ 1000 1.1	*Lysinibacillus boronitolerans*	1410	100	MH385002.1	99.87
CKJ 1000 1.2	*Lysinibacillus fusiformis*	1430	100	MT605500.1	100
CKJ 1000 2.2	*Bacillus proteolyticus*	1450	100	MT573794.1	100
CKJ 1000 3.1	*Pseudomonas stutzeri*	1432	100	MF125023.1	99.87
CKJ 1000 3.1.1	*Klebsiella variicola*	1395	100	MN725749.1	99.74
CKJ 1000 3.2.1	*Citrobacter freundii*	824	100	MH668092.1	97.69

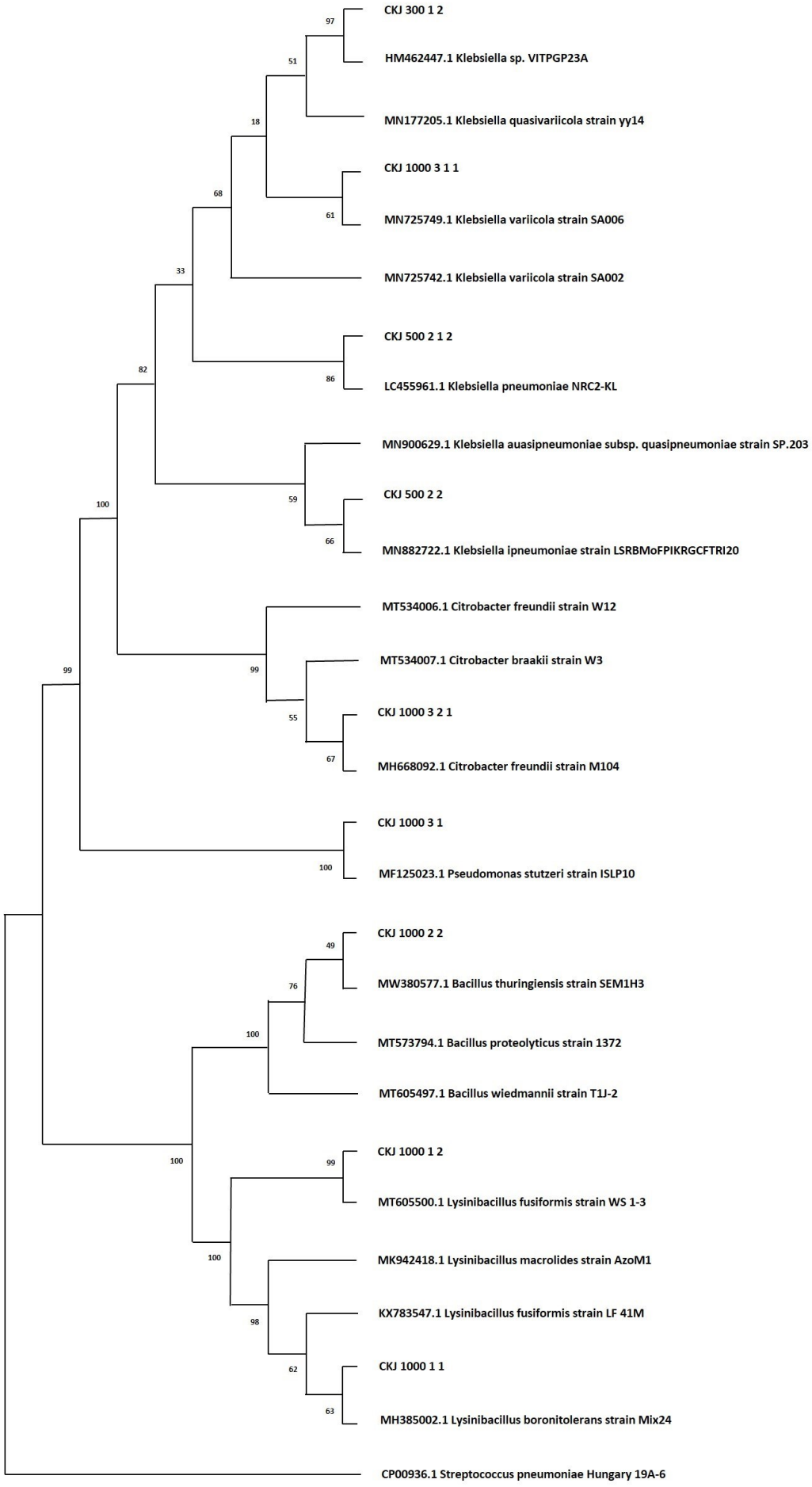

Figure 2. Phylogenetic tree based on 16S rDNA sequences of isolates CKJ 300 1.2, CKJ 500 2.1.2, CKJ 500 2.2, CKJ 1000 1.1, CKJ 1000 1.2, CKJ 1000 2.2, CKJ 1000 3.1, CKJ 1000 3.1.1, and CKJ 1000 3.2.1. This tree was made using the neighbour-joining method with Kimura two-parameters distances with the no-gap option. Number indicated the percentages of occurrence in 1000 boost traps trees.

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